5 September-October 1970
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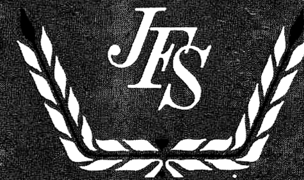

JOURNAL of FOOD SCIENCE

BASIC SCIENCE

519 Flavor chemistry of tomato volatiles–*S. J. Kazeniac and R. M. Hall*

531 Effects of pH and temperature on volatile constituents of caraway–*Y. Yin, N. Zarghami and D. E. Heinz*

533 Effects of pH and temperature on volatile constituents of cardamom–*C. P. Brennand and D. E. Heinz*

538 Volatile flavor components of coconut meat–*F. M. Lin and W. F. Wilkens*

540 Identification of dimethyl trisulfide as a major aroma component of cooked Brassicaceous vegetables–*F. T. Maruyama*

543 A rapid gas-liquid chromatographic determination for capsaicin in capsicum spices–*K. T. Hartman*

547 Comparison of Valencia essential oil from California, Florida and Israel–*A. Lifshitz, W. L. Stanley and Y. Stepak*

549 Cytological effects of juice or puree from irradiated strawberries–*S. T. Ross, M. V. Bradley and J. A. Oka*

551 On the nature of the association of protein in frozen-stored cod muscle–*M. L. Anderson and E. M. Ravesi*

558 Accelerated denaturation of myosin in frozen solution–*H. Buttkus*

563 Degradation of various meat fractions by tenderizing enzymes–*C. K. Kang and E. E. Rice*

565 Enzymatic deamination of adenosine monophosphate (AMP), adenosine and adenine by salmon, crab and scallop muscle extracts–*F. E. Stone*

568 Phosphocreatine and nucleotide changes in pig longissimus muscle during the development of rigor mortis under controlled environmental conditions–*G. R. Schmidt and E. J. Briskey*

571 Changes in tension and certain metabolites during the development of rigor mortis in selected red and white skeletal muscles–*G. R. Schmidt, R. G. Cassens and E. J. Briskey*

574 Relationship of calcium uptake by the sarcoplasmic reticulum to tension development and rigor mortis in striated muscle–*G. R. Schmidt, R. G. Cassens and E. J. Briskey*

577 Effect of contraction on tenderness of poultry muscle cooked in the prerigor state–*A. A. Klose, B. J. Luyet and L. J. Menz*

582 A new extraction method for determining 2-thiobarbituric acid values of pork and beef during storage–*V. C. Witte, G. F. Krause and M. E. Bailey*

585 Effect of Staphylococcus infection on acid and alkaline phosphatase in bruised poultry tissue–*S. S. Hussain and M. K. Hamdy*

590 Catalysts of lipid peroxidation in meats. 1. Linoleate peroxidation catalyzed by MetMb or Fe(II)-EDTA–*H. Liu*

593 Catalysts of lipid peroxidation in meats. 2. Linoleate oxidation catalyzed by tissue homogenates–*H. Liu*

596 Catalysts of lipid peroxidation in meats. 3. Catalysts of oxidative rancidity in meats–*H. Liu and B. M. Watts*

598 Formation of free radicals in dry milk proteins–*P. M. T. Hansen, W. J. Harper and K. K. Sharma*

601 Steroids in egg yolk–*C. Tu, W. D. Powrie and O. Fennema*

606 Rates of pH change and disappearance of glucose during low temperature pyrolysis–*R. H. Walter and I. S. Fagerson*

608 Pink discoloration in canned Williams' Bon Chretien pears–*A. Czerkaskyj*

612 Bound water defined and determined at constant temperature by wide-line NMR–*S. Shanbhag, M. P. Steinberg and A. I. Nelson*

615 Dehydration thermoprofiles of amino acids and proteins–*E. Karmas and G. R. DiMarco*

618 Growth patterns of selected psychrophilic microorganisms in cooked and uncooked aseptically procured turkey meat–*M. G. Mast and G. J. Mountney*

—CONTENTS CONTINUED (on the inside of the front cover) . . . . .

A PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS IFT

—CONTENTS CONTINUED—

from front cover

620 Recovery of Salmonellae from irradiated and unirradiated foods–*J. J. Licciardello, J. T. R. Nickerson and S. A. Goldblith*

624 Method for isolating viruses from ground beef–*R. Sullivan, A. C. Fassolitis and R. B. Read Jr.*

627 Some effects of ethylene oxide on Bacillus subtilis–*J. Marletta and C. R. Stumbo*

631 Ethylene oxide sterilization of Salmonella senftenberg and Escherichia coli: Death kinetics and mode of action–*G. T. Michael and C. R. Stumbo*

634 Soybean factors relating to gas production by intestinal bacteria–*J. J. Rackis, D. J. Sessa, F. R. Steggerda, T. Shimizu, J. Anderson and S. L. Pearl*

APPLIED SCIENCE and ENGINEERING

640 CCl_3F hydrate: Ability to inhibit oxidation of L-ascorbic acid in peas–*L. U. Thompson and O. Fennema*

641 Organic acid profiles of thermally processed spinach puree–*Yi Do Lin, F. M. Clydesdale and F. J. Francis*

645 Ascorbic acid and color changes in summer squash as influenced by blanch, pH and other treatments–*W. A. Sistrunk and J. N. Cash*

649 Production of frozen orange-juice concentrate from centrifugally separated serum and pulp–*M. Peleg and C. H. Mannheim*

652 Bacteriological contamination of some citrus oils during processing–*D. I. Murdock and G. L. K. Hunter*

655 Removal of oligosaccharides from soy milk by an enzyme from Aspergillus saitoi–*H. Sugimoto and J. P. Van Buren*

661 Bread from sorghum and barley flours–*M. R. Hart, R. P. Graham, M. Gee and A. I. Morgan Jr.*

666 Starch characteristics of selected grain sorghums as related to human foods–*O. H. Miller and E. E. Burns*

668 Effect of sodium nitrite on flavor of cured pork–*I. C. Cho and L. J. Bratzler*

670 Weight and color changes during storage of beef steaks packaged in clear plastic foam, and pulp trays–*E. M. Buck and B. A. Peters*

673 Cooling of porcine ham by oil immersion–*C. L. Kastner, R. L. Henrickson and B. L. Clary*

676 Effect of heating and cold storage on antithiamine activity in skipjack tuna–*N. Y. Tang and D. M. Hilker*

677 Effect of heat on the chemical and nutritive stability of fish protein concentrate (FPC)–*D. L. Dubrow and B. R. Stillings*

681 Radio-frequency pasteurization of cured hams–*N. E. Bengtsson, F. R. Del Valle and W. Green*

688 Effect of processing variables on stability and protein extractability of turkey meat emulsions–*G. L. Hargus, G. W. Froning, C. A. Mebus, S. Neelakantan and T. E. Hartung*

693 Effect of pH on the quality of chicken frankfurters–*R. C. Baker, J. Darfler and D. V. Vadehra*

695 Frozen whole eggs for scrambling–*K. Ijichi, H. H. Palmer and H. Lineweaver*

699 Comparison of frozen, foam-spray-dried, freeze-dried, and spray-dried eggs. 7. Soft meringues prepared with a Carrageenan stabilizer–*K. J. Morgan, K. Funk and M. E. Zabik*

702 A method for the determination of 5-hydroxymethyl-2-furaldehyde in the presence of 2-furaldehyde (furfural)–*R. O. B. Wijesekera and C. O. Chichester*

September-October 1970
Volume 35 : Number 5

JOURNAL of FOOD SCIENCE

Contents . . . . 1st and 2nd Covers
Abstracts: In this Issue iii
Page Charge Notice 626
Research Papers Begin 519

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Memo FROM THE SCIENTIFIC EDITOR

☐ THE INSTITUTE OF FOOD TECHNOLOGISTS' Council Policy Committee and Executive Committee have voted to discontinue publication of formal research papers in *Food Technology.* Effective January 1971, all acceptable research papers will be published in the *Journal of Food Science.* Those papers previously approved for publication in *Food Technology,* but scheduled for publication in 1971, have been transferred to the *Journal of Food Science.*

In order to accommodate and encourage the submission of research papers pertaining to applied food science and food engineering, which were previously published in *Food Technology,* the *Journal of Food Science* now contains two major sections—"Basic Science," and "Applied Science and Engineering." In addition, "Research Notes" will be included when the appropriate communications are available. Research Notes are given priority over other papers in the publication schedule.

It has become exceedingly clear that the *Journal of Food Science* must have a rapid publication schedule for acceptable papers. We now use a double reviewer system, with tight schedules for review and revisions. The thrust of our program is geared to the best interest of authors and to the solicitation of high quality papers. The Executive Committee and its Subcommittee on Publications have enthusiastically supported this improvement in schedule. Consequently by early 1971 we should be on a six month schedule—from receipt to publication—for papers of high quality. The technical difficulties, related to printing, have also been overcome, and the issues should be on schedule starting early in 1971.

I wish to also take this opportunity to express my warmest appreciation to the reviewers' who give unstintingly of their time to meticulously and promptly review papers which I submit to them. Their efforts are of primary importance in expediting the handling processes. Because a reviewer, out of devotion to his professional interest, is willing to cooperate so magnificently, we obviously expect the author to divert his efforts for the immediate revision of papers. It is our hope and our goal to continually improve the schedules and constantly raise the standards of the *Journal of Food Science.* This achievement would be in the best interest of the entire food industry.

Ernest J. Briskey

Scientific Editor

ABSTRACTS:

IN THIS ISSUE

FLAVOR CHEMISTRY OF TOMATO VOLATILES. S. J. KAZENIAC & R. M. HALL. *J. Food Sci.* **35**, 519–530 (1970)–Identification of 38 volatile compounds in tomato fruit is reported. Fruit maturity, the physical condition of the fruit, the type of fruit crushing, heat and oxygen were important in the development of some of these compounds. The quality of tomato volatiles was found to depend on interrelationships between enzymic and chemical reactions which occur during preparative treatments. Linolenic acid was indicated to be the precursor of *cis*-3-hexenal which developed during tissue maceration. In turn, *cis*-3-hexenal appeared to be the source of *trans*-2-hexenal. *cis*-3-Hexenal and 2-isobutylthiazole were found to be important in fresh tomato flavor and 2-isobutylthiazole was responsible for the characteristic flavor of certain tomato varieties.

EFFECTS OF pH AND TEMPERATURE ON VOLATILE CONSTITUENTS OF CARAWAY. Y. YIN, N. ZARGHAMI & D. E. HEINZ. *J. Food Sci.* **35**, 531–533 (1970)–The major volatile components of caraway seed oil were isolated and identified; and the effects of heat and pH on these volatile constituents were studied. Gas chromatographic analysis of the treated oil indicated that the two principal components, carvone and limonene, undergo little change even when subjected to harsh conditions. The chemical nature of the trace components changes only when the oil is subjected to high temperature or to solutions of varying pH.

EFFECTS OF pH AND TEMPERATURE ON VOLATILE CONSTITUENTS OF CARDAMOM. C. P. BRENNAND & D. E. HEINZ. *J. Food Sci.* **35**, 533–537 (1970)–Decorticated cardamom seeds were subjected to timed exposures to temperatures as high as 205°C and to solution pH's from 2–8. Chemical changes in the volatile constituents were followed by gas-liquid chromatographic analyses of the cardamom seed extracts. Temperatures above 149°C and increasing hydrogen ion concentration caused marked changes in composition of the volatile oils of this spice.

VOLATILE FLAVOR COMPONENTS OF COCONUT MEAT. F. M. LIN & W. F. WILKENS. *J. Food Sci.* **35**, 538–539 (1970)–Gas-chromatographic and mass-spectral techniques were employed in the isolation and identification of the volatile-flavor components of coconut meat. 15 compounds were positively identified. Odors of authentic compounds were described. Both delta-C_8, -C_{10} lactones and n-octanol were the major volatile components and responsible for the characteristic aroma of coconut meat. The contributions of other minor components to flavor and their significance were also described.

IDENTIFICATION OF DIMETHYL TRISULFIDE AS A MAJOR AROMA COMPONENT OF COOKED BRASSICACEOUS VEGETABLES. F. T. MARUYAMA. *J. Food Sci.* **35**, 540–543 (1970)–Aroma, retention time, and infra-red spectra of volatile components collected by simple distillation from 250g samples of vegetables and chromatographed in a dual detector gas-liquid chromatograph system were examined. Dimethyl trisulfide was identified as a major component. A mechanism for dimethyl trisulfide formation from S-methyl-l-cysteine sulfoxide is proposed.

A RAPID GAS-LIQUID CHROMATOGRAPHIC DETERMINATION FOR CAPSAICIN IN CAPSICUM SPICES. K. T. HARTMAN. *J. Food Sci.* **35**, 543–547 (1970)–The trimethylsilyl (TMS) derivative of capsaicin was prepared for standard capsaicin, weighed portions of oleoresins and the residues obtained from the ethyl acetate extraction of red peppers. The derivative was quantitatively measured using a gas-liquid chromatographic system equipped with an alkali flame detector approximately 25 times more sensitive to capsaicin than the unmodified flame ionization detector. Capsaicin recoveries from 5 fortified oleoresins ranged from 96.0–103.0%. Calculated and declared Scoville units showed good agreement (96.6–109.2%) for 6 capsicum spices.

COMPARISON OF VALENCIA ESSENTIAL OIL FROM CALIFORNIA, FLORIDA AND ISRAEL. A. LIFSHITZ, W. L. STANLEY & Y. STEPAK. *J. Food Sci.* **35**, 547–548 (1970)–Essential oils of Valencia oranges obtained from California, Florida and Israel were analyzed for the relative amount of C_8, C_9 and C_{10} aldehydes, as well as for their total carbonyl contents. The aldehyde patterns of the Florida and Israel oils were similar and were both different from that of the California oil. The carbonyl contents of the 3 oils were found to be statistically different from each other.

CYTOLOGICAL EFFECTS OF JUICE OR PUREE FROM IRRADIATED STRAWBERRIES. S. T. ROSS, M. V. BRADLEY & J. A. OKA. *J. Food Sci.* **35**, 549–550 (1970)–*Vicia faba* roots were treated 4 hr with undiluted juice or puree from strawberries irradiated with 200 or 400 Krad of gamma radiation from Co^{60} and from control berries. The differences between percentages of abnormal anaphases after treatments with juice or puree from irradiated and control berries were not significant at the 1% level ("t" test) in all the 7 experiments with 200 Krad as the radiation dose, and in 6 of 7 experiments when the dose was 400 Krad. In 1 experiment with a 400-Krad dose the difference between percentages of abnormal anaphases was barely significant at the 5% level of confidence.

ON THE NATURE OF THE ASSOCIATION OF PROTEIN IN FROZEN-STORED COD MUSCLE. M. L. ANDERSON & E. M. RAVESI. *J. Food Sci.* **35**, 551–558 (1970)–Decrease in readily extractable protein (REP) was related to free fatty acid (FFA) formation during storage of cod muscle at −82°C and −18°C. Aggregated protein extracted during the period of decrease in REP had properties similar to altered protein extracted from frozen-stored cod muscle in which FFA was formed largely during aging in ice. Change from a rapid rate of association and FFA formation to a slower rate during storage was attributed to change in solvent environment due to FFA formation. A model for the association process based on bonding potentials existing in the muscle on freezing and those developed during storage is presented.

ACCELERATED DENATURATION OF MYOSIN IN FROZEN SOLUTION. H. BUTTKUS. *J. Food Sci.* **35**, 558–562 (1970)–The aggregation of rabbit and trout myosins was studied in frozen solution at high ionic strength. Monomeric myosin aggregated into dimers and trimers during the initial stages of freezing. Aggregate formation increased as the temperature decreased below the freezing point and reached a maximum near the eutectic point of the myosin-potassium chloride-water solution (−11°). Ultrastructural studies showed that the aggregation proceeded in a side-to-side manner. Below the eutectic point the rate of aggregation and consequent insolubilization decreased and approached that observed at 0°. The effects of adding various substances on myosin aggregation at low temperature were also studied.

DEGRADATION OF VARIOUS MEAT FRACTIONS BY TENDERIZING ENZYMES. C. K. KANG & E. E. RICE. *J. Food Sci.* **35**, 563–565 (1970)–Beef muscle was separated into water soluble, salt soluble and insoluble fractions. These showed different degrees of hydrolysis, depending upon the enzyme used. Collagenase, bromelain and trypsin hydrolyzed the insoluble fraction more readily than the salt soluble fraction; whereas papain, Rhozyme P-11 and ficin hydrolyzed the salt soluble fraction more efficiently. In general, the water soluble fraction was more resistant to enzyme hydrolysis than the other fractions except with papain and Rhozyme P-11. However, the portion of the water soluble fraction that was hydrolyzed appeared as smaller peptides than the solubilized fragments of the other two fractions.

ABSTRACTS:

IN THIS ISSUE

ENZYMATIC DEAMINATION OF ADENOSINE MONOPHOSPHATE (AMP), ADENOSINE AND ADENINE BY SALMON, CRAB AND SCALLOP MUSCLE EXTRACTS. F. E. STONE. *J. Food Sci.* 35, 565–567 (1970)–Crude extracts of pink and coho salmon fillets contain both adenosine aminohydrolase (EC 3.5.4.4.) and 5′-AMP-aminohydrolase (EC 3.5.4.6.). Similar extracts from king and Tanner crab meat and from scallop adductor muscle contain adenosine aminohydrolase but not 5′-AMP-aminohydrolase. Adenine aminohydrolase activity was not detected in salmon, crab or scallops. These results suggest that the enzymatic deamination of 5′-adenosine monophosphate (AMP) will contribute to the 5′-inosine monophosphate (IMP) content of salmon fillets but will not contribute significantly to the IMP content of crab or scallop muscles.

PHOSPHOCREATINE AND NUCLEOTIDE CHANGES IN PIG Longissimus MUSCLE DURING THE DEVELOPMENT OF RIGOR MORTIS UNDER CONTROLLED ENVIRONMENTAL CONDITIONS. G. R. SCHMIDT & E. J. BRISKEY. *J. Food Sci.* 35, 568–570 (1970)–This study was conducted to compare the depletion of phosphocreatine (PC) and nucleotides (ATP and ADP) in strips of muscle which had been used for extensibility measurement in the rigorometer, with control strips of muscle which had been held unrestrained in the same environment. The rigorometer and control strips did not differ significantly ($P > .05$) in level of PC and ATP. Rigorometer strips from "stress-susceptible" pigs had less rapid and less extensive depletion of ADP than did control strips from the same animals. It appeared that extensibility remained in the tissue after the ATP had been depleted, but not after ADP reached a low level.

CHANGES IN TENSION AND CERTAIN METABOLITES DURING THE DEVELOPMENT OF RIGOR MORTIS IN SELECTED RED AND WHITE SKELETAL MUSCLES. G. R. SCHMIDT, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* 35, 571–573 (1970)–The relative time course of rigor mortis was compared in the vastus lateralis (red) and the longissimus (white) muscle from untreated and magnesium sulfate injected "stress-resistant" Chester White and "stress-susceptible" Poland China pigs. In the untreated animals the white muscle had a shorter time course of rigor mortis than did the red muscle. This difference disappeared when the animals were injected ante-mortem with magnesium sulfate, although both muscles from injected animals had a slower development of rigor mortis than the same muscles in control animals. Either magnesium sulfate had more of an effect on white than on red muscle post-mortem, or the differences between the post-mortem rates of glycolysis of red and white muscles were significantly minimized when struggle and stimulation associated with death had been eliminated. Red muscles in magnesium sulfate injected pigs developed more tension post-mortem than did white muscles. The development of isometric tension reached its maximum as the muscle lost all of its extensibility. Muscle from "stress-susceptible" pigs had a shorter time course of rigor mortis than the corresponding muscle from "stress-resistant" pigs of the same treatment. This difference occurred even when the two groups started with the same level of phosphocreatine and lactic acid post-exsanguination as is the case when treated with magnesium sulfate. Therefore, even though magnesium treatment can retard glycolysis sufficiently to prevent the development of the PSE condition, it does not necessarily standardize the post-mortem changes in all skeletal muscles of all pigs.

RELATIONSHIP OF CALCIUM UPTAKE BY THE SARCOPLASMIC RETICULUM TO TENSION DEVELOPMENT AND RIGOR MORTIS IN STRIATED MUSCLE. G. R. SCHMIDT, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* 35, 574–576 (1970)–Concentrations of phosphocreatine (PC), adenosine triphosphate (ATP) and lactic acid were determined in longissimus muscle from 7 "stress-resistant" Chester White pigs and 8 "stress-susceptible" Poland China pigs at several intervals during the loss of extensibility post-mortem. Calcium-binding ability of the sarcoplasmic reticulum and postmortem tension development of longissimus muscle also were determined. Loss of extensibility was completed sooner in longissimus muscle from stress-susceptible pigs than from stress-resistant pigs. For the most part, longissimus muscle samples from stress-susceptible pigs had lower ($P < .05$) levels of PC and ATP and higher ($P < .05$) levels of lactic acid at identical stages of change in extensibility than did samples from stress-resistant pigs. At identical stages of change in extensibility in "fast-" and "slow-glycolyzing" muscles, there were no significant differences in the ability of the sarcoplasmic reticulum preparations to bind calcium or in the muscle's ability to develop isometric tension.

EFFECT OF CONTRACTION ON TENDERNESS OF POULTRY MUSCLE COOKED IN THE PRERIGOR STATE. A. A. KLOSE, B. J. LUYET & L. J. MENZ. *J. Food Sci.* 35, 577–581 (1970)–Effects on tenderness caused by contraction of excised chicken muscles, induced by immediate post-mortem treatments consisting of electrical stimulation, beating, freeze-thawing, and heating, and followed by cooking in the prerigor state were measured. One member of each pair of muscles was held in restraint while exposed to the same conditions. In terms of percentage of original rest length, electrical stimulation reduced muscle length to 59%, and when followed by cooking to 44%; freeze-thawing reduced the length to 42%, and when followed by cooking to 40%; heating to 96%, and when followed by cooking to 52%; cooking alone to 48–53%. With the exception of the beating-heating combination, all contraction inducing treatments resulted in a reduction of the shear values (force per cross-sectional area) of cooked muscle to about one-half those of uncontracted controls. These results with chicken muscles on intense contraction followed by cooking in the prerigor state agree with recent similar observations on red meat muscles.

A NEW EXTRACTION METHOD FOR DETERMINING 2-THIOBARBITURIC ACID VALUES OF PORK AND BEEF DURING STORAGE. V. C. WITTE, G. F. KRAUSE & M. E. BAILEY. *J. Food Sci.* 35, 582–585 (1970)–2-Thiobarbituric acid (TBA) values of raw pork were determined by distillation and extraction methods and those of raw beef by extraction during storage at 4° and −20°C. TBA values of tissues were low compared with values usually reported for cooked tissue from these animals in similar environments. Differences in TBA values of beef and pork, and changes in TBA values of these tissues during storage at 4°C were significant whereas changes during storage at −20°C were insignificant. pH and TBA values were inversely related. Mathematical relationships between TBA values of pork and beef during storage at 4°C, between TBA values and pH and between results obtained by the two procedures for determining TBA values of pork during storage are discussed.

EFFECT OF STAPHYLOCOCCUS INFECTION ON ACID AND ALKALINE PHOSPHATASE IN BRUISED POULTRY TISSUE. S. S. HUSSAIN & M. K. HAMDY. *J. Food Sci.* 35, 585–589 (1970)–Increasing severity of trauma elicited marked differences in both acid and alkaline phosphatases. The former is a lysosomal enzyme; whereas, the latter is a nonlysosomal enzyme. The magnitude of enzymatic responses as a result of trauma was not the same for each enzyme. Acid phosphatase activity increased in the bruised tissue while alkaline phosphatase activity decreased. The bound form of acid phosphatase increased in the bruised tissue, a form of the enzyme absent in normal tissue. The following factors seem to play an important role on the activities of these enzymes in poultry tissues: age of trauma, severity of the bruise and staphylococcal infection. Rates of changes in the enzymatic activities after infection were directly related to the number of organisms in the infecting dose and induced an alteration in the activities of alkaline phosphatase in the bruised tissue from a decreased to an increased value compared to normal level.

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 1. Linoleate Peroxidation Catalyzed by MetMb or Fe(II)-EDTA. H. LIU. *J. Food Sci.* **35**, 590–592 (1970)–The hemoprotein MetMb accelerated linoleic acid peroxidation in pH range from 5.6–7.8, the catalysis increasing with pH. A complex of ferrous ion and ethylenediaminetetraacetic acid [Fe(II)-EDTA]–a non-heme iron model–in a 1 to 1 ratio accelerated peroxidation at lower pH; no catalysis took place above pH 6.4. Most chelating agents eliminated Fe(II)-EDTA catalysis, but had no effect on MetMb catalysis. Reducing agents, both ascorbic acid and thiols, on the other hand, accelerated Fe(II)-EDTA catalysis but inhibited MetMb catalysis.

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 2. Linoleate Oxidation Catalyzed by Tissue Homogenates. H. LIU. *J. Food Sci.* **35**, 593–595 (1970)–In beef tissue homogenate, both types of catalysts–hemoprotein and non-heme iron–are active catalysts of linoleate oxidation. Although the pH-dependent catalytic pattern of beef homogenate was similar to MetMb catalysis, the presence of non-heme iron could be identified by adding ascorbate or 8-OH-quinoline. Ascorbate-stimulated oxidation could be inhibited by chelating agents. Furthermore, lower concentrations of phosphate buffer rendered the non-heme iron more active at acidic pH. Linoleate oxidation was also catalyzed by H_2O_2-treated (heme-free) beef homogenates. The oxidation was accelerated either by thiols or by ascorbate.

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 3. Catalysts of Oxidative Rancidity in Meats. H. LIU & B. M. WATTS. *J. Food Sci.* **35**, 596–598 (1970)–After removal of MetMb by treating with H_2O_2, a significant lipid oxidation was demonstrated, especially at lower pH where non-heme iron is most active. The catalytic activity of hemoprotein is limited in raw meat. Oxygen can be removed from the tissues and MetMb reduced back to Mb by the reducing enzymes. This is especially true at higher pH. Possible limitations of the heme-catalyzed reactions in meat by high (inhibition) levels of myoglobin, or because of separation of reactants in cellular structures, are discussed. The effect of additives were in line with the interpretation that lipid oxidation is catalyzed by both non-heme and hemoprotein.

FORMATION OF FREE RADICALS IN DRY MILK PROTEINS. P. M. T. HANSEN, W. J. HARPER & K. K. SHARMA. *J. Food Sci.* **35**, 598–600 (1970)–The possible formation of free radicals in dry milk proteins by mechanical energy has been studied by electron spin resonance (ESR) spectroscopy. Grinding of various milk proteins with mortar and pestle for 5 min or longer yielded perceptible electron spin resonance signals. The signals did not exhibit characteristics of hyperfine structure and all had g-values close to that of a free electron (2.00). The intensity of the signals was particularly strong for the sulfhydryl-containing proteins, β-lactoglobulin and κ-casein, and increased with grinding time. The signals were considerably reduced by subsequent heating of the dry protein at 100°C/10 min. The grinding of whole casein was in all cases accompanied by the release of a gluey odor, suggesting that the gluey-flavor defect may be related to protein fragmentation.

STEROIDS IN EGG YOLK. C. TU, W. D. POWRIE & O. FENNEMA. *J. Food Sci.* **35**, 601–606 (1970)–This investigation was initiated to separate the unsaponifiable matter of egg yolk by thin layer chromatography (TLC) and attempt to identify the steroids by gas liquid chromatography (GLC). Three GLC liquid phases, differing in selective partition properties, were used to aid in the identification of unknown steroids. A selective method was also carried out to extract the estrogenic steroids in egg yolk if present. The results of thin layer and gas chromatographic analyses indicate that a large quantity of cholesterol and considerable amounts of lanosterol, desmosterol, Δ^7-cholestenol, cholestanol, Δ^7- and Δ^8-methostenol, 4α-methyl-$\Delta^{8,24}$-cholestenol and its Δ^7-isomer, 4,4α-dimethyl-$\Delta^{7,24}$-cholestenol, dihydrolanostenol, β-sitosterol and possibly ergosterol were present in egg yolk. Estrogenic steroids and other known steroid hormones were not found in egg yolk.

RATES OF pH CHANGE AND DISAPPEARANCE OF GLUCOSE DURING LOW TEMPERATURE PYROLYSIS. R. H. WALTER & I. S. FAGERSON. *J. Food Sci.* **35**, 606–607 (1970)–During low temperature pyrolysis (under 200°C), the disappearance of glucose and pH change of an aqueous solution of the pyrolyzate were found to approximate first order reactions. Since change of concentration of glucose and change of pH are directly related to the time, the mathematical equations describing the two occurrences were combined through this common variable. The resulting equation describes the residual concentration of glucose as a function of change of pH.

PINK DISCOLORATION IN CANNED WILLIAMS' BON CHRETIEN PEARS. A. CZERKASKYJ, *J. Food Sci.*, **35**, 608–611 (1970)–Pink discoloration in canned Williams' Bon Chretien pears is an occasional problem to the canning industry in the Goulburn Valley, Victoria. During 1963–1965, the relationship between skin blush on fresh fruit and pink discoloration after processing, effect of the level of applied nitrogenous fertilizer, and processing time were studied. There is a high positive correlation between the intensity of skin blush on the fresh fruit and pink discoloration of the fruit after processing. Only blushed fruits were prone to pink discoloration after processing. Blushed fruit from trees which had heavier application of nitrogen produced more intense pinking, but short overcooking showed no effect on pinking.

BOUND WATER DEFINED AND DETERMINED AT CONSTANT TEMPERATURE BY WIDE-LINE NMR. S. SHANBHAG, M. P. STEINBERG & A. I. NELSON. *J. Food Sci.* **35**, 612–615 (1970)–The NMR properties of bound water (BW) in wheat flour, corn starch and egg white at room temperature were studied using a wide-line NMR Spectrometer. At lowest radiofrequency attenuation, hydrogen nuclei from free water gave a negligible signal but the hydrogen from BW gave a strong signal, giving rise to a new definition for BW and a new method for its quantitative determination at room temperature. Within a given set of instrument parameters, the hydrogen nuclei from BW showed NMR properties independent of dry matter (DM) making it possible to derive a "universal" NMR calibration constant per unit weight BW. Weight of BW/unit weight DM increased with increasing total moisture content to a maximum, called Bound Water Capacity, which was independent of total moisture content but varied with the product.

DEHYDRATION THERMOPROFILES OF AMINO ACIDS AND PROTEINS. E. KARMAS & G. R. DiMARCO. *J. Food Sci.* **35**, 615–617 (1970)–Dehydration thermoprofiles representing water evapoation from aqueous solutions of 22 naturally occurring amino acids were determined. 14 amino acids exhibited peaks of "bound" water beyond the evaporation peak of "free" water. The acidic and basic amino acids indicated no water retention. Exceptional water retention properties, 30%–70% of the total water content as measured by peak areas, were exhibited by the nonpolar amino acids: isoleucine, leucine, methionine and valine. The methyl and methylene groups of these nonpolar structures seem to be responsible for the water retention properties probably due to hydrate formation. One explanation could be that as the temperature is raised to a critical point, the semicrystalline array around the nonpolar amino acid radicals collapses. Beef muscle tissue and egg albumin indicated strong water retention properties.

GROWTH PATTERNS OF SELECTED PSYCHROPHILIC MICROORGANISMS IN COOKED AND UNCOOKED ASEPTICALLY PROCURED TURKEY MEAT. M. G. MAST & G. J. MOUNTNEY. *J. Food Sci.* **35**, 618–620 (1970)–Sterile muscle tissue was procured from a turkey grown under commercial conditions. Growth patterns of selected microorganisms were compared in cooked and uncooked samples of this tissue held at 5°C and 20°C. Cooked tissue, in most instances, supported greater numbers of microorganisms than did uncooked tissue. Muscle tissue inoculated with a mixed culture (a *Flavobacterium* species and an *Alcaligenes* species) consistently contained greater numbers of bacteria than meat inoculated with a pure culture of *Pseudomonas fluorescens*. Uncooked sterile turkey meat remained in good condition, both in appearance and bacteriologically, for at least one year when stored at above freezing temperatures.

RECOVERY OF SALMONELLAE FROM IRRADIATED AND UNIRRADIATED FOODS. J. J. LICCIARDELLO, J. T. R. NICKERSON & S. A. GOLDBLITH. *J. Food Sci.* **35**, 620–624 (1970)–Maximum recovery of salmonellae from artificially contaminated irradiated and unirradiated foods was usually obtained on tryptic soy yeast extract agar, a nonselective medium. Recovery on various selective media depended, in general, upon the serotype and the substrate. Irradiated cells were definitely inhibited on *Salmonella-Shigella* agar or desoxycholate citrate agar, but only slightly, if at all, on brilliant green, bismuth sulfite or MacConkey agars. The highest count of irradiated cells of *Salmonella newport* by the MPN method was obtained with tetrathionate broth, yet this medium was inhibitory to *Salmonella oranienburg* and *Salmonella heidelberg*. In this latter situation, pre-enrichment in nutrient broth prior to inoculation into tetrathionate broth was beneficial.

ABSTRACTS:

IN THIS ISSUE

METHOD FOR ISOLATING VIRUSES FROM GROUND BEEF. R. SULLIVAN, A. C. FASSOLITIS & R. B. READ JR. *J. Food Sci.* 35, 624–626 (1970)–A method was developed to isolate viruses from ground beef. Antibiotics were used to suppress bacterial and fungal contaminants. A meat slurry was made in a standard salt and amino acid solution adjusted to pH 8.5. The slurry was filtered through cheese cloth and the filtrate examined for viruses by a plaque-forming unit (pfu) assay system. Approximately 75% of input coxsackievirus B-2 was recoverable. The method was used to examine market-purchased ground beef for viruses. 1 to 195 viral pfu/5 g were isolated from 3 of 12 loaves of meat. 1 loaf yielded poliovirus 1 and echovirus 6, 1 yielded poliovirus 2 and 1 yielded polioviruses 1 and 3.

SOME EFFECTS OF ETHYLENE OXIDE ON Bacillus subtilis. J. MARLETTA & C. R. STUMBO. *J. Food Sci.* 35, 627–631 (1970)–Spores of *Bacillus subtilis* were exposed to ETO and then suspended in distilled water overnight to determine if ETO treatment would affect release of protein, RNA, DNA, or DPA. Only DPA release was affected, it being appreciably greater from exposed than from unexposed spores. A comparison of lyophilized vegetative cells, germinated spores, and heat-activated spores revealed that the ETO resistance of germinated spores was much closer to that of heat-activated spores than to that of vegetative cells. ETO exposure resulted in no significant change in rate or efficiency of spore germination in either a rich medium or a minimal medium. However, ETO treatment notably increased the postgerminative phase of development.

ETHYLENE OXIDE STERILIZATION OF Salmonella senftenberg AND Escherichia coli: DEATH KINETICS AND MODE OF ACTION. G. T. MICHAEL & C. R. STUMBO. *J. Food Sci.* 35, 631–634 (1970)–Death of lyophilized *Salmonella senftenberg* and *Escherichia coli* when exposed to gaseous ethylene oxide (ETO) followed first order kinetics. *S. senftenberg* in whole egg powder was about twice as resistant as it was in the clean state. Inoculation of ETO treated cells into a minimal medium variously supplemented with a variety of nutritional materials indicated that the mode of action of ETO in causing loss of cell reproduction ability was that of alkylation of the guanosine triphosphate component of DNA.

SOYBEAN FACTORS RELATING TO GAS PRODUCTION BY INTESTINAL BACTERIA. J. J. RACKIS, D. J. SESSA, F. R. STEGGERDA, T. SHIMIZU, J. ANDERSON & S. L. PEARL. *J. Food Sci.* 35, 634–639 (1970)–An in vitro assay was used to show that toasted, dehulled, defatted soybean meal contains a gas-producing factor and a gas-inhibiting factor. The oligosaccharides–sucrose, raffinose and stachyose–are associated with the gas-producing factor when incubated in thioglycollate media with anaerobic bacteria of the intestinal tract of dogs. The phenolic acids in soybeans–syringic and ferulic acid–are effective gas inhibitors in vitro and in intestinal segments of dogs. The lipids, proteins and water-insoluble polysaccharides of soybean meal have no gas activity. During fermentation, gas production parallels the formation of monosaccharides by enzymatic hydrolysis of raffinose and stachyose. The amount and composition of gas produced from cottonseed and peanut meal were comparable to soybean meal.

CCl_3F HYDRATE: ABILITY TO INHIBIT OXIDATION OF L-ASCORBIC ACID IN PEAS. L. U. THOMPSON & O. FENNEMA. *J. Food Sci.* 35, 640–641 (1970)–Oxidation of L-ascorbic acid was measured at $-7°C$ in a closed system containing peas, water, CCl_3F hydrate and air. The presence of CCl_3F hydrate significantly inhibited oxidation of L-ascorbic acid to compounds devoid of vitamin C activity.

ORGANIC ACID PROFILES OF THERMALLY PROCESSED SPINACH PUREE. YI DO LIN, F. M. CLYDESDALE & F. J. FRANCIS. *J. Food Sci.* 35, 641–644 (1970)–Spinach puree was packed in TDT tubes and processed with an $F_o = 4.9$ at temperatures ranging from 240°F–300°F with 10°F increments. Analyses were carried out for organic acids, color and pH. The greatest changes in these quality parameters were noted at 240°F with such changes decreasing as temperature increased up to 300°F. A plateau was noted in the range of 270°–280°F since little improvement was gained above these temperatures. Acetic and pyrrolidone-carboxylic acids showed the most striking changes with increases of 129% and 132% respectively at 240°F.

ASCORBIC ACID AND COLOR CHANGES IN SUMMER SQUASH AS INFLUENCED BY BLANCH, pH AND OTHER TREATMENTS. W. A. SISTRUNK & J. N. CASH. *J. Food Sci.* 35, 645–648 (1970)–Ascorbic and dehydroascorbic acids were rapidly oxidized at 50°C. The reaction was slower at pH 5.0 than at pH 6.0 and 7.5. Color retention as measured by the CDM was greater at the higher pH levels. Time of blanch did not affect CDM a/b (hue) but did increase $\sqrt{a^2 + b^2}$ (chroma) and decreased the CDM L (lightness). Both color and ascorbic acid were lost very rapidly in unblanched squash puree under these conditions. Browning reactions had a major influence on CDM color values, even in blanched squash although the changes in carotenoid pigments were less pronounced.

PRODUCTION OF FROZEN ORANGE-JUICE CONCENTRATE FROM CENTRIFUGALLY SEPARATED SERUM AND PULP. M. PELEG & C.H. MANNHEIM. *J. Food Sci.* 35, 649–651 (1970)–The pulp was found to be compressible. Most of the essential oil was found in the pulp fraction and the residual oil content in the serum fraction showed linearity with serum turbidity. Concentrates produced by the separation method in question were stable and had superior organoleptic properties compared with those produced by the conventional method, using fresh cut-back juice. The essential oil content of the new concentrates was high enough to dispense with admixture of peel oil. It was found that stable concentrates can be produced without heat treatment of the feed juice.

BACTERIOLOGICAL CONTAMINATION OF SOME CITRUS OILS DURING PROCESSING. D. I. MURDOCK & G. L. K. HUNTER. *J. Food Sci.* 35, 652–655 (1970)–Tangerine, lemon and grapefruit oil emulsions undergo undesirable modification as a result of microbial growth. Alpha-terpineol is produced by the hydrolysis of d-limonene. The greatest microbial activity occurred in tangerine and lemon, and the least in grapefruit. Lemon oil emulsions containing less than 1% oil produced the highest concentration of alpha-terpineol. d-Limonene concentration in dilute lemon oil slurries decreased with a corresponding increase in alpha-terpineol.

REMOVAL OF OLIGOSACCHARIDES FROM SOY MILK BY AN ENZYME FROM Aspergillus Saitoi. H. SUGIMOTO & J. P. VAN BUREN. *J. Food Sci.* 35, 655–660 (1970)–A method for enzymatic removal of galacto-oligosaccharides from soy milk by a partially purified enzyme preparation from *Aspergillus saitoi* was investigated. Such sugars have been implicated in the tendency of soy milks to induce flatulence. The study of α-galactosidase in the enzyme preparation showed that it had its optimum pH between 5.0 and 5.5 and seemed stable above pH 4.0. Evaluations by means of thin-layer chromatography indicated that addition of small amounts of this enzyme preparation to soy milk resulted in complete hydrolysis of galacto-oligosaccharides. The method seems to be an economical procedure for the removal of oligosaccharides from soy milk.

BREAD FROM SORGHUM AND BARLEY FLOURS. M.R. HART, R.P. GRAHAM, M. GEE & A.I. MORGAN JR. *J. Food Sci.* 35, 661–665 (1970)–Various additives were examined as aids in making yeast bread from straight sorghum or barley flours. Good rise was achieved for both breads from a heavy batter of 45% solids. Several gums, especially 4000 cps methylcellulose, increased gas retention in sorghum bread and improved texture of both sorghum and barley breads. Several starches were successfully incorporated as a diluent in barley bread or used to improve texture and loaf volume of sorghum bread. The effect of several processing variables on sorghum bread was studied. 2 methods of sour fermentation were found compatible with sorghum bread.

STARCH CHARACTERISTICS OF SELECTED GRAIN SORGHUMS AS RELATED TO HUMAN FOODS. O. H. MILLER & E. E. BURNS. *J. Food Sci.* **35,** 666–668 (1970)–Seventeen varieties and hybrids of grain sorghum were analyzed for content of total starch, amylose, amylopectin, starch density, and starch granule diameter. Correlations among these attributes and organoleptic quality were made. Starches of regular-starch type varieties had higher densities than waxy starch varieties. Starch density was related directly to amylose content and inversely to total starch and amylopectin content. Amylose content was directly related to total starch content, starch density, and inversely related to amylopectin content. The varieties with high amylopectin content were preferred for sorghum bread. Organoleptic quality was directly related to amylopectin content and inversely related to total starch and amylose content.

EFFECT OF SODIUM NITRITE ON FLAVOR OF CURED PORK. I. C. CHO & L. J. BRATZLER. *J. Food Sci.* **35,** 668–670 (1970)–The effect of sodium nitrite on the flavor of cured pork was investigated by using the triangle and 2-sample taste tests. Sodium nitrite, through its reaction with pork muscle, contributes to the flavor of cured pork, in addition to the flavor due to salt, sugar and smoke.

WEIGHT AND COLOR CHANGES DURING STORAGE OF BEEF STEAKS PACKAGED IN CLEAR PLASTIC, FOAM AND PULP TRAYS. E. M. BUCK & B. A. PETERS. *J. Food Sci.* **35,** 670–672 (1970)–4 trials were conducted using 144 U.S. Choice-grade beef round steaks packaged in pulp, foam and clear plastic trays overwrapped with PVC film. Percent weight losses, at the end of 2 and 4 days of storage, were significantly greater for those steaks packaged in pulp trays as compared to clear or foam trays ($P < .01$). Evaporation losses from the various types of trays employed in this study were relatively uniform and differences in steak weight losses were primarily accounted for by absorbed or free juices in the tray. The color acceptability of all steaks declined during storage and there was no particular color advantage associated with any tray type. Visibility of steak undersides through clear plastic trays remained at an acceptable level throughout the storage period. The effect of stack position on shrinkage and color is discussed.

COOLING OF PORCINE HAM BY OIL IMMERSION. C. L. KASTNER, R. L. HENRICKSON & B. L. CLARY. *J. Food Sci.* **35,** 673–675 (1970)–12 swine were slaughtered and the hams removed from the "hot" carcasses. Individual paired hams from the same animal were placed in 1 of 2 cooling systems-(immersion in refrigerated light mineral oil or a forced-air system). Cooling curves and the F-test, in conjunction with the analysis of variance, indicated that the difference in cooling time between the 2 systems was nonsignificant. The objective of equating the heat-transfer coefficient was, therefore, accomplished. Chilling by oil immersion was 1.57 times faster than chilling in the forced-air system when both the air and oil velocities were 10.0 ft/sec. Moisture loss was 4.34 to 2.42 times greater in the air-chilling system than in the oil-immersion system.

EFFECT OF HEATING AND COLD STORAGE ON ANTITHIAMINE ACTIVITY IN SKIPJACK TUNA. N. Y. TANG & D. M. HILKER. *J. Food Sci.* **35,** 676–677 (1970)–The stability of antithiamine factor(s) in skipjack tuna *(Katsuwonus pelanus)* was tested after boiling by two methods, baking and cold storage at 10 and –10°C. The various heat treatments reduced the antithiamine activity from 55–86% of the control. Both frozen and refrigerated tuna, respectively, lost the antithiamine activity by 50–60% of the original level in 4 days.

EFFECT OF HEAT ON THE CHEMICAL AND NUTRITIVE STABILITY OF FISH PROTEIN CONCENTRATE (FPC). D. L. DUBROW & B. R. STILLINGS. *J. Food Sci.* **35,** 677–680 (1970)–To determine the heat stability of FPC (fish protein concentrate), we subjected FPC to one of two forms of heat: dry air (oven) or moist air (autoclave). Temperatures of the dry air were 100°, 120°, or 150°C; those of the moist air were 100° or 120°C. Exposure times for both treatments were 0, 30, 60, 120, or 240 min. Proximate composition, amino acid composition, and pH were determined. NPN (non-protein nitrogen) was also determined in autoclaved samples. PER (protein efficiency ratio) values were obtained for protein quality. Samples heated in dry air to 120°C for up to 4 hr showed little change, but those heated to 150°C for 60 min or longer decreased in lysine, arginine, available lysine, and PER. Samples heated in moist air increased in NPN, decreased in pH, and changed little in amino acid concentration. After 4 hr at 120°C, they decreased in protein quality.

RADIO-FREQUENCY PASTEURIZATION OF CURED HAMS. N. E. BENGTSSON, F. R. DEL VALLE & W. GREEN. *J. Food Sci.* **35,** 681–687 (1970)–For lean 2-lb hams, treatment time to reach the desired central temperature could be reduced to 1/3 by heating in a condenser field tunnel operating at 60 MHz, with substantial reduction of juice losses and a tendency to improve sensory quality compared with hot water processing. Treatment time could be further reduced by roughly the same extent by processing at 2450 MHz, but product thickness had to be reduced to secure sufficient heat penetration. Temperature gradients and juice losses were comparable to those in hot water treatment.

EFFECT OF PROCESSING VARIABLES ON STABILITY AND PROTEIN EXTRACTABILITY OF TURKEY MEAT EMULSIONS. G. L. HARGUS, G. W. FRONING, C. A. MEBUS, S. NEELAKANTAN & T. E. HARTUNG. *J. Food Sci.* **35,** 688–692 (1970)–White and dark turkey meat emulsions became increasingly more unstable with advancing chopping temperature. Stability of turkey meat emulsions was substantially increased when chopping was accomplished in a cold room. White turkey meat was observed to have stability superior to that of dark meat. Tensile strength of turkey emulsions was decreased with advancing chopping temperature and time. Decreases in protein solubility as chopping time and temperature increased indicated that protein denaturation may be a factor in decreased stability. Histological data further supported stability and tensile strength changes.

EFFECT OF pH ON THE QUALITY OF CHICKEN FRANKFURTERS. R. C. BAKER, J. DARFLER & D. V. VADEHRA. *J. Food Sci.* **35,** 693–695 (1970)–The effect of pH (4.6–8.6) on the tenderness, juiciness, flavor and preference of chicken frankfurters was studied. Results indicated that the normal pH (6.1–6.5) of chicken meat gave the best frankfurters.

FROZEN WHOLE EGGS FOR SCRAMBLING. K. IJICHI, H. H. PALMER & H. LINEWEAVER. *J. Food Sci.* **35,** 695–698 (1970)–Frozen whole eggs suitable for scrambling were prepared by the separate and combined use of additives and mechanical treatments. Various combinations of liquid skim milk, low levels of salt, sucrose and dextrose, and homogenization before freezing were tried. Products were developed that had low viscosity, undetectable sweetness and good appearance.

COMPARISON OF FROZEN, FOAM-SPRAY-DRIED, FREEZE-DRIED, AND SPRAY-DRIED EGGS. 7. Soft Meringues Prepared With a Carrageenan Stabilizer. K. J. MORGAN, K. FUNK & M. E. ZABIK. *J. Food Sci.* **35,** 699–701 (1970)–This study compared the quality characteristics of soft meringues prepared from frozen, foam-spray-, freeze- and spray-dried albumen. All meringues contained a carrageenan stabilizer. Ranked in order of increased average whipping times were meringues prepared from frozen, freeze-, foam-spray- and spray-dried albumen. No significant differences were found in objective measurements of drainage, evaporation, tenderness, color and height. Sensory evaluations showed no significant differences for most of the attributes evaluated. This study also compared soft meringues prepared from spray-dried albumen with and without carrageenan stabilizer. The data suggest the stabilizer decreases drainage and may improve the tenderness of meringues which are held for 20–22 hr of refrigerated storage.

A METHOD FOR THE DETERMINATION OF 5-HYDROXYMETHYL-2-FURALDEHYDE IN THE PRESENCE OF 2-FURALDEHYDE (FURFURAL). R. O. B. WIJESEKERA & C. O. CHICHESTER. *J. Food Sci.* **35,** 702–––– (1970)–A simple technique for the simultaneous assay of both 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde (5-HMF) was developed which overcomes the interference of 2-furaldehyde with the spectrophotometric measurements. After conversion of the two aldehydes to their 2,4-dinitrophenylhydrazones, followed by extraction and chromatography on silica gel plates, the respective spot areas can be measured and concentrations easily determined.

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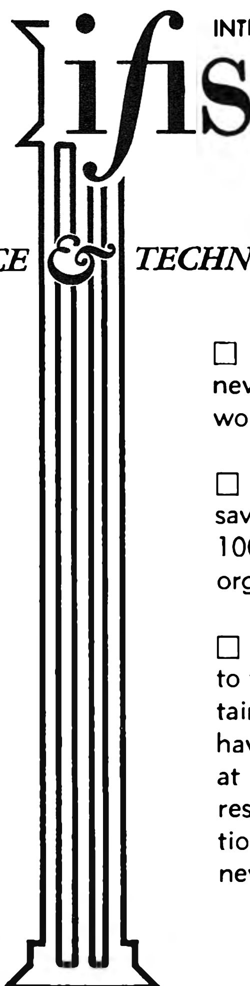

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S. J. KAZENIAC and R. M. HALL
Campbell Institute for Food Research, Camden, New Jersey 08101

FLAVOR CHEMISTRY OF TOMATO VOLATILES

SUMMARY—38 compounds were identified in tomato volatiles. Some of these had not been reported previously. Many factors, namely, fruit maturity, the physical condition of the fruit, the type of fruit crushing, the holding time of the juice or pulp after crushing, heat and oxygen were important for the development of certain tomato volatiles. Linolenic acid was indicated to be the precursor of cis-3-hexenal *which developed during tissue maceration. In turn,* cis-3-hexenal *appeared to be the source of* trans-2-hexenal. *Some of the compounds were found to be important to tomato flavor. 2-Isobutylthiazole and* cis-3-hexenal *appeared to intensify the fresh tomato flavor notes. 2-Isobutylthiazole was also responsible for the characteristic flavor of certain tomato varieties. The quality of tomato volatiles appeared to depend on the interrelationships of both enzymic and chemical reactions that occurred during the preparative treatments used to isolate the volatiles.*

INTRODUCTION

ACETALDEHYDE and ethanol were first reported in tomato volatiles by Gustafson (1934). Rakitin (1945) studied the changes in ethanol and acetaldehyde during tomato ripening. In the first important investigation of tomato volatiles, Spencer et al. (1954) identified acetaldehyde as the main carbonyl constituent along with isovaleraldehyde. Citral, citronellal and vanillin were suspected. Numerous other carbonyl compounds in minor amounts were also isolated though not identified. Acids, alcohols and esters were partially characterized, but the carbonyl compounds were found to constitute the largest class. Jacquin et al. (1955) reported that methanol was produced in tomatoes by demethylation of pectins via pectin esterase.

Acetone and furfuraldehyde were tentatively identified by Matthews (1961). Schormüller et al. (1962) found n-hexanal and 2-hexenal. Hein et al. (1963) presented evidence for the presence of diacetyl, citral, citronellal, alpha-pinene and limonene. Schormüller et al. (1965a) followed their earlier report with glyoxal, methylglyoxal, cinnamaldehyde and phenylpropanal in addition to the carbonyl compounds previously found in fresh tomato juice. Diacetyl, 2-butanone, 2-pentanone and 6-methyl-5-hepten-2-one were identified as important aroma substances in stored tomato juice. Several alcohols, namely, ethanol, butanol, isobutanol, isopentanol, *n*-pentanol, and *n*-hexanol, were also reported by Schormüller et al. (1965).

In an investigation evidently paralleling that of Schormüller et al. (1965a), Pyne et al. (1965) reported several new compounds in addition to many of those reported by Schormüller et al. The new compounds were ethyl acetate, *cis*-3-hexen-1-ol, 2-methylbutanol, n-propanol, 2 propanol benzaldehyde and methyl salicylate. Miers (1966) identified dimethyl sulfide and hydrogen sulfide in canned tomatoes and canned tomato juice and suggested that these compounds contributed to "cooked" tomato flavor.

Ryder (1966) reported 2,3-pentanedione, methional, phenylacetaldehyde, phenylethanol, pyridine, 2-methylpyrazine and 2,6-dimethylpyrazine in steam volatiles of tomatoes.

Giannone et al. (1967) found that several of the lower-boiling compounds varied widely in the juices from 10 different varieties of tomatoes. Propanal and ethyl propionate were 2 new compounds reported. Katayama et al. (1967) reported that the volatile compounds in juices from 4 varieties of tomatoes were about the same qualitatively but that there were some characteristic differences in the quantitative composition among the varieties. About 30 compounds were analyzed, 13 identified. Several of these were esters with methyl formate, propyl acetate and isoamyl acetate as new compounds. Johnson et al. (1968) reported that the amounts of iso- and active-amyl alcohols, n-pentanol and *cis*-3-hexen-1-ol varied with the variety of tomato and with the date of harvest. The 4 alcohols were the only volatiles examined.

Dalal et al. (1968) reported several new compounds, namely, isopentyl acetate, isopentyl butyrate, isopentyl isovalerate, butyl hexanoate and hexyl hexanoate. These volatiles were isolated from tomatoes canned and stored frozen prior to direct benzene extraction of juice made from the frozen tomatoes.

Evidence presented by Stevens (1968) indicated there were heritable differences in the concentration of eugenol, methyl salicylate and an unknown heterocyclic nitrogen compound in 2 tomato varieties, Campbell 146 and Campbell 1327. The flavor thresholds of these compounds indicated that they might be a factor in flavor differences between these varieties. Citral, 6-methyl-5-hepten-2-one and 6,10-dimethyl-5,9-undecadien-2-one also differed quantitatively among certain varieties. Buttery et al. (1968) reported 2,4-decadienal and 2-heptenal as well as eugenol and 6,10-dimethyl-5,9-undecadien-2-one as higher molecular weight compounds in tomato volatiles.

Nelson et al. (1969) indicated there were some quantitative differences in acetone, acetaldehyde, hexanal, isovaleraldehyde, methanol and ethanol among 3 different varieties of unprocessed tomatoes. No dimethyl sulfide was found in unprocessed tomatoes, but this compound showed the greatest increase in processed tomato juice. Methyl acetate was identified as a new compound; 2-hexenol and tert.-butanol were tentatively identified.

Shah et al. (1969) reported linalyl acetate and citronellyl and geranyl butyrates. Tentatively identified were 3-pentanol, *n*-nonanal, *n*-decanal, *n*-dodecanal and geranyl and citronellyl acetates. Quantitative differences in certain volatiles between artificially and field-ripened tomatoes also were indicated.

In a very extensive investigation, Viani et al. (1969) reported 46 compounds in tomato volatiles, among these a large number not previously reported. In this group were propionic acid, isovaleric acid, 3-methylbutyric acid, caprylic acid, γ-butyrolactone, γ-caprolactone, γ-octalactone, γ-nonalactone, 2-hydroxy-2,6,6-trimethylcyclohexylidene acetic acid γ-lactone, 1-penten-3-ol, benzyl alcohol, phenethyl alcohol, linalool, phenol, o-cresol, guaiacol, p-vinylguaiacol p-ethylphenol, o-hydroxyacetophenone, 2-hydroxy-3-butanone, 2-hydroxy-2,6,6-trimethylcyclohexanone, 6-methyl-*trans*-3,5-heptadien-2-one, β-ionone, 5,6-epoxyionone, *cis* 3-hexenal, *trans*-2,*trans*-4-hexadienal, *trans*-2,*cis*-4-heptadienal,

trans-2,*trans*-4-heptadienal, salicylaldehyde, phenylacetonitrile and 2-isobutylthiazole. The latter compound was described as having a strong green odor resembling that of tomato leaf.

In all these investigations on tomato volatiles aldehydes, ketones and alcohols occurred consistently. Where quantitative data were presented, large variations were found among many of the aldehydes and alcohols among different varieties. This paper reports on the isolation of some previously unidentified compounds in tomato volatiles, on factors related to their development and on the flavors of some of these compounds.

EXPERIMENTAL

Isolation and identification of volatile compounds

Fresh, firm, vine-ripened tomatoes of Campbell Variety 146 or Variety 1327 were washed and blended in a Waring Blendor at full speed for 1 min in 3-kg batches. The blended tomatoes or homogenates were transferred immediately to a distillation apparatus and steam distilled atmospherically. After numerous experiments, it was found that 85–90% of the total volatiles were recovered in the first 300 ml of distillate. Distillation of a 2nd 300-ml fraction, or a total of 600 ml, gave recoveries between 90 and 95%, but this 2nd fraction had strongly cooked tomato notes, lacking the fresh notes. Therefore, only the 1st 300-ml fractions were collected and used for preparing concentrates. Spencer et al. (1954) reported that 25% of the water, vacuum distilled from tomato juice, contained almost all the volatile compounds. Several batches of these fractions were combined, saturated with sodium chloride and extracted several times with small portions of chloroform. The total volume of solvent used was approximately equal to half the volume of distillate extracted. The chloroform extracts were dried with anhydrous sodium sulfate and the chloroform removed under vacuum to give about 2500-fold concentrates of volatiles. Chloroform was inefficient in extracting the polar, low-boiling compounds, but these were largely lost during vacuum concentration. The low-boiling compounds were isolated from aqueous concentrates, prepared by redistillation of steam distillates. Chloroform was far from an ideal solvent, but it was effective in removing the higher-boiling compounds. The concentrates from the chloroform extractions were separated with an Aerograph Model 90-P3 gas chromatograph using a 5-ft by 3/8-in. column containing 20% diethyleneglycolsuccinate on acid-washed, silanized 60–80-mesh Chromosorb W. The detector was a heated filament-type, thermal conductivity cell. Further preparative separations of the crude fractions from the DEGS column were made until the compound was found to be pure chromatographically on at least 2 columns with different polar properties. Other columns used were 20% Carbowax 20M and 20% silicone gum rubber S.E.30 on the same solid support as the DEGS column. Analytical purity of the compounds was checked either on a Perkin-Elmer Model 154D or an Aerograph 204 gas chromatograph. The columns used with the Perkin-Elmer instrument were 2-m by 1/4-in. coated with 15% DEGS on Chromosorb W and 15% Carbowax 1500 on Chromosorb W. Columns used on the Aerograph Model 204 gas chromatograph were 10-ft by 1/8-in. with 10% DEGS and 10% FFAP. Each compound shown in Table 12 had the same retention time as the authentic compound on at least 2 of the columns mentioned above.

Infrared spectra were determined with a Perkin-Elmer Model 221 Spectrophotometer equipped with a 4× KBr lens-type beam condenser, when the amount of sample was small. Sodium chloride micro cavity cells of 0.10-mm nominal path length were used. Spectra were obtained on pure material or in spectrograde carbon tetrachloride solution. The infrared spectra of the compounds isolated from tomatoes were compared with the appropriate known reference in all cases, except for *cis*-3-penten-1-ol. This spectrum was interpreted by comparison with the spectrum of *cis*-3-hexen-1-ol.

The 2,4-dinitrophenylhydrazine derivatives were prepared according to the procedure of Veibel (1954). The recrystallized derivative of the unknown was identical to that of the reference compound by melting point and light absorption in all cases. Attempts to prepare the derivative of *cis*-3-hexenal by this procedure gave the 2,4-dinitrophenylhydrazone of *trans*-2-hexenal. By using a dilute ethanolic solution of 2,4-dinitrophenylhydrazine containing acetic acid to a pH of 4, it was possible to prepare the *cis*-3-hexenal derivative with melting point of 100–101°C, identical to that reported by Winter et al., 1962b. Tomato volatiles with high amounts of *cis*-3-hexenal were obtained by rapidly stripping freshly prepared tomato homogenates by vacuum steam distillation. The distillates were extracted with chloroform. The chloroform extract was concentrated under vacuum and *cis*-3-hexenal was isolated by gas chromatography on a DEGS column, followed by rechromatography on an S.E. 30 column.

Isolation and identification of 2-isobutylthiazole

2-Isobutylthiazole was collected from a DEGS column along with a small amount of 6-methyl-5-hepten-2-ol. Rechromatography of this fraction on an S.E. 30 column gave good separation of the alcohol and thiazole. Synthetic 2-isobutylthiazole was prepared according to Didier et al. (1961) from chloroacetaldehyde and 3-methylbutanthioamide. The latter was also prepared according to Didier et al. (1961). Both the synthetic 2-isobutylthiazole and the compound isolated from tomato volatiles had identical retention times on DEGS, Carbowax 20M and S.E. 30 columns. The ultraviolet absorption of both compounds was the same with a molecular extinction coefficient of 5,050 at 237–238 mμ in absolute ethanol. The infrared spectra were also identical (Fig. 6).

The NMR spectrum of 2-isobutylthiazole had a doublet at 1.0 ppm, a complex multiplet at 2.1 ppm, a doublet at 2.9 ppm, a doublet at 7.2 ppm and a doublet at 7.7 ppm. The relative intensities of these peaks were 6, 1, 2, 1 and 1 hydrogen, respectively. The upfield assignments were 6 methyl hydrogens, 1 methine hydrogen and 2 methylene hydrogens, characteristic for the isobutyl group. The 2 downfield doublets were identical to the aromatic hydrogen substi-

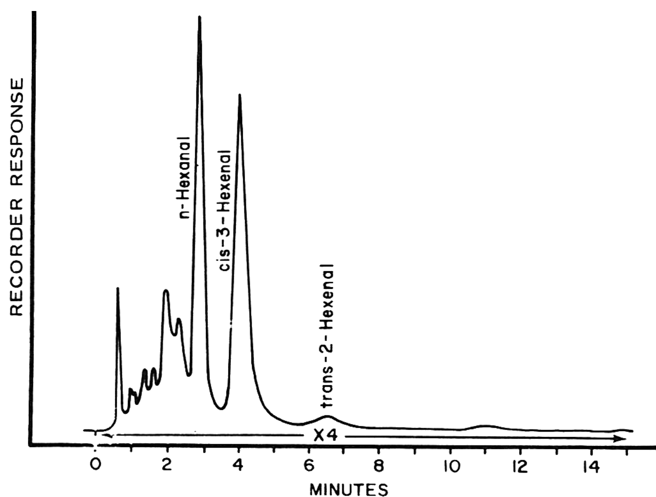

Fig. 1–Chromatogram of vapors over crushed ripe tomatoes, 10 min after blending. Column, 10 ft by 1/8 in., 10% FFAP on silanized Chromosorb W at 100°C isothermal. Nitrogen flow: 40 ml/min; sample size: 2.0 ml.

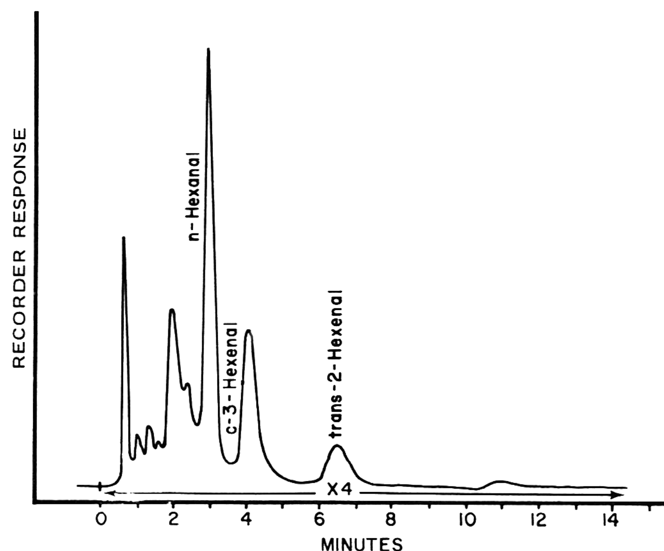

Fig. 2–Chromatogram of vapors over same sample of tomatoes as Figure 1, except 20 min after blending. Same GLC conditions.

Table 1–Effect of holding blended tomatoes at 40°C on aldehydes.

Holding time (min)	Peak areas in mm^2			Total hexenals
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal	
10	66	26	10	36
15	74	24	13	37
20	83	26	18	44
30	88	18	23	41
70	69	6	33	39

Table 3–Chemical conversion of cis-3-hexenal to trans-2-hexenal by tomato juice.

	Recovery of trans-2-hexenal	
	In first 25 ml distillate estimated by GLC	In total 250 ml distillate estimated by UV
	(mg)	
Control juice	Trace	0.95
Juice with 6.8 mg *cis*-3-hexenal added	3.25	4.9

Table 2–Effect of holding sliced tomatoes at 30–35°C on aldehydes.

Holding time (min)	Peak areas in mm^2			Total hexenals
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal	
0	84	35	11	46
20	52	24	9	33
40	23	17	5	22
60	16	14	3	17

tution pattern for 2-alkyl thiazoles. These absorption characteristics agree closely with the data of Cottet et al. (1967). The synthetic and the unknown compounds had identical NMR spectra.

The mass spectrum of 2-isobutylthiazole had a parent peak of 141 with a P+1 peak of 12.8% and a P+2 peak of 4.8% of the intensity of the parent peak. Losses of hydrogen and methyl fragments produced substantial peaks at m/e 140 and m/e 126. The intense base peak at m/e 99 indicates a resonance stabilized species after fragmentation of the bond beta to the ring (Fig. 5).

Vapor analysis

Tomatoes were blended at high speed for 1 min in a Waring Blendor and the homogenate immediately transferred to a 130-ml screw-capped jar equipped with a small rubber septum. A gas-tight syringe of 2-ml capacity was used for withdrawing the vapor sample. Where no times are specified in the tables, the blended tomato samples were held in a water bath at 40°C for 10 min, before sampling the headspace vapor. The vapor samples were injected into the gas chromatograph with as little delay as possible. Samples were chromatographed isothermally either on an Aerograph Model 204 or Perkin-Elmer Model 154-D instrument with a hydrogen flame detector.

Large quantitative variations in the volatile compounds between individual tomatoes within the same variety created serious problems in reproducibility of results. 2 approaches were used to minimize these errors. 1 method was to blend large samples of several hundred grams and take the appropriate aliquot from this large sample. The other procedure was to section individual tomatoes and use a section from each tomato to make up a composite sample. Results in the tables are reported as gas-chromatographic peak areas, since many of the experiments were made with different batches of tomatoes from different locations and harvest dates. Peak areas also differed from 1 instrument to the other.

Chemical conversion of cis-3-hexenal to trans-2-hexenal

6.8 mg chromatographically pure *cis*-3-hexenal were added to 2500 g of tomato juice enzyme inactivated by heat. This sample was steam distilled and a fraction of 25-ml distillate collected. Steam distillation was continued until a second fraction of 225 ml was collected, for a total of 250 ml. The same procedure was repeated using an aliquot from the composite sample of tomato juice without any added *cis*-3-hexenal. The 1st 25-ml fractions of distillates from both samples were analyzed by direct injection of the aqueous distillates into the gas chromatograph and also by ultraviolet absorption. The 2nd 225-ml fractions were analyzed by ultraviolet absorption only, because concentrations were too low to be detectable by gas chromatography. During this investigation it was found that around 90% of the ultraviolet absorption at 226 mμ of aqueous solutions of tomato volatiles was due to *trans*-2-hexenal. This conjugated monoenal has a molecular coefficient extinction of 18,200 at 226 mμ in water. Thus, the ultraviolet absorption served as a confirmatory test for *trans*-2-hexenal.

Flavor evaluations

The duo-trio test was used. The chromatographically pure compound was added to processed tomato juice or to a tomato paste diluted to solids approximately in the range found in juice. The whole juice or diluted paste without the added compound was used as a reference sample. The reference sample was identified and the taster asked to indicate which of the 2 unidentified samples was identical to the reference. The taster was not requested to state a flavor preference, but asked to describe the flavor of the sample he thought was different. The flavor panel consisted of 6 members screened for their ability to detect flavor differences in tomato juice. Half of the panel members had extensive experience in tasting tomato products. Significance at the 0.1% probability level was found for all the compounds reported except methyl salicylate, *n*-hexanol and 6-methyl-5-hepten-2-ol. These compounds had flavor differences significant at the 5% probability level.

RESULTS & DISCUSSION

Development of aldehydes

Volatiles can exist per se in the whole intact tomatoes or they can develop as artifacts. Many volatile compounds in tomatoes apparently exist in dynamic systems highly dependent on the treatment the tomato receives during its preparation for consumption or analysis. Differentiation between the 2 types is difficult. Figure 1 represents a gas chromatogram of the volatiles over crushed ripe tomatoes taken 10 min after blending. 2 of the major compounds formed were *cis*-3-hexenal and *n*-hexanal. The *cis*-3-hexenal was unstable both to heat and to the acidic pulp and juice and was isomerized to *trans*-2-hexenal upon standing or heating. A 3rd major peak appeared to be acetaldehyde. The decrease in *cis*-3-hexenal and the increase in *trans*-2-hexenal after 20 min of holding can be seen in Figure 2.

The conversion of *cis*-3-hexenal to *trans*-2-hexenal in freshly blended tomatoes upon standing is shown in Table 1. Total amounts of the 2 hexenals remained fairly constant during the whole holding period. *trans*-2-Hexenal can be readily recovered from ground, fresh tomatoes by distillation techniques. It is also possible to get *cis*-3-hexenal, but the isolation techniques require minimal heating and holding after grinding or blending. The positive identification of *cis*-3-hexenal was made through retention time data, infrared spectra and the 2,4-dinitrophenylhydrazone. If sliced tomatoes rather than blended tomatoes were held, the quantities of the aldehydes decreased rapidly in contrast to the blended samples (Table 2). We are presently trying to establish with ^{14}C-labeled compounds whether the aldehydes are converted to alcohols under these conditions.

Results of experiments designed to show the chemical conversion of *cis*-3-hexenal to *trans*-2-hexenal are given in Table 3. When *cis*-3-hexenal was added to processed tomato juice with no enzymic activity and distilled, *trans*-2-hexenal was recovered in high amounts in the distillate compared to the quantities found in the control sample. The unidentified compound suggested as being *cis*-3-hexenal by Pyne et al. (1965) probably was this compound. Their method of isolation should have resulted in the recovery of the large amounts of *cis*-3-hexenal and small amounts of *trans*-2-hexenal indi-

Table 4–Effect of oxygen on development of aldehydes in blended tomatoes.

	Peak areas in mm^2		
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal
Under air	264	200	13
Under nitrogen	Trace	22	Trace

Table 5–Effect of heating whole intact tomatoes for 1 hr at 65°C on development of volatiles.

	Peak areas in mm^2		
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal
Control–no heat	60	53	6
Heated tomatoes	3	2	Nil

cated on their chromatograms. Winter et al. (1962a) pointed out the possibility that aldehydes with a double bond in the *beta*-position such as 3-hexenal could undergo isomerization to the conjugated compound, namely, 2-hexenal. There was no evidence of any *cis*-2-hexenal which can form in chemical oxidations of unsaturated fatty acids (Duin et al., 1967). Separation of *cis*-2-hexenal from the *trans*-2-isomer is possible by gas chromatography.

Several factors were found to be important to production of *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal. Table 4 shows the effects of oxygen upon development of *cis*-3-hexenal and *n*-hexanal. When fresh tomatoes were blended under nitrogen in the absence of oxygen, *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal were not produced. Blending under nitrogen was done in a Waring Blendor with a specially constructed cover. This cover was air-tight and had connections that permitted exhaustion of the gases in the Blendor. Whole intact tomatoes were placed in the Blendor and the cover sealed air-tight. The Blendor was evacuated and then flooded with nitrogen. This evacuation and flooding procedure was repeated several times to ensure complete removal of air. When the vapors in the Blendor were oxygen-free by gas chromatography, the tomatoes were blended at the high speed for 1 min. Vapor samples were taken with a syringe through a rubber septum and analyzed with an Aerograph Model A-204 or Perkin-Elmer Model 154-D instrument with hydrogen flame detectors.

Schwimmer (1963) has reported that distinctly weaker odors resulted when string beans were treated with stringbean enzymes in vacuo than when under air. Nye et al. (1943) found that finely ground leaves of *Ailanthus glandulosa*, exposed to oxygen, gave the highest yields of 2-hexenal. An enzymic process was probable, since heat treatment of the leaves, prior to oxygen exposure, limited the amount of 2-hexenal formed. Similar evidence for the enzymic formation of 2-hexenal and *n*-hexanal in apples has been presented by Drawert et al. (1966). In cucumbers, 2,6-nonadienal and 2-nonenal, besides a trace amount of 2-hexenal were indicated to result enzymically upon cutting or mechanical rupture of the vegetable (Fleming et al., 1968).

Table 6–Effect of linolenic acid on development of aldehydes in enzyme-inactivated blended tomatoes.

	Peak areas in mm^2			
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal	Total hexenals
Control–no heat	141	38	15	53
Control–no heat with 750 ppm linolenic acid	120	72	21	93
Heated sample	38	Trace	24	24
Heated sample with 750 ppm linolenic acid	40	Trace	29	29

Table 5 shows the effects of enzyme inactivation by heat on development of *cis*-3-hexenal and *n*-hexanal. When whole ripe tomatoes with intact skins were heated in water at 65°C for 60 min, before blending, the aldehydes were formed in much smaller amounts. Such heating treatments were inefficient because of poor heat penetration through the whole tomato. Heating at higher temperatures caused excessive cracking and skin rupture. When the blended ripe tomatoes were heated at 85°C for 10 min and linolenic acid then added, no *cis*-3-hexenal was detectable and only slight increases in *trans*-2-hexenal. Heat would have converted *cis*-3-hexenal to *trans*-2-hexenal. These results, along with the changes in the aldehydes of nonheated controls, are presented in Table 6. Addition of linolenic acid to a tomato homogenate containing the active enzyme nearly doubled the amounts of the total hexenals. The reason for the somewhat lower quantities of *n*-hexanal caused by addition of linolenic acid is not clear. The data of Table 6 also indicate that linolenic acid was the probable precursor of *cis*-3-hexenal in tomatoes produced by enzymic reactions. Hoffmann (1961) isolated *cis*-3-hexenal from chemically oxidized soybean oil which contained linolenic acid. There were no indications in our experiments that *cis*-3-hexenal was formed chemically.

Table 7 shows how the addition of hydrogen peroxide inhibited the development of *cis*-3-hexenal and, therefore, *trans*-2-hexenal. The development of *cis*-3-hexenal and *n*-hexanal proceeded so rapidly it was difficult to inhibit the reaction completely. When whole tomatoes were blended slowly in aqueous solutions of hydrogen peroxide, the inactivation became more effective. Hydrogen peroxide is known to oxidize lipoxidases readily and inactivate them (Mitsuda et al., 1967).

Slight increases in *n*-hexanal were obtained by adding linoleic acid to tomatoes during blending. The amount of *n*-hexanal developed relative to the linoleic acid used was rather small. The same was true for *cis*-3-hexenal from linolenic acid. The reason for this was not determined. When linolenic acid was added in aqueous solutions containing sufficient ethyl alcohol to solubilize the linolenic acid, the reaction proceeded at a greater rate. Inadequate dispersion of the fatty substrate may have been involved (Tappel et al., 1952).

Table 7–Effect of hydrogen peroxide on development of aldehydes in blended tomatoes.

	Peak areas in mm^2		
	n-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal
Control	354	385	10
With 1,000 ppm hydrogen peroxide	49	66	4

Table 8–Effect of types of tomato crushing on development of trans-2-hexenal and methanol.

	(ppm)	
Treatment	*trans*-2-Hexenal	Methanol
Hand sliced	0.6	54
Coarsely diced	1.6	88
Blended 1 min at low speed	3.7	88
Blended 1 min at high speed	4.9	125

Freezing whole tomatoes in a blast freezer at −20°C or with dry ice or in liquid nitrogen affected the development of *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal. The −20°C samples, or "slow" freezing, gave practically none of these aldehydes upon thawing and blending; the dry-ice samples, very small amounts; the liquid nitrogen samples, somewhat higher quantities but still much lower than fresh tomatoes. Otherwise, the chromatograms showed patterns of volatiles qualitatively very similar to those of fresh tomatoes. Possible quantitative differences were indicated in somewhat higher amounts of alcohols, including *cis*-3-hexen-1-ol. Furfural and phenylacetaldehyde were also higher. Accurate quantitative comparisons were difficult, because of problems in fruit uniformity. Our samples were analyzed within 48 hr after freezing. Yu et al. (1968a) reported that an enzyme preparation extracted from tomatoes held frozen approximately 1 month at −20°C showed no activity towards leucine. Similar enzyme preparations prepared by them from fresh tomatoes formed 3-methylbutan-1-ol and 3-methylbutanal.

Tables 8 and 9 show how dependent the amounts of certain volatiles were on the method used to crush or macerate the tomatoes. The coarser degree of tissue rupture, the lower the amounts of aldehyde formed. Of the various procedures tried, grinding fresh tomatoes for 1 min at the high speed of a Waring Blendor produced the highest amounts of *trans*-2-hexenal and methanol in the volatiles. Because the amounts of *cis*-3-hexenal and *n*-hexanal developed by some of the treatments were too low to determine by gas chromatography, these samples were distilled and ultraviolet absorption used to determine the amounts of *trans*-2-hexenal and, therefore, indirectly, *cis*-3-hexenal. That small amounts of *trans*-2-hexenal and *n*-hexanal were developed by the coarser crushing was also confirmed by gas-chromatographic analyses of chloroform extracts of distillates of many of these samples. There were no indications that such a procedure seriously affected any of the other volatiles. Winter et al. (1962a) indicated that the amount of 2-hexenal that developed in raspberries depended on the oxygen concentration in the atmosphere at the time of crushing. Dolev et al. (1967b) showed that the oxygen introduced into the hydroperoxide molecules in lipoxidase reactions came from the gaseous phase and not from the aqueous phase. The more intimate mixing of air with the tomato particles by blending probably was the reason for the higher amounts of *trans*-2-hexenal developed in our blended sample. The maturity stage of the tomatoes was important to the amounts of *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal that developed. Rhodes et al. (1967) reported that lipids, lipases and lipoxidases were highly variable during the ripening stages of apples, reaching the highest amounts at the climacteric. Kapp (1966) found that total lipids varied with fruit maturity at harvest as well as with variety and storage treatments in tomato fruit pericarp. Linolenic, linoleic, oleic, stearic, palmitic and myristic acids increased during the period of greatest color development. Results in Table 10 show that the highest quantities of *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal were obtained from firm, fully ripe tomatoes. In Table 11 are presented data obtained by adding linolenic acid to tomatoes of varying degrees of ripeness. The enzyme(s) appeared to be at least 1 of the limiting factors in the development of *cis*-3-hexenal in green tomatoes.

Table 12 lists the compounds identified in tomato volatiles obtained by steam distillation of blended, fresh, ripe tomatoes. Figure 3 represents a gas chromatogram of the compounds isolated from an aqueous concentrate of tomato volatiles. Figure 4 shows a gas chromatogram of a concentrate of tomato volatiles, obtained as a chloroform extract of a steam distillate of blended, fresh, ripe tomatoes. The peak numbers on the chromatograms correspond to the peak numbers and identified compounds as listed in Table 12. As can be seen from Table 12, there were several other carbonyl compounds identified in tomato volatiles which could originate either from chemical or enzymic oxidation of lipids. Grosch (1967) reported that lipids isolated from fresh peas reacted with enzyme preparations, also obtained from peas, to produce a wide range of carbonyl compounds. In this group were acetaldehyde, propanal, *n*-butanal, *n*-pentanal, *n*-hexanal, *n*-nonanal, 2-butenal, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, 2-decenal, 2-undecenal, 2,4-heptadienal, 2,4-nonadienal, 2,4-decadienal and acetone. Fleming et al. (1968) indicated that 2-nonenal and 2,6-nonadienal were enzymically produced in cucumbers. Hamberg et al. (1965) reported that lipoxidases can have rather high degrees of specificity towards the oxidation of fatty acids. The diene systems starting at position 6 counted from the methyl ends and those beginning at position 9 counted from the carboxyl

Table 9—Effect of time of blending tomatoes on development of trans-2-hexenal and methanol.

Time (sec)	*trans*-2-Hexenal (ppm)	Methanol (ppm)
At high speed of Waring Blendor		
5	3.6	100
10	3.7	110
40	4.3	138
At low speed of Waring Blendor		
5	1.7	64
10	3.1	88
40	3.5	106

Table 10—Comparison of aldehydes in blended tomatoes of different stages of maturity.

	Peak areas in mm^2		
Stage of maturity	*n*-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal
Green	2	8	3
Half-ripe	50	80	4
Fully ripe	180	100	8

Table 11—Effect of linolenic acid on development of aldehydes in blended tomatoes at different stages of maturity.

	Peak areas in mm^2			
Stage of maturity	*n*-Hexanal	*cis*-3-Hexenal	*trans*-2-Hexenal	Total hexenals
Green	10	8	5	13
Green with 750 ppm linolenic acid	8	8	5	13
Half-ripe	40	19	7	26
Half-ripe with 750 ppm linolenic acid	30	28	11	39
Pink	76	32	9	41
Pink with 750 ppm linolenic acid	74	50	15	65
Fully ripe	141	38	15	53
Fully ripe with 750 ppm linolenic acid	120	72	21	93

ends are susceptible to the lipoxidase reaction. A mechanism for the reaction of linoleic acid with lipoxidase has been proposed by Dolev et al. (1967a). The enzyme formed exclusively 13-hydroperoxyoctadeca-9,11-dienoic acid, while chemical autoxidation of methyl linoleate gave equal amounts of the methyl 9- and 13-hydroperoxyoctadeca-9,11-dienoates.

Eriksson (1967) reported that lipoxidase catalyzed oxidation of *cis*-1-, *cis*-4-pentadiene systems in unsaturated fatty acids such as linoleic, linolenic and arachidonic by molecular oxygen. The hydroperoxides formed decomposed either spontaneously or catalytically in the presence of lipohydroperoxidase. Soy extracts contain specific lipohydroperoxidase, but green peas do not. Recently, Blain et al. (1968) have indicated that extracts from tomatoes exhibited reactions more characteristic of hematins than of the lipoxidase enzymes in their behavior towards carotene bleaching. Hematin catalysts have been reported to show optimal activity both for linoleate oxidation and crocin oxidation in the presence of linoleate between pH 3.5 and 4.5 (Dicks et al., 1966). Possibly, both types of enzyme systems are active in the development of volatiles in tomatoes. We found that *cis*-3-hexenal and *n*-hexanal were developed very rapidly, suggesting the spontaneous decomposition of hydroperoxides (Eriksson, 1967), and that these aldehydes were in very high amounts, indicating rather high specificity. We have estimated that about 90% of the total 2-enal and 2,4-dienal compounds in tomato volatiles was *trans*-2-hexenal. However, several other lipid-related aldehydes were found in tomato volatiles. Since hydroperoxides and free radical intermediates would be present in such systems, co-oxidation with unsaturated lipids could occur, to give the small amounts of these minor aldehydes. Similarly, carotenoids and other terpene-related compounds could be oxidized. In some of our experiments, 2,4-decadienal appeared to be lower, where oxygen was limited, suggesting chemical oxidation. It is not possible to tell whether 2-heptenal and 2,4-decadienal in the investigation of Buttery et al. (1968) were produced chemically or enzymically, because details of the sample preparations were not given. Further work is necessary to establish more definitely the origin of the carbonyl compounds and the extent of enzymic and chemical involvement in the development of volatiles in tomatoes.

Table 12–Identity of peaks on GLC chromatograms shown in Figures 3 and 4.

Peak no.	Compound	Means of identification
1	Acetaldehyde	RT, IR, 2,4-DNPH
2	2-Methylpropanal	RT, 2,4-DNPH
3	Methanol	RT, IR
4	*n*-Butanal	RT, 2,4-DNPH
5	Ethanol	RT, IR
6	3-Methylbutanal	RT, 2,4-DNPH
7	2-Methyl-3-buten-2-ol	RT, IR
8	2,3-Butanedione	RT, IR, 2,4-DNPH
9	2-Methylpropan-1-ol	RT, IR
10	*n*-Hexanal	RT, IR, 2,4-DNPH
11	*n*-Butanol	RT, IR
12	3-Methylbutan-1-ol	RT, IR
13	*trans*-2-Pentenal	RT, IR
14	*cis*-3-Hexenal	RT, IR, 2,4-DNPH
15	*n*-Pentanol	RT, IR
16	*trans*-2-Hexenal	RT, IR, 2,4-DNPH
17	*cis*-3-Penten-1-ol	IR
18	*n*-Hexanol	RT, IR
19	*cis*-3-Hexen-1-ol	RT, IR
20	6-Methyl-5-hepten-2-one	RT, IR
21	2-Isobutylthiazole	RT, IR, MS, NMR
22	1-Octen-3-ol	RT, IR
23	6-Methyl-5-hepten-2-ol	RT, IR
24	*trans*-2-Octenal	RT, IR
25	3-Methylmercaptopropanal	RT, IR
26	Furfural	RT, IR
27	Linalool	RT, IR
28	*trans*-2,*trans*-4-Heptadienal	RT, IR
29	Benzaldehyde	RT, IR
30	Phenylacetaldehyde	RT, IR
31	Geranial	RT, IR
32	Methyl salicylate	RT, IR
33	*trans*-2,*trans*-4-Decadienal	RT, IR
34	6,10-Dimethyl-5,9-undecadien-2-one	RT, IR
35	Guaiacol	RT, IR
36	Phenethyl alcohol	RT, IR
37	*beta*-Ionone	RT, IR
38	Eugenol	RT, IR

RT = agreement of known and unknown retention times on at least 2 columns with different polar properties, IR = identity of infrared spectra, MS = identity by mass spectrum, NMR = identity by nuclear magnetic resonance spectrum.

A 2nd group of aldehydes found in tomato volatiles appeared to be related to amino acids. Phenylacetaldehyde reported by Ryder (1966) and found in our investigation probably arose from phenylalanine. No details were given by Ryder on how his concentrate of tomato volatiles was obtained. However, the high amounts of phenylacetaldehyde, furfural and 3-methylmercaptopropanal shown in his gas chromatogram indicated that heat probably was a factor in the preparation. In our investigation, phenylacetaldehyde was produced at a fairly constant though low rate during the atmospheric steam stripping of volatiles from tomato homogenates or pulps.

3-Methylmercaptopropanal, also reported by Ryder (1966), has particular interest, because of its relationship to ethylene production in fruits and vegetables. Its formation could be heat-induced from methionine as shown by Ballance et al. (1961) or it could be present in raw tomatoes. Ku et al. (1967) have shown that ethylene can be produced from 3-methylmercaptopropanal either enzymically or at a much slower rate chemically with hydrogen peroxide alone. Lieberman et al. (1965) demonstrated similar results with hydrogen peroxide and ascorbic acid or cupric ions as the catalyst. Mapson et al. (1966) indicated that oxidative deamination by hydrogen peroxide and metal ions could occur with other amino acids as well as with methionine. Linolenate model systems also produced ethylene, requiring cupric ions and a strong oxidizing agent such as hydrogen peroxide or peroxidized linolenate. Earlier, Meigh (1962) had made somewhat similar studies with methionine and ethylene metabolism in tomatoes. Therefore, the presence of free 3-methylmercaptopropanal in ripe tomatoes would seem a good possibility. Its formation during the blending operation when high concentrations of hydroperoxides are present also would be probable.

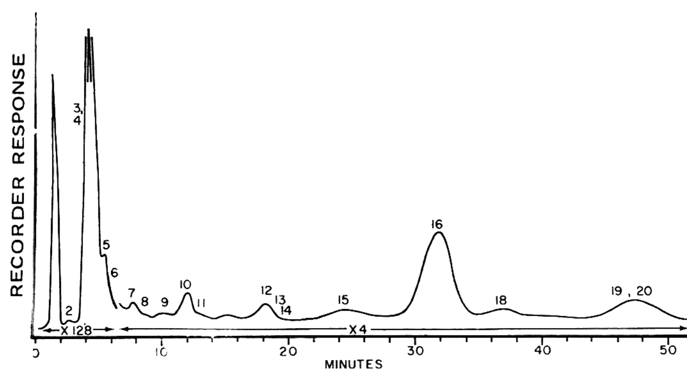

Fig. 3–Chromatogram of aqueous concentrate of steam volatiles from tomatoes. Column, 2 m by 1/4 in., 15% DEGS on Chromosorb W at 100°C isothermal. Helium flow: 20 ml/min; sample size: 1 μl.

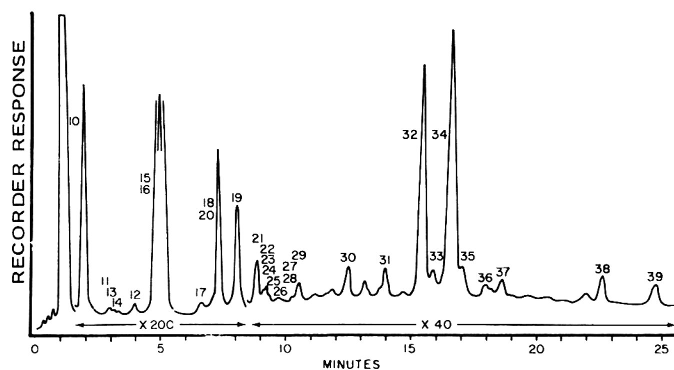

Fig. 4–Chromatogram of tomato concentrate of chloroform-extracted steam volatiles from tomatoes. Column, 10 ft by 1/8 in., 10% FFAP on silanized Chromosorb W; temperature programmed at 6°C/min from 90–200°C. Nitrogen flow: 40 ml/min; sample size: 5 μl.

Yu et al. (1968b) reported the enzymic production of 3-methylbutanal and the corresponding alcohol from ^{14}C-labeled L-leucine with crude enzyme extracts from tomatoes. *n*-Hexanal and *trans*-2-hexenal were not reported by these investigators. We found very low levels of 3-methylbutanal relative to the amounts of *trans*-2-hexenal and *n*-hexanal, as seen in Figure 3. Probably, the reason for this was the difference in the methods used in recovering the volatiles from the tomatoes. We have identified 2-methylpropanal but have not isolated 2-methylbutanal. Yu et al. (1968a) were unable to demonstrate the enzymic formation of 2-methylpropanal from valine as with 3-methylbutanal from leucine. The chemical formation of 2-methylpropanal and 3-methylbutanal from valine and isoleucine, respectively, has been shown by Casey et al. (1965).

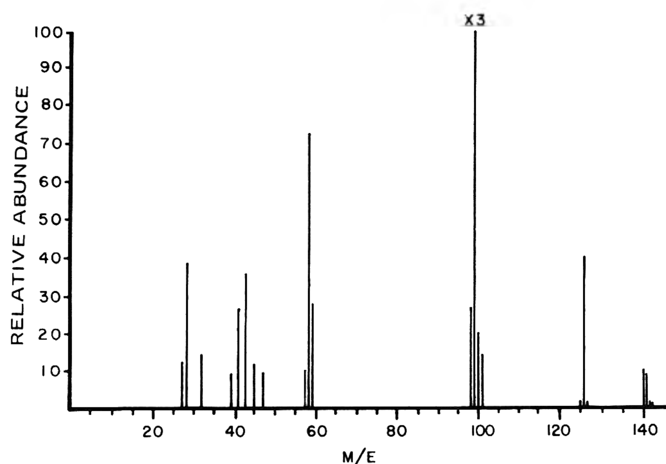

Fig. 5–Mass spectrum of 2-isobutylthiazole isolated from tomato volatiles.

Furfural, reported by Matthews (1961), Ryder (1966) and Dalal et al. (1968), was found to be temperature dependent in our experiments. Higher amounts of furfural and methanol were recovered from soft, over-ripe tomatoes, consistent with higher degrees of demethylation and furfural production. Heat was a definite factor. Jacquin et al. (1955) reported that methanol was produced in tomatoes from pectins via depolymerization and demethylation by pectic enzymes. The resulting uronic acids along with ascorbic acid would seem to be the most likely precursors for furfural.

Alcohols

Schormüller et al. (1965b) observed the aldehyde-alcohol relation in tomato volatiles; namely, that for acetaldehyde, 3-methylbutanal and *n*-hexanal there was a corresponding alcohol. They suggested that these aldehydes probably were reduced to the corresponding alcohols via diphosphopyridine nucleotide and an alcohol dehydrogenase. Meigh et al. (1966) showed that tomato tissues produced acetaldehyde, propanal and acetone enzymically from the corresponding alcohols. Eriksson (1967) reported that alcohol dehydrogenases were widely distributed in plants. With a dehydrogenase isolated from fresh peas he studied reaction rates and found that the rates for unsaturated alcohols were higher than those for the saturated alcohols except for 2-buten-1-ol. Rates for secondary, cyclic and aromatic alcohols were very low. Recently, Eriksson (1968) observed that when peas were subjected to various treatments, catabolic reactions became predominant, favoring alcohol formation. As can be seen from the compounds identified in Table 12, such a relationship between many of the aldehydes and alcohols would appear to exist in tomatoes. The equilibria between the aldehydes and alcohols, dependent on the many factors cited, control the volatile flavor or aroma of the tomato at the time it is consumed. Thus, tomato aroma can be highly variable.

We have some evidence that *cis*-3-hexen-1-ol is formed enzymically. The longer the time blended tomatoes were held, the greater the amount of *cis*-3-hexen-1-ol found. However, some small amount of *cis*-3-hexen-1-ol appeared to be present in whole fresh tomatoes. When whole ripe tomatoes were blended in the absence of oxygen, small amounts of *cis*-3-hexen-1-ol were recovered, although negligible quantities of *cis*-3-hexenal and *trans*-2-hexenal were found. Also, *cis*-3-hexen-1-ol was isolated from green tomatoes in appreciable amounts, although, again, only trace amounts of *cis*-3-hexenal and *trans*-2-hexenal were found.

Results of MacLeod et al. (1968) on cabbage volatiles and Ralls et al. (1965) on green-pea volatiles show similar aldehyde-alcohol relations. In our experiments, *trans*-2-hexen-1-ol appeared to be absent, despite the high levels of *trans*-2-hexenal found and the special attention devoted to its detection. This also seems to be the case with the volatiles in

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Table 13–Effect of temperature on development of methanol in sliced tomatoes held 20 min.

Temperature (°C)	Methanol (ppm)
25	116
40	138
50	183
60	204
68	216
75	210

Table 14–Amounts of 2-isobutylthiazole in different varieties of tomatoes.

Variety	Range (ppm)	Average (ppm)
Campbell 146	0.16–0.26	0.19
Campbell Accession No. 1306	0.06–0.11	0.09
Campbell Accession No. 1383	0.06–0.09	0.08
Campbell Accession No. 1402	0.07–0.08	0.07
Campbell 1327	0.05–0.08	0.06

Results represent a minimum of at least 3 different harvests of tomatoes during the 1963 season in the Camden, New Jersey, area.

cabbage (MacLeod et al., 1968) and in peas (Ralls et al., 1965). More recently, Murray et al. (1968) reported the tentative identification of *trans*-2-hexen-1-ol in unblanched frozen peas. This investigation was directed towards alcohols only and no other volatiles were reported. The use of unblanched peas, stored 6–8 months at −17.8°C might have been a factor. Nelson et al. (1969) have indicated the tentative identification of this alcohol in tomatoes. Their isolation procedure of direct extraction of macerated tomatoes with paraffin oil would appear to give a somewhat different pattern of volatiles than that found with distillation methods. Compounds such as *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexen-1-ol, prominent in our tomato volatiles, were absent in their analyses. Eriksson (1968) found that the equilibrium constants for *trans*-2-hexenal and *trans*-2-hexen-1-ol very strongly favored the aldehyde over the alcohol. *trans*-2-Hexen-1-ol has been reported in several fruits, namely, in apples (Flath et al., 1967); grapes (Stevens et al., 1966); peaches (Sevenants et al., 1966); apricots (Jennings et al., 1966). Possibly, the alcohol dehydrogenases vary among foods. Eriksson (1968) indicated that the alcohol dehydrogenase from yeast had notably different rates of activity towards some of the alcohols than the enzyme extracted from peas.

Mechanisms for the formation of 1-octen-3-ol from linoleic acid have been proposed (Hoffmann, 1962). This so-called mushroom alcohol has been reported in peas (Murray et al., 1968); snap beans (Stevens et al., 1967); black currants (Andersson et al., 1964); cranberries (Anjou et al., 1967). 1-Octen-3-ol was considered important in snap-bean flavor and in flavor differences between varieties (Stevens et al., 1967).

The amounts of ethanol in tomato volatiles were rather low, when fresh, firm ripe tomatoes were handled quickly. Ethanol was also low in the volatiles isolated by Pyne et al. (1965). The very high amounts reported by Katayama et al. (1967) in fresh tomato juice are difficult to understand. Possibly, ethanol was produced from the large amounts of acetaldehyde (Eriksson, 1968). Nelson et al. (1969) reported that acetaldehyde decreased while ethanol sharply increased in canned tomato juice of the Rutgers variety. They suggested the reducing atmosphere in the container might be the reason.

Tables 8 and 9 show that the amounts of methanol increased when the tomatoes were more finely chopped or blended. Holding tomato homogenates without enzyme inactivation also gave increased quantities of methanol. Heat also had a great effect on the amounts formed (Table 13). As indicated by Jacquin et al. (1955) and noted by Pyne et al. (1965), methanol most likely came from the demethylation of pectic substances. Methanol was determined gas chromatographically by direct injection of the aqueous distillates.

It appears that alcohols potentially related to amino acids, for example, 3-methylbutan-1-ol, 2-methylpropan-1-ol and phenethyl alcohol probably were present in the tomato. Yu et al. (1968b) have reported that 3-methylbutan-1-ol was produced enzymically from leucine with tomato extracts. Although the quantities of 3-methylbutan-1-ol and 2-methylpropan-1-ol were somewhat variable within samples of the same variety of tomatoes, the variations were much less than those found with *cis*-3-hexen-1-ol, for example. The amounts of 3-methylbutan-1-ol and 2-methylpropan-1-ol seemed more dependent on the physical condition of the fruit rather than the blending or grinding and isolation treatments. In general, the poorer the physical condition of the fruit, the higher the relative quantities recovered. In contrast to this, increases in the amounts of lipid-related alcohols, such as *cis*-3-hexen-1-ol and *n*-hexanol, at the expense of the corresponding aldehydes, were especially noticeable, if ground tomatoes were held without inactivation of the enzymes. With the lipid-related compounds, the quantities of aldehydes were higher than those of the corresponding alcohols. With 3-methylbutan-1-ol, the opposite was true. Therefore, 3-methylbutan-1-ol was either present in tomatoes or the enzymic formation of the alcohol was favored over the aldehyde.

Identification of 2-methylpropan-1-ol, 2-methylbutan-1-ol and 3-methylbutan-1-ol by Pyne et al. (1965) supports the idea that these alcohols were present in tomatoes. Furthermore, Eriksson (1968) indicated that methyl branching in a compound, such as 3-methylbutan-1-ol, gave low conversion rate of alcohols to aldehydes. The method for isolating volatiles, used by Pyne et al. (1965), minimized both enzymic reactions and chemical degradations.

Phenethyl alcohol also reported by Ryder (1966) seemed to follow a similar pattern. In our experiments, this alcohol was low and fairly constant, even in those experiments where phenylacetaldehyde was high. There was no evidence of benzyl alcohol in the volatiles, although Viani et al. (1969) have reported it. Possibly, the formation of both these alcohols was related to the low activity of alcohol dehydrogenase towards aromatic compounds (Eriksson, 1967), or benzyl alcohol was an artifact arising during distillation.

2-Isobutylthiazole

Even less is known about some of the other compounds that appeared to be present in tomatoes per se and were not developed by any treatment of the tomatoes. An important flavor compound in this group was 2-isobutylthiazole. The identity of this compound was rigorously established through comparisons of the unknown with synthetic material by mass, infrared, ultraviolet and nuclear magnetic resonance spectra. The amounts of 2-isobutylthiazole did not appear to be dependent on the crushing procedure or on oxygen. However, the concentrations of 2-isobutylthiazole varied widely from 1 variety of tomato to another, as can be seen in Table 14. 4,5-Substituted thiazoles are well known in thiamine biogenesis, but their biosynthesis is not yet clear. Methionine has been suggested as the sulfur precursor of the thiazole in thiamine (Johnson et al., 1966). The occurrence of this compound in tomato has also been observed by Viani et al. recently (1969).

Lignin-related compounds

The presence of several other com-

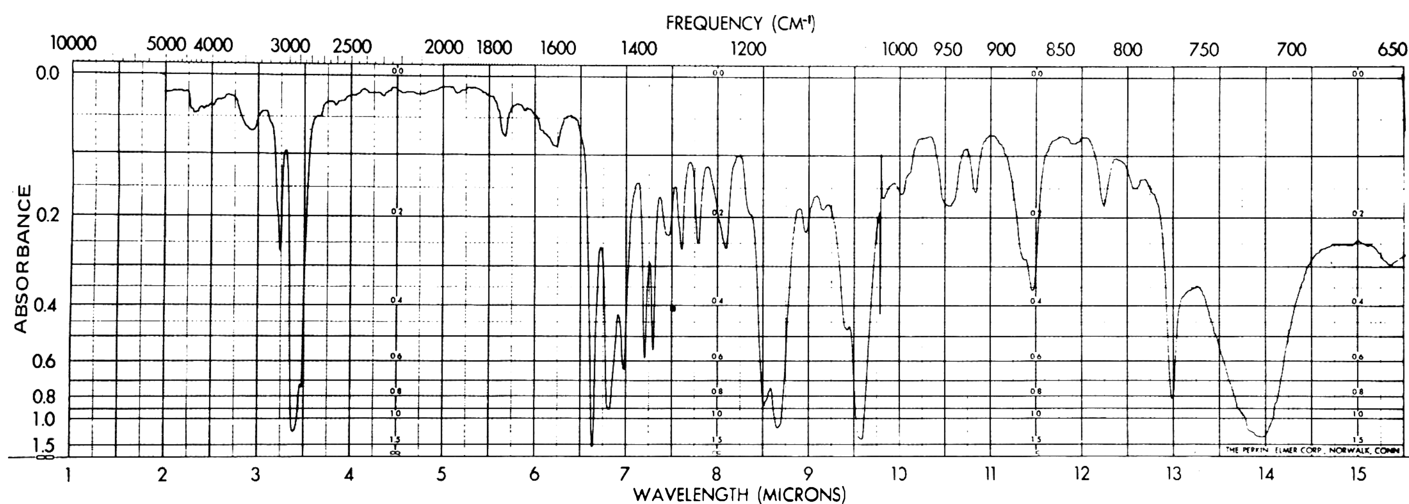

Fig. 6—Infrared spectrum of 2-isobutylthiazole isolated from tomato volatiles (liquid sample).

pounds related to phenylalanine would implicate lignin metabolism as an active system in tomato volatiles. Benzaldehyde could originate from mandelic acid or phenylglyoxylic acid through heating. Cinnamaldehyde, reported by Schormüller et al. (1965a), though not found in our investigation, can be oxidized to benzaldehyde during distillation. The conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase has been shown (Koukol, 1961). Other lignin-related compounds, namely, eugenol, guaiacol and methyl salicylate, have been identified in tomato volatiles. These phenolic compounds were recovered in about the same amounts, regardless of the treatment given the tomatoes. However, the amounts varied widely among varieties. Walker (1962) has also reported that phenolic acids, namely, cinnamic, ferulic and p-coumaric acids, occurred unbound in tomato wall tissue. Viani et al. (1969) have found several additional phenolic compounds in tomato volatiles. Finally, vanillin, suspected by Spencer et al. (1954), would be a possibility, though there was no evidence for this compound in our work. Their volatiles had been obtained from a commercial paste evaporator.

Terpene-related compounds

It can be seen that several terpenes or terpene-related compounds have been isolated from tomato volatiles (Table 12). Cole et al. (1957) have isolated 6-methyl-5-hepten-2-one and acetone from lycopene degradation during the heating of tomato pulp. Oxygen availability was the most important factor in color loss and lycopene oxidation. There were indications in some of our experiments that 6-methyl-5-hepten-2-one was recovered in lower concentrations when tomatoes were blended under nitrogen rather than under air. The blending procedure we used for grinding tomatoes introduced large amounts of air. The amounts of 6-methyl-5-hepten-2-ol found in tomato volatiles were low compared to the quantities of the corresponding ketone. The activity of the alcohol-dehydrogenase towards this alcohol and ketone would be expected to be low (Eriksson, 1968).

Peak 37 was identified as *beta*-ionone. This compound has been reported as an important flavor component in raspberries (Winter et al., 1962a). Possibly, *beta*-ionone was produced by oxidative degradation of *beta*-carotene in tomato homogenates by a reaction similar to that postulated for lycopene by Cole et al. (1957). Weurman (1961) was able to develop volatile compounds by treating raspberry substrates with various enzymes. However, none of the enzymes tried developed raspberry flavor. Lipases and alcohol dehydrogenases were used but not lipoxidase. Enzymic destruction of *beta*-carotene has been associated with concurrent oxidation of unsaturated fatty acids of the linoleate type by lipoxidase. Blain et al. (1962) have shown that *beta*-carotene can be degraded during the autoxidation of methyl linoleate in model systems.

Amounts of 6, 10-diemthyl-5, 9-undecadien-2-one found in tomato volatiles also appeared to be dependent on oxygen, decreasing when blending of tomatoes was done under nitrogen. Chemical oxidation of squalene yields 6,10-dimethyl-5,9-undecadien-2-one and 6-methyl-5-hepten-2-one (Pinder, 1960a). Possibly, similar oxidations could occur in tomato homogenates where hydroperoxides would appear to be prevalent. Squalene in tomatoes is well established (Beeler et al., 1963). Farnesyl derivatives are also present in tomatoes and might be precursors of these ketones. Farnesal was indicated to be in tomato volatiles by retention time data, but in very low amounts.

Geranial in tomato volatiles was not unexpected, because of the role of geranyl derivatives in the biosynthesis of terpene. The citral reported by Spencer et al. (1954) and Hein et al. (1963) probably was the same compound. Buttery et al. (1968) indicated that their 6,10-dimethyl-5,9-undecadien-2-one isomer had the geranyl form rather than the neryl form. Geranial has been reported in apricots (Jennings et al., 1966) and in oranges (Wolford et al., 1963), among other foods.

Peak 27 was identified as linalool. Geraniol would be expected, but so far we have not found it. Linalool isomerizes to geraniol under the influence of acids. However, this is a reversible anionotropic rearrangement (Pinder, 1960b). Linalool has been found in many foods; it was considered important in snap-bean flavor (Stevens et al., 1967).

Peak 7 was found to be 2-methyl-3-buten-2-ol. This compound has been reported in grape essence (Stevens et al., 1965) and in volatiles from black currants (Andersson et al., 1966). In these fruits, both linalool and geraniol were reported. Possibly, its precursor was linalool, although a rearrangement similar to that for the conversion of geraniol to linalool would merit consideration. The 3-methyl-3-butenyl derivatives are well established in terpene biosynthesis.

Peak 41 has been partially characterized but has not been identified. It would appear to be a terpene-related C-18 methyl ketone with structure similar to 6,10-dimethyl-5,9-undecadien-2-one, lacking the double-bond conjugation with the carbonyl group. It has a dry-hay or straw-like aroma and has not been evaluated for flavor. It may be important, however, since it appears to have heritable differences. It also appears to be oxygen dependent. It is possible that this compound is the 6,10,14-trimethylpenta-

deca-5,9,13-trien-2 one recently reported by Buttery et al. (1969).

Except for methyl salicylate, we did not find any esters in our investigation, but our steam distillation procedure might have been a factor. However, when 3-methylbutyl hexanoate was added to tomato juice and distilled, the ester was recovered in high yields, with only slight indication of hydrolysis. Possibly, the procedure used by Dalal et al. (1968) to prepare their extracts may have produced the esters.

Much more research must be done to define more clearly how volatiles develop in tomatoes. However, an over-all general picture of the reactions can be seen from the results known to date. Many of the volatiles isolated from tomatoes are related to the enzymic and chemical reactions that occur during the grinding or crushing of the tomatoes. Lipid-oxidizing enzymes react with fatty acids to produce hydroperoxides which break down into carbonyl compounds. The latter can be converted to the corresponding alcohols via alcohol dehydrogenases. Conversely, alcohols can be changed to aldehydes depending on the conditions which control the equilibria of such reactions. Hydroperoxides potentially can react with amino acids to produce aldehydes and ultimately alcohols corresponding to these aldehydes. Amino acids are susceptible to heat degradation and enzymic reactions as well. Both these types of reactions will produce aldehydes. Hydroperoxides can also attack the terpenes to form the terpene-related ketones and alcohols. Lignin derivatives, too, belong in this group of amino-related compounds but the pathways for their formation must still be worked out. Carbohydrates, principally through pectic substances and reducing sugars, play a minor role. It is difficult to classify 2-isobutylthiazole, which does not seem to fit anywhere in the spectrum of compounds found in tomato volatiles. Obviously, there are many more unidentified compounds that could alter this present concept of tomato flavor chemistry.

Flavor evaluation

Organoleptic evaluation of the flavors of volatile compounds in fresh fruits and vegetables is very difficult because of the accurate quantitation required and the many possible flavor interactions of the compounds. It is evident from the results described here that volatiles isolated for identification can differ grossly from those present when the fresh fruit or vegetable is actually consumed. Flavor evaluation of a pure compound in a pure medium can give important clues on threshold levels, but such a technique will not necessarily establish the flavor contribution of the compound in a food, because of possible synergistic effects, which actually can turn out to be negative. A good example of such flavor interactions was found with 2-isobutylthiazole. The pure compound in aqueous solution had a spoiled vine-like, slightly horseradish type flavor, which was rather objectionable. When it was added to canned tomato juice or tomato paste, it produced a more intense, fresh tomato-like flavor. Besides its characteristic aroma effects, this compound blended out the harsh notes and improved the mouth-feel properties of the juice or paste. It was effective in tomato juice at levels from 25 to 50 ppb dependent, of course, on the amounts of other volatiles already in the juice. At higher levels, its flavor became objectionable, being described by different tasters as rancid, medicinal or metallic. Its threshold value in water was 2 ppb. The characteristic aroma of 2-isobutylthiazole was distinctly detectable upon slicing firmly ripe tomatoes of the varieties high in this compound. This unique flavor was also evident in pulp and juice from tomatoes high in 2-isobutylthiazole.

Another important flavor component of fresh tomatoes was *cis*-3-hexenal. This compound improved the flavor of canned tomato juice and tomato paste in the fresh "green" flavor notes. Like 2-isobutylthiazole, *cis*-3-hexenal gave desirable blending and mouth-feel properties. It was effective in the range of 0.3–0.5 ppm in tomato juice. At levels of 1 ppm and higher, *cis*-3-hexenal produced strongly "green" rancid-type flavors which were objectionable. Winter et al. (1962b) have described the aroma of *cis*-3-hexenal as "green," fresh with very natural character. Hoffmann (1961) likened its flavor to that of green beans. Forss et al. (1962) pointed out that *cis*-nonconjugated unsaturation appeared responsible for a "green" or "plant-like" flavor. *cis*-3-Hexenal would appear important to the "fresh" flavors of tomatoes as well as many other fruits and vegetables.

The flavor effects of *trans*-2-hexenal were similar to those of *cis*-3-hexenal, but they were less intense and less fresh "green" in character. The *trans*-isomer also produced desirable blending or mouth-feel properties. The desirable range in canned tomato juice was 0.5–2 ppm. Above these levels, the rancid notes became pronounced and objectionable. It should be pointed out that this desirable range in canned tomato juice was well below the amounts of *trans*-2-hexenal found in fresh tomato homogenates, where the range was 3–10 ppm. Processed, canned tomato juice was low not only in *trans*-2-hexenal but also in many of the other volatile compounds. Therefore, the flavor effects of *trans*-2-hexenal were more conspicuous because there were less intense flavor interactions with other volatile compounds. Interestingly, *cis*-2-hexenal has much less desirable flavor notes than the *trans*-2-isomer, in contrast to the flavor association with *cis*-nonconjugated compounds.

n-Hexanal gave green-type flavors in tomato juice in the range of 0.1–0.5 ppm. However, the desirable flavor effects of *n*-hexanal were much less striking than those of *cis*-3-hexenal and *trans*-2-hexenal. Conversely, its off-flavor notes, typical of rancid vegetable fats, were readily evident and more objectionable at 0.5 ppm and higher. Similar flavor effects were found with tomato paste diluted with water to solids approximating those in juice.

trans-2-Pentenal showed fresh, "green" notes, but these were much less intense than those of *cis*-3-hexenal. Amounts of *trans*-2-pentenal found in tomato volatiles were very small, but these could be contributing to over-all flavor through additive effects.

As pointed out by Pyne et al. (1965), the "green" notes of *cis*-3-hexen-1-ol contributed significantly to tomato flavor. However, these notes lacked the flavor-enhancing properties of the aldehydes, especially the blending effects. It was difficult to evaluate the flavor of *cis*-3-hexen-1-ol in tomato juice, because the amounts in the juice were high enough to affect the flavor. In tomato paste where quantities of *cis*-3-hexen-1-ol were low, the effects were more noticeable and desirable. As indicated above, the amounts of *cis*-3-hexen-1-ol varied widely among tomatoes of the same variety and were especially dependent on the physical condition of the fruit and the method used for recovering the volatiles. Johnson et al. (1968) presented evidence which showed that differences in *cis*-3-hexen-1-ol and the C_5 alcohols were dependent on the tomato variety as well as the harvest.

Dalal et al. (1968) had presumed that *n*-hexanol and isovaleraldehyde gave tomatoes their "green leafy" aroma. In our flavor tests, *n*-hexanol did not produce any changes in the flavor of tomato juice or diluted paste until the concentration was 0.3 ppm, or about 3 times that found in tomato volatiles. At this concentration, there was a slight decrease in the tomato-like notes of the juice and paste.

Although 6-methyl-5-hepten-2-ol, 6-methyl-5-hepten-2-one and 6,10-dimethyl-5,9-undecadien-2-one had fruit-like aromas, their flavor effects in tomato juice and dilute paste were surprising. At concentrations on the high side of those found in tomato volatiles, each of these compounds decreased the tomato-like notes, giving a flat, insipid flavor. At concentrations around 0.75 ppm, 6-methyl-5-hepten-2-one acquired cooked, stewed tomato flavors. In tomato volatiles this compound ranged 0.3–0.5 ppm. Processed tomato juice developed heated paste notes with 6-methyl-5-hepten-2-ol

at 0.15 ppm and higher, compared to a range of 0.04–0.06 ppm in tomato volatiles. When the average concentration of 0.3–0.5 ppm of 6,10-dimethyl-5,9-undecadien-2-one was doubled, tomato juice acquired heated paste-type flavor notes, with slightly hay-like character. When a mixture of these 3 compounds was added, tomato juice acquired the typical flavor notes of heated, canned tomato pastes. Adding *n*-hexanal to such an off-flavored product considerably improved the flavor, introducing fresh "green" notes. These compounds produced similar decreases in the intensity of the tomato flavor in diluted tomato pastes adjusted to solids of tomato juice. However, 6,10-dimethyl-5,9-undecadien-2-one produced flavor notes strongly resembling those of spoiled watermelon at higher levels in diluted paste. Lycopene is very high in watermelon.

Stevens (1968) indicated that eugenol and methyl salicylate may be a factor in the flavor difference between the 2 tomato varieties he studied, based on the flavor thresholds of these compounds and the amounts isolated in the volatiles. At 0.03 ppm, the highest concentration found in tomatoes, methyl salicylate, decreased the tomato-like character of tomato juice and gave flavor notes of canned tomatoes opened and stored refrigerated for a few days. It is likely that methyl salicylate contributes to tomato flavor because of its relatively large quantitative differences between varieties, for example, 200–400 times.

2-Octenal in both tomato juice and diluted paste produced cardboard-type flavors in the 0.1-ppm range, or about twice the amounts found in volatiles from fresh tomatoes. Rather unique flavor properties were found with 2,4-decadienal. This compound produced very desirable mouth-feel or blending properties, smoothing out harsh, acidic-type notes of tomato juice and diluted paste at 2.5–10 ppb. This flavor property appeared to be characteristic of conjugated dienals, not only in tomatoes but in other foods as well. At higher concentrations, the rancid-like notes characteristic of 2,4-decadienal became objectionable.

The typical "fresh" flavors associated with tomatoes appeared to be related to volatile carbonyl compounds, especially *cis*-3-hexenal, *trans*-2-hexenal and *n*-hexanal, and to 2-isobutylthiazole. When the quantities of these compounds decreased so that their flavor effects were no longer detectable, the alcohols and other compounds became the predominant flavor contributors. Then, the flavor became "processed" or "enzymic." This type flavor was associated with tomato juices or pulps low in aldehydes and high in alcohols. Methanol was recovered in the highest amount of all the volatiles. Undoubtedly, the steam-distillation procedure contributed to the high levels. Methanol did not appear to contribute to the flavor of tomato juice or paste.

Frozen, whole tomatoes after thawing and slicing or blending had flavors considerably less desirable than sliced, nontreated, fresh tomatoes. The −20°C frozen samples had the poorest flavor. The liquid-nitrogen frozen tomatoes were somewhat better than either the −20°C samples or the dry-ice samples. The texture of all frozen tomatoes was poor. Dimick et al. (1956) had noted similar results with frozen strawberries.

The so-called heated or "cooked" tomato flavor has received some attention from Miers (1966). He reported that hydrogen sulfide and dimethyl sulfide were found in processed canned tomatoes and juices at concentrations above the flavor thresholds for these compounds. Similar large amounts of dimethyl sulfide were reported to develop in processed canned tomato juice by Nelson et al. (1969). In processed, canned tomato juices, the amounts of volatile compounds, especially those important to fresh "green" flavor were found to be quite low. Thus, the off-flavor notes of these sulfides would be much more noticeable and objectionable. Dimethyl sulfide produces rather unique flavor notes depending on its concentration and the food product in which it is used. Certain other compounds were found to be associated with "heated" or "cooked" tomato flavor. These compounds were furfural, 3-methylmercaptopropanal, 2,4-heptadienal, acetaldehyde and phenylacetaldehyde. Volatiles obtained by prolonged heating of tomato pulp or juice contained higher amounts of these compounds. 3-Methylmercaptopropanal showed unusual flavor characteristics. At concentrations of 1 ppm or above, it masked the typical flavor of tomato juice and produced an insipid flavor. Normally, its concentration in volatiles from fresh tomatoes recovered by steam distillation was estimated to be around 0.05 ppm.

Phenylacetaldehyde at 0.5 ppm lost its typical floral note and developed undesirable flavors in tomato juice. This compound also occurred around 0.1 ppm in tomato volatiles. Acetaldehyde also was found at high concentrations in volatiles with cooked flavor. Katayama et al. (1967) reported increased amounts of acetaldehyde in tomato juices heated at 80°C for 10 min compared to fresh juice. 2 varieties, identified as High-red and Daruma, showed very high changes in ethanol and acetaldehyde during this relatively short heating process. Similarly, Giannone et al. (1967) found high quantities of acetaldehyde in canned tomato juice.

Flavor evaluations of the other compounds in tomato volatiles are still continuing. Further chemical and organoleptic data are being obtained to establish which of the volatile compounds isolated from tomatoes are significant to flavor.

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YUSEN YIN, N. ZARGHAMI and D. E. HEINZ
Department of Consumer Sciences, University of California, Davis, Calif. 95616

EFFECTS OF pH AND TEMPERATURE ON VOLATILE CONSTITUENTS OF CARAWAY

SUMMARY—The major volatile components of caraway seed oil were isolated and identified; and the effects of heat and pH on these volatile constituents were studied. Gas chromatographic analysis of the treated oil indicated that the two principal components, carvone and limonene, undergo little change even when subjected to harsh conditions. The chemical nature of the trace components changes only when the oil is subjected to high temperature or to solutions of varying pH.

INTRODUCTION

CARAWAY, both the whole seed and its essential oil, is used primarily as a flavoring constituent in bakery goods, candies, condiment mixtures, and alcoholic liqueurs of the Kümmel type, and also for the manufacture of carvone for scenting of soaps. Caraway seed oil was reported by Guenther (1950) to contain two major constituents, carvone and limonene, and minor amounts of other compounds such as acetaldehyde, methyl alcohol, furfural, diacetyl, and such high-boiling fractions as dihydrocarvone, carveol, d-dihydrocarveol, l-dihydrocarveol, l-neodihydrocarveol, l-isodihydrocarveol, d-perillyl alcohol, and d-dihydropinol. Based on results from gas-liquid chromatography, Wheeler et al. (1961) reported that caraway oil contains 58% carvone. El-Deeb et al. (1962a), using column chromatographic analysis, showed the oil to contain carvone, limonene, α-phellandrene, α-terpinene, and citral. In addition, El-Deeb et al. (1962b) reported, using liquid chromatographic methods, methyl salicylate and eugenol. Ikeda et al. (1962) reported that the monoterpene hydrocarbons amounted to 38% of the caraway oil. Almost 100% of the amount was d-limonene with trace amounts of α-pinene, thujene, β-pinene, sabinene, Δ^3-carene, α-phellandrene, and unidentified compounds. Atal and Sood (1966) used gas-liquid chromatography to study Indian caraway and reported that this oil contained carvone, limonene, β-pinene, and p-cymene.

No work has been reported concerning the chemical changes which may take place in caraway oil during typical consumer utilization processes. However, a study of the volatile constituents of cardamom, another spice, indicated that drastic chemical changes can occur under conditions prevalent in consumer processing (Brennand and Heinz, 1970).

The present investigation was undertaken to determine if significant chemical changes take place in the volatile oil of caraway when subjected to typical handling conditions. Also, the major constituents in the volatile fractions of caraway were identified.

EXPERIMENTAL PROCEDURE

VOLATILE SAMPLES used were either a commercial caraway essential oil (Magnus, Maybee and Reynard, Inc., New York) or an oil obtained by extracting ground caraway seeds with hexane at room temperature. Whole caraway seeds (Spice Islands of Leslie Foods Inc.) were ground (10–30 mesh) in a Laboratory Wiley Micro Mill then stored in glass jars in the refrigerator. In the extraction procedure, 5g of the ground seeds were transferred into a disposable pipette which had glass wool at the tip. The seed material was then saturated with 3 ml hexane and extracted five times by passing 3 ml hexane through the sample. The extract was centrifuged to remove any residue, and the hexane fraction was evaporated to 0.5 ml under nitrogen flow. A portion (ca. 80 μl) of this extracted sample was analyzed directly utilizing gas-liquid chromatographic techniques.

The effect of heat and acid on the oil were studied in environments simulating the harshest utilization conditions normally to be expected.

Heat treatment: Samples (ca. 50 μl sealed in a 1/8 in. I.D. glass tube) of commercial caraway oil were heated in an oven at 93, 149, 177, 205, 233, 260, 288, or 316°C for 1 hr. 6 μl of the heat-treated samples were analyzed by gas chromatography and the height of each peak measured. Results were compared with those of the untreated commercial and extracted samples.

Acid treatment

5g of ground caraway seeds were soaked in 20 ml of buffer (McIlvaine's standard buffer solution, pH varying from 2.2–8) in a 100-ml flask for 24 hr. 15 ml of acetone and 15 ml of hexane were blended with the soaked seeds and the mixture was centrifuged. The organic fraction was washed several times with distilled water and then evaporated to 0.5 ml by passing a stream of nitrogen over its surface. 80 μl of each sample were directly analyzed by gas chromatography. Results were compared with those from an untreated, extracted sample and also with those from a sample from seeds soaked in 20 ml of deionized water.

A 25 ft × 1/4 in. O.D. stainless steel column packed with 4% FFAP on acid washed and DMCS treated Chromosorb G and an Aerograph Model 1520-1B, thermal conductivity, gas-liquid chromatograph were used in the analyses. A 5-in. packed column placed in the front of the normal column and renewed between different sets of analyses permitted simultaneous separation of the volatile oils from the fixed

Table 1—Volatile constituents in caraway seed oil.

Peak Number[a]	Compound	Identification method[b]	Previously reported by[c]
1	α-Pinene	RT	4
2	β-Pinene	RT	1, 4
3	Myrcene	RT, IR, UV	--
5	Limonene	RT, IR	1, 2, 3, 4
9	Terpinolene[d]	IR	--
10	p-Cymene[d]	RT, IR	1
23	*trans*-Dihydrocarvone	IR, MS	3
24	*cis*-Dihydrocarvone[e]	IR	–
25	l-*p*-Menthen-4-ol	IR	--
26	*cis*-8-*p*-Menthen-l-ol	IR	--
27	8-*p*-Menthen-2-ol (*neo*)	IR	3
28	8-*p*-Menthen-2-ol	IR	3
29	8-*p*-Menthen-2-ol (*iso*)	IR	3
29	Carvone	IR	1, 2, 3
30	8-*p*-Menthen-2-ol (*neo-iso*)	IR	–
31	*trans*-Carveol	IR	3
32	*cis*-Carveol	IR	–

[a]Peak numbers of chromatogram in Figure 1.

[b]RT, retention data; IR, infrared spectral data; UV, ultraviolet spectral data; MS, mass spectral data.

[c]1. Atal and Sood (1966); 2. El-Deeb et al. (1962a); 3. Guenther (1950) and references therein; and 4. Ikeda et al. (1962).

[d]These compounds were isolated and identified from the oil after it was exposed at 288°C for 1 hr.

[e]This compound was isolated and identified from the seeds after they were soaked at a pH of 7 for 24 hr.

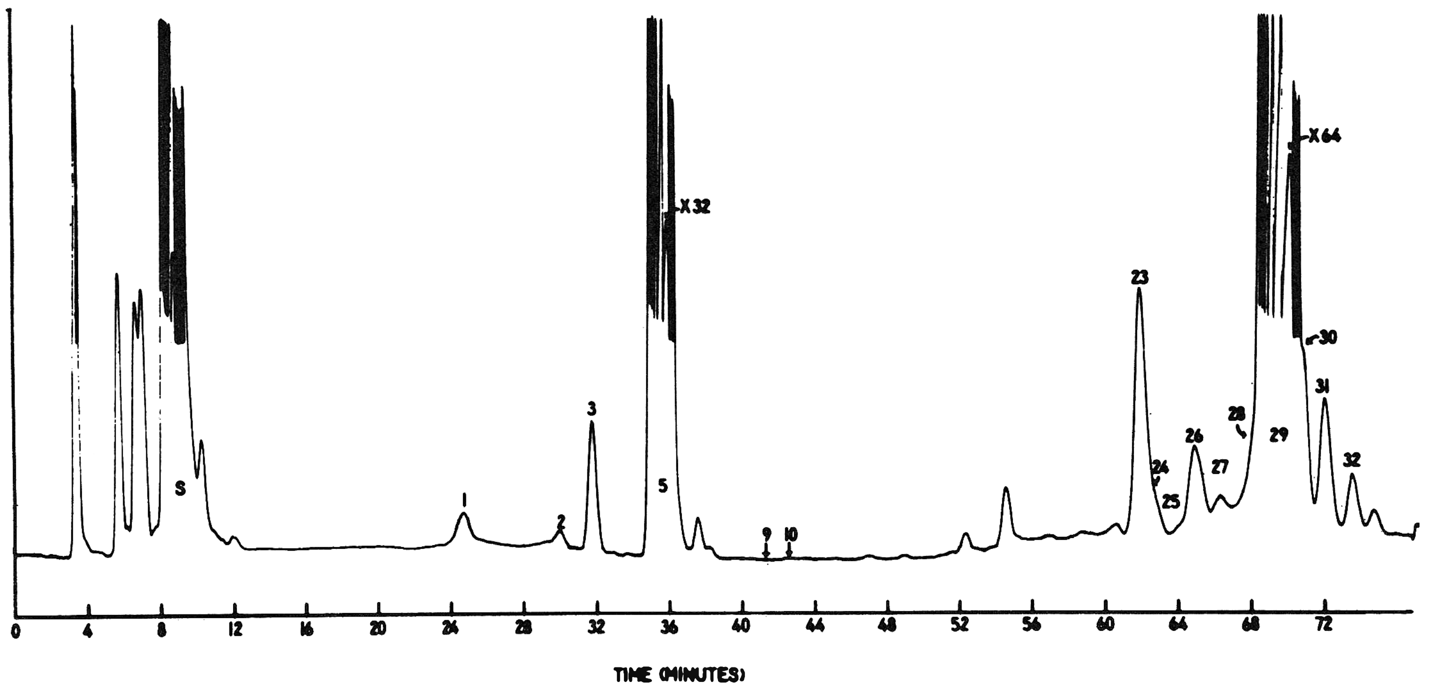


Fig. 1–A typical gas chromatogram of the hexane extracted oil obtained from caraway seeds.

oils and the analysis of these essential oils. Samples were chromatographed under the following conditions: injector temperature, 145°C; detector temperature, 250°C; column temperature, linearly programmed from 50°–240°C at 2°C/min; and a helium flow rate of 30 ml/min.

Changes in the relative amounts of the volatiles during each treatment were estimated using the peak height method (Essential Oil Association of U.S.A., 1965). Because all chromatographic parameters were kept constant between runs and only comparatively minor changes occurred in the individual treatments, comparable results were obtained.

Identification of the volatile constituents

Commercial caraway oil was used in the identification of the volatile constituents because of its lack of solvents and fixed oils and the apparent similarity between it and the extracted oils. Fractions eluting from the FFAP column were trapped in glass capillary tubes and further purified using an SE-30, 10 ft × 1/4 in. O.D., stainless steel column (5% packed on Chromosorb G, AW, DMCS). When necessary, further purification was accomplished using a 500 ft by 0.03 in. I.D. stainless steel capillary column coated with OV 17 or SF 96-50. Individual fractions were identified by comparing available infrared, ultraviolet, mass spectral, and retention data of the caraway isolates with those obtained from authentic samples or reported in the literature.

RESULTS & DISCUSSION

A TYPICAL GAS chromatogram of the volatile constituents of caraway oil extracted from the seeds, using hexane, is shown in Figure 1. The compounds which were identified as being under the major peaks in the chromatogram are listed in Table 1.

With the exception of α- and β-pinene, tentatively identified by retention data, the terpene hydrocarbons and ketones were identified by comparing their infrared or mass spectra or both with those of authentic compounds or with spectra reported by Mitzner et al. (1965). Terpene alcohols likewise were identified through comparison of their infrared spectra with those reported by Mitzner et

Table 2–Effect of heat and pH on the percentages[a] of carvone, limonene and total trace compounds in caraway seed oil.

	After exposure of oil for 1 hr at (Temperature °C)									
	26[b]	93	121	149	177	205	233	260	288	316
Carvone	51.3	51.8	51.4	50.6	54.6	52.2	51.6	51.0	49.7	45.3
Limonene	44.6	43.9	44.4	45.3	41.1	43.9	44.2	45.0	44.5	43.1
Total trace compounds	4.1	4.3	4.2	4.1	4.2	3.9	4.2	4.0	5.9	11.5

	After seeds were soaked 24 hr in solutions at pH								
	2.2	3.0	4.0	5.0	6.0	7.0	8.0	deionized H_2O	untreated seeds
Carvone	74.3	76.6	69.4	76.1	70.0	71.2	77.1	78.1	68.2
Limonene	24.0	21.8	29.1	22.2	28.3	26.8	21.1	20.2	29.4
Total trace compounds	1.7	1.6	1.4	1.7	2.7	1.9	1.7	1.8	2.4

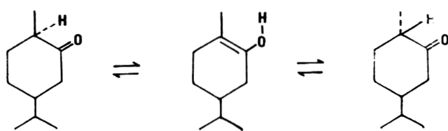
[a]Percentages determined by peak heights (see text).
[b]Represents untreated commercial caraway oil.

al. (1968). When compared to the commercial oil samples, the hexane-extracted samples contained more carvone (68% vs 52%) and less limonene (28% vs 44%). These variations may reflect differences in species, cultural environment or isolation procedures.

The chromatograms obtained from oil kept 1 hr at various temperatures from 24–260°C revealed no significant changes in the components (Table 2). In this temperature region, the characteristic flavor of caraway oil did not change. More significant changes in the volatile compounds occurred after exposure of the oil for 1 hr to temperatures of 288 or 316°C. At these temperatures, the percentage of limonene remained constant while that of carvone decreased slightly. The trace compounds increased to 11.5%. The latter increase was primarily due to increases in peak 8, an unknown oxygenated compound; peak 9, terpinolene; peak 10, p-cymene; and peak 24, dihydrocarvone. The infrared spectrum of peak 8 indicated no carbonyl or hydroxyl functional group, but the mass spectrum revealed fragments at m/e 43, 67, 69, 81, 97, and 139. Accurate mass values of fragments m/e 97 and 139 indicated them to be $C_6H_7O^+$ and $C_7H_{15}O^+$, respectively. The sample treated at 288°C had a slightly burnt, sweet perfume smell, but it still had the characteristic aroma of caraway. The sample treated at 316°C had a very strong burnt smell and lost much of the typical caraway aroma. Optical rotation data showed that the d-carvone present in the untreated caraway sample did not undergo racemization during this heat treatment.

Chromatograms of the acid-treated seed samples revealed random but insignificant changes in limonene and carvone over the pH range 2.2–8 (Table 2). The total concentrations of the trace compounds remained constant. However, minor increases or decreases in several individual trace compounds were noted. The changes in the various constituents may have been due to enzymatic reactions or to the absorption or extraction capacity of the seeds or their components at certain pH or in certain buffer solutions.

When the chromatogram of oil obtained from seeds treated with deionized water was compared with that of oil from seed soaked at a pH of 7, it was evident that the presence of a buffer system did not produce new compounds or cause a significant change in size of the peaks. However, when the chromatograms of oil obtained from untreated seeds were compared with those obtained from seeds soaked in either deionized water or buffer systems of various pH, a major change in the relative concentrations of the dihydrocarvones (peaks 23 and 24) was noted. Chromatograms of the oil obtained by extraction of the seeds prior to any treatment (Fig. 1) indicated the presence of only one of the two isomers (peak 23) at approximately the 0.7% level. In contrast, chromatograms of all the soaked seeds had both isomers at approximately the 0.3 and 0.2% levels. This might have been expected as these two isomers are in equilibrium with the intermediate enol tautomer in either acidic or basic conditions as:



Based on results obtained with gas-liquid chromatographic techniques, the volatile constituents of caraway oil are more stable than those of cardamom oil under comparable heat or acid treatments. Cardamom volatiles start changing above 149°C after 30 min of heating and undergo substantial changes in acidic or neutral solutions (Brennand and Heinz, 1969). In contrast, the caraway volatiles are stable even at temperatures of 260°C after 1 hr and undergo few changes when subjected to solutions of differing pH.

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C. P. BRENNAND[a] and D. E. HEINZ
Department of Consumer Sciences, University of California, Davis, Calif. 95616

EFFECTS OF pH AND TEMPERATURE ON VOLATILE CONSTITUENTS OF CARDAMOM

SUMMARY—Decorticated cardamom seeds were subjected to conditions typical of consumer utilization practices. These involved timed exposures to temperatures as high as 205°C and to solution pH's from 2–8. Changes in the volatile constituents were followed by gas-liquid chromatographic analyses of the cardamom seed extracts. Temperatures above 149°C and increasing hydrogen ion concentration caused marked changes in composition of the volatile oils of this spice. The effects of pH and temperature environments typical of products in which cardamom is used are discussed.

INTRODUCTION

SEEDS OF CARDAMOM are used as seasoning in a wide variety of products ranging from pickles to Danish pastry. The diversity of products results in major differences in the environmental conditions to which cardamom is exposed. The fact that spices change even during storage, as evidenced by the common problem of staling, indicates that significant changes could occur during usage. When added to foods, cardamom and other spices can be affected by the treatments that the product undergoes or by conditions inherent in the food product. Chemical changes which could affect flavor

[a]Present address: Department of Foods and Nutrition, Utah State University, Logan, Utah 84321.

might be particularly expected in cardamom, since the volatile oils which are credited as being responsible for the flavor of this spice consist largely of terpenoids. Terpenoids are generally unstable in the presence of acid, light, oxygen or heat, and are capable of undergoing hydrolysis, rearrangement, polymerization, and oxidative reactions.

The composition of the volatile oil of cardamom has been of interest to many investigators. Prior to the advent of gas chromatography, only seven compounds had been identified in the essential oil of cardamom: terpin hydrate, cineol, d-α-terpineol, terpinyl acetate, limonene, borneol, and sabinene (reported in Guenther, 1952). Terpin hydrate has not since been observed. After the advent of gas-liquid chromatography, Ikeda et al. (1962) reported 20.7% of the essential oils of cardamom are monoterpene hydrocarbons. Based on retention data, these workers identified them as: α-pinene, α-thujene, β-pinene, sabinene, myrecene, α-terpinene, d-limonene, γ-terpinene, and p-cymene. Nigam et al. (1965) used serial dilution techniques and infrared spectra of isolates to reaffirm the presence of the compounds reported earlier, with the exceptions of terpin hydrate, α-thujene, β-pinene, α-terpinene, and γ-terpinene. They further identified linalool, linalyl acetate, β-terpineol, geraniol, nerol, neryl acetate, nerolidol, and three ketones which they tenatatively identified as methyl heptenone, 2-undecanone, and 3-tridecanone. The composition of the volatile oils of cardamom varies quantitatively and qualitatively from variety to variety (Lewis et al., 1966). The differences in reported compositions most likely also reflect differences in methods of isolation.

Table 1–Constituents identified in the volatiles of cardamom

Peak no. (Fig. 1)	Constituent	Confirmed identity[a]	Reference[b]
1	α-Pinene	RD	2, 3
2	Sabinene	IR	1, 2, 3
3	Myrcene	RD, IR	2, 3
5	Limonene	RD	1, 2, 3
6	Cineol	RD, IR, MS	1, 3
	p-Cymene	RD, IR	2, 3
9	*trans*-Sabinene hydrate	RD, IR, MS	1[c]
11	Linalool	RD, IR, MS	3
15	1-Terpinenol-4	RD, MS	1
16	α-Terpineol	RD, IR, MS	1, 3
18	Linalyl acetate	RD, IR, MS	3
20	Nerol	RD, IR, MS	3
22	α-Terpinyl acetate	RD, IR, MS	1, 3
23	Neryl acetate	RD	3
31	Nerolidol	RD	3

[a]RD, GLC retention data; IR, infrared spectrum; MS, mass spectrum.

[b]Compounds previously reported in the literature: 1, Guenther, 1952; 2, Ikeda et al., 1962; and 3, Nigam et al., 1965.

[c]Reported in Guenther (1952) as an unknown solid with melting range 60–61°C. IR and MS were identical with spectrum of the synthesized product; Russell and Jennings (1970).

This investigation was undertaken to determine to what extent the essential oils of cardamom are affected by changes in pH, temperature, and temperature-pH combinations representative of conditions to which the spice is exposed during utilization.

EXPERIMENTAL

DECORTICATED SEEDS of cardamom (*Elettaria cardamomum*) obtained from Spice Islands of Leslie Food Inc. were stored in tightly capped brown glass bottles. Based on gas chromatographic analyses, there were no recognizable changes in the volatile oils isolated from these stored seeds at the start and after the conclusion of the experimentation.

Two model systems were established to study the effects of heat on the seeds. One system was open to the air during the heat treatment; in the other system, the seeds were kept in closed containers during the heating. The open system was used to determine if unlimited atmospheric oxygen had an effect while the closed system was used to prevent the loss of any volatiles during heating.

Samples of 2.50g of decorticated cardamom were used in the heat treatments. For the closed system the weighed samples were sealed in test tubes. For the open system, the samples were placed in uncovered beakers. Both systems

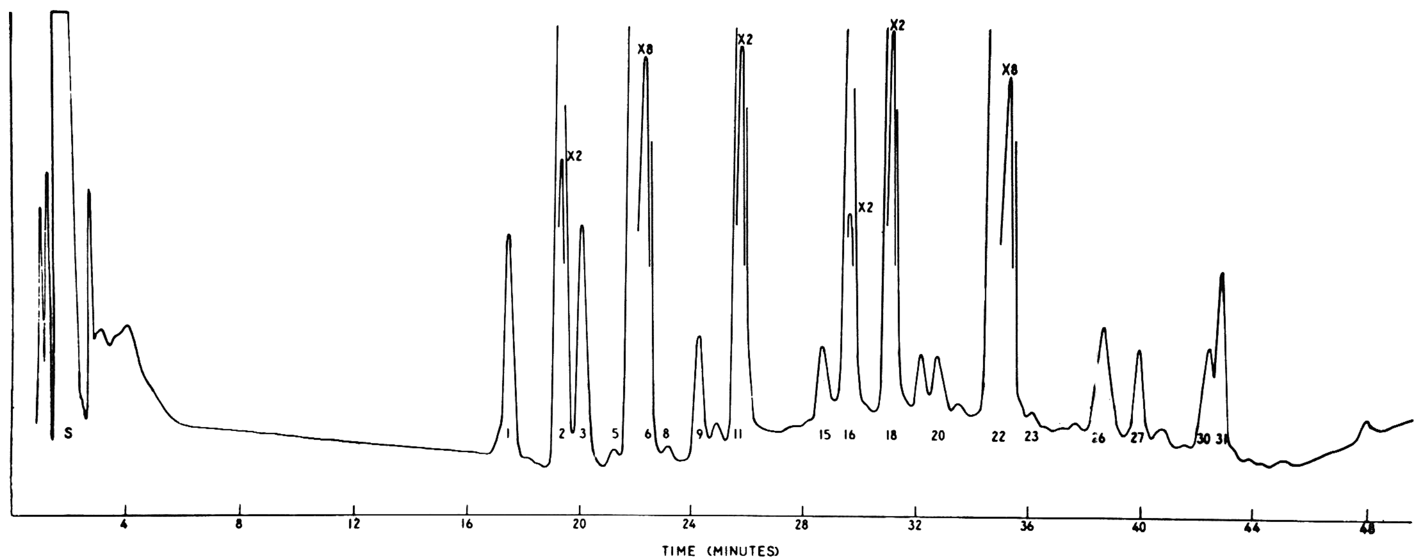

Fig. 1–Chromatogram of 200 µl of an ether extraction of cardamon seeds obtained on a 25 ft × 0.25 in. O.D. 1% SF 96 column.

Table 2–Relative peak heights reported as percentages of the sum of the heights of all peaks on chromatograms of the volatile oil of decorticated cardamom seeds heated in closed tubes.

Peak No.	Temperature °C									
	30 min								3 hr	15 hr
	Room	38	93	149	173	177	191	205	93	93
1	2.1	1.8	1.9	2.0	1.9	1.9	2.1	2.0	2.0	2.3
2	5.7	4.9	5.2	5.3	4.0	2.0	0.5	0.1	5.4	5.4
3	2.2	2.0	2.1	1.9	2.0	2.4	2.6	2.8	2.2	2.2
5	0.1	0.1	0.1	0.1	0.2	0.2	0.4	0.5	0.1	0.1
6	31.4	30.6	30.8	31.6	31.9	34.2	39.1	49.3	30.5	31.8
8	0.2	0.1	0.1	0.2	1.0	1.8	2.8	4.0	0.2	0.4
9	1.1	1.1	1.1	1.0	0.9	1.1	2.4	5.8	1.2	1.3
11	7.7	7.7	7.8	7.3	7.7	8.0	7.4	5.4	7.6	7.5
15	0.8	0.7	0.7	0.6	1.3	1.5	2.1	2.2	0.7	0.8
16	4.4	4.2	4.2	3.6	4.3	4.8	5.6	7.8	4.8	4.5
18	7.6	7.9	8.0	7.8	7.1	4.6	1.9	1.1	7.7	8.1
20	0.4	0.6	0.6	0.6	0.7	0.8	1.0	1.0	0.5	0.5
22	29.8	31.1	30.0	30.6	30.3	29.7	24.8	10.3	30.4	28.0
23[a]	s	s	s	s	0.2	0.5	1.4	1.5	s	s
26	1.0	1.1	1.1	1.1	0.6	0.2	0.1	0.1	0.8	1.0
27	0.8	0.9	1.1	1.0	0.8	0.5	0.4	0.6	0.6	0.7
30	1.0	1.2	1.1	1.2	1.1	1.1	1.1	1.3	1.2	1.4
31	1.7	1.9	1.9	1.8	2.0	1.9	1.6	1.0	1.9	2.1
TOTAL[b]	98.0	97.9	97.8	97.7	98.0	97.2	97.3	96.8	97.8	98.1

[a]s is a shoulder of a major peak.
[b]Trace peaks not listed account for the remaining percentage.

were heated in an oven at 38, 93, 149, 163, 177, 191 or 205°C ± 2° for 30 min. The effect of prolonged heating was investigated using closed-tube samples at 93°C for 30 min, 3 hr, and 15 hr, and open-beaker samples at 191°C for 30 min and for 4 hr.

After the heat treatment, each sample was quantitatively transferred with 15 ml reagent grade diethyl ether to a Virtis blender jar. The sample was macerated for 90 sec at high speed while the blender jar was immersed in a dry ice and ethanol bath. The resulting ether-cardamom slurry was centrifuged at 3,000 rpm for 1 min. A 200-μl quantity of the ether extract was taken directly from the centrifuge tube for gas chromatographic analysis.

Hydrogen ion concentrations typical of foodstuffs that include cardamom were found experimentally to be between pH 2 and 8, thus setting the parameters for the pH variable. Two temperatures were investigated in conjunction with the pH media, room temperature (21–26°C) and boiling temperature. Samples of decorticated cardamom (2.50g) in 15 ml dilute sodium phosphate buffer solution were macerated with the Virtis blender at high speed for 90 sec. The pH values used were: 2, 3, 4, 5, 6, 7, and 8. Resultant slurries were transferred to small reagent bottles and held at room temperature for 3 days prior to analysis. Duplicate sample sets were placed in a boiling water bath for 30 min. After removal from the boiling water, the samples were cooled in an ice bath and frozen overnight. They were analyzed the following day. Extraction of the soluble constituents was accomplished by adding 15 ml of diethyl ether to the slurry and manually shaking vigorously for 2 min. A portion of the resulting emulsion was centrifuged in capped tubes for 1 min at 3,000 rpm. Immediately afterwards, 200 μl of the ether layer was analyzed by gas chromatography.

All samples were chromatographed on an Aerograph Model 1520-1B, thermal conductivity, gas chromatograph. A 25 ft by ¼ in. O.D. stainless steel column packed with 70–80 mesh Chromosorb G, acid washed, DMCS treated, and coated with 1% SF-96(50), was used for the quantitative study. Samples were chromatographed under the following conditions: injector temperature, 175°C; detector temperature, 230°C; column temperature, 50°C until after the solvent peak was eluted (10 min), then linearly programmed at the rate of 4°C/min to a max of 250°C, where it was held isothermally until no more compounds were eluted. Helium was used as the carrier gas at a flow rate of 50–57 ml/min.

Fractions eluted from the gas chromatograph were collected by the procedure described by Jennings et al. (1964). Further purification was made on a similar column coated with 3% Carbowax 20 M. Identification of the purified constituents was accomplished by matching available infrared, mass spectral, and retention data with authenticated reference standards or published data.

Peak height percentages, as described by Essential Oil Association of U.S.A. (1965), were used to measure the relative changes in the volatile constituents. Each peak on the chromatogram was compared with the identical peak on chromatograms obtained from the different utilization conditions, but under the same chromatographic conditions.

RESULTS

A TYPICAL GAS chromatogram of the volatile constituents obtained from fresh cardamom seeds is shown in Figure 1. The compounds which were identified as being under the major peaks in the above chromatogram are listed in Table 1. Borneol, an often reported constituent of cardamom, did not elute precisely with any peak, but had a retention between those of the peaks numbered 15 and 16. The three ketones reported in the cardamom volatiles by Nigam et al. (1965) were not detected. 2,4-Dinitrophenylhydrazine have no visible precipitate with either the total extract or individual fractions as they eluted from the exit port of the gas chromatograph.

Changes brought about by the heat treatment became evident by comparing the relative heights of individual peaks on chromatograms representing the different treatments. Table 2 indicates the relative heights of the major peaks on chromatograms reflecting the effect of heat treatments in a closed system. These relative heights were determined as the percentage of the sum of all peak heights on a given chromatogram. The data in Table 2 represent all peaks which reached at least 1% of the total peak heights on a given chromatogram

In the closed system, differences in chromatograms of samples subjected to room temperature (21–26°C) and those held at 149°C were slight (Table 2). Even those samples kept at 93°C for 3 or 15 hr changed but little. Above 149°C the most drastic changes were the decreases in peaks 2, 18, 22, and 26, and the increases in peaks 6, 8, 9, 15, 16, and 23.

Changes in the cardamom samples which were heated in open beakers were less drastic than the changes in the closed system (Table 3). However, they followed the same trends as did changes in the corresponding fractions from the closed tubes. Heating for 4 hr at 191°C in the open beaker brought about greater changes than occurred in the 30-min sample heated at the same conditions.

Table 3–Relative peak heights reported as percentages of the sum of the heights of all peaks on chromatograms of the volatile oil of decorticated cardamom seeds heated in open beakers.

Peak No.	Temperature °C								
	30 min								4 hr
	room[a]	38[a]	93	149	173	177	191	205	191
1	2.0	1.8	1.9	2.0	2.0	1.9	2.1	1.9	1.9
2	5.5	5.1	5.8	5.4	5.3	5.0	5.2	4.4	2.3
3	2.2	1.9	2.2	2.1	1.9	1.8	1.8	2.0	1.7
5	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.4
6	31.2	31.2	30.0	31.5	32.1	32.5	33.2	35.2	40.8
8	0.1	0.1	0.2	0.1	0.2	0.2	0.3	0.5	0.9
9	1.0	1.0	1.1	1.0	1.0	1.0	1.4	1.0	1.1
11	7.2	6.9	7.7	7.0	6.7	7.1	6.6	6.9	6.0
15	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.6
16	3.9	3.8	4.3	3.6	3.4	3.3	2.9	3.0	2.9
18	7.6	7.4	8.0	7.5	7.4	7.5	7.2	5.6	2.4
20	0.5	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5
22	30.9	31.9	29.5	31.2	32.0	31.8	31.3	31.7	30.4
23[b]	–	–	s	s	s	s	0.2	0.8	1.8
26	1.1	1.2	1.2	1.1	1.0	0.9	0.8	0.5	0.3
27	0.9	1.0	1.2	0.9	0.9	0.8	0.7	0.5	0.4
30	1.1	1.2	1.1	1.1	1.2	1.1	1.2	1.3	1.4
31	1.8	1.9	2.0	1.8	1.6	1.4	1.4	1.3	1.2
TOTAL[c]	97.8	97.7	97.7	97.6	98.1	97.6	97.7	97.8	97.0

[a]–no peak observable under these circumstances.
[b]s is a shoulder of a major peak.
[c]Trace peaks not listed account for the remaining percentage.

Some of the peaks which changed in the closed system after 30 min at 191°C but did not change in the open system under the same conditions, did change in the latter with increased heating time.

The effect of hydrogen ion concentration on the volatile constituents of cardamom held at room temperature is indicated in Table 4. When the seeds are soaked in solutions buffered at different pH values, nine of the major peaks (2, 8, 9, 15, 16, 18, 26, 27, and 31) changed substantially. Samples in buffer held at 100°C for 30 min (Table 5) had even greater changes than those held at room temperature. In addition to the above peaks, peaks 5, 11, 22, and 23 also were affected by low pH when held at 100°C. With the possible exception of fraction 9 (*trans*-sabinene hydrate), all fractions that changed during both the temperature and pH studies changed in the same increasing or decreasing direction regardless of whether the change was induced by low pH or high temperature.

DISCUSSION

CHANGES CAN OCCUR in the volatile constituents of cardamom when these seeds are stored for long periods of time or are subjected to harsh conditions. Staling of spices during long periods of storage may be, at least in part, the result of evaporation of the volatile constituents. A 15-yr-old sample of cardamom analyzed in this laboratory had almost none of the volatiles remaining. However, results of this study indicate that when fresh cardamom is utilized under the same conditions as a seasoning is normally used, the changes which occur are primarily the result of hydrolysis, isomerization, and oxidative changes.

Decreasing trends of individual peaks in the open system exposed to prolonged heating, in which evaporation could occur, were the same decreasing trends as in the closed system, in which evaporation could not occur. Furthermore, the decrease in these fractions was less drastic in the open system than in the closed system. Therefore, the decreases in peaks 2, 18, 22, and 26 which occured during the heat treatment apparently were due to chemical changes rather than to evaporation. Comparison of the more volatile fractions which did not diminish under the conditions tested (e.g., peak No. 3) with some of the less volatile fractions which underwent drastic decreases (e.g., peak No. 22) supports this conclusion. The increases in peaks 6, 8, 9, 15, 16, and 23 which occurred above 149°C were the results of nonenzymatic changes since the harsh conditions precluded enzymatic changes. During the closed system heat treatment, peak No. 6 increased from 31.4 to 49.3% due to the formation of *p*-cymene. This increase in *p*-cymene is thought to have been, in part, the result of hydrolysis and oxidation of the major constituent, *α*-terpinyl acetate (peak No. 22), which decreased from 29.8 to 10.3%. The isomerization and oxidation of sabinene (peak No. 2) which decreased from 5.7 to 0.1% may also have contributed to the formation of *p*-cymene. Hydrolysis of *α*-terpinyl acetate occurred when the cardamom seeds were exposed to acid conditions for 30 min at 100°C, as evidenced by the simultaneous increase in

Table 4–Relative peak heights reported as percentages of the sum of the heights of all peaks on chromatograms of the volatile oil of decorticated cardamom seeds subjected to solutions of various pH's for 3 days at room temperature.

Peak No.	pH of Solution						
	8	7	6	5	4	3[b]	2[b]
1	1.1	0.9	1.3	1.1	1.3	0.8	1.0
2	3.4	3.1	3.5	3.5	3.6	2.2	0.1
3	1.5	1.4	1.6	1.6	1.7	1.2	1.7
5	0.1	0.1	0.1	0.1	0.1	0.1	0.1
6	29.2	29.4	29.6	30.2	30.4	31.0	31.2
8	0.2	0.2	0.3	0.4	0.5	1.0	1.4
9	1.6	1.5	1.5	1.1	0.8	0.3	0.6
11	7.9	7.8	8.1	7.7	7.6	7.7	7.7
15	1.2	1.2	1.4	1.6	1.9	2.4	4.7
16	4.6	4.6	5.1	4.4	4.4	4.7	6.0
18	7.4	7.6	7.0	7.6	7.4	5.9	4.2
20	0.6	0.6	0.5	0.6	0.5	0.4	0.9
22	32.4	33.0	31.4	31.5	32.1	35.4	34.6
23[a]	s	0.1	s	0.1	0.1	–	–
26	1.6	1.3	1.4	1.3	1.1	0.8	0.1
27	1.4	1.2	1.2	1.3	1.1	0.9	0.4
30	1.4	1.4	1.5	1.3	1.3	1.3	1.4
31	2.3	2.0	2.3	2.3	2.1	2.1	1.7
TOTAL[c]	97.9	97.4	97.8	97.7	98.0	98.2	97.8

[a]s is a shoulder of a major peak.
[b]–no peak observable under these circumstances.
[c]Trace peaks not listed account for the remaining percentage.

Table 5—Relative peak heights reported as percentages of the sum of the heights of all peaks on chromatograms of the volatile oil of decorticated cardamom seeds subjected to solutions of various pH's for 30 min at 100°C.

Peak	pH of Solution						
No.	8	7	6	5	4	3	2
1	0.8	0.9	0.9	0.6	0.9	1.0	0.7
2	2.6	2.8	2.5	1.7	1.8	0.5	0.8
3	1.1	1.0	1.2	0.9	1.2	1.4	1.3
5	0.1	0.2	0.2	0.2	0.3	0.5	1.4
6	33.3	32.4	30.6	27.5	31.8	31.2	36.4
8	0.2	0.3	0.5	0.5	0.7	1.2	1.9
9	1.3	1.1	0.8	0.5	0.6	0.6	4.7
11	8.2	7.7	7.7	7.5	7.5	7.8	1.0
15	1.1	1.0	1.4	1.9	2.2	3.3	2.2
16	4.9	4.6	4.6	4.6	4.8	5.5	21.2
18	3.1	3.7	3.6	3.6	3.5	3.2	0.5
20	0.5	0.6	0.6	0.8	0.6	0.4	0.4
22	35.4	36.7	37.9	43.0	37.0	36.5	15.8
23[a]	s	s	s	s	s	0.2	3.7
26	1.1	0.9	0.8	0.6	0.4	0.2	0.1
27	0.8	0.8	1.0	0.7	0.6	0.5	0.8
30	1.2	1.1	1.3	1.2	1.2	1.2	1.3
31	2.2	2.1	2.3	2.1	2.2	2.0	1.6
TOTAL[b]	97.9	97.9	97.9	97.9	97.3	97.2	95.8

[a]s is a shoulder of a major peak.

[b]Trace peaks not listed account for the remaining percentage.

its alcohol (Peak No. 16) from approximately 5% to 21.2%. The probable isomerization of linalyl acetate (peak No. 18) to nerol acetate (peak No. 23) illustrates another kind of chemical reaction possible under both the heat treatments and the pH conditions at 100°C. Such an isomerization would be expected to involve the internal return of the acetate moiety from its more hindered tertiary position in linalyl acetate to the primary carbon atom in nerol acetate simultaneously with double bond migration. If polymerization reactions are responsible for the loss of some of the constituents, the detection of the products would not be possible by the methods used in this experiment.

When cardamom seeds are used in food systems subjected either to high temperature (above 149°C) or to high acid concentration (below pH 4) at an elevated temperature, changes may occur in the volatile constituents. Many pastry products which utilize cardamom are exposed to oven temperatures from 149°C–205°C for 5–90 min. Some beverages utilizing cardamom have pH values as low as 2.8 and are exposed to simmering conditions for 15–25 min. Cardamom is frequently used as a seasoning in fruit dishes that have a pH range from 2.6 to 4.6. Despite the low pH of the fruits used in salads, changes occurring in the volatile constituents of cardamom should be slight since fruit salads are normally held only for short periods of time and at relatively cold temperatures. However, cardamom that is included in cooked fruit products such as jellies, chutneys, and marmalades which are heated for relatively long periods of time in a low pH medium is subjected to an environment extremely conducive to changes in its volatile constituents.

The importance to consumer acceptability of chemical changes occurring during the utilization of cardamom is yet to be established. A few of the changes can be predicted to be detrimental to the flavor and therefore, the acceptability of a product. The increase in peak 6 represents *p*-cymene, a terpene with a petroleum-like aroma. Further, this increase in *p*-cymene apparently occurs at the expense of the major constituent, α-terpinyl acetate, a compound which contributes to the desirable flavor of this spice. More subtle changes in the aroma of cardamom, for example that due to the isomerization of linalyl acetate (peak No. 18) to nerol acetate (peak No. 23), may be either desirable or undesirable to a consumer. Although changes in minor constituents are not discussed in this paper, it is possible that these changes may affect the aroma significantly if the constituents have low odor thresholds.

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FANG M. LIN[a] and WALTER F. WILKENS[a]
Department of Food Science and Technology
New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456

VOLATILE FLAVOR COMPONENTS OF COCONUT MEAT

SUMMARY—Gas, chromatographic and mass-spectral techniques were employed in the isolation and identification of the volatile-flavor components of coconut meat. 15 compounds were positively identified. Odors of authentic compounds were described. Both delta-C_8, -C_{10} lactones and n-octanol were the major volatile components and responsible for the characteristic aroma of coconut meat. The contributions of other minor components to flavor and their significance were also described.

INTRODUCTION

COCONUT, the kernels of nuts of the coconut palm *(Cocos nucifera)*, and its oil has been produced in the tropics and subtropics from the earliest times. Large amounts of coconut and coconut products are utilized for human consumption in the areas of production. Desiccated coconut meat, known as "shredded coconut" in the United States, is used as an ingredient in confectionery and bakery products.

Coconut possesses a pleasant, characteristic aroma which appears to be an acceptable flavor to most people. Wolf (1963) studied the organoleptic properties of grated coconut. Allen (1965) reported the presence of methylketones (C_7, C_9, C_{11}, C_{13}, C_{15}) and delta-lactones (C_6, C_8, C_{10}, C_{12}, C_{14}) in the volatile flavor constituents of coconut oil. The present work extends our knowledge of volatile constituents in coconut meat.

MATERIALS & METHODS

Sample preparation

Coconuts were obtained from a local grocery store. Fresh coconut meat was shredded and pressed with added water (1:1, w/v) in a cloth filter to remove coconut milk. 150 g of the shredded coconut meat were ground in a Waring Blendor with distilled water (1:5, w/v) at low speed for 3.5 min. The slurry was transferred to a 5-liter, round-bottomed flask placed in a water bath and the flask attached to a rotary flash evaporator subsequently connected to a Liebig condenser packed with glass beads, a spiral type (Friedrichs) condenser and a 500-ml collection flask in an ice bath. Water bath temperature was kept at 97 ± 1°C and vacuum applied with aspirator (36 mm Hg) until 500 ml of distillate was collected. 32 runs of above distillation were made and the distillates combined.

The volatiles were extracted from the combined distillates with redistilled CS_2 in a continuous liquid-liquid extractor.

Gas chromatography

Components of the CS_2 extract were fractionated with a Varian Aerograph Model 1520B gas chromatograph on a 10 ft by ¼-in. od aluminum column packed with 20% SE-30 on 60-70-mesh Anakrom AB at a helium flow rate of 60 ml/min. The temperature was programmed from 70 to 275°C at 1°C/min. 15 fractions were collected in 16-in. by 2-mm id glass tubes with dry-ice coolants. Each fraction was rechromatographed on a 10-ft by ¼-in. od aluminum column packed with 25% Carbowax 20M on 60-70-mesh Anakrom AB at a helium flow rate of 60 ml/min. The temperature was programmed from 50 to 250°C at 1°C/min. The subfractions were rechromatographed on a 10-ft by 1/8-in. od stainless steel column packed with 25% Carbowax on 60-70-mesh Anakrom AB at a helium flow rate of 30 ml/min with temperature programming 50–250°C at 1°/minute for relative retention time data against a series of ethyl ester standards.

Gas chromatography-mass spectrometry

The subfractions were rechromatographed under conditions similar to those used for retention time data. The effluent was passed into a heated (200°C) helium separator (Watson and Biemann, 1964) interfaced at the inlet of a Model 12-101A Bendix Time-of-Flight mass spectrometer. The elution of compounds was observed through a Bendix total output integrator and a Textronix Type 545B oscilloscope. The scan rate was set to give a 5-sec scan between m/e 40 and 150.

RESULTS & DISCUSSION

AN EXAMPLE of the chromatographic separation obtained from the volatile flavor of coconut meat on Carbowax 20 M column is shown in Figure 1. Relative retention indices (I_E) of the unknown compounds were calculated relative to a series of internal ethyl ester standards, where the carbon chain length of the acid function represents a whole number (ethyl hexanoate = 6.00) (van den Dool and Kratz, 1963).

Approximately 32 peaks were evidenced as shown in Figure 1. Not all of the peaks are identified because of mass spectral sensitivity limitation combined with overlapping peaks and background, especially at the higher temperature region of the GLC program. Definite identification of 15 volatile compounds was obtained as shown in Table 1. The authentic compounds of the identified volatiles were sampled in bottle by untrained panelists for odor description.

In addition to even-numbered delta-lactones and methyl ketones reported in coconut oil (Allen, 1965), 11 compounds, i.e., octanal, 2-heptanol, 2-octanol, 2-nonanol, 2-undecanol, hexanol, octanol, 2-phenylethanol, benzothiazole, delta-undecalactone, ethyl decanoate are for the first time found in coconut.

Relatively large amounts of delta-C_8 and -C_{10} lactones were present. Their characteristic, coconut-like aroma is responsible for the typical flavor of coconut meat.

Dimick et al. (1969) recently reviewed the occurrence and biochemical origin of aliphatic lactones in milk fat and proposed the existence of saturated fatty acid delta-oxidation in the ruminant mammary gland. Tang and Jennings (1968) found delta and gamma-C_8 lactones in apricot and pineapple; delta and gamma-C_{10} lactones in peach and apricot. Fioriti et al. (1967) reported both delta- and gamma-aliphatic lactones in highly peroxidized soybean and cottonseed oils but none of the lactones could be found in fresh, refined soybean oil. These lactones are postulated to arise from the high levels of hydroperoxides from the unsaturated fatty acids.

Delta-lactones but not gamma-lactones are found in coconut meat (Table 1) or its oil (Allen, 1965). Although catalytic pathways such as delta-oxidation of saturated fatty acids or delta-hydroxylation of unsaturated fatty acids may be involved in the formation of delta-lactones in coconut, it is also possible that the precursors of delta-lactones may arise from the intermediate compounds involved in the biosynthesis of higher fatty acids, and specific enzymes may be involved.

A large amount of n-octanol (Peak No. 9 of Fig. 1) with its lilac odor may also contribute significantly to the coconut meat flavor. The n-octanol probably arises from its acetate which serves as an intermediate compound in the biosynthesis of fatty acids and lactones in coconut. Kohashi et al. (1950) reported the preparation of n-octanol from the reduction of coconut-oil fatty acids at high temperature and pressure.

One of the distinct features of the coconut-meat flavor profile is the pres-

[a]Present address: Quaker Oats Company, 617 West Main Street, Barrington, Illinois 60010.

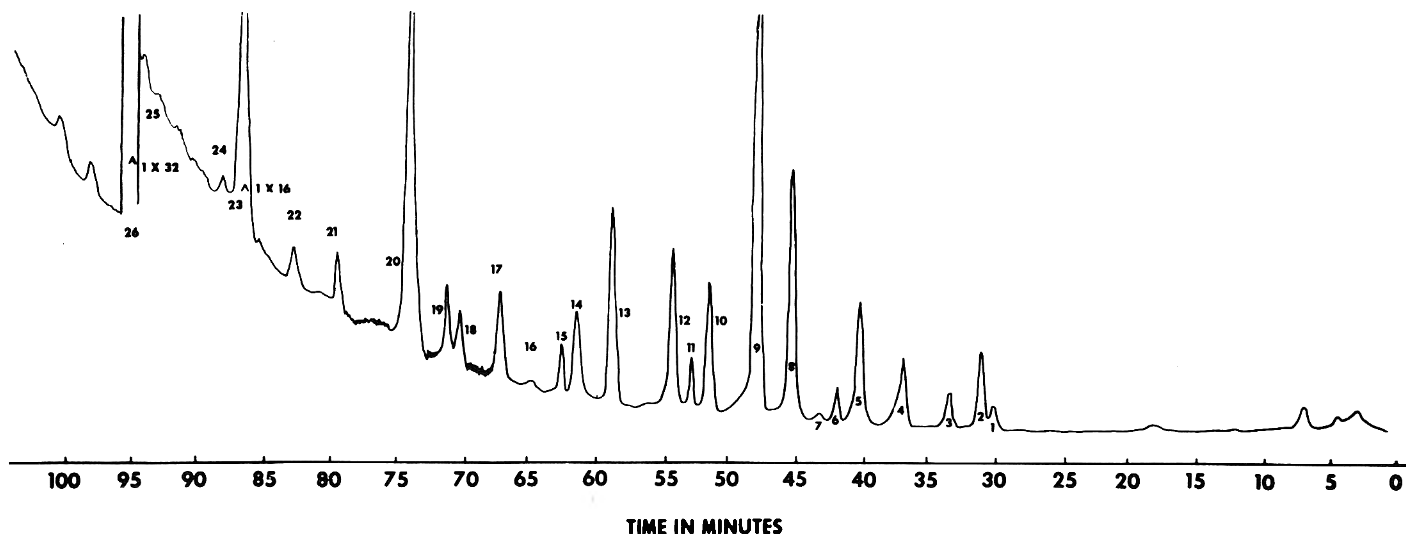

Fig. 1—Gas chromatogram of coconut meat volatiles on Carbowax 20 M column.

Table 1—Compounds found in the volatile flavor of coconut meat.

Compounds	I_E[1] Unknown	I_E[1] Authentic	Mass spectra identification	Odor description of the authentic compounds	Peak No. in Figure 1
Octanal	6.60	6.58	Positive	Heavy fruity, nutty, rancid	1
2-Heptanol	6.75	6.66	Positive	Over-ripe banana, slightly pleasant	2
Hexanol	7.10	7.00	Positive	Harsh, green, grassy, fruity	3
2-Nonanone	7.58	7.50	Positive	Pleasant, fruity	4
2-Octanol	8.00	7.66	Positive	Harsh, fruity	5
2-Nonanol	8.76	8.65	Positive	Slightly fruity, pungent	8
Octanol	9.13	9.03	Positive	Citrus, lemon, lilac, rose	9
2-Undecanone	9.63	9.56	Positive	--	10
Ethyl decanoate	10.00	10.00	Positive	Pleasant, fruity	12
2-Undecanol	10.73	10.55	Positive	--	13
2-Phenyl ethanol	12.54	12.55	Positive	Rose aroma	18
Benzothiazole	13.06	13.05	Positive	Harsh, medicinal	20
Delta-Octalactone	13.14	13.15	Positive	Buttery, coconut-like	20
Delta-Decalactone	15.31	15.35	Positive	Tropical fruit, coconut-like buttery	23
Delta-Undecalactone	16.40	16.39	Positive	Buttery, coconut-like	25
Dodecanoic Acid (Lauric Acid)	20.10	20.40	Tentative	Harsh, oily	--

[1] I_E = Relative retention time indices.

ence of secondary alcohols (C_7, C_8, C_9, C_{11}).

A small amount of benzothiazole was detected (part of peak No. 20). It has been reported in butter oil (Forss et al., 1967), stale milk (Arnold et al., 1966), and Swiss cheese (Langler et al., 1967). The origin and possible precursor of benzothiozole are not known.

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F. T. MARUYAMA
University of Minnesota, School of Home Economics, St. Paul, Minnesota 55101

IDENTIFICATION OF DIMETHYL TRISULFIDE AS A MAJOR AROMA COMPONENT OF COOKED BRASSICACEOUS VEGETABLES

SUMMARY—Aroma, retention time and infra-red spectra of volatile components from four vegetable varieties of Cruciferae Brassica oleracea *were examined. Components were separated by gas-liquid chromatography and characterized by differential chromatograms from a dual-detector system. Dimethyl trisulfide was identified as a major aroma component in cooked Brassicaceous vegetables. A mechanism for dimethyl trisulfide formation from precursors known to be present in cabbage is proposed. No evidence was found to indicate that new sulfur compounds develop as cooking time increases. It is proposed that the strong unpleasant aroma which is characteristic of overcooked Brassicaceous vegetables is due to the gradual loss of pleasant volatile components and resultant "unmasking" of the unpleasant sulfur components.*

INTRODUCTION

CRUCIFEROUS VEGETABLES are known to contain much organic sulfur. The characteristic flavor and odor of cooked vegetables of the genus *Brassica* have been attributed to volatile sulfur-containing compounds: hydrogen sulfide, dimethyl sulfide, dimethyl disulfide, allyl isothiocyanate, and allyl cyanide have been reported to be relatively abundant components of Brassicaceous vegetable volatiles (Simpson and Halliday, 1928; Dateo et al., 1957; Bailey et al., 1961; Hing and Weckel, 1964; MacLeod and MacLeod, 1968). Numerous other sulfur-containing compounds have been detected in Brassicaceous vegetable volatiles, but their importance with respect to vegetable flavor and aroma has not been established.

The amount of each compound present as a percentage of total volatile sulfur has been the criterion used to evaluate the role of volatile sulfur-containing compounds in cooked vegetable flavor and aroma. However, it is generally agreed that odor thresholds vary considerably for different chemicals. Volatile compounds present in trace amounts may contribute significantly to the characteristic flavor and aroma of fresh or cooked foods. This investigation was undertaken to evaluate the quality of volatile components from cooked Brassicaceous vegetables and to identify the chemical structure of important components. Aroma, retention time, differential chromatogram, and infra-red spectra of volatile components from four Brassicaceous vegetables were examined. Gas-liquid chromatography, using a combination of flame-ionization and electron-capture detection methods, was used to separate and characterize components. The flame-ionization detector responds almost equally to all organic compounds while the electron-capture detector is sensitive only to those compounds with electron affinity such as conjugated carbonyls, organic nitriles, and sulfur-containing compounds (Lovelock, 1963). Thus, a differential analysis of volatile components is made possible by use of the two-detector method. Moreover, since material passing through the electron-capture detector is not oxidized, the effluent can be evaluated for aromatic quality or collected for further identification.

EXPERIMENTAL

Materials

Fresh Brussels sprouts, cabbage, broccoli, and cauliflower were purchased at local retail markets and used within 3 days. The vegetables were trimmed, rinsed with water and cut into pieces for boiling: Brussels sprouts in quarters, cabbage in ½" shreds, and broccoli and cauliflower in 1" florets. 250g of vegetable with 1 liter of boiling distilled water were placed in a 2 liter boiling flask fitted with a simple distillation head and rapidly brought back to boiling. Distillates were collected for 5 min periods at −15°C.

Dimethyl trisulfide was synthesized (by Dr. Robert J. Petersen, Northstar Research and Development Institute, Minneapolis, Minnesota), according to the method described by Westlake et al. (1950), from dimethyl disulfide and sulfur using dibutylamine as a catalyst. Other sulfur-containing compounds were obtained from Eastman Organic Chemicals Company and used without further purification as reference standards.

Apparatus

A Beckman GC-2 gas chromatograph with hydrogen-flame detector and a Barber Colman model 5060 Chromatograph with electron-capture detector were used. Stainless steel ¼" o.d. by 6' columns packed with 60/70 mesh diatomaceous earth (Anakrom ABS) coated with 10% Carbowax 20 M were used in both chromatographs at 70°C. Helium with a flow rate of 70 ml per min was the carrier gas in the Beckman system and attenuation 5×10^2. Nitrogen was used in the Barber Colman system with attenuation 30×10^2 and detector voltage 35–55 D.C.

Infra-red spectra were analyzed in a Perkin-Elmer 521 Grating infra-red spectrophotometer with a micro attachment, using the 0.75 mm die for KBr pellets.

Procedure

Distillates from the boiling vegetables were collected during the initial 5 min of boiling and again between the 20th and 25th min and between the 40th and 45th min. 10 ml of vapor were drawn into a Hamilton gas tight syringe from above a 10 ml portion of vegetable distillate held 15 min at 75°C in a foil capped 250 ml Erlenmeyer flask, and injected into the chromatographs. Odors were evaluated by the investigator as the vapor components emerged from the electron capture detector and descriptive terms applied. These terms and the intensity of the odors were confirmed informally by 2–3 other laboratory personnel during replicate runs. Formal odor panel study of collected material was not done. The separate chromatograms from the 2 chromatograph systems were normalized by using an adjustment factor determined daily from the relative retention times of a reference compound, usually ethyl disulfide, in the 2 systems. Distillates from at least 6 retail lots of each vegetable were analyzed chromatographically and odor evaluations were replicated 3 times by the same observer. Composite chromatograms were then drawn from the replications.

For comparison of infra-red spectra volatile components were collected as they emerged from the electron-capture detector in 10 cm lengths of 3 mm o.d. pyrex tubing. A 1" thick piece of dry ice with a 3 mm diameter hole drilled in the center was used as a condenser. The ends of the tube were sealed in an oxygen flame and the samples were stored frozen until analyzed, usually within a week.

RESULTS & DISCUSSION

THE NORMALIZED composite chromatograms of volatiles from vegetable distillates collected during the initial 5 min of boiling are presented in Figures 1 to 4.

A significant feature of the chromatograms of all four vegetables was peak a occurring at 3.2 min accompanied by intense unpleasant aroma and peak b occurring at 30 min accompanied also by

Table 1—Retention time of sulfur compounds.

Compound	Retention time (minutes)
dimethyl sulfide	1.0
dimethyl disulfide	5.0
allyl isothiocyanate	27.5
diethyl disulfide	11.2
ethyl sulfide	2.0
n-butyl sulfide	15.5
n-butyl isothiocyanate	32.0

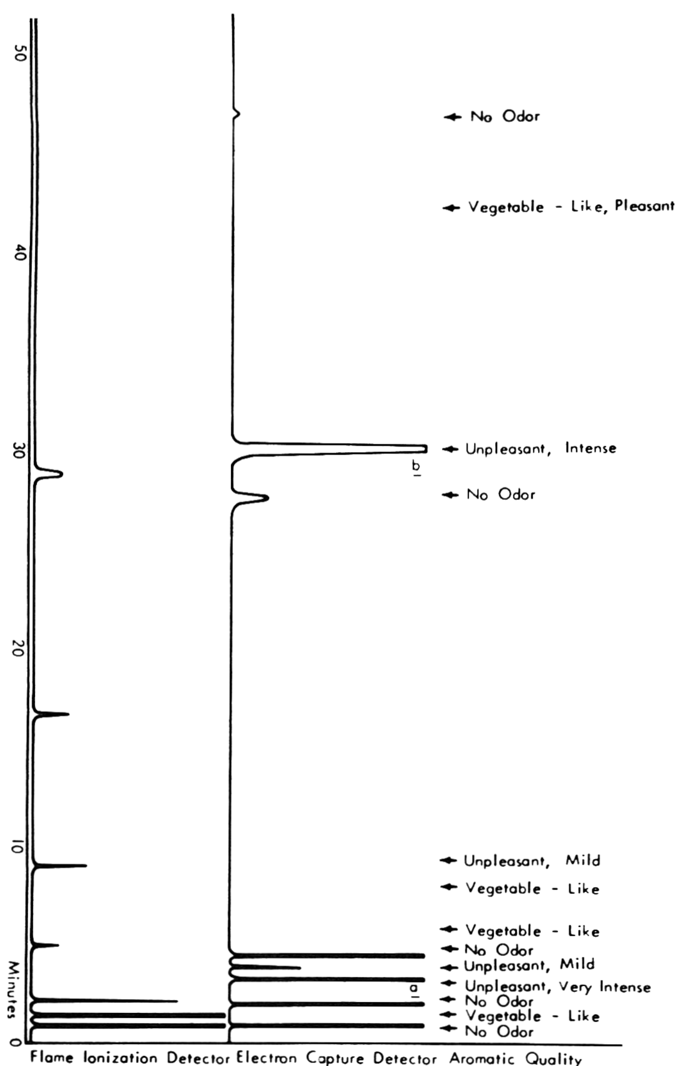


Fig. 1–Chromatograms of vapor from boiled Brussels sprouts.

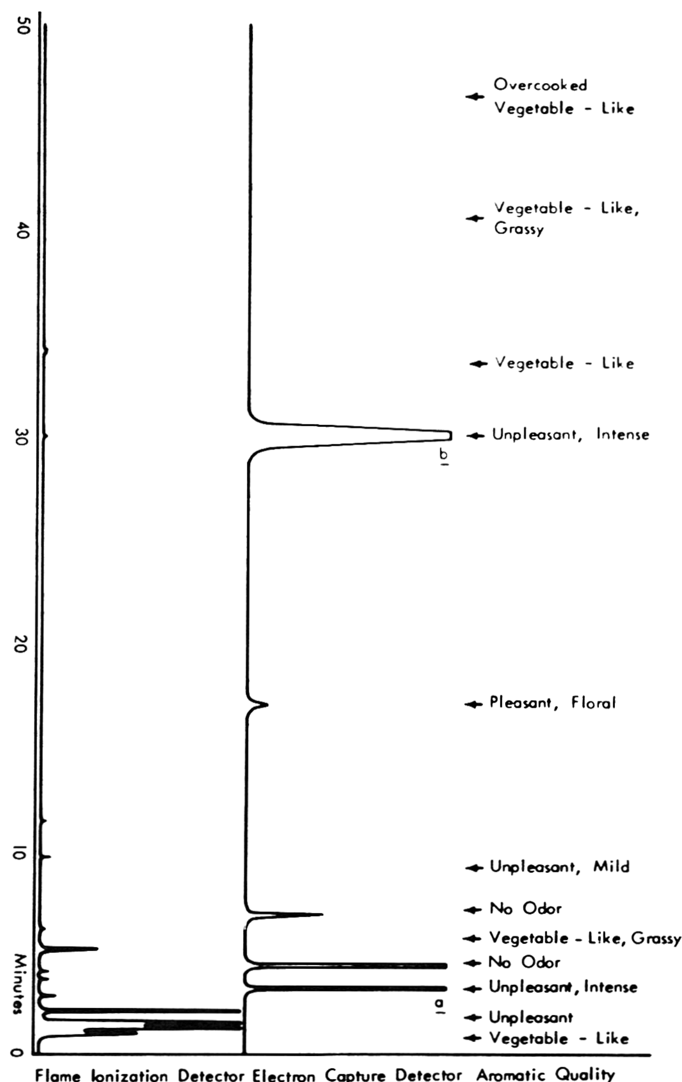


Fig. 2–Chromatograms of vapor from boiled broccoli.

intense unpleasant aroma. Because of their intense unpleasant aroma, the compounds responsible for peaks a and b were considered important aroma components and further study was done in order to establish their chemical identity. Comparison of retention times with those of selected organic sulfur-containing compounds (Table 1) showed that the compounds represented by peaks a and b were not those commonly attributed to Brassicaceous vegetables.

Absence of peaks a and b in the flame-detector system chromatogram indicated that the compounds responsible for these peaks had an unusual electron affinity which activated the electron-capture detector but failed to activate the flame-ionization detector because an insufficient quantity was present. An impurity in the dimethyl disulfide standard with a retention time and aromatic characteristics similar to the component responsible for peak b was noted. The impurity was suspected to be dimethyl trisulfide as disulfides have been reported to disproportionate upon standing to trisulfides and monosulfides (Oaks et al., 1964). Dimethyl trisulfide was synthesized and compared to component b. The following characteristics were found: (1) aromatic quality was similar to that of component b; (2) retention time was identical; (3) infra-red spectra were identical when chromatographed and collected under similar conditions. Based on these properties, component b has been identified as dimethyl trisulfide.

The chemical identity of component a remains unestablished at this time. The relative sensitivities of the electron-capture detector and flame-ionization detector were found by Oaks et al. (1964) to be < 0.48 for mercaptans, < 0.04 for monosulfides and < 3.3 for saturated disulfides (relative sensitivity for dimethyl trisulfide = 240). The fact that component a was detected by the electron-capture detector but not by the flame-ionization system indicates that component a is not a mercaptan, monosulfide, or saturated disulfide. The short retention time of component a indicates that it is a low molecular weight compound.

Based upon retention time in the electron-capture system, the presence of allyl isothiocyanate in cabbage and Brussels sprouts volatiles was indicated but not in sufficient quantity to contribute to aroma. There was also indication in the flame-ionization system chromatogram that Brussels sprouts volatiles contain dimethyl disulfide, but under the conditions of this study, it was not an aromatically important component. Presence of dimethyl sulfide was not indicated in the chromatograms of volatiles from the four vegetables studied. This may be explained by the fact that the distillates were collected at −15°C and the highly volatile dimethyl sulfide may have been lost. Others (Bailey et al., 1961; MacLeod and MacLeod, 1968) who have reported dimethyl sulfide in cabbage

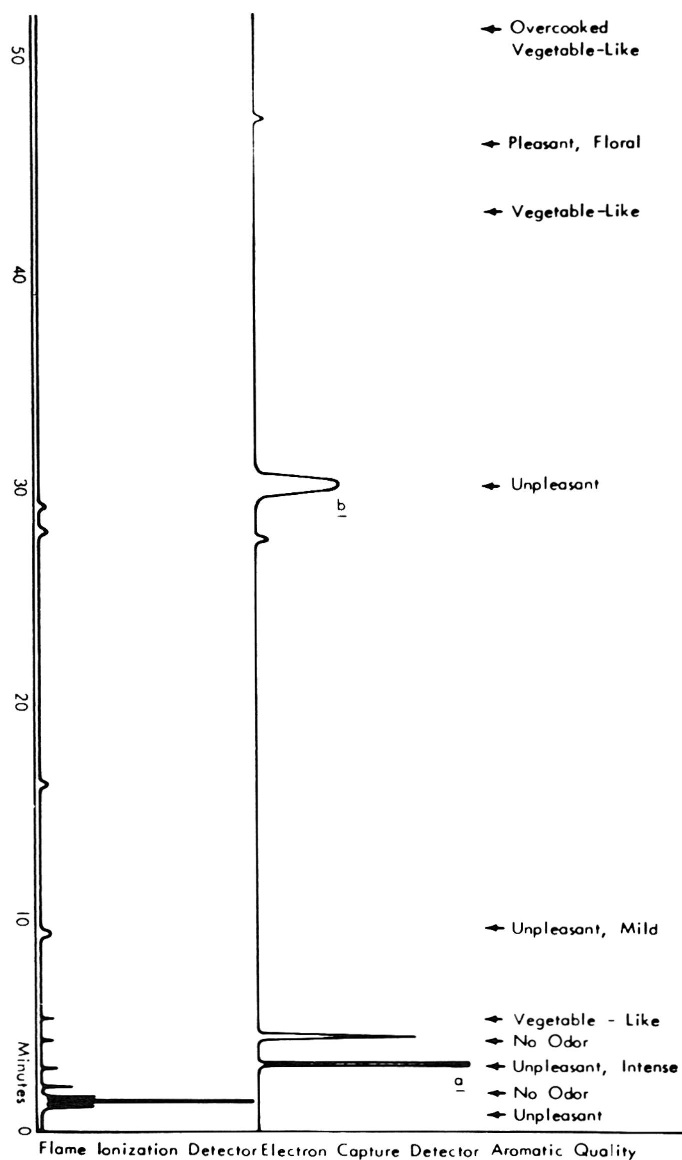


Fig. 3–Chromatograms of vapor from boiled cabbage.

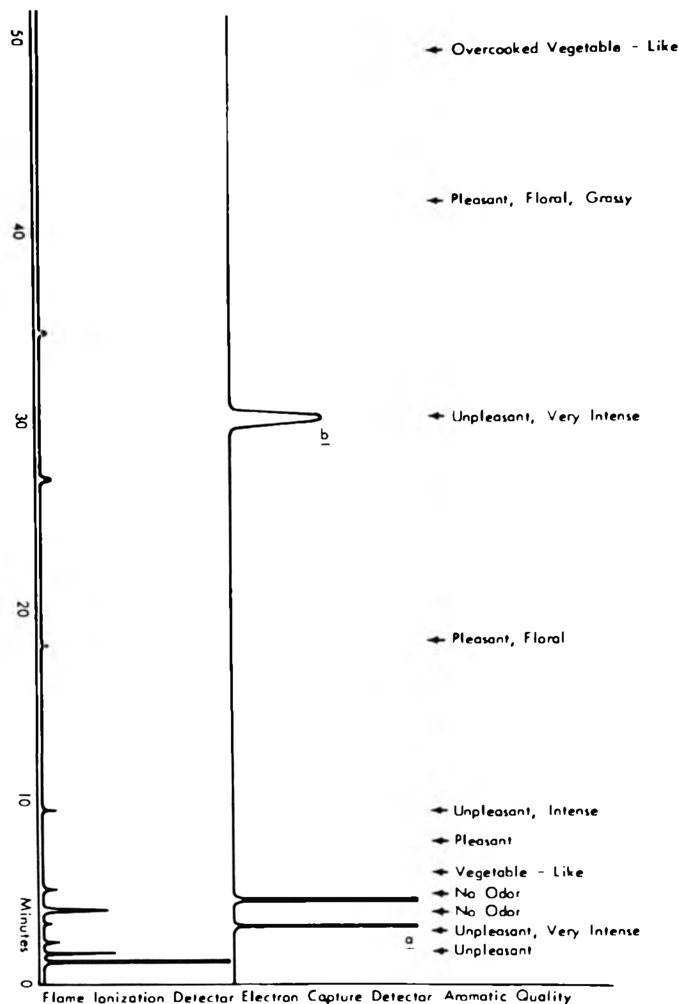


Fig. 4–Chromatograms of vapor from boiled cauliflower.

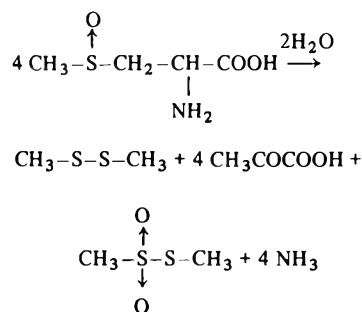
volatiles used liquid nitrogen to condense the volatiles and larger amounts of vegetables than were used in these studies.

No evidence was found for the development of new sulfur-containing compounds as cooking time increased; chromatograms of distillates collected at 20–25 min and at 40–45 min indicated no additional peaks. The amounts of all components except three diminished as cooking progressed as evidenced by decrease in peak size. The three exceptions were components a and b and a mildly unpleasant component appearing at 9+ min, detected only by the flame-ionization system. Dimethyl trisulfide is therefore an important aroma component because of its unpleasant quality and its continued volatilization during cooking of these vegetables. Thus it appears that the strong unpleasant aroma which is characteristic of overcooked Brassicaceous vegetables is due to the gradual loss of the more pleasant components and resultant "unmasking" of the unpleasant sulfur-containing components.

Although dimethyl trisulfide has been demonstrated to be an onion flavor component (Carson and Wong, 1961), its contribution to aroma of cooked vegetables of the cabbage family has been unrecognized, probably due to its presence in extremely low concentration. Using gas chromatography in combination with mass spectrometry, Bailey et al. (1961) detected in fresh cabbage dimethyl trisulfide as well as numerous isothiocyanates, sulfides, and disulfides; however, they did not evaluate the relative importance of the various sulfur-containing compounds with respect to fresh cabbage aroma. The results here not only confirm the presence of dimethyl trisulfide in cabbage volatiles but attribute to it an important role in cooked cabbage aroma because of its quality and intensity and its continued volatilization during prolonged cooking. In addition, these studies indicate that dimethyl trisulfide is a major aroma component in cauliflower, broccoli and Brussels sprouts. Quantitatively, dimethyl trisulfide is not as prominant as dimethyl disulfide, dimethyl sulfide, and other sulfur-containing compounds reported by others, but its aromatic role in cooked Brassicaceous vegetables appears to be important.

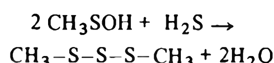
The formation of dimethyl trisulfide can be explained on the basis of precursors and reaction products already known to exist in cooked Brassicaceous vegetables. The mechanism of dimethyl disulfide formation from S-methyl-l-cysteine sulfoxide, a free amino acid first isolated and characterized by Synge and Wood

(1956), was proposed by Ostermayer and Tarbell (1960) as follows:



The reaction proceeds through an unstable intermediate CH_3SOH (sulfenic acid) forming $CH_3\text{-}\overset{\uparrow}{S}\text{-}S\text{-}CH_3$ (thiosulfinate) which rearranges to the disulfide and thiosulfonate.

From thermodynamic considerations, the presence of a trisulfide is not unexpected when the disulfide is present. The formation of dimethyl trisulfide may result from the unstable sulfenic acid reacting with hydrogen sulfide as follows:



The evolution of H_2S during cooking of cabbage is well known and has been reported to account for 25% of the total volatile sulfur of cabbage (Simpson and Halliday, 1928). Further experimentation is needed to test the validity of the proposed mechanism.

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KENNETH T. HARTMAN
Frito-Lay Research Department, 900 North Loop 12, Irving, Texas 75060

A RAPID GAS-LIQUID CHROMATOGRAPHIC DETERMINATION FOR CAPSAICIN IN CAPSICUM SPICES

SUMMARY–The trimethylsilyl (TMS) derivative of capsaicin was prepared for standard capsaicin, weighed portions of oleoresins and the residues obtained from the ethyl acetate extraction of red peppers. The derivative was quantitatively measured using a gas-liquid chromatographic (GLC) system equipped with an alkali flame detector approximately 25 times more sensitive to capsaicin than the unmodified flame ionization detector. Capsaicin recoveries from 5 fortified oleoresins ranged from 96.0–103.0%. Calculated and declared Scoville units showed good agreement (96.6–109.2%) for 6 capsicum spices. Vanillyl N-nonoylamide and vanillyl N-decoylamide were snythesized and the TMS derivatives prepared. Chromatograms obtained for these synthetic analogs of capsaicin showed that they would be detected by the described technique if used as adulterants in capsicum spices.

INTRODUCTION

THRESH (1876) first crystallized the pungent principle of capsicum spices and named it capsaicin. The structure of capsaicin was later shown by Nelson and Dawson (1923) to be the vanillylamide of isodecylenic acid.

Various methods have been employed for determination of the capsaicin strength of capsicum spices. In the United States, the most common means of estimating the pungency of capsicum spices is an organoleptic procedure introduced by Scoville (1912). However, the accuracy of this method is limited and often exhibits poor reproducibility between laboratories.

A number of ultraviolet and colorimetric spectrophotometric procedures have been reported for the determination of capsaicin. A comprehensive review of these techniques was published in the first (1959) and second (1964) report of the Joint Committee of the Pharmaceutical Society. It was found that these procedures do not differentiate between capsaicin and its synthetic analogs.

The amides of vanillylamine and fatty acids are made easily and cheaply and offer attractive adulterants for capsicum spices. Nakajima (1946) has reported the toxic properties of such synthetic amides. Likewise, these amides do not have the same pungency as capsaicin and cause poor correlation of chemical and organoleptic procedures.

A variety of techniques has been used in an attempt to detect the differences between the amides of capsaicin and those of saturated fatty acids. Todd (1958) reported a procedure capable of distinguishing between these amides; however, this technique would not distinguish between the vanillylamine amides of unsaturated fatty acids and capsaicin. Todd and Perun (1961) later reported a gas-liquid chromatographic method based on the detection of the methyl ester of the isolated fatty acid of capsaicin. Morrison (1967) recently reported the direct gas-chromatographic detection of capsaicin.

All of the previously listed methods involve lengthy isolation steps before determination of the capsaicin content of capsicum spices. A rapid and sensitive method has been developed which does not require isolation of capsaicin from capsicum oleoresins or the ethyl acetate extracts of ground red peppers. This procedure involves silanization of capsaicin to improve its gas-liquid chromatographic properties. The level of capsaicin

Table 1–Method reproducibility tests.

Capsicum spice	% Capsaicin found Assay 1	Assay 2	% Reproducibility[1]
African Capsicum oleoresin (1)	2.84	2.85	99.6
African Capsicum oleoresin (2)	1,83	1.82	100.5
Capsicum oleoresin (3)	1.76	1.77	99.4
Capsicum oleoresin (4)	1.69	1.61	105.0
Capsicum red pepper oleoresin (5)	0.960	0.990	97.0
Capsicum red pepper oleoresin (6)	0.465	0.470	98.9
Ground cayenne red pepper (1)	0.232	0.239	97.1
Ground cayenne red pepper (2)	0.127	0.120	105.8
Ground red pepper (3)	0.0592	0.0584	101.4
Chili powder (4)	0.00587	0.00568	103.3

$$1 = \frac{\text{\% Capsaicin by assay 1}}{\text{\% Capsaicin by assay 2}} \times 100.$$

Table 2–Recovery of capsaicin from fortified Capsicum oleoresins.

Capsicum spice	% Capsaicin added	% Capsaicin after fortification	% Recovery
African Capsicum oleoresin (2)	1.47	3.29	96.7
Capsicum oleoresin (3)	1.40	3.16	103.0
Capsicum oleoresin (4)	1.39	2.94	96.5
Capsicum red pepper oleoresin (5)	0.70	1.68	101.3
Capsicum red pepper oleoresin (6)	0.70	1.16	96.0

detection is greatly improved by using an alkali flame detector instead of a standard flame ionization detector.

EXPERIMENTAL

Preparation of capsaicin standard

A modification of the procedure of Todd (1958) was used for the isolation of capsaicin from capsicum oleoresin. The isolated capsaicin was further purified using the hexane crystallization procedure of Todd et al. (1961).

Isolation of capsaicin

600 ml of oleoresin (500,000 Scoville units) and 300 ml of 70% acetic acid were shaken and allowed to separate overnight. The acid layer was drawn off and washed 3 times with 150-ml portions of petroleum ether (30–60°C boiling range) and filtered; 600 ml of water was added to the washed acid layer and it was extracted 3 times with 150-ml portions of methylene chloride. The methylene chloride was combined, washed 3 times with water to remove traces of acid and evaporated to about 150 ml. The solution was cooled to room temperature, 1 g of Norit A (acid-washed) decolorizing carbon added and the preparation mixed by shaking. Decolorizing carbon was removed by filtering. Remaining color was further removed by twice repeating the decolorizing step. The solvent was evaporated and pure capsaicin obtained from the oily residue by crystallization from hexane.

Crystallization of capsaicin

A saturated solution of capsaicin in hexane was prepared by boiling the residue in hexane for several minutes (about 300 mg of amide to 100 ml of hexane). The saturated hexane was decanted from undissolved residue and added to an equal volume of boiling hexane. This mixture was allowed to stand until it cooled to room temperature and then stored overnight at 0°C. (Seeding the solution with a few crystals of capsaicin prior to cooling to 0°C prevents difficulties sometimes encountered during crystallization of the amide.) Capsaicin purified in this manner was found to have a purity of 98+% (m.p. 65.8–66.6°C).

Preparation of vanillyl N-nonoylamide and vanillyl N-decoylamide

Vanillylamine HCl was obtained commercially (Aldrich Chemical Co.) and the free base isolated by the procedure of Nelson (1919), except that the amine was dried for 48 hr at 1 mm in a vacuum desiccator as described by Challis and Clemo (1947). Nonoic and decanoic acids were obtained commercially (Applied Science Laboratories) and the acyl chlorides prepared by the procedure of Challis and Clemo (1947). Vanillyl N-nonoylamide and vanillyl N-decoylamide were prepared from these compounds with the method of Nelson (1919).

Apparatus

An F&M Model 1609 gas chromatograph equipped with an alkali flame detector was used for the determination of capsaicin. The modification of the flame ionization detector to an alkali flame detector was similar to that described by Giuffrida (1964), except that the platinum helix was coated with potassium chloride instead of sodium chloride. The helix was prepared from a single piece of 20-gauge platinum iridium wire and consisted of a lower loop made to slip over the jet orifice and an upper loop to be located, off center, above the jet orifice (Fig. 1). The lower loop was formed by wrapping around a 3-mm o.d. glass rod 3 times. The upper loop was formed by wrapping 4 times around a 5-mm o.d. glass rod. The upper loop of the helix was coated with a saturated aqueous solution of pure KCl (Corning saturated KCl solution for pH meters). This loop was dipped into the solution and moved slowly in and out of the flame of a Bunsen burner until all water had evaporated. Then the loop was held in the flame until the KCl melted. The helix was then removed from the flame and the KCl allowed to solidify. This procedure was repeated until a 1- to 1.5-mm layer of fused salt had deposited on the interior of the 5-mm i.d. loop of the helix. (Substantial loss of KCl occurred when water was evaporated too rapidly from the helix after it had been dipped in the coating solution. Very little loss occurred when the water was slowly evaporated from the dipped helix by passing it very slowly in and out of the flame of a Bunsen burner.) The coated helix was positioned in the F&M Model 1609 flame ionization detector as illustrated in Figure 1.

A 1-m by 4.5-mm i.d. aluminum column packed with 3% SE 30 on 80–100-mesh Gas Chrom Q was used with the following operating conditions:

Temperatures: (a) Column–210°C. (b) Detector–275°C. (c) Injector–275°C; Gas flow rates: (a) Carrier gas–50 ml per minute helium. (b) Air–400 ml per minute. (c) Hydrogen–Adjusted to provide a baseline current of 6×10^{-9} amps; Sensitivity: (a) Range–100. (b) Attenuator–4, 8 or 16. Chart speed–0.5 in. per minute.

Determination of capsaicin in capsicum oleoresins

Oleoresin (100–200 mg for high pungency levels or 200 to 400 mg for low levels) was accurately weighed into a calibrated 10-ml glass-stoppered graduated cylinder, the oleoresin diluted to 4.00 ml with dry tetrahydrofuran (Baker's Analyzed Reagent Grade–dried with anhydrous sodium sulfate) and 600 μliters each of trimethylchlorosilane and hexamethyldisilzane added in this sequence with mixing after addition of each reagent. An accurately measured aliquot (3–7 μliters) of the solution was injected into the gas chromatograph using a

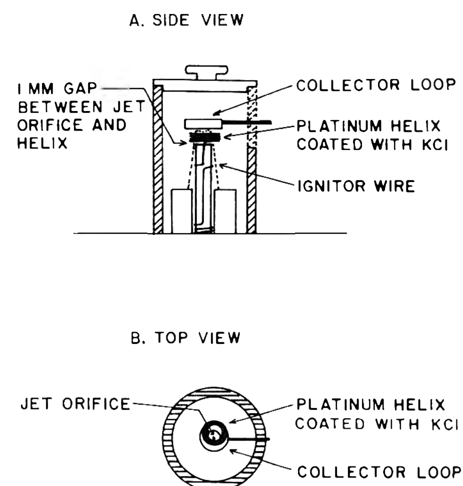

Fig. 1–Modification of the F&M Model 1609 flame ionization detector to an alkali flame detector.

Table 3—Comparison of calculated and declared Scoville units.

Capsicum spice	% Capsaicin found[1]	Scoville units Calculated	Scoville units Declared	% Agreement of Scoville units
African Capsicum oleoresin (1)	2.84	482,800	500,000	96.6
Ground cayenne red pepper (1)	0.236	40,120	40,000	100.3
Ground cayenne red pepper (2)	0.128	21,760	20,000	108.8
Ground red pepper (3)	0.0588	9,996	10,000	99.9
Chili powder (4)	0.00578	983	900	109.2

[1] Average of 2 determinations.

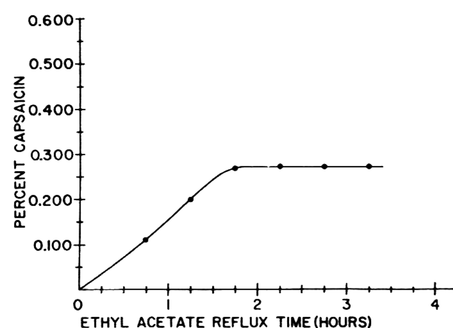

Fig. 2—Ethyl acetate extraction of capsaicin from ground Capsicum pepper.

10-μliter Hamilton syringe. After about 10 min an aliquot of a similarly prepared standard solution (0.387 mg capsaicin per ml) was injected. The capsaicin content of the capsicum oleoresin was determined by comparison of the peak area of the amide obtained from the sample and the standard solutions.

6 capsicum oleoresins were analyzed in duplicate. Portions of 5 of these oleoresins were fortified with capsaicin and again analyzed. Recovery values were calculated from the concentration of capsaicin determined before and after fortification.

Determination of capsaicin in ground capsicum red peppers

Ground red pepper, 5.00 g, was weighed into a 125-ml glass-stoppered boiling flask, 80 ml of ethyl acetate (Baker's Analyzed Reagent Grade) added and the mixture refluxed for 2.5 hr. The mixture was allowed to cool and the solvent decanted into a 100-ml glass-stoppered volumetric flask. The extracted ground pepper remaining in the boiling flask was washed with 2, 10-ml portions of ethyl acetate and each washing decanted into the 100-ml volumetric flask. The extract was made to volume with ethyl acetate and mixed. 5 g of anhydrous sodium sulfate was added to the solution, it was again mixed and the salt allowed to settle. The mixture was filtered, the first 5 ml of filtrate discarded and 50–60 ml of the filtrate collected. Solvent was removed from an accurately measured aliquot of the filtrate (25–50 ml) using a 50 ± 5°C water bath and reduced pressure. The residue was quantitatively transferred to a 10-ml calibrated glass-stoppered graduated cylinder with sufficient dry tetrahydrofuran to provide a final volume of 4.00 ml. The capsaicin TMS derivative was prepared and treated in the same manner as described for weighed oleoresin after dilution to 4.00 ml with tetrahydrofuran.

Calculation of Scoville units from capsaicin content

Todd (1958) reported a Scoville unit rating of 17,000,000 for pure capsaicin. This value was used to calculate the Scoville units for the capsicum spices.

RESULTS & DISCUSSION

CAPSAICIN analyses of 6 samples of capsicum oleoresins and 4 samples of ground capsicum red pepper showed a reproducibility range of 97.0–105.8% (Table 1).

A recovery range of 96.0–103.0% was obtained for 5 samples of capsicum oleoresins fortified with capsaicin. Since these oleoresins contained capsaicin prior to fortification, recovery values were obtained by analyzing portions of each sample before and after fortification (Table 2).

Scoville units calculated from the capsaicin content of 1 capsicum oleoresin and 4 samples of ground capsicum red pepper ranged from 96.6–109.2% of the declared values (Table 3).

During the analyses of the previously described samples it was observed that addition of the TMS reagents resulted in sample and standard solutions that were cloudy. A small amount of precipitate settled from preparations after 2–3 hr. This condition was especially evident with residues obtained from the ethyl acetate extraction of ground red pepper. To assure homogeneous solutions, each preparation was mixed by shaking prior to removal of an aliquot for gas chromatography. The periodic analysis of sample preparations allowed to stand for up to 8 hr after formation of the TMS derivative indicated that the precipitate does not increase the method error. Recovery values, as well as close agreement of calculated and declared Scoville units, also indicated that the formation of the precipitate had no significant effect on the method's accuracy. Additional tests showed that the TMS reagent can tolerate 500 mg of oleoresin or the residue obtained from the ethyl acetate extract of ground capsicum red pepper without affecting the accuracy of the method.

All analytical results were based on alternately injecting a sample and standard TMS capsaicin solution at 10-min intervals. Several injections each of sample and standard solution were made prior to each day's analysis to assure optimum column performance.

The extraction rate of capsaicin from ground capsicum red pepper during refluxing with ethyl acetate is shown in Figure 2. These data indicate that a 2.5-hr reflux time is sufficient to quantitatively extract capsaicin.

To obtain the maximum response for capsaicin with the alkali flame detector, tests were conducted to determine the optimum position of the potassium chloride-coated helix. The position of the helix shown in Figure 1 provided the maximum sensitivity for capsaicin. The sensitivity of the alkali flame detector was found to be about 25 times the capsaicin response of the standard F&M Model 1609 flame ionization detector.

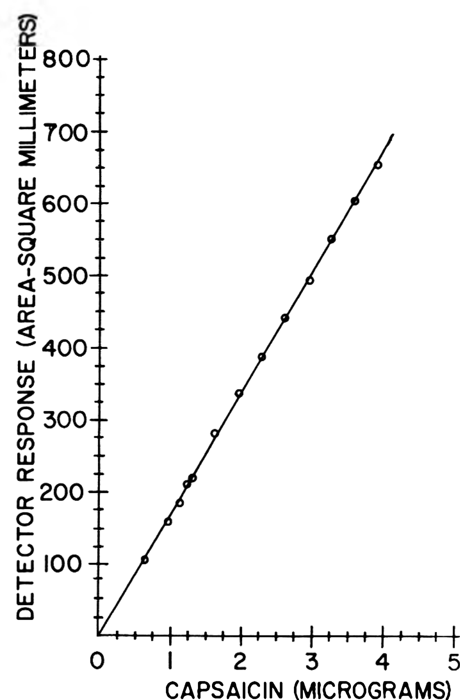

Fig. 3—Linearity plot for the alkali flame detection of the TMS derivative of capsaicin.

A plot of the alkali flame detector response versus quantity of capsaicin injected showed a linear relationship over the range of 0.5–4 μg (Fig. 3).

Standard capsaicin used for quantitation was isolated and purified as previously described. This standard was found to have a purity of 98.2% by the Spectrophotometric Difference Method as described in the second report of the Joint Committee of the Pharmaceutical Society

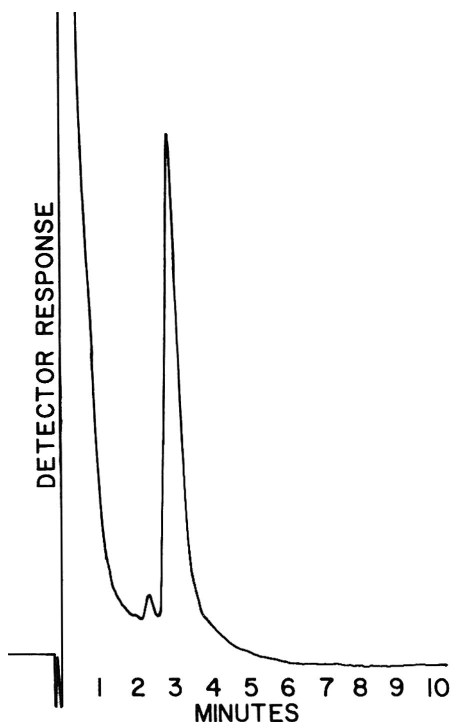

Fig. 4–Typical chromatogram for the TMS derivative of standard capsaicin. 1-m by 4.5-mm i.d. aluminum column packed with 3% SE 30 on 80–100-mesh Gas Chrom Q. Column: 210°C; detector: 275°C; injector port: 275°C; flow-rate: 50 ml per minute helium; sensitivity: 3×10^{-9} amps full scale deflection. Sample size injected: 3.8 µliters representing 0.387 mg capsaicin per ml.

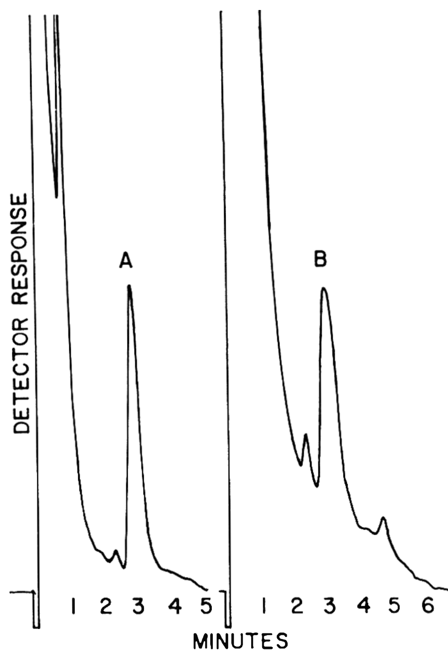

Fig. 5–Typical chromatograms for Capsicum oleoresin and the residue from the ethyl acetate extraction of ground Capsicum red pepper. GLC conditions same as listed in Figure 4, with the exception of sensitivity. Chromatogram A: Oleoresin (287,000 Scoville units). Sample size injected: 6.9 µliters containing 0.0253 g oleoresin per ml. Sensitivity: 6×10^{-9} amps full-scale deflection. Chromatogram B: Ground red pepper (10,000 Scoville units). Sample size injected: 5.9 µliters representing 0.240 g of pepper per ml. Sensitivity: 3×10^{-9} amps full-scale deflection.

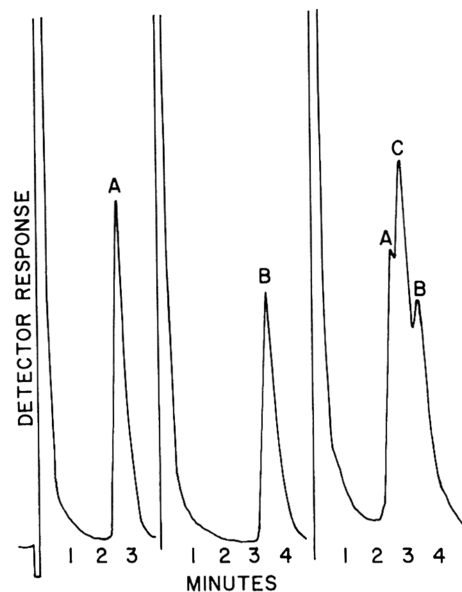

Fig. 6–Chromatograms of the TMS derivative of vanillyl N-nonoylamide and vanillyl N-decoylamide. GLC conditions, same as listed in Figure 4. Chromatogram A: 3.8 µliters representing 0.375 mg vanillyl N-nonoylamide per ml. Chromatogram B: 3.8 µliters representing 0.267 mg vanillyl N-decoylamide per ml. Chromatogram ACB: 5.7 µliters representing 0.096, 0.184 and 0.067 mg, respectively, of vanillyl N-nonoylamide (A), capsaicin (C) and vanillyl N-decoylamide (B) per ml.

(1964). A typical chromatogram of the TMS derivative of capsaicin standard is illustrated in Figure 4. 2 peaks were evident on the chromatogram. 1 peak was found to have an area representing 2.5% of the other peak area. The small peak had a relative retention time of 0.84 to the large peak. A similar chromatogram was obtained for a second capsaicin standard (93.4% purity) prepared by the procedure of Suzuki et al. (1957). Calculations for the capsaicin content of capsicum spices were based only on the area of the large peak (capsaicin); therefore, the identification of the small peak was not determined. However, the relative peak area obtained for the compounds using the nitrogen-sensitive detector, isolation techniques used to prepare the tested standards, purity analysis of the 1 capsaicin standard (98.2%) and the relative retention time of the small peak to the large peak together indicated the possibility of a second amide in the standard closely related to capsaicin. These observations were found to agree with those reported by Todd and Perun (1961). They have indicated the possibility of a second amide in capsicum spices and suggested that the amide may be the fatty acid homologous to isodecylenic acid, but 1 carbon shorter.

Typical chromatograms for the capsaicin TMS derivative in capsicum oleoresins and the residue from the ethyl acetate extraction of ground capsicum red pepper are illustrated in Figure 5. These chromatograms also exhibit the large peak preceded by a smaller peak similar to that previously described for the standard capsaicin.

The GLC column used in the described method was tested to determine its ability to separate other TMS derivatives of amides closely related to capsaicin. Vanillyl N-nonoylamide and vanillyl N-decoylamide, 2 closely related analogs of capsaicin, were synthesized as previously described. These compounds showed melting points and pungency characteristics similar to those reported by Nelson (1919). These amides were chromatographed both individually and in a mixture with capsaicin. A relative retention time to capsaicin of 0.88 was obtained for vanillyl N-nonoylamide and 1.21 for vanillyl N-decoylamide. These data show that complete separation was not obtained for all 3 of the amides on a 1-m, 3% SE 30 GLC column. However, sufficient separation was achieved between capsaicin and the synthetic amides to indicate their presence in capsicum spices (Fig. 6).

GLC columns used for the described method have exhibited long life with good efficiency when the glass wool plug in the inlet end of the column was replaced periodically (after every 40–50 analyses). Continual accumulation of nonvolatile product material on the glass wool plug eventually resulted in peak broadening if not removed periodically from the column.

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A. LIFSHITZ, W. L. STANLEY and Y. STEPAK
Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel

COMPARISON OF VALENCIA ESSENTIAL OIL FROM CALIFORNIA, FLORIDA AND ISRAEL

SUMMARY—Samples of essential oils from California, Florida and Israel, all of Valencia orange, were analyzed for percent area in GLC of C_8, C_9 and C_{10} aldehydes, as well as for their total carbonyl content. It was found that the aldehyde patterns of the Florida and Israel oils are similar and are both different from that of the California oil. As for the carbonyl content, the 3 oils were found to be statistically different from each other.

INTRODUCTION

ORANGES of the Valencia species are grown in various countries of the world; the differences in soil, climate, etc., have an influence on the composition of the essential oil extracted from the fruit. Stanley and Vannier (1959) have shown that there exists a marked difference between the oil of lemons grown in California and in Arizona, and even between the oil of lemons, grown in different parts of California. On the occasion of Dr. Stanley's visit to Israel, he brought with him results of analyses performed on Valencia essential oils of California and Florida origin. It was found valuable to compare his findings with analyses of Israel oil of the same Valencia species.

EXPERIMENTAL

EACH OF the 18 California and 19 Florida samples examined was a composite of daily samples taken from commercial production of 2 processing plants in the respective states, for each working month during 1959, 1960 and 1961. They were examined for the following parameters:

a) Following separation, according to the Girard method (Stanley et al., 1961), the aldehyde fraction was injected into a gas chromatograph and the percent area from the total aldehyde area of octanal, nonanal, decanal, neral, geranial and 2 unidentified aldehydes was measured.

b) The terpene fraction was injected into a gas chromatograph and the percent area from the total terpene area of α-pinene, sabinine, myrcene and d-limonene was measured.

c) Total carbonyls, according to the method of Fritz et al. (1959).

d) Total hydrocarbons, according to the method of Ikeda et al. (1962).

e) Citral content, according to the method of Stanley et al. (1958).

The 29 Israel oils were chosen statistically from the 300 samples taken from 10 factories situated in different areas of Israel during 1968. They were analyzed by direct injection into a

Table 1—Essential oils from Florida and California.

	Florida			California		
Parameters examined	Average	Standard deviation	Coeff. of variation	Average	Standard deviation	Coeff. of variation
Percent area of octanal[1]	35.43	5.58	0.158	28.89	5.84	0.202
Percent area of nonanal[1]	6.08	1.10	0.181	5.36	1.09	0.203
Percent area of decanal[1]	41.43	5.48	0.133	47.85	5.55	0.115
Percent area of unknown aldehyde[1]	0.38	1.28	3.37	1.74	2.57	1.47
Percent area of undecanal[1]	0.59	0.45	0.73	2.07	1.39	0.67
Percent area of unknown aldehyde[1]	0.16	0.38	2.37	0.60	1.25	2.07
Percent area of dodecanal[1]	5.15	1.64	0.32	7.58	2.20	0.29
Percent area of neral[1]	1.00	0.77	0.76	0.76	0.74	0.905
Percent area of geranial[1]	9.55	3.38	0.35	4.94	3.37	0.68
Total hydrocarbons (percent)	90.70	2.53	0.027	89.38	2.38	0.027
Percent area of pinene	0.10	0.16	1.60	0.26	0.21	0.81
Percent area of sabinene	0.09	0.18	1.95	0.17	0.23	1.35
Percent area of myrcene	1.26	0.48	0.38	1.27	0.38	0.305
Percent area of d-limonene	98.51	0.70	0.007	98.28	0.70	0.007
Total carbonyls (percent)	1.53	0.19	0.12	1.20	0.21	0.183
Percent citral	0.22	0.08	0.35	0.11	0.02	0.22

[1] Percent area from total aldehydes.

Table 2–Essential oils from Israel, Florida and California.

	Israel			Florida			California		
Property	Average	Standard deviation	Coeff. of variation	Average	Standard deviation	Coeff. of variation	Average	Standard deviation	Coeff. of variation
Percent area of octanal[1]	45.50	6.31	0.138	42.20	6.49	0.159	35.10	6.72	0.192
Percent area of nonanal[1]	7.90	0.64	0.081	7.45	1.16	0.156	6.53	1.31	0.201
Percent area of decanal[1]	46.60	3.13	0.06	50.35	6.10	0.12	58.37	6.90	0.118
Percent of total carbonyl	1.37	0.167	0.122	1.53	0.19	0.124	1.20	0.21	0.175

[1] Percent area from octanal + nonanal + decanal.

Table 3–Analysis of variance of total carbonyls.

	Florida	Israel	California
Average	1.53	1.37	1.20
Standard deviation	0.19	0.167	0.21
No. of samples	19	29	18
F ratio	9.72		
		8.75	

Table 4–Analysis of variance of percent area of octanal.

	Florida	Israel	California
Average	42.2	45.5	35.1
Standard deviation	6.72	6.30	6.72
No. of samples	19	29	18
F ratio	2.98		
		28.8	

Table 5–Analysis of variance of percent area of nonanal.

	Florida	Israel	California
Average	7.45	7.90	6.53
Standard deviation	1.15	0.64	1.31
No. of samples	19	29	18
F ratio	3.03		
		17.8	

gas chromatograph without previous separation and were examined only for the percent area in GLC of octanal, nonanal and decanal, as well as the total carbonyl, as a statistical factor analysis of the American oils has shown that only these 4 parameters are valuable for comparison.

RESULTS & DISCUSSION

RESULTS of the American oils examined for 16 parameters are shown in Table 1.

To determine the parameters most suitable for comparison, a statistical evaluation of the American results was made. The criteria were:

a) Parameters exhibiting the smallest coefficient of variation

b) Parameters not season-dependent

c) Parameters giving fairly high results

d) Parameters not strongly correlated to other parameters, so that independent factors are measured.

It was found that only 4 parameters are adequate. Table 2 gives the particulars of the comparison.

As the analyses had been performed in different years and at different places using different instruments and working conditions, the percent area in GLC and not the actual areas were compared.

Analysis of variance of the results is shown in Tables 3, 4 and 5. It can be seen from Tables 4 and 5 that Israel and Florida oils are similar in their C_8, C_9 and C_{10} aldehyde ratios and that both oils are significantly different from the oil of California. These facts should be further commented on by agricultural chemists as to what factors are dominant, when influencing the properties of the fruit constituents. The climate in Israel is considered much more similar to that of California than of Florida, yet the essential oil pattern, as shown, is more similar to that of Florida.

As for the total carbonyl content, Table 3 shows that the oils of different states are diffferent. The Florida oil is richest in carbonyl compounds, whereas the California is poorest, with the Israel oil falling between them. These values are not necessarily indicative of the fragrance or the economical value of the essential oil.

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SARAH T. ROSS, MURIEL V. BRADLEY and JANET A. OKA
Department of Pomology, University of California, Davis, California

CYTOLOGICAL EFFECTS OF JUICE OR PUREE FROM IRRADIATED STRAWBERRIES

SUMMARY—Vicia faba *roots were treated 4 hr with undiluted juice or puree from strawberries irradiated with 200 or 400 Krad of gamma radiation from* Co^{60} *and from control berries. 200 Krad would be the highest feasible dose for strawberries as a fungicidal treatment to extend shelf life without causing unfavorable changes in fruit quality. The differences between percentages of abnormal anaphases after treatments with juice or puree from irradiated and control berries were not significant at the 1% level ("t" test) in all the 7 experiments with 200 Krad as the radiation dose, and in 6 of 7 experiments when the dose was 400 Krad. In 1 experiment with a 400-Krad dose, the difference between percentages of abnormal anaphases was barely significant at the 5% level of confidence.*

INTRODUCTION

THE FEASIBILITY of irradiating fruits or fruit juices to prolong shelf life has been investigated for several years. Of all the fruits studied in this respect, the strawberry shows the greatest promise for practicable use of and economic benefit from irradiation as a fungicidal treatment. The required fungicidal dose, 200 Krad of gamma radiation, does not cause adverse changes in fruit quality, and extends shelf life by 3–6 days (Sommer and Maxie, 1966).

As a consequence of projected use of irradiation as a food preservative, concern has been expressed that radiomimetic substances might be formed in irradiated foods and be injurious to the consumer. A number of reports have subsequently dealt with effects on chromosomes of irradiated food substances, chiefly carbohydrates or foods containing large quantities of them. Only 2 studies, to our knowledge, have been concerned with effects of juice from irradiated fruits or of irradiated fruit juice. Chopra et al. (1963) reported chromosome aberrations in 14.3% of barley root tip cells and in 8.5% of onion root tip cells after 4 hr of treatment with orange juice irradiated with 200 Krad of gamma radiation, followed by 24 hr on water-soaked filter paper. They noted also 9.1% aberrant mitoses in barley roots treated 4 hr with irradiated (200 Krad) apple juice, then 24 hr on wet filter paper. Mäkinen et al. (1967) reported 8.0% of chromosome breaks in 300 cells after 4 hr of treatment with juice from pineapples given a dose of 100 Krad of gamma radiation, and 17.3% chromosome breaks when the radiation dose was 500 Krad. All the above percentages are above the respective control values. Since commercial irradiation of the strawberry is technically promising, it seemed desirable to obtain evidence concerning possible radiomimetic agents in irradiated strawberries. Reported here are the effects on *Vicia faba* root tip chromosomes of treatments with juice or puree from irradiated and nonirradiated strawberries.

MATERIALS & METHODS

STRAWBERRIES, obtained from a commercial wholesale source, represented samples throughout most of the fruiting season in Central California. Aside from time in transit to the laboratory, to and from the radiation facilities and during irradiation, the berries were kept at 32°F. The radiation dosages were 200 and 400 Krad. Conditions of irradiation were: gamma rays from Co^{60} delivered at a rate of 5.5 Krad/min by an AEC Mark II irradiator, temperatures of 70–76°F in the chamber and air circulated through it at a rate of 500–600 ml/min. The experiments were completed within 24 hr after irradiation ended. After irradiation, berries were washed, hulled and pureed in a blender. Juice was obtained by centrifuging the puree at 4,500 rpm for 1 hr, then centrifuging the supernatant at 12,500 rpm for ½ hr.

Seedlings of *V. faba* cv. Bell were grown under aseptic conditions at 23°C. 12 seedlings were suspended over each treatment dish with primary roots immersed in 30 ml of undiluted, unsterilized juice or puree. The juice was too viscous to be filter-sterilized and autoclaving would have altered some natural properties. The dishes were kept in a 23° incubator during the 4-hr treatments. Immediately before and again after treatment, the pH and soluble solids of each solution were determined. The root tips were fixed for 24 hr in 3:1 95% ethanol-glacial acetic acid. Fixation numbers were coded. After fixation the root tips were hardened at least 5 days in 70% ethanol. Untreated root tips provided data for the base control.

The pH of the puree and juice was relatively low, ranging from 3.5 to 4.15 for different lots of berries. Previously, we had found that sucrose solutions adjusted to similar low pH values cause more damage to chromosomes than at pH 4.5 and higher (Bradley et al., 1968). We wished to determine, therefore, how berry juice adjusted to higher pH values would affect percentages of aberrant mitoses. Use of phosphate or citrate buffers was not desirable, for we found that when added to sucrose solutions, they damaged the root tips and increased chromosome abnormalities. Moutschen and Matagne (1965) had also reported unfavorable effects of such buffers on chromosomes. The effect of 1 N KOH was, therefore, tested as a means of increasing the pH of the berry juice. Such large quantities were required to raise the pH, however, that the resulting mixture bore little resemblance to the natural juice. The plan to test the effects of berry juice at higher pH values, was, therefore, abandoned. Because of

Table 1—Percentages of abnormal anaphases in Vicia faba *root tips treated 4 hr with juice or puree from irradiated (200 Krad) and control strawberries.*

Experiment No.	pH	% Soluble solids	Radiation dosage	Mean No. anaphases per replicate	Mean % abnormal anaphases per replicate	Mean no. anaphases per replicate	Mean % abnormal anaphases per replicate
				Irradiated berries		Control berries	
				Juice			
1	3.65	7.0	200 Krad	292	11.2	309	10.2
2	3.72	10.77	200 Krad	665	8.7	579	8.2
3	4.15	8.5	200 Krad	384	9.0	346	8.5
4	3.65	9.0	200 Krad	259	12.5	375	9.8
5	3.5	10.0	400 Krad	332	12.8[1]	474	7.6
6	3.5	9.35	400 Krad	166	8.3	183	11.0
7	3.65	8.6	400 Krad	198	11.2	238	8.8
8	3.55	8.51	400 Krad	186	6.5	223	8.8
				Puree			
9	3.65	9.93	200 Krad	352	5.3	289	8.0
10	3.7	10.68	200 Krad	222	9.6	293	10.6
4[2]	3.65	9.0	200 Krad	236	8.2	346	8.0
6[2]	3.5	9.35	400 Krad	166	9.0	151	12.2
7[2]	3.65	8.6	400 Krad	122	10.4	155	10.0
8[2]	3.55	8.51	400 Krad	119	8.5	96	7.5

Base control: 9% abnormal anaphases.

[1] Significantly different from the control at the 5% level.

[2] The same shipment of berries was used for the juice experiment having the same number.

the low pH of berry juice and puree, it was necessary to obtain data concerning chromosome aberrations from propionocarmine-stained anaphases instead of Feulgen-stained metaphases, which would have enabled critical analyses of aberration types and frequencies per cell.

After root tip treatment with low pH solutions, the chromosomes cannot be contracted by any of the usual contracting agents, nor can they be stained by the Feulgen method. To offset in some measure the variability among root tips in percentages of naturally occurring abnormal anaphases, the terminal 1-mm regions of all 12 roots per treatment were squashed in a few drops of stain and mixed into a slurry. Thus, material from all 12 root tips was represented on each of 12 slides. All anaphases in 6 preparations (more than 100 per treatment, except in 1 case) were scored as to presence or absence of chromosome abnormalities. The aberrations included chromosomes which were not on the spindle, bridges, and fragments of different sizes but most frequently the large satellite typical of the largest *Vicia* chromosome. No anaphases showing signs of undue pressure in squashing were included in the scores.

Each experiment included 4 or 5 replicate treatments with irradiated and nonirradiated berry juice or puree, or both.

RESULTS & DISCUSSION

DATA from all the experiments are shown in Table 1. The percentages of abnormal anaphases after treatments with juice and puree from irradiated berries were compared to those from the corresponding controls by the "t" test. When the radiation dose given the berries was 200 Krad, the differences between the 2 percentages were not significant at the 1% level of confidence in the 7 experiments with juice or puree. When the radiation dose was 400 Krad, the differences in percentages were not significant at the 1% level in 6 of the 7 experiments. In experiment No. 5, however, the difference in percentages of abnormal anaphases after treatments with juice from irradiated and from control berries was barely significant at the 5% level. Results of these experiments thus indicate that there should be no more cause for concern about the possibility of chromosome-breaking substances being present in strawberries irradiated with 200 Krad (the dose that would be used commercially to prolong shelf life) than about their presence in nonirradiated strawberries.

The pH and soluble solids of juice and puree from berries of the same lot were identical. However, juice and puree of different lots varied considerably in pH and soluble solids, probably reflecting differences in environmental conditions during maturation and ripening. Irradiation of berries caused no change in pH or percent soluble solids of juice or puree.

During the treatment period the roots induced very little change in pH of the strawberry juice or puree from either irradiated or control berries, rarely as much as 0.05 unit. This was in contrast to pH changes during 4-hr treatments of *V. faba* root tips with irradiated (200 Krad) and control 2% sucrose solutions, adjusted with KOH or HCl before treatment to pH values comparable to those of the berry juice (Bradley et al., unpublished data). In those solutions the root tips raised the pH by 1/2 to 2 or 3 units. The strawberry obviously contains an effective buffering system. In spite of the root tips' inability to raise the pH of juice or puree toward a value ordinarily more favorable for growth, the roots were in much better condition after treatment with berry juice or puree than after low pH sucrose treatments.

No correlation was found between percentages of abnormal anaphases with either pH or soluble solids levels (Table 1). Thus, the diversity in percentages of abnormal anaphases among like experiments cannot be attributed to differences in pH or soluble solids. It seems to be related to differences in incidence of abnormal anaphases in untreated root tips, as shown by the following: In untreated root tips from the shipment of seeds used in these experiments (the base control), the range of abnormal anaphases varied from 3–18%, with both mean and mode at 9.0%. The means of the mean percentages of abnormal anaphases per replicate for all treatments with juice or puree from irradiated berries and for all controls were 9.3 and 9.2%, respectively. This is taken as evidence that the variability among percentages of abnormal anaphases is an expression of chance assortments of 12 root tips per replicate, each root conditioned to exhibit 3 to 18% abnormal anaphases. It would follow then that the root tips were affected only slightly, if at all, by differences in factors such as pH, soluble solids, irradiation of berries or 4-hr immersion in an alien medium.

The absence of evidence of radiomimetic substances in juice and puree from irradiated strawberries is thus contrary to the reports of Chopra et al. (1963) and Mäkinen et al. (1967) on effects of treatments with irradiated apple and orange juice, and with juice from irradiated pineapples, respectively. In experiments designed to show anaphases in earlier and perhaps more sensitive stages of the interphase when treatments began than the anaphases studied here, percentages of abnormal anaphases in treatments with juice from irradiated strawberries might be somewhat higher. Such experiments were attempted, but the roots were severely damaged by treatment plus post-treatment and would not have yielded reliable data. In any case, if radiomimetic substances sufficient to cause percentages of aberrant mitoses of the order reported by the above authors were present in irradiated strawberry juice, it would seem that they would have become apparent after the treatments alone, particularly when the radiation dose was 400 Krad.

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MARGARET L. ANDERSON and ELINOR M. RAVESI
USDC National Marine Fisheries Service, Gloucester Fishery Products Technology Lab, Gloucester, Massachusetts 01930

ON THE NATURE OF THE ASSOCIATION OF PROTEIN IN FROZEN-STORED COD MUSCLE

SUMMARY—Decrease in readily extractable protein (REP) paralleled by an increase in free fatty acid (FFA) occurred more rapidly in cod muscle during the first 8–10 wk of storage at −12°C and −18°C than during subsequent storage. After 32 wk, FFA appeared to be approaching a lower maximum at −18°C. Aggregated protein was extracted during the period of decrease in REP. It was similar in extraction and sedimentation properties to altered protein obtained from frozen-stored cod muscle in which FFA was formed largely during storage in ice. Increase in cohesiveness of the myofibrils was the only alteration in microstructure observed with phase microscopy. It was concluded that change to a more hydrophobic solvent environment due to FFA formation plays an important part in the rate change and in the level of FFA reached as a function of storage temperature. A model for the association process based on bonding potentials existing in the muscle on freezing and those developed during storage is presented.

INTRODUCTION

INVESTIGATION OF THE causes of frozen-storage-induced deterioration of fish muscle has been hampered by a number of difficulties. It was not possible, for example, to test independently the two main theories that had been proposed—the "salt denaturation" and the "lipid hydrolysis" theories (Connell, 1964)—since the effects on muscle proteins of continued exposure to concentrated salts in the liquid phase of frozen muscle could not be separated from the effects of exposure to free fatty acids (FFA) formed during storage. Another troublesome problem in finding the molecular basis of this type of deterioration was the lack of a method for studying protein that had become inextractable during frozen storage. It has now been shown that the deterioration process can be dissected (Anderson and Ravesi, 1969) and that altered protein can be extracted from muscle in which deterioration occurred (Anderson and Ravesi, 1970). Incidental findings of these studies suggested that the technique used to extract altered protein would be useful in studying the normal frozen-storage-induced deterioration process where lipid hydrolysis with FFA production is accompanied by a decrease in protein extractability. In these studies, muscle of varying FFA content was produced by aging it in ice, and the effects of holding it in the frozen state were determined after 11 mo storage at −29°C. A significant decrease in extractable protein occurred in muscle that had been aged in ice 1 day postmortem. Thus, deterioration had begun in this muscle; this deterioration was the normal frozen-storage-induced variety, since the FFA present (less than 50 mgs/100g muscle) had been produced largely during frozen storage. However, most of the protein in this muscle could be extracted with overnight extraction followed by additional blending. Moreover, aggregated protein was detectable in ultracentrifugal patterns of protein extracted under these conditions. It appeared, then, that the pathway to the association process in muscle in which FFA formation took place largely during frozen storage would be amenable to study.

This paper describes a study of the deterioration at −12°C and −18°C of 1-day-iced cod muscle using the reblending technique to extract altered protein. Deterioration at two temperatures was followed to look for changes in microstructure that might be temperature dependent, since it had been postulated previously (Anderson et al., 1965) that storage temperature would influence the geometry and distribution of the liquid pools where deterioration was taking place.

EXPERIMENTAL

MYOTOMES DISSECTED from fifteen 20–25 lb gutted cod, *Gadus morhua*, that had been held in ice 1 day after capture were pooled, mixed, weighed into 10g samples and packaged in threes as in a previous study (Anderson and Ravesi, 1969). They were placed in cartons in a room at −29°C for 12 hr and then stored at −12°C and −18°C.

Procedures for preparing protein and lipid extracts, assaying protein and FFA contents, obtaining ultracentrifugal patterns of protein extracted and photomicrographs of residues of centrifuged homogenate were the same as in previous studies (Anderson and Ravesi, 1969 and 1970).

Protein readily extractable in 0.05 and 0.5 ionic extractants and protein extractable after prolonged (overnight) extraction in 0.5 ionic extractant with and without reblending were

Table 1—Protein contents of 0.05 ionic extracts and ratios of FFA to protein extracted from cod muscle during storage at −12°C and −18°C.

Weeks in	Storage at −12°C		Storage at −18°C	
Storage	Protein[1]	Ratio[2]	Protein[1]	Ratio[2]
0	.701	5.6		
1			.663	18.6
2	.637	16.3		
3	.557	22.1	.665	27.7
4	.567	28.4		
5	.534	26.4	.611	31.8
6	.531	22.8		
7	.545	23.3	.610	38.0
8	.566	26.0		
9	.537	22.7	.638	36.3
10	.505	21.6		
11	.552	16.5	.603	24.0
12	.491	21.0		
13			.578	19.4
15	.472	11.2	.587	
17	.488	13.3	.535	17.0
19			.545	16.7
21	.504	13.3	554	12.1
23			.555	22.9
28	.528	19.5	.490	12.7
32	.438	12.1	.511	15.6

[1] g protein N/100g muscle extracted.
[2] Mg FFA/g protein N extracted.

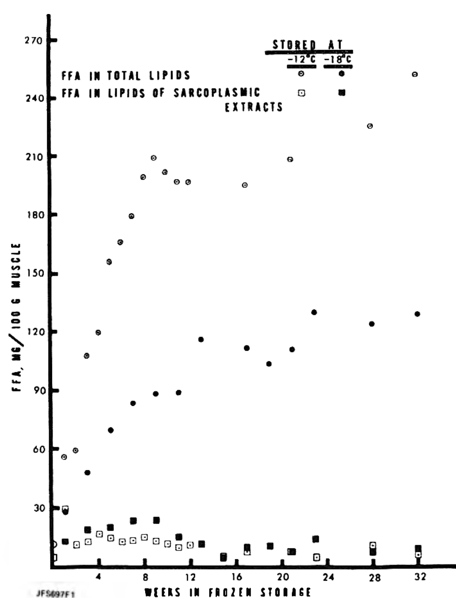

Fig. 1–FFA contents of cod myotomes and their sarcoplasmic protein extracts during storage at −12°C and −18°C.

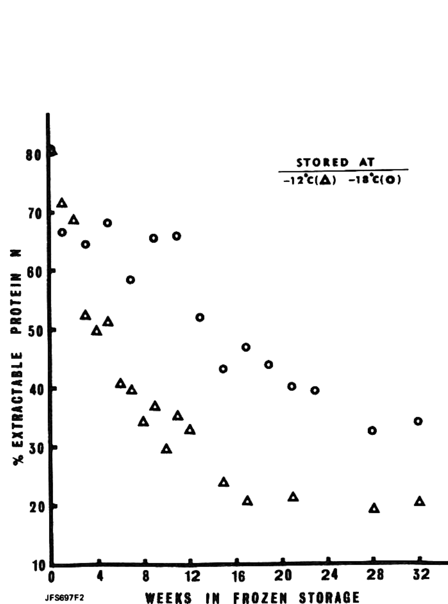

Fig. 2–Readily extractable protein contents of cod muscle during storage at −12°C and −18°C.

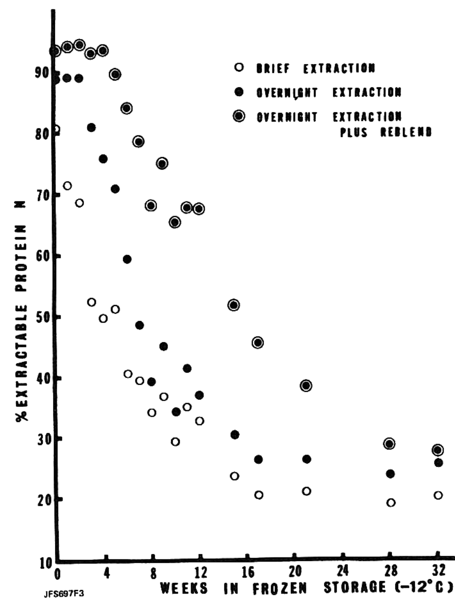

Fig. 3–Change in percentage protein extractable with three techniques from cod muscle stored at −12°C.

determined weekly in samples of myotomes stored at −12°C and biweekly in samples stored at −18°C for the first 3 mo of storage and at less frequent intervals after this, through 32 wk storage. Protein extractability in the presence of bovine serum albumin (BSA) was determined (Anderson and Ravesi, 1968) for muscle stored at −12°C for 1–12 wk and at −18°C for 13–19 wk. Inextractable residues obtained in the preparation of extracts were examined by phase microscopy, and representative portions were photographed. Ultracentrifugal patterns were obtained of double-strength (muscle to extractant ratio 20:380) protein extracts prepared with prolonged extraction time plus reblending. Patterns for a few of the extracts of muscle that had been stored at −12°C were obtained after the extracts had been stored at 23°C.

The FFA contents of lipid extracts of whole myotomes and the lipids associated with protein extracted in 0.05 ionic extractant were determined at the same storage intervals.

RESULTS

Extraction studies

FFA in muscle. The amounts of FFA recovered from muscle stored at −12°C increased rapidly (Fig. 1) during the first 8 wk of storage to about 200 mg/100g myotomes. During the remaining 24 wk, increase in amount of FFA extracted occurred at a slower rate. The rate of increase in FFA in muscle stored at −18°C was much slower, about 90–100 mg/100g muscle during the first 8–10 wk. However, in this muscle, too, a phase of much slower increase in FFA occurred during the next 24 wk.

FFA associated with sarcoplasmic protein. The amount of FFA associated with the sarcoplasmic protein of muscle was, as in the case of muscle aged in ice (Anderson and Ravesi, 1968), lower than the amount in the whole muscle. It reached a maximum after about 4 wk storage at −12°C and 8 wk at −18°C. Both the amount of FFA extracted (Fig. 1) and its ratio to the amount of sarcoplasmic protein extracted (Table 1) show that, in general, more FFA was associated with the sarcoplasmic protein of muscle stored at −18°C than with that of muscle stored at −12°C.

Protein extracted in low ionic strength extractant. The soluble protein content of extracts prepared in 0.05 ionic extractant (sarcoplasmic extracts) with 1-day-iced muscle before freezing was equivalent to 23–24% of the total protein of the muscle (2.95g protein N/100g muscle). As shown in Table 1, the amount of protein readily soluble in low ionic strength extractant decreased during frozen storage. The rate of decrease was greater in extracts prepared from muscle stored at −12°C than from muscle stored at −18°C. In the former muscle, it decreased to a value equivalent to 15% of the total protein of the muscle. It is likely that it had not reached a minimum in muscle stored at −18°C. The decrease in sarcoplasmic protein readily extracted is explainable as the result of increased resistance of the muscle to homogenization. There has been general agreement that there is no decrease in the extractability of sarcoplasmic protein during frozen storage (Dyer and Dingle, 1961; Connell, 1968) In some cases (Dyer, 1953; Connell, 1962), this is an assumption based on indirect methods for determining sarcoplasmic protein contents.

Protein extracted in 0.5 ionic extractant. Percentage protein readily extractable from muscle stored at −12°C decreased rapidly (Fig. 2) during the first 8 wk of storage. Comparison of the data for this muscle in Figures 1 and 2 shows a correlation of onset of a slower rate of FFA production with a slower rate of decrease in extractable protein. Percent protein readily extractable continued to decrease for the next 9 wk or so and then remained rather constant at about 20% during the rest of the storage period. Since the readily extractable total protein plateaued at a higher level than the readily extractable sarcoplasmic protein (15%), the extraction of other protein in addition to sarcoplasmic protein was indicated. Decrease in readily extractable protein occurred more slowly in muscle stored at −18°C (Fig. 2). Minimum extractability was probably not reached during the storage period. It is not as easy to detect the point of onset of a slower rate of decrease in muscle stored at the lower temperature as it was in muscle stored at the higher temperature. There is, nevertheless, a correlation between decrease in percentage protein extractable and increase in FFA content.

For all practical purposes, the curves for decrease in readily extractable protein in this study were exponential at both storage temperatures. This is in agreement with some investigators, but in disagreement with others, as reviewed by Connell (1968). Failure to find the initial lag in protein extractability observed by Dyer and Fraser (1959) in cod stored at −12°C

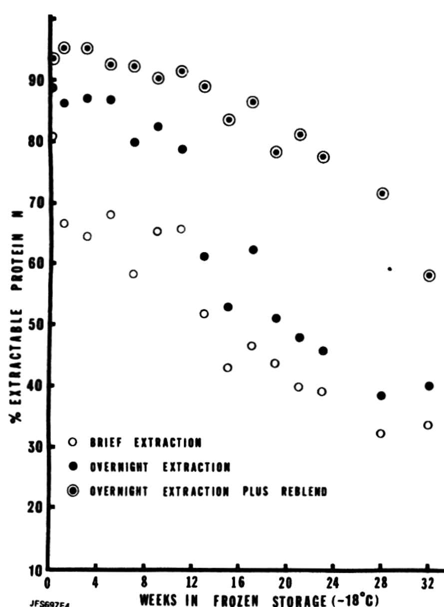

Fig. 4–Change in percentage protein extractable with three techniques from cod muscle stored at –18°C.

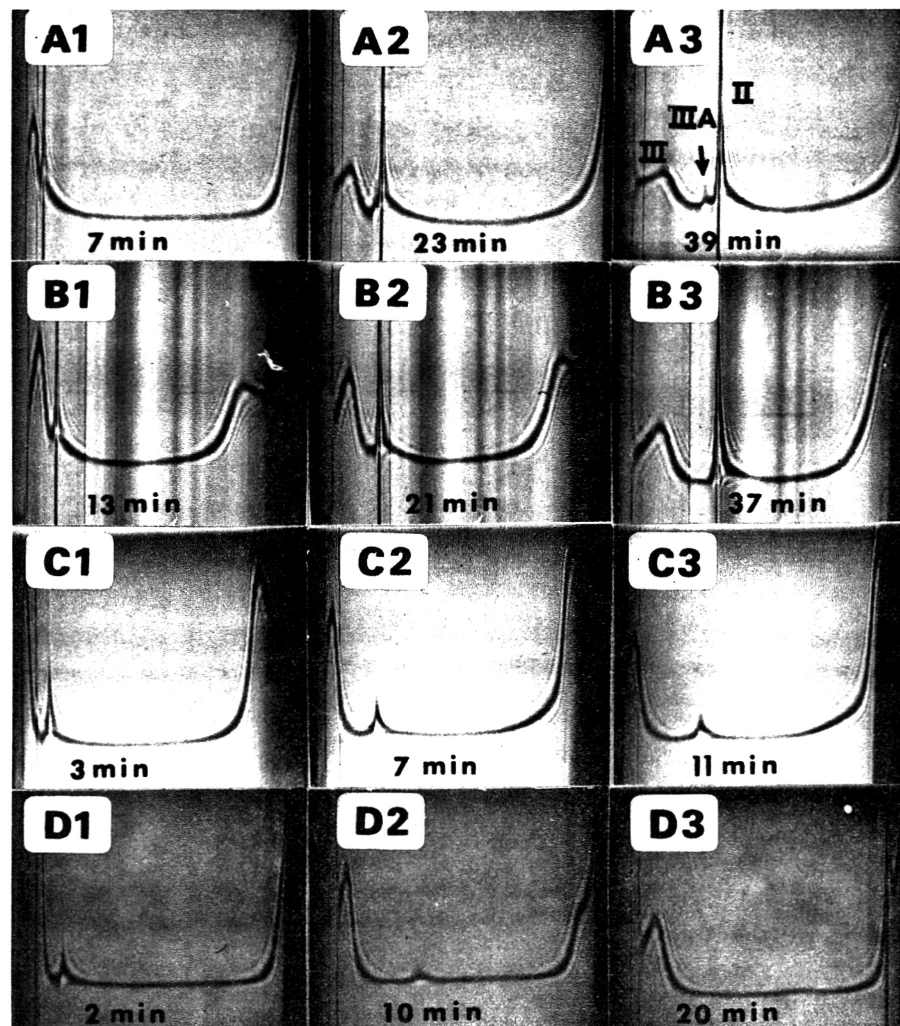

Fig. 5–Sedimentation patterns of double strength protein extracts prepared with overnight extraction plus reblending from cod muscle stored at –12°C for: A) 0 days; B) 6 wk; C) 11 wk; D) 15 wk. Protein concentrations (mg protein N/ml) were: A) 0.959 (extract was diluted 2:1 with extractant before centrifugation); B) 1.33; C) 0.966; D) 0.698. Photographs were taken at a phase plate angle of 55° and at the intervals indicated after reaching 50, 740 rpm. Components are identified by Roman numerals in the order of decreasing sedimentation rates (Anderson and Ravesi, 1970).

is attributed to differences in sensitivity of extraction techniques (Ravesi and Anderson, 1969).

Effect of prolonged extraction. During the first part of the storage period, much additional protein was extracted with overnight exposure of the homogenized muscle to extractant from muscle held at both storage temperatures (Fig. 3 and 4). After the 5th wk of storage, there was a sharp decrease in the amount of protein extractable with this technique from muscle stored at 12°C (Fig. 3). Thereafter, the amount of additional protein extracted with the longer extraction time was generally similar to that from 1-day-iced muscle that had not been frozen. Toward the end of the storage period, however, there was a tendency toward smaller amounts. Increased yield due to overnight extraction of muscle held at –18°C began to decline after the 9th storage wk (Fig. 4). In general, a gradual narrowing of the gap between the protein contents of the two types of extracts occurred until the 19th storage week, after which the differences were rather uniform and roughly equivalent to that in 1-day-iced muscle.

Anderson and Ravesi (1970) have suggested that increased yields of extractable protein on overnight exposure of cod muscle stored at –29°C after aging in ice is due to slow dissolution of unaltered protein from areas in the microstructure not involved in the association process at the time of examination, but not readily accessible to the extractant because of resistance to fragmentation. In this study, the extraction of significant amounts of additional protein with this technique from frozen-stored cod muscle of relatively low FFA content substantiates the suggestion.

Effect of prolonged extraction plus additional homogenization. When the muscle-extractant homogenate was reblended briefly after standing overnight after preparation, more than 90% of the protein was extractable through the 4th storage week from muscle stored at –12°C (Fig. 3) and the 11th storage week from muscle stored at –18°C (Fig. 4). When the amount of protein extractable with this technique dropped below 90%, it decreased more rapidly in muscle held at –12°C. A narrowing of the gap between percent protein readily extractable and that extractable with this technique from muscle held at –12°C was very definite (Fig. 3). After 32 wk storage, the difference was small. In muscle held at –18°C, however, narrowing of the gap had just begun (Fig. 4). The curves for decrease in protein extractability with the two techniques are similar, however, reflecting the differences in sensitivity of the extraction techniques.

Effect of bovine serum albumin. There were no significant differences in percentage protein extractable when extraction was carried out in the presence and absence of BSA. This was true for extraction with the three techniques used and, also, when homogenates were stored for an additional 24 hr after the 2nd homogenization. This additional exposure to salt solution for 24 hr did not result in significant changes in extractable protein in extracts with and without BSA. This is

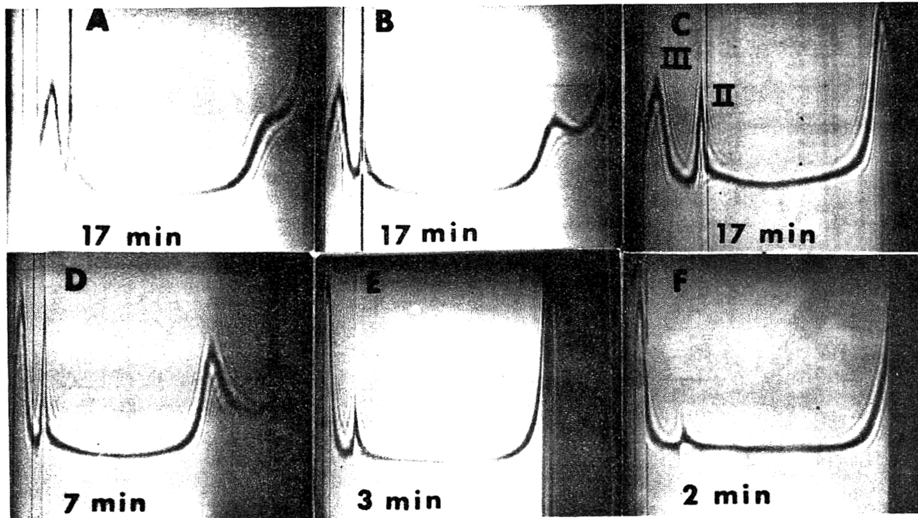

Fig. 6–Sedimentation patterns of double strength protein extracts prepared with overnight extraction plus reblending from cod muscle stored at −18°C for: A) 9 wk; B) 11 wk; C) 15 wk; D) 19 wk; E) 28 wk; F) 32 wk. Protein concentrations (mg protein N/ml) were: A) 1.28; B) 1.38; C) 1.29; D) 1.16; E) 1.06; F) 0.886. Photographs were taken at a phase plate angle of 55° after reaching 50, 740 rpm at the intervals indicated.

in contrast to the effect of BSA on protein extractability in muscle aged in ice (Anderson and Ravesi, 1968). Failure to find increases in amount of protein extracted in the presence of BSA or when reblended homogenates were stored for an additional 24 hr suggested that in the frozen-stored muscle, significant disaggregation did not occur. The stability of the association in neutral salt solution indicated that the interaction, initially electrostatic (Anderson et al., 1965; Anderson and Ravesi, 1968), was followed by the formation of non-ionic bonds.

Ultracentrifugal patterns. Sedimentation data have been treated grossly since the usual analysis is not applicable to the patterns obtained (Anderson and Ravesi, 1969). Although there was no change in the protein content of double-strength extracts prepared with overnight extraction plus reblending from muscle held at −12°C through 4 wk (Fig. 3), ultracentrifugal patterns of extracts showed changes in composition of the protein extracted. There were decreases in peaks II and IIIA and some evidence for the presence of higher molecular weight aggregates–larger areas under the turn-up of the pattern toward the bottom of the cell and larger pellet areas (not illustrated). Evidence for aggregation and polydispersity of proteins in these extracts increased with further storage of the muscle (Fig. 5). When compared with the patterns for unfrozen 1-day-iced muscle (A1-3), the patterns for muscle frozen 6 wk (B1-3) showed definite peak formation on the turn-up, and a markedly larger pellet area. Peak II had decreased and peak IIIA was no longer seen.

Ultracentrifugal patterns for muscle stored for 9–21 wk at −12°C (C1-3, D1-3) reflected the marked changes in percentage protein extractable with the reblending technique during this part of the storage period (Fig. 3). Progressive decreases in peak II, the fast peak and pellet areas were consistent with a shift toward greater association of contractile protein in the muscle. The fast peak was not seen in patterns for muscle frozen for more than 15 wk. In patterns for muscle frozen 28 and 32 wk, peak II was not seen, but in each instance there was a substantial peak III (not illustrated), suggesting that peak III (myosin) was still, to some extent, extractable when protein extractability had reached a minimum. Changes in the ultracentrifugal patterns of extracts of muscle stored at −18°C (Fig. 6) occurred more slowly than in the patterns of muscle stored at −12°C. Fast peak formation was definite beginning with the 9th-wk pattern (A).

With further storage of the muscle, the fast peak and pellet area increased in size and then decreased while peak II continued to decrease (B–F). In 11- and 13-wk patterns, the fast peak was demonstrable at storage intervals where the percentage protein extractable (Fig. 4) was still high and, because of its marked concentration dependence (Anderson and Ravesi, 1970), could be viewed as it separated from peak II and grew at its expense (not illustrated). In patterns for muscle stored 17–32 wk, very rapidly sedimenting components separated from the boundary as a peak before speed was reached (not illustrated). In the 32-wk pattern, the pellet area was still rather large and both the fast peak and peak II were still seen.

Effect of storing the extracts at 23°C. When compared with patterns for extracts that had been kept at 1°C until centrifuged, patterns for extracts stored at 23°C for 2 or 2-½ hr showed greater polydispersity (Fig. 8). For example, in the patterns for muscle stored 2 wk at −12°C (A,B), the smaller, faster sedimenting peak II and the larger pellet area in the pattern for the extracts stored at 23°C are evident. In patterns for muscle in which a greater degree of association had taken place, (illustrated for one sample of muscle in C), peak II was smaller, and the fast peak and pellet area were larger when the extract had been stored at 23°C. Increase in polydispersity in protein extracts held for short periods at 23°C implicated hydrophobic interaction, a likely stabilizer of the association. It also suggested a possible relation of the fast peak to peak I of Ellis and Winchester (1959), since these investigators carried out their analytical ultracentrifugation at temperatures above 0°C–as high as 20°C in some instances.

Microscopic examination of residues of muscle extractant homogenates. After 1 and 2 wk storage at −12°C (Fig. 8), small bundles of myofibrils in residues of homogenates prepared with 0.05 and 0.5 ionic extractant and centrifuged immediately after preparation (A,B) were consistent with the beginning of resistance both to fragmentation and extraction of protein. When exposure to 0.5 ionic extractant was prolonged, the residue was smaller in volume, but fragments were similar in appearance to those seen with brief extraction. Fragments in reblended homogenates were small, smudged, and appeared to be lacking in banding pattern (C).

In 0.5 ionic residues of muscle stored 4–5 wk at −12°C, much larger fragments made up of bundles of chopped-off myofibrils were seen along with many small, smudged fragments (D). There were also fragments of full fiber width in which there was much distortion of the banding pattern (E). This raised the question of whether the distortion had occurred *in situ* or on exposure of the fragments to the extractant, a point of considerable interest since it was postulated that the deterioration process occurred in liquid pools within the frozen muscle. After careful search, several severed fibers were found in which it could be seen that myofibrils were spilling out of the fiber where the sarcolemma was not intact. Whole sections of myofibrils still within the fiber were oriented toward the spill-

ing (not illustrated). Thus it appeared that the distortion was an artifact of extraction and was seen where extraction of myofibrillar protein was in progress, presumably the protein that was extractable with overnight exposure to the extractant.

With further storage at −12°C, bundles of myofibrils were larger in residues prepared in both 0.05 and 0.5 ionic extractants. By the 9th storage week, fragments in 0.05 ionic homogenates were mostly of full fiber width; and after the 11th storage week, no single myofibrils were seen. The banding pattern in the observable outer layers of the transversely severed fibers appeared to have no alterations detectable with phase microscopy. However, there was little evidence for separation of the surface of these fragments into myofibrils and the banding pattern appeared to be continuous across the fragment. This was in contrast to the appearance of bundles of myofibrils from fresh muscle, obtainable by blending briefly in 0.05 ionic extractant muscle from which the connective tissue had not been removed. In these bundles distinct separation into myofibrils was observable in many places on the surface and at the edges (F).

Fragments of less than full fiber width in residues from stored muscle also showed lack of separation into myofibrils (D). However, there were many bundles of myofibrils having areas where the banding pattern appeared to be distorted (G,H). These distorted areas were found both in the presence (G) and absence (H) of extractant for contractile protein, indicating that the distortion was not entirely explainable as the result of partial dissolution of protein. Fragments in residues of reblended homogenates were always smaller than in residues examined after the initial homogenization and consisted mostly of amorphorous debris (I).

In residues of muscle stored 15–32 wk at −12°C, fragments of less than full fiber width in freshly prepared homogenates increased in size according to duration of storage. Lack of banding pattern in these fragments and in fragments of full fiber width was seen only where the fiber had been torn in the blending process (J,K,L), leading to the conclusions that lack of perceptible banding patterns in fragments can be explained as the result of tearing apart the fibers of muscle which the association process has made resistant to fragmentation, and that the only alteration due to frozen storage that was detectable with phase microscopy was increased cohesiveness of the microstructure. This is in agreement with the findings of other investigators, as reviewed by Connell (1968).

When muscle was stored at −18°C, changes in microscopic appearance of residues of homogenized muscle were similar to those for muscle stored at −12°C, but occurred more slowly. At the end of the storage period, they had not become as extensive as in muscle stored at the higher temperatures. For example, single myofibrils were seen in residues of low ionic strength homogenates until the 28th wk of storage at −18°C. No other differences were noticed.

DISCUSSION

EXTRACTION of additional protein from frozen-stored 1-day-iced muscle with the same reblending technique used to extract altered protein from cod muscle stored at −29°C after aging in ice (Anderson and Ravesi, 1970) suggested that the proteins extracted had similar properties. Indeed, similarities in sedimentation patterns of protein extracted from muscle both frozen fresh and frozen after aging in ice indicated that the association process that occurs in frozen-stored cod muscle in which the FFA is formed largely during frozen storage is similar to that which occurs in frozen-stored muscle in which the FFA is formed largely during aging in ice, i.e., prior to freezing and subsequent storage.

The curves for FFA formation are essentially logarithmic at both storage temperatures and, as far as they go, are in agreement with the results of Olley et al. (1962, 1969). Olley et al. (1969) observed an initial rapid rate of FFA formation followed by a much slower rate. They also found that the asymptotes for FFA formation decreased in general with decreasing temperature of storage. They explained their results in terms of the effects on lipid hydrolysis of different amounts of free water in the muscle as a function of storage temperature. The results of the study reported here have led us to present a different interpretation for our own findings and for those of Olley et al. (1969) and to propose a model for the deterioration process in frozen-stored muscle.

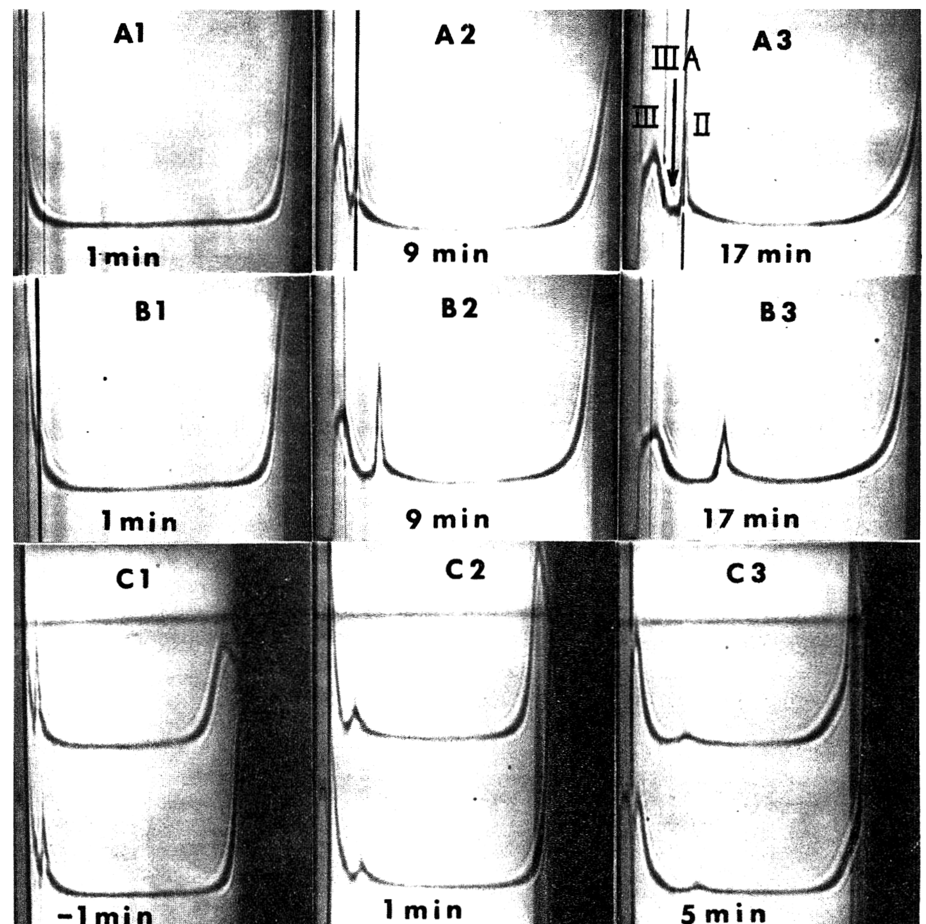

Fig. 7–Sedimentation patterns of double strength protein extracts prepared with overnight extraction plus reblending showing the effects of storing the extracts at 23°C. Muscle had been stored (A and B) for 2 wk at −12°C and (C) for 2 wk at −18°C followed by 2 wk in a small self-defrosting freezer set at −12°C. Extracts were: kept at 1°C until centrifuged (A and C, upper pattern); stored at 23°C before centrifugation for 2½ hr (B) and 2 hr (C, lower pattern). Photographs were taken at a phase plate angle of 55° and at intervals before and after reaching 50, 740 rpm as indicated.

Our interpretation is based on effects of change in solvent environment due to FFA formation during frozen storage. We propose that the ratio of FFA to available water in the frozen muscle rather than the amount of available water determines the extent of lipid hydrolysis. Our interpretation accounts not only for the differing asymptotes, but also for the change from an initial rapid rate of lipid hydrolysis and decrease in protein extractability during the first 8 wk or so of storage to a much slower rate. We have shown that the association of contractile protein in cod muscle aged in ice involves protein-FFA complex formation (Anderson and Ravesi, 1968); that holding the muscle in the frozen state favors the association (Anderson and Ravesi, 1969); and now that a similar association is involved in cod muscle frozen-stored without aging in ice. By definition a complex is physical and "held together by van der Waals, electrostatic, charge transfer and 'solvophobic' type forces" (Sinanoğlu, 1968). We propose that hydrophobic bonding, a "solvophobic" force, plays a significant role in the observed association of protein in cod muscle. Non-polar side groups of certain amino acid side chains of the proteins involved and the hydrocarbon tails of the FFA are among these groups that participate in hydrophobic bonding (Némethy, 1968). At the beginning of the storage period, the solvent is largely aqueous and hydrophobic binding is favored. With storage and the formation and binding of FFA to protein, the solvent environment becomes increasingly non-polar and the tendency for hydrophobic bonding decreases (Schnell, 1968). This concept explains both the initial rapid rate of FFA formation and decrease in protein extractability and the later slower rate. It follows that in the presence of a given amount of FFA in the muscle, the solvent environment would become non-polar sooner in the muscle stored at −18°C than in the muscle of higher free water content stored at −12°C. This explains the temperature dependence of the asymptotes. It suggests also that either directly or indirectly, combination of FFA with proteins is a driving force for lipid hydrolysis. The evidence for decrease in protein extractability due to an association of protein in which hydrophobic bonding plays an important role is consistent with Connell's (1965) finding that the bonds formed in frozen-stored muscle are sensitive to sodium dodecyl sulphate.

The concept of change of solvent environment during storage offers a possible explanation for our finding that the amount of FFA associated with the sarcoplasmic proteins increased to a maximum early in the storage of muscle frozen without aging in ice and then decreased with further storage (Table 1), and in muscle stored at −29°C after aging in ice (Ravesi and Anderson, 1969), was approximately one-half that before freezing. This is due to decreased bonding of FFA to sarcoplasmic protein through hydrophobic forces as the solvent environment becomes more non-polar. In the muscle frozen after aging in ice, change to a more non-polar environment occurs rapidly with freezing.

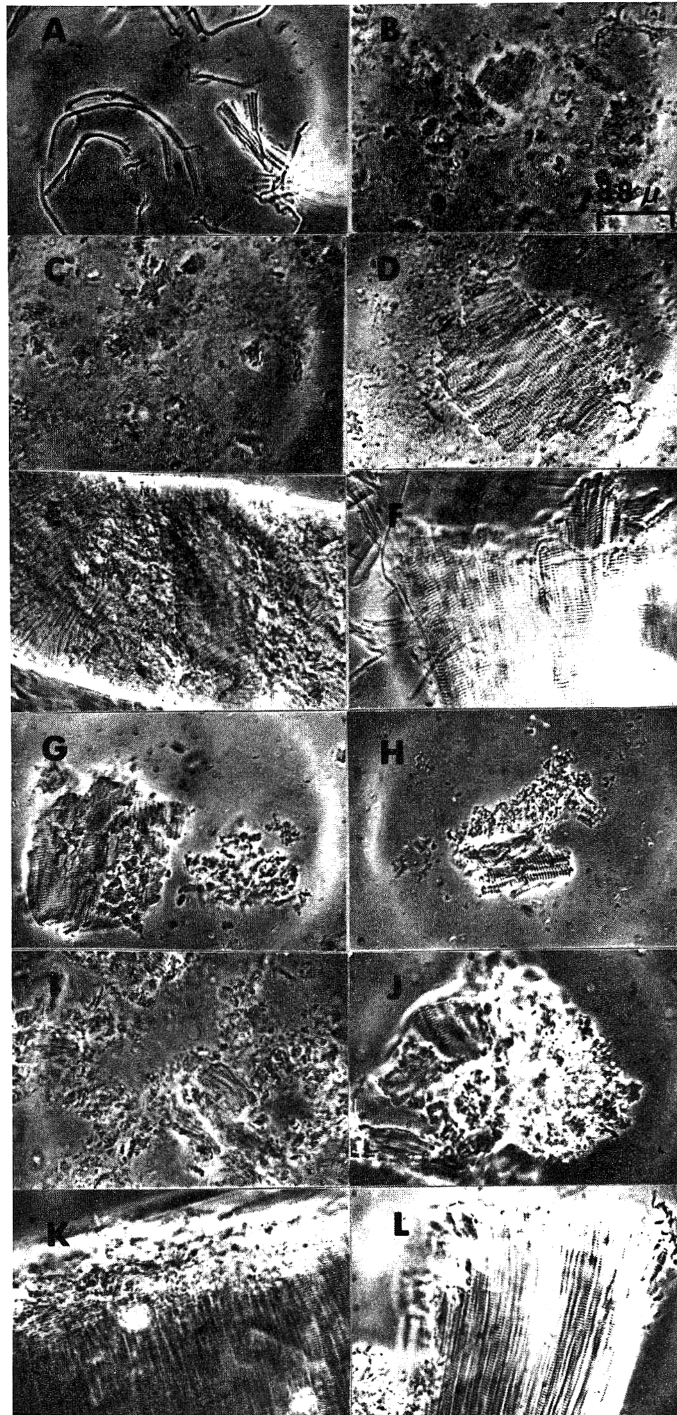

Fig. 8—Phase contrast micrographs of residues of homogenates of muscle stored at −12°C for: A) 2 wk; B and C) 1 wk; D) 5 wk; E) 4 wk; F) 0 wk; G and H) 12 wk; I) 9 wk; J–L) 28 wk. Muscle was homogenized in 0.05 ionic extractant (A,F,H,K,L) and in 0.5 ionic extractant (B,C,D,E,G,I,J). Residues were from homogenates centrifuged immediately after preparation except C and I, which were from homogenates centrifuged after storage for 20 hr at 0°C and reblending. Magnification was as in B.

The authors realize that the interpretation given is an oversimplification. Solute-solvent interactions involving all three classes of solutes (Némethy, 1968) must take part in the association process. They wish simply to emphasize the evidence for the role of hydrophobic bonding in the observed association of contractile protein in frozen-stored cod muscle. It was previously postulated (Anderson et al., 1965) that in frozen-stored muscle the geometry and distribution of the liquid phase as a function of storage temperature play an important role in the rate of FFA-protein interaction. Now, as investigation of the deterioration process has progressed and new information concerning the rate of association has come to light–acceleration of reaction rate in the frozen state (Anderson and Ravesi, 1969) and the effects of change in solvent environment–the rate of association is seen to be even more complicated. Other portions of the earlier working hypothesis can be developed more fully in the light of recent findings. There was nothing in the earlier model, for example, to explain how a reaction taking place in a liquid phase that makes up only small fractions of the frozen muscle could eventually involve the whole muscle tissue. A continuing process where the liquid phase would move on to involve new areas of microstructure would be a necessary part of the model. The concept of change in solvent environment makes this possible.

Where liquid pools containing solutes concentrated as a result of freezing are adjacent to protein filaments in the frozen state, binding of salts occurs and proteins are bathed in the liquid phase. Dissolution of protein in the sense of being driven apart into molecular dispersion need not take place, and microscopic studies indicated that no rearrangement of structure detectable at the level of light microscopy occurs. Penetration of the liquid phase into areas of the microstructure containing protein residues is all that is necessary.

As FFA are formed and diffuse by virtue of the liquid pools, they are bound to protein, leading to association with (1) rearrangement of the molecular surface; (2) loss of ordered water following rearrangement (Némethy, 1968), a probable source of the water that is not re-absorbed when the muscle is thawed (Connell, 1968) and which contributes to the well-known thaw drip of muscle after prolonged storage; and (3) decreased competition for binding of available salt. Because of loss of salt binding capacity, the altered protein is frozen. Salts in the liquid phase are then available to bind to more unaltered protein and the liquid phases moves in to bathe the protein filaments, thus making possible a continuing process which eventually involves the whole microstructure.

The observation that there is little change in microstructure as detected by phase microscopy suggests that when association takes place in a given area, it involves protein molecules that are close to each other. Thus, whether or not actin molecules are associated with myosin molecules in the muscle before and after freezing, there is the potential for association of like molecules or portions of molecules in the filaments. This is in keeping with the observation that there are changes in interfilament spacing after frozen storage (Connell, 1968). It offers a possible explanation for the finding that myosin is preferentially extractable (Connell, 1962) under conditions where actomyosin is no longer extractable.

Objections have been raised to the theory that FFA formation is related to the association of protein in frozen-stored muscle on the grounds that (1) there are species differences in the extent of deterioration in the presence of similar amounts of FFA (Olley et al., 1962) and (2) deterioration as a result of freezing or frozen storage takes place in muscle of low FFA content (Olley et al., 1967; Connell, 1968). Participation of neutral and unhydrolyzed polar lipids as "non-polar type" forces would explain species differences in the extent of association occurring in the presence of a given amount of FFA formation. Moreover, association is favored when the muscle is frozen (Anderson and Ravesi, 1969), and some association can take place on freezing even in muscle of very low FFA content due to the various bonding potentials that already exist in the muscle prior to freezing. This concept can explain decrease in protein extractability in muscle that has been (1) frozen and thawed without prior storage in ice; (2) stored in ice for 1 day and more and then frozen or frozen-stored (Notevarp and Heen, 1940; Love, 1962; Love et al., 1965; Connell, 1968; Anderson and Ravesi, 1969), where in the frozen-stored muscle, association would be due to bonding resulting from potentials developed during storage as well as those existing on freezing; (3) frozen initially at temperatures much lower than those of commercial practice and then frozen-stored at higher temperatures (Love and Elerian, 1963; Love, 1968), where the bonding resulting from the initial lowering of temperature and that resulting from new bonding potential developed in storage are additive; and (4) subjected to fluctuations in storage temperature, including temperature changes without thawing (Dyer et al., 1957A,B; Love, 1962) and with thawing and refreezing followed by storage (Dyer et al., 1962; Peters et al., 1968) where, in both instances, FFA formation occurs more rapidly at the higher holding temperatures and in muscle thawed and refrozen, reacts more favorably in the frozen state. The literature on the effects of freezing without storage on the protein extractability of fresh muscle is not in agreement (Ironside and Love, 1958; Suzuki et al., 1965; Connell, 1968). This may be due to differences in sensitivity of extraction techniques or to differences in the state of rigor and time of storage in ice post-mortem of fish that is considered fresh post-rigor muscle. For muscle frozen initially at low temperatures and then stored at higher temperatures, differences in distribution of the liquid phase resulting from reaching temperatures closer to that required for vitrification and then rewarming would also have a significant influence on the rate of the association process.

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H. BUTTKUS
Fisheries Research Board of Canada
Vancouver Laboratory, 6640 N.W. Marine Drive, Vancouver 8, B.C.

ACCELERATED DENATURATION OF MYOSIN IN FROZEN SOLUTION

SUMMARY—The aggregation of rabbit and trout myosins was studied in frozen solution at high ionic strength μ = 0.50 KCl—potassium phosphate buffer pH 6.9 between 0 and −30°. During the initial stages of freezing, monomeric myosin $S^{\circ}_{20,w}$ = 6.5 S aggregated to form dimers and trimers with sedimentation rates $S^{\circ}_{20,w}$ = 10 and 12 S, respectively. The higher aggregates sedimented at low centrifugal fields and were insoluble in molar salt solutions at pH 8. Solubilization was, however, achieved in solvents known to disrupt hydrophobic and hydrogen bonds in addition to conditions which will reduce disulfide bonds. The nature of the sulfhydryl groups of myosin was reinvestigated and, in accordance with their behavior, a mechanism for the aggregation reactions has been proposed which involves disulfide-sulfhydryl exchange reactions between activated myosin molecules and aggregates. Previous kinetic and chemical data for myosin denaturation are in agreement with the proposed mechanism. The rate of formation of the insoluble, high molecular weight protein aggregates in myosin solutions increased as the temperature decreased below the freezing point and reached a maximum near the eutectic point of the myosin-potassium chloride-water solution (− 11°), due to concentration effects. Below the eutectic point, at −20 and −30° where only water bound to the protein remains unfrozen, aggregation and consequent insolubilization decreased again and approached the rate observed at 0°. The general pattern of myosin solubility at different freezing temperatures was similar to the decreasing protein solubility during storage of whole muscle, with the difference that denaturation of purified myosin solutions was accelerated. At the most critical temperature, around −10°, the rate of denaturation of a 0.7% myosin solution was reduced by the enzyme substrates 0.02 M adenosinetriphosphate (ATP) or tripolyphosphate (P_3) and to a lesser degree by pyrophosphate (P_2). Substances such as glycerol or magnesium chloride which lowered the eutectic point also lowered the rate of denaturation at −10°. N-ethylmaleimide was not effective in reducing the rate of denaturation and mercaptoethanol led to the formation of gels. Electronphotomicrograph studies showed that aggregation of the monomeric myosin molecules proceeds in a side-to-side manner. Loss of solubility was also produced when myosin solutions contained a cross-linking reagent such as malonaldehyde, or detergents which interfere with the hydrophobic bonding of the native molecule. The different mechanisms responsible for the denaturation or insolubilization of myosin and its increasing molecular weight are briefly discussed.

INTRODUCTION

AGGREGATION of rabbit myosin in solution at 25° has been shown to proceed from the monomer to a dimer to a trimer and as far as an octamer within 48 hr (Holtzer, 1956; Holtzer and Lowey, 1959). The initial mechanism up to the trimer was also shown to be operative in the aggregation of cod myosin around 0° and at freezing temperatures (Connell, 1959; 1963). However, while the earlier work was concerned mainly with the initial aggregates, up to the trimer, and their sedimentation characteristics in the ultracentrifuge, rate of formation of the high molecular weight aggregates and their chemical properties have not been investigated.

In the present study the rate of formation of myosin aggregates greater than M_3 was measured between 0 and −30°. These high molecular weight aggregates sediment at low centrifugal fields and are not soluble in M salt solutions. Their behavior in solvents known to disrupt bonds responsible for structural protein conformations was also investigated and a general comparison between the properties of rabbit myosin and the present status of fish myosins has been made.

EXPERIMENTAL

RABBIT myosin was prepared according to the procedure of Szent-Györgyi (1951) and further purified as suggested by Dreizen et al. (1965). Myosins from trout and halibut muscle were mostly prepared by the method of Mackie and Connell (1964), including a final centrifugation at 100,000 × g for 90 min. However, for comparative purposes these fish myosins were also prepared by the Szent-Györgyi method above. Sedimentation velocity measurements were made in a Spinco Model E Ultracentrifuge and results calculated as described by Schachman (1957).

Freezing of myosin solutions was carried out in stoppered plastic tubes at temperatures ranging from −5 to −30°. The frozen solutions (4–5 ml) were thawed at 24-hr intervals by immersing the tubes for about 45 min in a shal-

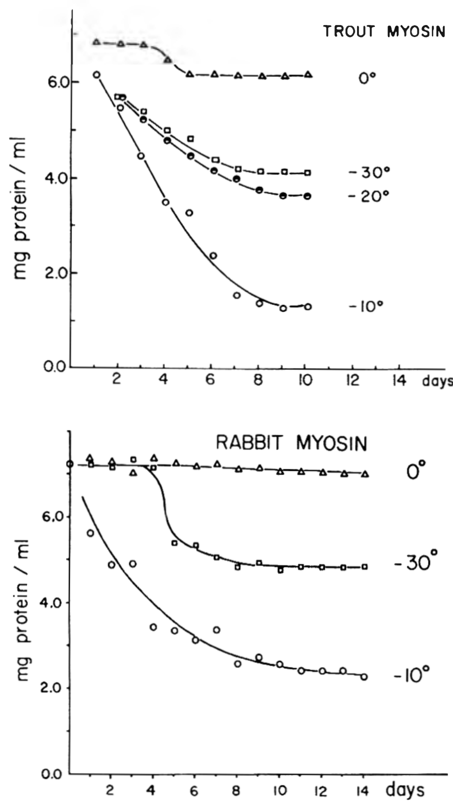


Fig. 1 and 2—Relative rates of decreasing protein concentration in myosin solutions stored at 0, −10, −20, −30° (trout myosin, Fig. 1) and at 0, −10 and −30° (rabbit myosin, Fig. 2) for up to 14 days. The myosin solutions were about 0.7% in protein 0.45 M KCl, buffered with 0.026 M potassium phosphates at pH 6.9. After freezing, the protein solutions were thawed in 4–5° water and centrifuged for 7 min at 12000 × g and 0° before the protein remaining in solution was determined; the solution was refrozen at the respective temperature and kept until the following day, when the above procedure was repeated.

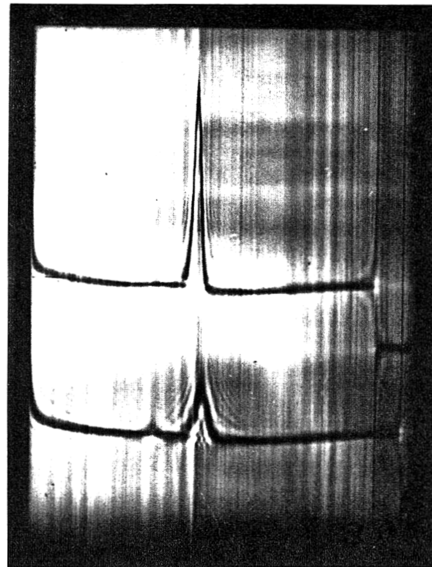


Fig. 3—Ultracentrifugation patterns of rabbit myosin. The top pattern shows the monomeric myosin remaining after 5 days of storage at 0°. The lower pattern shows the dimers and trimers remaining in solution after 5 days of storage at −10°. Protein concentrations 0.3%, μ = 0.5, pH 6.9. The picture was taken 175 min after reaching full speed at 47660 rpm, temperature 5°. The sedimentation constants, $S^{\circ}_{20\,w}$ for the monomer, dimer and trimer were calculated and found to be 6.5 S, 10 S and 12 S, respectively.

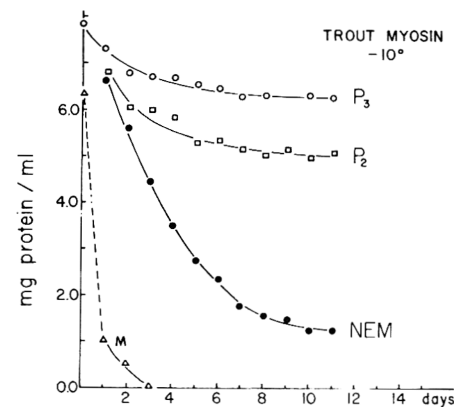


Fig. 4—Relative rates of decreasing protein concentration in solutions of trout myosin made 0.02 M in pyrophosphate (P_2), tripolyphosphate (P_3) or N-ethylmaleimide (NEM), or 0.001 M in malonaldehyde, and stored at −10°. All reagents were adjusted to pH 7 before being added to the protein solutions. Other conditions were 0.45 M KCl, 0.026 M potassium phosphates, pH 6.9.

low pan containing 4 liters of water at 4–5°. The tubes were then centrifuged at 12,000 × g for 10 min. The control sample kept at 0° was treated identically. After centrifugation the protein remaining in solution was measured by the biuret method and the samples refrozen at the respective temperatures in air. The insoluble protein aggregate formed during the freezing process was centrifuged to the bottom of the tube and washed with 0.5 M KCl for further experiments. Reagents such as ATP, pyrophosphate, N-ethylmaleimide, etc., added to the protein preparation before a freezing experiment, were adjusted to pH 6.8–7.0. Sulfhydryl determinations were carried out with N-ethylmaleimide and p-chloromercuribenzoate in 8 M urea (Boyer, 1954; Alexander, 1958).

Lipid was extracted according to Bligh and Dyer (1959) and the free fatty acids separated on silicic acid columns (Baron and Hanahan, 1958).

Electronphotomicrographs of frozen myosin solutions and those kept at 0° were prepared as follows: Preparations containing about 16–17 mg protein/ml were diluted with M ammonium acetate buffer, pH 7.2 to a concentration of about 1.7 mg/ml, then dialyzed for 24 hr in a thin dialysis bag against 2 changes of ammonium acetate buffer at 0°. Further dilution was made by taking about 1 ml out of the dialysis bag and by making it up to 30 ml with M ammonium acetate buffer to give a concentration of about 0.05 mg of protein per ml. This solution was then sprayed on to collodion-covered copper grids and shadowed with palladium. The electronphotomicrographs were taken with a Philips instrument.

RESULTS & DISCUSSION

WHEN myosin was frozen in a solution 0.45 M in KCl, 0.026 M in mono- and dipotassium phosphate, pH 6.9, μ = 0.5, large molecular weight aggregates formed which could be centrifuged out of solution at low centrifugal fields. Plotting the decreasing protein concentration in solution against time of storage, rate curves of the denaturation of rabbit and trout myosins were obtained as shown in Figures 1 and 2. By comparison, trout myosin formed insoluble polymers more rapidly, particularly when frozen at −10°, but also at 0° a somewhat lower stability was noted for the fish myosin. A striking feature for rabbit and trout myosins was that the maximum rate of denaturation was around −10°. Ultracentrifugation studies showed indeed that rabbit myosin stored at 0° remained in the monomeric state, whereas polymers readily formed when the myosin solutions were frozen, particularly at −10° (Fig. 3). When the storage temperature was decreased further to −20 or −30° the rate of denaturation decreased and approached the minimum at 0°. Formation of insoluble precipitate at −5° was also less than at −10°. The general pattern of myosin denaturation displayed in Figures 1 and 2 bears a striking resemblance to the protein solubility curves obtained during frozen storage of cod muscle as well as muscle from other fish and general experience with other tissues (Dyer and Fraser, 1959; Dyer, 1951; Love, 1958); with the difference, however, that myosin in solution is denatured at a faster rate than when the proteins are in their undisturbed cellular arrangement.

Attempts to stabilize myosin and to reduce the rate of its aggregation were successful when the freezing solutions were made 0.02 M in pyrophosphate or in tripolyphosphate, the latter being more effective (Fig. 4). The effect of 0.02 M adenosine triphosphate, Figure 5, in reducing the aggregation of myosin is similar to that of the tripolyphosphate, both compounds being substrates for myosin in the presence of Ca^{+2} (Kielley, 1961). When the myosin solution was made 0.001 M in malonaldehyde, before freezing to −10°, the rate of insolubilization of the protein was greatly increased and an elastic, porous product was formed (Fig. 4). Malonaldehyde, similarly to glutaraldehyde, reacts with α- and ϵ-amino as

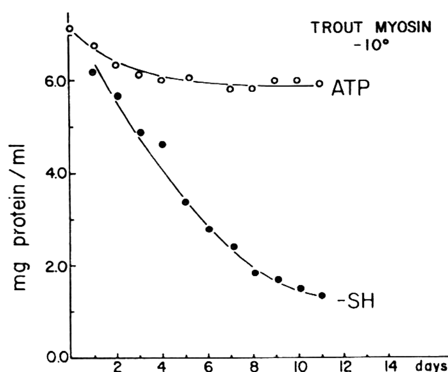


Fig. 5–Relative rates of decreasing protein concentration in solutions of trout myosin made 0.02 M in adenosine triphosphate (ATP) or 0.03 M in mercaptoethanol (-SH) and stored at –10°. Other conditions as cited under Figures 1–4.

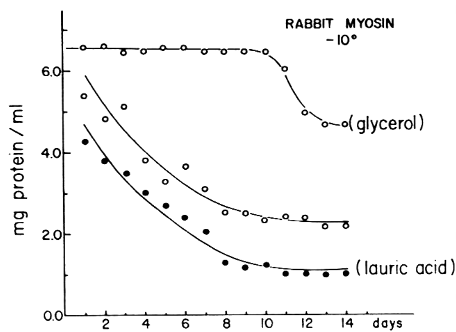


Fig. 6–Relative rates of decreasing protein concentration in solutions of rabbit myosin adjusted to 5.4 M glycerol or 0.001 M in lauric acid and stored at –10°. The initial protein concentrations were 0.65%, 0.45 M KCl, buffered with 0.026 M potassium phosphates to pH 6.9. The middle curve represents the decrease in protein concentration of a myosin solution frozen at –10° without additives.

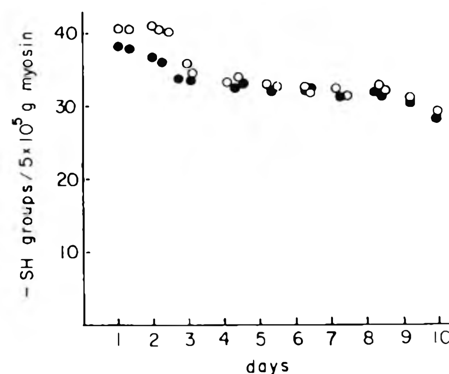


Fig. 7–Decrease in titrable sulfhydryl groups of rabbit myosin (open circles) and trout myosin (closed circles) at 0.45 M KCl, 0.026 M potassium phosphate buffer pH 6.9, when stored in air at 0°. The sulfhydryl determinations were begun 20–24 hr after starting extraction of the muscle, and were carried out on 3.6 mg of protein each.

well as sulfhydryl groups of proteins and amino acids and is able to act as a cross-linking reagent between different monomers (Buttkus, 1969; Habeeb and Hiramoto, 1968). N-ethylmaleimide at a concentration of 0.02 M did not seem to affect the rate of aggregation appreciably, because in the native protein not all sulfhydryl groups are readily available to the reagent. Addition of mercaptoethanol at low concentrations, 0.03 M, led to the formation of gels when the myosin solutions were frozen at –10° and no significant reduction in insolubilization was achieved (Fig. 5).

Mercaptoethanol is frequently and successfully used to keep sulfhydryl enzymes in the native or reduced state; however, in the present case, use of mercaptoethanol led to an increase in viscosity and formation of gels in the myosin solutions. This observation led us to reinvestigate the nature of the sulfhydryl groups of myosin and it was found that in freshly prepared myosin, i.e., 24 to 28 hr after killing the animal, 42–43 sulfhydryls per 5×10^5 g of protein were determined in 8 M urea. When stored at 0° in air, the total number of free sulfhydryl groups soon decreased to 30–33 and after 10 days reached a value of 28 (Fig. 7). The initial decrease indicated that about 10 of the sulfhydryl groups of the myosin molecule are very reactive and seem to oxidize readily to form intramolecular disulfide bonds, since myosin solutions up to 5 days at 0° can still be homogeneous in the ultracentrifuge and have the characteristic sedimentation coefficient $S^{\circ}_{20,w}$ = 6.5 (Fig. 3). The aggregation and oxidation of sulfhydryl groups in myosin from trout was somewhat faster in comparison with that from rabbit; however, it did not seem as fast and extensive as aggregation reported for myosin from cod muscle.

The high molecular weight myosin aggregates formed in myosin solutions, with or without additives, during the freezing process were examined for their solubility characteristics in different solvents. The aggregates were found to be insoluble in M NaCl, 8 M urea, 6 M guanidine hydrochloride or detergents, all of which were adjusted to pH 8 if necessary. Solubilization of all the aggregates except the myosin-malonaldehyde polymer was, however, accomplished in 6 M guanidine hydrochloride solution, which was 0.5 M in mercaptoethanol and at pH 8, or 0.3 M in sulfite or cyanide. Dissolution of the aggregate suspended in 6 M guanidine hydrochloride was also achieved by reduction with sodium borohydride. These experiments provide experimental proof that the sulfhydryl groups must have been directly involved in the polymerization of the monomeric myosin molecules.

The second requirement to dissolve the aggregate, the presence of guanidine, was an indication for the involvement of hydrophobic and possibly also hydrogen bonds in its formation (Kauzmann, 1959; Tanford, 1964). On the basis of the observed behavior of the sulfhydryl groups in the monomeric myosin molecule and the effect of reducing conditions on the high molecular weight aggregates, a general mechanism for the polymerization can be proposed: About 10 of the very reactive sulfhydryl groups, diagrammatically represented in the top portion of the myosin molecule (M) in Figure 8, are first oxidized. The molecule with the intramolecular disulfide bonds (M*) is now in an activated state being susceptible to sulfhydryl-disulfide interchange reactions with molecules of its own kind to form M^*_2 or with molecules being already multiples of M* having a molecular weight of M^*_x.

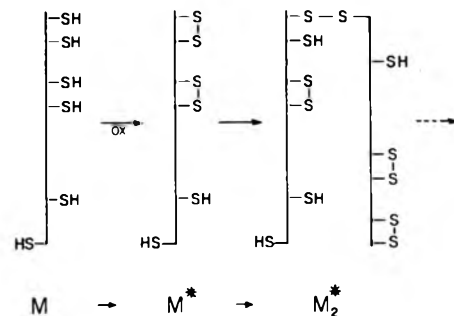
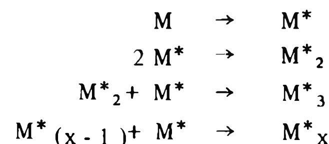


Fig. 8–Schematic representation of the oxidation and the consequent sulfhydryl-disulfide exchange reactions of myosin which ultimately lead to the formation of large molecular weight myosin polymers. The closely spaced sulfhydryl groups on top represent 10 very reactive groups whereas the lower 2 represent 32 SH groups of the myosin molecule more stable to oxidation. Note that the sulfhydryl-disulfide interchange reactions proceed without a decrease in the total number of free sulfhydryl groups.



Also, M and M* could probably form M - M* which would then oxidize to M^*_2.

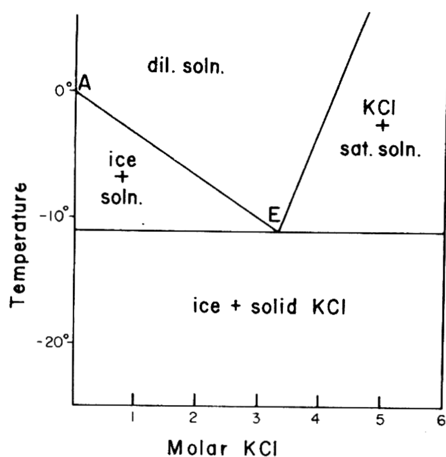
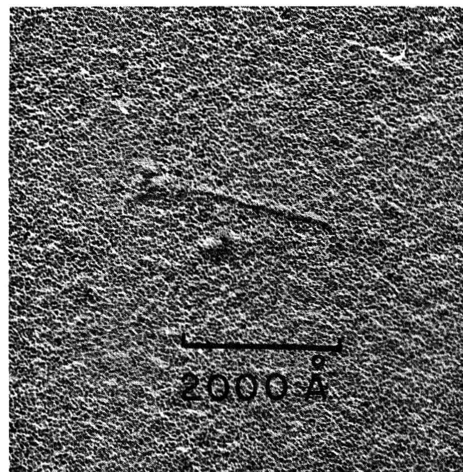


Fig. 9—Temperature-composition phase diagram for the potassium chloride-water system. The line AE represents the equilibrium freezing point curve. As the temperature of a dilute KCl solution is lowered to a point on AE, pure ice separates and the remaining solution is concentrated in solute. At point E, the eutectic point, ice and potassium chloride crystals separate out together, forming the eutectic mixture, i.e., the solution freezes without change in composition. A greater initial concentration in the unfrozen solution will only result in a greater total volume of liquid phase in the frozen solution.



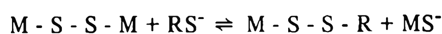
(a)

(b)

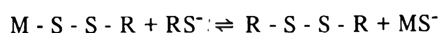
Fig. 10a—Electronphotomicrograph of 2 monomeric myosin molecules. According to current knowledge, a myosin molecule consists of 2 polypeptide chains in an α-helical coiled coil and each of these chains ends in a globular head. The double head is particularly well recognizable in the lower molecule. b—Electronphotomicrograph of myosin molecules which aggregated during storage for 5 days at $-10°$ in a side-to-side manner.

The important point is that the initial reactions are bimolecular and could be expected to follow second-order kinetics. This has indeed been shown to be true for rabbit as well as cod myosins (Johnson and Rowe, 1961; Connell, 1963). However, at more advanced stages of aggregation, when the reactive centers become limiting or the movement of the relatively large molecules becomes rate determining, due to precipitation or increases in viscosity at higher protein concentrations, deviations from second-order kinetics must be expected. Interaction by hydrophobic and hydrogen bonds then probably follows the interlinking of molecules by -S-S- bonds.

A possible involvement of sulfhydryl groups in the aggregation process of myosin has been investigated in several laboratories and was rejected because the number of sulfhydryl groups did not seem to change during storage or in solutions which had undergone some notable aggregation or because mercaptoethanol in low concentrations accelerates aggregation (Holtzer and Lowey, 1959; Connell, 1959). However, from Figure 8 we see that the total number of free sulfhydryl groups, indeed, remains the same during the sulfhydryl-disulfide interchange reactions and at low thiol concentrations mixed myosin disulfides M*-S-S-R can be formed and due to steric reasons are probably more reactive in the sulfhydryl-disulfide exchange reaction. At excess thiol concentrations and in the presence of 6 M guanidine at pH 8 the disulfide aggregates could be reduced to the monomeric state



$$M - S - S - M + RS^- \rightleftharpoons M - S - S - R + MS^-$$



$$M - S - S - R + RS^- \rightleftharpoons R - S - S - R + MS^-$$

The question then arises: Why was the maximum rate of denaturation of the myosin solutions around $-10°$? (Fig. 1 and 2.) Realizing that the major component, on a molar basis, in the myosin solution was potassium chloride (0.45 M), the freezing of this solution can be compared to that of a dilute potassium chloride-water solution and the equilibrium diagram for this binary system is shown in Figure 9. The initial freezing of the solution will result in the separation of ice and a liquid phase containing the solute(s), despite the fact that the solution has a solid appearance. As the

temperature decreases along the line AE, more ice separates and the solution becomes more concentrated until below point E, ice and solid KCl separate. For potassium chloride the eutectic point E is at –11° and the concentration of the liquid phase 3.31 M KCl. If other solutes such as myosin are present in the solution, they too are concentrated in the liquid regions of the frozen solution, resulting in an acceleration of the predominantly bimolecular aggregation reaction. The major mineral component in muscle tissue is also potassium chloride (Hamoir, 1961) and the eutectic point would, therefore, be in the region of –11°. In this temperature region at high potassium chloride concentrations, some solubilization of myosin molecules out of the thick filaments would be feasible and consequently lead to aggregation reactions. Below the eutectic point the liquid regions solidify and separate as ice and solid KCl, except for the water bound to protein or some inorganic hydrates. The mobility of the reacting compounds is thus greatly reduced and the rate of reaction declines as the temperature is lowered further. To check if the high salt concentration in the frozen solution had any effect on the accelerated aggregation reaction at –10°, myosin solutions were made up to 3.3 M in KCl pH 6.9 and stored at 0° for 13 days without any significant increase in aggregation compared to a control 0.45 M in KCl. Gross pH changes in freezing solutions were eliminated by using potassium phosphate buffers (pH 6.9) which show the least change in pH during freezing (van den Berg and Rose, 1959). The thawing and refreezing process did not have any effect on the aggregation of the protein, since a sample thawed and refrozen to –30° each day for 10 days had the same amount of soluble protein as a sample stored for 10 days at –30° without interruption.

If the eutectic point of the myosin solutions is lowered by making glycerol or magnesium chloride the major constituents, the maximum concentration effect is delayed until –47 or 33.6°C, respectively. Myosin solutions kept at –10° in the presence of 5.4 M glycerol are stabilized and denaturation is reduced to a minimum, comparable to solutions held at 0° (Fig. 6). After 10 days of storage, however, the protecting effect of glycerol seemed to decline somewhat. Myosin solutions made 0.1 M in $MgCl_2$ and frozen at –10° appeared to be protected from aggregation as well, possibly by a lowering of the eutectic point, but a chelation effect of the sulfhydryl groups on the magnesium ions cannot be excluded as a possible reason.

A different form of denaturation of proteins is brought about by the interaction with fatty acids or detergents which can interfere with the hydrophobic bonding of the native molecule or form micelles on the surface and change the charge of molecules, etc. Figure 6 shows that lauric acid at 0.001 M in the myosin solution during freezing at –10° increases the over-all rate and extent of denaturation.

A general comparison of the properties of fish and rabbit myosins was also carried out. Although trout and rabbit myosins appear very similar physically and chemically (Buttkus, 1966; 1967), trout myosin was more susceptible to the effects of freezing and aggregated more rapidly (Fig. 1 and 2). Further analysis showed that fish myosins (trout and halibut) prepared either by the Mackie and Connell (1964) or according to the Szent-Györgyi (1951) procedure still possess acetylcholinesterase activity and 15 units of adenylic acid deaminase activity/mg protein at 25°. Rabbit myosin prepared by presently accepted methods (Szent-Györgyi, 1951; Dreizen et al., 1965) did contain 18 units deaminase activity/mg protein at 25°, but no acetylcholinesterase activity, although the latter was at one time proposed to be a natural part of myosin (Köver et al., 1964). The free fatty acid content of rabbit myosin preparations was also found to be less than that of trout and halibut myosins, 0.015–0.020 and 0.090–0.10 g of fatty acids (mol wt 300) per 100 g of protein, respectively.

Electronphotomicrographs of rabbit myosin preparations following dialysis against ammonium acetate buffer are shown in Figure 10. 2 myosin molecules (Fig. 10a) from a preparation not frozen but stored and dialyzed at 0° is comparable with the general features of earlier, more extensive work on the structure of myosin (Slayter and Lowey, 1967). The myosin structures seen in Figure 10a are in agreement with the concept that myosin consists of 2 major polypeptide chains in an α-helical coiled coil, each ending in a head of globular conformation from which 2 smaller subunits can be reversibly dissociated at high pH (Frederiksen and Holtzer, 1968). The aggregates (Fig. 10b) are of comparable dimensions of the monomers observed (1500–2400 Å) and indicate that polymerization did occur in a side-to-side manner, as predicted from physical studies (Holtzer, 1956). The distribution in length of the different particles can be accounted for by breakage of some of the particles during the application of the protein solution to the grids.

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CHUNGHEE K. KANG and ELDON E. RICE
Swift & Company, R&D Center, 1919 Swift Drive, Oak Brook, Ill. 60521

DEGRADATION OF VARIOUS MEAT FRACTIONS BY TENDERIZING ENZYMES

SUMMARY—Beef round muscle was separated according to solubility characteristics into water soluble, salt soluble and insoluble fractions. These showed different degrees of hydrolysis when reacted with an enzyme. The magnitude varied with nature of the enzyme. Collagenase, bromelain and trypsin showed stronger solubilizing activity on the insoluble fraction than on the salt soluble fraction; whereas papain, Rhozyme P-11 and ficin showed strong activity on the salt soluble fraction and hydrolyzed the insoluble fraction less efficiently. In general, the water soluble fraction was more resistant to enzyme hydrolysis than the other fractions, except with papain and Rhozyme P-11. However, the portion of the water soluble fraction that was hydrolyzed appeared as smaller peptides than the solubilized fragments of the other two fractions.

INTRODUCTION

PROTEOLYTIC ENZYMES are known to increase the tenderness of meat when properly used and the tenderness produced by them is undoubtedly a result of protein breakdown; yet there is relatively little information available regarding the fraction of meat which is changed during enzyme tenderization. When Wang et al. (1957) made microscopical observations of the structural nature of muscle tissue, they were able to identify the sites of enzyme action and show that enzymes of different origins preferentially attack different muscle components. However, it was not possible to define the exact relation between tenderization and protein digestion.

A chemical investigation of the relative effect of proteolytic enzymes of plant, animal and microbial origins has been reported by Miyada and Tappel (1956) using ground beef muscle. They found that the greatest change among the various protein fractions occurred in the transformation of soluble protein nitrogen to non-protein nitrogen due to hydrolysis, mainly of actomyosin.

Our present study was undertaken to compare the susceptibility of various fractions of meat to enzyme treatment and, if possible, to correlate these findings with the tenderizing effect of the enzymes. It was thought that differences in the responses of the various meat fractions might help identify the portion of meat most intimately involved during tenderization.

EXPERIMENTAL

Preparation of meat fractions

Lean beef round was ground and extracted repeatedly with water to provide water soluble (sarcoplasmic) protein, then with 0.67M sodium chloride to yield salt soluble (myofibrillar) protein, and water and salt insoluble (stroma) fractions similar to those of Hegarty et al. (1963). After preparation, each fraction was lyophylized. The nitrogen content of all fractions was determined by Kjeldahl analysis and portions of each fraction containing 19.6 mg of nitrogen were weighed into vials and hydrated overnight in a cold room (5°C) by additions of 8 ml of 0.05M phosphate buffer (pH 7.0).

Enzymes

The enzymes selected for the experiment were bromelain, ficin, papain, and trypsin. These are well known, readily available, potent, proteolytic enzymes. Preliminary assays were made to determine the amount of each enzyme which would digest a standard amount of the salt soluble fraction to give the same number of free amino groups. The estimated amounts for bromelain, ficin, papain, and trypsin were 0.29, 0.09, 0.19 and 0.40 mg respectively. Rhozyme P-11 and collagenase were also included because of their reported selective degradation properties and 14.4 mg of Rhozyme P-11 and 1 mg of collagenase were added to give a similar magnitude of activity.

Enzyme degradation of meat fractions

1 ml of standardized enzyme solutions were pipetted into two series of duplicate sets of vials containing 8 ml of the hydrated substrates. One set of duplicate vials was then incubated for 90 min at 60°C for bromelain, ficin, papain and trypsin, and for 3 hr at 38°C for collagenase and Rhozyme P-11. The other was incubated for 110 min at temperatures increasing gradually from 25°C to 80°C. This slow rise of temperature simulates cooking conditions. At the end of the reaction time, one series of samples from each set was treated with 1 ml of 1M perchloric acid to stop the enzyme reaction and to provide acid soluble end products; the other series was heated for 15 min in a boiling water bath to stop the enzyme reaction and to

Table 1—Increases in solubilized components of various meat fractions by enzyme treatment

		Soluble in pH 7 Phosphate Buffer		Soluble in 0.1M Perchloric Acid	
Enzymes	Substrates[a]	Soluble Nitrogen (μg/ml)	Free Amino Groups (μg/ml)	Soluble Nitrogen (μg/ml)	Free Amino Groups (μg/ml)
None (Control)	W[b]	558	101	472	82
	S[b]	291	25	199	14
	I[b]	94	3	8	3
Bromelain (0.29 mg)	W	634	142	474	101
	S	1280	138	708	144
	I	1959	82	1016	80
Collagenase (1.00 mg)	W	272	18	70	21
	S	562	69	261	55
	I	1320	38	795	38
Ficin (0.09 mg)	W	160	48	156	6
	S	953	120	806	120
	I	667	37	523	29
Papain (0.19 mg)	W	509	106	173	36
	S	1251	108	660	104
	I	303	27	237	20
Rhozyme P-11 (14.40 mg)	W	749	199	742	292
	S	1016	237	1010	311
	I	467	123	408	139
Trypsin (0.40 mg)	W	689	109	519	108
	S	1367	119	891	112
	I	1546	95	1008	110

[a]W = Water soluble fraction; S = salt soluble fraction; I = insoluble fraction. Each substrate contained 19.6 mg of nitrogen and was made up to a final volume of 10 ml for incubation at 60°C for 1½ hours or, for collagenase and Rhozyme, at 38°C for 3 hr. The amount of dry enzyme powders used per tube are shown in parentheses.

[b]Values for the controls represent the amounts of the components determined when substrates were carried through the procedure without addition of enzyme. These values were subtracted from those found for the enzyme treated substrates to give the increases tabulated in the remainder of the table.

Table 2–Percent of meat fractions solubilized by treatment with enzymes[a]

	Meat Fractions[b]		
Enzymes	Water Soluble (%)	Salt Soluble (%)	Insoluble (%)
Bromelain	16	33	51
Collagenase	13	26	61
Ficin	9	54	37
Papain	25	60	15
Rhozyme P-11	34	45	21
Trypsin	19	38	43

[a]Data refer to the non-heat-coagulable nitrogen in solution after enzyme treatment, as a percent of the total nitrogen of the sample.

[b]To facilitate comparisons between enzymes, data have been adjusted to enzyme levels which would produce the same total amount of solubilized nitrogen from the three fractions.

Table 3–Comparison of degree of hydrolysis of three meat fractions after enzyme degradation.

		Soluble in pH 7 phosphate buffer			
Enzymes	Substrates	Soluble nitrogen (μg/ml)	Free amino nitrogen (μg/ml)	Free amino nitrogen / Soluble Nitrogen (X 100)	Estimated[a] Avg. Peptide Chain Length (No. of A.A.)
Bromelain	W	634	125	19.7	5.1
	S	1280	121	9.5	10.5
	I	1959	72	2.7	37.0
Collagenase	W	272	16	5.9	17.0
	S	562	61	10.9	9.2
	I	1320	33	2.5	40.0
Ficin	W	160	42	26.2	3.8
	S	953	106	11.1	9.0
	I	667	33	4.9	20.4
Papain	W	509	93	18.3	5.5
	S	1251	95	7.6	13.2
	I	303	24	7.9	12.7
Rhozyme P-11	W	749	175	23.4	4.3
	S	1016	209	20.5	4.8
	I	467	108	23.1	4.3
Trypsin	W	689	96	13.9	7.2
	S	1367	105	7.7	13.0
	I	1546	84	5.4	18.5

[a]Estimated from the equation:

$$\frac{\text{Free amino nitrogen}}{\text{Soluble nitrogen}} = \frac{1}{\text{Avg. peptide chain length}}$$

provide non-heat-coagulable and phosphate buffer soluble products of the meat fractions. A milliliter of water was added to each vial of the boiled samples to make the final volumes 10 ml for all samples. Control samples of each of the substrates were run through the procedures without additions of enzymes.

After cooling, the digests were centrifuged and the supernatants filtered. The residues were washed with 10 ml of buffer and filtered through the same paper to combine with the first filtrate.

Measurements

Total nitrogen in the initial substrates was measured by Kjeldahl analyses and the nitrogen contents of phosphate buffer or acid soluble filtrates were determined by nesslerization following sulfuric acid digestion.

The degree of hydrolysis was measured by analyses of terminal amino groups with trinitrobenzene sulfonic acid (Habeeb, 1966).

RESULTS AND DISCUSSION

THE INCREASES in soluble components of various meat fractions during enzyme treatment are shown in Table 1. Data in the left half of the table relate to non-heat-coagulable materials soluble in 0.05M phosphate buffer (pH 7.0) after the various enzyme treatments; data in the right half of the table refer to identical treatments except that they represent materials soluble in 0.1M perchloric acid. As expected, smaller amounts of soluble nitrogen were found for the perchloric acid filtrates than for the buffer filtrates.

The percent of nitrogen solubilized was obtained by dividing the amount of nitrogen in each filtrate by the amount of nitrogen in the substrate subjected to enzyme treatment, and multiplying the result by 100. To facilitate comparisons among enzymes, data have been adjusted to enzyme levels which would produce the same total increase in soluble nitrogen from the three fractions, as shown in Table 2. Figures in any one line of Table 2 thus reflect the relative degree of hydrolysis of the several types of protein in meat by an enzyme acting under the conditions specified. In a similar manner, figures in any one column reflect the relative effectiveness of the several enzymes for each type of protein. However, values on the different lines do not represent relative potencies per gram of enzyme–the amounts of enzymes were adjusted to give solutions similar in overall hydrolytic ability. As indicated in the table, the three fractions of meat show a varying degree of susceptibility to enzyme degradation and, also, each enzyme had its specific pattern of hydrolytic effect. All of the enzymes tested in this experiment showed substantial activity on the salt soluble and insoluble fractions and variable activity on the water soluble fraction.

Figure 1 is a graphical presentation of data reflecting increases in the nitrogen content of non-heat-coagulable materials from sarcoplasmic and stroma proteins when treated with enzymes under conditions that would result in solublization of 25% of the nitrogen of the myofibrillar fraction. Collagenase, bromelain, and trypsin showed stronger solubilizing activities on the stroma fraction than on the salt soluble fraction, whereas papain, Rhozyme P-11 and ficin showed more activity on the salt soluble fraction than on the insoluble fraction. In general, the water solubles were more resistant to enzyme degradation than the other two fractions and ficin showed the least activity on this water soluble fraction.

The ratio of free amino nitrogen over total soluble nitrogen was calculated and, using this ratio, the average peptide chain length was estimated from the percent of free amino nitrogen, considering one free amino nitrogen for each peptide (ignoring the effects of epsilon-amino group). The peptide chain lengths are inversely proportional to the percents of free amino nitrogen. These are shown in Table 3. While these are only approximations they show the general effects of the several enzymes. For all of the enzymes tested except collagenase the water soluble fraction showed the greatest percent of free amino nitrogen, indicating that the non-coagulable material produced from the water soluble fraction consisted of smaller peptides than those derived from the insoluble fraction. When hydrolysis of the same substrate by a different enzyme was considered, different pictures were obtained. For example, the insoluble fraction of meat was broken down to smaller peptides with Rhozyme P-11 than with the other enzymes.

Substrates and enzymes incubated at the programmed temperatures showed more or less the same relative activities, although trypsin was slightly more active

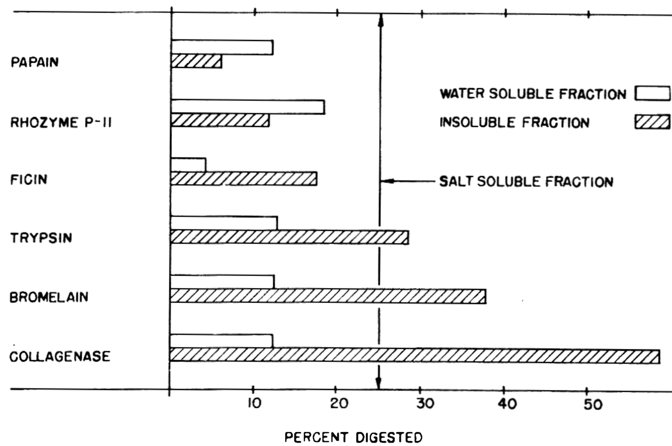

Fig. 1—Relative activity of enzymes on three fractions of meat.

due to a longer period at its optimum temperature range.

Numerous attempts have been made to correlate chemical and physical changes taking place in specific structural components of meat during post mortem aging with tenderization. As pointed out in the literature, modification of connective tissue is desirable to reduce toughness of meat since the quantity and degree of cross linkage of connective tissue affects tenderness of meat (Cover and Smith, 1956; Irvin and Cover, 1959; Goll et al. 1964a and 1964b), and yet little or no change in connective tissue has been observed during natural aging, (Steiner, 1939; Wierbicki et al., 1954; de Fremery and Streeter, 1969) whereas a convincing body of evidence indicates changes within the fibrous components of muscle during aging. Davey and Gilbert (1968a and 1968b) showed that aging processes are associated with an increased extractability of actin as well as with the disruption of Z bands in myofibrils. A similar change in fine structure, i.e., disruption of Z bands, was caused by a brief treatment of myofibrils with trypsin (Stromer et al., 1967).

The proteolytic enzymes tested in our experiment showed hydrolytic activity both on myofibrillar protein and on connective tissue, and the degree of hydrolysis of these fractions varied with the type of enzyme used. Collagenase, bromelain and trypsin degraded the stroma fraction more strongly than the myofibrillar fraction. In contrast, papain, Rhozyme P-11 and ficin showed more activity on the myofibrillar fraction than on the other. These differences in hydrolytic characteristics may lead us to a better understanding of mechanisms of enzymatic tenderization and to a more desirable tenderness of meat.

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FREDERICK E. STONE
Bureau of Commercial Fisheries, Technological Laboratory, Ketchikan, Alaska 99901

ENZYMATIC DEAMINATION OF ADENOSINE MONOPHOSPHATE (AMP), ADENOSINE AND ADENINE BY SALMON, CRAB AND SCALLOP MUSCLE EXTRACTS

SUMMARY—Crude extracts of pink and coho salmon fillets contain both adenosine aminohydrolase (EC 3.5.4.4.) and 5'-AMP-aminohydrolase (EC 3.5.4.6.). Similar extracts from king and Tanner crab meat and from scallop adductor muscle contain adenosine aminohydrolase but not 5'-AMP-aminohydrolase. Adenine aminohydrolase activity was not detected in salmon, crab or scallops. These results suggest that the enzymatic deamination of 5'-adenosine monophosphate (AMP) will contribute to the 5'-inosine monophosphate (IMP) content of salmon fillets but will not contribute significantly to the IMP content of crab or scallop muscles.

INTRODUCTION

THE DEGRADATION products of the adenine nucleotides, particularly inosine monophosphate (IMP) and hypoxanthine, have been correlated with the quality and flavor of swordfish (Dyer et al., 1966), cod (Jones and Murray, 1964) and irradiated fillets (Spinelli and Miyauchi, 1968). The build-up and subsequent decline of IMP in fish during storage has been attributed to a rapid deamination of adenosine 5'-monophosphate (AMP) to IMP, followed by a slow dephosphorylation of IMP to inosine (Jones and Murray, 1964; Tarr, 1966).

Pathways for the degradation of the adenine nucleotides in the invertebrates have not been clearly established. Arai (1966) found no IMP in certain marine invertebrates and demonstrated that, in many species, AMP is dephosphorylated

Table 1–Total activities[1] of the aminohydrolases detected in salmon, crab and scallop muscle extracts.

Species	Extraction buffer	AMP	Substrate Adenosine	Adenine
Pink salmon	Lee[2]	4.6	0.08	None
	0.1 M Succinate pH 6.5	0.6	0.12	None
	0.6 M KCl	0.5	0.09	None
Coho salmon	Lee[2]	4.0	0.07	None
	0.1 M Succinate, pH 6.5	0.8	0.12	None
	0.6 M KCl	0.6	0.08	None
King crab	Lee[2]	None	0.33	None
	0.1 M Succinate, pH 6.5	None	0.43	None
	0.6 M KCl	None	0.39	None
Tanner crab	Lee[2]	None	0.09	None
	0.1 M Succinate, pH 6.5	None	0.14	None
	0.6 M KCl	None	0.12	None
Weathervane scallop	Lee[2]	None	0.14	None
	0.1 M Succinate, pH 6.5	None	0.16	None
	0.6 M KCl	None	0.11	None

[1] Total activity is the number of micromoles of substrate deaminated per minute per gram of moist tissue at 25°C. Conditions for extraction and assay are given in the experimental section.

[2] 0.3 M KCl, 0.09 M KH_2PO_4 and 0.6 M K_2HPO_4, adjusted to pH 6.5.

to adenosine, which is then deaminated to inosine. The crustaceans have been reported to contain either or both pathways. Arai (1966) observed that in prawns deamination of AMP predominated at 19°C whereas deamination of adenosine predominates at −6°C, and suggested that a similar situation might occur in king crab. A different situation was observed in lobster (Dingle et al., 1968) in which AMP aminohydrolase but no adenosine aminohydrolase activity was detected.

The purpose of this study was to determine if the deaminases of AMP, adenosine and adenine are present in various extracts from the muscles of pink salmon, coho salmon, king crab, Tanner crab and weathervane scallops. Detection of these substrate specific enzymes would indicate which of the pathways for the degradation of AMP are possible in these species.

MATERIALS & METHODS

Sample preparation

Pink salmon *(Oncorhynchus gorbuscha)* and coho salmon *(Oncorhynchus kisutch)* were caught near Ketchikan, Alaska, in August, 1968, and held in ice less than 24 hr before preparation. Composite samples of each salmon species were prepared by blending several fillets in a prechilled grinder. These were stored at −50°C until analyzed.

Hard-shell male king crabs *(Paralithodes camtschatica)* and Tanner crabs *(Chionoecetes bairdi)* were caught near Kodiak, Alaska, in September, 1968. They were butchered alive and the leg sections immediately frozen in dry ice and flown to the laboratory, where they were stored at −50°C. The shells were removed prior to analysis and the leg and body meat from several crabs blended together in a prechilled grinder.

Weathervane scallop *(Platinopecten caurinus)* adductor muscles were collected in the Gulf of Alaska in September, 1968, and held on ice less than 48 hr before being flown to the laboratory. Upon arrival, composite samples were prepared by blending the muscles in a prechilled grinder, then stored at −50°C until analyzed.

Extraction techniques

To compare species differences in the extractability of the aminohydrolases, 3 extraction buffers were used for each species. The 3 extraction buffers selected were: a) 0.3 M KCl, 0.09 M KH_2PO_4 and 0.06 M K_2HPO_4 adjusted to pH 6.5 (Lee, 1957); b) 0.1 M potassium succinate adjusted to pH 6.5, and c) 0.6 M KCl (Dingle et al., 1968).

Extracts for enzyme assay were prepared from each species examined by homogenizing 10–20 g of frozen muscle tissue in a Waring Blendor with 7.0 parts of cold extraction buffer, then stirring for 1 hr at 3°C.

The extracts were centrifuged at 14,000 × g for 30 min, forming a firmly packed residue which contained no deaminase activity. At least 80% of the salmon extracts and 90% of the crab and scallop extracts could be decanted free of undispersed material. Insoluble particles were removed by pouring the supernatant liquid through 2 layers of cheese cloth.

Enzyme assay

The enzyme extracts were diluted with the appropriate extraction buffer to a range measurable by Kalckar's (1947) method of differential spectrophotometry. The aminohydrolase activities of each of the diluted extracts were determined, using 3 different substrates. The substrates selected were: a) 15 mg/liter AMP in 0.1 M potassium succinate adjusted to pH 6.5; b) 12 mg/liter adenosine in 0.05 M phosphate adjusted to pH 7.5, and c) 8 mg/liter adenine in 0.05 M phosphate adjusted to pH 7.0.

The reaction was carried out in a quartz cuvette containing 0.1 ml of diluted enzyme extract and 3.0 ml of the appropriate substrate. The rate of decrease in absorbance at 265 mμ and 25°C was determined using a Gilford Model 2000 Automatic Recording Spectrophotometer. The initial slope of the absorbance vs. time curve was converted to micromoles of substrate deaminated per minute per gram of moist tissue by multiplying by the reaction volume (liters) and dividing by the difference between the molar absorbancies of the substrate and products at 265 mμ and by the weight (g) of moist tissue represented. Differences in molar absorbance between substrate and product used was 8860 for AMP and IMP (Smiley et al., 1967), 7900 for adenosine and inosine (Hoagland and Fisher, 1967) and 5100 for adenine and hypoxanthine (Heppel et al., 1957).

RESULTS & DISCUSSION

THE AMINOHYDROLASE activities of extracts from pink salmon, coho salmon, Tanner crabs, king crabs and weathervane scallops with AMP, adenosine and adenine as substrates are given in Table 1. Total activities for each aminohydrolase in the crude extract were reported as micromoles substrate deaminated per minute per gram of moist tissue. Further purification of the enzymes may increase the activity per gram of protein extracted, but would decrease the yield per gram of moist tissue.

Makarewicz (1969) reported the effects of various nucleotides, nucleosides and inorganic ions on the rate of AMP deamination in extracts of muscle from elasmobranch fish. He reported that AMP-aminohydrolase from this source is substrate specific and has a broad pH optimum at pH 6.6 in 0.1 M succinate buffer. His data support the observation of Kalckar (1947) that the initial rate of deamination is a function of the substrate concentration in the range of the present experiment. Any variation in the assay method, particularly in the substrate concentration, will change the observed enzyme activity. Since the conditions for assay are not identical to those in the intact muscle tissue, it is unlikely that there will be a correlation between the observed rate of enzyme activity and the rate of formation of product in the intact animal.

Furthermore, since the degradation of the adenine nucleotides involves a sequence of reactions, the rate at which the products of deamination accumulate in muscle tissue depends upon both the activities of the enzymes which form

these products and the activities of the enzymes which further change these products. For these reasons, in the present work, the detection of enzyme activity, rather than the accumulation of products, was used to suggest probable pathways for deamination.

The 3 buffers described in Table 1 extracted 5′-AMP-aminohydrolase from pink and coho salmon. Creelman and Tomlinson (1960) reported that the IMP found in salmon is slowly dephosphorylated to inosine. The detection of 5′-AMP aminohydrolase is evidence that AMP is a source of IMP in salmon. Storage conditions which favor the activity of this enzyme while inhibiting the dephosphorylation of IMP should be beneficial to the quality of salmon.

Adenosine aminohydrolase activity was detected in all 3 extracts from each of the species investigated. Although Creelman and Tomlinson (1960) found no adenosine in salmon, detection of adenosine aminohydrolase suggests that part of the inosine which they observed was derived by the deamination of adenosine. In this respect, pink and coho salmon are similar to lingcod, in which Tarr and Comer (1964) observed both AMP aminohydrolase and adenosine aminohydrolase.

Under the conditions used in this report, no deamination of AMP to IMP could be demonstrated in king crab, Tanner crab or weathervane scallops. All 3 buffers failed to extract detectable amounts of 5′-AMP aminohydrolase from these species. Further examination failed to disclose this enzyme in any of the extracts either before or after centrifugation, or in the precipitated residues. Adenosine aminohydrolase activity, however, was detected in every extract from these species.

These findings suggest that the principal pathway for the degradation of the adenine nucleotides in king crab, Tanner crab and weathervane scallops involves the formation of inosine from adenosine rather than the formation of IMP from AMP. In this respect, king crab and Tanner crab are different from some of the other crustaceans. Dingle et al. (1968) found AMP-aminohydrolase but no significant amount of adenosine aminohydrolase in lobster muscle extracts. King crab and Tanner crab are also different from prawns, in which Arai (1966) found evidence for both AMP-aminohydrolase and adenosine aminohydrolase.

Furthermore, Arai (1966), Porter (1968) and Groninger and Brandt (1969) found that the IMP content of king crab is less than the flavor threshold value. Groninger and Brandt (1969) also reported that AMP rather than IMP accumulated in king crab during processing and during the storage of the raw tissue on ice.

The buffers used in this experiment failed to extract a detectable amount of adenine aminohydrolase activity. Tarr and Comer (1964) report that lingcod muscle possesses a very weak adenine deaminase activity but suggest that the deamination of this substrate might be caused by the adenosine deaminase present. Failure to detect adenine aminohydrolase in any of the extracts suggests that the degradation of AMP by way of adenine is insignificant in the 5 species investigated.

Unlike king crabs and Tanner crabs, which are processed live, the adductor muscles of weathervane scallops are commonly held on ice in the holds of ships for periods up to 10 days and sometimes even longer. Although Kitagawa and Tonomura (1957) and Arai (1966) found no IMP in scallops, they did not extend their studies to include ice-held scallops. Failure to detect 5′-AMP-aminohydrolase activity in scallops, however, implies that the formation of IMP from AMP would be insignificant.

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Use of trade names is for descriptive purposes and does not imply endorsement.

G. R. SCHMIDT and E. J. BRISKEY
Department of Meat and Animal Science, University of Wisconsin, Madison, Wisconsin 53706

PHOSPHOCREATINE AND NUCLEOTIDE CHANGES IN PIG LONGISSIMUS MUSCLE DURING THE DEVELOPMENT OF RIGOR MORTIS UNDER CONTROLLED ENVIRONMENTAL CONDITIONS

SUMMARY—This study was conducted to compare the depletion of phosphocreatine (PC) and nucleotides (ATP and ADP) in strips of muscle which had been used for extensibility measurements in the rigorometer with control strips of muscle which had been held unrestrained in the same environment. There were no significant differences in PC and ATP between the rigorometer and control strips. Rigorometer strips from "stress-susceptible" pigs had less rapid and less extensive depletion of ADP than did control strips from the same animals. It appeared that extensibility remained in the tissue after the ATP had been depleted, but not after ADP reached a low level.

INTRODUCTION

CHEMICAL AND PHYSICAL changes thought to be associated with the development of rigor mortis have been recently summarized by Newbold (1966): (1) phosphocreatine (PC) and pH start to decrease immediately after exsanguination; (2) adenosine triphosphate (ATP) level remains virtually constant until the PC content has been almost depleted; and (3) extensibility decreases sharply as the ATP level approaches depletion. The loss in extensibility has been observed in the muscles from animals of various species and has been found to be generally associated with the decrease in level of ATP (Newbold, 1966). However, some disagreement has been reported on the association of ATP and extensibility in pig muscle. Lawrie (1960) reported that the major loss in extensibility begins when ATP has decreased to 87%, while Bendall et al. (1963) found that this rapid change in extensibility occurred when ATP had decreased to 30% of its initial level. Additionally, it is not known whether the chemical changes in muscle strips, which have been subjected to loading and unloading, as is done during the measurement of rigor mortis, parallel the changes in excised control strips which have been held in the same environment, without the application of weight.

The present study was conducted to determine whether the extensibility measurement per se alters the phosphocreatine and nucleotide content of the muscle. Muscles of "stress-susceptible" and "stress-resistant" animals (Judge et al., 1968; Forrest et al., 1968) were selected so that the evaluations could be made over a wide range in rate of metabolism post-mortem (Kastenschmidt, 1966).

EXPERIMENTAL

Muscle used

Pig muscle (longissimus) was obtained from 12 "stress-susceptible" Poland China and 7 "stress-resistant" Chester White pigs which had been stunned with a captive bolt pistol and bled by severing the anterior vena cava. Samples were excised within 5 min of immobilization and exsanguination.

Measurement of extensibility changes

Twelve strips of 5 cm long and 1 cm square in cross section, with the fibers running longitudinally, were excised from the longissimus of each pig. Six strips were placed on the isotonic-isometric rigorometer as described by Schmidt et al. (1968), with a loading-unloading cycle of 2 min on and 2 min off. The test strips remained under a permanent load of about 10g, and a further 50g (the "applied" load) was added and removed periodically.

Environment and sample preparation

All samples were maintained in a water saturated nitrogen atmosphere. In addition to the six strips used to measure the losses in extensibility as described above, six additional strips were similarly suspended in the same environment, but without the application of weight. Six channels were available for isotonic measurement, enabling the comparison of six strips from the same muscle at six different time periods post-mortem. In the first experiment samples were removed at 30-min intervals and in the subsequent experiment, samples were removed at 60-min intervals (Fig. 1).

Chemical estimations

A portion of the sample (longissimus) was frozen in liquid nitrogen within 5 min of excision. As samples were removed from the rigorometer, they were frozen in liquid nitrogen. All samples were stored at liquid nitrogen temperatures until the analyses could be performed. In the initial experiments, samples were analyzed only for PC and ATP. In the subsequent experiment, samples were analyzed for PC, ATP and adenosine diphosphate (ADP) (Bergmeyer, 1963).

Definition

The samples removed from the carcass and frozen in liquid nitrogen within 5 min of excision are referred to as "initial" samples. The extensibility of the muscle strips at 30 min after stunning is referred to as "initial extensibility." "Extensibility" refers to the total increase in length during 2 min under an applied load.

In the preliminary experiment, muscle strips were incubated at 25°C, whereas in the subsequent experiment, muscle strips were incubated at 37°C.

RESULTS

RESULTS OF THE preliminary experi-

Table 1—Phosphocreatine and adenosine triphosphate levels in rigorometer and control samples of Longissimus muscle (stress-susceptible)[a] at various times post-mortem and in relation to loss in extensibility (25° C).

Metabolite and sample source	Time post-mortem (min)					
	30	60	90	120	150	180
PC						
Rigorometer	0.30[b] ±0.14	0.12 ±0.10	0.18 ±0.14	0.32 ±0.17	0.16 ±0.10	0.01 ±0.00
Control	0.20 ±0.10	0.05 ±0.00	0.11 ±0.10	0.07 ±0.00	0.05 ±0.00	0.00 ±0.00
ATP						
Rigorometer	1.30 ±0.44	1.02 ±0.24	0.79 ±0.33	0.50 ±0.32	0.58 ±0.36	0.10 ±0.00
Control	1.43 ±0.33	1.17 ±0.36	0.87 ±0.32	0.75 ±0.30	0.40 ±0.20	0.30 ±0.26
Extensibility	Percent initial extensibility					
	100[c]	87 ±5	75 ±15	50 ±13	41 ±13	25 ±10

[a]Six "stress-susceptible" Poland China pigs.

[b]Values given are the mean (μmoles/gm tissue) ± the standard error of the mean.

[c]The extensibility of the muscle strips at 30 min after stunning is referred to as initial extensibility.

ment are shown in Table 1. Very low initial values of PC and ATP were observed. The rate of depletion of ATP and PC and the loss of extensibility were relatively slow in these muscle strips. The extensibility decreased to 25% of its initial capacity and ATP levels decreased to very low values by 180 min post-mortem Since the extensibility had decreased to only 25% of the original extensibility, while the ATP decreased to very low levels, the following changes were made in the subsequent experiment: Incubation temperatures were raised to 37°C and samples were removed at 1-hr intervals for 6 hr post-mortem (Fig. 1). All incubated strips of muscle were analyzed for PC, ATP and ADP. Six "stress-susceptible" Poland China and seven "stress-resistant" Chester White pigs were utilized in this second experiment.

The results of the second experiment are shown in Tables 2 and 3. Very small differences were observed in PC and ATP levels between rigorometer and control strips of muscles from both strains of pigs. Only slight differences were seen in ADP levels between rigorometer and control strips of muscles from "stress-resistant" pigs (Table 3). However, after 1 hr post-mortem significant ($P < .01$) differences were seen in ADP levels between rigorometer and control strips of muscles from "stress-susceptible" pigs (Table 2). The control strips of muscle from "stress-susceptible" pigs had lower ($P < .01$) ADP levels after 1 hr post-mortem than did the strips from the same animals which were being subjected to the loading and unloading process.

DISCUSSION

WHEN COMPARED with experiment 2, the somewhat lower initial levels of ATP and PC in experiment 1, as well as a subsequently slower decrease in metabolite level, was thought to be the result of variation in the initial rate of glycolysis and the incubation temperature. The data presented demonstrate that muscle strips remain extensible in the absence of detectable levels of ATP. Several previous investigators (Marsh, 1954; Marsh et al., 1958; Bendall et al., 1963; Cassens et al., 1967; Nauss et al., 1966; Erdos, 1943; Weber et al., 1954) concluded that rigor mortis occurred when ATP was almost depleted. However, Cassens et al. (1967) observed that ox muscle at 1°C did not have a low ATP value or reach an ultimate pH until sometime after the change in extensibility was complete. Lawrie (1960) also observed high ATP values at completion of changes in extensibility in pig longissimus muscle at 37°C. Recent work by Kushmerick et al. (1968), using frog sartorius muscle treated with 2,4-dinitrofluorobenzene (DNFB), and observed during thaw rigor, was interpreted to show that the development of thaw rigor required the absence of both ADP and ATP.

When comparing the data from the rigorometer strips with the control strips (Tables 2 and 3), the only consistent significant difference ($P < .05$) observed between the two strips (control and rigorometer) was in ADP values of muscle from "stress-susceptible" pigs after 1 hr post-mortem. One is only able to speculate on reasons for this difference. Perhaps since the muscle strip was in a stretched condition for 50% of its time in the rigorometer, the actin and myosin were only able to interact over a smaller portion of their length than the control

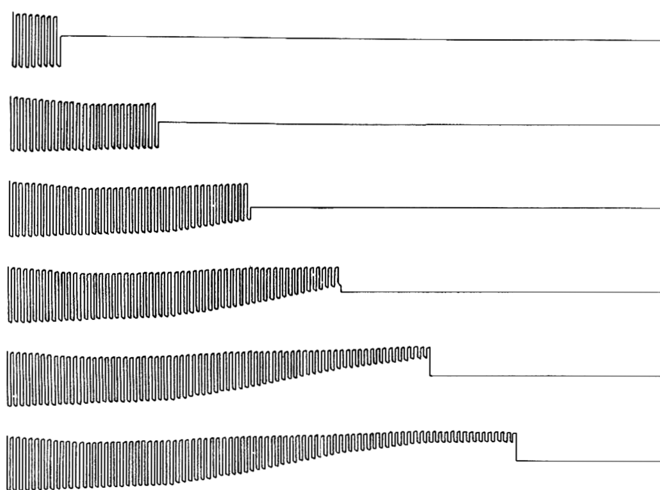

Fig. 1—Example of isotonic recordings of muscle post-mortem with samples removed for analysis at 1 hr intervals.

Table 2—Phosphocreatine, adenosine triphosphate and diphosphate levels in rigorometer and control samples of Longissimus muscle (stress-susceptible)[a] at various times post-mortem and in relation to loss in extensibility (37°C).

Metabolite and sample source	Time post-mortem (hr) 0	1	2	3	4	5	6
PC							
Rigorometer	0.78 ±0.53	0.03[b] ±0.03[c]	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Control		0.53 ±0.53	0.37 ±0.25	0.11 ±0.11	0.06 ±0.06	0.02 ±0.02	0.05 ±0.05
Significance		NS	<.05	NS	NS	<.05	NS
ATP							
Rigorometer	1.96 ±0.22	0.30 ±0.15	0.16 ±0.16	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Control		0.27 ±0.03	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Significance		NS	NS	NS	NS	NS	NS
ADP							
Rigorometer	1.188 ±0.096	0.880 ±0.035	0.758 ±0.014	0.653 ±0.065	0.622 ±0.054	0.588 ±0.037	0.519 ±0.056
Control		0.828 ±0.014	0.606 ±0.077	0.508 ±0.055	0.492 ±0.052	0.402 ±0.065	0.385 ±0.060
Significance		NS	<.01	<.01	<.01	<.01	<.01
Extensibility		Percent initial extensibility[d]					
		44 ±9	3 ±3	1 ±1	0 ±0	0 ±0	0 ±0

[a]Six "stress-susceptible" Poland China pigs.
[b]Mean value (µmoles/gm tissue).
[c]Standard error of the mean.
[d]100% extensibility was taken at 30 min post-mortem.

Table 3–Phosphocreatine, adenosine triphosphate and diphosphate levels in rigorometer and control samples of Longissimus muscle (stress-resistant)[a] at various times post-mortem and in relation to loss in extensibility (37°C).

Metabolite and sample source	Time post-mortem (hr) 0	1	2	3	4	5	6
PC							
Rigorometer	1.49[b] ±0.65[c]	0.60 ±0.23	0.44 ±0.09	0.09 ±0.03	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Control		0.56 ±0.11	0.58 ±0.10	0.21 ±0.12	0.08 ±0.00	0.00 ±0.00	0.00 ±0.00
Significance		NS	NS	NS	NS	NS	NS
ATP							
Rigorometer	2.22 ±0.61	1.95 ±0.50	1.34 ±0.38	0.51 ±0.55	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Control		1.83 ±0.43	1.34 ±0.50	0.60 ±0.31	0.37 ±0.04	0.00 ±0.00	0.00 ±0.00
Significance		NS	NS	NS	<.01	NS	NS
ADP							
Rigorometer	0.680 ±0.114	0.832 ±0.138	0.693 ±0.085	0.826 ±0.045	0.739 ±0.057	0.820 ±0.066	0.788 ±0.102
Control		0.843 ±0.138	0.752 ±0.071	0.734 ±0.061	0.731 ±0.070	0.699 ±0.050	0.734 ±0.088
Significance		NS	NS	NS	NS	<.01	NS
Extensibility		Percent initial extensibility[d]					
		77 ±6	52 ±14	37 ±14	24 ±12	9 ±9	8 ±7

[a]Seven "stress-resistant" Chester White pigs.
[b]Mean value (μmoles/gm tissue).
[c]Standard error of the mean.
[d]100% extensibility was taken at 30 minutes post-mortem.

strips which were free to shorten. Thus, the amount of stretch which a muscle endures may play an important role in determining ultimate ADP values when the muscle is inclined to shorten to a large extent soon after death, as in "stress-susceptible" pigs, but has little effect on muscle from "stress-resistant" pigs which are less inclined to shorten quickly after death.

Since the levels of PC and ATP did not differ between the control (unrestricted) and rigorometer samples, it is considered to be a valid procedure to use samples which are not on the rigorometer to compare PC and ATP to extensibility changes in duplicate samples, as long as all samples are held in the same environment and the temperature is rigidly controlled.

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G. R. SCHMIDT, R. G. CASSENS and E. J. BRISKEY
Department of Meat and Animal Science, University of Wisconsin, Madison, Wisconsin 53706

CHANGES IN TENSION AND CERTAIN METABOLITES DURING THE DEVELOPMENT OF RIGOR MORTIS IN SELECTED RED AND WHITE SKELETAL MUSCLES

SUMMARY The relative time course of rigor mortis was compared in the vastus lateralis (red) and the longissimus (white) muscle from untreated and magnesium sulfate injected "stress-resistant" Chester White pigs and "stress-susceptible" Poland China pigs. In untreated animals the white muscle had a shorter time course of rigor mortis than did the red muscle. This difference disappeared when the animals were injected ante-mortem with magnesium sulfate although both muscles from injected animals had a slower development of rigor mortis than the same muscles in control animals. Either magnesium sulfate had more of an effect on white than on red muscle post-mortem, or the differences between the post-mortem rates of glycolysis of red and white muscles were significantly minimized when struggle and stimulation associated with death had been eliminated. Red muscles in magnesium sulfate injected pigs developed more tension post-mortem than did white muscles. The development of isometric tension reached its maximum as the muscle lost all of its extensibility. Muscle from "stress-susceptible" pigs had a shorter time course of rigor mortis than the corresponding muscle from "stress-resistant" pigs of the same treatment. This difference occurred even when the two groups started with the same level of phosphocreatine and lactic acid post-exsanguination, as is the case when treated with magnesium sulfate. Therefore, even though magnesium treatment can retard glycolysis sufficiently to prevent the development of the PSE condition (Sair et al., 1970), it does not necessarily standardize the post-mortem changes in all skeletal muscles of all pigs.

Table 1—Biochemical and mechanical properties of selected red and white muscles from control and magnesium sulfate injected "stress-resistant" and "stress-susceptible" pigs.

	µmoles/gram tissue[a]			min[c]	grams[d]
	PC	ATP	Lactic acid	Time	Tension
Control "stress-resistant" pigs (n = 5)					
Longissimus	1.45	3.52	37.89	143	22
	± 50[b]	±.41	±.58	±21	±3
Vastus lateralis	1.58	3.02	21.53	260	21
	±.45	± 21	±2.36	±31	±3
Significance	NS	NS	P<.01	P<.01	NS
Control "stress-susceptible" pigs (n = 6)					
Longissimus	.57	1.15	50.61	83	–
	±.38	± 37	±5.87	±16	
Vastus lateralis	1.18	1.62	36.86	138	–
	±.61	± 47	±1.96	±33	
Significance	NS	NS	P<.01	P<.05	–
Magnesium sulfate injected "stress-resistant" pigs (n = 6)					
Longissimus	12.70	3.05	12.54	327	15
	±1.45	±.50	±3.57	±44	±2
Vastus lateralis	11.56	2.33	8.90	344	47
	±4.32	±0.14	±2.20	±43	±1
Significance	NS	NS	NS	NS	P<.01
Magnesium sulfate injected "stress-susceptible" pigs (n = 5)					
Longissimus	12.31	2.33	11.17	241	14
	±1.04	±0.71	±2.24	±19	±6
Vastus lateralis	11.16	2.77	9.72	263	40
	±.57	±.63	±2.61	±27	±1
Significance	NS	NS	NS	NS	P<.05

[a]Mean value (samples were frozen in liquid nitrogen within 5 min of excision).
[b]Standard error of the mean.
[c]Time refers to minutes required to complete extensibility changes at 37°C.
[d]Maximum number of grams tension developed post-mortem.

INTRODUCTION

NUMEROUS STUDIES have been conducted on certain biochemical and biophysical aspects of rigor mortis in skeletal muscle. The results of these studies, however, have not been conclusive. A high concentration of myoglobin (red muscle) has been associated with a high capacity for aerobic resynthesis of energy-rich phosphate and a high activity of the succinic dehydrogenase-cytochrome system. It has also been associated with a low capacity of anaerobic glycolysis, low adenosine triphosphatase (ATPase) activity, and low energy-rich phosphate stores (Lawrie, 1953a, 1953b). Using light and dark portions of the semitendinosus, Beecher et al. (1965) found that the light portion had a lower pH and glycogen content immediately after exsanguination, but a longer delay phase of rigor mortis and equal contents of lactic acid 24 hours post-exsanguination. However, Milo et al. (1964) concluded that rigor mortis developed more quickly in white than in red muscle, but their evaluations were based on in situ estimations. Forrest et al. (1967) observed no significant difference between red and white muscles in response to electrical stimulation or in the time course of rigor mortis.

Beecher et al. (1965) anesthetized a number of animals with sodium pentobarbital to eliminate the death struggle and minimize stimulation during excision of the sample. They found that in comparison with the control animals, there was a greater increase in the length of the delay phase of rigor mortis in light muscle portions than in dark muscle portions. Sair et al. (1970) showed that the post-mortem levels of phosphocreatine (PC) could be greatly increased by ante-mortem intravenous injections of a solution of magnesium sulfate.

Studies were conducted (1) to determine the relative time course of rigor mortis in a red muscle (vastus lateralis) and a white muscle (longissimus) from strains of pigs which normally have slow-glycolyzing muscles ("stress-resistant" Chester White) and fast-glycolyzing muscles ("stress-susceptible" Poland China); (2) to evaluate the time course of rigor mortis in muscle in "stress-resistant" Chester White pigs and "stress-susceptible" Poland China pigs with and without ante-mortem injection of magnesium sulfate; and (3) to investigate the relation

between the development of tension and the loss of extensibility in muscles varying widely in the time course of rigor mortis.

EXPERIMENTAL

Untreated controls

Five "stress-resistant" Chester White pigs and six "stress-susceptible" Poland China pigs were utilized as untreated controls.

Magnesium treatment

Six "stress-resistant" Chester White and five "stress-susceptible" Poland China pigs were intravenously injected with 20 to 25 mg of magnesium sulfate per kilogram body weight. Approximately one-half of this dose was injected to produce narcosis and the other half of the dose was infused at a slow rate to maintain this state of narcosis over a 20-min period prior to exsanguination.

Sampling

All animals were stunned with a captive bolt pistol and bled by severing the anterior vena cava. Muscle samples were excised from the longissimus and vastus lateralis within 5 min of immobilization and exsanguination.

Chemical estimations

Within 5 min of excision portions of the samples were frozen and stored in liquid nitrogen until analysis could be performed. Samples were analyzed for phosphocreatine (PC), adenosine triphosphate (ATP), and lactic acid according to the procedures described by Bergmeyer (1963).

Mechanical estimations

Extensibility and tension changes were determined on muscle samples, 1 cm^2 in cross section and 5 cm long, at 37°C in a water saturated nitrogen atmosphere with the use of an isotonic-isometric rigorometer (Schmidt et al., 1968). A microdisplacement myograph (Part No. 91-100-80, E & M Physiograph Company) was used to measure the development of tension. A sensitivity of 10g per centimeter pen deflection was used. Changes in extensibility were determined with a motion myograph (Part No. 91-100-73, E & M Physiograph Company). A 50g weight was applied and removed at 2-min intervals.

Table 2–Biochemical and mechanical properties of selected red and white muscles of control and magnesium sulfate injected "stress-resistant" and "stress-susceptible" pigs.

	μmoles/gram tissue[a]			min[c]	grams[d]
	PC	ATP	Lactic acid	Time	Tension
Control–Longissimus					
"Stress-resistant" pigs (n = 5)	1.45	3.52	37.89	143	22
	±.50[b]	±.41	±.58	±21	±3
"Stress-susceptible" pigs (n = 6)	.57	1.15	50.61	83	–
	±.38	± 37	±5.87	±16	
Significance	P<.05	P<.01	P<.01	P<.01	–
Control–Vastus lateralis					
"Stress-resistant" pigs (n = 5)	1.58	3.02	21.53	260	21
	±.45	±.21	±2.36	±31	±3
"Stress-susceptible" pigs (n = 6)	1.18	1.62	36.86	138	–
	±.61	±.47	±1.96	±33	
Significance	NS	P<.01	P<.01	P<.01	–
Magnesium sulfate injected longissimus					
"Stress-resistant" pigs (n = 6)	12.70	3.05	12.54	327	15
	±1.45	±.50	±3.57	±44	±2
"Stress-susceptible" pigs (n = 5)	12.31	2.33	11.17	241	14
	±1.04	±0.71	±2.24	±19	±6
Significance	NS	NS	NS	P<.01	NS
Magnesium sulfate injected vastus lateralis					
"Stress-resistant" pigs (n = 6)	11.56	2.33	8.90	344	47
	±4.32	±0.14	±2.20	±43	±1
"Stress-susceptible" pigs (n = 5)	11.16	2.77	9.72	263	40
	±.57	±.63	±2.61	±27	±1
Significance	NS	NS	NS	P<.01	P<.01

[a] Mean value (Samples were frozen in liquid nitrogen within 5 min of excision).
[b] Standard error of the mean.
[c] Time refers to minutes required to complete extensibility changes at 37°C.
[d] Maximum number of grams tension developed post-mortem.

RESULTS

CERTAIN BIOCHEMICAL and mechanical properties of muscle from control and magnesium injected 'stress-resistant" and "stress-susceptible" pigs are shown on Table 1. Although there were no significant differences in the initial levels of PC and ATP between red and white muscles of any of the groups of pigs, the longissimus muscles (white) in the control "stress-resistant" and "stress-susceptible" pigs had significantly ($P < .01$) higher initial levels of lactic acid than the vastus lateralis (red) muscles of the same animals. However, when injected with magnesium sulfate, there were no significant differences in the initial levels of lactic acid in the muscles of "stress-susceptible" and "stress-resistant" pigs.

The longissimus samples from the control "stress-resistant" and "stress-susceptible" pigs lost all of their extensibility in a significantly ($P < .05$) shorter post-mortem period than did the vastus lateralis samples. This difference was not observed in the magnesium sulfate injected pigs. Isometric tension data were not available for the muscles from the control "stress-susceptible" pigs. However, in the magnesium sulfate injected pigs the longissimus muscle of both groups developed significantly ($P < .05$) less isometric tension than did the vastus lateralis.

The effect of "stress-susceptibility" on post-mortem metabolism is shown in Table 2. Within the control animals, the longissimus muscle samples from "stress-resistant" pigs had higher ($P < .05$) initial levels of PC, higher ($P < .01$) initial levels of ATP, lower ($P < .01$) levels of lactic acid, and longer ($P < .01$) time course of rigor mortis than did longissimus samples from "stress-susceptible" pigs. The vastus lateralis samples from control "stress-resistant" pigs had the same initial levels of PC, significantly higher ($P < .01$) initial levels of ATP, lower levels of lactic acid, and a longer time course of rigor mortis than did vastus lateralis samples from the control "stress-susceptible" pigs.

Within the magnesium sulfate injected animals, there were no significant differences in initial levels of PC, ATP, or lactic acid between "stress-resistant" pigs and "stress-susceptible" pigs in either the longissimus or vastus lateralis. However, the magnesium sulfate injected "stress-resistant" pigs had a significantly ($P < .01$) longer time course of rigor mortis in both the longissimus and vastus lateralis than did magnesium sulfate injected ' stress-susceptible" pigs. Vastus lateralis muscles, from the magnesium sulfate injected "stress-resistant" pigs, produced significantly ($P < .01$) greater isometric tension than did the same muscle from the magnesium sulfate injected "stress-susceptible" pigs.

Figure 1 shows the concurrent loss of extensibility and development of tension for longissimus and vastus lateralis samples. The maximum amount of tension developed at or near the time of complete loss in extensibility in the duplicate strip.

DISCUSSION

THE VASTUS LATERALIS samples

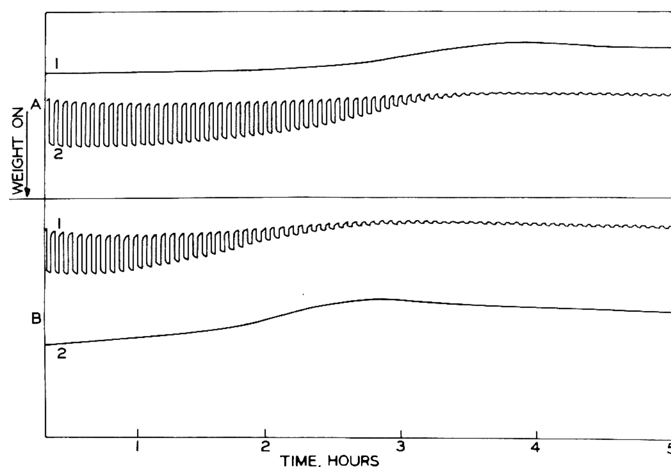

Fig. 1—The time course of isometric and isotonic changes in the vastus lateralis (red) and longissimus (white) muscles. (A) Vastus lateralis muscle: 1. Isometric change post-mortem; and 2. Isotonic change post-mortem. (B) Longissimus muscle: 1. Isotonic change post-mortem; and 2. Isometric change post-mortem.

from the control animals had a longer delay before the loss of extensibility started to occur and also proceeded to rigor completion more slowly than did the longissimus samples from the same animals. However, when animals were injected ante-mortem with magnesium sulfate, no difference could be detected in the time course of rigor mortis between these red and white muscles. Forrest et al. (1967) also found no difference between red and white muscles in the time course of rigor mortis, whereas Beecher et al. (1965) and Briskey et al. (1962) found that the dark portion of the semitendinosus had a more rapid time course of rigor mortis than the light portion of the same muscle. Beecher et al. (1965) found that anesthesia (sodium pentobarbital) delayed the development of rigor in the light, but not in the dark portion of the semitendinosus. Sair et al. (1970) demonstrated that the intravenous injection of magnesium sulfate, ante-mortem, prevented the post-mortem development of pale, soft, and exudative (PSE) porcine muscle. Magnesium is known to block neuromuscular transmission (del Castillo et al., 1954). However, the exact mechanisms whereby magnesium sulfate affects muscle metabolism post-mortem are not known.

White and red muscles from magnesium sulfate injected "stress-susceptible" pigs had respectively similar levels of PC, ATP and lactic acid to white and red muscles from "stress-resistant" magnesium injected pigs. These muscles, however, from the magnesium sulfate injected "stress-susceptible" pigs still had a shorter time course of rigor mortis than did the corresponding muscles from the "stress-resistant" pigs. It appears, therefore, that if the muscles are potentially fast-glycolyzing (Kastenschmidt et al., 1968; Lister et al., 1970; Sair et al., 1970) they will have a faster development of rigor mortis, even though they have been forced, through magnesium sulfate injections, to have similar initial levels of PC. This implies that either the magnesium ions have a longer lasting effect post-mortem in the muscles of "stress-resistant" animals, or that the muscles of "stress-susceptible" animals are inherently affected by anaerobic conditions to a greater extent, than are muscles of "stress-resistant" animals. When under the influence of magnesium the red muscle developed more isometric tension than the white muscle. The discussion of this finding must await further studies on the mechanism of action of magnesium.

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G. R. SCHMIDT, R. G. CASSENS and E. J. BRISKEY
Muscle Biology Laboratory, University of Wisconsin, Madison, Wisconsin 53706

RELATIONSHIP OF CALCIUM UPTAKE BY THE SARCOPLASMIC RETICULUM TO TENSION DEVELOPMENT AND RIGOR MORTIS IN STRIATED MUSCLE

SUMMARY—Concentrations of phosphocreatine (PC), adenosine triphosphate (ATP) and lactic acid were determined in longissimus muscle from 7 "stress-resistant" Chester White pigs and 8 "stress-susceptible" Poland China pigs at several intervals during the loss of extensibility post-mortem. Calcium-binding ability of the sarcoplasmic reticulum and postmortem tension development of longissimus muscle also were determined. Loss of extensibility was completed sooner in longissimus muscle from stress-susceptible pigs than from stress-resistant pigs. For the most part, longissimus muscle samples from stress-susceptible pigs had lower (P < .05) levels of PC and ATP and higher (P < .05) levels of lactic acid at identical stages of change in extensibility than did samples from stress-resistant pigs. At identical stages of change in extensibility in "fast-" and "slow-glycolyzing" muscles, there were no significant differences in the ability of the sarcoplasmic reticulum preparations to bind calcium or in the muscle's ability to develop isometric tension.

INTRODUCTION

THE BIOCHEMICAL changes which occur in a particular muscle post-mortem vary considerably among animals within a species. It has been observed (Judge et al., 1968; Forrest et al., 1968) that the muscles of "stress-susceptible" pigs have a fast rate of glycolysis, a short-time course of rigor mortis and become pale, soft and exudative (PSE) post-mortem. Conversely, the muscles of "stress-resistant" pigs have a slow rate of glycolysis, a long-time course of rigor mortis and retain a normal color and gross morphology post-mortem. Ability of the sarcoplasmic reticulum to bind calcium decreases with time post-mortem (Greaser et al., 1969). The muscles which have the most rapid loss of calcium-binding ability also become PSE (Greaser et al., 1969). Muscle which becomes PSE usually has a short-time course of rigor mortis (Briskey, 1964).

Muscle remains in a relaxed state while the sarcoplasmic reticulum maintains the sarcoplasmic concentration of calcium ions below a critical level of about 1×10^{-7} M (Feinstein, 1966). Consequently, a measure of tension development should indicate how successful the sarcoplasmic reticulum has been in binding calcium ions (Davies, 1963). A change in muscle elasticity and extensibility has been classically associated with the time course of rigor mortis (Bate-Smith, 1939; Bendall, 1960). It has been established that the contraction which occurs during the development of rigor mortis also utilizes adenosine triphosphate (ATP) (Marechal, 1960; Hsu, 1950).

Studies were made (1) to determine the association of the calcium-binding ability of the sarcoplasmic reticulum to certain other biochemical and mechanical changes which occur in muscle post-mortem, (2) to compare the changes in and levels of these biochemical constituents with the development of tension in muscle samples varying widely in the time course of rigor mortis.

EXPERIMENTAL

Muscle used

Pig longissimus muscle was obtained from animals stunned with a captive-bolt pistol and bled by severing the anterior vena cava. Samples were removed within 3 min of immobilization and exsanguination.

Measurement of calcium ion-binding ability

A modified method of Greaser (1969) was used to measure the calcium ion-binding ability of the sarcoplasmic reticulum. Samples were homogenized with 4 volumes of ice-cold 0.1 M KCl and 5 mM histidine (pH 7.2) for 2 min in a Waring Blendor with 10- to 15-sec bursts. Myofibrils, connective tissue, mitochondria and undisrupted fiber segments were removed by centrifugation at 8,000 × g for 20 min. The resulting supernatant was then subjected to centrifugation at 30,000 × g for 1 hr and the sediment was referred to as heavy sarcoplasmic reticulum. This sedimented material was resuspended in 0.1 M KCl-5 mM histidine and the protein content was determined with the biuret

Table 1—Time course of extensibility change[1] in strips of longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	Extensibility (percent initial)			
Sample source	100[3]	75	50	0
Stress-resistant		79[4]	133	214
		±7[5]	±6	±11
Stress-susceptible		51	66	124
		±3	±5	±6
Significance		P<.01	P<.01	P<.01

[1] Experiment 1.
[2] Strips were incubated in a water-saturated nitrogen atmosphere at 37°C.
[3] 100% Extensibility was taken at 30 min post-mortem.
[4] Mean value.
[5] Standard error of the mean.

Table 2—Metabolites[1] of longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	Extensibility (percent initial)			
Metabolite and sample source	100[3]	75	50	0
PC				
Stress-resistant	1.31[4]	.72	.32	.09
	±.47	±.18	±.16	±.08
Stress-susceptible	.92	.11	.00	.00
	±.33	±.06	±.00	±.00
Significance	NS	P<.01	P<.01	P<.05
ATP				
Stress-resistant	3.74	2.71	1.61	1.20
	±.33	±.28	±.31	±.30
Stress-susceptible	2.78	1.62	1.06	.70
	±.40	±.35	±.36	±.12
Significance	P<.01	P<.01	NS	P<.05
Lactic Acid				
Stress-resistant	35.78	57.81	67.42	82.25
	±5.87	±4.45	±2.41	±3.72
Stress-susceptible	44.38	70.00	80.99	95.54
	±3.90	±6.50	±3.74	±6.62
Significance	P<.05	P<.05	P<.01	P<.05

[1] Experiment 1.
[2] Strips were incubated in a water-saturated nitrogen atmosphere at 37°C.
[3] 100% Extensibility was taken at 30 min post-mortem.
[4] Mean value (μmoles/g tissue) ± standard error of the mean.

Table 3–Calcium accumulation[1] by the heavy sarcoplasmic reticulum fraction from longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	Extensibility (percent initial)			
Sample source	100[3]	75	50	0
Stress-resistant	4.19[4]	3.01	2.10	1.39
	±.47	±.71	±.36	±.30
Stress-susceptible	4.00	2.67	2.24	1.63
	±.41	±.47	±.52	±.43
Significance	NS	NS	NS	NS

[1]Experiment 1.

[2]Strips were incubated in a water-saturated nitrogen atmosphere at 37°C.

[3]100% Extensibility was taken at 30 min post-mortem.

[4]Mean value (μmoles Ca^{++}/mg protein) ± standard error of the mean.

Table 4–Metabolite levels[1] in longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	μmoles/gram tissue		
	Phosphocreatine	ATP	Lactic Acid
Stress-resistant	.82[3]	2.26	40.16
	±.26[4]	±.12	±3.12
Stress-susceptible	.00	.93	44.16
	±.00	±.41	±9.80
Significance	P<.01	P<.01	NS

[1]Experiment 2.

[2]All samples were frozen in liquid nitrogen within 5 min of exsanguination.

[3]Mean value.

[4]Standard error of the mean.

procedure (Gornall et al., 1949) using bovine serum albumin as a standard. Measurement of calcium ion uptake was performed in a medium consisting of 0.1 M KCl, 5 mM histidine (pH 7.2), 5 mM $MgCl_2$, 5 mM ATP and 0.2 mM $CaCl_2$ (containing 0.1 μc of $^{45}Ca^{++}$). The approximate protein concentration of the heavy sarcoplasmic reticulum as used in these experiments was 0.04 mg/ml. Following addition of the resuspended fractions to the assay medium and incubation for 15 min at 22–24°C, the particulate material was removed by filtration through millipore filters (type HA), average pore diameter 0.45 μ (Martonosi and Feretos, 1964). The radioactivity of an aliquot of the filtrate was determined by liquid scintillation counting and compared with the appropriate standard to determine the amount of calcium bound by the protein.

Chemical estimations

A portion of the sample (longissimus) was frozen in liquid nitrogen immediately after excision. As samples were removed from the rigorometer, they were also frozen in liquid nitrogen. All samples were stored at liquid nitrogen temperatures until analysis could be performed. Samples were analyzed for phosphocreatine (PC), adenosine triphosphate (ATP) and lactic acid according to procedures of Bergmeyer (1963).

Mechanical estimations

Extensibility and tension changes were determined on muscle samples using an isotonic-isometric rigorometer (Schmidt et al., 1968). A micro-displacement myograph (Part No. 91-100-80, E & M Physiograph Co.) was used to measure the development of tension. A sensitivity of 10 g/cm pen deflection was used. Changes in extensibility were determined using a motion myograph (Part No. 91-100-73, E & M Physiograph Co.). A 50-g weight was applied and removed at 2-min intervals. Samples were maintained at 37°C in either (1) a water-saturated nitrogen atmosphere, or (2) a nitrogen-purged isotonic 0.9% sodium chloride solution until removed for analysis. A series of previous experiments showed that either method is valid as long as comparisons are made within a method.

Experiment 1. 4 stress-resistant Chester White pigs and 5 stress-susceptible Poland China pigs were utilized in experiment 1. 3 portions of the longissimus were removed immediately after exsanguination: Portion 1 was frozen in liquid nitrogen, portion 2 was utilized for measurement of calcium uptake and portion 3 was divided into 6 strips of parallel fibers (1 cm^2 by 5 cm) and placed in a water-saturated nitrogen atmosphere at 37°C for isotonic measurement of the development of rigor mortis. At 75, 50 and 0% of initial extensibility (as set at 100% at 30 min post-mortem), duplicate strips of muscle were removed from the isotonic-isometric rigorometer. One of the duplicates was frozen in liquid nitrogen for chemical analysis and the other was used to isolate the sarcoplasmic reticulum for assay of its calcium-binding ability.

Experiment 2. 3 stress-resistant Chester White pigs and 3 stress-susceptible Poland China pigs were utilized in experiment 2. 3 portions of the longissimus were removed from each animal immediately after exsanguination: Portion 1 was frozen in liquid nitrogen, portion 2 was utilized for the measurement of calcium ion uptake and portion 3 was divided into 3 strips of parallel fibers (1 cm^2 by 5 cm) and placed in a nitrogen-purged 0.9% sodium chloride solution. 1 of the strips of portion 3 was used for isotonic and 2 used for isometric measurements. At 50 and 0% of initial extensibility (set at 100% at 30 min post-mortem), strips of muscle were removed from the isometric myographs. The strips were then used for the isolation of the sarcoplasmic reticulum for assay of its calcium-binding ability.

Results, experiment 1. Results for experiment 1 are shown in Tables 1, 2 and 3. The muscle samples from stress-resistant Chester White pigs lost extensibility more slowly than samples from stress-susceptible Poland China pigs (Table 1). At identical stages of change in extensibility, muscle from stress-resistant pigs had higher levels of PC and ATP and lower levels of lactic acid (Table 2) than did muscles of stress-susceptible pigs. No significant differences were noted between the muscles of stress-susceptible and stress-resistant pigs in the ability of the sarcoplasmic reticulum to bind calcium at identical stages of change in extensibility of the muscle (Table 3).

Results, experiment 2. Muscle samples taken immediately after exsanguination were analyzed for PC, ATP and lactic acid. Samples from stress-resistant animals had higher (P < .01) levels of PC and ATP than did the samples from stress-susceptible animals (Table 4). No significant differences in lactic acid levels were detected between muscles of the 2 animal groups. As also seen in experiment 1, samples from stress-resistant pigs took a significantly (P < .01) longer time to reach 0% extensibility than did samples from the stress-susceptible pigs (Table 5). Ability to bind calcium was the same in all muscles at identical stages of change in extensibility (Table 6). The grams of tension developed at 50 and 0% of initial extensibility were not significantly different for the muscles from the 2 strains of pigs (Table 7).

DISCUSSION

DURING development of rigor mortis, muscle becomes inextensible, loses its sarcoplasmic reticulum's ability to bind calcium ions, utilizes PC and ATP, produces lactic acid and develops tension. Muscles which differ widely in the rate of change in extensibility post-mortem also differ in a like manner in the time course of the change in calcium-binding ability of the sarcoplasmic reticulum, PC depletion, ATP depletion, lactic acid production and tension development. At identical stages of change in extensibility, however, there were no significant differences (P > .05) between the stress-susceptible and stress-resistant pigs in the ability of their muscles to develop tension or sarcoplasmic reticulum preparations to bind calcium.

Although initial samples were frozen in liquid nitrogen within 5 min after exsanguination, the longissimus muscle from stress-susceptible pigs of experiment 1 had significantly (P < .01) lower values of ATP and higher values of lactic acid than did the muscles from stress-resistant pigs. This difference implies that muscle from the stress-susceptible pigs had utilized a greater amount of high-energy phosphate compounds than muscles from stress-resistant pigs within 5 min of exsanguination. Our "initial" extensibility readings were taken at 30 min post-mortem. This period of time was necessary to excise the sample, isolate the strip of parallel fibers and adjust the rigorom-

Table 5–Time course of extensibility change[1] in strips of longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	Extensibility (percent initial)		
Sample source	100[3]	50	0
Stress-resistant		80[4]	143
		±28[5]	±40
Stress-susceptible		31	53
		±8	±16
Significance		P<.05	P<.01

[1]Experiment 2.
[2]Strips were incubated in a nitrogen-purged 0.9% NaCl solution at 37°C.
[3]100% Extensibility was taken at 30 min post-mortem.
[4]Mean value.
[5]Standard error of the mean.

Table 6–Calcium accumulation by the heavy sarcoplasmic reticulum[1] fraction from longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.

	Extensibility (percent initial)		
Sample source	100	50	0
Stress-resistant	2.71[2]	1.00	.38
	±1.17[3]	±.38	±.11
Stress-susceptible	2.01	1.09	.59
	±.10	±.34	±.39
Significance	NS	NS	NS

[1]Experiment 2.
[2]Mean value (μmoles Ca^{++}/mg protein).
[3]Standard error of the mean.
NS–Nonsignificant.

Table 7–Isometric tension development[1] in strips of longissimus muscle from "stress-resistant" and "stress-susceptible" pigs.[2]

	Extensibility (percent initial)		
Sample source	100[3]	50	0
Stress-resistant		10[4]	14
		±2.2[5]	±4.2
Stress-susceptible		13	21
		±.9	±3
Significance		NS	NS

[1]Experiment 2.
[2]Strips were incubated in a nitrogen-purged 0.9% NaCl solution at 37°C.
[3]100% Extensibility was taken at 30 min post-mortem.
[4]Mean value (g tension).
[5]Standard error of the mean.
NS–Nonsignificant.

eter. Since the muscles from stress-susceptible pigs were metabolizing at a rapid rate, shown by low initial levels of ATP and high initial levels of lactic acid, a large amount of their potential extensibility could have been lost before 30 min post-mortem.

Similar levels of calcium-binding ability were reached in the sarcoplasmic reticulum from the muscles of the 2 strains of animals at identical stages of loss in extensibility. The efficiency of the sarcoplasmic reticulum as a calcium ion sink is greatest when there is an ATP regenerating system (such as PC) to maintain a high level of ATP and keep the level of adenosine diphosphate (ADP), which is an inhibitor of the calcium pump (Hasselbach and Makinose, 1962), at very low levels. When the ATP level falls or the ATP generating system is exhausted, the calcium ions leak out of the sarcoplasmic reticulum vesicles (Weber et al., 1964; Ohnishi and Ebashi, 1963).

These data reflect the extreme difficulty in associating the mechanical parameters of muscle post-mortem to the biochemical changes in that muscle. At a given point of loss in extensibility, in muscles which vary widely in the time course of rigor mortis, it is difficult to predict the level of biochemical intermediates. It is not clear whether the sarcoplasmic reticulum inactivation is the cause or effect of contraction during the development of rigor mortis. It is clear, however, that there is a contraction associated with rigor mortis and that this contraction takes place within a shorter period of time in muscle from stress-susceptible pigs. The increase in free calcium in the sarcoplasm undoubtedly causes the contracture during development of rigor mortis. That rigor mortis can develop under conditions where there is little loss in calcium-binding ability of the sarcoplasmic reticulum per se, perhaps explains why there is potentially such a wide range in degree of contraction that can occur during development of rigor mortis.

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A. A. KLOSE
USDA, Western Regional Research Laboratory, ARS, Albany, California 94710
B. J. LUYET and L. J. MENZ
American Foundation for Biological Research, Madison, Wisconsin

EFFECT OF CONTRACTION ON TENDERNESS OF POULTRY MUSCLE COOKED IN THE PRERIGOR STATE

SUMMARY—Effects on tenderness caused by contraction of excised chicken muscles, induced by immediate post-mortem treatments consisting of electrical stimulation, beating, freeze-thawing, and heating, and followed by cooking in the prerigor state were measured. One member of each pair of muscles was held in restraint while exposed to the same conditions. In terms of percentage of original rest length, electrical stimulation reduced muscle length to 59%, and when followed by cooking to 44%; freeze-thawing reduced the length to 42%, and when followed by cooking to 40%; beating to 96%, and when followed by cooking to 52%; cooking alone to 48–53%. With the exception of the beating-heating combination, all contraction-inducing treatments resulted in a reduction of the shear values (force per cross-sectional area) of cooked muscle to about one-half those of uncontracted controls. These results with chicken muscles on intense contraction followed by cooking in the prerigor state agree with recent similar observations on red meat muscles.

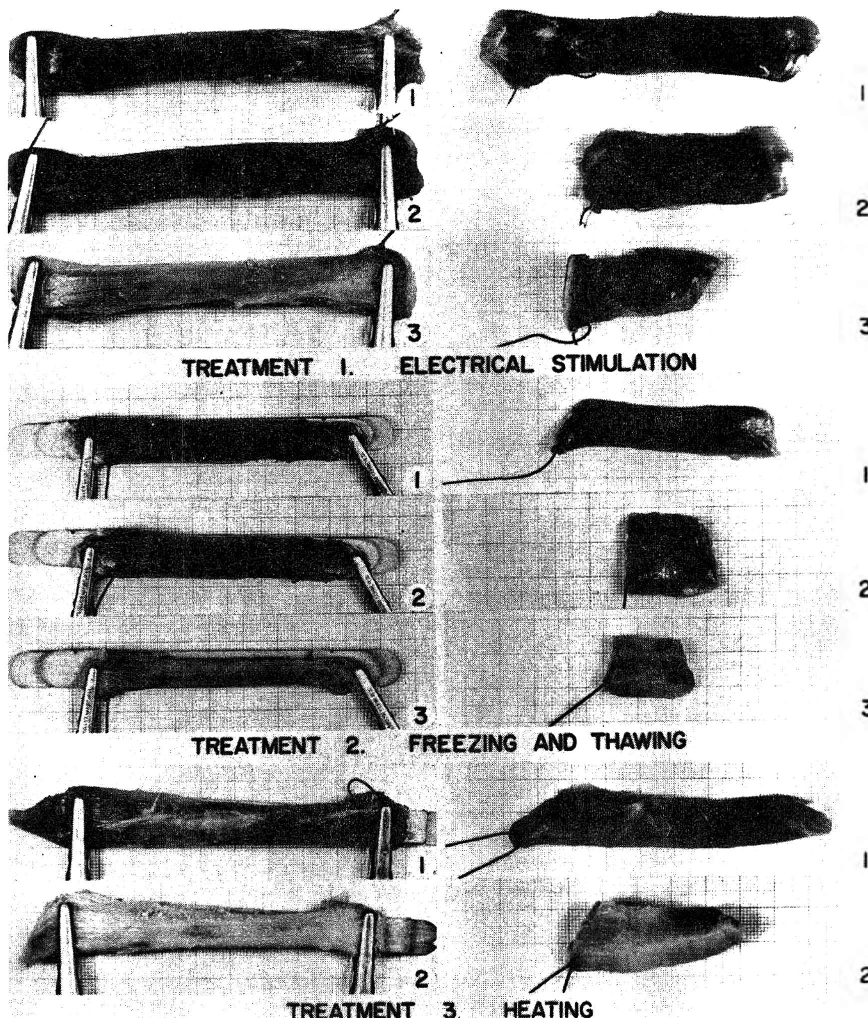

Fig. 1—Chicken thigh muscles, subjected immediately post-mortem to the treatments indicated, either under restraint, on the left-hand side, or free to contract, on the right. Sartorius muscles were used for Treatment 1; semimembranosus for Treatment 2; and semitendinosus for Treatment 3. For each treatment (1) shows the muscles before treatment; (2) taken at completion of the contraction-inducing treatment indicated; and (3) show muscles after being heated (cooked). (Background is 1 mm graph paper.)

INTRODUCTION

CONTRACTION is one of the most obvious effects of certain physical agents, such as heat, on prerigor poultry muscle. The relation between such contraction and tenderness of the cooked muscle is of theoretical and practical interest. Most studies on the effect of contraction on tenderness have been made on muscles cooked after passing through rigor; in general contraction has been associated with a decrease in tenderness (Lowe, 1948; Locker, 1960). However, the effects of contraction do not all fall into a simple consistent pattern. Weidemann et al. (1967) reported that ox muscle cooked prerigor in an unrestrained condition (contraction developed) was more tender than the restrained controls, while the reverse was true when the two were cooked in the post rigor state. Also, Marsh and Leet (1966) found that, for beef neck muscles allowed to contract in the cold, 20% contraction was associated with a fair degree of tenderness; between 20% and 40% contraction was associated with a rapid decrease in tenderness to a minimum; and between 40% and 60% contraction gave increasing tenderness back to the level found at 20% contraction.

The purpose of this study was to compare in chicken muscles excised immediately post-mortem and cooked in the prerigor state the effect on tenderness of various contraction-inducing treatments, including electrical stimulation, freezing and thawing, and heating. One member of a pair of muscles was permitted to contract while the other was subjected to the same treatments while restrained to its rest length. Effects of the treatments on the ultrastructure as revealed by electron microscopy were also observed and will be reported in separate publications by the coauthors.

MATERIALS & METHODS

Experimental material

Commercial-type chickens 10–15 wk old were used in these studies. In order to minimize struggling during slaughter, each bird was placed in a glass chamber containing carbon dioxide gas for 3–4 min, then bled by cutting the neck arteries; the skin was removed, and selected muscles were excised at their origin and insertion. The sartorius, semitendinosus, and semimembranosus muscles of the legs were

used. To the extent possible, important comparisons were made between the right and left counterparts of the same muscle from the same bird. There was necessarily a time lag between slaughter and treatment of the excised muscles, ranging from 3–20 min. However, the order of application of the various treatments to the successively excised muscles was randomized between birds to average out the effect of the time lag. Original excised lengths of the muscles ranged from 55–104 mm.

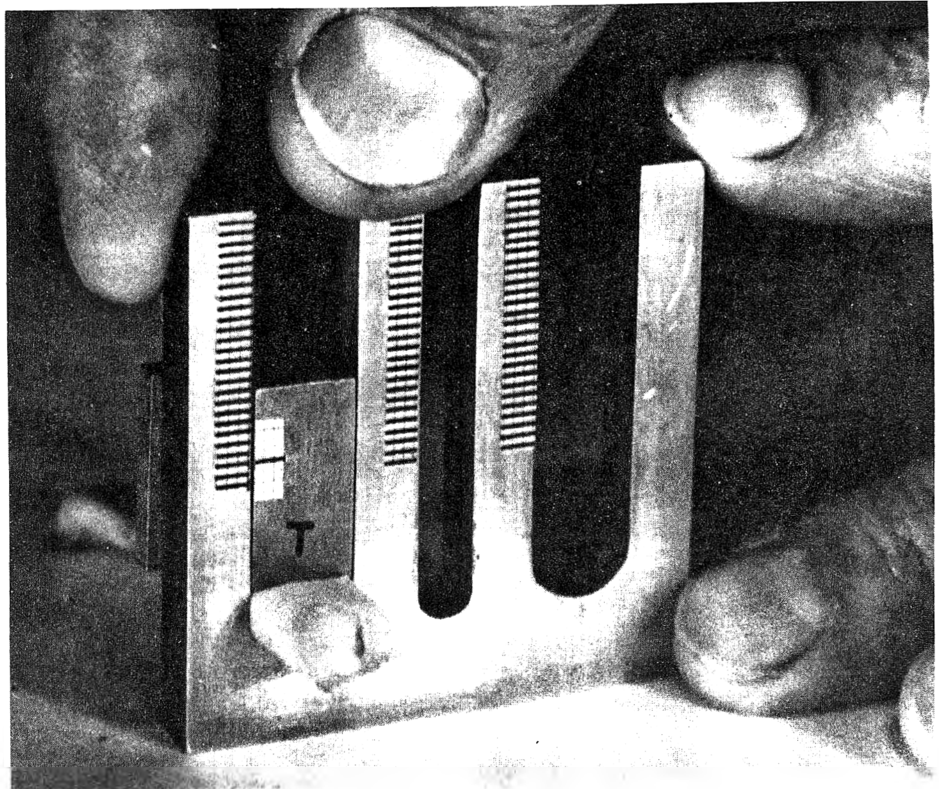

Fig. 3–Gauge constructed for the measurement of cross-sectional areas of a given thigh muscle. "T" indicates T-shaped slide, shown in measuring position directly above muscle.

Treatment of muscles

For measurement of the extent of contraction caused by the various treatments and of its effects, one muscle of a pair was allowed to contract freely, while the other was restrained at approximately its rest length by securing it at each end to a flat wooden stick by means of metal clamps, the so-called alligator clamps (Fig. 1). Overall contraction of the whole muscle was thus prevented, although it should be emphasized (and will be discussed in a subsequent paper on electron microscopical structural changes by Menz and Luyet) that this does not necessarily prevent localized areas of contraction along some parts of the muscle, compensated by stretching and tearing of the structure in other parts.

For electrical stimulation, the muscles to be contracted to tetanus in an unrestrained condition, and those to be electrically stimulated in a restrained condition, were exposed for 15 sec to the electric impulses delivered by the secondary coil of a Phipps and Bird inductorium of which the primary was connected to a 20 volt DC power supply. The stimulated muscles were then transferred immediately into boiling or hot water as described below for the heating procedures.

For the freezing and thawing treatment, muscles were immersed directly into an isopentane bath precooled in dry ice. Typical freezing curves indicated that the temperature of the muscle remained in the freezing plateau region for about 10 sec. For thawing, the frozen muscles were placed on a piece of filter paper saturated with saline solution in a covered glass dish maintained at room temperature. Rewarming and thawing required about 30 min, after which the thawed muscles were immediately immersed in boiling or hot water as described below for the heating procedure.

Beating of the muscle, in simulation of the physical action of mechanical defeathering machines on the musculature of the bird, was accomplished within 15 sec after excision by giving the muscle 60 blows during a period of 30 sec with a hard rubber mallet. The blows were not heavy enough to crush or visibly damage the tissue.

Two methods were used for heating or cooking the treated muscles. The first method was to immerse the muscle in boiling water and leave it there until the temperature in its center reached 82°C (Heating Method A). Initial use of this heating method revealed certain questionable features: there was a steep temperature gradient between surface and center of muscle, outer portions were exposed to temperatures above 82°C for a relatively long time, and most important, the contracted and restrained samples required different times to reach 82°C due to widely different cross-sectional areas. Therefore, for the remainder of the studies, the heating method was modified to consist of immersion of the muscle in water at 82°C where it was held until its central temperature reached 80°C and 10 min thereafter; this procedure reduced, percentagewise, the difference in times between restrained and unrestrained muscles. Such a modification (Method B) resulted in total immersion times ranging approximately from 12–14 min. Heating curves illustrating the two methods are shown in Figure 2 (graph B represents a case in which the heating bath was 84°C).

In addition to normal muscles, glycogen depleted muscles, which go into rigor quickly but are relatively tender even when cooked immediately after slaughter, were included in these contraction studies. For the purpose of ante mortem glycogen depletion, birds received an injection of 0.5 ml. of a solution of epinephrine (10 mg/ml in 0.06 M NaCl) about 15 hr prior to slaughter. Removal of the muscles and their treatment were accomplished within 25 min after slaughter. Depletion of glycogen in the muscle was tested by a determination of the pH of a muscle slurry; in fact the pH remained at abnormally high values in the vicinity of 6.8.

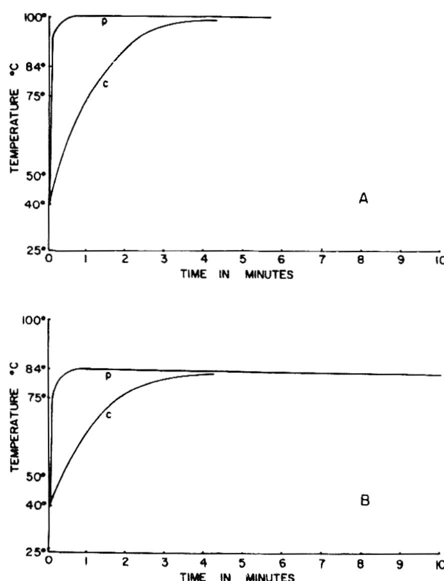

Fig. 2–Temperatures during cooking of interior center (c) and periphery (p) of muscles immersed in boiling water (A), and 84°C water (B).

Procedures for measuring effects of treatments

To determine the degree of contraction and the changes in shape resulting from the treatments, the muscles were photographed on a graduated scale and the dimensions before and after treatment, or some of the successive phases, were measured (Fig. 1).

For determination of tenderness of the heated (cooked) muscles, measurements were made of the maximum force required to shear through measured cross sections of the whole muscle or of longitudinally cut slices of the muscle. A standard Warner-Bratzler shearing machine was used. To measure the area of each

Table 1–Contraction of muscles by various treatments.

Bird No.	Length as percent of original	
	Electrical stimulation followed by heating[1]	
	After stimulation	After stimulation and heating
1	74	59
2	69	51
3	47	30
4	58	36
5	45	41
6	61	46
Average	59	44
	Freezing and thawing followed by heating[1]	
	After freezing and thawing	After freezing, thawing, and heating
1	50	50
2	42	39
3	36	36
4	46	46
5	40	35
6	38	33
Average	42	40
		After heating[1] only
1		59
2		56
3		55
4		50
5		57
6		38
Average		53
	Beating and heating[1]	
	After beating	After beating and heating
7a[2]	89	53
7b	95	48
8a	98	55
8b	100	51
Average	96	52

[1] Heating for all treatments by Method A, described in text.
[2] The a and b represent different muscles from same bird.

Table 2–Contraction of muscles by heating.[1]

Bird No.	Sartorius Length after heating as % of original	Semitendinosus Length after heating as % of original	Semimembranosus Length after heating as % of original
9	47	47	45
10	42	52	44
11	56	50	50
12	57	48	52
Average	50	49	48

[1] Heating by Method B, described in text.

sheared section, a gauge was constructed (Fig. 3) which consists of a brass plate ¼ inch thick, with three slots of dimensions calculated to accommodate muscles of different sizes. A muscle is placed in the slot of the most appropriate size, a T-shaped slide is applied gently against the muscle with one's finger, and the area of the particular cross section is read from the graduated scale. Each muscle was sheared at regular successive intervals along its length, the number of transverse cuts per slice ranging from 3–6. Muscles which through contraction became too thick to be handled conveniently in the Warner-Bratzler apparatus were sliced lengthwise to give several strips, each of which was measured for cross-sectional area and then sheared transversely as described above.

In a limited study to confirm, under our conditions, the already well established relation between shear force and subjective estimates of tenderness, one series of 18 single muscle samples (series 1 of Table 5, ranging in shear from 20–135 g/mm²) were scored once, at the time they were sheared, by a panel of four persons for tenderness (1=most tender, 5=least tender). These muscles had been cooked by immersion in water thermostated at 82°C and by holding in the water until the central temperature of the muscle reached 80°C and 10 min thereafter (about 14 min in all), and then cooling to room temperature before shearing or chewing.

While a complete and detailed description of the histological procedures used will be presented in a separate paper on structural changes, a brief outline of the techniques used to estimate density of filaments is included: Muscle bundles were immersed in 3% glutaraldehyde (M/15 phosphate, pH 7.4), rinsed in phosphate buffer, treated in 2% uranyl acetate, dehydrated in a graded alcohol series, embedded in an epoxy mixture, and then sections were cut on a Porter-Blum microtome MT-1, post-stained with lead citrate, and examined in a RCA-EMU-3E electron microscope.

RESULTS

Extent of contraction by various treatments

The general nature of the induced contractions is illustrated in Figure 1. Individual and average data from six birds are given in Table 1. Electrical stimulation or freeze-thawing of prerigor muscle gave substantial contractions which were increased only slightly by subsequent cooking. Heating (cooking) alone resulted in reduction to 53% of original length. Data for two birds in Table 1 indicate that beating as employed did not have any immediate appreciable effect on contraction. Data in Table 1 were obtained with samples heated by Method A, that is, immersion in boiling water until center temperature reached 82°C. When heating Method B (immersion in 82°C water until center temperature reached 80°C and 10 min thereafter) was applied to the same kind of muscles, degrees of contraction given in Table 2 were obtained. The difference between the overall averages for the two heating methods, 53 vs 49, is not appreciable, nor are the differences between birds or between kinds of muscle.

Influence of contraction on tenderness

Table 3 presents comparative shear values for contracted and restrained muscles from four series of treatments three of which produced substantial contraction. Expressed as grams of force per square millimeter (g/mm²) of cross-sectional area, the shear forces averaged about twice as high for the restrained samples as for their contracted counterparts. However, the treatment beating followed by heating gave only a slightly but consistently higher value for the restrained than for the contracted samples, the restrained samples having much lower values than found for other treatments. Heating by Method B for 3 muscle pairs from each of 4 birds yielded about twice as great shear values (Table 4) for restrained as for contracted samples, in agreement with the data for other treatments presented in Table 3.

In a series of tests with normal and glycogen depleted muscles (Table 5), stimulated by either electrical current, freeze-thawing or beating before heating (cooking), restrained muscles again had higher shear values, expressed as g/mm², than unrestrained, contracted muscles,

Table 3–Influence of contraction on shear values.

Bird No.	% of original length after treatment	Shear values (g/mm²) Contracted sample	Restrained sample	Ratio of shear values restrained to contracted
		Treatment: Electrical stimulation, heating[1]		
13	66	43	76	1.78
16	46	34	64	1.90
2	51	54	54	1.00
3	30	28	64	2.24
4	36	37	81	2.17
5	41	21	57	2.66
6	46	62	109	1.75
Average				1.93
		Treatment: Freezing, thawing, heating[1]		
13	40	142	275	1.94
14	44	144	186	1.29
15	34	102	157	1.54
16	37	56	66	1.19
2	39	45	76	1.66
3	36	42	115	2.77
4	46	46	184	4.03
5	35	46	90	1.95
6	33	64	68	1.06
Average				1.94
		Treatment: Heating only[1]		
13	44	55	97	1.75
1	59	40	56	1.39
2	56	25	42	1.66
3	55	25	61	2.48
4	49	34	72	2.13
5	57	43	84	1.96
6	38	32	69	2.17
Average				1.93
		Treatment: Beating, followed by heating[1]		
7a	53	39	43	1.10
7b	48	41	47	1.15
8a	55	34	51	1.49
8b	51	42	55	1.30
Average				1.26

[1] Heating for birds 1–8 by Method A and for birds 13–16 by Method B, described in text.

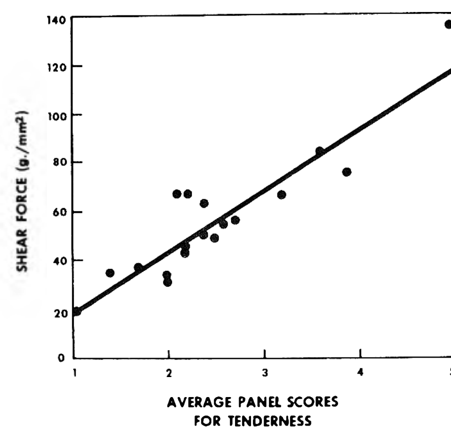

Fig. 4–Relation between shear force and average panel scores for tenderness (1 most tender, 5 least tender). Data plotted from Series 1 in Table 5.

but many of the ratios were not as high as 2:1, and in 2 of 27 pairs, there was an apparent reversal. Compared to the normal muscles, the glycogen depleted muscles did not have the much lower shear values to be expected from results previously reported by other investigators (de Fremery and Pool, 1963). Reasons for this discrepancy are not apparent, but the overall effectiveness of epinephrine injections administered for lowering the muscle glycogen level is to be questioned. Muscles of Series 1 in Table 5 were scored for tenderness (the most tender being rated 1, the least tender, 5) by a taste panel of 4 persons. The degree of correlation between shear force values in g/mm² and average panel score is shown in Figure 4. The calculated correlation coefficient was 0.91, which is significant at the 0.001 level.

DISCUSSION

IN GENERAL, shear forces are expressed in units of force per unit of area in the shearing plane, and results in Tables 3 to 5 have been expressed in this manner. In the one experiment for which taste panel data were obtained there was fair correlation between shear force values expressed in these units and average tenderness scores (Fig. 4). However, there is some question of the validity of this procedure in the present study. We are comparing muscles of equivalent areas (right and left muscles from the same bird) but of different structural composition, due to contraction in the unrestrained member of the pair. This contraction is reflected in the number of filaments per unit of cross-sectional area, which was measured in one case for electrically stimulated muscle. In limited histological examinations of relatively small areas of the whole muscle, in the non-restrained (contracted) muscle, electron microscopy showed 710 myosin filaments per square micron in the A band and 1550 actin filaments per square micron in the I band; the H band was absent (Huxley and Hanson, 1957). In the restrained muscle, there were 1150 myosin filaments per

Table 4–Influence of contraction induced by heating on shear values.[1]

Bird No.	Muscle	% of original length after treatment	Shear values (g/mm²) Contracted sample	Restrained sample	Ratio of shear values restrained to contracted
9	Sartorius	47	36	77	2.17
	Semitendinosus	47	44	67	1.52
	Semimembranosus	45	48	99	2.08
10	Sartorius	42	34	104	3.02
	Semitendinosus	52	35	70	2.01
	Semimembranosus	44	46	125	2.70
11	Sartorius	56	20	54	2.65
	Semitendinosus	50	31	62	2.04
	Semimembranosus	50	42	71	1.68
12	Sartorius	57	19	40	2.12
	Semitendinosus	48	28	52	1.86
	Semimembranosus	52	44	68	1.55
Average					2.12

[1] Heating by Method B, described in text.

Table 5–Effect of contraction on shear force (g /mm ^{2}) in stimulated normal and glycogen-depleted muscles.[1]

Stimulation before beating	Normal						Glycogen depleted					
	Electrical Stim.		Freeze-thawing		Beating		Electrical Stim.		Freeze-thawing		Beating	
	Contr.	Restr.	Contr.	Restr.	Contr.	Restr.	Contr.	Restr.	Contr.	Restr.	Contr.	Restr.
Series												
1	56	84	(135	49)[2]	50	75	37	67	32	63	45	67
							33	54	20	35	43	66
2	63	119	(102	74)[2]	71	135	69	147	98	99	82	153
							56	94	54	78	60	99
3	33	64	44	68	33	62	51	67	58	76	39	72
							33	60	35	41	37	69

[1] Heating by Method B, as described in text.
[2] Unexplained reversals of general trend.

square micron in the A band (including the H band) and 2530 actin filaments per square micron in the I band. Thus, there was essentially a reduction in the density of filaments proportional to the extent of contraction, so that if shear force were expressed as force per filament, the difference between contracted and restrained samples would largely disappear.

The relation of Warner-Bratzler shear force to cross-sectional area of the samples from a uniform piece of meat (if such there be) has been studied to a limited extent (Paul and Bratzler, 1955; Pool and Klose, 1969). The latter authors found that the shear forces were most nearly proportional to the cross-sectional area raised to the power 0.6, rather than to the power 1.0 assumed in most calculations. This suggests that the proportionality of the force is more nearly with the equivalent diameter than with the area of the cross section. Expressing data in this paper as force per unit of equivalent diameter still gives smaller values for contracted than for restrained muscles, but the differences are not as great as when the values are expressed as forces per unit area.

Implications of these results in terms of the sliding filamentary model of the muscle fiber and the structural basis of the resistance to transverse shear are not clear. However, we may speculate, as many have, that in the extreme state of contraction developed in the stimulated non-restrained muscles the actin filaments have slid into the H zone of the sarcomere, and the conditions have been changed in such a way that, after cooking, the adhesiveness between the adjacent filaments and the other structural components of the sarcomeres result in a myofibril more susceptible to a shearing stress.

From a practical standpoint, the results suggest the importance for tenderness of the degree of extension and contraction of the various muscles on the carcass during the early post-mortem period. It seems that the way in which the slaughtered bird is suspended, tumbled, and beaten (by feather picking machines) during early processing could profitably bear further investigation.

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This is the first of three papers reporting investigations covering food science, preservation by freezing, and ultrastructure, respectively.

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VERNON C. WITTE, GARY F. KRAUSE[a] and MILTON E. BAILEY
Department of Food Science and Nutrition and Department of Statistics
University of Missouri, Columbia, Missouri

A NEW EXTRACTION METHOD FOR DETERMINING 2-THIOBARBITURIC ACID VALUES OF PORK AND BEEF DURING STORAGE

SUMMARY—2-Thiobarbituric acid (TBA) values of raw pork were determined by distillation and extraction methods and those of raw beef were determined by the latter method during storage at 4° and −20°C. TBA values of these tissues were low compared to values usually reported for cooked tissue from these animals in similar environments. There were significant animal differences in TBA values of beef and pork; there were significant changes in TBA values of these tissues during storage at 4°C, but changes during storage at −20°C were insignificant. pH and TBA values were inversely related. Mathematical relationships between TBA values of pork and beef during storage at 4°C, between TBA values and pH and between results obtained by the two procedures for determining TBA values of pork during storage are discussed. The extraction method is an acceptable method for determining TBA values and is easier to use than the distillation method. The TBA test has limited use for meat samples that have been frozen.

INTRODUCTION

THE QUALITY OF the raw beef and pork is of major importance in the manufacture of sausage and similar comminuted meat products. Production of fresh and cooked sausage and canned meat products in 1967 exceeded 21 billion pounds (Pietraszek, 1968b), as compared with a total meat production of over 33 billion pounds (Pietraszek, 1968a). The quality of these products must be kept high to insure repeated sales and market demand.

Many sausage items are manufactured from raw meat which has been stored at cooler or freezer temperatures. During this time, deterioration occurs and the meat constituents may become oxidized. A measure of this oxidative change would be a valuable tool in assessing the condition of the raw ingredients.

The TBA test has been used successfully by several investigators (Tims and Watts, 1958; Younathan and Watts, 1960; Ramsey and Watts, 1963; Marion and Forsythe, 1964; Keskinel et al., 1964) to measure lipid oxidation during short term storage of cooked meats, but its use as a measure of oxidative change in fresh meats, has not been fully explored.

The objective of this research was to study changes in TBA values of raw beef and pork during storage and to compare results obtained by two different methods of TBA values.

EXPERIMENTAL METHODS

Meat processing

Pork. Five different experiments were carried out on 210 ± 10 pound hogs slaughtered and dressed packer style at the University abattoir. The carcasses were chilled overnight at 4°C and separated into wholesale cuts as described in the 1952 Proceedings of the Reciprocal Meats Conference. The separable lean tissues from the ham, picnic, Boston butt and loin from the left side of each carcass was cut into approximately 1/2 in. cubes, mixed thoroughly and 1/2 lb samples packaged in polyethylene bags. These samples were sealed with metal clips and stored as described later.

Beef. Forequarters from cutter grade cows were obtained from a local packer after overnight chilling at 4°C. Separable lean tissue from the forequarter was ground through a 1/2 in. plate, thoroughly mixed and re-ground through a 1/8 in. plate and mixed. 1/2 lb samples from each forequarter were packaged as described for pork. This was repeated on tissue from five different animals. Each cycle involving tissue from one animal was completed prior to initiating another cycle.

Sample storage following processing

Storage treatments were designed to include various combinations of 4°C (chilled) storage and −20°C (frozen) storage. Forty-eight samples of both pork and beef were randomly as signed to 4° and −20°C storage treatments Eight samples were chosen for each of six groups stored at 4°C for 2, 3, 4, 5, 6 and 7 days. After initial storage at 4°C, the eight samples in each storage group were then assigned to a −20°C storage-treatment for 0 through 7 days.

Immediately before analysis, each sample was ground through a 1/4-in. plate, mixed thoroughly, extracted or distilled and then analyzed.

TBA value analyses

All pork samples were analyzed for TBA value by two methods. One method was that of Tarladgis et al. (1960). The other was as follows: 20g of comminuted meat was blended full speed for 1.5 min in a chilled stainless steel Waring Blendor cup with 50 ml of 4°C-extracting solution containing 20% trichloroacetic acid in 2M phosphoric acid. The resulting slurry was transferred quantitatively to a 100 ml volumetric flask with 40 ml water. The sample was diluted to 100 ml with water and homogenized by shaking. A 50 ml portion was filtered through Whatman No. 1 filter paper. 5 ml of filtrate was transferred to a test tube (15 × 200 mm) followed by 5 ml of 2-thiobarbituric acid (0.005M in distilled water). The tube was stoppered and the solution mixed by inversion and kept in the dark for 15 hr at room temperature (Tarladgis et al., 1964). The resulting color was measured at 530 nm in a Beckman DU spectrophotometer.

TBA values of the beef samples were determined only by the extraction procedure. All TBA values were determined on two different portions of each sample. Two replicate colorimetric analyses were made of each portion.

In computing results from the distillation method, Tarladgis et al. (1960) multiplied the sample absorbance by a constant (K) to obtain the TBA value. A 10g sample and 68% recovery of standard from meat resulted in a K value of 7.8 for the distillation method (Tarladgis et al., 1960).

The TBA value used to express the results of

Table 1—Mean TBA values of pork.

Animal no.	n	Mean[1] TBA value Distillation[2]	Extraction[3]
1	192	0.534 A	0.306 A
2	192	0.195 BE	0.112 B
3	192	0.134 BC	0.083 C
4	192	0.326 DE	0.154 D
5	192	0.165 BE	0.073 E

[1] Mean values during cooler and freezer storage.

[2] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.192).

[3] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.008).

Table 2—Effect of 4°C storage on mean TBA values of pork.

Days of storage	n	Mean TBA value Distillation[1]	Extraction[2]
2	160	0.182 A	0.103 A
3	160	0.236 BA	0.102 A
4	160	0.240 BA	0.132 B
5	160	0.268 B	0.156 CB
6	160	0.297 B	0.173 C
7	160	0.401 C	0.208 D

[1] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.017).

[2] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.026).

[a] Department of Statistics, Univ. of Mo., Columbia, Mo.

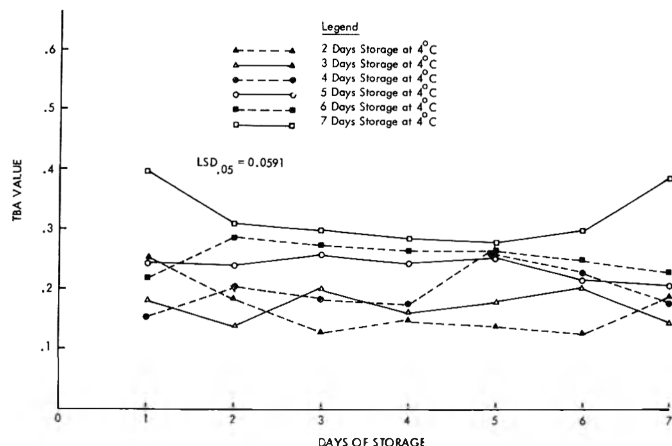

Fig. 1–Effect of –20°C storage on mean TBA values (distillation) of pork following chilling at 4°C.

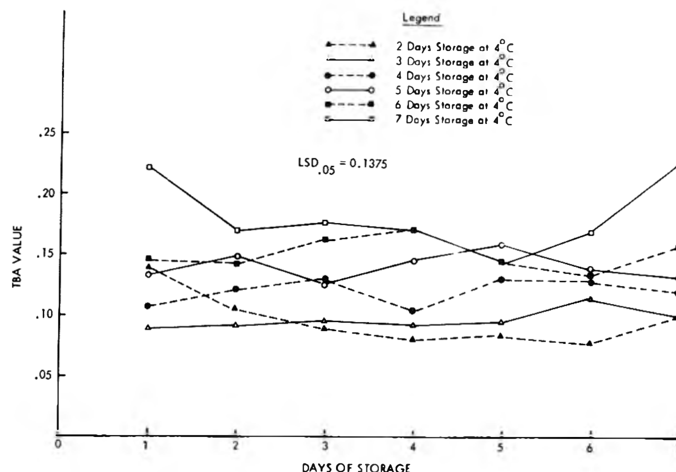

Fig. 2–Effect of –20°C storage on mean TBA values (extraction) of pork following chilling at 4°C.

the extraction method was calculated by multiplying the absorbance by the K value for extraction which was calculated from standard curves and known dilutions as follows:

$$K\ (\text{extraction}) = \frac{S}{A} \times MW \times \frac{10^6}{E} \times \frac{100}{P}$$

where S = Standard concentration (1×10^{-8} moles 1,1,3,3-tetraethoxypropane)/5 ml.
A = Absorbance of standard.
MW = Molecular weight of malonaldehyde.
E = Sample equivalent.
P = Percent recovery.

A standard containing 1×10^{-8} moles 1,1,3,3-tetraethoxypropane/5 ml resulted in an absorbance of 0.147. Recovery of the same concentration of standard added to authentic samples of fresh meat was 94%. The same equivalent (E) for a 20g sample was 1 (20g diluted to 100 ml and 5 ml analyzed). These constants resulted in a K value of 5.2. Therefore, TBA values under conditions used were calculated by multiplying absorbance by 5.2.

The pH of two separate lean samples of pork and beef in the various storage environments was determined by the official A.O.A.C. procedure (1960).

Table 3–Effect of –20°C storage on mean TBA values of pork.

Days of storage	n	Mean TBA value	
		Distillation[1]	Extraction[2]
1	120	0.241 A	0.140 A
2	120	0.228 A	0.130 A
3	120	0.226 A	0.131 A
4	120	0.215 A	0.128 A
5	120	0.234 A	0.127 A
6	120	0.222 A	0.129 A
7	120	0.224 A	0.141 A

[1] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.065).

[2] Means in the same column followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.024).

Statistical analyses

Analysis of variance and linear regression were calculated as outlined by Snedecor (1956). Significance of difference between means was determined by the method of Least Significance Difference (LSD) as used by LeClery (1957). Correlation coefficients were computed as described in Ezekiel (1950).

RESULTS & DISCUSSION

TBA values of pork

Animal variation. Compilation of results from individual pork carcasses during storage revealed that there were significant ($P < 0.05$) differences between mean TBA values for the individual animals. Table 1 contains data from both distillation and extraction analyses of pork. TBA values (distillation) of tissues from animals 1 and 4 were higher ($P < 0.05$) than those from other animals. The value for animal 1 was higher ($P < 0.05$) than the others. Differences in TBA values measured by the extraction method were also significantly different ($P < 0.05$). Animals number 1 and 4 yielded tissue which had higher ($P < 0.05$) TBA values than the other animals. Animals 1 and 4 had been fed a slightly different diet compared to the other animals which may account for the somewhat higher values. Animal variation in TBA values may also be attributed to differences in pH.

Effect of pH. During storage there was an inverse relationship between TBA values and pH. The regression equation relating pH and TBA values determined by distillation was:

$$\hat{Y} = 1.8099 - 0.2830\ X$$

where $\hat{Y}$ is the TBA value and X the sample pH. The regression equation relating pH and TBA value determined by extraction was:

$$\hat{Y} = 1.6141 - 0.2636\ X$$

For each unit increase in pH, the TBA values (distillation and extraction) decreased, on the average, 0.28 and 0.26 units respectively. Correlation coefficients between pH and TBA values (n = 420) were −0.159 ($P < 0.01$) and −0.239 ($P < 0.01$) for distillation and extraction methods respectively. The relationship between TBA values and pH confirms results reported by Keskinel et al. (1964).

Influence of 4°C storage. The effect of cooler storage at 4°C on TBA values of the 5 pork carcasses is shown in Table 2. There was a continuous increase in TBA values during storage, and values after 7 days storage were significantly ($P < 0.05$) higher than those after 2 days storage. All values were extremely low compared to those obtained during similar storage of cooked pork (Younathan and Watts, 1960).

Simple regression equations for TBA values of pork during 4°C storage for the distillation and extraction method respectively were:

$$\hat{Y} = 0.0733 + 0.0328\ X, \text{ and}$$

$$\hat{Y} = 0.0491 + 0.0175\ X$$

where $\hat{Y}$ is the TBA value and X the days of storage at 4°C. TBA values of pork increased approximately 0.03 and 0.02 units respectively for each day of storage at 4°C. Correlation coefficients between TBA values and days of storage at 4°C for the distillation and extraction methods respectively were 0.372 and 0.335 (n = 420).

Storage at −20°C. Changes in mean TBA values of pork during storage at −20°C were minor and insignificant (Table 3). The interactions of TBA values determined by the two different methods during storage at 4° and −20°C are shown in Figures 1 and 2. These are plots of TBA values and days of storage at −20°C for samples following storage for various time periods at 4°C. Results in these figures clearly show that TBA val-

Table 4–Mean TBA values of beef.

Animal no.	n	Mean[1] TBA value (extraction)
1	192	0.306 A
2	192	0.112 B
3	192	0.083 C
4	192	0.154 D
5	192	0.073 E

[1] Means followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.008).

Table 5–Effect of 4°C and –20°C storage on mean TBA values of beef.

Days of storage	Mean TBA value (extraction) 4°C[1]	–20°C[2]
1	–	0.118 A
2	0.102 A	0.122 B
3	0.108 A	0.129 B
4	0.121 B	0.126 B
5	0.132 C	0.129 B
6	0.142 D	0.127 B
7	0.153 E	0.129 B

[1] n = 160. Means followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.008).

[2] n = 120. Means followed by the same letter are not significantly different ($LSD_{0.05}$ = 0.008).

Table 6–Influence of freezing on mean TBA value of raw pork and beef following storage at 4°C.

Days of storage at 4°C	Pork TBA value[1] Distillation U[2]	F[3]	Pork TBA value[1] Extraction U[2]	F[3]	Beef TBA value[1] Extraction U[2]	F[3]
2	0.284	0.246	0.142	0.139	0.111	0.098
3	0.669	0.182[4]	0.134	0.089	0.112	0.097
4	0.522	0.153	0.210	0.108	0.120	0.110
5	0.458	0.242	0.257	0.137	0.130	0.128
6	0.575	0.222	0.329	0.144	0.135	0.134
7	0.933	0.301	0.375	0.223	0.168	0.143

[1] n = 20.

[2] Samples were not frozen.

[3] Samples were frozen and stored at –20°C for 1 day prior to analysis.

[4] Means underscored by the same line were significantly ($P < 0.05$) different.

ues of raw pork change little during storage at –20°C except for samples stored for 7 days at 4°C prior to freezing. Storage of fresh meat past 6 days at 4°C may be detrimental to quality during subsequent frozen storage.

TBA values of beef

Animal variation. The mean TBA values determined by extraction during storage of samples from individual beef forequarters are given in Table 4. All values for the different samples are significantly ($P < 0.05$) different. Variation between samples from various beef carcasses may be important in regard to use of TBA value as an index of quality, although all values for these samples were of low magnitude. As pointed out for pork, pH of individual beef carcasses may also influence TBA values.

Effect of pH. The regression equation inversely relating TBA values (extraction) to pH was:

$$\hat{Y} = 0.9128 - 0.1401\ X$$

where $\hat{Y}$ is the TBA value and X the pH. Each unit increase in pH resulted in approximately 0.14 unit decrease in TBA value. The correlation coefficient between TBA value and pH for beef samples was –0.245 (n = 480).

Storage at 4° and –20°C. As with pork, there was a gradual increase in TBA values of beef as storage progressed at 4°C (Table 5). After three days storage at this temperature, all changes were significant ($P < 0.05$). The simple regression of TBA value with 4°C storage of beef was:

$$\hat{Y} = 0.0741 + 0.0097\ X$$

and the coefficient of correlation was 0.332 (n = 480); where $\hat{Y}$ is the TBA value and X the days of storage at 4°C.

Changes in TBA values of beef during storage at –20°C were insignificant. The interactions between storage at 4°C and at –20°C on TBA values of beef were similar to those shown for pork stored under similar conditions.

Influence of freezing on TBA values of pork and beef

The influence of freezing on TBA values of raw pork and beef following storage at 4°C is shown in Table 6. TBA values of all samples were decreased by freezing but changes were greatest in pork samples analyzed by the distillation method. There were significant ($P < 0.05$) decreases in TBA values of most pork samples analyzed and in the beef samples frozen following storage for 7 days at 4°C. These differences were less apparent in values determined by the extraction method than those determined by the distillation method.

The formation of carbonyl addition products would possibly account for the apparent loss in malonaldehyde during freezing as confirmed during the course of these studies and mentioned by Chang et al. (1961). The rate of reaction between α-amino acids of myosin and malonaldehyde is greater at –20°C than at 0°C (Buttkus, 1967). There also could be loss of malonaldehyde through its reaction with guanidine to form 2-aminopyrimidine (Brown, 1962), with urea to form 2-hydroxypyrimidine (Hamberg et al., 1968) or with arginine to form non-volatile derivatives (King, 1966), although the possibility that these derivatives would be dissipated by the heat of distillation is remote.

Due to the large difference between unfrozen and frozen pork samples (Table 6), regression and correlation computations were made on data from the frozen samples. Since little difference was noted between unfrozen and frozen beef samples, data from both storage conditions was included in regression equations and correlation coefficients.

Relationship between TBA values of pork determined by the distillation and extraction methods

The regression equation of TBA values of pork as determined by the distillation method relative to TBA values of the same samples determined by extraction was:

$$\hat{Y} = 0.0358 + 0.3917\ X$$

where $\hat{Y}$ is TBA value as determined by the extraction method and X the TBA value determined by the distillation method. Mean TBA values determined by the distillation method were approximately twice as large as those determined by the extraction method. Either the heat of distillation increased the quantities of aldehyde from lipid precursors, or heat disrupted certain carbonyl addition products thought to occur by reactions between malonaldehyde and amino acids, pyrimidine or protein (Buttkus, 1967). Kwon et al. (1965) specified that heating was required to free malonaldehyde from its bound state with protein and the acid extraction method used in these experiments did not involve heating. It is unlikely that the low values obtained in the extraction method were due to color development with TBA at room temperature rather than by heating since it has been shown that the E_M^{530} of color developed by the former method is greater than that developed by the latter method (Tarladgis et al., 1964).

The overall correlation between TBA values as determined by the extraction method relative to these determined by

the distillation method for duplicate analyses of two separate portions from 240 individual pork samples (n = 960) was 0.845.

The extraction method has the advantage of simplicity and ease of useability in laboratories not equipped with distillation equipment. It also has the advantage of being more specific and selective for carbonyl compounds as described by Tarladgis et al. (1962) since it does not require heating. The major disadvantage of the extraction method compared to the distillation method is its lower sensitivity.

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S. S. HUSSAIN and M. K. HAMDY

Food Science Department, University of Georgia, Athens, Georgia 30601

EFFECT OF STAPHYLOCOCCUS INFECTION ON ACID AND ALKALINE PHOSPHATASE IN BRUISED POULTRY TISSUE

SUMMARY—Increasing severity of trauma elicited marked differences in both acid and alkaline phosphatases. The former is a lysosomal enzyme; whereas, the latter is a nonlysosomal enzyme. The magnitude of enzymatic responses as a result of trauma was not the same for each enzyme. Acid phosphatase activity increased in the bruised tissue while alkaline phosphatase activity decreased. The bound form of acid phosphatase increased in the bruised tissue, a form of the enzyme absent in normal tissue. The following factors seem to play an important role on the activities of these enzymes in poultry tissues: age of trauma, severity of the bruise and staphylococcal infection. Rates of changes in the enzymatic activities after infection were directly related to the number of organisms in the infecting dose and induced an alteration in the activities of alkaline phosphatase in the bruised tissue from a decreased to an increased value compared to normal level.

INTRODUCTION

WHILE normal tissue is assumed to be sterile in the absence of manifest disease, McCarthy et al. (1963) revealed that small numbers of bacteria are present in normal poultry tissue whereas bruised tissues permit relatively large populations of both aerobic and anaerobic bacteria to persist. Among the predominant organisms isolated from poultry bruises were gram-positive cocci, identified as *Staphylococci*, of which 48% were *Staphylococcus aureus* and 52% *S. epidermidis*. Hamdy et al. (1964) showed that the gut, air sac and skin of birds were possible sources of these bacteria and may serve as a portal-of-entry site to the traumatized areas. Hamdy and Barton (1965; 1966) established that traumatized tissue immediately following contusion and infection with pathogenic *S. aureus* or nonpathogenic *Escherichia coli* K-12 supported and stimulated the growth of these organisms equally well for 9 days and that *S. aureus* and *E. coli* were able to persist in bruised tissue for a long time (18 days) even in the absence of noticeable infection.

Among the interesting changes occurring in poultry tissue were those dealing with the various lysosomal enzymes. Activities of all these enzymes increased as a result of tissue trauma, reached a maximum between the 3rd and 5th day and then declined during healing (Brown and Hamdy, 1964; 1965). It was also noted that the maximal metabolic activities occurring in the injured tissues coincided with the maximal microbial aerobic and anaerobic counts (McCarthy et al., 1963). Do new antimicrobial or stimulatory factors appear in this situation, or is the action of normal tissue agents changed in the bruise site? Very little definitive information is available on which to base an answer. However, preliminary evidence indicated that the microenvironment of the tissue may contribute to the activity of the organisms found in the bruised tissue. The present investigation describes experiments conducted to determine the effect of trauma and staphylococcal infection on tissue microenvironment in poultry. 2 phosphatase enzymes, acid and alkaline phosphatase, were selected because of their function in maintaining the concentration of intracellular inorganic phosphate for bone formation (Roche, 1950), in the control of metabolism by dephosphorylation (Atkinson and Morton, 1960) and possibly in synthesis of some esters (Morton, 1958).

MATERIALS & METHODS

Experimental birds and bruising procedure

Normal white Leghorn chickens 8–10 weeks old, weighing 3–4 lb and kept in bat-

teries in a constant-temperature house (22°C) were used in this study. They were offered standard rations and water ad libitum. The feathers over the breast muscle (the pectoralis major) were plucked prior to bruising and, unless otherwise stated, bruising was contused using the standard technique developed by Hamdy et al. (1961). Symmetrically located areas on different chickens were used as control (nonbruised).

Cultures

A virulent marker stain (MS) of *S. aureus* (coagulase-deoxyribonuclease-positive, mannitol-negative and phage-type 52/50 A/80) was used. This culture is similar to other *S. aureus* isolated from bruised tissue except for its negative action on mannitol (a marker characteristic) and thus can be differentiated from other staphylococci that may be present in tissues. The test culture was activated by repeated transfer in liver infusion broth (Difco). The active culture was then inoculated into flasks containing 500 ml of this broth and incubated for 18 hr at 37°C. The cells were harvested by centrifugation, washed 3 times and resuspended in sterile saline to the desired concentration. The latter was checked by plating on mannitol salt agar.

Experimental infection

The staphylococcus saline suspension (0.25–0.5 ml) containing the appropriate number of viable cells was injected intramuscularly (IM) into the pectoralis major muscle of bruised and control chickens. This IM injection was always performed at the center of the traumatized area 0.5–1 hr after trauma (Hamdy and Barton, 1965).

Tissue sampling

At various intervals following trauma and infection of the chicken, several of these birds were sacrificed by exsanguination. The skin of the birds was removed, using aseptic techniques, and the desired tissues (normal, bruised and infected) were excised, held at 2–3°C in precooled petri dishes and used for enzyme analysis witin 1 hr.

Enzyme assay: Preparation of tissue homogenates and extracts

Homogenates of the tissues were prepared at 5°C by blending (for 2 min) 4 g of minced tissues with 16 ml of 0.25 M sucrose solution for determination of both alkaline phosphatase and free acid phosphatase. The tissue homogenate for total acid phosphatase was prepared in a manner similar to that for the free acid phosphatase, except that the sucrose solution contained 0.2% Triton X-100. Triton X-100 has been shown by Wattiaux and de Duve (1956) to liberate lysosomal enzymes from liver tissues. The homogenization was performed at 16,000 rpm in 50-ml precooled stainless steel Sorvall Omni-Mixer cups and the homogenate kept at 2°C and used within 30 min. The insoluble residues in the homogenate were removed by centrifugation at 3°C at 12,000 × g for 10 min and the supernatant solution (i.e., tissue extract) used for the following assay methods:

1. Total, free and bound acid phosphatase. Total activities in the free form (not enclosed in the lysosome particles) were measured directly in the tissue extracts using the procedure described by Lowry and Lopez (1946). The reaction mixture contained 0.5 ml of the tissue extract, 0.5 ml of 0.0125 M disodium p-nitrophenyl-phosphate and 1.0 ml of sodium acetate buffer (pH 5.0). The mixture was incubated for 10 min at 37°C, after which time the enzyme activity was stopped with 5.0 ml of 2% phosphotungstic acid prepared in 0.1 N HCl. After centrifugation, the absorbence of 1.0 ml of supernatant in 3.0 ml of 0.66 N NaOH was measured at 410 mμ in Bausch and Lomb Spectronic 20. This was corrected for the substrate and tissue extract control absorbences, then compared to a standard curve for p-nitrophenol to determine the activity of the enzyme. Total acid phosphatase activities were measured in a like manner except for the homogenization prepared in the presence of 0.2% Triton X-100 (Wattiaux and de Duve, 1956; Brown and Hamdy, 1964; 1965). The concentration of 0.2% of Triton X-100 was found to have no effect on chicken muscle phosphatase (Brown and Hamdy, 1964). Total bound acid phosphatase activities were calculated by subtracting the total free from the total activity of Triton-freed form. The specific activities of these 3 forms of enzyme were expressed as mμmoles of p-nitrophenol liberated per mg protein in tissue extract per 10 min incubation at 37°C.

2. Alkaline phosphatase. The assay for this enzyme was similar to that used for free acid phosphatase, except the reaction mixture contained 1.0 ml of 0.1 M glycine buffer (pH 10.5), 0.5 ml of tissue extract and 0.5 ml of 0.0125 M p-nitrophenyl phosphate. Protein concentration in tissue extracts was determined with Biuret reagent (Gornall et al., 1949) with crystalline egg albumen as the standard protein.

Table 1—Activities of acid and alkaline phosphatases in a 2-day-old bruise. Results are reported as average specific activity (mμmoles p-nitrophenol/mg protein/10 min incubation).

Tissue sample examined	No. of birds	Enzyme specific activities: Acid phosphatase Total	Free	Bound	Alkaline phosphatase
Control (nonbruised)	10	32.0	32.0	0.0	3.2
		±9.5[1]	±9.5	0.0	0.5
Bruised	10	46.5	40.9	5.4	2.9
		±8.9	±10.6	±1.2	±0.8

[1] Standard deviation.

Table 2—Effect of moderate (3 blows) and severe (5 blows) trauma on the distribution of total, free and bound acid phosphatase in poultry tissues.[1] Results of enzyme specific activity is reported as average mμmoles-p-nitrophenol per mg protein/10 min incubation.

Age of bruise (days)	Moderate bruise: No. of birds	Total	Free	Bound	Severe bruise: No. of birds	Total	Free	Bound
0	6	30.6	26.0	4.6	6	29.8	24.5	5.3
1	6	34.4	29.8	4.6	6	32.8	29.4	3.4
2	6	46.5	40.9	5.6	6	38.8	36.4	2.4
3	6	61.5	51.6	9.9	6	65.4	56.9	8.5
4	6	73.5	64.4	9.1	9	81.4	69.9	11.5
5	6	74.0	67.0	7.1	9	82.3	71.6	10.7
6	6	69.4	65.3	4.1	6	74.7	65.6	9.2
7	6	59.9	55.9	4.0	6	59.9	55.9	4.0
8	6	39.4	35.2	4.2	6	47.0	44.1	3.0
9	6	41.4	39.1	2.3	6	48.6	43.9	4.7

[1] Specific activity of free acid phosphatase in 60 control (nonbruised) birds was 32.8 ± 4.6.

RESULTS

Effect of tissue trauma on phosphatase activities

10 birds were breast-bruised on the pectoralis major muscle using 3 blows (moderate trauma). 2 days after contusion, the centers of the traumatized tissues were excised and assayed for the lysosomal enzyme acid phosphatase (total, free and bound) and for alkaline phosphatase, a nonlysosomal enzyme (de Duve, 1959). Tissues of 10 normal (nonbruised) birds were assayed for the same enzymes and served as controls. The results, reported as average specific activity ± standard deviation (Table 1), showed that trauma affected all the activities of these enzymes, resulting in a slight decrease in the alkaline phosphatase and a marked increase in total, free and bound acid phosphatase. It is of interest to point out that bound acid phosphatase was found in the homogenates obtained from bruised chicken muscle and was completely absent in normal tissue. In the absence or presence of Triton X-100, the activity of acid phosphatase was the same in the control nonbruised birds (Table 1).

Table 3—Effect of moderate (3 blows) and severe (5 blows) trauma on alkaline phosphatase in poultry tissue.[1] Enzyme specific activity is designated as mμmoles-p-nitrophenol per mg protein per 10 min incubation.

Age of bruise (days)	Moderate trauma		Severe trauma	
	No. of birds	Specific activity	No. of birds	Specific activity
0	6	3.5 ±1.6[2]	6	3.1 ±1.0
1	6	1.5 ±0.3	6	0.4 ±0.0
2	6	1.1 ±0.4	6	0.9 ±0.7
3	6	2.1 ±0.4	6	1.8 ±0.4
4	6	2.3 ±0.3	9	2.0 ±0.7
5	6	2.6 ±0.4	9	1.9 ±0.3
6	6	2.8 ±1.8	6	2.2 ±0.3
7	6	3.0 ±0.3	6	2.4 ±1.6
8	6	3.1 ±1.8	6	2.5 ±1.2
9	6	3.1 ±0.8	6	2.8 ±0.6

[1] Specific activity of alkaline phosphatase in 60 control (nonbruised) birds was 3.0 ± 0.6.
[2] Standard deviation.

Table 4—Effect of mild (10^3 cells) and severe (10^8 cells) staphylococcal infection on the distribution of total, free and bound activity of acid phosphatase in moderate and severe bruised and in control-infected poultry tissue.[1] Enzyme specific acitivity is expressed as mμmoles-p-nitrophenol per mg protein/10 min incubation.

No. of birds	Age of bruise (days)	Specific activity of acid phosphatase: Moderate bruise infected			Severe bruise infected			Control infected
		Total	Free	Bound	Total	Free	Bound	Free
Mild infection								
6	0	24.5	24.5	None	36.4	27.8	8.6	38.6
6	1	17.5	17.5	None	25.6	22.4	3.2	30.9
6	2	20.0	20.0	None	26.2	23.8	2.4	31.3
6	4	35.6	35.6	None	42.4	40.3	2.0	25.5
6	7	48.0	48.0	None	57.2	55.1	2.1	31.5
6	9	32.5	32.5	None	48.7	46.7	2.0	22.5
Severe infection								
6	0	37.8	28.1	9.6	38.3	19.1	19.2	26.0
6	1	31.2	24.5	6.8	36.1	32.0	4.1	33.3
6	2	41.9	26.5	15.4	47.4	38.9	8.5	28.5
6	3	59.3	53.4	5.9	60.7	48.9	11.8	25.9
6	4	71.4	57.8	13.6	79.5	55.0	24.5	48.2
6	7	111.2	94.3	16.9	86.9	75.2	11.7	48.3
6	9	63.9	44.1	19.8	83.0	66.9	16.2	47.4

[1] Specific activity of free acid phosphatase in 60 control (noninfected) birds was 32.8 ± 4.6.

Effect of age and severity on acid and alkaline phosphatase

The activity of alkaline phosphatase and the distribution of the various forms of acid phosphatase (total, free and bound) were determined in tissue obtained from 60 moderately bruised birds (3 blows) and from 66 severely bruised birds (5 blows) as well as from 60 control (nonbruised) birds. The results (Table 2) showed that immediately following trauma, the free acid phosphatase activity decreased in both moderate and severe bruises as compared to control level. This was followed by a gradual increase to reach a maximum level (above control) on the 5th day and a gradual decline thereafter toward normalcy by the 9th day. It was also noted that increasing the severity of trauma (from 3–5 blows) exerted slight effect on the free form of the acid phosphatase. However, the data on the distribution of the different forms of acid phosphatase (total, free and bound) activities revealed that trauma elicited the release of the bound form in these tissues and that the magnitude of the activities of these aforementioned forms of acid phosphatase was directly related both to the age and to the severity of trauma. It was also noted that the maximum activity of all the 3 forms of acid phosphatase was evident on the 4th to 5th day followed by a gradual decline to the 9th day, but above control level. Most of the acid phosphatase activities in the bruised tissue were in free form. However, bound form of this enzyme increased between the 3rd and 6th day post-trauma and accounted for almost 15 – 20% of the total activities.

The data shown in Table 3 depict the effect of age and severity of trauma on alkaline phosphatase in the bruised tissue. Pronounced decreases in the enzyme activities were noted to correlate with the severity of trauma. Increasing the number of blows from 3–5 decreased the activity of this enzyme to reach a low level within 1–2 days. This was followed by a gradual increase (but below control at all times) to reach the normal (control) value on the 9th day, which paralleled the morphological healing of the tissue.

Effect of bruising and staphylococcal infection on acid phosphatase

(a) **Mild infection.** 3 groups of 60 birds each were used in this experiment. Birds of the 1st group were bruised using 3 blows (moderate trauma), birds of the 2nd group received 5 blows (severe trauma) and the 3rd group served as control. Each bird in the 3 groups was mildly infected using a small number (10^3 cells) of *S. aureus*, and at various intervals the tissues of these infected birds were assayed for all forms of acid phosphatase. The results (Table 4) indicated that infection of control tissues (nonbruised) altered the activity of this enzyme compared to control (noninfected), especially immediately following infection, where the activity increased, and on the 4th and 9th days, where the activity decreased. It was also noted that these control-infected tissues had no bound activity. In both moderate and severe bruises, mild infection reduced the activity of the free acid phosphatase to a level below control value for the first 3 days post-infection. Thereafter the activities increased above control (noninfected) on the 4th day and persisted at this high level to the 8th day, declining to normalcy on the 9th day. No explanation can be given at the present time for the low bound activity of acid phosphatase in the severe-bruised-infected tissue compared to severe-bruised noninfected tissue (see Table 2), and for the absence of this bound form in the moderate-bruised-infected tissues (Table 4). Increased activity in both total and free acid phosphatase was observed in severe-bruised-infected compared to moderate-bruised-infected tissues.

(b) **Severe infection.** The previous experiment was repeated on 3 groups (each containing 60 birds) except for the number of staphylococcal cells used for the infection of these groups. The latter was accomplished by IM injection of 1.6×10^8 cells of *S. aureus* (severe infection); the results are also summarized in Table 4. Again, it was observed that severe-infection decreased the activity of the free form of acid phosphatase in both bruised and control tissues immediately following infection and also enhanced the free form of this enzyme on the 3rd to the 9th day post-trauma and infection in both moderate and severe bruises and on the 4th to the 9th in the control-infected birds. No bound activity was detected in the control-infected tissue, but this type of infection induced an increase in both the total and the bound forms of acid phosphatase in the severe bruised-infected

Table 5–Effect of mild infection (10^3 cells) and severe infection (10^8 cells) on the specific activity of alkaline phosphatase in moderate and severe and in control-infected tissues. Specific activity is expressed as average mμmoles-p-nitrophenol per mg protein/10 min incubation.

No. of birds	Age of infection (days)	Specific activity in infected tissues: Moderate trauma		Severe trauma		Control-infected	
		Mild-inf.	Severe-inf.	Mild-inf.	Severe-inf.	Mild-inf.	Severe-inf.
6	0	2.6	2.2	1.9	2.7	1.2	2.1
		±0.3[2]	±0.7	±1.5	±0.6	±0.1	±0.3
6	1	2.2	0.8	1.4	2.1	1.6	2.7
		±0.9	±0.1	±2.2	±0.6	±0.2	±0.4
6	2	2.6	2.9	2.0	2.9	2.4	3.2
		±1.2	±0.8	±0.8	±0.2	±1.0	±1.0
6	3	3.5	7.1	5.9	11.3	1.1	3.4
		±0.8	±1.7	±1.6	±1.7	±0.2	±1.5
6[1]	4	2.2	9.3	2.2	6.8	1.1	7.2
		±0.9	±1.4	±1.0	±0.8	±0.3	±0.8
6	5	2.2	10.3	2.4	7.3	1.2	4.4
		±0.7	±1.5	±1.5	±1.4	±0.2	±2.2
6	6	1.5	7.6	1.6	7.4	0.7	3.4
		±0.7	±3.9	±0.7	±1.2	±0.6	±0.4
6	7	2.2	6.1	2.5	4.9	0.8	3.3
		±0.7	±0.7	±2.4	±0.7	±0.1	±0.8
6	8	2.5	5.5	1.7	3.7	1.5	3.4
		±0.6	±1.0	±3.6	±2.1	±0.1	±1.0
6	9	1.9	4.5	1.9	4.8	1.6	3.1
		±0.9	±0.2	±0.6	±2.3	±1.1	±0.6

[1] 6 birds were used for control-infected and for moderate trauma, whereas 9 birds were used for severe trauma.

[2] Standard deviation.

tissues. The pattern of free activity of acid phosphatase during the entire experiment was somewhat similar, but varied in magnitude, in the 3 groups of birds up to the 2nd day post-infection. This was followed by a slight increase in the severe-bruised-infected tissues on both the 5th and 6th day compared to the moderately bruised-infected tissues. Maximum activity of this form of enzyme was noted on the 7th day for moderate and severe infected bruises, respectively.

Effect of bruising and infection on alkaline phosphatase

Tissues of bruised-infected and control-infected birds used for the determination of acid phosphatase were also employed for the assay of alkaline phosphatase. Therefore, the protocol for the experimental design used to examine the effect of mild and severe staphylococcal infection on the activities of acid phosphatase in moderate and severe bruises and in control-infected tissues were the same for alkaline phosphatase.

(a) Mild infection. Results of the effect of mild infection (10^3 cells) on the alkaline phosphatase activities in the moderate (3 blows) and in the severe (5 blows) as well as in the control-infected tissues are recorded in Table 5. It is evident that the infection slightly decreased the activity of this enzyme in all the tissues examined compared to control (Table 3) and that the control-infected tissues appeared to exhibit the lowest level during the entire experimental period. Very little difference was also noted between moderate-infected and severe-infected bruises at all times, except on the 3rd day post-trauma and infection, where the alkaline phosphatase activity in the severe-bruised-infected tissues drastically increased above the moderate-infected bruise as well as the control-infected tissue.

(b) Severe infection. Results of the effect of severe infection (10^8 cells) on the alkaline phosphatase activities in the tissues (Table 5) indicate that this type of infection is indeed associated with significant increases in the activities of alkaline phosphatase and that the magnitude and the time for maximal activity in the infected tissues were also affected by severity of the trauma. Maximum specific activity of the enzyme was noted on the 3rd and the 5th day post-infection in the severe and in the moderate trauma, respectively. This value is about 3.5-fold the level of this enzyme in the normal tissue. It was also observed that the alkaline phosphatase activity in all infected tissues decreased gradually towards normalcy on the 9th day.

DISCUSSION

DATA obtained in this investigation have shown that bruising affected the microenvironment of the tissues, as evidenced by the changes in the activities of alkaline and all forms of acid phosphatase. However, the magnitude and the time these changes occur were affected by factors such as age and severity of the trauma and the degree of staphylococcal infection.

During healing of a bruise, many synthetic activities must take place in the region of regeneration. For example, both acid and alkaline phosphatase split off phosphate from such substrates as hexose phosphate, glycerol 2-phosphate, phenyl phosphate and other phosphate esters with optimal activity in the acid and alkaline pH ranges, respectively. The increase in acid phosphatase and the decrease in alkaline phosphatase activities in the bruised poultry tissue during the degenerative and regenerative processes confirm the results previously reported by Brown and Hamdy (1964). The increase of the acid phosphatase (total, free and bound) may be due to infiltration of leucocytes into the bruised areas as suggested by Cahn and Hirsch (1960), or may be due to active synthesis of this enzyme at the site of the bruise induced by the degradation products of the dead or nicrotic tissues as speculated by Tappel et al. (1962). Decreased alkaline phosphatase activity in the moderate bruise and as a result of increasing the severity of trauma may be due to the shift of pH affected by lactic dehydrogenase and inhibition of alkaline phosphatase accordingly.

Staphylococcal infection and the number of viable cells in the infecting dose indeed were important factors affecting the microenvironment of both bruised and nonbruised tissues (control). This effect was more pronounced in bruised-infected compared to control-infected birds. Significant enzymatic changes in various tissue enzymes, including alkaline phosphatase and all forms of acid phosphatase, were altered as a result of staphylococcal infection. In this type of infection there has been a tendency to stress the role of virulence or noxious factors of the bacteria as being the primary mediators of tissue injury. However, results of this study as well as those reported in a previous publication (Hamdy and Barton, 1965) indicated that altered tissue microenvironment by bruising also played an important role in stimulating and supporting growth of these bacteria. The increase of both acid and alkaline phosphatase after 2–3 days of infecting the bruises appeared to be of tissue origin rather than microbial via lysis of the staphylococcal cells. This was substantiated by the results obtained, which showed that when a constant number of bacterial cells was used for infection, the enzyme activities increased as the severity of trauma changed from 3 to 5 blows. Other experiments (unpublished results) with washed cell suspensions of *S. aureus*, cell-free supernatants and ruptured cell

preparations showed that microbial acid and alkaline phosphatases were of low enzymatic activity. Since bruised tissues have a rapid cell turnover, particularly during the regeneration phase (3 days post-trauma), it may be that the observed increase in the specific activities of acid and alkaline phosphatase in the tissues is related in some manner to a more rapid cell turnover during staphylococcal infection.

Gilfillan et al. (1956) reported marked changes in several enzymes of the tricarboxylic acid cycle in chicks during infection with *Salmonella pullorum*, and Woodward et al. (1954) have shown that infection with *Francisella tularensis (Pasteurella tularensis)* stimulated a depletion of free amino acids in the blood of white rats. Again, Woodward et al. (1969) showed that infection of these animals with *F. tularensis* and *Salmonella typhimurium* and exposure to the endotoxin of *S. typhimurium* stimulated significant increases in various serum enzymes including aldolase, lactic dehydrogenase and other enzymes. These authors also established that the rates of changes in enzymatic activity after infection were directly related to the size of the infecting dose and the type of infective agent used. Brown and Hamdy (1964) reported that both the timing and the degree of maximal activity of acid phosphatase were directly related to the number of blows applied for inflicting bruises.

There have been a number of studies conducted on acid phosphatase in response to viral infection. For example, Mallucci and Allison (1965) showed that fowl phage virus activated acid phosphatase in chick embryo cells. It seems that the host's reaction to infection, whether due to bacterial, virus or injury, is related to the adrenal stimulation and the protein-catabolic reaction following trauma. Lust and Beisel (1967) reported that the intracellular function of alkaline phosphatase is presently not well defined and that the enzyme is found in high concentrations in the small intestines, bone and kidney, whereas other tissues have appreciably less, with brain and muscle having very low activity. Again, these authors reported that the level of alkaline phosphatase decreased in the mouse liver as the infection wtih *Diplococcus pneumonia* progressed, while a marked increase occurred in the small intestine. Counts et al. (1961) observed a rapid increase in serum alkaline phosphatase to accompany experimental staphylococcal sepsis in mice.

Data obtained in the present investigation showed that tissue trauma decreased the activities of alkaline phosphatase and that this decrease was pronounced in severely traumatized tissues. On the other hand, staphylococcal infection in bruised tissue reversed this effect significantly, leading to a higher level (above normal value) on the 4th to the 9th day post-infection. This observation is a new finding and may be due to the reaction of the host to staphylococcal infection, regardless of the infective dose employed. While these studies still leave unanswered the specific question of why virulent staphylococcus bacteria persist in the bruised tissue, nevertheless, they provide additional information that enzyme alterations do occur in bruised tissue during an acute staphylococcal infection and that these changes may be responsible for the survival and persistence of these organisms in the injured tissues.

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HSIAO-PING LIU[1]
Department of Food and Nutrition, Florida State University, Tallahassee

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 1. Linoleate Peroxidation Catalyzed by MetMb or Fe(II)-EDTA

SUMMARY—The possibility of catalysis of unsaturated fat oxidation by both hemoprotein and non-heme iron components of meat has been pointed out. Model systems of these 2 catalysts have been compared. The hemoprotein MetMb accelerated linoleic acid peroxidation in pH range 5.6–7.8, the catalysis increasing with pH. A complex of ferrous ion and ethylenediaminetetraacetic acid [Fe(II)-EDTA]—a non-heme iron model—in a 1 to 1 ratio accelerated peroxidation at lower pH; no catalysis took place above pH 6.4. Most chelating agents eliminated Fe(II)-EDTA catalysis, but had no effect on MetMb catalysis. Reducing agents, both ascorbic acid and thiols, on the other hand, accelerated Fe(II)-EDTA catalysis but inhibited MetMb catalysis. Other sulfur compounds with -S- or -S-S- groups, except thiodipropionic acid (TDPA) at concentration of 6×10^{-3} M, showed no effect on either type of catalysis.

INTRODUCTION

ALTHOUGH the catalytic effect of hemoglobin (Hb) and other iron porphyrins on lipid oxidation is a generally accepted phenomenon, it is still not certain that this is the main oxidative catalyst in meat. Hematin-catalyzed lipid peroxidation has been demonstrated as an important deterioration reaction in unsaturated fat (Tappel, 1955), precooked meats (Younathan and Watts, 1959) and dehydrated foods (Bishov et al., 1960). However, inorganic ferrous ion has also been demonstrated to be a catalyst of unsaturated lipid peroxidation in mitochondria (Ottolenghi, 1959) and microsome (Robinson, 1965), as well as in pure unsaturated lipids (Wills, 1965). The prooxidant contribution of metals in meat and meat products has not been widely investigated.

Wills (1966) presented evidence that both types of catalysts—hemoprotein and inorganic iron—functioned in rat tissues. The 2 types could be differentiated by their relative activities at different pH and in the presence of chelating agents, ascorbic acid and thiol compounds. Inorganic iron, either as ferrous or ferric ion, was a very weak catalyst unless activated by reducing agents. Iron-catalyzed oxidation was pH-sensitive and most active under acid conditions. Hemoprotein-catalyzed oxidation was little affected by pH and inhibited by reducing agents.

Wills (1966) not only offers an experimental approach to the problem of identifying the main type of catalytic action in meats; he also points up the importance of such an identification for practical problems of meat handling. The effects on lipid oxidation of many meat components, treatments or additives not only may be different in degree but even opposite in direction with the 2 types of catalysts.

Before undertaking the investigation of lipid oxidation catalysts in meats, the properties of both types of catalysts in model systems should be studied systematically. The development of suitable model systems is the first step.

Heme compounds are known to inhibit rather than accelerate lipid oxidation when they are present in high concentrations relative to the unsaturated fatty acid (Lewis and Wills, 1963). In developing a model system using MetMb as a catalyst, various concentrations of the heme were tried. MetMb (6×10^{-7} M) was found to catalyze 7×10^{-3} M linoleic acid at a moderate rate. These concentrations were, therefore, used throughout.

The non-heme iron model for lipid peroxidation developed for these studies consisted of a mixture of Fe(II) and EDTA, each present at a concentration of 1.5×10^{-4} M. Such iron chelates of EDTA have been used to produce free radicals in various oxidative reactions (Udenfriend et al., 1954; Norman and Smith, 1964; Staudinger et al., 1964; Orr, 1967), but the complex has not previously been tried on lipids. With higher ratios of EDTA to iron, i.e. 2:1, iron catalysis is inhibited rather than accelerated (Grinstead, 1960; Barber, 1966).

Thiols, ascorbic acid and chelating agents were expected to have different effects on hemoprotein versus non-heme iron catalysis. Addition of these compounds therefore might help to differentiate the 2 systems. Several sulfur-containing compounds have been shown to retard lipid oxidation in fats and oils but have not been investigated in meat. Thiodipropionic acid (TDPA) showed effective retardation of peroxide formation in soybean oil (Schwab, 1953) and has been patented as a sterilizer for fats and oil (Searle, 1955). Other sulfur-containing compounds, i.e., methionine and oxidized glutathione (GSSG) or cystine, containing either -s- or -s-s- groups, were considered of interest in this investigation in view of the work of Holdsworth et al. (1964) demonstrating peroxide reduction by similar compounds.

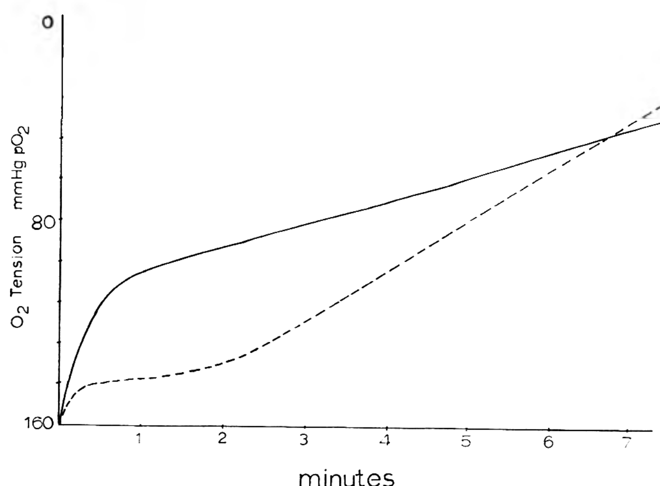

Fig. 1—Change to O_2 tension to linoleate emulsion catalyzed by MetMb or Fe(II)-EDTA at pH 5.6. Linoleic acid 7 $\times 10^{-3}$ M; MetMb 6×10^{-7} M; Fe(II)-EDTA (1.5×10^{-4} M: 1.5×10^{-4} M).

———— *Fe(II)-EDTA catalysis*

-------- *MetMb catalysis*

[1] Present address: 76-10 31st Avenue, Jackson Heights, New York 11370.

Table 1—Effect of chelating agents on Fe(II)-EDTA catalyzed linoleate peroxidation at pH 5.6 and 6.2.

		Rate of O_2 uptake (mm Hg pO_2/min)	
		pH 5.6	pH 6.2
Fe(II)-EDTA		9.6	3.2
+ EDTA	6×10^{-4} M	0	0
	6×10^{-5} M	0	0
+ 8-OH-Quinoline	6×10^{-4} M	0	0
	6×10^{-5} M	0	0
+ $Na_3P_5O_{10}$	6×10^{-4} M	0	0
	6×10^{-5} M	0	0
+ Citric Acid	6×10^{-4} M	1.6	0
	6×10^{-5} M	4.8	1.6
+ Xanthine	6×10^{-4} M	2.4	0
	6×10^{-5} M	6.4	2.4

Table 2—Effect of 6×10^{-3} M TDPA on linoleate peroxidation catalyzed by MetMb.

	Rate of O_2 uptake (mm Hg pO_2/min)			
	5.6	6.2	7.0	7.8
MetMb	20.8	16.0	32.0	61.8
+ TDPA	64.0	56.0	16.0	48.0
Fe(II)-EDTA	9.6	3.2	0	0
+ TDPA	9.6[1]	25.6	0	0

[1] A lag period occurred before oxidation began.

EXPERIMENTAL

Materials

Pure linoleic acid was obtained from the Hormel Institute. Crystalline lyophilized horse metmyoglobin was obtained from Nutritional Biochemicals Corporation. All other reagents were of standard quality. The water used for the preparation of all solutions was passed through a deionizing column.

Methods

Emulsions of linoleic acid were used as substrates for peroxidation studies and were always prepared immediately before use by a slight modification of Surrey's method (1964). 1 g of linoleic acid was added drop by drop to 20 ml water in which 1 ml Tween 20 was dissolved. The contents were thoroughly mixed to disperse the acid into a fine emulsion. Then, 1 N KOH was added and the mixture once again agitated with a magnetic stirrer until a clear transparent solution was obtained. To this solution 200 ml of 0.2 M phosphate buffer were added. A few drops of concentrated HCl were used to adjust to the desired pH. The final volume was made up to 400 ml with H_2O. The resulting solution contained approximately 9×10^{-3} M linoleic acid in 0.1 M phosphate buffer.

To 20 ml of the emulsion were added the catalysts and test solutions desired and the total volume made up to 25 ml in which the concentration of linoleic acid was 7×10^{-3} M. Changes in oxygen tension were recorded, using the Beckman Oxygen Analyzer 777 calibrated at 160 mm Hg, the partial pressure of oxygen in air. The rates were linear after the first few minutes. The oxygen utilization was then calculated from the recording by measuring the linear slope over a 3-min period and expressing the values as mm Hg pO_2/min.

RESULTS

Comparative activity of the 2 catalysts

In non-heme iron catalysis, O_2-consumption of 16 recordings from 1 fatty acid preparation ranged from 8.0–11.2 mm Hg pO_2/min with an average of 9.4 ± S.D. 7.5% at pH 5.6. MetMb was also compared under the same conditions, except that the phosphate concentration was 0.1 M. The O_2-consumption of 12 recordings from 1 preparation ranged from 16–19.2 mm Hg pO_2/min with an average of 17.5 ± S.D. 3.7%. Since the heme iron concentration used is less than one one-hundredth of the non-heme iron, obviously the heme is a much more potent catalyst.

The form of the recordings shown in Figure 1 was highly reproducible. A sudden initial drop of O_2 tension with the Fe(II)-EDTA catalysis and a lag period before MetMb catalysis began at pH 5.6 were characteristic of the 2 types of catalyst.

Effect of additives on the Fe(II)-EDTA system

Fe(II)-EDTA was active only at acidic pH; no catalysis took place above pH 6.4. The complex neither inhibited nor accelerated linoleate oxidation at neutral or alkaline pH. It has also been pointed out by Wills (1965) that the optimum pH for inorganic iron plus either ascorbic acid or cysteine was at pH 5.5.

The effects of various chelating agents on Fe(II)-EDTA catalysis at pH 5.6 and 6.2 are shown in Table 1. Each additive in this study was run at least 3 times, and the differences from the control were reproducible and significant. Most chelating agents inhibited Fe(II)-EDTA catalysis. The inhibition was complete with EDTA, 8-OH-quinoline and $Na_3P_5O_{10}$. Citric acid and xanthine showed less effect.

Among the sulfur-containing compounds, GSSG and L-methionine, in concentrations ranging from 6×10^{-5} M to 6×10^{-3} M, did not have any effect on Fe(II)-EDTA catalysis. TDPA had no effect at concentrations less than 6×10^{-4} M. At 6×10^{-3} M, it significantly accelerated the catalysis at pH 6.2 but not at 5.6, 7.0 or 7.8 (Table 2). A short lag period was found before catalysis began at pH 5.6.

Ascorbic acid and thiols in the concentration range from 6×10^{-5} M to 6×10^{-3} M accelerated Fe(II)-EDTA catalysis over the entire pH range investigated. Ascorbic acid has long been recognized as an inorganic iron activator, but the thiols seem not to have been compared in such systems. Data are shown in Table 3 for the reducing agents at a concentration of 6×10^{-4} M. Reduced glutathione accelerated much less than the other reducing agents.

Effect of additives on MetMb-catalyzed systems

MetMb catalyzed much faster and without a lag period at more alkaline pH. The oxygen uptake at pH 7.8 was approximately 4 times greater than at pH 6.2 (Table 4). However, at pH 7.8, as the oxidation progressed, rate of oxygen up-

Table 3—Effect of thiols and ascorbic acid on linoleate peroxidation catalyzed by Fe(II)-EDTA.

	Rate of O_2 uptake (mm Hg pO_2/min)			
	5.6	6.2	7.0	7.8
Fe(II)-EDTA	9.6	3.2	0	0
+ 6×10^{-4} M Ascorbic Acid	64.0	40.0	27.2	16.0
+ 6×10^{-4} M L-Cysteine	32.0	25.6	28.8	24.0
+ 6×10^{-4} M Merceptoethylamine	25.0	9.6	11.2	51.2
+ 6×10^{-4} M Reduced glutathione	16	9.6	3.2	1.6

Table 4—Effect of thiols and ascorbic acid on linoleate peroxidation catalyzed by MetMb.

	Rate of O_2 uptake (mm Hg pO_2/min)			
	5.6	6.2	7.0	7.8
6×10^{-7} M MetMb	12.8	11.2	20.8	44.8
+ $6 \times ^{-4}$ M Ascorbic Acid	12.8	11.2	12.8	9.6
+ 6×10^{-4} M Reduced glutathione	8.0	4.0	9.0	5.6
+ 6×10^{-4} M Mercepto-ethylamine	6.4	5.3	3 6	6.4
+ 6×10^{-4} M L-Cysteine	8.0	3.2	6.4	4.8

Table 5–Effect of pH and additives on lipid oxidation catalyzed by Fe(II)-EDTA versus MetMb.

Additives and conditions	Fe(II)-EDTA	MetMb
pH	5.6 > 6.2 > 7.0 = 7.8	7.8 > 7.0 > 5.6 > 6.2
Chelating agents (6×10^{-4} M)		
EDTA, $Na_3P_5O_{10}$, 8-OH-Quinoline	Strong inhibition	No effect
Citric Acid, xanthine	Slight inhibition	No effect
Ascorbic Acid (6×10^{-4} M)	Acceleration	Inhibition
Sulfur-containing compounds (6×10^{-4} M)		
Thiols	Acceleration	Inhibition
GSSG, L-methionine	No effect	No effect
TDPA (6×10^{-4} M)	No effect	No effect
TDPA (6×10^{-3} M)	Acceleration at pH 6.2	Acceleration at pH 5.6, 6.2; slight inhibition at pH 7.0, 7.8

take decreased, probably because of the destruction of the hematin catalyst (Tappel, 1953).

Most sulfur-containing compounds, except GSSG and L-methionine, inhibited MetMb catalysis (Table 4). The extent of inhibition tended to increase with higher pH. TDPA (6×10^{-4} M) again had no effect on MetMb catalysis, but at the 6×10^{-3} M concentration it accelerated the catalysis at pH 5.6 and 6.2, while slightly inhibiting it at pH 7.0 and 7.8 (Table 2). Chelating agents did not show any effect on MetMb catalysis, except for 8-OH-quinoline, which gave a lag period before MetMb catalysis commenced. The lag period was longer at lower pH than at higher pH.

DISCUSSION

FE(II)-EDTA complex in 1 to 1 ratio catalyzed linoleate peroxidation at constant rate. Thus, it can be suggested as a non-heme iron model for free radical formation studies in model systems prior to application in foods. It must be remembered that although Fe(II)-EDTA was used as a model to represent non-heme iron catalysis, this particular iron complex obviously does not occur in food systems. Other iron complexes which might be present in meat may react differently to additives.

Table 5 summarizes the effect of pH and various additives on the 2 types of catalysts in the model system. Fe(II)-EDTA catalyst was active only at acidic pH, whereas MetMb was more active at alkaline pH. Wills (1965) has also found similar results. Although he indicated that the rate of Hb-catalyzed oxidation was little affected by change in pH over the range 5.5–8.0, the rate of oxidation of linoleic acid tended to increase as the pH was made more alkaline.

Chelating agents inhibited Fe(II)-EDTA catalysis but not MetMb catalysis. The iron in the porphyrin ring was not susceptible to further chelation. The induction period resulting from addition of 8-OH-quinoline to MetMb-catalyzed linoleate peroxidation may be the phenolic type antioxidant reaction. It is suggested that the active hydrogen of 8-OH-quinoline reacted with 1 ROO-radical to give a relatively stable semiquinone radical.

Among the sulfur-containing compounds, thiols greatly enhanced the Fe(II)-EDTA catalysis. They may be acting as reducing agents to regenerate the ferrous iron from the ferric form during the catalytic oxidative reaction, as has been suggested for ascorbic acid, or they may act as chelators to render the non-heme iron more active. Bernheim (1964) demonstrated that the metal-cysteine chelate was catalytic, whereas MEA chelate might either sequester the metal or behave as a chain breaker. His experiment was carried on at pH 6.7. This may explain why Fe(II)-EDTA plus MEA was less active than Fe(II)-EDTA plus cysteine or ascorbic acid at that pH value (Table 3). The inhibition of ascorbic acid and thiols on MetMb catalysis was demonstrated. When non-heme iron was absent, they were probably acting as chain breakers.

Alkyl sulfides are believed to reduce hydroperoxides to stable, nonradical products (Holdsworth et al., 1964). In this experiment, GSSG, L-methionine and TDPA with either -s- or -s-s- groups showed no effect on either Fe(II)-EDTA or MetMb-catalyzed linoleate peroxidation. Rate of peroxide decomposition by these catalysts is presumably much greater than peroxide reduction by alkyl sulfides. From the results summarized in Table 2, TDPA would seem to have no usefulness in food if either heme or non-heme iron acts as catalyst.

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HSIAO-PING LIU[1]
Department of Food and Nutrition, Florida State University, Tallahassee

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 2. Linoleate Oxidation Catalyzed by Tissue Homogenates

SUMMARY—In beef tissue homogenate, both types of catalysts—hemoprotein and non-heme iron—are active catalysts of linoleate oxidation. Although the pH-dependent catalytic pattern of beef homogenate was similar to MetMb catalysis, the presence of non-heme iron could be identified by adding ascorbate or 8-OH-quinoline. Ascorbate-stimulated oxidation could be inhibited by chelating agents. Furthermore, lower concentrations of phosphate buffer rendered the non-heme iron more active at acidic pH. Linoleate oxidation was also catalyzed by H_2O_2-treated (heme-free) beef homogenates. Oxidation was accelerated either by thiols or by ascorbate. Both EDTA and 8-OH-quinoline abolished catalysis, but xanthine and citric acid further accelerated. An active non-heme iron chelate thus is indicated. In shrimp tissue homogenate, the evidence suggested that non-heme metal complexes were playing a dominant role in catalysis. Except for the anomalous behavior of thiols, the effect of pH changes and various additives was similar to that in Fe(II)-EDTA model systems.

INTRODUCTION

LINOLEATE peroxidation catalyzed by hemoprotein versus a non-heme iron chelate in a model system was described in the preceding paper. Before extending this study to meats, observations on a semimodel system, utilizing tissue homogenates as catalysts, were considered desirable.

Lipid peroxide formation in meats may be affected by differences in the substrate, i.e., the unsaturated fatty acid content of the tissue, as well as by the catalytic system. To overcome this difficulty the catalytic components in tissue can be studied by addition of excess substrate in the form of emulsions of unsaturated fatty acid to tissue homogenates. Furthermore, lipid oxidation in the semimodel system can be followed by use of the oxygen analyzer as in model systems, a much more convenient and time-saving method than those currently used in meats, which involve extraction and analysis of oxidized decomposed products from the lipid.

Peroxidation of the endogeneous unsaturated fatty acids in mitochondrial and microsomal fractions can be induced by incubation with a variety of redox agents such as ferrous ion (McKnight et al., 1965), hemoprotein (Tappel, 1955a), ascorbate (Ottolenghi, 1959), GSSG and especially by mixtures of GSH and GSSG (Hunter et al., 1964). Barber (1966) demonstrated that the catalytic role of iron and ascorbic acid was an important nonenzymic mechanism for lipid oxidation in tissue. He also indicated that sufficient iron was present in tissue particulates to bring about such catalysis. Lipid peroxidation could be inhibited by EDTA. Robinson (1965) tried several different metals and found only iron was capable of increasing lipid peroxidation with ascorbate or cysteine in microsomal suspension. Both heme or non-heme iron-containing enzymes present in the microsomal fractions could catalyze lipid peroxidation if the iron were made accessible through some change in tertiary structure (Utley et al., 1967).

Homogenates of rat liver, spleen, heart and kidney can also actively catalyze peroxide formation in emulsions of linoleic or linolenic acid (Wills, 1966). He pointed out that in the mitochondrial and microsomal fractions, hemoproteins were likely to be more important, since ascorbic acid or thiols inhibited peroxidation. The inhibition increased with increasing pH. On the other hand, ascorbic acid strongly stimulated catalysis by 100,000 g supernatant and, presumably, non-heme iron was important in this fraction.

In this study, beef muscle homogenate is assumed to contain both heme and non-heme iron components. To differentiate non-heme iron from hemoprotein, Wills (1966) gave the tissue a prior treatment with H_2O_2 to destroy all the catalytic functions of hemoprotein and liberate the inorganic iron, so that non-heme iron would be the only catalyst. Shrimp tissue contains no hemoprotein other than cytochromes. Shrimps and H_2O_2 treatment beef muscle were, therefore, selected as heme-poor sources to be compared with the heme-rich sources, i.e., beef. Effects of various treatments on these 3 types of catalyzed linoleate peroxidation may, therefore, shed light on the catalytic systems in these foods.

EXPERIMENTAL

EMULSIONS of linoleic acid were prepared as in the preceding paper. Homogenates from beef and shrimp were used as the catalysts. Eye of the round (Semitendenosus) beef, and shrimp, were purchased at local retail markets.

Beef was trimmed, ground and mixed thoroughly, to obtain a homogeneous product. Shrimp was shelled and cleaned before homogenizing. 50 g of ground beef were homogenized for 2 min in a Virtis blender with 100 ml 0.25 M cold sucrose solution, then filtered through 4

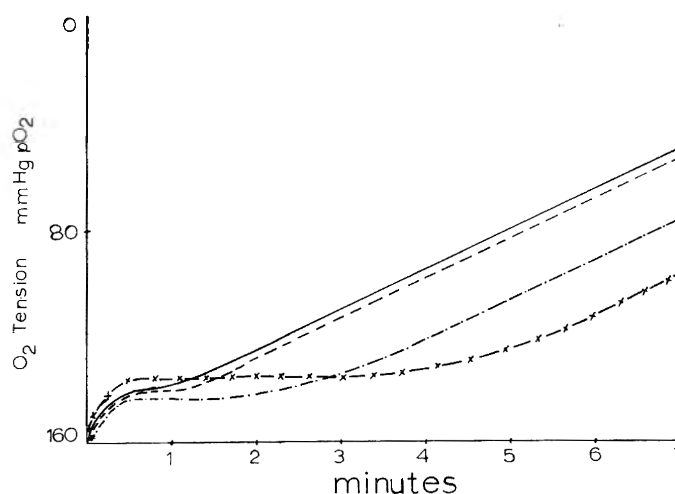

Fig. 1—Effect of 8-OH-quinoline on linoleate peroxidation catalyzed by MetMb or beef homogenate at pH 5.6. Linoleic acid 7 × 10^{-3} M; MetMb 6 × 10^{-7}M; beef homogenate (1.2%; w/v); phosphate buffer 0.1 M; 8-OH-quinoline 6 × 10^{-4} M. ——— MetMb catalysis. – – – – – Beef homogenate catalysis. —·—·—·— MetMb plus 8-OH-quinoline. x-x-x-x Beef homogenate plus 8-OH-quinoline.

[1] Present address: 76-10 31st Avenue, Jackson Heights, New York 11370.

Table 1–Effect of ascorbic acid and thiols on linoleate peroxidation catalyzed by beef homogenate.

	Rate of O_2 uptake (mm Hg pO/min)			
	pH			
	5.6	6.2	7.0	7.8
Homogenate (1.2%; w/v)	32.0	28.8	41.6	72.8
+ 6×10^{-4} M Ascorbic Acid	49.6	24.0	8.0	1.6
+ 6×10^{-4} M L-cysteine	20.8	9.6	11.2	4.8
+ 6×10^{-4} M Mercaptoethylamine	19.2	9.6	8.0	1.6
+ 6×10^{-4} M Reduced glutathione	27.2	16.0	25.6	40.0

Table 2–Effect of phosphate concentrations on linoleate peroxidation catalyzed by homogenate at pH 5.6.

	Ratio of O_2 uptake of experimental to control			
	Phosphate concentration			
	0.1 M	0.05 M	0.01 M	H_2O
Control (mm Hg pO_2/min)	16	19.2	27.2	28.8
+ 6×10^{-4} M Cysteine	0.8	0.66	1.0	1.0
+ 6×10^{-4} M Ascorbic Acid	3.8	2.75	1.6	1.3
+ 6×10^{-4} M EDTA	1.0	1.0	1.0	1.0
+ 6×10^{-4} M Citric Acid	1.0	1.37	1.8	2.0

layers of cheese cloth. The filtrate was collected and stored in the refrigerator not longer than 30 min. It was brought to room temperature before using. Shrimp homogenate was prepared in the same way, except that less tissue (10 g) was used to prevent bubble formation.

To separate the non-heme iron, beef homogenate was treated drop by drop with 30% H_2O_2 until the pigment was decolorized. The treated tissue showed no absorption peaks in the range 400–700 mμ when analyzed by reflectance spectrophotometry. 1 ml (1.2 %; w/v) of the homogenate was added to the linoleic acid emulsion and additives, keeping the total volume at 25 ml. The catalytic activity was again measured by using the oxygen analyzer as in the preceding paper.

In all experiments reported, duplicate determinations made on the same preparations agreed within the confidence limits indicated by the preceding paper. Similar patterns of activity were also shown by the additives tested on more than one homogenate preparation.

RESULTS & DISCUSSION

IT WAS expected that both heme and non-heme iron would contribute to catalysis by beef homogenate. However, the pattern of homogenate catalyzed oxidation over a pH range 5.6–7.8 (Table 1) is similar to that of MetMb catalysis in the model system. It may be that since the Mb in the homogenate is dissolved in the supernatant, it may make better contact than non-heme iron with added substrate. Although the pattern remained the same, the extent of catalysis varied in homogenates in the same muscle from different animals. Variable composition of the meat or different amount of peroxides produced during preparation of linoleate emulsions could account for this.

Addition of 6×10^{-4} M chelating agents and -S- or -S-S- compounds to the system produced the same effect as if hemoprotein were the only catalyst present; neither type of additive showed any effect on homogenate-catalyzed linoleate peroxidation over pH 5.6–7.8. As with MetMb catalysis in model systems, thiols and ascorbic acid inhibited the oxidation catalyzed by homogenates at alkaline pH. However, unlike the MetMb model, at pH 5.6, 6×10^{-4} M ascorbic acid greatly accelerated the homogenate catalysis, indicating that non-heme iron components may play a role in tissue homogenates at this pH. Again, 8-OH-quinoline (6×10^{-4} M) caused an induction period before homogenate catalysis commenced. The induction period was longer than with MetMb catalysis. An example at pH 5.6 is given in Figure 1. It is suggested that non-heme iron may also be functioning in beef homogenate.

Orr (1967) discussed the effect of ionic strength on the Cu (II) catalytic reaction. An increase in the ionic strength of phosphate buffer from M/2150–M/105 completely prevented the reaction, suggesting that metals can be effectively removed from solution at the higher phosphate concentration. Beef homogenates in the above experiment were tested in 0.1 M phosphate buffer. To find out whether the phosphate concentration would affect the activity of non-heme iron in tissue, 3 different concentrations of phosphate buffer as well as H_2O were compared in fatty acid emulsions at pH 5.6, where non-heme iron is most active. The homogenate catalytic rate increases as the phosphate concentration decreases (Table 2). Cysteine slightly inhibited the catalysis at 0.1 and 0.05 M phosphate buffer concentrations, but the inhibitions decreased at lower concentrations. Citric acid, on the other hand, had no effect at 0.1 M but accelerated the homogenate catalysis at lower buffer concentrations, where trace metals are more active. Although citric acid has been used to retard lipid oxidation by complexing with trace metal catalysts, it is quite possible that citrate, like EDTA, can form active (catalyzing) chelates under some conditions. It is not clear why ascorbic acid decreased its catalytic activity on homogenate catalysis in lower concentrations of phosphate buffer, particularly since it was shown in the preceding paper to have a greater effect with increasing concentrations of Fe(II)-EDTA.

Further evidence for the presence of non-heme iron in beef homogenates was obtained by adding chelating agents to ascorbate-stimulated homogenate catalysis at pH 5.6 in both 0.1 and 0.01 M phosphate buffer emulsion (Table 3).

Among the chelating agents tested, the best inhibitors with the homogenate catalyst, as with the Fe(II)-EDTA, were EDTA and $Na_3P_5O_{10}$. Variable inhibitions were obtained with the other chelating agents tried.

As H_2O_2 can completely destroy

Table 3–Effect of chelating agents on linoleate oxidation catalyzed by ascorbate and homogenate at pH 5.6.

	Ratio of O_2 uptake of experimental to control	
	Phosphate concentration	
	0.1 M	0.01 M
Control (mm Hg pO_2/min)	16	20.8
+ 6×10^{-4} M Ascorbic Acid (I)	3.8	1.8
(I) + 6×10^{-4} M EDTA	0.5	0.77
(I) + 6×10^{-4} M $Na_3P_5O_{10}$	0.6	0.62
(I) + 6×10^{-4} M 8-OH-Quinoline	0.8	1.00
(I) + 6×10^{-4} M Xanthine	3.6	1.00
(I) + 6×10^{-4} M Citric Acid	2.0	1.85

Table 4–Effect of ascorbic acid, reduced glutathione or L-cysteine on linoleate peroxidation catalyzed by H_2O_2-treated beef homogenate.

	Rate of O_2 uptake (mm Hg pO_2/min)			
	pH			
	5.6	6.2	7.0	7.8
H_2O_2-treated beef homogenate (1.2%; w/v)	4.8	3.2	2.4	1.6
+ 6×10^{-4} M Ascorbic Acid	64.0	22.4	6.4	1.6
+ 6×10^{-4} M Reduced glutathione	11.2	4.8	4.0	1.6
+ 6×10^{-4} M L-cysteine	6.4	4.8	8.0	1.6

Table 5—Effect of chelating agents on linoleate peroxidation catalyzed by H_2O_2-treated homogenate with ascorbate or cysteine at pH 5.6.

	Ratio of O_2 uptake of experimental to control	
	Phosphate buffer concentration	
	0.05 M	0.005 M
Control (mm Hg pO_2/min)	4.8	6.4
+ 6×10^{-4} M Ascorbic Acid (I)	5.7	1.5
(I) + 6×10^{-4} M EDTA	0.33	0.25
(I) + 6×10^{-4} 8-OH-Quinoline	0.33	0.38
(I) + 6×10^{-4} M Xanthine	8.6	3.75
(I) + 6×10^{-4} M Citric Acid	8.0	4.25
+ 6×10^{-4} M L-cysteine (II)	1.1	1.5
(II) + 6×10^{-4} M EDTA	0.5	0.25
(II) + 6×10^{-4} M 8-OH-Quinoline	0.66	0.25
(II) + 6×10^{-4} M Xanthine	1.66	1.0
(II) + 6×10^{-4} M Citric Acid	1.66	2.0

Table 6—Effect of ascorbic acid, sulfur-compounds and chelating agents on linoleate peroxidation catalyzed by shrimp homogenate.[1]

	Ratio of O_2 uptake of experimental to control			
	pH			
	5.6	6.2	7.0	7.8
Control (mm Hg pO_2/min)	8.0	6.4	4.8	3.2
6×10^{-4} M Ascorbic Acid	2.0	1.2	0.5	0.6
6×10^{-4} M L-cysteine	0.66	0.4	0.5	0
6×10^{-4} M Reduced glutathione	0.66	0.6	0.5	0
6×10^{-4} M L-cystine	0.8	1.0	1.0	1.0
6×10^{-4} M L-methionine	1.0	1.0	1.0	1.0
6×10^{-4} M EDTA	0	0	0	0
6×10^{-4} M 8-OH-Quinoline	0	0	0	0
6×10^{-4} M_3 Na P_5O_{10}	1.0	1.0	1.0	0.5
6×10^{-4} M Citric Acid	1.0	1.0	1.0	1.0

[1] Linoleate emulsion was prepared in 0.05 M phosphate buffer.

hemoprotein and liberate all the non-heme iron into the homogenate, H_2O_2-treated beef homogenate was prepared to test its non-heme iron catalytic properties. Over a pH range 5.6–7.8, linoleate peroxidation catalyzed by H_2O_2-treated homogenate followed a pattern similar to that of the model system Fe(II)-EDTA (Table 4). However, the extent of O_2 uptake was less at pH 5.6 and the decrease with pH was not so rapid. Even at pH 7.8, slight catalytic activity still existed. The reducing agents ascorbic acid, GSH and L-cysteine again accelerated at acidic pH but, unlike the Fe(II)-EDTA system, there was almost no effect at pH 7.8. L-cysteine showed a maximum acceleration at pH 7.0 rather than at acidic pH; this hump also occurred in the model system. As expected, the acceleration induced by reducing agents can be abolished by adding EDTA and 8-OH-quinoline.

A comparative study of various chelating agents on linoleate peroxidation catalyzed by ascorbic acid or cysteine and H_2O_2-treated homogenate at pH 5.6 is shown in Table 5. Among these chelators, only EDTA and 8-OH-quinoline abolished the acceleration. Xanthine and citric acid further accelerated. It is again indicated that chelating agents can act either as metal complex-activator or metal complex-inactivator, depending on the conditions of the reaction.

Shrimp, considered to have no Mb present, was tested for its non-heme iron catalytic reaction on linoleate peroxidation (Table 6). Shrimp homogenate followed more or less the same pattern as H_2O_2-treated beef homogenate catalysis. The chelating agents $Na_3P_5O_{10}$, xanthine, citric acid and the -S- or -S-S-group-containing compounds L-cystine and L-methionine did not show any effect on the catalysis. Thiol compounds L-cysteine and GSH and the chelating agents EDTA and 8-OH-quinoline inhibited the catalysis. Again, ascorbic acid accelerated the catalysis at pH 5.6 and 6.2.

In shrimp tissue, non-heme components were playing the dominant role. The complete inhibition by EDTA and 8-OH-quinoline and the high acceleration by ascorbate at acidic pH support this. The possible hemoprotein catalyst cytochrome c is present in a very slight amount (approximately 1.0 mg/100 g). In this semimodel system, this concentration was far below the catalytic range. Other metal complexes, such as the copper-containing protein hemocyanin, the O_2-transporting protein in some invertebrates, has been considered as an active catalyst (Tappel, 1955b). The fact that, unlike their activity in beef, thiols increased inhibition in shrimp as pH increased and showed no acceleration, may indicate that either different types of iron-complexes or other metal complexes may be present in shrimp tissue.

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HSIAO-PING LIU[1] and BETTY M. WATTS
Department of Food and Nutrition, Florida State University, Tallahassee, Florida

CATALYSTS OF LIPID PEROXIDATION IN MEATS. 3. Catalysts of Oxidative Rancidity in Meats

SUMMARY—Data presented in this paper can be interpreted as evidence that both heme and non-heme iron are functioning as catalysts of lipid oxidation in meat. The most direct evidence comes from cooked meat, since there the picture is not complicated by interfering enzymes. After removal of MetMb by treating with H_2O_2, a significant lipid oxidation was demonstrated, especially at lower pH where non-heme iron is most active. The catalytic activity of hemoprotein is limited in raw meat. Oxygen can be removed from the tissues and MetMb reduced back to Mb by the reducing enzymes. This is especially true at higher pH. Possible limitations of the heme-catalyzed reactions in meat by high (inhibition) levels of myoglobin, or because of separation of reactants in cellular structures, are discussed. The effects of additives were in line with the interpretation that lipid oxidation is catalyzed by both non-heme and hemoprotein. In raw meat, lipid oxidation could be slightly accelerated by adding TDPA or cysteine but inhibited by adding ascorbic acid or EDTA. It is considered that EDTA inhibited the non-heme iron catalysis at the natural acidic pH, whereas ascorbic acid prevented Mb oxidation and thus indirectly retarded the rancidity developed.

INTRODUCTION

OXIDATIVE deterioration of the lipids in meat has been ascribed largely to catalysis by hematin compounds (Watts, 1961; Tappel, 1962). Added or contaminating metals have been shown to be pro-oxidants. For example, Moskovits and Kielsmeier (1960) demonstrated that the contaminating iron in sausage exerted a powerful pro-oxidant activity. MacLean and Castell (1964) found that trace amounts of iron or copper ion added to whole or blended cod fillets produced a serious off-odor ranging from "seaweedy" to that of strongly rancid fish oil. However, this published work does not assess the catalytic activity of the metal originally present in meat or fish.

Several observations in the literature on the effect of pH and various additives could be interpreted as evidence for non-heme iron catalysis in meats. Keskinel et al. (1964) found an inverse relationship between the pH of meat samples and the TBA number which would be expected if non-heme iron is playing a significant role. On the other hand, this effect of pH could result from increased effect of reducing enzymes in raw tissues at higher pH (Stewart et al., 1965b).

Phenolic antioxidants would, of course, be expected to delay rancidity in meat regardless of whether heme or non-heme iron was the catalyst. Butyl hydroxyanisole and propyl gallate (Greene, 1966) have been demonstrated to protect meat and fish. However, the protective effect of polyphosphates (Tims and Watts, 1958) is difficult to explain if heme is the only catalyst, since these phosphates do not inhibit heme-catalyzed reactions, but presumably act by sequestering trace metals. The strong pro-oxidant activity of added cysteine in blended cod muscle (Castell et al., 1966), its enhancement by ascorbic acid and inhibition by EDTA, also suggest non-heme iron catalysts.

From the accompanying papers on model systems and homogenates (Liu, 1970a; 1970b), the points of greatest contrast between the 2 types of catalysts were the opposite effects of pH changes, reducing agents and metal chelators. Ascorbic acid accelerated non-heme iron catalysis but inhibited heme iron catalysis. EDTA, on the other hand, showed the highest inhibition of non-heme catalysis among the chelating agents tested, but had no effect on heme catalysis. These observations have now been applied to meat.

EXPERIMENTAL

BEEF ROUND and fresh pork loin were used. The meat was trimmed of visible fat, ground and mixed thoroughly. 3 ml 0.1% chlorotetracycline-HCl (CTC) was added to every 100 g meat to prevent spoilage by bacteria during storage (Stewart et al., 1965b). After addition of various test solutions or water to the control, an amount equivalent to 50 g of meat was placed in a polyethylene bag and stored in the refrigerator at 3°C until analysis.

The extent of lipid oxidation was determined by distilling malonaldehyde (MA) from the meat sample as described by Tarladgis et al. (1960). The distillate was then analyzed for MA by U.V. spectrophotometry (Kwon and Watts, 1963). This is preferable to thiobarbituric acid reagent for MA estimation, since other compounds can give colored complexes with TBA. Wills (1964) showed that Fe(III) reacted under test conditions.

The meat to be analyzed by reflectance spectrophotometry for MetMb formation must be quickly stirred to a uniform color before determination. The percentage of the pigment present as MetMb was determined from the ratio: $\frac{K/S\ 572\ m\mu}{K/S\ 525\ m\mu}$ (Stewart et al., 1965a).

RESULTS & DISCUSSION

EFFECTS of 2 widely different pH values on rancidity in raw beef were compared. The natural pH of the piece of beef round used was 5.5. Half of the meat was adjusted to pH 7.8 by adding 5 N NH_4OH. This is far outside the normal range of meat, but the catalytic activity of Mb is high at this pH according to both model and homogenate systems. Ascorbic acid and EDTA were then added to these 2 samples. MA formation was determined over a 5-day storage period.

At pH 7.8, neither controls nor treated samples increased in MA during storage (Table 1). All samples were a deep dark-red color. This color did not fade until the last day of the experiment. On the other hand, the control samples held at pH 5.5 showed increasingly high rancidity over the storage period. Either ascorbic acid or EDTA added separately inhibited MA formation. Protection was increased when both of them were added together to the meat. As for the pigment, the sample to which ascorbic acid was added maintained a bright-red color until the fifth day of storage. Ascorbic acid plus EDTA also gave this desirable appearance.

Table 1—Effect of ascorbic acid and EDTA on MA formation.

	mg MA/1000 g meat					
	pH 5.5			pH 7.8		
	Days			Days		
	1	3	5	1	3	5
Control	8.8	10.2	13.6	1.1	1.1	0.8
4×10^{-4} moles Ascorbate	4.5	5.5	7.5	0.8	0.6	0.6
2.5×10^{-4} moles EDTA	4.5	4.3	4.9	1.0	0.8	1.2
4×10^{-4} moles Ascorbate + 2.5×10^{-4} moles EDTA	3.6	3.7	3.1	1.6	1.9	1.2

[1] Present address: 76-10 31st Avenue, Jackson Heights, New York 11370.

Table 2–Effect of EDTA, cysteine, TDPA and ascorbic acid on MA formation in beef and pork.

	mg MA/1000 g meat			
	Pork (pH 5.65)		Beef (pH 5.7)	
	Days		Days	
	1	4	1	4
Control	0.6	1.2	2.0	3.6
1×10^{-4} moles EDTA	0.4	0.8	1.0	1.6
2.2×10^{-4} moles TDPA	0.6	1.4	2.1	4.6
3.3×10^{-4} moles Cysteine	0.4	1.2	1.7	4.3
8×10^{-5} moles Ascorbic Acid	–	–	1.3	2.7

Table 3–Effect of H_2O_2 on MA formation in cooked meat.

	mg MA/1000 g meat					
	H_2O_2 treated			Untreated		
Days	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7
1	2.3	1.9	1.7	6.0	7.0	6.7
3	5.6	4.0	3.3	9.5	11.2	9.8
5	7.2	4.9	4.2	14.0	14.0	12.0

Both EDTA and control samples were pale brown.

Superficially, there seems to be a discrepancy between these results and those found in the previous papers. At pH 7.8, according to both model and homogenate systems, hemoprotein was the main catalyst for linoleate peroxidation; but in meat, no oxidation occurred. Other factors in the complex meat system are believed to be responsible for the lack of lipid oxidation. At this high pH, enzymatic reducing systems in meat are very active, utilizing the available oxygen and maintaining Mb in the reduced form, believed to be inactive as a catalyst.

At pH 5.5, catalysis by non-heme iron was expected. EDTA inhibited the rancidity, indicating the presence of non-heme iron in meat. The inhibition caused by ascorbic acid is interpreted as an effect on the pigment Mb rather than on the non-heme iron moiety. The effect of ascorbic acid on maintaining a good color in meat has been discussed (Caldwell et al., 1960). Thus, ascorbic acid does not affect the rancidity directly, but indirectly through inhibiting MetMb formation.

TDPA has been shown in previous literature to retard peroxide formation in fats and oils, but it did not show any effect on heme or non-heme iron catalysis in either model or homogenate systems. Furthermore, it accelerated both types of catalysis, especially the heme iron catalysis at pH 5.6, at a concentration of 6×10^{-3} M. Cysteine, like ascorbic acid, inhibited heme iron catalysis but accelerated non-heme iron catalysis. When these sulfur compounds were applied to beef and pork (Table 2), they showed no effect on pork but slightly accelerated the rancidity in beef. These 2 pieces of meat had a relatively low rate of MA formation.

The accelerations by cysteine and TDPA and the inhibition by EDTA, especially in beef, indicated that at the natural acidic pH of meat, heme and non-heme iron were both important catalysts. In this experiment, ascorbic acid concentration was only one-fifth that of the last experiment. Again, it inhibited MA formation in beef. With respect to MetMb formation in this piece of beef, ascorbic acid was the only additive which gave a better color to the meat. On the fourth day, the percentage of MetMb formation was zero in the meat sample containing ascorbic acid, while the control contained 15% MetMb. Other additives did not give any pigment protection (15–20% MetMb).

In cooked meat, reducing enzymes are completely inactivated by heat and all the heme iron is converted to the ferric form without any chance to reduce back. The ferric heme has always been considered as the active form in catalyzing lipid oxidation. One experiment was set up to determine the oxidative rancidity developed at various pH values in cooked meat before and after H_2O_2 treatment to destroy hemes (Table 3).

MA values were high and little affected by pH in the untreated cooked meat. In the H_2O_2-treated meat, although less MA formed during storage, the amount was still of practical significance, considering that off-odors are easily detectable in meats when MA values exceed 1. As would be expected, with a non-heme iron catalyst, oxidation was greater at lower pH values. It is recognized that in meat, as in the semimodel system, the destruction of hemes increases the non-heme iron present. This is probably one source of non-heme iron in cooked stored meats, where loss of heme pigments and fading of color are frequently observed.

Considering the much greater effectiveness of heme compounds as compared to the non-heme iron chelates as catalysts of lipid oxidation, it might seem surprising that non-heme iron could have a significant role in the deterioration of meats. A possible factor in lessening the relative importance of heme as a catalyst in tissue is that when the concentration of heme to that of polyunsaturated fatty acid at any particular site exceeds a fixed ratio, the heme acts as an antioxidant rather than a pro-oxidant (Lewis and Wills, 1963). These workers demonstrated that hemoglobin, hemin, cytochrome c and tissue homogenates inhibit lipid oxidation at high concentrations. Kendrick and Watts (1968) found increasing acceleration of linoleate oxidation by metmyoglobin in model systems up to a linoleate to heme molar ratio of about 250 to 1. At this point, a doubling of the heme (or a corresponding decrease in the fatty acids) produces complete inhibition of oxidation.

If one makes a rough calculation of polyunsaturated fatty acids and Mb in meats, the ratio would normally fall in the catalytic range. However, the structural relations of cells tend to separate Mb and polysaturated fatty acids. Mb is in solution in the cytoplasm, while the polyunsaturated fatty acids are mainly in membranes and particulates. Under these circumstances, Mb could be inhibiting in localized areas of the cell and out of contact with fatty acids in other areas.

The nature of non-heme iron complexes responsible for catalyzing lipid oxidation in meat is not known. Among the non-heme iron components of muscle tissue, transferrin, ferritin and a number of enzymatically active compounds of the respiratory chain in mitochondria have been identified. Other non-heme iron components may also be present. Since variable amounts of reducing agents. i.e., ascorbic acid or cysteine, and chelators, i.e., citric acid, amino acids, etc., are naturally present in meat tissue, the catalytic activity of non-heme iron would be expected to show wide variations in different samples of meat. The catalysis is certainly important from a practical standpoint.

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P. M. T. HANSEN, W. J. HARPER and K. K. SHARMA
The Ohio State University and
The Ohio Agricultural Research and Development Center, Columbus, Ohio 43210

FORMATION OF FREE RADICALS IN DRY MILK PROTEINS

SUMMARY–The possible formation of free radicals in dry milk proteins by mechanical energy has been studied by electron spin resonance (ESR) spectroscopy. Grinding of various milk proteins with mortar and pestle for 5 min or longer yielded perceptible electron spin resonance signals. The signals did not exhibit characteristics of hyperfine structure and all had g-values close to that of a free electron (2.00). The intensity of the signals was particularly strong for the sulfhydryl-containing proteins, β-lactoglobulin and κ-casein, and increased with grinding time. The signals were considerably reduced by subsequent heating of the dry protein at 100°C/10 min. The grinding of whole casein was in all cases accompanied by the release of a gluey odor, suggesting that the gluey-flavor defect may be related to protein fragmentation.

INTRODUCTION

THE FOOD uses of casein and caseinates have been limited by the development of a flavor defect generally described as gluey. Ramshaw and Dunstone (1969) recently suggested that the gluey off-flavor develops during the early stages of the nonenzymic browning reaction between reducing sugar and milk proteins. However, their observation that irradiation of casein with UV light could also induce glueyness suggests that alternative pathways may exist involving perhaps free radicals. Dunlop and Nicholls (1965) have demonstrated the presence of free radicals in a number of ultraviolet-irradiated proteins, including casein.

Input of mechanical energy into high molecular weight substances has been shown to result in chemical degradation. For example, it has been shown by Steurer and Hess (1944) that the dissipation of energy during ball-milling of cellulose is sufficient to rupture covalent bonds. Assarson et al. (1959) observed that carbon-carbon and carbon-oxygen linkages in sawdust were broken due to mechanical degradation, and the formation of free radicals during wood grinding and pulp beating has subsequently been reported by Kleinert and Morton (1962). Urbanski (1967) has shown that free radicals are formed by grinding solid materials, such as anthracene, acridine, cellulose and polyvinyl chloride. The production of long-lasting, free radicals in powdered bone material has been reported by Marino and Becker (1968). The mechanical breakdown of polystyrene by ball-milling has been studied by Eckert et al. (1968), who also investigated some of the possible reactions of the free radicals thus created.

The purpose of this study was to investigate the possible development of free radicals in dry milk proteins as a result of mechanical forces.

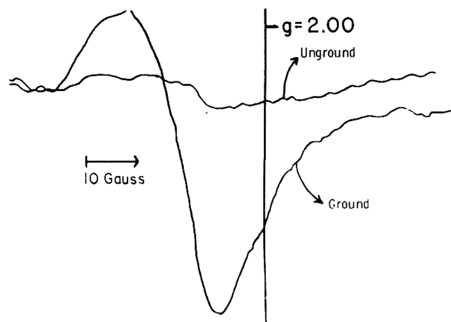

Fig. 1–First derivative ESR spectrum of ground and unground isoelectric casein. Field: 3400 Gauss (g = 2.00).

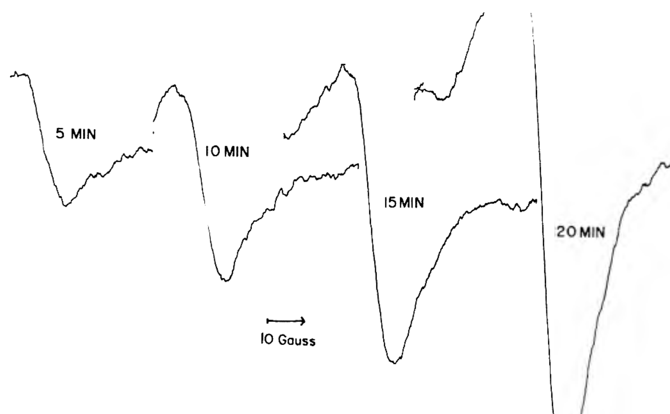

Fig. 2–Effect of grinding time on the intensity of free radical signals in sodium caseinate.

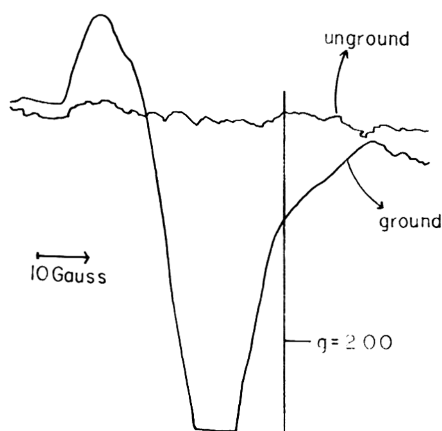

Fig. 3–Development of free radical in ground, crystalline β-lactoglobulin. Field: 3400 Gauss (g = 2.00), 10-min grinding.

EXPERIMENTAL

Preparation of samples

Whole, dry sodium caseinate was prepared by isoelectric precipitation of casein from skimmilk at pH 4.6 using 1 N HCl. After washing twice with water, the casein was dissolved in 0.5 N NaOH, keeping the pH below 8.0 at all times. The sodium caseinate solution was dialyzed in water for 24 hr at 4°C. The dialyzed caseinate was freeze dried and stored at –15°C. α- and β-Caseins were prepared by the method of Hipp et al. (1952), α_S-casein was prepared from α-casein by the method of Zittle and Custer (1963) and κ-casein by Zittle's method (1962). β-Lactoglobulin (Nutritional Biochemicals Corporation, Cleveland, Ohio) was 3 times crystallized. Samples of casein/whey protein coprecipitates (Muller et al., 1967) were supplied by L. L. Muller, CSIRO, Division of Dairy Research, Melbourne, Australia, and had been previously ground either to a coarse granular form or to the grain size of table salt. These samples were packed under vacuum and were stored for approximately 9 months at 5°C prior to this investigation. Agarose was obtained from Marine Colloids, Inc., Rockland, Maine.

Test samples were ground manually by mortar and pestle for different time intervals immediately before obtaining the electron spin resonance spectra. Some difficulty was encountered in grinding the freeze-dried caseins but this problem was overcome by pressing the caseins in a Carver Laboratory Press, Model B, prior to grinding. A small portion of the dry protein was then filled into quartz capillaries packed to a depth of approximately 1.5 in. by light tapping. Measurements were performed at room temperature. The instrument used was a Model E-3 Varian EPR spectrometer employing 100 kc magnetic field modulation.

RESULTS

A SAMPLE of dry isoelectric casein was ground vigorously for 10 min; the electron spin resonance (ESR) spectra obtained in the field range of 3400 Gauss are shown in Figure 1. The spectrum for the ground casein contained a characteristic, first derivative signal for organic, free radicals which was absent in the unground control.

Fig. 4–Development of free radical in ground agarose. Field: 3375 Gauss (g = 2.01), 10-min grinding.

The effect of varying the grinding time on the magnitude of the free radical signal for sodium caseinate is shown in Figure 2. The spectra revealed a gradual increase in the absorbance when the treatment was prolonged. In all cases, only a single, somewhat asymmetric signal was observed in the spectra, but attempts to resolve hyperfine structure were unsuccessful. The slow return of the recorded spectra to the baseline in this as well as in subsequent spectra may possibly have been caused by adsorbed oxygen which affects line widths (Snipes and Keith, 1970).

The development of free radical was not limited only to ground casein and caseinates, and Figure 3 illustrates the effect of grinding crystalline β-lactoglobulin. The signal was more intense and considerably more asymmetric than those recorded for the isoelectric casein and sodium caseinate. Only a weak signal was observed for the linear carbohydrate polymer, agarose, as shown in Figure 4. No free radicals resulted from the grinding of the amino acids tryptophan, tyrosine, methionine, cystine and glycine, or of glutathione.

A more detailed analysis was made on individual proteins of whole caseinate and Figure 5 shows the spectra for ground α_S-, β- and κ-caseins. The free radical signals for α_S- and β-casein were weak as compared to the powerful signal response of κ-casein. When ground κ-casein was heated to 100°C for 10 min the free radical signal almost vanished.

The spectra for stored, commercially manufactured casein and milk protein coprecipitates did not differ appreciably from one another. Factory-ground samples which had not been exposed to additional grinding at the time of analysis still exhibited weak free radical signals after 9 months of storage. These signals could in all cases be amplified on subsequent grinding and the resulting spectra were of a pattern similar to Figure 1. No differences were detected in the spectra due to the presence or absence of whey protein or calcium or to small variations in lactose content.

All whole caseinate samples released a gluey odor on grinding and the intensity increased with grinding time. This odor was not observed in ground β-lactoglobulin.

DISCUSSION

RESULTS of this study demonstrate that paramagnetic properties are induced in dry milk proteins by grinding treatments and that the intensity increases with grinding time. Apparently, the mechanical stresses imposed upon the protein in this manner were sufficient to cause a homolytic breakage of covalent bonds which generated free radicals (Ayscough, 1967). Thus, our observations confirm earlier findings that macromolecules are subject to chemical degradation arising from purely mechanical forces (Kleinert and Morton, 1962; Bachman and Devries, 1969).

Although no attempt was made to identify the free radicals, the spectra showed g-values close to 2.00 and similar to those reported for some UV-irradiated proteins. Dunlop and Nicholls (1965) found that irradiation of casein produced

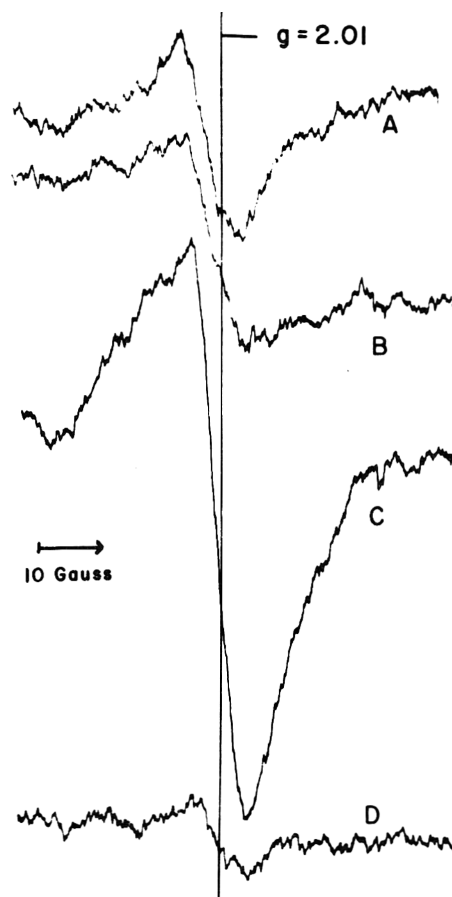

Fig. 5–Relative intensity of free radicals in fractions of whole casein. Field: 3385 Gauss (g = 2.01).

A. β-casein, 10-min grinding. B. α_S-casein, 10-min grinding. C. κ-casein, 10-min grinding. D. κ-casein, 10-min grinding, followed by 10-min heating at 100°C.

a spectrum of rather broad line width, for which hyperfine structure could not be resolved. They could not detect any of the distinctive features of cystine degradation in casein, such as occurred in insulin and egg white, but found that the casein spectrum resembled that of irradiated gelatin. In the present study it was observed that the cysteine-containing proteins, β-lactoglobulin and κ-casein, yielded much more intense free radical signals than α_S- and β-casein. Therefore, it is possible that these sulfur amino acids may present centers for the formation of macroradicals. Alternatively, the presence of sulfur bridges in the protein may result in a rather more rigid structure which would be increasingly vulnerable to grinding, so that radicals may be generated in other segments of the amino acid chain. The presence of high-polymer structure was found to be a prerequisite for this reaction, since no effect of grinding was observed for any of the individual amino acids tested.

The stability of the free radicals formed appeared to be temperature dependent, since the signals were greatly diminished upon heating of the ground samples. This observation was in agreement with the findings of Backman and Devries (1969), who measured the surface density of free radicals in several fractured polymers and found a very marked temperature dependence. Similar findings have been made by Kleinert and Morton (1962) in their study of free radicals in ground wood. On the other hand, the free radicals exhibited appreciable stability at moderately low temperature. For example, the ESR signals persisted for many hours in ground casein held at room temperature. Weak signals could also be detected in 9-month-old, commercial casein stored at approximately 5°C and were presumably a lingering effect of the grinding treatment used at the time of manufacture.

Results of early experiments in Australia by Loftus-Hills and Thiel (1960) showed that ball-milling of casein invariably produced a gluey odor which upon prolonged treatment could become almost fecal in character. This observation has been confirmed in the present study and possibly links the gluey off-flavor development to free-radical chain reactions. Virtually all casein and caseinate products have been exposed to some degree of abrasion during their manufacture so that free radicals would always be expected in the final product. The report by Ramshaw and Dunstone (1969) that UV-irradiation of casein could produce elements of the off-flavor would be consistent with a concept of free radical processes (Dunlop and Nicholls, 1965). The findings by Ramshaw and Dunstone (1969) that the development of gluey flavor is accelerated by heat may perhaps be explained on the grounds that the secondary reactions of the free radicals are vastly speeded up by increased temperature.

Because of the high reactivity of free radicals, attention should be given to their role and ultimate fate in dry food proteins. Eckert et al. (1968) have pointed out that the free radicals will be concentrated in the surface of finely divided particles; therefore, their number will be small compared to the bulk of material. Urbanski (1967) has suggested that mechanically induced, free radicals may be responsible for the caking of some powder substances and for the explosive nature of dust, particularly coaldust. For proteins it seems likely that viscosity and solubility properties would change markedly as the result of the rupture of covalent bonds. However, the biological and nutritional effects of these free radical reactions remain obscure.

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C. TU[a], W. D. POWRIE[b] and O. FENNEMA
Department of Food Science, University of Wisconsin, Madison, Wisconsin

STEROIDS IN EGG YOLK

SUMMARY—This investigation was initiated to separate the unsaponifiable matter of egg yolk by thin layer chromatography (TLC) and attempt to identify the steroids by gas liquid chromatography (GLC). Three GLC liquid phases, differing in selective partition properties, were used to aid in the identification of unknown steroids. A selective method was also carried out to extract the estrogenic steroids in egg yolk if present. The results of thin layer and gas chromatographic analyses indicate that a large quantity of cholesterol and considerable amounts of lanosterol, desmosterol, Δ^7-cholestenol, cholestanol, Δ^7- and Δ^8-methostenol, 4α-methyl-$\Delta^{8,24}$-cholestenol and its Δ^7-isomer, 4,4α-dimethyl-$\Delta^{7,24}$-cholestenol, dihydrolanostenol, β-sitosterol and possibly ergosterol were found to be present in egg yolk. Estrogenic steroids and other known steroid hormones were not found in egg yolk.

INTRODUCTION

NUMEROUS RESEARCHERS have reported that egg yolk contains a chick-growth factor. According to Denton et al. (1954), the addition of yolk to a chick ration brought about a weight gain of 18% greater than that for chicks at 4 wk of age on a basal diet. The growth factor in yolk has been reported to be not identical with the fish factor (Arscott, 1956; Denton et al., 1954; Menge et al., 1957). Although the yolk factor has not been chemically identified, Menge et al. (1957) demonstrated that it is present in the unsaponifiable matter of an ether extract. These researchers also noted that hot but not cold saponification destroyed the yolk growth factor. In addition to the growth-promoting effect, yolk in the diet of mice has been shown by Szepsenwol (1961) to accelerate sexual development. Circumstantial evidence from animal assays has been forwarded by several researchers for the presence of an estrogenic compound(s) in egg yolk (Altmann and Hutt, 1938; Marlow and Richert, 1940; Riboulleau, 1938).

According to Szepsenwol (1959), egg yolk in the diet of mice can induce malignancies of the lungs and lymphoid tissue. Szepsenwol (1964) further reported that carcinogens in yolk were lipid in nature, one being soluble in alcohol while the other was ether-soluble. However, no other studies have confirmed these observations. Cholesterol, being present in the yolk at very high concentration (Pihl, 1952), might be considered to have a carcinogenic effect. Mice, on a diet containing cholesterol, developed a high incidence of lung adenocarcinoma (Szepsenwol, 1966). On the other hand, both mammary cancer and lung adenocarcinoma were induced by a diet containing lard (Szepsenwol, 1966). Oxidation products of cholesterol, rather than cholesterol, were considered by Bischoff (1963) to be highly carcinogenic when injected subcutaneously into mice and rats.

The yolk growth factor, accelerators of sexual development and possible carcinogens, being soluble in lipid solvents. may be steroids. So far, little information has been reported on the types of steroids in yolk other than cholesterol. Thus this study was initiated to separate the unsaponifiable matter of yolk by thin layer chromatography (TLC) and attempt to identify the steroids by gas liquid chromatography (GLC).

EXPERIMENTAL

Materials

Yolk from infertile hen's eggs about 2 days old was prepared by a method similar to that of Powrie et al. (1963).

Steroids used as reference standards were from various sources. Δ^8-methostenol and zymostenol were kindly provided by Dr. A. A. Kandutsch and Dr. G. J. Schroepfer, Jr., respectively. Δ^7-methostenol was obtained from Dr. W. W. Wells. 14α-methyl-5α-cholest-7-en-3β-ol was kindly supplied by Dr. J. C. Knight. Cholesterol, cholestane, androstane, 5α-cholestane-3β-ol, desmosterol, β-sitosterol, stigmasterol, cholestan-3-one, cholesteryl palmite, progesterone, estradiol, estrone, androstan-17-one, testosterone and androsterone were obtained from Applied Science Laboratories, State College, Pa. Lanosterol, dihydrolanosterol, 4-cholesten-3-one, 3,5-cholestadien-7-one, ergosterol, 5-androsten-17α-methyl-3β,17β-diol, cholestane-3β,5α,6β-triol, 5α-pregnan-3,20-dione, and 5α-pregnan-21-ol-3,20-dione were purchased from Steraloids Inc., Pawling, N.Y. Cholest-7-en-3β-ol was obtained from IKAPHARM, Israel. Squalene was purchased from Eastman Kodak, Rochester, N.Y. N-octacosane and 4-androsten-3,17-dione were obtained from ANALABS, Hamden, Conn. α-tocopherol and ubiquinone-30 were purchased from SIGMA, St. Louis, Mo.

Lipid extraction

Lipid extraction of egg yolk was carried out by a method similar to that outlined by Tu et al. (1967). A mixture of 50g of egg yolk and 54g of water were blended in a Waring Blendor with 200 ml of methanol and 100 ml of chloroform for 4 min. About 100 ml of chloroform was added to this mixture and blending was continued for 1 min; thereafter, 100 ml of distilled water was blended into the mixture for 30 sec. The mixture was filtered through a No. 42 Whatman filter paper in a Büchner funnel with slight suction. After rinsing the residue with 50 ml of chloroform, the residue was re-extracted with 350 ml of chloroform by blending the mixture for 5 min. After filtration, the combined filtrates were placed in 1000 ml graduated cylinder to measure the volume of the chloroform layer after separation of the two phases. The aqueous alcoholic upper layer was removed by suction.

Cold saponification

Solvent in each lipid extract was evaporated under reduced pressure as a flask rotated in a water bath at 50°C. Each lipid residue was mixed with 10% KOH in ethanol (about 19 ml KOH soln/g lipid) and the mixture held under nitrogen at 25°C for 14 hr with occasional stirring. Water, twice the volume of added KOH solution, was added to the mixture and the resulting solution was extracted three times with 0.7 volume of ethyl ether. The combined ether extracts were washed three times with 0.5N KOH to remove any remaining free fatty acids and several times with water to neutrality. The washed extract was dried over anhydrous sodium sulfate. The solvent in the extract was evaporated under pressure and an appropriate volume of chloroform was added to the unsaponifiable matter for thin layer chromatography.

Thin layer chromatography (TLC)

Preparative and analytical TLC was carried out on 20 × 20 cm glass plates, each coated with a 0.5 mm layer of silica gel (Adsorbosil-2, Applied Science Laboratories, Inc.). A slurry was prepared by mixing 1 part silica gel powder and 1.5 parts water. Rhodamine 6 G (Allied Chemical Corp., New York) was usually added to the water to make a 0.1% dye solution prior to slurry preparation. The inclusion of Rhodamine 6 G was helpful for visualization of spots on developed plates. The slurry was applied to the plates by a Desaga-Brinkmann adjustable applicator. Each plate was activated at 110°C for 1 hr. The preparative TLC plates were spotted at 0.5 cm intervals with a chloroform solution of unsaponifiable matter by a Hamilton microsyringe. Each plate contained about 17 mg of the unsaponifiable fraction. A solvent system of benzene-ethyl acetate (10:1, v/v) was used to develop the plates. The plates were dried at 25°C. Spots on the Rhodamine 6 G plates were visualized with the aid of UV light as pink-yellow fluorescent areas or as dark areas. Spot positions on dye-free TLC plates were determined by spraying a narrow region at the ends with 50% H_2SO_4 and then visualizing with UV light.

The developed plates were divided into 9 zones (Fig. 1), each eluted for subsequent GLC analyses. The silica gel in each zone was scraped into a sintered-glass funnel for elution of the

[a]Post-doctoral Research Associate. Present address: Faculty of Food Science, University of Toronto, Toronto, Canada.

[b]Present address: Department of Food Science, University of British Columbia, Vancouver, Canada.

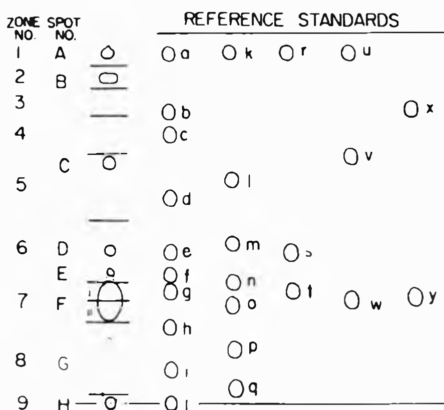

Fig. 1–*Rhodamine 6 G-silica gel thin layer chromatogram of unsaponifiable matter for egg yolk. Solvent system: Benzene-ethyl acetate (10:1 v/v). Reference standards: a, squalene; b, cholesteryl acetate; c, $\Delta^{3,5}$-cholestadiene-7-one; d, cholestane-3-one; e, lanosterol; f, Δ^8-methostenol and Δ^7-methostenol; g, cholesterol; h, estrone; i, estradiol; j. cholestane-3β,5α,6β-triol; k, cholesteryl palmitate; l, androstane-17-one; m, Δ^4-cholestene-3-one; n, β-sitosterol; o, Δ^7-cholstenol; p, progesterone; q, testosterone; r, cholestane; s, dihydrolanosterol; t, desmosterol; u, n-octacosane; v, ubiquinone; w, cholestanol; x, α-tocopherol; y, ergosterol.*

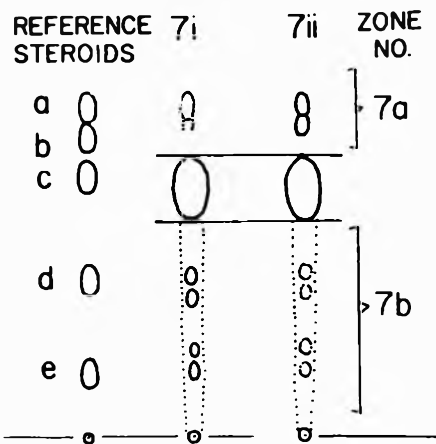

Fig. 2–*$AgNO_3$ thin layer chromatogram of zone 7 (Fig. 1) eluate for egg yolk. 7i = eluate from upper half of zone 7; 7 ii = eluate from lower half of zone 7. Solvent systems: Chloroform-acetone (95:5, v/v). Reference standards: a, cholestanol; b, Δ^7-cholestenol; c, cholesterol; d, desmosterol; e, ergosterol.*

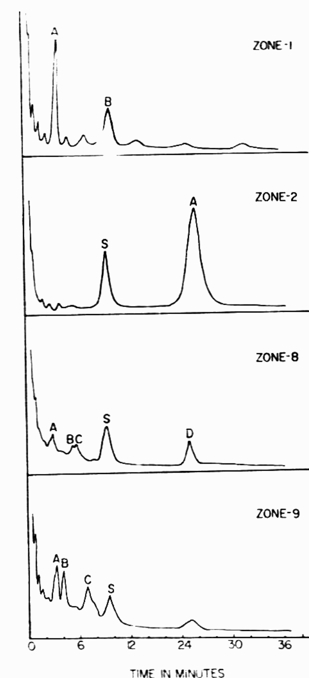

Fig. 3–*Gas chromatograms of egg yolk unsaponifiables in zone eluates from TLC plate (Fig. 1). Operating conditions: 6 ft × 4 mm I.D. column with 1% SE-30 on Gas-Chrom Q, 80/100 mesh; column temperature, 211°C; 95 ml/min N_2 carrier gas; hydrogen flame detector. S represents the peak of added cholestane as reference standard.*

unsaponifiable components. The silica gel with Rhodamine 6 G from the upper 7 zones was extracted three times with 15 ml of chloroform. The Rhodamine 6 G remained on the silica gel. With dye-free TLC plates, the components from zones 8 and 9 were eluted from silica gel with chloroform-methanol (1:1, v/v). After evaporation of the solvent under reduced pressure, the residues from zone 1 through 7 were dissolved in methylene chloride and the residues from zone 8 and 9 were dissolved in methylene chloride-methanol (9:1, v/v) for GLC studies.

Silver nitrate-silica gel TLC plates were prepared to separate compounds structurally-related to cholesterol. A slurry was prepared by mixing 60g of silica gel powder (Adsorbosil-2) with 90 ml of 12.5% silver nitrate solution. This amount of slurry was sufficient to coat five 20 × 20 cm chromatoplates, each 0.5 mm thick. The chromatoplates were allowed to dry at 25°C for more than 5 hr in the dark. Each plate was activated at 110°C for 1 hr. For preparative TLC, the plates were spotted at 0.5 cm intervals. A solvent system of chloroform-acetone (95:5, v/v) was used to develop the plates. The plates were dried at 25°C, and sprayed with 50% H_2SO_4 for visualization of spots. Spots could be detected nondestructively by lightly spraying each dried plate with distilled water. When the plate was viewed against a dark background, the spots appeared white as the plate dried.

A method similar to that of Truswell and Mitchell (1965) was used for steroid extraction. Absorbent in each zone was scraped into a 15 ml conical centrifuge tube and extracted with 3 portions of 6 ml 50% ethanolic ammonia. The tubes were centrifuged at about 1000 × G after each extraction and the extractant was removed. The combined extracts were diluted with an equal volume of water and extracted three times with 15 ml redistilled n-hexane. The combined hexane extracts were washed four times with 25 ml of water and dried over anhydrous sodium sulfate. The solvent in the extract was evaporated under reduced pressure and the residue was dissolved in methylene chloride for GLC analysis.

R_c is defined as the ratio of distance of the sample spot and distance of the cholesterol spot from the origin. Cholesterol was used as an internal standard for all plates. Preliminary identification of yolk steroids was carried out by comparison of R_c values of unknown spots on TLC plates with R_c values of authentic steroids mentioned under MATERIALS and by spot color (after H_2SO_4 spraying) comparisons.

Gas liquid chromatography (GLC)

GLC was carried out with a Barber-Colman Model 10 gas chromatograph with hydrogen flame ionization detector. Pyrex glass columns (U-shaped, 6 ft. long, 4 mm I.D.) were packed with 1% SE-30 (methyl silicone polymer). QF-1 (fluorinated alkyl silicone polymer) and NGS (neopentyl glycol succinated polyester) on Gas Chrom Q, 80/100 mesh. The following operating conditions were used: nitrogen gas flow rate, 95 ml/min for SE-30, 120 ml/min for QF-1 and 130 ml/min for NGS; column temperature, 211°C; detector temperature, 255°C; flash evaporator temperature, 280°C. Samples of 1 to 2 μl were injected into the column with a 10 μl Hamilton syringe.

Relative retention times (RRT) were calculated relative to cholestane. Cholestane was used in all samples as an internal standard. The steroid number (SN) was calculated by the method of VandenHeuvel and Horning (1962). Identification of compounds in gas chromatographic peaks was based on the comparison of RRT and SN values of unknown and authentic steroids. A non-selective liquid phase (SE-30) was used to obtain a rough estimation of the number of carbon atoms in each peak compound. Two selective phases (QF-1 and NGS) were used for further confirmation of the structure of each unknown. Purified steroids employed as standards for identification are listed under MATERIALS.

For the quantitative estimation of identified steroids in yolk by gas chromatography, purified authentic standards were used to obtain plots of the area of a peak vs. steroid concentration with the exception that Δ^8-methostenol and lanosterol were employed as substitutive standards for dehydromethostenol and 4,4α-dimethyl-$\Delta^{7,24}$-cholestadiene-3β-ol, respectively. The area under the peak was calculated by the triangulation method.

Cholesterol determination

The total cholesterol content of egg yolk was determined by the method outlined by Tu et al. (1967) except that 0.5 ml aliquot of lipid extract (total volume of 520 ml) from 50g of egg yolk was used for saponification, and 1 ml aliquot of the petroleum ether layer containing unsaponifiables was taken for color development.

Extraction of free esterogenic steroids

A method similar to that of Eleftheriou et al. (1966) was used for the extraction of free estrogenic steroids. The lipid residue obtained from 100g of egg yolk as outlined previously was dissolved in 100 ml of toluene. The solution was extracted three times with 100 ml of 5% NaOH solution to extract steroids. The NaOH extracts were pooled and backwashed

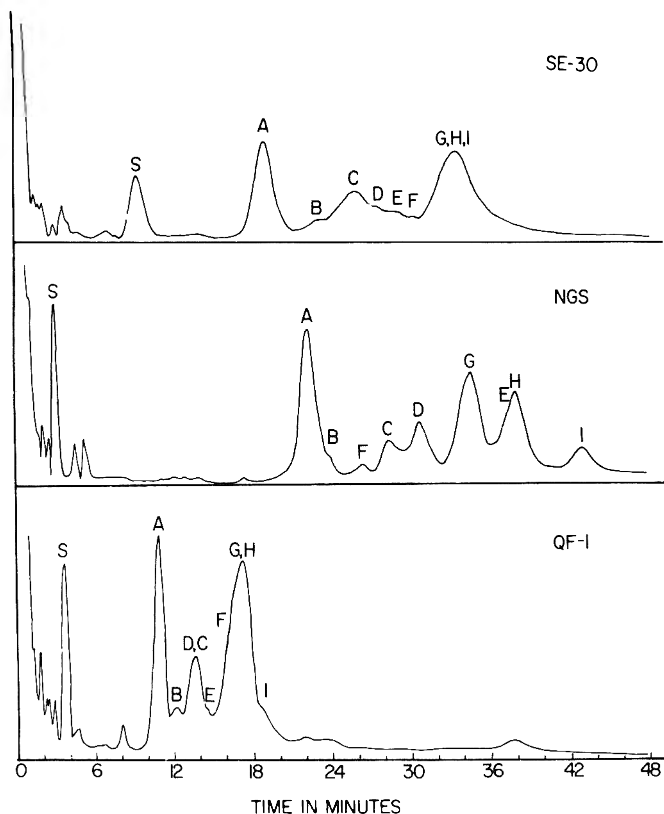

Fig. 4—Gas chromatograms of egg yolk unsaponifiables in zone 6 eluate from TLC plate (Fig. 1). Operating conditions: 6 ft × 4 mm I.D. column with 1% SE-30, NGS or QF-1 on Gas-Chrom Q, 80/100 mesh; column temperature, 211°C; 95 ml/min (for SE-30), 130 ml/min (for NGS) or 120 ml/min (for QF-1) N_2 carrier gas; hydrogen flame detector. Steroid peaks: A, cholesterol; B, Δ^8-methostenol; C, Δ^7-methostenol; D, 4α-methyl-$\Delta^{8,24}$-cholestadiene-3β-ol; E, 4α-methyl-$\Delta^{7,24}$-cholestadiene-3β-ol; F, dihydrolanosterol; G, lanosterol; H, β-sitosterol; I, 4,4α-dimethyl-$\Delta^{7,24}$-cholestadiene-3-β-ol; S, added cholestane as reference standard.

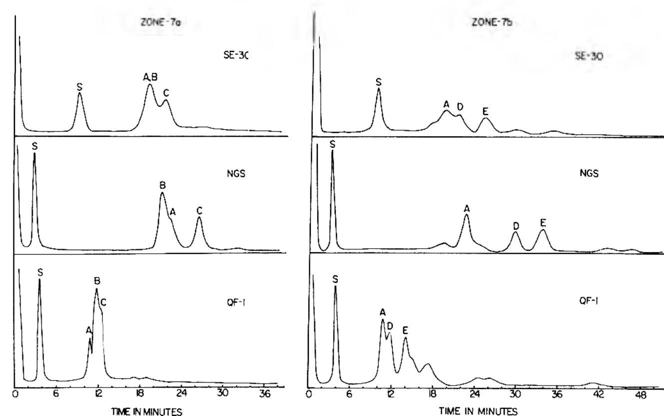

Fig. 5—Gas chromatogram of egg yolk unsaponifiables in zone 7a and 7b eluates from $AgNO_3$ TLC plate (Fig. 2). Operating conditions: 6 ft × 4 mm I.D. column with 1% SE-30, NGS or QF-1 on Gas Chrom Q, 80/100 mesh; column temperature, 211°C; 95 ml/min (for SE-30), 130 ml/min (for NGS) or 120 ml/min (for QF-1) N_2 carrier gas; hydrogen flame detector. Steroid peaks: A, cholesterol; B, cholestanol; C, Δ^7-cholestenol; D, desmosterol; E, ergosterol; S, added cholestane as reference standard.

three times with 100 ml of toluene. The aqueous phase, adjusted to about pH 8 with 6N H_2SO_4 solution, was extracted three times with 300 ml of benzene. The combined benzene extracts were taken to dryness under reduced pressure at 40°C. The residue was dissolved in methanol-chloroform (1:4, v/v) for TLC using a solvent system of ethyl acetate-hexane (1:1, v/v). For spot visualization, the developed plates were sprayed with 50% H_2SO_4 solution and heated at 100°C for 30 min. Zones, where estrogenic steroids would be located if present, were scraped from preparative plates into a sintered-glass funnel and extracted three times with 15 ml of chloroform-methanol (1:1, v/v). After the solvent was evaporated under reduced pressure, the residue was dissolved in chloroform-methanol (4:1, v/v) and subjected to GLC analysis.

Proximate analyses

Moisture and lipid contents were determined by the methods outlined by Tu et al. (1967).

RESULTS & DISCUSSION

ACCORDING TO Menge et al. (1957), the unidentified growth factor in the unsaponifiable fraction of egg yolk was destroyed when a refluxing temperature was used for saponification. Unsaponifiable matter for this study was therefore prepared by cold (25°C) saponification to avoid any chemical alterations.

The average amount of unsaponifiable matter in two samples of liquid egg yolk (about 52% moisture and 35% lipid) was 1.92%. This value is close to that given by Pennock et al. (1962) who reported a value of 1.62% as unsaponifiable lipid in liquid egg yolk. Cholesterol is the major component of the yolk's unsaponifiable fraction. In this study the average cholesterol content of two samples was 1404 mg/100g wet yolk. Pihl (1952) reported a cholesterol content of 1560 mg/100g for yolk.

When analytical chromatoplates were developed with benzene-ethyl acetate (10:1, v/v), the unsaponifiable compounds were resolved into 7 distinct spots and one tailing spot (spot G) near the origin (Fig. 1). Each preparative TLC plate was divided into 9 zones (Fig. 1) prior to elution and GLC analysis. Spot positions of a variety of reference compounds have been included in Fig. 1 for aiding in the tentative identification of unsaponifiable compounds. The compound(s) in spot A at the solvent front was considered to be a hydrocarbon(s). Identical R_C value and purple spot color were obtained for spot A and squalene. The presence of some brown color in spot A suggests the occurrence of other hydrocarbons. Although squalene has been identified in biological tissues (Horlick and Avigan, 1963; Williams and Pearson, 1965; Channon, 1926), it has not been reported as a constituent of egg yolk. Squalene in the diet can be converted to cholesterol (Channon, 1926; Langdon and Bloch, 1953). Spot B with a red hue (dull blue under UV) was presumed initially to be a cholesterol esters which were not hydrolyzed during cold saponification. This spot was not visible in the chromatogram with unsaponifiables after hot saponification. After comparing the mobilities and colorations of spots C (light red color on Rhodamine 6 G TLC plate) and D with those of reference compounds, the major compounds in spots were considered to be ubiquinone (spot C) and lanosterol and dihydrolanosterol (spot D). Pennock et al. (1962) reported that egg yolk contained about 116 μg of ubiquinone-50. The R_C value and correlation of spot E were similar to those for Δ^7- and Δ^8-methostenol. The light red tailing from spot E on H_2SO_4-sprayed chromatoplate was considered to be β-sitosterol.

With a predominance of cholesterol in spot F, any other structurally-related

Table 1–GLC data from Fig. 4 of steroids in TLC zone 6 eluate for egg yolk.

		SE-30 RRT[a]			NGS RRT		QF-1 RRT		
Steroids	Peak no.	Experimental peak	Reference peak	Steroid no.	Experimental peak	Reference peak	Experimental peak	Reference peak	Average steroid content (μg/100g wet egg yolk)
Cholesterol	A	2.02	2.03	29.3	7.48	7.51	3.03	3.04	–
Δ^8-methostenol	B	2.53	2.56	30.0	7.89	7.86	3.44	3.45	small quantity
Δ^7-methostenol	C	2.75	2.75	30.3	9.55	9.52	3.78	3.80	713
4α-methyl-$\Delta^{8,24}$-cholestadiene-3β-ol	D	2.89	(2.84[b])	30.5	10.22	(10.30)	3.78	(3.75)	small quantity
4α-methyl-$\Delta^{7,24}$-cholestadiene-3β-ol	E	3.08	(3.05)	30.7	–	(12.50)	4.12	(4.12)	small quantity
Dihydrolanosterol	F	3.22	3.25	30.9	8.81	8.86	–	4.48	361
Lanosterol	G	3.56	3.58	31.2	11.58	11.62	4.82	4.85	1,550
β-sitosterol	H	3.56	3.58	31.2	12.70	12.60	4.82	4.88	3,326
4,4α-dimethyl-$\Delta^{7,24}$-cholestadiene-3β-ol	I	3.56	(3.59)	31.2	14.42	(14.40)	5.13	(5.07)	520

[a]Retention time relative to cholestane = 1.

[b]When a reference compound was not available, the relative retention time in parenthesis for the proposed structure was calculated as described in the text.

steroids would be completely masked. As shown in Figure 1, cholestanol, Δ^7-cholestenol, desmosterol and ergosterol have chromatographic mobilities similar to that of cholesterol. To determine the presence of these steroids, the cholesterol band on each preparative TLC plate was subdivided into two equal sections, 7i and 7ii. The eluate from each section was applied to preparative $AgNO_3$-silica gel chromatoplates which were subsequently developed with a chloroform-acetone (95:5, v/v) system. The analytical chromatogram of 7i and 7ii eluates are presented in Figure 2. In zone 7a of the $AgNO_3$ TLC plate sprayed with H_2SO_4, two distinct spots were obtained with the 7ii eluate but spots in the 7i chromatogram were only slightly visible. These spots had typical R_C values and coloration of cholestanol (light yellowish brown) and Δ^7-cholestenol (light pinkish brown). Although brown tailing was noted in zone 7b of each of the chromatograms, distinct spots especially for the 7i eluate were in the desmosterol and ergosterol locations. The compounds in zones 7a and 7b on preparative plates were eluted for GLC analysis.

The tailing spot G in Figure 1 was colored orange on dye-free, unsprayed silica gel plates. After H_2SO_4 spraying, the spot became light green within a few minutes at about 25°C. Upon heating the chromatogram plates at 110°C, the spot gradually became brown. Such color transformation is typical for carotenoids. No visible spots were present in the tailing spot on H_2SO_4-sprayed, heated chromatoplates. The compounds in spot H apparently have comparatively high polarities. Most of the common hormonal steroids, if present, would be located in zones 8 and 9.

An extraction method similar to that of Eleftheriou et al. (1966) was used to selectively concentrate estrogenic steroids from egg yolk. When the extract was examined by TLC, extensive tailing (light brown) with no distinct spots was noted on the H_2SO_4-sprayed plates. Common estrogenic steroids on chromatoplates generally turn orange with H_2SO_4 spraying and heating at 110°C. Hertelendy et al. (1965) were unable to detect estrogenic steroids in hen's yolk by TLC. In our study, eluate from assumed estrogenic steroids zones on preparative TLC plates was further subjected to GLC.

Eluates from each of the 9 zones on preparative TLC plates (Fig. 1) of egg yolk unsaponifiables were subjected to GLC with nonselective SE-30 as the liquid phase. The steroid numbers (SN) were calculated from the relative retention time (RRT) values with the SE-30 column to obtain a rough estimation of the number of carbon atoms in each peak compound and to aid in the identification of steroids. Figure 3 shows gas chromatograms of egg yolk unsaponifiables from zones 1, 2, 8, and 9. Zones 3, 4, and 5 have not been included since very minor peaks were obtained. In the case of zone 1 eluate, the chromatogram had two major peaks, A (SN 24.3) and B (SN 27.3), along with numerous minor peaks. Authentic squalene (C_{30}) had the same RRT value as peak B compound. The presence of squalene in egg yolk was further confirmed with NGS and QF-1 columns. The SN value of 29.4 for the major peak A in zone 2 chromatogram was lower than that of cholesteryl acetate (30.8) and apparently could not be cholesterol ester. None of the peak compounds in zones 3, 4, and 5 was identified. Although spot C in the TLC chromatogram (Fig. 1) was considered to be possibly ubiquinone, this compound could not be detected under our GLC conditions.

With zone 6 eluate, numerous peaks with SN values of 29.3 and above were found in the gas chromatogram (Fig. 4). The high SN values were indicative of cholestane derivatives. Analytical TLC (Fig. 1) also indicated that zone 6 consisted of methyl-steroids. The results of GLC analysis with SE-30 showed that nine steroids were possibly involved in zone 6. The RRT and SN values of yolk steroids in zone 6 and of reference compounds are tabulated in Table 1. The compound in peak A had RRT value of 2.02, almost identical to that of cholesterol. The peak B had a RRT value of 2.53 which is close to that for authentic Δ^8-methostenol. On the basis of the RRT value, the component in peak C was indistinguishable from Δ^7-methostenol. Comparisons of the RRT values revealed that 4α-methyl-$\Delta^{8,24}$-cholestadiene-3β-ol and its Δ^7-isomer were the components of peak D and E, respectively. These two steroids were not available in this study and their RRT values were derived by the method outlined by Clayton et al. (1963). The RRT value of 4α-methyl-$\Delta^{8,24}$-cholestadiene-3β-ol was calculated as 2.84 (RRT value of 2.56 for Δ^8-methostenol times a retention factor of 1.11 for Δ^{24}-bond) and for the Δ^7-isomer, the RRT value was 3.05 (RRT value of 2.75 for Δ^7-methostenol times a retention factor of 1.11). The RRT value of peak F was almost identical to that of dihydrolanosterol whereas the large peaks G, H, I could be a mixture of lanosterol, β-sitosterol and 4,4α-dimethyl-$\Delta^{7,24}$-cholestadiene-3β-ol due to the similar RRT values. The RRT value of 4,4α-dimethyl-cholestadiene-3β-ol was derived as the product of the RRT value (2.56) for $\Delta^{7,24}$-cholestadiene-3β-ol and a retention factor (1.406) for 4,4α-dimethyl group.

Table 2–GLC data from Fig. 5 of steroids in $AnNO_3$ TLC zone 7a and 7b eluates for egg yolk.

		SE-30			NGS		QF-1		Average
		RRT[a]			RRT		RRT		steroid
Steroids	Peak no.	Experimental peak	Reference peak	Steroid no.	Experimental peak	Reference peak	Experimental peak	Reference peak	content (μg/100g wet egg yolk)
Cholesterol	A	2.02	2.00	29.3	7.50	7.51	3.01	3.03	1,404,000
Cholestanol	B	2.05	2.05	29.4	7.07	7.06	3.28	3.31	4,870
Δ^7-cholestenol	C	2.29	2.27	29.8	8.85	8.85	3.48	3.48	3,170
Desmosterol	D	2.21	2.21	29.6	9.82	9.81	3.28	3.28	7,570
Ergosterol	E	2.64	2.58	30.1	11.16	11.38	3.94	3.78	3,670

[a]Retention time relative to cholestane = 1.

In order to confirm further the proposed structures for peak components in zone 6, the zone 6 eluate was subjected to GLC with two selective liquid phases, NGS and QF-1. As shown in Figure 4, gas chromatographic resolution of steroids was good when NGS was used. Such a pattern was not surprising since NGS has a selective retention effect for carbon-carbon unsaturation (VandenHeuvel and Horning, 1962). The RRT values of peak compounds separated on NGS and QF-1 are also presented in Table 1 along with the RRT values for compounds with assigned structures. Comparison of the RRT values with those standards confirm the presence of cholesterol, Δ^8- and Δ^7-methostenol, Δ^7- and Δ^7-isomers of dehydromethostenol, dihydrolanosterol, lanosterol, 4,4α-dimethyl-$\Delta^{7,24}$-cholestadiene-3β-ol, and β-sitosterol. A compound other than 4α-methyl-$\Delta^{8,24}$-cholestadiene-3β-ol may be present in peak D of the NGS chromatogram since peak D for SE-30 has a much smaller area.

When the eluate from zone 7 (Fig. 1) was examined by GLC, only one large, broad-spreading cholesterol peak was formed. Obviously, minor steroids would have been masked if they were present. Thus, the components in 7a and 7b zones of $AgNO_3$ chromatoplates (Fig. 2) were eluted for GLC analyses with all three liquid phases. The gas chromatograms are presented in Figure 5. The results indicate that cholesterol, cholestanol, Δ^7-cholestenol, demosterol and possibly ergosterol (experimental RRT values in NGS and QF-1 deviated slightly from those of ergosterol reference) were found to be present in this zone. The RRT values of sterol peaks are presented in Table 2 along with the RRT values for the corresponding steroid references. With SE-30 column, only a few minor chromatographic peaks were present for zone-8 eluate whereas three major peaks were noted in the zone 9 chromatogram (Fig. 3). The SN values, being 25.9 and below indicated that if the peak compounds were steroids, they would be androgen, pregnane and estrane derivatives. No common hormonal steroids were identified in these zones with GLC of three phases. In the case of the preparative TLC plates with NaOH extracts of estrogenic steroids from egg yolk, the zones where steroids would be present were eluted for GLC analysis. The chromatogram did not have any estrogen peaks.

The quantitative estimation of steroids found in zone 6 and 7 was carried out by GLC on a NGS column. The steroid contents in egg yolk are presented in Tables 1 and 2. Although cholesterol is by far the only major steroid, considerable amounts of minor steroids were also present in egg yolk.

Some non-hormonal steroids other than cholesterol have been found in yolk by previous investigators. Cholestanol and Δ^7-cholestenol were identified by Nakanishi et al. (1953). $\Delta^{3,5}$-cholestadiene-7-one and ergosterol were found to be constituents of yolk by Pennock et al. (1962). According to Boorman and Fisher (1966), β-sitosterol was found in eggs from hens given a diet supplemented with maize sterols, but eggs from hens not receiving dietary maize sterols contained no such sterol. Undoubtedly β-sitosterol found in the yolk used in our study was derived from the feed. Ground corn, being part of the diet of hens laying the eggs for our study, contains an abundant quantity of β-sitosterol (Kritchevsky and Tepper, 1961).

Since yolk has an estrogenic activity (Altmann and Hutt, 1938; Riboulleau, 1938; Marlow and Richert, 1940) and a growth-promoting effect (Denton et al., 1954; Menge et al., 1957), the presence of estrogenic steroids would be expected. However, our negative results confirm those of Hertelendy and Common (1965) who were unable to detect estrogenic steroids in yolk by TLC. Hertelendy and Common (1965) have suggested that estrogenic activity of yolk may be attributed to unstable estrogens or a number of estrogens at concentrations lower than detectable amounts.

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R. H. WALTER[a] and I. S. FAGERSON
Department of Food Science and Technology, University of Massachusetts, Amherst, Massachusetts 01002

RATES OF pH CHANGE AND DISAPPEARANCE OF GLUCOSE DURING LOW TEMPERATURE PYROLYSIS

SUMMARY–During low temperature pyrolysis (under 200°C), the disappearance of glucose and pH change of an aqueous solution of the pyrolyzate were found to approximate first order reactions. Since change of concentration of glucose and change of pH are directly related to time, the mathematical equations describing the two occurrences were combined through this common variable. The resulting equation describes the residual concentration of glucose as a function of change of pH.

INTRODUCTION

THE KINETICS of caramelization of invert sugar solutions have been described as autocatalytic at neutral pH and 95°C, and linear at alkaline pH and room temperature (Doss and Ghosh, 1949). Ramaiah et al. (1956) suggested the possibility that first and second order reactions jointly control the development of color in basic glucose solutions.

Song et al. (1966) explained kinetic data derived from studies on the Maillard reaction by proposing a mechanism whereby, throughout the induction period, major intermediates from glucose accumulate to a steady-state concentration. Thereafter, browning becomes a linear function of time.

Carbon monoxide, carbon dioxide and water evolving from catalyst-free glucose pyrolysis were found to be governed by first order reactions, independent of temperature. A zero order reaction preceded the first order reactions in cellulose decomposition (Greenwood, 1967).

Shafizadeh (1968) found that the carboxyl content of cotton linters heated in oxygen at 170°C increased linearly with time, and that during pyrolysis of cellulose in nitrogen at 288°C the rate of decomposition proceeded exponentially after a prolonged period of zero-order degradation.

At low pyrolysis temperatures, only trace amounts of volatile compounds are produced from glucose. The dominant reactions are chemical dehydration leading to unsaturation, anhydro-ring formation, and polymerization (Puddington, 1948; Sugisawa and Edo, 1966; Greenwood, 1967).

The production of acids in heated carbohydrate solutions is a well-known chemical occurrence, and the degree of acidity is a measure of the completeness of "burn" in the production of caramel (Peck, 1955). An acid medium catalyzes the formation of furan-like structures from glucose in solution (Singh et al., 1948). The natural acidity of certain foods is believed also to catalyze the degradation of naturally occurring sugars (Reynolds, 1965; Stadtman, 1948).

In this paper, observations on low temperature pyrolysis of glucose are presented, and a mathematical relationship is shown to exist between the change of pH and the reduction of concentration of glucose.

EXPERIMENTAL

The disappearance of glucose

Pairs of pre-heated (150°C) 150 × 16 mm test tubes, each containing 10 mg finely powdered anhydrous D-glucose (Baker analyzed reagent, dried for 48 hrs over P_2O_5; hereafter called glucose) and a thermometer were heated at 180 ± 1°C in a silicone oil bath for 15, 30, 60, and 90 min. After each heating period, the test tubes were immediately frozen at −10°C to arrest glucose decomposition. Undecomposed glucose in each test tube was silylated by the method of Sweeley et al. (1963), and 0.1 μl samples of the clear supernatant were gas chromatographed. The peak areas corresponding to the silyl ethers for each heating interval were measured and totaled. The average total from each pair of test tubes was plotted as a decimal fraction of unity (unheated glucose). This fraction was taken as the glucose concentration (C).

Change of pH versus temperature

Pairs of pre-heated test tubes containing 1 g samples of glucose were heated for 5 min, at increments of 10 degrees, from 100°C. After heating and freezing, 20 ml distilled water was added to each test tube, the solids dissolved with the aid of a Vortex mixer, and the pH of each solution measured with a Leeds & Northrup Model 7401 pH indicator. The pH of a similar, unheated solution (pH_o) was also measured. The average reading of each pair of test tubes (pH_t) was used to plot change of pH ($dpH = pH_o - pH_t$) as a function of temperature.

Change of pH versus time

One-gram samples of glucose were heated as previously described at 140 ± 1°C, for 1, 2, 3, and 5 min, and thereafter, for increments of 5 min, to 30 minutes. These were subsequently frozen, dissolved in distilled water, and the pH measured. The average reading of each pair of test tubes was plotted as dpH versus time

RESULTS & DISCUSSION

IN CONTRAST with withdrawals from a batch process, the method of separately heating small samples of glucose in pre-heated test tubes reduced the possibility of a pseudo-induction period due to slow heat transfer (Greenwood, 1967).

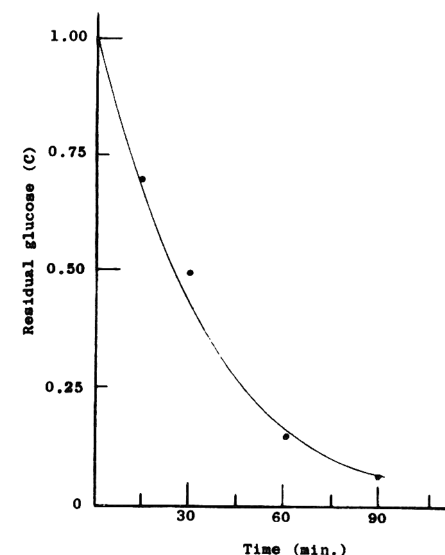

Fig. 1–Residual glucose as a function of heating time at 180°C.

[a]Present address: International Flavors & Fragrances, Inc., Union Beach, New Jersey 07735

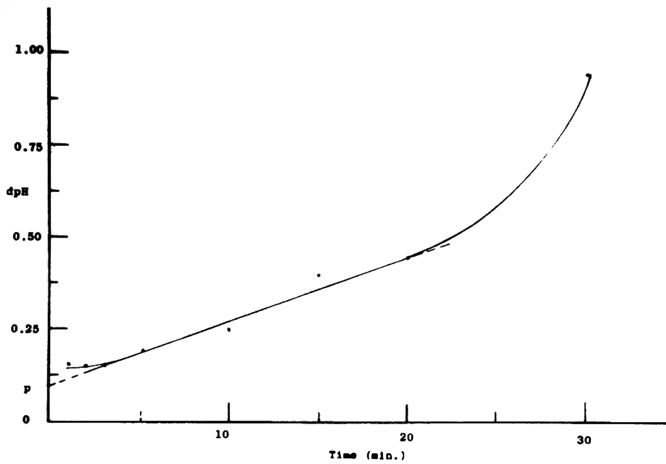

Fig. 3–Change of pH (dpH) as a function of heating time at 140°C ($dpH = 6.35 - pH_t$).

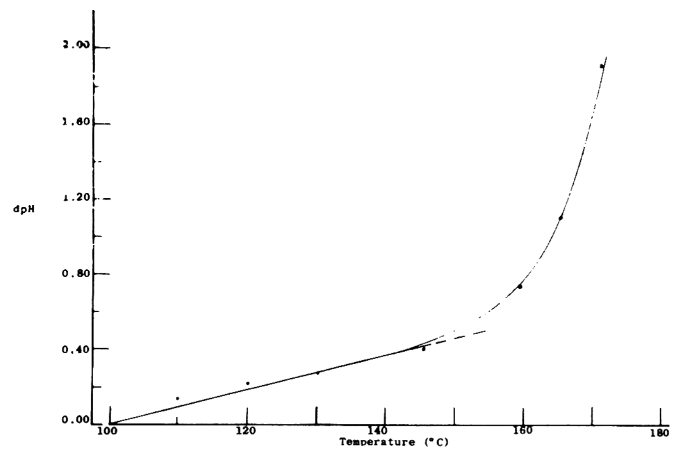

Fig. 2–Change of pH (dpH) as a function of temperature, for a heating period of 5 min ($dpH = 6.55 - pH_t$).

The disappearance of glucose

Figure 1 resembles an exponential plot of the general form $C_t = C_0e^{-kt}$, where, in this experiment, C_t is the concentration of glucose at time t; C_0 is the concentration at t = 0, and k is the rate constant. The relative constancy of k calculated at different points on the curve (Table 1) indicates that the disappearance of glucose above the melting point is indeed, an exponential function of time. The linear equation describing this function is written:

$$\log C_0 - \log C_t = \frac{k}{2.30} t \qquad (1)$$

Change of pH versus temperature

Figure 2 indicates the importance of rigid temperature control at temperatures above 150°C since small temperature variations result in large pH differences in this region.

Change of pH versus time

From the data of the dpH versus temperature experiment, 140°C was chosen for this experiment. Figure 3 illustrates a rapid drop in pH (hence at t = 0, dpH $\neq$ 0). During the period 1–5 min, there was no appreciable change in the level of acidity. Subsequently, there was a constant rate of acidification during the period 5–20 min, until finally, after 20 min, the rate of production of hydrogen ions advanced to a higher order of reaction, possibly as a result of secondary effects involving decomposition products.

The upper segment of the curve (not shown) approached a maximum limit as dpH increased beyond 2.0.

The observation that the straight line segment of Figure 3 does not extrapolate to zero is similar to observations on starch in which water of hydration allegedly interfered with the kinetics of degradation (Greenwood, 1967).

Since by definition, pH = $-\log C(H^+)$, in equation (1), in terms of pH, $\log C_0 = -pH_0$ and $\log C_t = -pH_t$. Equation (1) may therefore be written:

$$-dpH = \frac{k'}{2.30} t + p \qquad (2)$$

This modified form describes the straight line segment of Figure 3 in which p is the intercept, and the negative sign denotes decreasing pH (increasing acidity).

By substituting in (1) the expression for t derived from (2), the following equation is obtained:

$$\log C_0 - \log C_t = -K(dpH + p) \qquad (3)$$

K is the ratio of the slope of (1) to the slope of (2). When $C_0 = 1$, (3) may be written:

$$\log C = K(dpH + p) \qquad (4)$$

The provision that $K < 0$ (Taylor, 1960) is satisfied by the negative slope of (1).

Equation (4) is valid at any temperature at which there is linearity between log C and t, and dpH and t. This equation should make it possible to follow quantitatively the course of glucose conversion during pyrolysis, by pH measurements, once k and k′ have been predetermined under identical conditions.

Equation (4) has the advantage of non-dependence on absolute measurements of concentration, and the ease of determining pH differences.

Table 1–Rate constants (k) for residual concentration of glucose (C) versus time (t).

t (Min.)	C	$k \times 10^{-2}$ (Min. $^{-1}$)
7.5	0.83	2.6
15	0.68	2.6
30	0.44	2.8
45	0.28	2.8
60	0.18	2.8
75	0.11	2.9
90	0.072	2.9

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A. CZERKASKYJ
Horticultural Research Station, Tatura, Victorian Department of Agriculture, Australia, Postcode 3616

PINK DISCOLORATION IN CANNED WILLIAMS' BON CHRETIEN PEARS

SUMMARY—Pink discoloration in canned Williams' Bon Chretien pears is an occasional problem to the canning industry in the Goulburn Valley, Victoria, Australia. During 1963–1965, the relationship between the skin blush on fresh fruit and pink discoloration after processing, effect of the level of applied nitrogenous fertilizer, and the processing time were studied. There is a high positive correlation between the intensity of skin blush on the fresh fruit and pink discoloration of the fruit after processing. Only blushed fruits were prone to pink discoloration after processing. Blushed fruit from trees which had heavier application of nitrogen produced more intense pinking, but short overcooking showed no effect on pinking.

INTRODUCTION

THE MAIN PEAR variety used for canning in Australia is the Williams' Bon Chretien (Williams), known as Duchess in South Australia and Bartlett in the U.S.A. The Goulburn valley region of Victoria, with well over a million trees, is the main center for its production in Australia.

The canned product is normally white to slightly creamy. However, pears grown in the Goulburn Valley occasionally develop a pink discoloration ("pinking") of the flesh when canned. Although pink fruit retains a true flavor, it is undesirable commercially because of its effect on the appearance of the main content of the can.

Joslyn (1941), Joslyn and Peterson (1956), Chandler et al. (1959), (unpublished), Luh et al. (1960), van der Merwe (1963), and Nortjé (1964), associated the pink discoloration in canned Bartlett pears with the leucoanthocyanin content in the fresh fruit. These research workers indicated that accumulation of leucoanthocyanins in Bartlett fruit depended on a complex of environmental conditions, and was highly associated with low pH, high titratable acidity and a high content of soluble solids in the fresh fruit. These colorless compounds turn red, similar to cyanidin when it is heated. Excessive heating and delayed cooling after cooking were considered to be the processing factors associated with pinking in canned pears. These workers concluded that one way to prevent pink color development in canned Bartlett pears, is to check fresh fruit for these properties before canning; to remove fruit which is susceptible to pinking from the processing line; and to watch the canning operation closely. However, at the same time it was found that fruits differ in composition, not only between trees grown in different environmental conditions (Luh et al., 1960), but also between trees in the same orchard (Merwe, 1963) or even from the same tree (Nortjé, 1964). In a previous experiment with Williams' pears (unpublished), the author found that soluble solids and titratable acidity values are affected by level of applied nitrogenous fertilizer. Thus, laboratory tests on fruit for soluble solids, titratable acidity and pH could be helpful in locating regions or orchards producing fruit potentially more susceptible to pinking, but would be difficult to use to locate such fruit within the orchard. It would be desirable to define other more readily recognizable characteristics of pears having a high pinking potential which would make field identification possible.

In most crops of Williams' pears, it is usual to find a small proportion with a splash of red blushed skin, contrasting sharply with the deep green ground color. The red blush is usually confined to one half of the fruit, and on fruits exposed to direct sunlight. It was thought that this skin blush could be related to subsequent pinking in the canned product—an aspect apparently not considered by previous workers. This paper reports a series of experiments between 1963 and 1965 which investigated the association between the presence and severity of skin blush (usually only on one side) and the occurrence of pinking after processing. Effects of different levels of nitrogenous fertilizer and of processing times on fruit in relation to skin blush intensity were also studied.

MATERIALS & METHODS

Fruit samples

The fruits used for these investigations were grown on heavy soil (Goulburn clay loam) under irrigation at the Horticultural Research Station, Tatura. Fruit samples were picked from randomly selected trees during normal commercial harvest. The fresh fruit was selected within size range 6.4–7.0 cm diameter, and classified according to intensity of the red skin blush:

Blush class
- A = fruit without blush (control)
- B = fruit with very light blush (trace of red pigment)
- C = fruit with light blush
- D = fruit with medium blush
- E = fruit with dense blush
- F = fruit with very dense blush

Selected fruit samples were stored at 0°C for 7 days, then ripened at 20° ± 1°C and 85% ± 5% relative humidity until the same firmness range 1.10 ± 0.25 kg was reached (Ballauf penetrometer with 7.9 mm diameter plunger).

Treatments

Blush-pinking relationship. Blushed fruits were cut in half to separate the unblushed and blushed halves, giving two samples per group: B1, C1, D1, E1 and F1 comprised the unblushed halves, and B2, C2, D2, E2 and F2 the blushed halves. Both halves of unblushed fruit (A) were mixed and canned together.

In 1963, comparisons were limited to B2, E1, E2 and A, there being four replications of these treatments. In the following years the relationship of fruit skin blush was coupled with other factors suspected of contributing to pinking.

Nitrogenous fertilizer level. In 1964, fruit was sampled from trees under three different nitrogenous fertilizer levels to study the effect of nitrogen on the incidence and degree of pink discoloration in the canned product. Fruit halves B2, E1, E2 and A were considered, with

Table 1—Chemical composition of fresh and canned samples—1965

		Soluble Solids %		Titratable Acidity %		pH	
Fresh Fruit	Canned Sample[1]	Ripe Fruit	Canned Fruit	Ripe Fruit	Canned Fruit	Ripe Fruit	Canned Fruit
Unblushed (control)	A	13.8	19.2	0.34	0.19	4.40	4.36
Light blush	C1	15.1	19.9	0.31	0.19	4.45	4.31
	C2	15.3	20.0	0.30	0.18	4.45	4.34
Medium blush	D1	15.1	19.8	0.31	0.19	4.40	4.34
	D2	15.6	20.2	0.28	0.18	4.45	4.36
Dense blush	E1	16.1	20.2	0.26	0.19	4.55	4.31
	E2	16.7	20.7	0.24	0.18	4.60	4.34
L.S.D. (P = 0.05)		0.18	N.S.	0.01	N.S.	N.S.	N.S.
(P = 0.01)		0.25		0.02			

[1] C1 D1 E1 = Unblushed halves of blushed fruit; C2 D2 E2 = blushed halves.

3 replications of these 12 treatments.

The fertilizer levels, 2.25, 4.50 and 9.00 kg (N2, N4, N9) of ammonium sulphate per tree per year, had been applied for the previous 12 years.

Processing time. In 1965, fruit halves C1, C2, D1, D2, E1, E2 and A were compared over three cooking times, there being four replications of these treatments. All samples were precooked (exhausted) for 18 min at 92° ± 0.5°C. The cans were then sealed and cooked at 100°C for 10, 15 or 20 min. Normally 15 min cooking resulted in ideal firmness and flavor.

Physical and chemical tests

10 fruits per plot were selected at random for tests of fruit firmness, soluble solids, titratable acidity and pH in both green and ripe fruit before canning. 1 can per plot was kept to assess these qualities in canned fruit. Methods described by Leonard et al. (1954) were used.

Canning

The fruit was peeled and cored by hand, and canned in No. 2½ cans with 20% (1964) or 30% (1963, 1965) sucrose syrups. Fruit during preparation was held immersed in tap water to minimize enzymic browning, and spray rinsed strongly before being placed into cans. The 524.5 ± 3.0g of halved fruit (7–8 halves) were packed in each can, then filled with 326.0 ± 3.0g of hot syrup.

In each year, fruit samples were precooked for 18 min at 92° ± 0.5°C. The cans were then sealed and the fruit cooked in boiling water in a stationary cooker (at 100°C) for a set time, cooled rapidly in a cold water bath for 10 min, and stored at ambient temperature.

Visual assessment of pinking color

After 6 months' storage at ambient temperature, the incidence and the degree of pinkness in each can were assessed visually by a panel of 3 persons of "superior color discrimination" as determined by the Farnsworth-Munsell 100 Hue Test for color vision. Fruit was assessed under constant lighting conditions.

The value of pink color was expressed as: no pink = 0; very slight pink = 1; slight pink = 2; medium pink = 3; pronounced pink = 4; and very pink = 5. Very slight pink discoloration (1) would not be noticed by the consumer; slight pink color (2) could be considered as being barely noticeable; and medium pink (3) was easily noticeable.

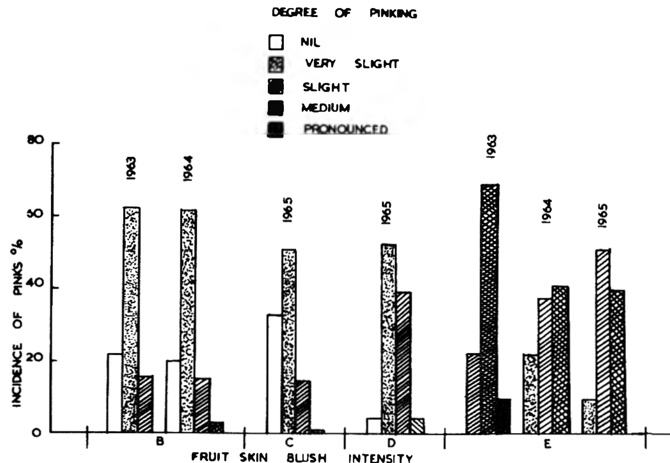

Fig. 1–The incidence and degree of pink discoloration in canned blushed halves of Williams' pears 1963–1965.

The degree of pinkness given for each treatment was the mean value for pink halves only.

Test for pink color precurser

In a separate test (1965) the relative concentrations of pink color precurser in both blushed and unblushed halves of the same fruit over a range of blush severity, were determined. The method of Luh et al. (1960) modified for fresh fruit was used.

Two uniform fruit of each blush group (A to F) were halved longitudinally into blushed and unblushed samples, and one core of tissue (2 cm dia × 1 cm long) taken from the equatorial flesh of each half was chopped finely with a knife. Duplicate 10g samples were taken into 150 ml beakers, and extracted with 1.5 ml of NaCl, 2 ml of 6N HCl, and 50 ml of n-butanol, with constant stirring for 15 min. The supernatant was poured off into a test tube and heated in a boiling water bath for 15 min, after which it was cooled rapidly to 20°C ± 1°C and centrifuged for 30 min at 5,000 R.P.M. The EEL large tubes (16 mm dia, 8 ml capacity) were filled up, and the light absorbance at 520 nm (green filter No. 624) measured by an EEL colorimeter Model A. Distilled water was used to adjust the zero reading of the instrument. No attempt was made to convert the EEL panel reading into absolute units (mg pigment) because suitable calibration standards were not available at that time. Soluble solids, titratable acidity and pH, were determined for each sample. The remains of each fruit sample were cooked in glass beakers with distilled water for 33 min at 100°C so the development of pink color in fruit could be observed.

RESULTS

AS THE UNBLUSHED fruit, A, did not produce any pink halves in the can under any conditions, it was not included in statistical analyses.

A general picture of fresh and canned fruit composition in relation to skin blush is given in Table 1. It shows that unblushed fresh fruit (A) has significantly lower soluble solids and higher titratable acidity than blushed fruit. Moreover, the unblushed halves have significantly lower soluble solids and higher titratable acidity than blushed halves of the same fruit. These trends increase with blush severity. However, the differences were no longer significant after processing.

There was no difference in color of the flesh between the unblushed and blushed fruit before cooking.

Table 2–Effect of blush intensity on incidence and degree of pinking in canned Williams' pears–1963–1965.

Blush Intensity	Canned half[1]	Incidence of pink halves %			Degree of pinkness (score 1-5)		
		1963	1964	1965	1963	1964	1965
Unblushed	A	0	0	0	–	–	–
Very light	B1	–	–	–	–	–	–
	B2	78.0	80.0	–	1.20	1.25	–
Light	C1	–	–	41.8	–	–	1.00
	C2	–	–	67.0	–	–	1.22
Medium	D1	–	–	68.5	–	–	1.20
	D2	–	–	94.0	–	–	1.49
Dense	E1	100.0	96.0	95.6[2]	1.63	1.76	1.86
	E2	100.0	100.0	100.0[2]	2.88	2.15	2.31
L.S.D. (P = 0.05)		–	–	10.9	0.22	0.40	0.24
(P = 0.01)		–	–	14.7	0.34	0.55	0.33

[1]B1, C1, D1, E1 = Unblushed halves of blushed fruit; B2, C2, D2, E2 = Blushed halves.
[2]Excluded from statistical analysis.

Blush intensity and pink discoloration

Data on the relationship between skin blush and pinking for the 3 yr are shown in Table 2 and Figure 1. The unblushed fruit (A) did not produce any pink discolored halves after processing.

Only blushed fruit was prone to pinking and the blushed half of any single fruit was more susceptible than the unblushed half. The more intense the blush, the greater the degree of pinkness and the greater the percentage of pink halves in the sample. (See also Table 4.)

Effect of nitrogenous fertilizer

The amount of nitrogenous fertilizer applied had no effect on the incidence of pink pears in the total. However, the degree of pinkness in the affected fruit

Table 3–Effect of nitrogenous fertilizer level on incidence and degree of pinking in canned Williams' pears–1964

Nitrogen fertilizer (applied) kg/tree	Incidence of pink halves[1] %	Degree of pinkness[1] (score 1–5)
2.25	67.7	1.20
4.50	69.3	1.81
9.00	69.6	2.14
L.S.D. (P = 0.05)	–	0.40
(P = 0.01)	–	0.55

[1] Data presented and analyzed from B2, E1 and E2 samples only; no interaction between blush intensity and nitrogen level.

increased significantly ($P < 0.01$) for the fruit from the more heavily fertilized trees (Table 3). Fruit from N2 treated trees was only slightly discolored (1.20) whereas from N9 it was in the medium pink range (2.14).

Effect of processing time

The final cooking of Williams' pears for 10, 15 or 20 min after can sealing did not show any differences between the treatments, either in the incidence or degree of pink discoloration of halves in the can (Table 4), except the 10 min cook–unblushed halves. The relationship between fruit blush severity and pink discoloration in canned product was evident within each treatment.

Pink color precursor

The acidified n–butanol extract of fresh fruit, after heating for 15 min in a boiling water bath, developed a distinct pink color. The faint pink color increased sharply in intensity from the unblushed fruit (A) to the very slightly blushed fruit (B), and then it increased gradually in intensity, until it became dark red for fruit F (Fig. 2). The color intensity of the extract was highly correlated with the blush intensity on the fruit ($r = +0.98$, Fig. 2), and with soluble solids ($r = +0.97$, Fig. 3), titratable acidity ($r = -0.96$, Fig. 4), and pH ($r = +0.94$, Fig. 5) of the fresh fruit.

The intensity of pink color developed in the remains of the fruit samples cooked for 33 min in distilled water, was of the same pattern as in n-butanol extract for blushed fruit, except for control (A) which did not turn pink. Also the intensity of pink color developed in canned blushed fruit followed a similar pattern (Fig. 1).

DISCUSSION

ONLY PEARS with a red skin blush were susceptible to pink flesh discoloration after processing, and the incidence and degree of pinkness were highly associated with the blush intensity. It was evident, when the pinking was very light, that this did not detract much from the general appearance or acceptibility of the fruit, as it would be impossible for the consumer to detect such a single half when served in the normal way. On the other hand, while a very slight pinkness of every fruit half would be of no great worry, the presence of even one fruit piece showing a medium or stronger pinkness would spoil the general appearance of the lot markedly. Thus a small proportion of fruit with high pinking potential mixed with normal fruit could cause great damage to the appearance of the canned product. This could come only from the fruit with more intense skin blush (Fig. 1). Our investigation in 1965 on the skin blush development of Williams' pears, showed that 30% of fruits were blushed, but only 4% had a medium or more intense blush (unpublished data). Since the pinking data showed that only medium and higher blushed fruit should be considered as fruit with higher pinking potential (Fig. 1), incidence of pink fruit could be minimized or avoided by removing all medium and higher blushed fruit from the processing line. Such rejected fruit, of good aromatic flavor, could be used for cocktails, canned with other mixed fruits, canned separately as a "specials" pink pears, or used for the fresh fruit market.

The data on acidified n-butanol fruit extracts (Fig. 2) showed a high correlation ($r = +0.98$) between the fruit skin blush and pink color development in the extract as measured by light absorption in an EEL colorimeter, indicating that the amount of pink color precursor in fruit increased with the blush intensity. At the same time, it was shown that pinking in canned pears was associated with skin blush on fresh fruit–the denser the blush the higher the pinking. Luh et al. (1960), van der Merwe (1963) and Nortjé (1964) in their investigations found that pink discoloration in canned Bartlett pears was highly correlated to leucoanthocyanin content of the fresh fruit, which in turn was correlated with soluble solids and acidity level of such fruit. Therefore, it could be concluded that red skin blush on Williams' pears grown in Goulburn Valley, is an indicator of leucoanthocyanin content in the fruit.

There was a high correlation ($r = +0.98$) between the soluble solids content and the fruit blush development. Table 1 shows that unblushed fruit (A) had the lowest content of soluble solids (13.8%), whereas fruit even with light red skin pigment (C) showed 15.1%, increasing to

Table 4–Effect of cooking time on the incidence and degree of pinking in canned Williams' pears–1965.

Fruit half	Cooking time[1] (min.)	Incidence of pink halves % in fruit of light + medium blush[2]	Incidence of pink halves % in fruit of dense blush	Degree of pinkness (score 1–5) in fruit of light blush	Degree of pinkness (score 1–5) in fruit of medium + dense blush[2]
Unblushed	10	37.9	94.5	1.00	1.57
(C1, D1, E1)	15	63.1	95.5	1.00	1.69
	20	64.6	96.9	1.00	1.33
Blushed	10	77.2	100.0	1.20	1.94
(C2, D2, E2)	15	84.8	100.0	1.30	1.88
	20	79.4	100.0	1.16	1.88
L.S.D. (P = 0.05)		13.3	–	–	0.30
(P = 0.01)		17.9	–	–	0.40

[1] Fruit was precooked at 92°C for 18 min before can sealing.
[2] No interaction between cooking time and blush intensity.

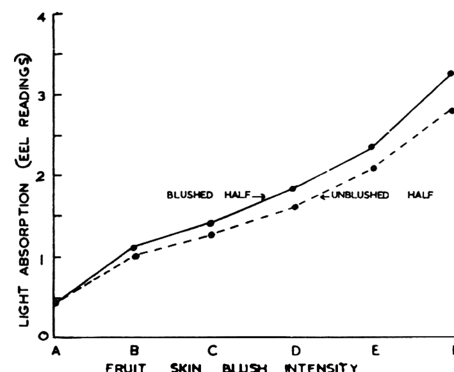

Fig. 2–The relationship between development of pink color in raw fruit extract and intensity of blush of the fruit, 1965.

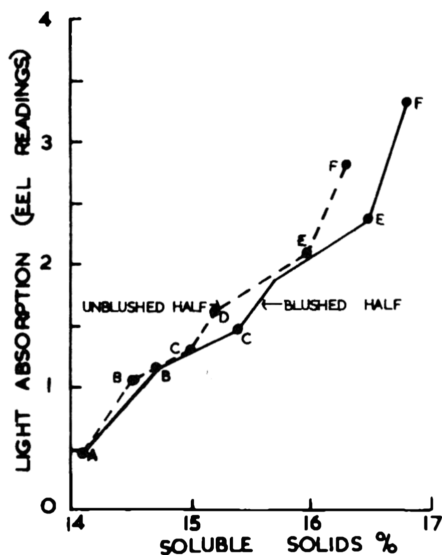

Fig. 3–Relationship between development of pink color in acidified n-butanol raw fruit extract and soluble solids of raw Williams' pears–1965. (A, B, C, D, E, F = fruit blush intensity).

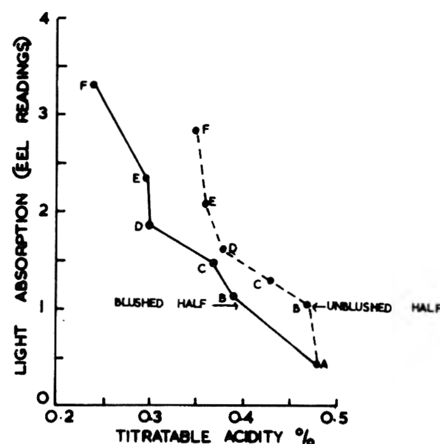

Fig. 4–Relationship between development of pink color in acidified n-butanol raw fruit extract and titratable acidity of raw Williams' pears–1965. (A, B, C, D, E, F = fruit blush intensity)

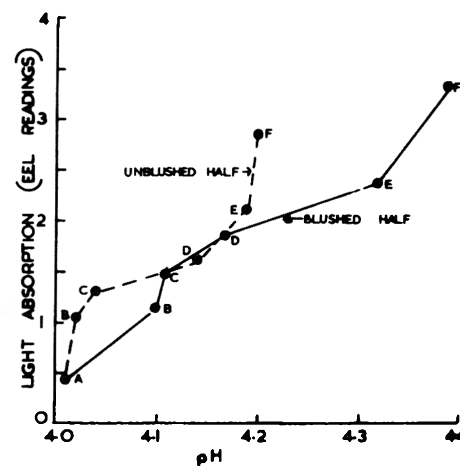

Fig. 5–Relationship between development of pink color in acidified n-butanol raw fruit extract and pH value of raw Williams' pears–1965. (A, B, C, D, E, F = fruit blush intensity)

16.3% in more blushed fruit (E). A significant decrease in titratable acidity in the fruit with more intense blush was evident (Table 1).

While the positive correlation between pink discoloration in canned fruit, (or optical density of acid n-butanol fruit extract) and the soluble solids content in fresh fruit (Fig. 3) was in full agreement with the result of van der Merwe (1963) for Bartlett pears, the apparent negative correlation with titratable acidity (Fig. 4) and positive correlation with pH (Fig. 5) were of reverse pattern to the data of Luh et al. (1960) and van der Merwe (1963). The reason for this is not known. As all fruits for this experiment were grown under the same field conditions and were selected for uniformity of size and ripeness, fruit maturity or environment were not contributing factors.

The fact that acidified n-butanol fruit extract of unblushed Williams' pear (A) turned to a faint pink color after heating for 15 min (Fig. 2), while the same fruit cooked in distilled water or similar pears canned in the normal way did not produce any discolored pink fruit (Table 2), suggested that probably only a small part of the total leucoanthocyanins present in the fruit turns pink when heated during normal fruit processing, or the amount present in unblushed fruit is too small and too diluted to have any visible effect under these conditions.

Luh et al. (1960) and van der Merwe (1963) reported the effect of fruit processing time as a factor inducing pink color development in canned Bartlett pears. They found that prolonged cooking times produced pink fruit. Presumably the processing time (35–40 min at 100°C) in their experiment was much longer than that involved in commercial processing. In our experiment, blushed fruit was pink after 10 min at 100°C (considered undercooked), whereas unblushed fruit did not discolor even after 20 min when it was considered to be 5 min overcooked. These results suggest that the cannery practice of increasing cooking time by up to 5 min for harder fruit is not likely to induce pinking in unblushed fruit, or increase the incidence or degree of pink discoloration in canned blushed Williams' pears, provided they are cooled immediately after processing.

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SUDHAKAR SHANBHAG, M. P. STEINBERG and A. I. NELSON
Department of Food Science, University of Illinois, Urbana

BOUND WATER DEFINED AND DETERMINED AT CONSTANT TEMPERATURE BY WIDE-LINE NMR

SUMMARY—The purpose of this work was to study the NMR properties of bound water (BW) in wheat flour, corn starch and egg white at room temperature and to develop a method for quantitative determination of BW in a material and its maximum BW capacity. Each sample was studied over the 5 to 85% moisture range, using a wide-line NMR Spectrometer. At the lowest radiofrequency attenuation, hydrogen nuclei from free water gave a negligible signal but the hydrogen from BW gave a strong signal, giving rise to a new definition for BW and a new method for its quantitative determination, both at room temperature. Within a given set of instrument parameters, the hydrogen nuclei from BW showed NMR properties independent of dry matter. Therefore, a "universal" NMR calibration constant per unit weight BW could be derived for these food products. The weight of bound water per unit weight of dry matter increased with increasing total moisture content to a maximum, called Bound Water Capacity, which was independent of total moisture content but varied with the product.

INTRODUCTION

THE WATER in a material can be categorized into two portions known as free and bound. This concept is of crucial importance to many food problems such as dehydration, microbial spoilage, chemical degradation and texture. Bound Water (BW) is that which shows physical properties different than those of water by itself, e.g., lower freezing point, higher boiling point, lower vapor pressure and higher density (Kuprianoff, 1958; and Gur Arieh et al., 1967). One of the best definitions of BW is water that does not freeze (Meryman, 1966).

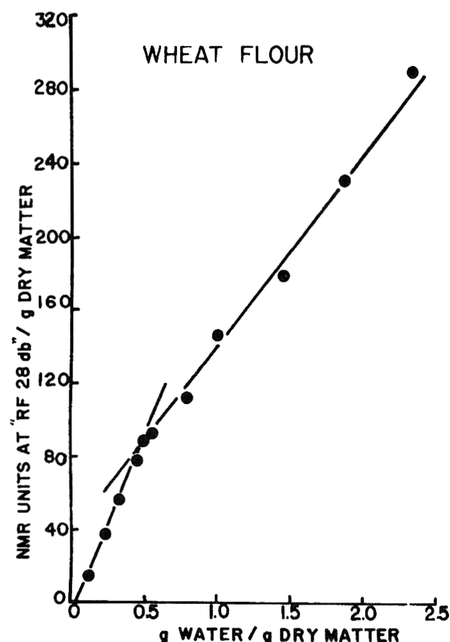

Fig. 1—Calibration curve showing the moisture content of wheat flour as measured by a wide-line NMR spectrometer.

Kuprianoff (1958) concluded that the most reliable determination of BW is to measure the water that persists in the liquid phase at sub-freezing temperatures. This can be done by calorimetric determination of the ice formed; BW is total water minus the ice. Using this method Mannheim et al. (1957) showed that 35% of the water in bread remains unfrozen at 0°F. This method is indirect, its accuracy leaves much to be desired, and it requires much time and labor for a single determination.

Toledo et al. (1968) applied the freezing technique using a PA-7 Process Analyzer with high attenuation (RF of 28 db) which measures liquid water but not ice. They cooled the sample from room temperature to −60°F and determined BW directly at 0°F. Thus, the results were based on a specific sub-freezing temperature and were therefore empirical. The determination may be affected by the presence of salts and sugars which depress the freezing point. Also, binding of water may be temperature dependent. In most cases we wish to know water binding at room temperature rather than at 0°F.

Therefore, it was desired to develop a technique using wide-line NMR to determine BW at the temperature of interest and the maximum capacity of a given material to bind water.

EXPERIMENTAL

THE WIDE-LINE NMR Spectrometer used for this work was a Model PA-7 Process Analyzer (Varian Associates, Inc., Palo Alto, California) equipped with an integrator (V-4221), a variable temperature controller (V-4540) and a 2.5 ml variable temperature probe.

Commercial samples of wheat (cake) flour, corn starch, and dehydrated egg white were equilibrated to desired moisture contents by placing at 72°F for 24 hr in desiccators containing sulfuric acid solutions giving the correct relative humidity. Samples with moisture contents higher than obtained by holding over water (i.e., moisture content of above 19% for flour, 20% for starch and 26% for egg white) were prepared by adding the calculated amount of water and mixing thoroughly.

Moisture content of samples under 30% was determined by the AOAC (1955) vacuum oven method. For samples over 30%, the moisture content was calculated from the amount of water added to the low moisture material.

About 0.5 g sample was added to a tared 2.5 ml sample tube; sample weight was determined to 0.1 mg and dialed into the integrator. The sample tube was inserted into the magnet and "swept" at the desired radiofrequency attenuation expressed in decibels (db). This could be

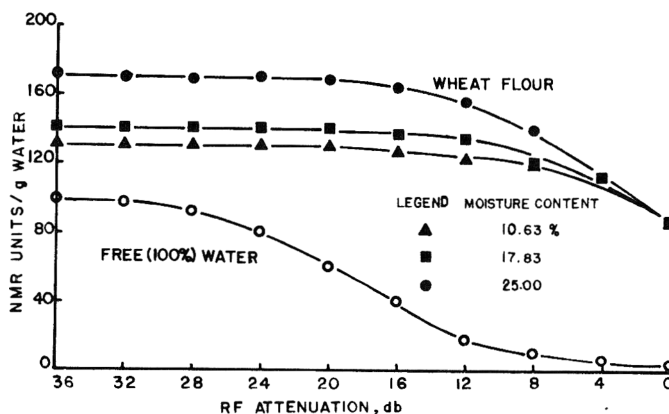

Fig. 2—Effect of radiofrequency attenuation on the NMR signal from water in wheat flour containing only bound water. Curve for distilled water shown for comparison.

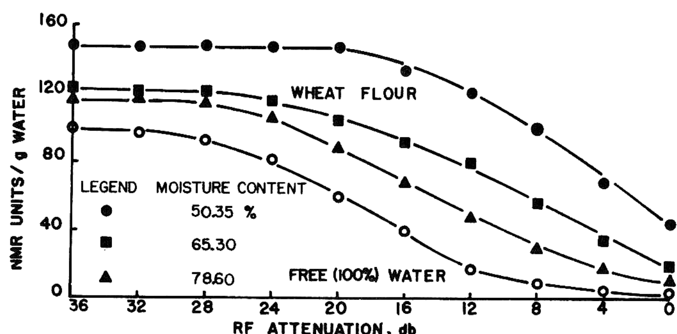

Fig. 3–Effect of radiofrequency attenuation on the NMR signal from water in wheat flour containing both bound and free water. Curve for distilled water shown for comparison.

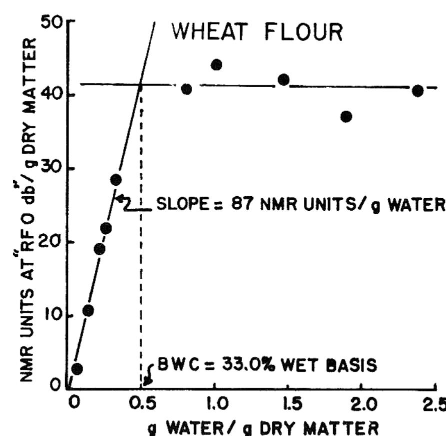

Fig. 4–Relation between NMR signal at lowest attenuation and moisture content, both on a dry matter basis, for wheat flour.

adjusted at 4 db intervals from 0–60 db. All the measurements were made at 72°F.

Instrument parameters used for all observations were:

TC–0.5 sec	SEN–200	SM–1
ST–0.5 min	MOD–0.5 Gauss	RM–20
SA–2 Gauss	TH–0.1 mv	

Integrator Readout–1000 equals 1.0 g

At each db level the instrument recorded a single value, NMR units per g of sample. This value was converted to NMR units per g water or per g dry matter by means of the experimentally determined moisture content of the sample.

Statistical analyses of the data, including regression line, coefficient of determination and confidence interval for the calibration constant, were performed according to Steel and Torrie (1960) using the 5% level of significance.

RESULTS & DISCUSSION

MEASUREMENT of the total water in a food product with the PA-7 Wide-Line NMR instrument is usually done at RF 28 db. This attenuation is chosen because at any level above this we do not obtain a straight line relation between moisture content and NMR reading while at levels below this the sensitivity decreases because the amount of signal obtained per g water is smaller.

Figure 1 shows the signal obtained in NMR units at RF 28 db per g dry matter (DM) plotted against g water per g DM for wheat flour. A broken calibration curve with the break at about 0.5 g water per g DM was found. The significance of this critical moisture content will be elucidated below. Evidently this is the type of curve alluded to by Rockland (1969).

NMR signals per g of free (distilled) water obtained by measurement at different RF attenuations from 36–0 db are shown by the bottom curves on Figures 2 and 3. As the attenuation decreased, i.e., RF level increased, NMR signal per g water generally decreased. The NMR signal was only 0.5 units/g at 0 db as compared to 92 units/g at RF 28 db. Ice gave no signal at any attenuation.

Similar RF attenuation experiments were performed with wheat flour samples at moisture contents of 10–78% moisture. The data were grouped into two families of curves. One family, shown at the top of Figure 2, represented flour samples at 25% moisture and below. It showed an increasing signal with increasing moisture at the high attenuation levels with convergence to a common point at RF 0 db. The ordinate for this common point was 87 NMR units per g water. In contrast, free water gave a signal of only 0.5 NMR units per g (Fig. 2 and 3).

The second family of curves, shown at the top of Figure 3, showed decreasing signal with increasing moisture from 50–78% with a general parallelism at all RF levels. Similar curves, not shown, were obtained at 36 and 70% moisture.

When all the NMR data for wheat flour at RF 0 db obtained from the instrument on a g sample basis were converted to a DM basis and plotted against moisture content of each sample, also on a DM basis, Figure 4 was obtained. This shows two straight lines intersecting at slightly below 0.5 g water per g DM, the same point as the intersection in Figure 1. As the moisture content was increased above zero, the signal at RF 0 db increased linearly with a slope of 87 units per g water until the critical moisture content (33.0% wet basis) was reached and thereafter remained essentially constant.

Calculation of the same data in terms of total sample instead of DM for both parameters and plotting signal against moisture content gave Figure 5. As the moisture content was increased above zero, the signal increased linearly, again with a slope of 87 units per g water, to a maximum (again at 33.0% moisture wet basis) and then decreased linearly to a negligible signal for 100% water.

Corn starch and egg white were tested in the same way and the data from each gave a series of plots similar to that shown for flour. The data are summarized in Table 1. The slopes of the ascending regression lines were 87 for flour, 85 for corn starch and 84 for egg white. The 95% confidence interval for each slope is in Table 1. The coefficient of determination (Table 1) for each of the three regression lines showed excellent agreement of the points.

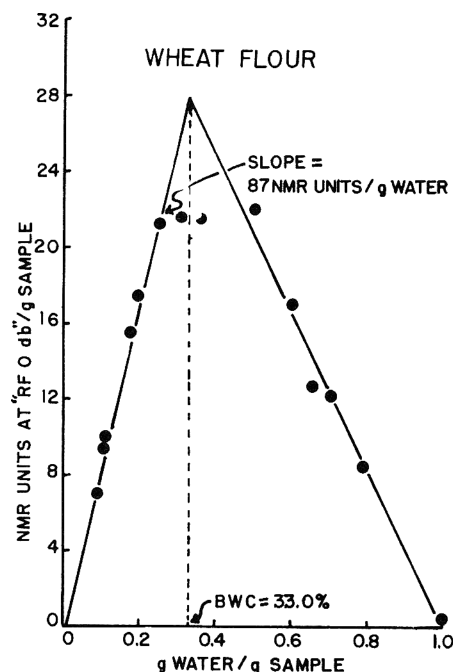

Fig. 5–Data for Figure 4 plotted on a sample weight basis instead of on a dry matter basis.

The slope was closely the same in each case so the data from all three products were pooled to calculate a single ascending regression line which showed a slope of 85.0 (Table 1). The coefficient of determination for this line was 0.96, showing excellent agreement among the data for all three products. The

Table 1—NMR calibration constants at RF 0 db for bound water and bound water capacity for three food products from regression equations for ascending and descending lines from plot of NMR readings (Y) against moisture content, both on a sample weight basis.

	Food Products			Pooled Data from 3 Products
	Wheat Flour	Corn Starch	Egg White	
Ascending regression line				
b, slope, calibration constant	86.9	84.9	84.2	85.04
95% confidence interval for calibration constant	84.83–89.03	82.76–87.10	82.46–86.00	81.22–88.86
a, Y intercept	−0.67	−1.03	−1.89	−1.15
r^2, coefficient of determination	0.970	0.993	0.978	0.964
Descending regression line				
b, slope	−42.1	−28.2	−41.7	--
a, Y intercept	41.90	28.43	42.51	--
r^2, coefficient of determination	0.983	0.967	0.996	--
Intersection point of the two regression lines or Bound Water Capacity (BWC) expressed as percent water	33.0	26.0	35.3	--

confidence interval for this slope was calculated to be 3.82, so it may be concluded with 95% confidence that the constant, NMR units at RF 0 db per g BW, lies between 81.22 and 88.86. In calculating the 95% confidence interval, all the variation was ascribed to NMR readings. It is highly probable that much of this variation was due to vacuum oven moisture determination. The relative errors involved should be determined for each application of this technique under the specific laboratory conditions involved.

This finding of a "universal" BW calibration constant (85 NMR units/g BW) for these three products, indicates that one g BW, irrespective of the substrate or total moisture content, gives the same signal at RF 0 db. This enables us to determine the amount of BW in any product which is shown by investigation to behave similarly to these products. It should be cautioned that the absolute value of the constant as found here is valid only for the instrument and measuring parameters used here. However, the principle and techniques should be generally valid so that a calibration curve for the material being studied should readily give the constant needed.

The question arises whether the signal obtained at RF 0 db is due to mobilization of hydrogen from the starch and protein by the water present rather than to BW itself. This was answered by experimenting with silica gel which contains no hydrogen, in place of the flour. A similar set of curves was obtained and the slope of the ascending curve was 82.6 which was within the 95% confidence range found with the food products. Therefore, it was concluded that the signal obtained at RF 0 db is due to BW.

The Y-intercept of each ascending regression line was at a slight negative value instead of passing through the origin as expected. This may be readily ascribed to errors in vacuum oven moisture determination. Also, this may mean that with the instrument parameters used here, moisture contents below about 2% could not be detected.

The equations for the ascending and descending regression lines for each product (Table 1) were solved simultaneously for their intersection point. The moisture content for each point, expressed as percent water (Table 1) was 33.0 for flour, 26.0 for starch and 35.3 for egg white. Each value represents the maximum amount of water the product could bind. These critical moisture contents (Fig. 1, 4 and 5) are therefore termed Bound Water Capacity (BWC). The values given here may be compared with the following literature values obtained by determination of water not frozen at low temperature: wheat flour 22.5% at −18°C (Toledo et al., 1968); whole corn 33.8% at −20°C and egg white 31% at −70°C (Kuprianoff, 1958).

The points at the apex of this intersection in case of Figure 5 did not fall on either line. These points represent samples prepared by adding the calculated amount of water to low moisture samples. Evidently not all the added water could be bound by the DM in the short time, 2 hr, allowed for equilibration. This short time was chosen to avoid fermentation. Although the same technique was used to prepare samples giving points on the high moisture line, in these cases there was so much free water present that equilibrium was achieved in the time allotted.

The ascending and descending curves (Fig. 5) are compatible with each other as shown by the following calcuation: At an ordinate of 12 in Figure 5, the abscissa for the ascending curve is 0.146 g BW per g sample. From the intersection point in Figure 5,

$$\text{BWC} = 33.0\%\ \text{water} = \frac{33.0\ \text{g BW}}{67.0\ \text{g DM}} = 0.493\ \frac{\text{g BW}}{\text{g DM}}$$

$$\text{Then, } \frac{0.146\ \text{g BW/g sample}}{0.493\ \text{g BW/g DM}} = 0.296\ \frac{\text{g DM}}{\text{g sample}}$$

so the calculated abscissa is

$$1-0.296 = 0.704\ \frac{\text{g total water}}{\text{g sample}}.$$

At an ordinate of 12, the high moisture curve of Figure 5 shows an abscissa of 0.700 thus corroborating the calculated value.

The data presented here may be interpreted on the basis that all the water, both bound and free, gives a signal at RF 28 db (Fig. 1), but that only BW is measured at RF 0 db (Fig. 2). Thus, all flour samples containing only BW (25% or less) gave a uniform reading of 87 units/g water at RF 0 db (Fig. 2).

At higher RF values such as 28 db, the signal per g BW varied with moisture content of the flour. This shows that the low moisture calibration curve in Figure 1 is not a perfect straight line as drawn. At high RF attenuation the curves (Fig. 2) for BW are horizontal due to the RF saturation effect, and a straight line relation between signal and BW cannot be expected, as explained at the beginning of this section.

As the free water level increases (total water above 50%) the RF attenuation curves (Fig. 3) show a shorter horizontal portion at the high RF attenuation and, in general, approach the level and shape of the 100% water curve. The reason for this is the diluting effect that the free water has on the BW at the high total moisture contents. Thus, the BW remains a function only of the amount of solid matter present so that BW can be determined at any moisture content.

For instrument calibration as well as determination of BWC, a minimum of four samples at moisture levels in the BW range and four in the free moisture range must be examined as indicated in Figure 4 or 5. However, once the calibration constant has been established for the instrument as well as the material being studied, the BWC can be determined by a single determination as follows:

1. Take a sample at a moisture content known to be above the BWC.
2. Take the NMR reading at RF 28 db.
3. Find g total water per g sample using a calibration curve similar to Fig. 1.
4. Calculate g DM/g sample = $1 - \frac{\text{g total water}}{\text{g sample}}$
5. Take the NMR reading at RF 0 db.

6. Calculate g BW/g sample = $\frac{\text{NMR units at RF 0 db/g sample}}{\text{85.04 NMR units/g BW}}$

7. Calculate $\frac{\text{g BW/g sample (step 6)}}{\text{g DM/g sample (step 4)}}$ =

$\frac{\text{g BW}}{\text{g DM}}$ = BWC

If total moisture content is known (e.g., vacuum oven moisture) steps 2 and 3 may be eliminated. A sample calculation may be made using the flour sample at the highest moisture content, 78.6%.

Step 4. $1 - 0.786 \frac{\text{g total water}}{\text{g sample}} = 0.214 \frac{\text{g DM}}{\text{g sample}}$

Step 5. NMR reading at RF 0 db = 8.50 NMR units per g sample.

Step 6. $\frac{\text{8.50 NMR units/g sample}}{\text{85.04 NMR units/g BW}} = 0.0999 \frac{\text{g BW}}{\text{g sample}}$

Step 7. $\frac{\text{0.0999 g BW/g sample}}{\text{0.214 g DM/g sample}} = 0.467 \frac{\text{g BW}}{\text{g DM}}$

or 31.9% wet basis BWC

This compares with 33.0% BWC as shown in Table 1. Thus, for better accuracy a number of samples at various moisture contents should be analyzed and the "tepee" curve used to determine BWC.

It may be noted that we have here only three variables in case of a material at a moisture content above the BWC: Total Water Content, BWC and NMR reading at RF 0 db. From any two of these, the third may be calculated.

This new concept for measuring BW in food products should prove quite valuable as a measure of one extremely important product characteristic in studying the effect of process and storage variables on the product. This method is direct, rapid, accurate, nondestructive and can be calibrated for the temperature of interest to the worker.

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ENDEL KARMAS and G. ROBERT DiMARCO
Department of Food Science, Rutgers–The State University, New Brunswick, N.J. 08903

DEHYDRATION THERMOPROFILES OF AMINO ACIDS AND PROTEINS

SUMMARY–Dehydration thermoprofiles representing water evaporation from aqueous solutions of 22 naturally occurring amino acids were determined. 14 amino acids exhibited peaks of "bound" water beyond the evaporation peak of "free" water. The acidic and basic amino acids indicated no water retention. Exceptional water retention properties, 30%–70% of the total water content as measured by peak areas, were exhibited by the nonpolar amino acids: isoleucine, leucine, methionine and valine. The methyl and methylene groups of these nonpolar structures seem to be responsible for the water retention properties probably due to hydrate formation. One explanation could be that as the temperature is raised to a critical point, the semicrystalline array around the nonpolar amino acid radicals collapses. Beef muscle tissue and egg albumin indicated strong water retention properties.

INTRODUCTION

IN RECENT YEARS, differential enthalpic analysis (DEA), also referred to as differential microcalorimetry, has been utilized extensively to measure quantitative energy changes when a substance undergoes programmed heating (Watson et al., 1964). However, the references in the thermo-analytical literature concerning water-protein and water-amino acid systems are scarce.

By using differential thermal analysis (DTA), Mishin and Garbuzov (1951) found that several proteins, including egg albumin, show a definite endothermic change in the heating curves between 110°C and 140°C caused by the loss of water. They predicted that DTA could be used for the identification of proteins. Perkins and Mitchell (1957) confirmed the latter statement publishing the DTA curves of 12 amino acids and 3 proteins. Felix et al. (1963) used DTA to analyze the water binding of keratins; the heating curves showed an endotherm at 130°–145°C as ascribed to vaporization of bound water. Similar experimental conditions were applied by Vinson et al. (1965) to dried and rehydrated keratin. The principal phase transitions were observed at 77°–88°C, 103°C, 114°C, and 135°C. They concluded that the two latter temperatures indicated water binding sites. Hence DTA or DEA seem to be uniquely suited for characterization of proteins with respect to water binding or retention. Not only are the phase transition temperatures defined but also the energetics of these transitions.

The present investigation was undertaken to use differential microcalorimetry to study the water binding aspects of all the natural amino acids and some globular proteins.

EXPERIMENTAL

FOR THE FIRST part of this study, 22 naturally occurring amino acids (L-configuration) of analytical grade were used. Regardless of the solubility, 25 mg of each amino acid was mixed in a glass vial with 475 μl of distilled water. The stoppered vials were allowed to stand in a refrigerator for several days with occasional shaking. Some amino acids were soluble and thus yielded 5% aqueous solutions. Others were less soluble and yielded a saturated clear supernatant with insoluble deposits on the bottom of the vial. By means of a microsyringe, 3 μl aliquots were taken from the clear solution or supernatant, transferred to an open aluminum sample pan, equilibrated isothermally at 37°C in the sample holder of a Differential Scanning Calorimeter, Model DSC-1B (The Perkin-Elmer Corp., Norwalk, Conn.), for exactly 20 sec, and subsequently scanned from 37°C upward at the 0.032 cal/sec output sensitivity for a full-scale deflection and 10°C/min heating rate. At least five trials were performed: two were run to the endpoint–sublimation, charring, or decomposition–of the dehydrated material; the others were run only to completion of dehydration. The information recorded included: dehydration thermoprofiles, per cent of water retained beyond the primary peak as estimated from the peak areas, and decomposition characteristics of the amino acids.

Dehydration thermoprofiles of proteins were studied on beef muscle tissue and egg albumin under similar conditions using 2.5–3.0 mg as the sample size.

Table 1–Dehydration data of aqueous L-amino acid solutions as estimated from thermoprofiles.

L-amino acid	Avg. water retention area (%)	Remarks
Alanine	5	Sublimation at 230°C
Arginine		Charring at (230°–) 250°C
Asparagine	1	Charring at (240°–) 265°C
Aspartic Acid		Charring at (265°–) 355°C
Cysteine	2	Sublimation at 228°C
Cystine		Charring at (225°–) 275°C
Glutamic Acid		Charring at (225°–) 270°C
Glutamine		Charring at (235°–) 257°C
Glycine	6	Sublimation with very small charred residue at 260°C
Histidine		Charring at (280°–) 300°C
Hydroxyproline	7	Decomposition (?) at (280°–) 300°C
Isoleucine	50	Sublimation at 205°C
Leucine	70	Sublimation at 210°C
Lysine		Charring at (350°–) 400°C
Methionine	60	Sublimation at 227°C
Phenylalanine	15	Sublimation at 227°C
Proline	2	Sublimation at 240°C
Serine	3	Decomposition at (235°–) 280°C
Threonine	6	Charring at (245°–) 300°C
Tryptophan	10	Dark brown liquid residue at 285°C. with very disagr. aroma
Tyrosine		Sublimation with very small charred residue at 288°C
Valine	30	Sublimation at 205°C

RESULTS & DISCUSSION

PRELIMINARY STUDIES revealed that higher concentrations of amino acids influenced neither the general appearance of the thermoprofiles nor the proportion or percentage of the retained water significantly. The choice of 5% aqueous solution of amino acids was based on the reasoning to obtain possibly maximum details in the thermoprofiles without diminishing the significant role of water by keeping it at or in excess of 95%. As a matter of fact, the total areas of thermoprofiles ranged from 95% up for amino acid solutions to 100% for pure water, implying that the energy measured by the areas was entirely used to evaporate the water present. This conclusion was fortified by the fact that when pure crystalline amino acids were run under identical experimental conditions, only smooth baselines resulted without any dehydration peaks.

The thermoprofiles representing water evaporation from dilute aqueous amino acid solutions, and pure water as control, from 37°C–140°C are presented in Figure 1. All show a primary endotherm whose peak maximum is in the vicinity of 70°C. Apparently enough energy was supplied to evaporate the "free" water at about 70°C due to relatively high vapor pressure of water, the high surface-to-volume ratio of the sample, and the dynamic nitrogen atmosphere. The evaporation of water "bound" at various degrees was delayed beyond the primary peak.

For primary peaks, an empirical baseline was drawn between the 37°C point (when the temperature program was started) and the intercept with the baseline at about 70°C as indicated with a dashed line. (See alanine, Fig. 1). The corresponding areas were assumed to represent the energy required to evaporate the free water. The baselines for all thermoprofiles beyond the primary peak continued at a uniform slope. Where the water retention peaks appeared in the thermoprofiles, empirical baselines were drawn as illustrated by dashed lines in Figure 1. The corresponding areas represent the energy required to evaporate the modified or retained water. All calculations, entirely empirical in nature, were based on these areas of free and retained or modified water.

The thermoprofiles of the acidic amino acids (aspartic and glutamic acid) and basic amino acids (arginine, histidine, and lysine) as well as cystine, glutamine, and tyrosine did not display any other peaks following the primary peak up to at least 200°C, indicating that all the water had been given off at the single large peak. Five of these amino acids, except the basic amino acids, showed a very sharp peak with an almost perpendicular recovery to the baseline, very similar to that of pure water (Fig. 1). One may assume that no water binding whatsoever was exhibited by these five amino acids.

The remaining 14 amino acids showed water retention endotherms beyond the primary peak. The average water retention figures are tabulated in Table 1. For alanine, asparagine, cysteine, glycine, hydroxyproline, proline, serine, theronine, and tryptophan, the figure was relatively small, from 1% up to 10%.

The thermoprofiles of the basic amino acids in the hydrochloride form differed very little from the free base form. Only histidine-HCl and lysine-HCl indicated a small water retention peak (about 1% of the total area) at 95°C and 120°C, respectively.

Four nonpolar amino acids–isoleucine, leucine, methionine, and valine–exhibited exceptional water retaining properties, from 30%–70% of the total area (Fig. 1). It is interesting to note that these nonpolar amino acid residues consist of methyl and methylene groups which seem to be responsible for the exceptional water binding. However, this does not follow the classical concept, because methyl and methylene groups do not form ions in water nor do they accept hydrogen bonds; there is very little attraction between them and the water molecules. The only explanation seems to be hydrate formation (Buswell and Rodebush, 1956; Karmas, 1968). Hence, the exceptional water retention of these nonpolar amino acids may be due to semicrystalline water structures which did not evaporate at temperatures of the "free" water but were made to collapse at higher temperatures thus accounting for the water retention peaks in the amino acid thermoprofiles.

As previously mentioned, acidic and basic amino acids exhibited no water retention at all. The polar groups, such as carboxyl groups, may have caused multilayer adsorption of water molecules, whereas hydrogen bonding between the polar groups and the water molecules was probably as strong as that among the water molecules themselves. This possibility has also been postulated by Warner (1961). Although water molecules are bound by adsorption, it seems to be a weaker force than that involved in semicrystalline water cages; hence, no water retention peaks were displayed. For the same reason, the hydroxyl group of tyrosine must have had a detrimental effect on the water retaining property of the benzyl group of phenylalnine. In the same fashion, the interaction of polar and nonpolar residual groups of other amino acids may be explained and correlated

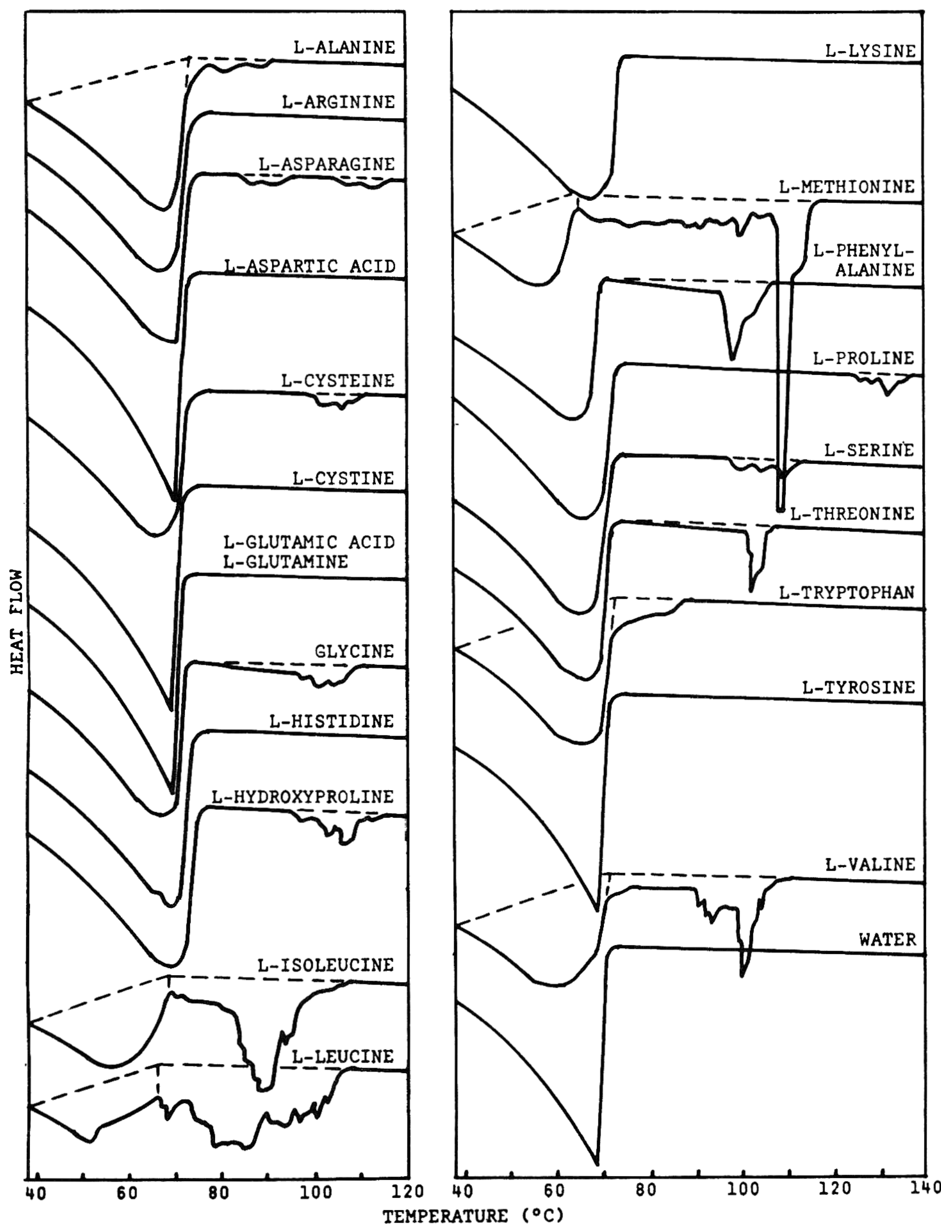

Fig. 1–DSC thermoprofiles representing water evaporation from aqueous amino acid solutions subjected to a 10°C per minute temperature increase.

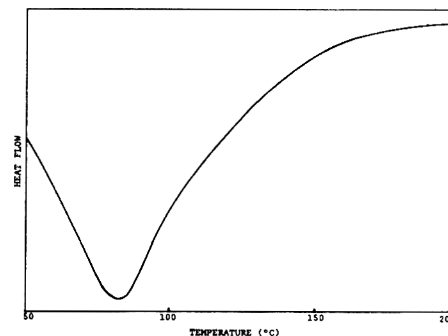

Fig. 2–A DSC thermoprofile representing water evaporation from raw beef muscle tissue subjected to a 10°C per minute temperature increase.

with water retention properties.

Although Table 1 gives information about the ultimate fate–sublimation, decomposition, or charring–of the dehydrated amino acids when the temperature was programmed up to as high as 400°C, the thermoprofiles were not included here. The corresponding transition endotherms and exotherms on the chart were identified by visual observation in the DSC-1B sample holder. An interesting observation was that amino acids with no water retention did not sublime, while the five amino acids which exhibited the greatest water retention properties all sublimed at a relatively low temperature: 205°–227°C. It may be that a compound which has the least attraction between its molecules escapes charring by sublimation when the kinetic content of the compound has reached a certain level.

Dehydration thermoprofiles were determined also for proteins. Two entirely different animal proteins were chosen: beef muscle tissue, a water-protein system exhibiting highly-developed structural features; and egg albumin, an aqueous solution of a number of proteins.

The results were similar for both proteins with no significant deviation in thermoprofiles. A representative thermoprofile of water evaporation from raw beef muscle tissue is illustrated in Figure 2. Although only one dehydration peak was apparent, this peak indicated a very strong water retention for both raw beef muscle tissue and egg albumin. Water retention extended well beyond 150°C in the thermoprofiles.

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M. G. MAST and G. J. MOUNTNEY[a]
Ohio Agricultural Research & Development Center, Columbus, Ohio 43210

GROWTH PATTERNS OF SELECTED PSYCHROPHILIC MICROORGANISMS IN COOKED AND UNCOOKED ASEPTICALLY PROCURED TURKEY MEAT

SUMMARY—Sterile muscle tissue, removed aseptically from the breast of a turkey reared under commercial conditions, was inoculated with psychrophilic microorganisms capable of causing spoilage. The growth patterns of these microorganisms were compared in the cooked and uncooked samples of this sterile meat when stored at 5°C and 20°C. Similar growth patterns were exhibited in both types of meat. At points on the growth curves where significant differences did occur between the two types of meat, levels of growth in the cooked meat were higher. The growth pattern of a mixed culture comprised of an Alcaligenes *species and a* Flavobacterium *species was compared to that of* Pseudomonas fluorescens *in both types of meat stored at both temperatures. Muscle tissue inoculated with the mixed culture consistently contained greater numbers of bacteria than meat inoculated with the pure culture. Uncooked sterile turkey meat remained in good condition, both in appearance and bacteriologically, for at least one year when stored at above freezing temperatures.*

INTRODUCTION

SPOILAGE OF POULTRY meat is caused mainly by the development of certain psychrophilic microorganisms, especially members of the genera *Pseudomonas* and *Acinetobacter.* In order to determine the contribution of a specific microorganism to the rate of spoilage, a pure culture of the organism in question must be introduced into sterile, nondenatured poultry meat. By evaluating the independent effects of a single species of bacteria we are better able to understand its role in spoilage.

Two basic methods are used to obtain sterile meat for such studies. One involves rearing an animal under germ-free conditions and then removing the meat aseptically. Various aspects of germ-free studies and gnotobiology have been discussed in detail by Gordon (1959), Reyneirs (1959), and Luckey (1963). The second method involves procuring the meat aseptically from an animal which has not been reared under gnotobiotic conditions. This procedure involves the assumption that meat from normal, healthy animals is sterile at the time of slaughter. Researchers have had varying degrees of success in procuring sterile meat from such animals, partially depending on the techniques utilized in removing the meat. Zender et al. (1958), using a surgical room technique, successfully procured sterile meat from rabbits and lambs. Sharp (1963) obtained sterile rabbit tissue by submerging the excised tissue in alcohol and then igniting it. By painting the flamed tissue with a dye solution, he further reduced the percentage of contaminated samples.

van den Berg et al. (1963; 1964) and Khan and van den Berg (1964) procured sterile chicken meat by dipping the meat into solutions of chlortetracyline and sodium hypochlorite. Ockerman (1966) and Ockerman et al. (1969) obtained sterile bovine muscle samples by using a surgical isolator technique.

Frazier (1967) reported that changes which occur in meat during cooking may render the product more susceptible to attack by microorganisms. However, little work has been reported comparing microbial growth in sterile uncooked and cooked poultry meat.

The objectives of this study were (1) to develop a method for obtaining sterile meat from poultry, (2) to study the growth rates of selected psychrophilic microorganisms in aseptically procured poultry meat, and (3) to compare the growth rates of these microorganisms in cooked and uncooked sterile meat.

[a]Present address: Cooperative State Research Service, USDA, Washington, D.C. 20250.

MATERIALS & METHODS

A 20-LB WHITE TURKEY hen was used as the source of meat for these experiments. Preliminary trials were conducted with two chicken broilers weighing approximately 3 lb each to test procurement techniques. However the microbiological experiments reported here were conducted with turkey muscle tissue because of the large quantity of meat obtainable from one bird.

Slaughter procedure

The slaughter procedure involved suspending the bird head down, swabbing the neck area with ethanol, severing the jugular vein and then piercing the brain. The feathers were removed without scalding, and the neck and hocks were removed with shears. The bird was then placed in a stainless steel container, rinsed with ethanol, covered with aluminum foil, and taken to the laboratory for further processing. All surfaces and utensils coming into contact with the sample material were presterilized. Aseptic procedures were utilized throughout the study.

In the laboratory the surface of the bird was thoroughly scrubbed with towels saturated with ethanol. The carcass was completely submerged in ethanol for 15 sec, allowed to drain, and ignited. While still flaming the bird was placed into an isolator. To reduce the possibility of contamination from the air inside the isolator and on the skin of the bird, the carcass was left in the closed isolator for 20 min under ultraviolet light.

Aseptic sample procurement

The meat was obtained from breast muscle on both sides of the sternum of the turkey. The

Table 1—Growth of microorganisms in turkey muscle tissue stored at 20°.

	Culture[1,3]		Treatment of sterile tissue[2,3]	
Storage time in days	Pure culture of *Pseudomonas fluorescens*	Mixed culture of *Flavobacterium* sp. + *Alcaligenes* sp.	Cooked	Uncooked
0	4.7774	4.8713	4.8611	4.7876
½	5.9314	6.2540	6.3910	5.7944
1	8.7713*	9.0464*	9.1161**	8.7016**
2	9.7160	10.2675	9.9231	10.0612
3	9.9693	10.2534	10.3100	9.9127
4	10.0257	9.9396	9.7087	10.2566
5	9.3930	10.258	9.9165	9.6823

[1]Each logarithmic value represents the mean of six determinations; three trials in uncooked turkey muscle tissue and three trials in cooked turkey muscle tissue.

[2]Each logarithmic value represents the mean of six determinations; three trials using *Pseudomonas fluorescens* and three trials using a mixed culture of a *Flavobacterium* sp. and an *Alcaligenes* sp.

[3]Means in the same row having one asterisk are signficantly different at $P < 0.05$; means having two asterisks are significantly different at $P < 0.01$.

breast skin was cut with a scalpel and pulled away from the incision with forceps. Small portions of the outer muscles were excised and placed into sterile containers. Care was taken to avoid cutting into the air sacs separating the layers of muscle in the breast, since the air sacs could be sources of contamination. Tissue from the area surrounding the air sacs was kept separate from the outer muscle tissue.

Sterility testing

Small samples of tissue were taken from each container and placed into 16 × 150 mm tubes containing Fluid Thioglycollate Medium (BBL). These tubes were incubated at 35°C for 72 hr and visually observed for turbidity as an indication of microbial growth. A control sample of tissue inoculated with microorganisms was used as a positive reference. 1 ml aliquots of broth were removed from the tubes after 48 hr of incubation and transferred to Tryptone Glucose Extract Agar (TGEA), Difco. These plates were incubated at 35°C for 48 hr and were then examined for microbial growth.

The containers of sterile tissue were also placed in an incubator at 20°C for 48 hr after which samples were again removed and tested for sterility by the method outlined above. After the sterility of the samples was established, the tissue was frozen and held at −18°C for 7 days.

Individual sample preparation

The frozen tissue was subsequently removed from the freezer and allowed to thaw at 5°C. Samples of tissue weighing approximately 5g each were transferred in the isolator to preweighed, 20 ml screw-capped vials. One-half of these tissues were autoclaved (121°C, 15 psi for 15 min), and then stored at 5°C. Small portions of tissue were removed from each of the remaining vials which had not been autoclaved, and checked for sterility. The vials were then weighed and stored at 5°C.

Microorganisms

The microorganisms used in this study were (1) *Pseudomonas fluorescens*, ATCC Culture No. 12633, and (2) a mixed culture consisting of a *Flavobacterium* species and an *Alcaligenes* species cultured from a broiler carcass which showed signs of spoilage.

Both cultures of organisms were grown in Nutrient Broth (Difco) for 24 hr at 20°C. To obtain rapidly proliferating organisms for the inocula, two subsequent transfers were made to Nutrient Broth. The cells in the final cultures were separated from the media by centrifugation and were rinsed with a sterile peptone-salt medium (0.1% peptone, Difco; 0.5% NaCl). After washing and centrifuging the cells two more times, the cell suspensions were diluted so that an optical density of 0.25 was obtained when measured on a Coleman Spectrophotometer (Model 14, Coleman Instrument, Inc.) adjusted to a wavelength of 660 mμ, as suggested by Ingraham (1958). This suspension was then diluted with the peptone-salt medium so that when an appropriate amount was added by pipette to the sterile muscle tissue samples, approximately 4.4×10^4 microorganisms per gram would be present.

Experimental design

Samples of sterile cooked and uncooked turkey muscle tissue were inoculated with either a mixed culture (*Flavobacterium* species and *Alcaligenes* species) or a pure culture (*Pseudomonas fluorescens*) and were held at either 5°C or 20°C following inoculation. Samples held at 5°C were examined after 0 days (a control, analyzed immediately after being inoculated with the microorganisms), and 1, 2, 5, 10, 15, and 20 days. Samples held at 20°C were examined after 0, ½, 1, 2, 3, 4, and 5 days. An uninoculated control sample was held with each series of vials and examined with the final inoculated sample.

Procedure for bacterial analysis

Total plate counts were performed in triplicate on each sample at the end of the storage period. The entire contents of each vial were aseptically transferred to a sterile blendor jar. Enough sterile peptone-salt diluent was added to make a 1:10 dilution. The mixture was then blended for 30 sec at low speed (8,000 rpm) with a Waring Blendor (Waring Blendor Corp.). TGEA was used as the medium for all plate counts.

All plates were incubated at 20°C for 48 hr, since Peterson and Gunderson (1960), Olsen and Jezeski (1963), and Keenan et al. (1967) reported 20–21°C to be the optimum temperature for growth of *Pseudomonas fluorescens*. Colonies were counted on a Quebec Colony Counter (Spencer Lens Co.).

Interpretation of data

Bacterial counts were converted to logarithms and growth curves were plotted. The data were analyzed by analysis of variance according to Steel and Torrie (1960).

RESULTS & DISCUSSION

USING THE PROCEDURES outlined above, sterile muscle tissue was successfully procured from poultry grown under commercial conditions. All of the turkey muscle tissue which was procured was sterile, and 95% (34 of 36 samples) of the muscle tissue from two broiler type chickens was sterile. The presence of bacteria in two samples in the preliminary trials were attributed to contamination in handling and not to inherent microorganisms in the tissue. These results are in agreement with those of Zender et al. (1958), Sharp (1963), van den Berg et al. (1963; 1964), Khan and van den Berg (1964), Ockerman (1966), and Ockerman et al. (1969), all of whom obtained sterile muscle tissue from normal animals which had not been reared under gnotobiotic conditions. However, these results disagree with reports of Boyer (1926), Burn (1934), Lepovetsky et al. (1953), and McCarthy et al. (1963), who consider bacteria to be an inherent part of normal tissue.

The growth patterns of the microorganisms (mixed culture consisting of a *Flavobacterium* sp. and an *Alcaligenes* sp., and a pure culture of *Pseudomonas fluorescens*) in aseptically procured turkey muscle tissue (cooked and uncooked) are illustrated in Figure 1 (20°C) and Figure 2 (5°C). The microorganisms grew more rapidly at 20°C than at 5°C, as would be expected. However at both 20°C and 5°C, the same number (approximately 10^{10}) of bacterial cells were present at the maximum stationary phase of the growth curve. This stationary phase was reached after 2 days when the samples were held at 20°C and after 10 days when they were held at 5°C.

Samples of muscle tissue inoculated with the mixed culture consistently contained greater numbers of bacteria than did samples inoculated with a pure culture of *Pseudomonas fluorescens* (Tables 1 and 2). Several factors which may account for this difference are (1) the organisms in the mixed culture may have been better adapted for growth in poultry tissue than *Pseudomonas fluorescens* since they were originally cultured from a chicken carcass; (2) the organisms in the mixed culture may have faster growth rates than *Pseudomonas fluorescens* at the temperatures tested; and (3) the two species in the mixed culture may have had beneficial effects on each other.

Cooked muscle tissue generally sup-

Table 2—Growth of microorganisms in turkey muscle tissue stored at 5°C.

	Culture[1,3]		Treatment of sterile tissue[2,3]	
Storage time in days	Pure culture of *Pseudomonas fluorescens*	Mixed culture of *Flavobacterium* sp. + *Alcaligenes* sp.	Cooked	Uncooked
0	4.7774	4.8713	4.8611	4.7876
1	5.0927	5.2143	5.2633*	5.0437*
2	5.4776*	6.0696*	6.0164*	5.5309*
5	7.4196**	9.2784**	8.4152	8.2828
10	9.8307*	10.4938*	9.9177	10.4068
15	10.1602	10.4517	10.2076	10.4043
20	10.0392	10.1542	10.0314	10.1621

[1] Each logarithmic value represents the mean of six determinations; three trials in uncooked turkey muscle tissue and three trials in cooked turkey muscle tissue.

[2] Each logarithmic value represents the mean of six determinations; three trials using *Pseudomonas fluorescens* and three trials using a mixed culture of a *Flavobacterium* sp. and an *Alcaligenes* sp.

[3] Means in the same row having one asterisk are significantly different at $P < 0.05$; means having two asterisks are significantly different at $P < 0.01$.

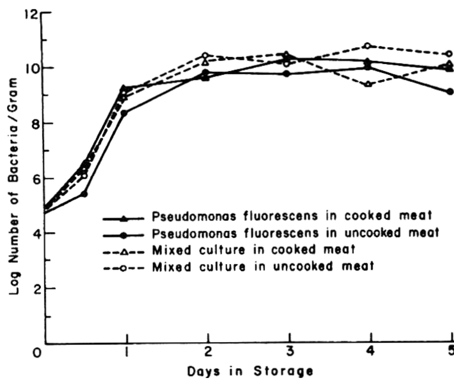

Fig. 1–Growth patterns of microorganisms in aspetically procured turkey muscle tissue stored at 20°C.

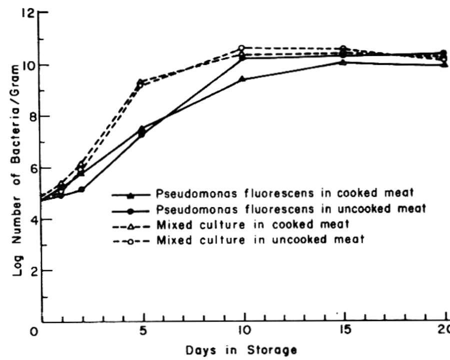

Fig. 2–Growth patterns of microorganisms in aseptically procured turkey muscle tissue stored at 5°C.

ported greater numbers of bacteria than did uncooked muscle tissue, although these differences were usually not significant ($P < 0.05$; Tables 1 and 2). This observation supports the conclusions of Frazier (1967) that heat processing may alter the composition of a food rendering it more readily available to some organisms than it was in the native state, and thus increase the rate of spoilage in the food.

A portion of the sterile turkey muscle tissue procured in this study was stored in 20 ml screw-capped vials held at 5°C for over 1 yr. Eighteen samples of the uncooked meat, with a mean weight of 7.04g/sample, had a mean loss in weight of 3% (0.21g/sample) during this period. There was no accumulation of moisture as "weepage" in any of the containers.

Samples of this meat were found by bacteriological tests to be sterile after this storage period. No off-odor was detected in these samples.

Visual comparisons of the uncooked sterile tissue and the cooked tissue demonstrated the advantages of preserving meat in an undenatured state. The uncooked tissue maintained the appearance of fresh turkey tissue, whereas the cooked product was pale in color and shrunken in size.

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J. J. LICCIARDELLO, J. T. R. NICKERSON and S. A. GOLDBLITH
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

RECOVERY OF SALMONELLAE FROM IRRADIATED AND UNIRRADIATED FOODS

SUMMARY–Maximum recovery of salmonellae from artificially contaminated irradiated and unirradiated foods was usually obtained on tryptic soy yeast extract agar, a nonselective medium. Recovery on various selective media depended, in general, upon the serotype and the substrate. Irradiated cells were definitely inhibited on Salmonella-Shigella *agar or desoxycholate citrate agar, but only slightly, if at all, on brilliant green, bismuth sulfite or MacConkey agars. The highest count of irradiated cells of* Salmonella newport *by the MPN method was obtained with tetrathionate broth, yet this medium was inhibitory to* Salmonella oranienburg *and* Salmonella heidelberg. *In this latter situation, pre-enrichment in nutrient broth prior to inoculation into tetrathionate broth was beneficial.*

INTRODUCTION

CHOICE of a selective medium and the analytical procedure for detection or quantitation of salmonellae depend upon the serotype, the concentration of salmonellae, the concentration of competitive or interfering microorganisms and the composition of the food (Banwart and

Table 1—Quantitative recovery of Salmonella oranienburg on various media after irradiation in chicken meat.

	Salmonellae per gram after a dose of							
Medium	0 krad	60 krad	120 krad	180 krad	240 krad	300 krad	360 krad	420 krad
	Direct plate count							
TSYE agar	160×10^5	51×10^5	115×10^4	130×10^3	118×10^2	74×10^1		
Minimal agar	120×10^5	30×10^5	85×10^4	140×10^3	138×10^2	70×10^1		
BGS agar	35×10^5	8×10^5	3×10^4	14×10^3	15×10^2	22×10^1		
BiS agar	105×10^5	37×10^5	106×10^4	112×10^3	105×10^2	62×10^1		
	MPN count							
SC broth	330×10^5	33×10^5	68×10^4	220×10^3	240×10^2	24×10^1	160	1.1
TT broth	230×10^5	13×10^5	68×10^4	33×10^3	33×10^2	17×10^1	1	0.2
Nutrient broth → TT broth	68×10^5	33×10^5	130×10^4	350×10^3	110×10^2	92×10^1	24	3.3

Ayres, 1953; Silliker and Taylor, 1958; Taylor et al., 1958). This complication is further compounded when one also considers the enumeration of salmonellae which have been stressed by freezing, dehydration, freeze-drying or heat (Hartsell, 1951; North, 1961; Sinskey et al., 1964; Clark and Ordal, 1969).

It has been proposed to eliminate or reduce the level of *Salmonella* contamination in egg products or poultry by ionizing radiation. However, any salmonellae surviving this process might be in a weakened or debilitated state and inhibited by the selective agents in the various media employed for isolation of *Salmonella*. Mossel (1960) reported there was no difference in recovery of salmonellae from irradiated egg on either nutrient agar, crystal violet neutral red bile lactose mannitol agar, TDYM agar, or through Muller's tetrathionate broth and selenite bile brilliant green sulfapyridin medium. Freeman (cited by Bridges, 1963) also found no effect of post-irradiation media such as nutrient agar, MacConkey agar or desoxycholate agar on recovery of *Salmonella*. However, Ley et al. (1963) reported that recovery of salmonellae after irradiation was poor on desoxycholate citrate agar. In a study by Corry et al. (1969), it was found that the recovery of irradiated *Salmonella typhimurium* was dependent upon the enrichment broth, the temperature and duration of incubation and the plating medium. They recommended enrichment in tetrathionate broth at 37°C and plating on brilliant green agar or bismuth sulfite agar for confirmation. However, since their investigation was concerned with only one *Salmonella* serotype, their recommended procedure cannot be applied unequivocally to other serotypes.

The purpose of our investigation was to compare the efficiency of various commercially available selective media on the quantitative recovery of several *Salmonella* serotypes from some artificially contaminated irradiated foods, with particular emphasis on the method and media recommended by the U.S. Public Health Service (Lewis and Angelotti, 1964) for enumeration of salmonellae in foods.

Table 2—Quantitative recovery of Salmonella typhimurium on various media after irradiation in liquid whole egg.

	Salmonellae per gram after a dose of					
Medium	0 krad	40 krad	80 krad	120 krad	160 krad	200 krad
	Direct plate count					
TSYE agar	34×10^5	31×10^3	46×10^2	33×10^1	30	–
BGS agar	18×10^5	4×10^3	22×10^2	21×10^1	10	–
BS agar	13×10^5	12×10^3	27×10^2	52×10^1	40	–
	MPN count					
SC broth	92×10^5	33×10^3	35×10^2	–	49	2.2
TT broth	54×10^5	17×10^3	13×10^2	–	7	0.8
TSYE broth → SC broth	54×10^5	49×10^3	24×10^2	–	240	2.3
Nutrient broth → TT broth	35×10^5	23×10^3	92×10^2	–	33	1.4

MATERIALS & METHODS

Cultures

The *Salmonella* serotypes used throughout this investigation were *S. typhimurium* ATCC 7823, *Salmonella thompson* ATCC 8391, *Salmonella newport* ATCC 6962, *Salmonella heidelberg* ATCC 8326, *Salmonella oranienburg* ATCC 9239 and *Salmonella anatum* ATCC 9270. Stock cell suspensions were prepared by growing cultures on the surface of tryptic soy agar (Difco) enriched with 5 g per liter yeast extract (TSYE agar) for 3–4 days at 37°C, washing off the cells with phosphate buffer pH 7, centrifuging and resuspending in buffer. Cells were held at 2.2–4.4°C until used.

Inoculation of samples

The test substrates were chicken meat, whole egg magma, trypticase soy broth (BBL) supplemented with 5 g per liter yeast extract (TSYE broth) and 0.013 M phosphate buffer pH 7. Approximately 600 g chicken meat (white and dark) were inoculated with 15 ml of a *Salmonella* cell suspension while the meat was being comminuted in a laboratory model Hobart silent cutter. The final concentration of salmonellae was about 10^7 per gram of meat. 1-oz (28.3-g) snap-cap polyethylene vials were filled to capacity with the inoculated meat, then briefly centrifuged to eliminate airpockets. In egg magma, fresh hen's eggs were broken out and the contents (about 300 g) pooled. This was mechanically blended at a low speed to a uniform magma while adding 5 ml of a cell suspension. The resulting salmonellae concentration was about 10^7 cells per gram whole egg. Culture tubes (30 by 200 mm) were filled with 20-ml portions and plugged. A quantity of phosphate buffer or TSYE broth was also inoculated to a level of about 10^7 salmonellae per ml and 10-ml amounts apportioned into culture tubes (18 by 150 mm). This unnaturally high inoculum level was necessary, to obtain a measurable number of survivors following an irradiation treatment (200–400 krad) that may be considered feasible for liquid whole egg or poultry.

Irradiation

Sealed samples were irradiated in an ice-water bath (0°C) in a U.S.A.E.C. Mark 1 Cobalt-60 Food Irradiator. The dose rate as determined by Fricke dosimetry was approximately

Table 3–Efficiency of different media with reference to TSYE agar in recovering various Salmonella serotypes from inoculated unirradiated chicken, egg magma or phosphate buffer.

	Direct plate count								MPN count			
											Nutrient broth ↓	TSYE broth ↓
Serotype	TSYE agar	Minimal agar	BG agar	BGS agar	BiS agar	MC agar	SS agar	DC agar	SC broth	TT broth	TT broth	SC broth
	Percent recovery from chicken meat											
S. typhimurium	100	103	–	38	91	106	6	89	96	96	160	–
S. oranienburg	100	75	–	22	66				200	145	43	–
S. thompson	100	–	38	45	60				88	50	125	–
S. newport	100	71	–	11	83				69	69	69	–
S. heidelberg	100	–	69	81	80				41	58	108	116
	Percent recovery from egg magma											
S. typhimurium	100	–	–	53	38				270	160	103	160
S. oranienburg	100	82	–	94	116				158	220	158	–
S. thompson	100	–	–	78	–				65	92	315	100
S. newport	100	–	49	45	9				28	67	185	110
S. heidelberg	100	–	–	85	35				105	125	67	125
	Percent recovery from phosphate buffer											
S. typhimurium	100	–	–	49	110	90	12	–	–	–	–	–
S. thompson	100	–	–	85	96	93	1	–	–	–	–	–
S. newport	100	–	–	70	88	87	6	23	–	–	–	–
S. heidelberg	100	85	–	73	69	95	20	31	–	–	–	–
S. anatum	100	83	–	0	88	69	15	18	–	–	–	–

5000 rad/min. Rather than compare the recovery on the various media at just 1 arbitrary dose, it was considered more informative to irradiate at several different doses, so that the inactivation rates determined on each different medium could be compared. Poultry samples were irradiated in duplicate in increments of 50–75 krad, depending on the radioresistance of the inoculated serotypes, and 7 different doses were administered. Egg samples were irradiated to a maximum dose of 200 krad in increments of 40 krad, and broth samples to a maximum dose of 120 krad in increments of 30 krad.

Enumeration of salmonellae

A quantitative count of salmonellae in broth samples was made immediately after irradiation, whereas with poultry or egg samples counts were made the folowing day, with an interim storage of 2.2–4.4°C.

A 1/10th dilution was made by mechanically blending 25 g of chicken meat with 225 ml chilled 0.013 M phosphate buffer pH 7, or by mixing 10 ml egg magma with 90 ml chilled buffer. Further decimal dilutions were made with chilled buffer.

Because the natural level of contamination of foods with *Salmonella* is usually too low to be detected by direct plating, the most probable numbers (MPN) technique is employed in actual practice for estimating *Salmonella* counts. However, in this study, in addition to the MPN method, the direct plating procedure was also included, because with the concentration of salmonellae being used it could be feasibly employed, thus enabling a check to be made on the accuracy of the MPN counts; secondly, since a direct plating method, because of its simplicity, would be more preferable in experimental studies concerned with the radiation inactivation kinetics of *Salmonella*, it would be of interest to compare the recovery of irradiated salmonellae on the various solid plating media.

For a direct plate count, 0.1-ml portions of various sample dilutions were streaked in duplicate using a bent glass rod on the surface of prepoured agar plates, which were then incubated 2–3 days at 37°C. The agar media included TSYE agar, a nonselective, complete medium; ammonium-glucose-salts minimal medium (Anderson, 1946) for determining the fraction of metabolically injured cells (Straka and Stokes, 1959); brilliant green (BG) agar (Difco); brilliant green sulfadiazene (BGS) agar for suppressing growth of pseudomonads (Galton et al., 1954); bismuth sulfite (BiS) agar (Difco); MacConkey (MC) agar (Difco); desoxycholate citrate (DC) agar (Difco); and *Salmonella-Shigella* (SS) agar (Difco). The level of inoculation of the food samples with salmonellae was about 10^3 to 10^4 times the concentration of the natural microbial flora and for this reason a nonselective medium such as TSYE agar could be employed.

For the most probable numbers (MPN) determination selenite-cystine (SC) broth (Difco) and tetrathionate (TT) broth base (Difco) with added iodine were used. A series of 5 tubes was inoculated with serial dilutions of the samples and after a 2-day incubation at 37°C a loopful from each tube was streaked on BG agar for confirmation as *Salmonella*. The effect of pre-enrichment was studied by inoculating a 5-tube series of either nutrient broth or trypticase soy yeast extract (TSYE) broth with the sample, incubating 2 days at 37°C, then subculturing 0.1-ml portions from each tube into tubes of either SC or TT broth.

RESULTS & DISCUSSION

TABLES 1 and 2 represent a typical tabulation of the results for the recovery of 2 serotypes (*S. oranienburg* and *S. typhimurium*) on various media from irradiated and unirradiated chicken meat or whole egg. The space required to reproduce all the other tables of similar data would be prohibitive (5 serotypes × 3 substrates). Therefore, Tables 1 and 2 are presented solely to illustrate the intervals of radiation dose used and the range in cell concentration recovered.

Recovery from unirradiated food

In Table 3 is shown the percent recovery of various *Salmonella* serotypes from inoculated unirradiated chicken, egg magma or phosphate buffer on different media with reference to recovery on TSYE agar. The effect, if any, of the food substrate on the recovery by a particular medium can be determined by comparison to the recovery from phosphate buffer. An attempt will be made only to generalize the results, since the recovery on a particular medium depended not only on the serotype but also on the food substrate. This observation has been reported by many other investigators. As expected, the highest recovery was usually with TSYE agar. BGS agar was not considered to be any more inhibitory than BG agar. Galton et al. (1954) also made the same observation; however, Raj (1966) concluded that BGS was definitely inhibitory to *S. typhimurium* in fish samples.

In the present study it was indicated that BGS was less inhibitory in recovering salmonellae from egg magma than from chicken meat. BiS agar was as good or better than BGS agar in recovering salmo-

Table 4—Decimal reduction dose (krad) for Salmonella serotypes in various substrates as determined by recovery on different media.

	Direct plate count								MPN count			
Serotype	TSYE agar	Minimal agar	BG agar	BGS agar	BiS agar	MC agar	SS agar	DC agar	SC broth	TT broth	Nutrient broth ↓ TT broth	TSYE broth ↓ SC broth
					D_{10} value in chicken meat							
S. typhimurium	52	48	–	52	48	47	40	47	45	45	45	–
S. oranienburg	71	72	–	71	73	–	–	–	68	61	75	–
S. thompson	68	–	60	56	63	–	–	–	60	60	60	–
S. newport	50	50	–	59	48	–	–	–	50	57	52	–
S. heidelberg	71	–	70	68	67	–	–	–	69	62	67	67
					D_{10} value in egg magma							
S. typhimurium	28	–	–	28	32				26	23	30	29
S. oranienburg	37	37	–	30	37				38	29	35	–
S. thompson	48	–	–	31	47				24	27	24	32
S. newport	16.7	–	19.2	17.2	35				17.5	21	16	16
S. heidelberg	31	–	–	32	36				31	26	34	31
					D_{10} value in TSYE broth[1]							
S. typhimurium	15.5	–	–	13	14	13.4	12.2	–				
S. thompson	25.5	–	–	23.5	22.2	20.6	–	–				
S. newport	11	–	–	17	13	11	9.8	–				
S. heidelberg	17.5	12.6	–	15.2	13.6	14.5	17	13.8				
S. anatum	13.4	12	–		11.6	11.5	10.4	9.2				

[1] *Salmonella heidelberg* and *S. anatum* done in phosphate buffer.

nellae from inoculated chicken, but only as good or poorer in recovering salmonellae from inoculated egg magma. There did not seem to be any important difference in recovery on these 2 media from phosphate buffer. Addition of 5 g per liter of yeast extract enhanced recovery on BGS agar but not on BiS agar. It is not recommended that yeast extract be added to BGS agar without determining its effect on selectivity of the medium. If the BiS agar plates were more than 24 hours old when used, even though refrigerated, the medium often tended to be very inhibitory. Therefore, in this investigation the BiS plates were used within 24 hours of having been poured. Difficulty was occasionally encountered with some batches of BGS agar and no recovery of cells occurred at the dilutions plated. Read and Reyes (1968) reported a variation in the recovery of salmonellae on BGS agar due to lot differences and serotype sensitivities. They recommended that a particular lot of this agar be tested before used. Recovery was good on MC agar but poor on DC and SS agar. Banwart and Ayres (1953) also reported poor recovery of salmonellae on desoxycholate citrate lactose sucrose agar and on SS agar.

The MPN count in most cases ranged from one-half to twice the direct plate count on TSYE agar. There was no overwhelming evidence that SC broth was superior to TT broth. Although it may have appeared in some cases that recovery was improved by pre-enrichment prior to inoculation into 1 of the selective broths, in general the value of the practice seemed to be questionable for the examination of unirradiated fresh food.

Recovery from irradiated food

To determine the efficiency of the various media in recovering salmonellae from irradiated foods, the decimal reduction dose (D_{10}), which is the reciprocal of the inactivation rate, was compared for each serotype on the various media. These values are shown in Table 4. In most instances the highest D_{10} was obtained using TSYE agar as the recovery medium. With BGS agar, BiS agar or MC agar the D_{10} value ranged from about the same to slightly less than that with TSYE agar, with one exception—the D_{10} for *S. thompson* was relatively lower in the food substrates using BGS agar. In egg magma a higher D_{10} resulted using BiS agar compared to BGS agar. In those few cases where BG and BGS were compared, the D_{10} was slightly higher on BG agar; however, the difference may not have been significant.

In some cases a higher D_{10} was obtained with 1 of the selective media than with TSYE agar. This was because recovery on the selective medium may have been low for the unirradiated sample but comparable to recovery on TSYE agar for irradiated samples; or recovery on the selective medium improved with increasing radiation dose because a larger aliquot of the food substrate was being included in the inoculum for plating and may have neutralized some of the inhibitory properties of the medium.

The lowest D_{10} values were usually obtained with SS or DC agar. Both of these contain high concentrations of surface-active agents probably inhibitory to radiation-injured cells. On desoxycholate lactose agar, which contains only 1/10th as much sodium desoxycholate as DC agar, recovery of irradiated or unirradiated cells was comparable to that on other selective media.

The value of D_{10} obtained with minimal medium was equal or slightly lower than that on TSYE agar. This would indicate that metabolic damage as a result of irradiation was, at most, slight. However, that some type of radiation damage, probably physical, must have occurred is evident, in that with increasing irradiation treatment the cells became more sensitive in media with high concentrations of surface-active agents.

The D_{10} values obtained with the MPN broths were generally comparable with, or in a few cases lower than, those obtained with TSYE agar. SC broth was superior to TT broth in recovery of irradiated *S. oranienburg* or *S. heidelberg*. Pre-enrichment into nutrient broth prior to inoculation into TT broth was beneficial in these 2 cases. For irradiated *S. newport*, however, TT broth was the better medium.

CONCLUSIONS

IN CONCLUSION, it can be stated that reasonably satisfactory recovery of salmo-

nellae can be obtained from irradiated or unirradiated poultry or egg magma using BG, BiS or MC agar plates. It should be realized that recovery on these media will probably be less than maximum and in the present study it was found, in most cases, to range from 40–85% of the maximum. Since many workers (Taylor et al., 1958; Galton and Boring, 1964) have concluded that of these 3 media BG agar has the best selectivity and capability for distinguishing *Salmonella*, it would be considered the solid plating medium of choice for recovering salmonellae from irradiated or unirradiated foods known to be highly contaminated.

However, in practical applications it is expected that the MPN method would be employed for enumerating the number of salmonellae in irradiated foods, because the number of survivors would most probably be below the level detectable by direct plating. In this case, it is indicated that pre-enrichment in a nonselective broth, such as nutrient broth, followed by subculture into TT broth and confirmation on BG agar, would give satisfactory results.

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ROBERT SULLIVAN, ALEXANDER C. FASSOLITIS and RALSTON B. READ JR.
U.S. Department of Health, Education and Welfare, Public Health Service
Food and Drug Administration, Division of Microbiology, Cincinnati, Ohio

METHOD FOR ISOLATING VIRUSES FROM GROUND BEEF

SUMMARY–A method was developed to isolate viruses from ground beef. 5, 1-g samples were taken from each loaf of meat and each sample dispersed in 4 ml of Eagle's minimum essential medium. Elimination or reduction of bacterial and fungal contaminants was accomplished by adding antibiotics to the medium to give final concentrations per ml of 1,000 units of penicillin G, 1,000 μg of streptomycin, 50 μg of tetracycline hydrochloride and 5 μg amphotericin B. The meat slurry was adjusted to pH 8.5 with NaOH, shaken at room temperature (23°C) for 1 hr, pH readjusted to 8.0 and the slurry stored at 4°C for at least 16 hr. After this period, the slurry was reshaken for 1 hr at room temperature, filtered through a double layer of cheese cloth and the filtrate assayed for viruses by a plaque-forming unit (pfu) procedure. By repeated experiments, this method recovered approximately 75% of input coxsackievirus B-2. The method was used to examine market-purchased ground beef for viruses. 1 to 195 viral pfu/5 g were isolated from 3 of 12 loaves of meat. 1 loaf yielded poliovirus 1 and echovirus 6, 1 yielded poliovirus 2 and 1 yielded polioviruses 1 and 3.

INTRODUCTION

THERE WERE 345 reported outbreaks of food-borne illness in the United States in 1968, involving 17,567 people. The causative factors in 85 of these outbreaks could not be determined and in 15 of these 85 beef was suspected as the implicated food. Similar significant numbers of food-borne illneses of unknown etiology were reported in previous years (U.S. DHEW, 1969). This situation prompted us to develop techniques for the assay of foods for viruses. The limited data on viruses in foods suggested that they may play an important role in food-borne diseases.

Milk has been implicated in at least 5 outbreaks of poliomyelitis (Aycock, 1927; Dingman, 1916; Goldstein et al., 1946; Knapp et al., 1926; Lipari, 1951). In all these outbreaks, it is probable that the milk was contaminated with poliovirus during improper handling under unsanitary conditions in the dairies. Bovine strains of parainfluenza 3 virus have been isolated from milk from mastitic cows (Kawakami et al., 1966). Etiological agents of teat lesions in cattle have been shown to include viruses of the pox group, herpes group and papova group (Huck, 1968).

Poliovirus was recovered from blowflies 15 days after experimental contamination (Dave et al., 1965a) and cockroaches harbored the viruses for 51 days (Dave et al., 1965b). This indicates the possibility of mechanical vectoring of these agents into foods. Polioviruses and echoviruses have been isolated from the soil of fields irrigated with sewage, and some of the vegetables grown in these fields were contaminated with cytopathic agents (Bagdasar'yan, 1964a). Echoviruses and coxsackieviruses survived on vegetables stored under household conditions, and polioviruses survived on radishes for over 2 months (Bagdasar'yan, 1964b). Hoof-and-mouth disease virus has

survived in meat for 73 days (Dimopoullos, 1960). It was possible to detect poliovirus and coxsackieviruses in various experimentally contaminated frozen foods for up to 5 months (Lynt, 1966). The virus (or viruses) of infectious hepatitis is another likely candidate for food-borne disease. Cliver's (1969) review of food-associated viruses cites epidemiological evidence in many outbreaks for probable viral contamination of food by handlers.

Application of heat is 1 method of inactivating viruses. Fortunately many, if not most, viruses are inactivated within 5 min when heated to 65°C. This indicates that properly cooked foods and properly pasteurized milk should present no public health problem. There are many other factors which may limit occurrences of overt viral diseases. For instance, individuals may be immune to certain viruses as a result of previous clinical or subclinical infections. Because of the ubiquitousness of viruses, however, they may enter foods during preparation, thereby creating primary foci for epidemics (Berg, 1964). This becomes particularly important when foods are prepared in 1 location and shipped to another. The consuming population may have little or no immunological experience with viruses that might be present in foods.

Although proper heating or cooking of foods, cleanliness in their preparation and the immunological status of the consumers act as barriers to virus-caused diseases, it is necessary to determine what and how many viruses are in various foods. To achieve this objective a relatively simple procedure is needed in order to examine a large number of specimens. This communication describes such a procedure developed in this laboratory along with the results of its application to a small number of market-purchased samples of ground beef.

MATERIALS & METHODS

Meat

Ground beef was purchased from refrigerated, self-service counters in packages containing 1–3 lb of meat. The packages were intact and the meat appeared to be of good quality.

Preparation of meat for assay

At least 5, 1-g portions were taken from different parts of a loaf of meat. These were combined and a small loaf formed at the bottom of a 7.5- by 18.5-cm sterile plastic bag. Formation of the loaf was conveniently done by placing the bag on a hard surface and moving the meat with a wooden tongue depressor applied to the outside of the bag. The bag and its contents were placed at −60°C until the loaf was frozen. The frozen loaf was returned to room temperature (23°C) and 1-g portions made by cutting through the plastic and the meat. The pieces of plastic were removed and each meat portion placed in individual 17- by 100-mm polystyrene tubes with friction-fit polyethylene caps. The tubes contained 4 ml each of Eagle's (1959) minimum essential medium (MEM), with nonessential amino acids in Hanks' balanced salt solution (Hanks et al., 1949) without phenol red. This medium contained antibiotics in concentrations of penicillin G, 1,000 units/ml; streptomycin sulfate, 1,000 μg/ml; tetracycline hydrochloride, 50 μg/ml and amphotericin B, 5 μg/ml; the pH had been adjusted to 8.5 with NaOH. Each tube with its contents was shaken by hand so that the meat was dispersed in the medium and pH readjusted to 8.0 with NaOH. The tubes, tightly capped, were placed in a test-tube rack in a horizontal position and the rack secured to a reciprocating shaker. The mixtures were agitated vigorously for 1 hr at room temperature, then placed at 4°C at least 16 hr. The meat slurries were returned to room temperature and again agitated for 1 hr. They were then filtered through a double layer of cheese cloth. This filtration of each 5-ml slurry was easily done by placing the cheese cloth in the bottom of a barrel of the syringe. The plunger of the syringe was used to force the liquid portion of the mixture through the cheese cloth into a polystyrene tube. The tubes that had contained the meat slurries were rinsed twice with 2.5 ml of MEM at pH 7.5 and the 5 ml of rinse forced through the cheese cloth containing the meat debris. The rinse filtrate was added to the meat filtrate so that the volume of material from each 1-g portion to be assayed was approximately 10 ml.

The 10 ml of filtrate was inoculated into 5, 6-oz prescription bottles containing confluent monolayers (45 cm^2) of primary cell cultures from *Cercopithecus aethiops* (African Green) monkey kidneys. An Ionagar No. 2-Eagles medium overlay, as previously described (Sullivan et al., 1968), was used in the viral plaque-forming unit (pfu) assay system.

Method evolution

Coxsackievirus B-2 VR 29 was used as an indicator of recovery efficiency as various procedures were studied. Samples of ground beef shown to be virus-free, at least by the procedure in its stage of development at that time, were inoculated with approximately 7,000 viral pfu as determined from titrations of the virus in Eagle's MEM.

High-speed blenders were used to make meat slurries. Air-pressure filtration through coarse fiber-glass pads and filtration through cotton and cheese cloth were investigated. Antibiotic levels used to reduce or eliminate the unwanted bacterial contamination in the assay of milk for viruses (Sullivan et al., 1968) were adequate for the meat assay. 5 μg of amphotericin B per g of meat was necessary for fungal suppression. Optimum pH levels for elution of viruses from magnetic iron oxide slurries (Rao et al., 1968) were used for the meat slurries and found satisfactory.

RESULTS & DISCUSSION

Method evolution

Comment is warranted on some largely mechanical difficulties encountered in developing the method. Direct inoculation of meat or untreated meat slurries on cell sheets caused cellular necrosis. Use of high-speed blenders for making ground-beef slurries was objectionable because of the number of blenders needed and the difficulties in harvesting the meat slurries from the blenders. Sterile plastic bags were convenient to use and, if necessary, could be sent to the location of an outbreak of food-borne illness for collection of suspected food samples. The bags containing Eagle's MEM at pH 8.5 with the high levels of antibiotics could be shipped in ice from the laboratory, the food added at the field site and reshipped back to the laboratory in ice. Ground beef is easily dispersed in the MEM by kneading and shaking.

Attempts to filter meat slurries by air pressure through coarse fiber-glass discs resulted in rapid clogging. Cotton, both absorbent and nonabsorbent, placed in the bottom of a syringe required high hand pressures for filtrations. When glass syringes were used, breakage was common; this presented a safety problem. Disposable syringes eliminated this hazard and were less expensive, but still filtration was difficult. The combination of a double layer of cheese cloth and a disposable syringe gave rapid, convenient filtration. The adjustment of the pH with $NaHCO_3$ resulted in gel formation and subsequent clogging of the cheese cloth. This problem was solved by using NaOH for pH adjustment.

The evolved method has been used in this laboratory for over a year in a study of the radioresistance of coxsackievirus type B-2 in ground beef. A 75% average recovery of this virus from the meat has been achieved, if viral recovery from Eagle's MEM is used an an indicator of 100% recovery. The sensitivity of the method is essentially the same for recoveries of 10, 50 and 7,000 pfu of inoculated coxsackievirus B-2 from gram amounts of ground beef. The data to illustrate this are presented in Table 1.

Assay of market meat

To test the usefulness of this method, 12 packages of ground beef purchased at different times from 12 different markets were assayed for the presence of viruses. Results are summarized in Table 2. As shown, 3 loaves of meat were found to contain viruses. All of the viral isolates were serologically identified by the Enterovirus Infections Unit, National Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia. The 12 packages of meat were purchased during June through February. The first positive meat was purchased in October and contained 2 viruses identified as poliovirus 1 and echovirus 6; there was an average of 195 viral pfu/5 g of this meat. The second isolate, identified as poliovirus 2, was found in meat purchased in January; there was an average of 6 viral pfu/5 g meat. The third positive meat was purchased in February and contained poliovirus 1 and poliovirus 3; there was an average of 1 viral pfu/5 g of this meat. More testing must be done before any significance is attributed to possible seasonal variation of viruses in ground

Table 1–Recovery of virus from ground beef inoculated with coxsackievirus B-2.

Average input (pfu[1]/g)	1-g samples	Average recovery (pfu/g)	Percent
7700	50	6200	80
50	50	42	84
10	50	6	60
Over-all average recovery			75

[1] Viral plaque-forming units.

Table 2–Viral isolation from ground-beef samples purchased from markets.

Source	Number of meat loaves examined	Average pfu[1]/5 g	Virus identified
9 different markets	9	0	–
3 different markets	1	195	Poliovirus 1 and echovirus 6
	1	6	Poliovirus 2
	1	1	Poliovirus 1 and poliovirus 3

[1] Viral plaque-forming units.

beef, or the extent of their distribution in the supply of ground beef.

The route by which the viruses entered the meat remains open to speculation. Insofar as we could determine from the literature, it is not known whether human enteroviruses are capable of infecting cattle. Neither is it known what the fate of these viruses would be in the alimentary canal of these animals. That coliform bacteria in ground beef are not unusual indicates that this meat could be contaminated with enteroviruses during processing or handling.

Fortunately, studies done in this laboratory on thermal inactivation of viruses likely to be food-borne indicate that these viruses are inactivated within 5 min at temperatures above 65°C. The long-established procedures for production and preparation of foods in properly managed food-processing plants act as significant barriers to viral food-borne disease. These procedures include use of healthy animals, scrupulous cleanliness in all phases of food preparation and, finally, their proper cooking (U.S. DHEW, 1962).

The simplicity of the method described for the isolation of viruses in ground beef meat makes it possible to survey this commodity at different stages of production. The method could be applied to meats involved in outbreaks of illnesses in which no causative factor can be determined by standard biological and chemical analysis.

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J. MARLETTA[a] *and C. R. STUMBO*
Department of Food Science and Technology, University of Massachusetts, Amherst, Massachusetts 01002

SOME EFFECTS OF ETHYLENE OXIDE ON BACILLUS SUBTILIS

SUMMARY–A number of factors of possible significance in elucidating the mechanism of inactivation of spores exposed to the alkylating agent, ethylene oxide (ETO), were investigated in this study. The sterilant system consisted of a mixture of ethylene oxide (12%) and dichlorodifluoromethane (88%). Spores were exposed to ETO and suspended in distilled water overnight to determine if the ETO treatment would affect release of protein, RNA, DNA, or DPA. Only DPA release was affected, it being appreciably greater from exposed than unexposed spores. Pretreatment of spores with agents postulated to affect the intactness of the spore coat was noted to alter ETO resistance characteristics. The resulting survivor curve exhibited an unusually long death lag period followed by an increased first-order death rate. Such an alteration, however, could be counteracted by increasing the time between pretreatment and ETO exposure. A comparison of lyophilized vegetative cells, germinated spores, and heat-activated spores revealed that the ETO resistance of germinated spores was closer to that of heat-activated spores than to that of vegetative cells. To obtain a logarithmic death rate for heat-activated spores, it was necessary to precondition at about 98% R.H. prior to the normal preconditioning at 33% R.H. Vegetative cells and germinated spores, however, did not require preconditioning other than at 33% R.H. The effect of increasing time of ETO exposure on germination and postgerminative and vegetative development of spores in a rich medium and in a deficient medium was evaluated. No significant change in rate or efficiency of germination was observed for either medium. However, the postgerminative phase of development of treated spores was notably lengthened over that of untreated spores in either medium. Treated spores which were incubated in the rich medium entered the vegetative phase of growth before those incubated in the deficient medium, this difference becoming more pronounced with increasing periods of exposure.

INTRODUCTION

VAPOR PHASE sterilization has been studied with considerable interest during the past 30 years as a means of reducing to an acceptable level, contaminating microbial populations on or within materials which are heat- and moisture-sensitive. Certain chemicals which are microbicidal in the vapor phase, notably the epoxides, remain effective at relatively low temperatures and low humidities. In these respects, methods of sterilization employing such chemicals have certain distinct advantages when compared to conventional methods employing moist or dry heat.

Ethylene oxide, (ETO) the simplest of the epoxide compounds, has been shown to possess microbicidal properties in the aqueous as well as in the gaseous phase. An adequate explanation of the inactivation mechanism by which microorganisms are rendered sterile when exposed to such a sterilant has been lacking. Much of the difficulty in this respect lies in the fact that ETO is capable of reacting with many components in microbial cells provided a nucleophilic moiety is present. Phillips (1952) proposed that alkylation by ETO of free carboxyl, amino, hydroxyl or sulfhydryl groups on bacterial proteins served to disrupt metabolic processes, eventually causing death of the organism. He felt that the greater resistance evidenced by spores over that of vegetative cells could be explained by a folding of protein molecules in spores to provide greater protection of sulfhydryl groups of key enzyme systems.

More recently, evidence has been presented which suggests that reaction with nucleic acids is the significant reaction in sterilization by a variety of alkylating agents (Brookes and Lawley, 1960; Fraenkel-Conrat, 1961; Alexander et al., 1961). Lawley and Brookes (1968) studied alkylation in sensitive and resistant strains of *E. coli*. They hypothesized that resistant strains were better able to withstand alkylation due to the influence of enzymatic DNA repair processes which allow recovery of damaged cells.

Almost no attention has been given to similar studies as the above using spore forms. Shull (1963) suggested that certain peptides supplied in the recovery medium for spores which had been treated in a solution of ETO served as agents in the repair of synthetic mechanisms disrupted as a result of alkylation. The present study was undertaken to contribute additional knowledge of the effects of ethylene oxide on spores, germinated spores and vegetative cells of *Bacillus subtilis*.

MATERIALS & METHODS

Microorganism

B. subtilis ATCC 9524 was selected for this study. The culture's identity and purity were confirmed prior to its use.

Production and harvesting of spore crop

The sporulation medium consisted of standard nutrient agar with 10 ppm added manganous sulfate. After steaming to insure uniformity, a 250 ml quantity was placed in each of a large number of 1–qt, rectangular orange juice bottles. These bottles were then autoclaved for 20 min at 121°C, slanted and allowed to cool.

A broth culture of the organism was obtained by inoculating 100 ml of nutrient broth with cells from a typical colony and incubating at 30°C for 24 hr. (An incubation temperature of 30°C was used throughout for culturing *B. subtilis*.) 1 ml of this culture was pipetted onto each of 3 bottle slants and spread over the agar surface by tilting. These were then incubated for 4 days to achieve maximal sporulation.

20 ml of cold (2°C) sterile distilled water (CSDW) and 10–15 sterile, galvanized, half-inch machine nuts were added to one of the incubated slants. Using a gentle shaking motion, sporulated growth was very quickly suspended without contamination by bits of agar. The resulting suspension was poured into a chilled (2°C) container, leaving the machine nuts behind on the slant. Another 20 ml of CSDW was added to this slant to suspend any remaining growth. This suspension and the machine nuts were transferred to another of the slants. Growth on this was suspended as described above and combined with that from the first slant. A second washing was performed as for the first slant and the whole procedure repeated for the third slant. The pooled suspension was heated at 80°C for 20 min to activate the spores. 1 ml of this was added to each of a large number of bottle slants, which were then incubated as before. The steps outlined above for the first set of slants were followed to obtain the final spore crop.

Cleaning of spores

Since repeated washings did not eliminate all vegetative cells from the crude spore suspension, lysozyme treatment was considered appropriate if it proved to have no effect on spore resistance to ETO.

Before lysozyme treatment of all of the crude spore crop, a small quantity was removed and divided into two portions, one for lysozyme treatment and the other for sonication. Complete lysis of vegetative cells was effected by the addition of 0.3 mg of lysozyme per ml and incubation for 2.5 hr at 2°C. Spores were freed of vegetative cells by sonication in a model U–20 Biosonik[b] operating at a frequency of 20 Kc for 1 hr.

Spores so treated were then washed three times, heat-activated after adjustment of concentration to about 10^8 spores per ml, deposited on paper discs, humidity equilibrated, and exposed to ETO to determine their comparative resistance. Results, which will be presented later, indicated that the lysozyme treatment had no effect on resistance; therefore, it

[a]Present address: Bio-Marine Research Laboratory, State Fish Pier, Gloucester, Mass. 01930

[b]Bronwill Scientific Co., Rochester, N.Y.

was applied to the remainder of the crude suspension. The treated suspension was then centrifuged in the cold at 8000G for 15 min, the supernatant discarded and spores in the resulting pellet resuspended in CSDW. The spores were washed twice more in this fashion.

Lyophilization of spore crop

After the final washing, the spores were resuspended in 150 ml of CSDW and heated at 80°C for 20 min. The spore suspension was then divided equally among six 50 ml polyethylene centrifuge tubes and the tubes centrifuged (8000G) at 2°C. After centrifugation, the supernatants were discarded, and the spore pellets frozen and lyophilized in the centrifuge tubes. The spore powder so obtained was distributed in ampoules, sealed under vacuum, and stored at −20°C.

Obtaining samples of heat-activated spores, germinated spores, and vegetative forms for comparative resistance studies

Before lyophilization of the washed spore crop obtained earlier, a portion of this spore pellet was placed into 0.02M K_2HPO_4, centrifuged, and the resulting pellet lyophilized.

A crop of germinated spores was obtained by inoculating nutrient broth with the lyophilized spores and following germination spectrophotometrically until germination was complete. These germinated spores were then centrifuged, washed with 0.02M K_2HPO_4, and lyophilized.

Vegetative cells were produced in nutrient broth, centrifuged, washed with 0.02M K_2HPO_4, and lyophilized as above. The use of 0.02M K_2HPO_4 for suspending *B. subtilis* cells was recommended by Demain (1958).

Preparation of test population for exposure

For studies involving spores deposited on absorbent paper discs and exposed to ETO, lyophilized spores were resuspended in distilled water to give about 10^8 spores per ml. As carriers, paper discs 3/8 inch (antibiotic assay discs) were placed on pyrex discs and sterilized. Each paper disc was then inoculated with 0.01 ml of the test suspension. These samples were allowed to equilibrate overnight at 33% R.H.

When larger quantities of spores were needed, lyophilized spores were deposited in a thin layer over the bottom of a sterile petri plate and the samples allowed to equilibrate as above prior to ETO exposure.

Exposure apparatus

The apparatus used for exposure was the same as that described by LIU et al. (1968).

Relative humidity, temperature and ethylene oxide concentration

A relative humidity of 33%, a temperature of 40°C, and an ethylene oxide concentration of 700 mg per liter were the exposure parameters used. The sterilant consisted of a gaseous mixture containing 12% ethylene oxide and 88% dichlorodifluoromethane by weight.

Exposure procedure

Essentially, exposures were made in the same manner as described by LIU et al. (1968). After exposure, samples exposed on paper discs were placed in individual 10 ml dilution blanks containing 1% Darvan No. 1 (a dispersant). The tubes were then agitated on a shaker until the paper discs were pulverized. After appropriate dilutions in CSDW, triplicate pour-plates were prepared for each blank. Nutrient agar containing 0.1% soluble starch, to aid in germination of treated spores, was employed throughout as the recovery medium.

When samples were exposed as a freeze-dried powder, a portion of the exposed sample (heat-activated spores, germinated spores, or vegetative cells) was placed into one of two types of diluent. Heat-activated spores were placed in 1% Darvan No. 1, while germinated spores and vegetative cells were placed in 0.02M K_2HPO_4. After shaking to disperse clumped cells, each suspension was poured into a colorimeter tube. The optical density of each sample was then adjusted with the appropriate diluent to a value of 0.20, resulting in a cell concentration of 10^7-10^8 per ml. These adjusted suspensions were diluted and replicate plates prepared as described above.

Construction of survivor curves and calculation of death rates

During each exposure period, three samples were exposed and each was diluted and plated in triplicate. Colony counts were made after 2–3 days incubation. The colony counts were averaged and the logarithms of the averages taken. Logarithms of average survivor values thus obtained were plotted against corresponding times of exposure to obtain survivor curves. Three such determinations were generally carried out. The method of least squares was used to determine the lines of best fit for straight line portions of the curves, and the resulting regression coefficients were used to determine the D values (times required to destroy 90% of the cells present).

Leakage of spore components after ETO exposure

Lyophilized spores were severely exposed (3 hr) to the sterilant at 33% R.H., 40°C, and at an ETO concentration of 700 mg per liter. A control was prepared by exposing spores for 3 hr at 33% R.H., 40°C, and at an air pressure of 5 psig (the same as the sterilant gas pressure used for the treated sample). Both were placed into CSDW and left on a shaker at 2°C overnight. The suspensions were then centrifuged at 8000G for 15 min and the supernatant decanted and saved for analysis.

Preliminary to chemical analysis, a UV absorption spectrum was determined to approximate extent of leakage. The following chemical analyses were carried out on each supernatant:

1. Protein: Lowry et al. (1951).
2. RNA: Ceriotti (1955).
3. DNA: Burton (1956).
4. DPA: Janssen et al. (1958).

Spore coat disruption

To rupture disulfide bonds and generally "loosen" macro-molecular structure of the spore coat, the technique of Gould and Hitchins (1963) was employed. Spores were suspended in a 1% (v/v) solution of thioglycollic acid in 8M urea, after which, the suspension was heated at 70°C for 1/2 hr. After centrifugation and five washings, 0.01 ml quantities of suspension were added to paper discs and the inoculated discs humidity equilibrated (24 hr at 33% R.H. and 2°C).

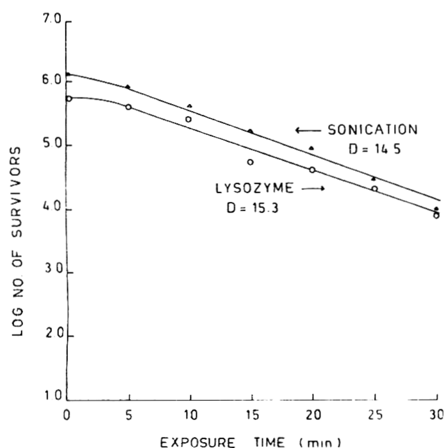

Fig. 1–Survivor curves for B. subtilis spores cleaned by sonication and by lysozyme.

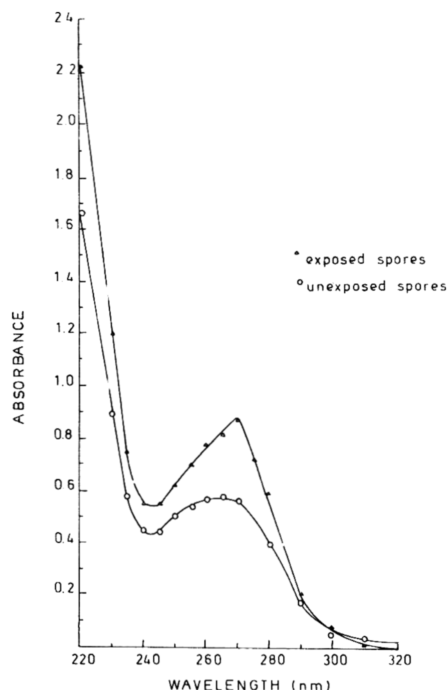

Fig. 2–Ultraviolet absorption spectra of supernatants from control spores of B. subtilis and spores exposed to ETO.

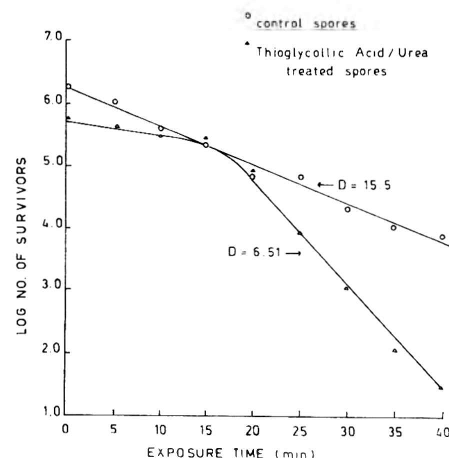

Fig. 3–Survivor curves for B. subtilis control spores and spores treated in thioglycollic acid plus urea.

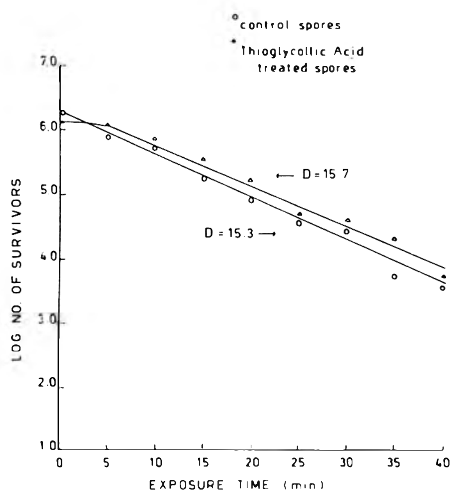

Fig. 4—Survivor curves for B. subtilis control spores and spores treated in thioglycollic acid plus urea, but preconditioned at 33% R.H. for 7 days prior to exposure to ETO.

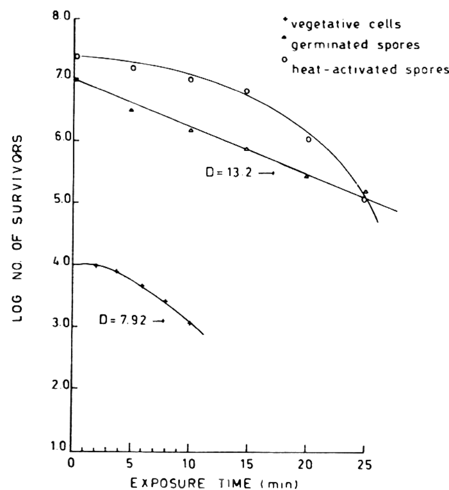

Fig. 5—Survivor curves for heat-activated spores, germinated spores, and vegetative cells of B. subtilis.

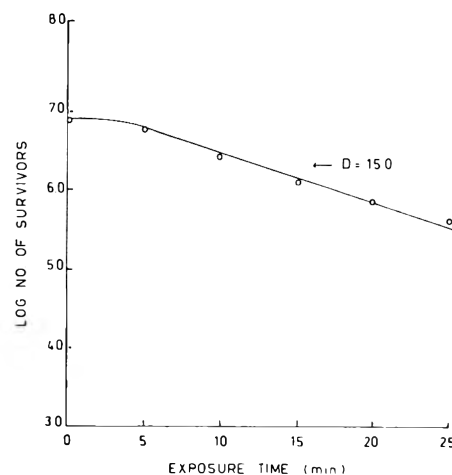

Fig. 6—Survivor curve for heat-activated spores of B. subtilis preconditioned at 98% R.H. followed by preconditioning at 33% R.H.

Post treatment development of spores

To observe the various stages of development of ETO treated spores, two types of liquid media were utilized: 1) nutrient broth plus 0.1% soluble starch, and 2) minimal salts broth for spores (Demain, 1958).

A quantity of lyophilized spores was placed in the appropriate medium in a Klett flask and the optical density adjusted to 0.32 for each sample with the desired growth medium. After addition of spores, the flasks were kept in an ice-water bath until all samples were ready so that initiation of germination would be synchronized. The flasks were placed on a reciprocating shaker in a 30°C incubator and the optical density measured periodically.

RESULTS & DISCUSSION

Resistance of spores cleaned by sonication versus lysozyme

In spite of concerted efforts to promote maximal sporulation, a large number of vegetative cells remained in the spore suspensions obtained after harvesting and washing. It was felt that ETO resistance results might be affected by these vegetative cells offereing a protective barrier for spores against sterilization. Removal of these cells by either lyozyme treatment or sonication was considered, the former technique being preferred. Therefore, an experiment was designed to determine if there would be any difference in ETO resistance of spores cleaned in either manner, and if in either case, the resistance was different from that reported by Liu et al. (1968) for spores which had been water washed only. They reported a D value of 14.7 min for the same organism under identical exposure conditions.

Upon exposure to ETO of spores cleaned by sonication and spores cleaned by lysozyme, no significant difference in resistance was noted (see Fig. 1). It may be noted that the D value reported by Liu et al. (1968) lies between the two found here. Therefore, lysozyme treatment was adopted as the cleaning method in this study.

Release of materials from exposed spores

To determine if leakage of various spore components had occurred as a result of ETO treatment, the supernatants obtained after removal of control spores and exposed spores by centrifugation were analyzed both spectrophotometrically and by standard colorimetric assays. It may be noted from examination of the curves in Figure 2 that UV-absorbing materials were present in both supernatants, and additionally, that differences in DPA release may be indicated by the relatively higher peak at 270 nm for the supernatant from exposed spores.

When absorbance values obtained in the assay for protein, RNA, DNA, and DPA were referred to standard curves, it was found that a considerably greater quantity of DPA had been released from exposed spores than from control spores (0.37% of spore dry weight as opposed to 0.23% for the control). Nearly equal amounts of the other components tested for were found in the supernatants from exposed and control spores.

Effect of thioglycollic acid/urea treatment on resistance

Pretreatment of spores with agents postulated to affect the intactness of the spore coat was noted to alter ETO resistance characteristics so that the survivor curve exhibited what appeared to be an unusually long death lag period followed by an increased first-order death rate in relation to the control (see Fig. 3). However, if after the pretreatment, spores were left to humidity equilibrate for 7 days and then exposed, a typical survivor curve was obtained (Fig. 4). It was theorized that reformation of tertiary structure in the spore coat by oxidation of sulfhydryl groups formed in the pretreatment to the original disulfide linkages may have occurred during prolonged equilibration in order to reinstate typical resistance to ETO.

Comparative resistances of lyophilized heat-activated spores, germinated spores, and vegetative cells

Heat-activated spores which had been humidity equilibrated at 33% R.H. and exposed to ETO displayed non-logarithmic death characteristics (Fig. 5), but this could be reversed by preconditioning at 98% R.H. for 7 days prior to preconditioning at 33% R.H. for 3 days. (Fig. 6). The necessary preconditioning periods are indicated in the sorption and desorption curves of Figure 7.

Germinated spores and vegetative cells, however, displayed a logarithmic death rate with only the 33% R.H. preconditioning. It can be seen from comparing D values in Figures 5 and 6 that the resistance of germinated spores is much closer to that of heat-activated spores than to that of vegetative cells.

It appears that incomplete rehydration of spore components critical to sterilization resulted in the non-logarithmic death of heat-activated spores preconditioned at 33% R.H. (Gilbert et al., 1964, found that preconditioning at a high R.H. was necessary for complete rehydration of highly desiccated spores.) The reason

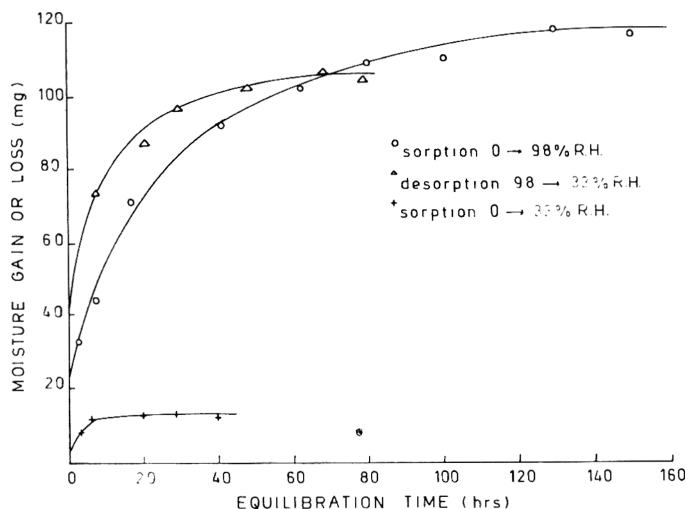

Fig. 7–Rate of sorption and desorption of moisture by lyophilized heat-activated spores of B. subtilis equilibrated at various relative humidities and at a temperature of 2°C.

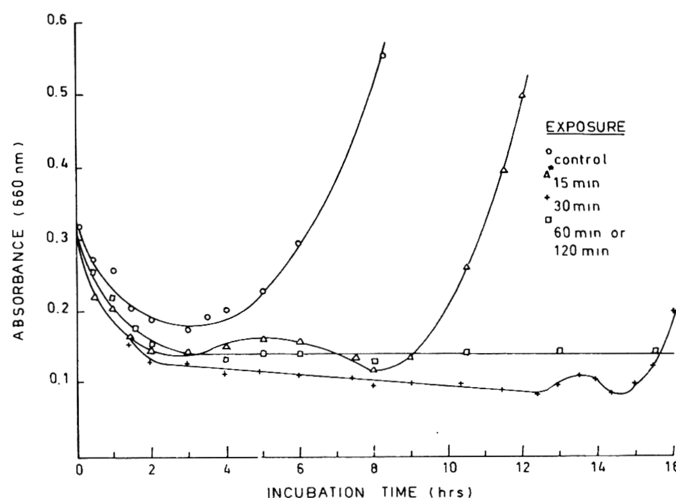

*Fig. 8–Behavior of B. subtilis spores incubated at 30°C in minimal salts broth after exposure for various periods of time to ETO at 33% R.H., 40°C, and at an ETO concentration of 700 mg per liter. *(Germinative phase of spores exposed for 30 min is also represented by this symbol.)*

germinated spores and vegetative cells were not as moisture-sensitive was very likely due to the greater permeability of these organisms.

The finding that germinated spore resistance was so close to that of heat-activated spores was not expected in view of the variety of physiological and metabolic changes which occur during germination. Evidently, although many reactive sites become available upon germination, critical sites to sterilization, such as nucleic acids, which are located within the core, may continue to resist alkylation to the extent that resistance still resembles that of heat-activated spores.

Behavior of treated spores in nutrient broth and minimal salts broth

The effect of increasing time of ETO exposure on germination and postgerminative and vegetative development of spores in a rich medium and in a deficient medium was evaluated (Fig. 8 and 9). In these curves, the germinative phase refers to that period during which the initial absorbance decreases and finally levels off; the vegetative phase is characterized by a steady increase in absorbance indicating emergence of new vegetative cells and later, cell division; and the postgerminative phase is that period of growth between the end of the germinative phase and the beginning of the vegetative phase.

No appreciable change in rate or efficiency of germination was found for spores in either medium as severity of exposure increased. These findings are in agreement with those of Shull (1963) who treated spores of *B. subtilis* in a water solution of ETO. However, the postgerminative phase of development of treated spores was notably lengthened over that of untreated spores in either medium. Treated spores which were incubated in the rich medium entered the vegetative phase of growth before those incubated in the deficient medium, this difference becoming more pronounced with increasing periods of exposure. During the postgerminative phase in the minimal medium, it was noted, for spores which had been ETO treated for only 15 min, that some vegetative cells began to emerge from treated spores; but, soon after, these cells apparently lysed causing absorbance of the suspension to decrease.

The observed differences for treated spores in the two kinds of growth media may be explained if the metabolic processes leading to formation of vegetative cells are considered to be inhibited in damaged spores. Thus, such inhibition should be magnified in a medium deficient in nutrients essential for repair and recovery.

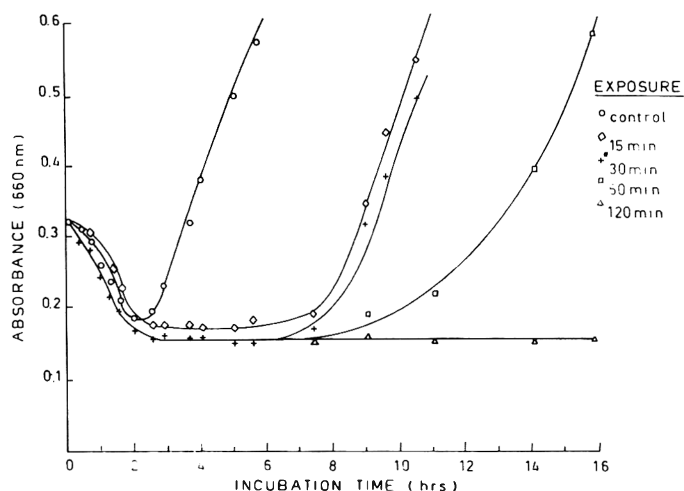

*Fig. 9–Behavior of B. subtilis spores incubated at 30°C in nutrient broth after exposure for various periods of time to ETO at 33% R.H., 40°C, and at an ETO concentration of 700 mg per liter. *(Germinative and postgerminative phases of spores exposed for 60 min and 120 min are also represented by this symbol.)*

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G. T. MICHAEL[a] *and C. R. STUMBO*

Department of Food Science and Technology

University of Massachusetts, Amherst, Massachusetts 01002

ETHYLENE OXIDE STERILIZATION OF SALMONELLA SENFTENBERG AND ESCHERICHIA COLI: DEATH KINETICS AND MODE OF ACTION

SUMMARY—Death of lyophilized Salmonella senftenberg *and* Escherichia coli *when exposed to gaseous ethylene oxide (ETO) followed first order kinetics.* S. senftenberg *in whole egg powder was about twice as resistant as it was in the clean state. Supplementation of a minimal growth medium with amino acids, organic acids, the base components of DNA and RNA, vitamins and nucleic acid sugars demonstrated that guanine and guanosine triphosphate were the only supplements which would promote reproduction of cells surviving severe ETO treatment. Treatment of guanine and guanosine triphosphate with ETO prior to adding them to the minimal medium caused guanosine triphosphate, but not guanine, to lose the ability to promote reproduction, indicating that under the conditions of this experiment, only the guanosine triphosphate was alkylatable; and that, it was alkylation of this component of DNA that caused the bacterial cells to lose their power of reproduction.*

INTRODUCTION

IT HAS BEEN well established that certain chemicals in the vapor phase are effective microbicides. Of the chemicals thus far tested, ethylene oxide (ETO) appears to be the most promising for widespread use in the sterilization of many materials that are so sensitive to dry or moist heat as to preclude the use of these agents in their sterilization. Moreover, for many applications, sterilization with ETO has been found more convenient and economical, even though heat or some other agent might well be employed.

During the past forty years, ETO vapor phase sterilization has been studied by a number of investigators, including Griffith and Hall (1938), Kaye (1949), Kaye and Phillips (1949), Phillips (1949, 1952, 1968), Opfell et al. (1959), Ernst and Shull (1962), Vondell (1962), Gilbert et al. (1964), Doyle and Ernst (1967), LIU et al. (1968), Kuzminski et al. (1969), Blake and Stumbo (1970) and others. It has been established that death kinetics of microorganisms subjected to ETO are influenced by a number of factors, principally by temperature, ETO concentration in the sterilant atmosphere, relative humidity of the sterilant atmosphere, water activity of the microorganisms to be killed, level of contamination, microbial species to be eliminated, and physical and chemical nature of the material to be sterilized.

Most studies to date have been with spores more resistant to ETO than most vegetative forms. The reason for this is most logical—any sterilization treatment which will destroy the most resistant microbial forms will generally free the material of all less resistant forms. However, there are numerous occasions wherein the primary object is to free materials of vegetative forms, particularly pathogens, with no real concern about the more resistant spore forms. This is true in the case of certain medical supplies and equipment and of certain food ingredients to be used in formulated products which receive no additional sterilization treatments.

Salmonellosis is today the predominant food-borne infection. Foods commonly implicated in salmonellosis outbreaks are those which in their preparation are not heated sufficiently to destroy Salmonellae, or may become contaminated subsequent to any heat process given. ETO sterilization of some dry foods and food ingredients is now carried out. Notwithstanding the gravity of the salmonellosis problem, and the promise of ETO sterilization in dealing with it, there has been virtually nothing reported on the kinetics of death of Salmonellae subjected to ETO.

Regardless of the many years of rather extensive work with ETO as a sterilant, there remains a paucity of information regarding the mechanism by which ETO kills microorganisms. There is little doubt that the action is alkylation (see Phillips, 1952; Ross, 1958; Wheeler, 1962; Alexander et al., 1957, 1961). However, exactly what is alkylated to cause the microbial cell to lose reproductive ability has remained in doubt.

Therefore, the present study was undertaken to study the death kinetics of *Salmonella senftenberg* and *Escherichia coli*; and, to further elucidate the mechanism by which ETO kills microorganisms.

[a]Present address: Colonial Provision Co., Inc., Boston, Mass.

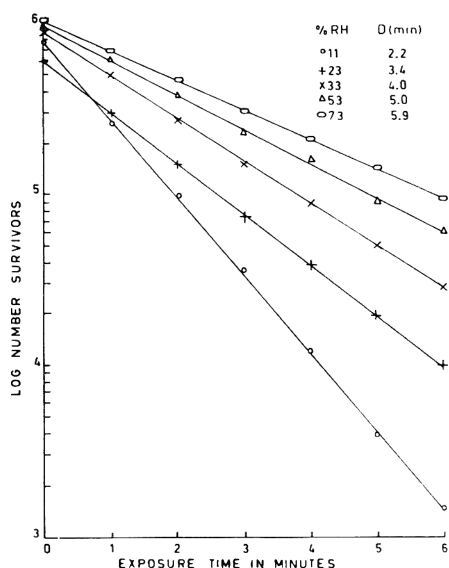

Fig. 1–Survivor curves for lyophilized cells of S. senftenberg preconditioned at different relative humidities.

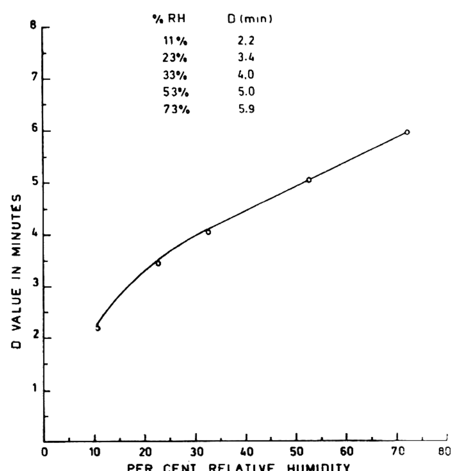

Fig. 2–Influence of relative humidity on ETO resistance of lyophilized cells of S. senftenberg.

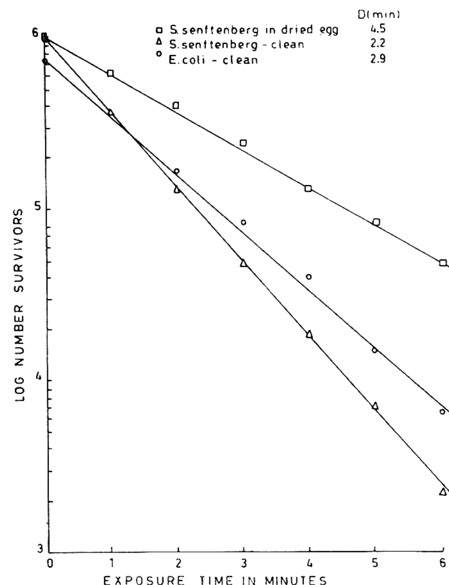

Fig. 3–Survivor curves for lyophilized cells of S. senftenberg and E. coli and for S. senftenberg lyophilized in whole egg. All test samples were preconditioned at 11% R.H.

MATERIALS & METHODS

Test organisms

A culture of *S. senftenberg* 775W which was morphologically smooth (S) was obtained from the Department of Veterinary and Animal Science at the University of Massachusetts which secured the culture from M. M. Galton, C.D.C., Atlanta, Georgia. A culture of *E. coli* K12 was obtained from the Department of Microbiology, University of Massachusetts.

Media employed

Trypticase Soy Agar supplemented with 0.5% Yeast Extract (TSY Agar) was used as the recovery medium for the test organism after treatment with ETO. Trypticase Soy Broth supplemented with 0.5% yeast extract (TSY Broth) was used for the production of the test microorganisms. This broth was also used to observe the growth pattern at 37°C of ETO exposed and unexposed cells.

A Minimal Salts (MS) Broth containing $(NH_4)_2SO_4$ (2g/l), KH_2PO_4 (6g/l), K_2HPO_4 (14g/l), Na Citrate (lg/l), $MgSO_4 \cdot 7H_2O$ (0.2g/l), $MnSO_4 \cdot H_2O$ (0.25 mg/l), and glucose (5g/l) was used to observe the growth pattern at 37°C of ETO exposed and unexposed cells. The same broth plus 1.5% agar was used as a plating medium.

Preparation of clean lyophilized cells

Producing harvesting and cleaning. A loopful of cells maintained on a TSY Agar slant was inoculated in TSY Broth (10–20 ml) contained in culture tubes and incubated for 18–20 hr at 37°C. These actively growing cells were then added to 500 ml of TSY Broth and incubated for 18–20 hr while shaken at 37°C. The resulting culture was centrifuged at 5000G for 15 min and the pellet of cells redispersed in cold, sterile, trypticase buffer (pH 7.0; M/15 KH_2PO_4, and M/15 Na_2HPO_4 plus 0.1% trypticase) and recentrifuged. This procedure was repeated 5 times. The cells were suspended in cold, sterile, trypticase buffer and refrigerated at 4°C until lyophilized (within 3 days).

Lyophilization. A 0.5 ml volume of the washed cell suspension was transferred to each of a number of vials, shell frozen and lyophilized. The vials were hermetically sealed by a torch directly from the freeze-drying manifold to prevent entry of air which could have damaged the lyophilized cells during refrigerated storage at 4°C. A spark coil tester was used to verify that a vacuum seal had been obtained in each vial of lyophilized cells. Those samples containing air were discarded.

Preparation of preconditioned cells

A vial of lyophilized cells was shaken on a Vortex-Genie Mixer for two minutes to produce a finely powdered sample. The dried cells were evenly distributed on a sterile petri dish which was placed in a desiccator, containing a particular solution to maintain a desired relative humidity, at 4°C. To obtain relative humidities of 11, 23, 33, 53 and 73% saturated solutions of lithium chloride, potassium acetate, magnesium chloride, magnesium nitrate, and sodium chloride, respectively, were used. The desiccator and its contents were allowed to equilibrate under refrigeration overnight to precondition the cells to the equilibrium relative humidity of the sterilization exposure atmosphere.

Preparation of lyophilized whole egg containing S. senftenberg.

A washed suspension of *S. senftenberg* cells was uniformly mixed with whole eggs. The inoculated slurry was then lyophilized and powdered with a sterile mortar and pestle. Prior to exposure to ETO this inoculated egg powder was placed in a sterile petri dish and preconditioned overnight, under refrigeration, at 11% R.H.

Exposure system and procedure

The exposure system and procedure used by Liu et al. (1968) were employed.

Sterilant gas

A mixture of ethylene oxide (12%) and dichlorodifluoromethane (88%), by weight, was used as the sterilant gas.

Exposure chamber relative humidity

Relative humidities of 11, 23, 33, 53 and 73% were employed in the exposure chamber. The ideal gas law was applied to calculate the theoretical weight of water to be introduced into the exposure chamber. In addition, relative humidity during any exposure period was continuously monitored with an electric hygrometer, and adjustments made if indicated.

Exposure chamber ETO concentration

A concentration of 700 ± 20 mg/liter of ETO was employed in the exposure chamber.

Temperature

The exposure temperature was 40°C.

Exposure times

The times of exposure were 0, 1, 2, 3, 4, 5, and 6 min.

Estimation of number of surviving cells

Immediately after exposure, 3 mg of the lyophilized clean cells were aseptically transferred to 9 ml of cold, sterile buffer solution. The suspension was then agitated for 2 min on a Vortex-Genie Mixer. Appropriate dilutions were made and pour plates prepared with TSY or MS Agar. Counts of colonies on TSY Agar developing from survivors were made after 2 days incubation at 37°C; while counts of colonies on MS Agar were made after 4 days incubation at 37°C.

In the case of inoculated egg, immediately after exposure, 10 gm of the whole egg powder were added to 90 ml of cold, sterile, pH 7.0 buffer diluent and the mixture blended. TSY Agar pour plates were then made and incubated at 37°C. Colony counts were made after 2 days incubation.

Effect of ETO on cell reproduction, glucose utilization, protein synthesis, RNA synthesis and DNA synthesis

Lyophilized cells of *S. senftenberg* and *E. coli* were preconditioned at the relative humidity which gave the maximum destruction rate (of *S. senftenberg* cells) and exposed to ETO of the same relative humidity at 40°C and 5 psig until about 90% of the population was destroyed. One portion of a treated batch of cells was appropriately diluted and plated in TSY Agar and in MS Agar. Another portion of the same batch of exposed cells and unexposed control cells were added to sterile TSY Broth and sterile MS Broth contained in four flasks which were mechanically shaken while being incubated at 37°C. During the period of incubation, optical density (O.D.), glucose uptake (Glucostat Reagent), protein (Lowry et al. 1951), RNA (Ceriotti, 1952), and DNA (Burton, 1956) determinations were made at selected time intervals to ascertain the effects of gaseous ETO treatment on cellular metabolism.

Nutritional study of the repair process

In order to study the repair process of damaged cells which were previously exposed to ETO, individual flasks of MS Broth containing damaged cells were supplemented with amino acids, organic acids, the base components of DNA and RNA, vitamins, and nucleic acid sugars, respectively, and incubated at 37°C to observe the growth pattern of the treated cells. The damaged cells were also cultured in TSY Broth along with the unexposed control cells cultured in MS Broth and TSY Broth at 37°C. O.D., glucose uptake, protein, DNA, and RNA determinations were made at selected intervals.

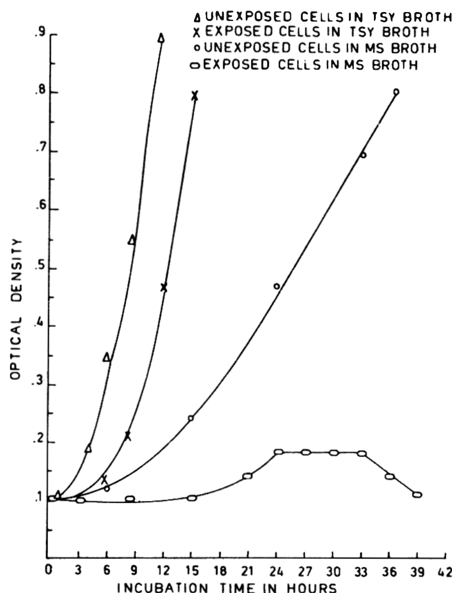

Fig. 4–Growth curves for exposed and unexposed cells of S. senftenberg.

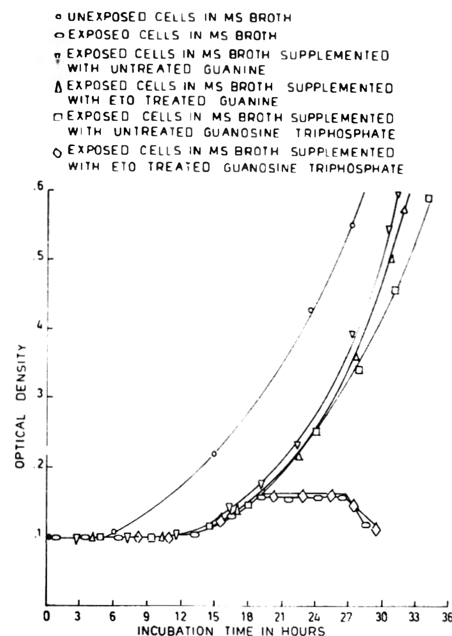

Fig. 5–Effect of ETO treated and untreated guanine and guanosine triphosphate on growth of exposed and unexposed S. senftenberg.

RESULTS & DISCUSSION

LYOPHILIZED CELLS of *S. senftenberg* preconditioned at 11, 23, 33, 53, and 73% relative humidities and exposed to ETO at the same relative humidities, 40°C, and 5 psig were found to be more resistant to the gaseous sterilant as the relative humidity increased from 11 to 73% (see Fig. 1 and 2). In Figure 3 is shown a comparison of the resistance of lyophilized cells of *S. senftenberg* and of *E. coli*; and of *S. senftenberg* cells lyophilized in whole egg powder. It will be noted that *S. senftenberg* cells in whole egg powder were about twice as resistant as were the clean cells. Protection from the ETO, by occlusion in the egg powder may have been a factor; however, had it been a major factor a straight-line survivor curve should not have been obtained. A more logical explanation for the greater resistance of *S. senftenberg* in whole egg would seem to be competition for ETO by alkylatable components in the egg, of which there are many.

Growth curves for cells of *S. senftenberg* which were preconditioned in the lyophilized state at 11% R.H. and exposed to ETO for 1 D value at 40°C, 11% R.H. and an ETO concentration of 700 mg/l, and the growth curves of the unexposed controls are depicted in Figure 4. Almost identical results were obtained with *E. coli*. It was observed that the unexposed cells of *S. senftenberg* and *E. coli* inoculated in TSY Broth grew up first during incubation at 37°C; while ETO exposed cells inoculated in TSY Broth grew up before the unexposed cells in MS Broth. The cells which were exposed to ETO and inoculated into MS Broth appeared, according to O.D. readings, to initiate growth, then level off. Subsequently the O.D. decreased. During the limited increase in O.D. of the exposed cells in MS Broth, the viable cell count on TSY Agar did not change; the cell mass increased; the protein content increased; the RNA content increased; the glucose content decreased; but the DNA content did not change. The decrease in O.D. was due to the lysis of the cells. Therefore, it appeared that the metabolic glycolytic pathways were not completely inhibited. ETO treatment inhibited the DNA synthesis but protein and RNA synthesis were not inhibited. However, the nutrient rich TSY Broth allowed the exposed cells to recover from ETO damage and display normal reproduction.

The surviving ETO treated cells produced colonies on TSY Agar but not on MS Agar. However, after the surviving organisms formed colonies on TSY Agar, cultures from the individual colonies streaked on MS Agar formed colonies after incubation at 37°C. It appeared that the ETO treated cells needed a rich nutrient medium in order to repair their damage.

By supplementing MS Broth with guanine or guanosine triphosphate, the surviving ETO treated cells were able to "repair" and reproduce. (The other supplements tried had no detectable effect on repair and reproduction.) When MS Broth was supplemented with ETO treated guanine, the ETO treated cells showed recovery and reproduction. However, when the MS Broth was supplemented with ETO treated guanosine triphosphate, the ETO treated cells did not recover and reproduce (see Fig. 5 for *S. senftenberg*). Again, almost identical results were obtained with *E. coli*. These results indicate that it is alkylation of phosphated guanine in the cell that is primarily responsible for loss of cell reproduction ability. The alkylated phosphated guanine may be that in the cell guanine pool as well as that in the cell DNA.

It might be concluded that one need look no further than the inactivation of nuclear DNA for an explanation of the lethal effect of alkylating agents such as ETO. Results of the present study tend to support this view. However, because only two bacterial species were represented in this study, generalization with respect to other microbial species, particularly spore formers, should be made with extreme caution pending further work. It has been shown that alkylating agents can react with a number of biologically important cellular components (nucleophiles) which are vital to cell metabolism and reproduction. These include cellular compounds as vitamins and co-factors such as nicotinamide-adenine-dinucleotide (NAD) *para-*

aminobenzoic acid (PABA), folic acid and pyridoxal; adennine as well as guanine of DNA and RNA; and proteins, particularly enzymes.

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J. J. RACKIS and D. J. SESSA
Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604
and
F. R. STEGGERDA, T. SHIMIZU, J. ANDERSON and S. L. PEARL
Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

SOYBEAN FACTORS RELATING TO GAS PRODUCTION BY INTESTINAL BACTERIA

SUMMARY–An in vitro assay showed that toasted, dehulled, defatted soybean meal contains a gas-producing factor and a gas-inhibiting factor. The oligosaccharides–sucrose, raffinose and stachyose–are associated with the gas-producing factor when incubated in thioglycollate media with anaerobic bacteria of the intestinal tract of dogs. The phenolic acids in soybeans–syringic and ferulic acid–are effective gas inhibitors in vitro and in intestinal segments of dogs. The lipids, proteins and water-insoluble polysaccharides of soybean meal have no gas activity. During fermentation, gas production parallels the formation of monosaccharides by enzymatic hydrolysis of raffinose and stachyose. The amount and composition of gas produced from cottonseed and peanut meal were comparable to soybean meal.

INTRODUCTION

EXPERIMENTS in which human subjects consumed various soybean products (Steggerda et al., 1966) showed that the gas-producing factor resides mainly in the low molecular weight carbohydrate fraction that includes such oligosaccharides as sucrose, stachyose and raffinose. It was also shown that when navy beans were consumed in amounts equal to that of dehulled, full-fat and defatted soybean meal, gas production was about 2.5 times greater. It was suggested that the difference might be related either to the much greater protein content of soybeans or to a greater quantity of the gas-producing carbohydrate fraction in navy beans.

In another series of experiments, using dogs, an attempt was made to locate the site of gas production along the gastrointestinal tract by placing known quantities of navy bean homogenates in various loops of the small intestine and the colon, then measuring the amount of gas produced over definite time intervals (Richards and Steggerda, 1966). These experiments showed that although some gas could be produced in the duodenum and jejunum, the major production was in the ileum and colon. In this same report, it was also shown that gas production could be inhibited by combinations of neomycin and sulfathalidine, as well as a bacteriostatic agent, Vioform. Results of all these findings strongly suggested that the interaction of the intestinal microflora with constituents in the bean homogenate was the major factor in gas production. It was also observed that the class of bacteria most responsible for the reaction was the gram-positive anaerobic spore-forming clostridia type, normally inhabiting the gastrointestinal tract of man and animals (Bornside and Cohn, 1965). The characteristic reaction between bean products and the anaerobic flora is described by Anderson (1924) as being an "explosive rate of gas production with the liberation of exceptionally high concentrations of CO_2 and H_2." It is believed that this type of in vivo reaction in the small intestine and colon of the dog also occurs in the gastrointestinal tract of man when various bean products are consumed (Steggerda, 1968).

The major purpose of this report will be threefold: First, to screen a number of chemical fractions, as well as related compounds, found to exist in some way or another in soybeans for their specific ability to contribute to or inhibit gas production; secondly, to show by alcoholic extraction methods the manner in which the gas-producing and gas-inhibiting factors reside in the soybean and,

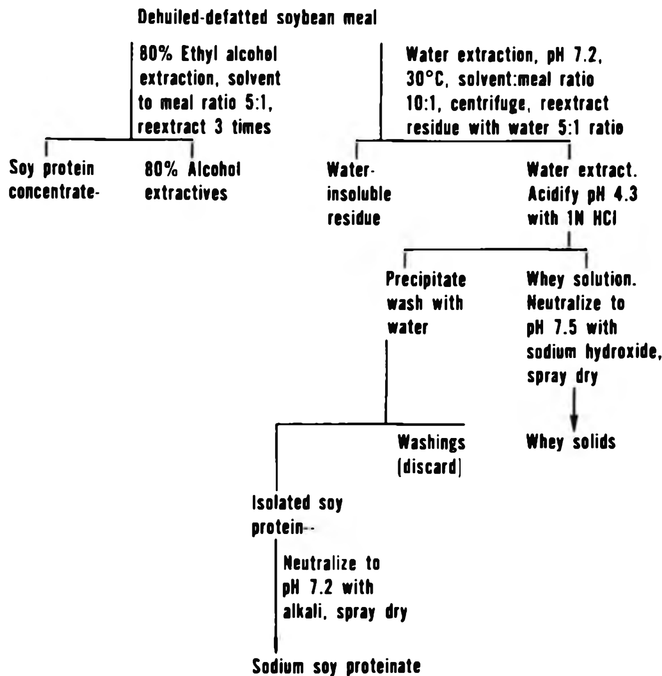


Fig. 1—Preparation of soybean products for in vitro gas production by intestinal bacteria.

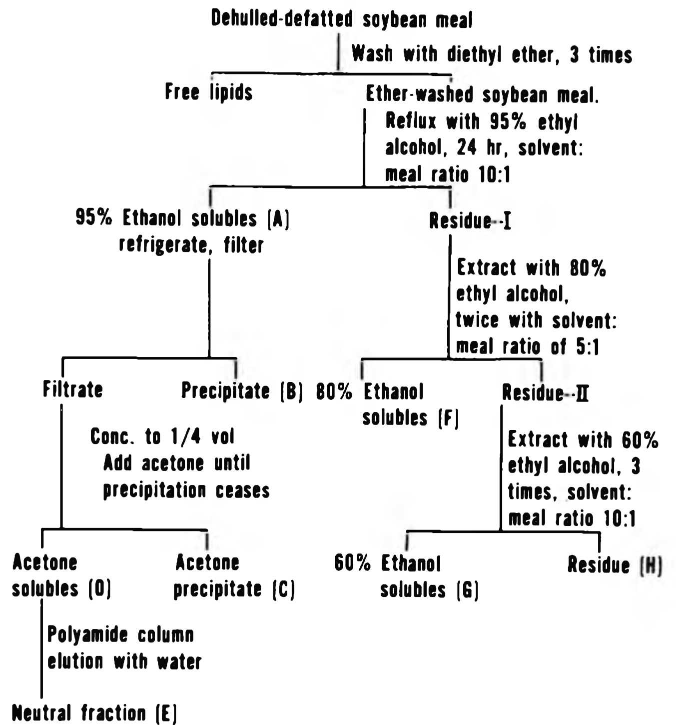


Fig. 2—Isolation of the gas-producing and gas-inhibiting factors.

finally, to propose a theory concerning gas production in the intestine and colon when soybean products are ingested.

MATERIALS & METHODS

2 METHODS were used to prepare various fractions for study, the first dealing with the preparation of high-protein fractions by either 80% ethanol or water extraction, the second designed to separate low molecular weight constituents. The first procedure (Fig. 1) is primarily one used commercially in the processing of soybeans into high-protein food products; the 80% ethyl alcohol extraction yields a 70% protein concentrate and the water-extraction process eventually yields sodium soy proteinate, which contains about 98% protein (ash-free dry basis). These products were prepared from Kay-Soy-56-7, a raw dehulled, defatted soybean meal manufactured by Archer-Daniels Midland Company, Decatur, Illinois. The prepared products were then treated with live steam for 40 min at 100°C to inactivate antinutritional constituents (Rackis, 1965). This treatment with live steam is referred to as toasting.

The second isolation procedure consisted of first washing dehulled, defatted soybean meal prepared in the laboratory from certified, seed-grade soybeans with diethyl ether to remove free lipids, then extracting the washed meal with a series of aqueous ethanol solutions: 95, 80 and 60%, respectively. A diagram of the manner in which the extractions and isolations of the various fractions was made is given in Figure 2. The letters on the diagram identify the different fractions used in testing for the presence or absence of the gas-producing factors. To remove solvent, the extracts were first concentrated to a thick syrup on a rotary evaporator at 40–45°C and then freeze-dried. Toasted, dehulled, defatted cottonseed and peanut meals (also tested for their gas-producing ability) were obtained from the Southern Regional Research Laboratory, New Orleans, Louisiana.

The in vitro technique used for making the studies on gas production has been previously described (Richards et al., 1968). In utilizing the in vitro tests, it must be emphasized that by far the best results are obtained when fresh 24-hr cultures of the anaerobic intestinal microflora are used. Cultures of 48-hr standing are not satisfactory. It is also important to point out that the cultures and incubation media must be free of any trace of oxygen. Gas production from the oligosaccharides, stachyose and raffinose, is difficult to obtain without complying with these criteria.

RESULTS

Effect of low molecular weight carbohydrates on gas production

As indicated earlier (Steggerda et al., 1966), the gas-producing factor in soybeans resides principally in the low molecular weight carbohydrate fraction. The oligosaccharide content of dehulled, defatted soybean meal, according to Kawamura et al. (1965), is approximately 15% (8% sucrose, 5% stachyose and 2% raffinose). Only trace amounts of verbascose and glucose are present. Small amounts of carbohydrates are also present as glycosides of isoflavones, sterols and sapogenins.

To determine which low molecular weight carbohydrate could most effectively react with the clostridia type of organism to produce the characteristically high concentrations of CO_2 and H_2, the following experiments were performed: To 1 cc of uniformly cultured anaerobic bacteria in a 30-cc syringe was added 9 cc of a nongas-producing thioglycollate medium containing a 1% concentration of the carbohydrate to be tested. Each carbohydrate was run in triplicate and the gas produced recorded at intervals of up to 24 hr. After the gas volumes were determined for each test, a sample of the gas was collected and analyzed for its CO_2 and H_2 content. Average results for each carbohydrate tested are recorded in Table 1. As will be noted, glucose gave the most gas during 24 hr. The glucose-anaerobic reaction also gave the most measurable amount of gas in 6 hr. The oligosaccharides—sucrose, raffinose and stachyose—were among the lowest producers and, except for sucrose, gas production occurred at a slower rate than for the monosaccharides. From other tests it was learned that if the experiments had continued for 36 or 48 hr the amount of gas produced from the raffinose and stachyose fractions would equal that observed for glucose. However, the ratio of CO_2 and H_2 was essentially the same in all cases. These results suggest that glucose may be the form in which the anaerobic bacteria can most effectively use carbohydrate to produce CO_2 and H_2, and that the response to raffinose and stachyose is delayed because hydrolysis has to occur before they can be used efficiently. Support for this concept will be discussed later.

Table 1—Anaerobic gas production with carbohydrates. Anaerobic cultures isolated from dog colon biopsies.[1]

Substrate	Total gas produced (cc) 1 hr	6 hr	12 hr	24 hr	Gas composition (%) CO_2	H_2
Monosaccharides						
Glucose	0	23	38	51	37	62
Maltose	0	19	38	43	38	61
Fructose	0	10	20	37	41	58
Galactose	0	8	20	36	38	61
Oligosaccharides						
Sucrose	0	19	27	30	32	68
Raffinose	0	9	25	30	35	65
Stachyose	0	10	27	31	37	63
Control	0	1.5	1.5	2.0	..	..

[1] To 10 cc of thioglycollate-anaerobic bacteria medium, 0.1 g of substrate was added.

Table 2—Anaerobic fermentation in vitro of soybean products and other oilseed meals. Anaerobic cultures isolated from dog colon biopsies.[1]

Product	Sample[2]	Gas volume (cc/24 hr)	Composition of gas % CO_2	% H_2
I	Soybean meal (dehulled-defatted)	40	44	45
II	Ether-washed soybean meal	46	52	43
III	Whey solids	40	47	48
IV	80% Alcohol extractives	39	34	61
V	Water-insoluble residue	3	No gas for analysis	
VI	Sodium soy proteinate	0	No gas for analysis	
VII	Free lipids	3	No gas for analysis	
VIII	Fatty acids (C_{16}–C_{18})	0	No gas for analysis	
IX	Egg albumen	0	No gas for analysis	
X	Sodium caseinate	0	No gas for analysis	
XI	Cottonseed flour	43	48	46
XII	Peanut flour	38	52	43

[1] To 10 cc of thioglycollate-anaerobic bacteria medium, 0.5 g of sample was added.
[2] See Figure 1 for preparation.

Influence of specific soybean products and other oilseed meals in gas production

Steggerda et al. (1966) observed previously that whey solids isolated by water extraction techniques and 80% alcohol extractives produced large amounts of gas in human subjects, whereas the sodium soy proteinate fraction had no effect on gas production.

To ascertain whether there was a combination of factors in the intestine responsible for the results in humans, or whether these responses in humans were unique for the specific fraction being tested, an in vitro technique was used to reproduce these responses in the test tube. To make these in vitro tests, 0.5 g of each soybean product was added to the thioglycollate-anaerobic culture medium and incubated at 37°C. For 24 hr, gas production was recorded periodically and gaseous composition determined at the end of the experiment.

The data reported in Table 2 represents an average of at least 2 assays, each run in triplicate. An assay required that the bacteria and culture media used were always from the same batch to allow for proper comparison. The results show that the gas-producing factor is present only in soybean products I through IV. The water-insoluble residue (V), sodium soy proteinate (VI) and lipid fractions (VII and VIII) did not contribute to gas production.

According to Aspinall et al. (1967), soybean meal contains about 18% polysaccharide consisting of a complex mixture of acidic polysaccharides, arabinogalactan and small amounts of cellulosic material. These carbohydrates remain with the water-insoluble residue, which contains 75% polysaccharide and 25% protein. The small amount of gas produced with the free lipids can be accounted for by the presence of sterol glucosides and oligosaccharides. Like soy protein, egg albumen and sodium caseinate had no activity. The in vitro results correlate well with previously reported experiments with humans (Steggerda et al., 1966). Preliminary tests also demonstrated that with the same products there is good correlation between in vitro gas activity and in vivo flatus activity in the colon of anesthetized dogs. Therefore, fat, protein and polysaccharide components of soybeans do not contribute to gas production. Further, low molecular weight carbohydrates are the active flatus principle.

As shown in Table 2, the amount and composition of gas produced by anaerobic fermentation of toasted cottonseed and peanut flours were comparable to soybean meal. Similar results were obtained by in vivo fermentation in the ileum and colon of the dog. Cottonseed and peanut flours have not yet been tested to determine whether flatulence would occur in human subjects.

All the products that produced gas in quantities large enough to measure gave the same high characteristic concentrations of CO_2 and H_2. As Table 2 indi-

Table 3—Effect of phenolic acids on gas production. Anaerobic culture of the dog colon was used for the in vitro experiments.

Soybean meal + phenolic acid (mg) as substrate[1]	In vitro technique: Total gas produced (cc) 6 hr	12 hr	24 hr	In vivo technique[2]: Ileal loop Gas volume (cc in 6 hr)	Colon loop Gas volume (cc in 6 hr)
Soybean meal	29	34	48	78	30
Syringic, 0.01	1	3	5		
Syringic, 0.03	0	0	1		
Syringic, 5				45	36
Syringic, 15				2	6
Soybean meal	15	21	26		
Ferulic, 0.01	15	20	24		
Ferulic, 0.03	1	3	7		
Ferulic, 0.05	0	0	0		
Ferulic, 30				30	2
Chlorogenic, 0.05	7	21	35		

[1] In vitro, 0.5 g meal; 6 g meal in 150 ml of water homogenate per loop in vivo.
[2] Milligrams phenolic acid per kilo of body weight.

Table 4—Effect of syringic acid on bacteria growth.[1]

Syringic Acid added[2] (g)	Light transmission at 620 mμ (%)						
	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr
Blank[3]	100	100	100	100	100	100	100
Control[4]	95.7	92.0	76.5	53.0	29.7	25.7	21.5
0.002	99.0	95.4	46.8	28.2	26.7	25.3	21.8
0.009	99.7	93.7	46.6	28.5	25.3	24.8	21.5
0.037	98.7	96.0	53.0	33.8	29.5	29.0	26.2
0.150	98.3	99.7	97.8	79.5	61.5	56.5	54.3
0.300	98.3	98.5	98.8	98.2	99.0	98.0	94.3
0.600	98.7	98.0	98.7	98.5	99.0	98.2	95.3

[1]Growth as a function of the percent transmittance of the inoculated culture.

[2]Gram per 100 cc of thioglycollate–0.5% glucose medium.

[3]Medium without bacteria and acid.

[4]Medium with bacteria and no acid.

Table 5—Effects of syringic acid on gas production.

Syringic Acid added[1] (g)	Total gas produced (cc)					% CO_2
	2 hr	4 hr	6 hr	8 hr	24 hr	
Blank	0.0	0.0	0.0	0.0	0.0	No gas collected
Control	0.0	3.3	17.3	27.3	47.5	50.4
0.005	0.0	2.9	17.1	25.9	47.9	48.4
0.019	0.0	3.0	18.0	27.0	48.2	51.9
0.038	0.0	1.4	15.8	24.3	45.5	50.1
0.080	0.0	1.9	10.1	16.5	38.6	47.9
0.150	0.0	0.0	2.9	7.0	24.3	41.9
0.300	0.0	0.0	0.0	0.0	0.0	No gas collected
0.600	0.0	0.0	0.0	0.0	0.0	

[1]Gram per 100 cc of thioglycollate-0.5% sucrose medium.

cates, the amount of CO_2 present in relation to the H_2 was, in general, higher (44–52%) than observed when the isolated carbohydrate fractions were studied (32–41%, see Table 1). At present no significance can be attached to this observation.

Evidence for inhibitory factors for gas production in soybeans

In experiments on humans, Steggerda et al. (1966) postulated that soy protein isolates had an inhibitory effect on flatulence. This same response was observed in experiments in which soy protein was introduced into isolated loops of the lower intestine or colon of the intact, anesthetized dog. Several nonprotein constituents are present in soy protein isolates (Nash et al., 1967). Isoflavones—genistein and daidzein—and their glucoside derivatives are found in various protein fractions. In search of a clue for the mechanism of the flatus inhibitory activity of the protein fraction, the effect of these phenolic compounds on gas production was studied. Other phenolic compounds found in decreasing amounts in soybeans are syringic, ferulic and chlorogenic acids (Arie et al., 1966). For the in vitro experiments, the phenolic compounds were added to a culture originally taken from a dog's colon and incubated in thioglycollate medium containing soybean meal. A limited number of experiments were also performed to test their effectiveness as inhibitors in the ileal and colon loop of the dog (in vivo).

Table 3 shows that when 0.5 g of soybean meal alone was tested for gas production by the in vitro technique, a marked response occurring within the first 6 hr of recording continued to increase through 12 and 24 hr. In the in vivo experiment, when 6 g of the soybean meal was mixed in 150 cc of water and then inserted into the ileal and colon loops, the gas production within a 6-hr collection was 78 and 30 cc, respectively. In the in vitro experiments in which 0.01 or 0.03 mg of syringic acid was added to the tube containing 0.5 g of soybean meal, an inhibition of gas production occurred that was nearly complete at the 0.01-mg level. If syringic acid (15 mg/kilo of body weight) was added to the soybean homogenate at the time of insertion into the ileum or colon, the inhibition of gas production was nearly complete.

In a similar series of experiments, ferulic acid was less effective. To inhibit in vitro gas production completely, 0.05 mg was required. For the in vivo tests it took a dose of 30 mg/kilo of body weight before any significant inhibition occurred. Chlorogenic acid had only a slight inhibitory effect even at the 0.05-mg level when tested along with the ferulic series with the in vitro technique. Although the data are not reported in Table 3, the major isoflavone glucoside, genistin, had no inhibitory effect on flatus production in the ileum and colon of the dog, even at a concentration of 200 mg/kilo of body weight/loop. Also, the presence of genistin did not increase gas production compared with the control. The difference between in vitro gas production in the syringic and ferulic acid series can be accounted for, because concentration of the anaerobic cultures varied at the time of the experiment. These experiments definitely support the premise of a gas-inhibitor factor in soybeans.

Mechanism of inhibition of gas production by syringic acid

To test the manner in which the phenolic acids inhibit gas production, rate of growth of the anaerobic bacteria was studied. This rate study was done by measuring the density of the clostridia culture growing on an appropriate thioglycollate-glucose medium containing known amounts of syringic acid (1 cc of culture plus 9 cc of media). The culture growth was recorded as percent light transmission at 2-hr intervals for the first 12 hr, with a final reading 24 hr after time of inoculation.

Table 4 contains the average data obtained from an experiment in which each concentration of syringic acid was tested in triplicate. Growth of the bacteria in the control culture, to which no syringic acid was added, is rapid after 4 hr of incubation, reaching a maximum in about 24 hr. This maximum indicated that either the effectiveness of the thioglycollate-glucose medium as a source of growth material had been exhausted and no further growth occurred, or that the clostridia culture had changed to a spore-forming state and had become dormant.

In the presence of 0.002–0.037% syringic acid, initial growth of the bacteria was apparently stimulated, as indicated by the transmission readings obtained at only 6–8 hr of incubation. After 24 hr of incubation, however, these cultures containing the low levels of syringic acid reached a level of growth comparable to that of the control. Bacterial growth was inhibited in media containing 0.15% syringic acid, with complete inhibition occurring in media containing 0.30% syringic acid.

The inhibition of growth appears to be bacteriostatic instead of bactericidal, since growth resumes after the inactivated anaerobic bacteria are transferred to a fresh thioglycollate-glucose environment. The bacteria not only assume their original state, but also produce gas just as effectively as before the syringic acid treatment.

To test further whether there was a correlation between growth inhibition and the inability to produce gas, varying amounts of syringic acid were added to 1 cc of bacterial culture plus 9 cc of thioglycollate medium containing 1% sucrose as the carbohydrate source. This added sucrose was sufficient to maintain good gas production even after 24 hr of incubation (Table 5).

Table 6–Extractability of flatus factors from soybean meal with aqueous ethanol.

Fraction	Soybean fraction[1]	Yield (g/100 g meal)	Carbohydrate content as % glucose	Gas volume (cc/24 hr)
	Soybean meal	...	33[2]	40
A	95% Ethanol solubles	18.1	82	2
B	Cold precipitate	3.9	93	50
C	Acetone precipitate	6.5	86	11
D	Acetone solubles	7.7	80	0
E	Neutral fraction	4.8	80	80
F	80% Ethanol solubles	3.4	55	16
G	60% Ethanol solubles	4.2	22	55
H	Residue	74.3	25[3]	2

[1] See Figure 2 for details, 0.5-g sample used for assay.

[2] 15% Oligosaccharide (Kawamura et al., 1965), 18% polysaccharide (Aspinall et al., 1967).

[3] Contains 72% protein (N × 6.25), 3% ash and 25% polysaccharide by difference.

The amount of gas produced by the bacteria in media containing 0.005–0.038% syringic acid was comparable to that of the control cultures at each of the collection periods between 4 and 24 hr of incubation. Slight inhibition of gas production occurred with 0.08% addition of syringic acid, with complete inhibition occurring at the 0.30% level. Since the amount of CO_2 present in all the collected gas samples was the same as that observed in earlier experiments, the concept is supported that gas production is truly a clostridia-carbohydrate reaction.

When data in Tables 4 and 5 are compared, it is seen that growth inhibition is shown to parallel the decrease in gas production.

Isolation of gas-producing and gas-inhibiting factors from soybean meal

Our results show that the gas-producing factor is associated with water-soluble oligosaccharides and that certain phenolic acids can inhibit gas production. The in vitro assay was then used to follow extraction of the gas-producing and gas-inhibiting factors from soybean meal at various concentrations of aqueous ethyl alcohol (Fig. 2). The gas produced by the various soybean meal fractions is given in Table 6.

Although fractions A–E contained 80–93% carbohydrate as determined by the procedure of DuBois et al. (1956), the amount of gas produced by each fraction varied widely. The 95% ethanol solubles (A) and acetone solubles (D) produced little or no gas, whereas the intermediate fractions B and C produced 50 and 11 cc of gas per 24 hr.

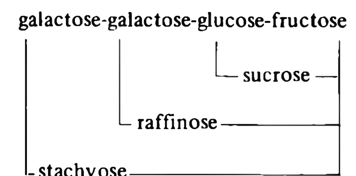
Constituents in the acetone-soluble fraction which completely inhibit gas production can be removed by polyamide column chromatography. The neutral fraction (E) obtained by elution with water contains 80% carbohydrate and smaller amounts of amino acids and peptides. The gas-inhibiting factor, together with most of the ultraviolet absorbing and fluorescing constituents, remained tightly bound to the column. Fraction E was highly active, producing 88 cc of gas per hr compared with only 40 cc for soybean meal. In a repeat experiment, fraction E was further purified by charcoal column to obtain a sample containing nearly 100% oligosaccharide, as sucrose, stachyose and raffinose, in about the same ratio as that in the meal (8:5:1.5, respectively). When retested, the purified sample was even more active. Preliminary tests have also indicated that the neutral fraction in small amounts stimulated gas production with monosaccharide substrates.

The data also show that when the residue fraction of the 95% ethanol extraction was further extracted with 80 and 60% ethanol, considerable amounts of the gas-producing factor were obtained (see F and G). The residue fraction (see H) had no activity, and chemical analysis showed that it contained 25% polysaccharide, a complex mixture of acidic polysaccharides, arabinogalactan and small amounts of cellulose material (Aspinall et al., 1967). The carbohydrate content of the fractions shown in Table 6, extracted with 95, 80 and 60% ethanol, respectively, included sugars of the saponins, isoflavone and sterol glucosides, as well as the oligosaccharides. The oligosaccharides account for most of the carbohydrate content of these fractions. As indicated previously, the isoflavones did not affect gas production. Saponins, isolated according to Birk et al. (1963), produced gas in these in vitro tests; however, since soybean meal contains only 0.5% saponin, its contribution to the gas-producing ability of dehulled-defatted soybean meal is negligible. Sterol glucoside content is also very low. The total carbohydrate content of soybean meal is about 33%, consisting of 15% oligosaccharide and 18% polysaccharide.

Further chemical analyses, silica gel and paper chromatographic studies indicated that the extracts also contained a large number of minor components, including amino acids, peptides, phospholipids and various constituents of nucleic acids. As shown in Table 6, total yield of material extracted from soybean meal with aqueous alcohol was 25.7 g/100 g meal, with the carbohydrates accounting for 70% of the yield. By analysis, the acetone-soluble fraction (D) and the 80 and 60% ethanol extractives all contained ultraviolet absorbing and fluorescing components. However, these fractions were not tested for the presence of gas-inhibiting factors.

DISCUSSION

FROM THE experiments performed, it appears that the gas-producing factor is closely associated with the oligosaccharides or low molecular weight carbohydrate fraction in soybeans. As shown in Table 1, glucose in the presence of anaerobic clostridia type of bacteria produces gas at a faster rate than either fructose or galactose. The sequence of sugars in the oligosaccharides is, respectively:



This suggests that a breakdown of the oligosaccharides to monosaccharides occurs before the gas-producing mechanism can take place. Then it is understandable that a longer delay must occur before the onset of gas production with such oligosaccharides as stachyose and raffinose, particularly if glucose is the preferred substrate.

To further support this concept, a limited number of experiments were performed in which paper chromatography was employed for recording changes that can occur in the amounts of oligosaccharides and monosaccharides in the presence of anaerobic bacteria. The procedure was to incubate 1 cc of an anaerobic culture medium with 1 cc of a 1% solution of either stachyose or raffinose in an anaerobic thioglycollate media at 37°C for 48 hr. Then, along with the recording of gas production, 1.5-cc samples of media were withdrawn from each tube at 3-, 6-, 12-, 18-, 24- and 48-hr intervals of incubation and analyzed for carbohydrate distribution via paper chromatography using butanol:acetic acid:water (3:2:1). At the end of each time period the incubation mixtures were

first centrifuged to remove the bacteria and then the protein was precipitated with 5% trichloroacetic acid (TCA). After extracting TCA with ether, the samples were dried. With time, stachyose and raffinose gradually disappeared. Intermediate hydrolysis products corresponding to di- and trisaccharides reached a maximum value in about 6 hr, followed by formation of maximum amounts of glucose, fructose and galactose in about 12 hr. Rate of gas production paralleled the enzymatic formations of monosaccharides.

These results lend strong support to the proposal that one source of gas in the gastrointestinal tract is the breakdown of oligosaccharides to monosaccharides by the bacteria themselves before they can effectively produce gas.

That the inhibiting factor for gas production in soybeans is associated with certain phenolic compounds and acts by way of causing an inhibition of the activity of the anaerobic bacteria is demonstrated by in vitro experiments. Whether a similar inhibitory mechanism occurs in the gastrointestinal tract of the intact animal or man has not been tested. A previous report (Steggerda et al., 1966), showed that gas production in man following navy bean ingestion is twice that observed for soybeans. Whether these differences in gas production can be attributed to either gas inhibitors or gas accelerators is not known. Additional research is needed to establish whether other soybean components can inhibit gas production in the gastrointestinal tract.

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Mr. L.D. Kirk of the Northern Laboratory assisted in the preparation of some of the soybean products.

APPLIED SCIENCE and ENGINEERING

LILIAN UMALE THOMPSON[a] and O. FENNEMA
Department of Food Science, University of Wisconsin, Madison, Wisconsin 53706

CCl_3F HYDRATE: ABILITY TO INHIBIT OXIDATION OF L-ASCORBIC ACID IN PEAS

SUMMARY—Oxidation of L-ascorbic acid was measured at −7°C in a closed system containing peas, water, CCl_3F hydrate and air. The presence of CCl_3F hydrate significantly inhibited oxidation of L-ascorbic acid to compounds devoid of vitamin C activity.

INTRODUCTION

GAS hydrates are ice-like clathrate compounds, stable at temperatures above 0°C (Mandelcorn, 1959; 1964). In simple systems, the hydrates of CCl_3F, CCl_2F_2 and C_3H_8, all existing in a Type II structure, were found to exert a highly significant inhibitory effect on oxidation of L-ascorbic acid (Thompson et al., 1971). Type II gas hydrates contain cavities of 2 sizes, only the larger occupied by hydrate-forming chemicals of the kinds just mentioned. The antioxidant property of Type II gas hydrates arises from their ability to entrap oxygen in the small, normally unfilled cavities of the hydrate structure.

The purpose of this study was to determine if CCl_3F hydrate can effectively inhibit oxidation of L-ascorbic acid in a complex natural system such as green peas.

MATERIALS & METHODS

FRESH green peas were shelled, blanched in boiling water for 45 sec and cooled in cold distilled water for 1 min. Approximately 25 g of peas were mixed with 20 ml of distilled water and 6 ml of CCl_3F (redistilled, DuPont) in a closed, cylindrical 125-ml glass bottle, and hydrate was formed at 0°C as described for simple systems (Thompson et al., 1971). The 6-ml volume of CCl_3F was superimposed upon the atmosphere existing in the headspace of the container, so as to avoid loss of air. All samples were equilibrated at −7°C for 1 hr and the conclusion of this period was regarded as zero time. Control samples were handled in an identical manner except CCl_3F was omitted. Duplicate samples were removed for analysis at zero time and at various additional times over 12 weeks. As each sample reached the end of its reaction period, 50 ml of 6% HPO_3 was added to stop further oxidation of ascorbic acid and to facilitate melting. The ascorbic acid (AA) and dehydroascorbic acid (DHA) contents of the peas and surrounding liquid were analyzed in general accordance with the 2,6-dichlorophenolindophenol visual titration method described by the Association of Vitamin Chemists (1966).

RESULTS & DISCUSSION

FROM Figure 1 it is apparent that AA retention beyond 1 wk of storage at −7°C was consistently greater in the samples containing CCl_3F hydrate than in corresponding control samples. DHA contents of the samples were small (rarely exceeded 4 mg/100 g of peas), exhibited a slight tendency to decrease during storage at −7°C and were not influenced significantly by the presence or absence of CCl_3F hydrate.

Since the AA lost during storage at −7°C did not accumulate as DHA, it must have been oxidized further to diketogulonic acid (DKG) and its degradation products. Oxidation of DHA to DKG and its products is irreversible and results in a total loss of vitamin C activity. Thus, the changes shown in Figure 1 depict actual changes in vitamin C activity rather than a reversible and relatively unimportant oxidation of AA to vitamin-C-active DHA.

It should be mentioned that CCl_3F is sparingly soluble in water and can not penetrate peas or any nonfluid aqueous system in significant amounts (Van Hulle et al., 1966). Thus, the hydrate of CCl_3F would have formed almost exclusively in the water surrounding the peas. This would be advantageous from the standpoint of subsequent separation of CCl_3F from the peas.

Based on results obtained previously with AA oxidation in simple systems, it would be expected that other gas hydrates of Type II or partially filled Type I structures (for examples, see Barduhn et al., 1960) would inhibit oxidation of AA in tissues under circumstances similar to those employed in this study (Thompson et al., 1971).

Commercial use of gas hydrates with foods imposes several requirements on the chemicals (hydrate formers) employed for hydrate formation, i.e., economy (as compared to other methods of controlling oxidation, such as vacuumizing or inert gas packaging), nontoxicity and compatibility with accepted organoleptic properties of the product. In addition, formation and stabilization of the hydrate must be possible at reasonable pressures. Although none of the hydrate formers capable of forming Type

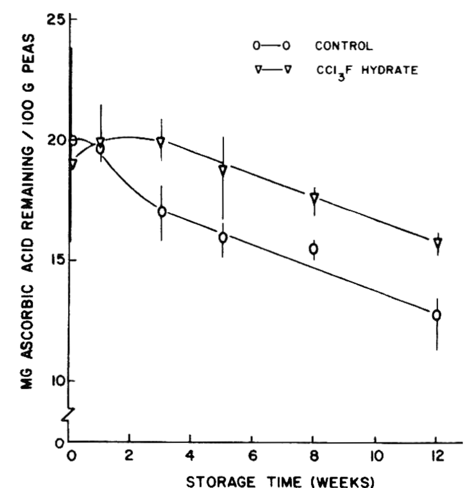

Fig. 1—Influence of CCl_3F hydrate on the stability of L-ascorbic acid in peas stored at −7°C. Each range represents 4 replicates.

[a]Present address: Department of Food Science, University of Toronto, Toronto, Canada.

II or partially filled Type I gas hydrates can satisfy all of these requirements, CF_2Cl_2 (fluorocarbon-12) has perhaps the most favorable combination of properties.

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YI DO LIN, F. M. CLYDESDALE and F. J. FRANCIS

Department of Food Science and Technology, University of Massachusetts, Amherst, Massachusetts 01002

ORGANIC ACID PROFILES OF THERMALLY PROCESSED SPINACH PUREE

SUMMARY Deaerated spinach puree was packed into TDT tubes, flushed with nitrogen and sealed. The tubes were divided into batches and processed with an F_0 = 4.9 at temperatures ranging from 240°–300°F with 10°F increments. Analysis for organic acids was carried out by means of an Automatic Organic Acid Analyzer (AOAA) and confirmed by paper chromatography. Oxalic acid showed very poor resolution on the AOAA; therefore, a chemical method was used for analysis. pH measurements were taken and color was measured on a HunterLab Model D25 Color Difference Meter. The acids identified were acetic, formic, fumaric, lactic, succinc, α-ketoglutaric, pyrrolidone-5-carboxylic, malic, citric, and oxalic. Acetic and pyrrolidone-carboxylic acids showed the most striking changes with increases at 240°F of 129% and 132% respectively. The greatest changes in the quality parameters evaluated in this study were noted at 240°F with such changes decreasing as temperature increased up to 300°F. A plateau was noted in the range of 270°–280°F since little improvement was gained above these temperatures.

INTRODUCTION

THE MAINTENANCE OF color in thermally processed green vegetables has been and remains a problem in food processing. Many attempts have been made to stabilize the color in past years with limited success (Clydesdale et al., 1968).

One of the major problems has been the decrease in pH caused by processing. This leads to the replacement of the magnesium in chlorophyll with hydrogen and the formation of pheophytin. However, one method which has been used with some success has been the use of high-temperature short-time processing (HTST) (Gupte and Francis, 1964; Luh et al., 1964; Tan and Francis, 1962; Epstein, 1959). Clydesdale (1966) noted that when spinach puree was processed by conventional and HTST methods the HTST pack showed no change in pH after processing, while the conventional pack showed a 10% decrease. More recently, Chen et al. (1970) found a similar situation in strained green peas processed by HTST and retort methods.

A decrease in pH upon retort processing is not restricted to green vegetables. Shallenberger et al. (1959) found that pyrrolidone-carboxylic acid (PCA) was formed during the production of beet puree and Luh et al., 1969 found a decrease in pH of retort processed strained carrots along with the formation of PCA. PCA is thought to cause a bitter flavor in processed foods. Shallenberger et al. (1958) has shown a relationship between PCA and off-flavor in beet puree. Also Mahdi et al. (1961) investigated the effect of PCA on the flavor of 22 processed fruits and vegetables.

As well as their effect on quality factors, organic acid formation and subsequent changes in pH could be an important public health factor in thermally processed foods. Esselen and Pflug (1956) collected thermal resistance data for P.A. 3679 spores in the process temperature range of 250° to 290°F. They found that spore destruction times tended to follow a semi-logarithmic destruction rate but it appeared that there may be some changes in the destruction rate at temperatures above 270°F. They suggested that such changes in spore destruction rates may be due to the influence of chemical changes occurring in food during heating.

Obviously the formation of acids during processing is an important parameter in the final quality and safety of a processed fruit or vegetable. With the increase in processing by HTST methods it becomes necessary to investigate chemical changes which occur at elevated temperatures. This investigation was initiated to study the effect of the time-temperature parameters of processing on organic acid profiles, pH, and color of pureed spinach. It was decided that differences in packs processed at only one conventional retort temperature and one HTST temperature did not present an adequate study of such changes. For this reason spinach puree was processed with F_0 = 4.9 at temperatures ranging from 240°F–300°F with increments of 10°F. Both the fresh and processed purees were then analyzed to determine organic acid profiles, color, and pH in view of their importance in thermal processing, appearance, and nutritional value.

MATERIALS & METHODS

SPINACH PURCHASED from a local market was comminuted, deaerated and packed into TDT tubes with a syringe immediately after the tubes had been flushed with nitrogen. After filling, the head space in the tubes was flushed with nitrogen and then sealed with an oxygen flame.

The tubes were divided into batches of 50 each and processed with an F_0 = 4.9 at temperatures ranging from 240°F to 300°F with 10°F increments as calculated by Gupte and Francis (1964). After processing the tubes were frozen at −20°F until analyses for organic acids, color and pH were performed.

Organic acid analysis

Classically organic acids have been estimated by the use of ion exchange resins and paper or column chromatography. In general the column chromatography has relied upon an acidified inert support material such as silica gel and a gradient elution system of nonpolar plus polar solvents. Customarily this separation has been preceded by a reasonably elaborate sample preparation and followed by titration of fractions collected with dilute alkali. However, in this investigation organic acids were quantitatively analyzed by an automatic organic acid analyzer (AOAA) (Waters Associates, Inc., Framingham, Mass.). The principles on which this analyzer is based are described by Kesner and Muntwyler (1966).

The column used in this investigation consisted of oven-dried (110°C, overnight) silica gel (Malinchrodt, Acid Silicic A. R., 100 mesh suitable for chromatographic analysis by the

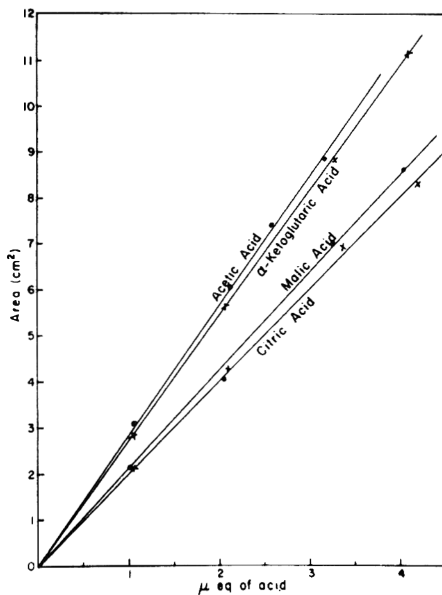

Fig. 1–Calibration curves for citric, malic, α-ketoglutaric and acetic acids.

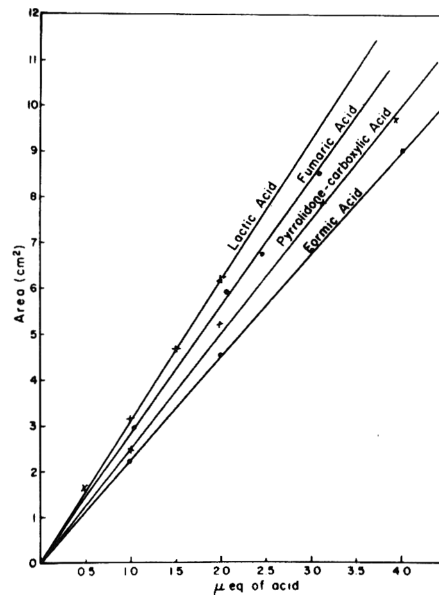

Fig. 2–Calibration curves for formic, pyrrolidone-carboxylic, fumaric and lactic acids.

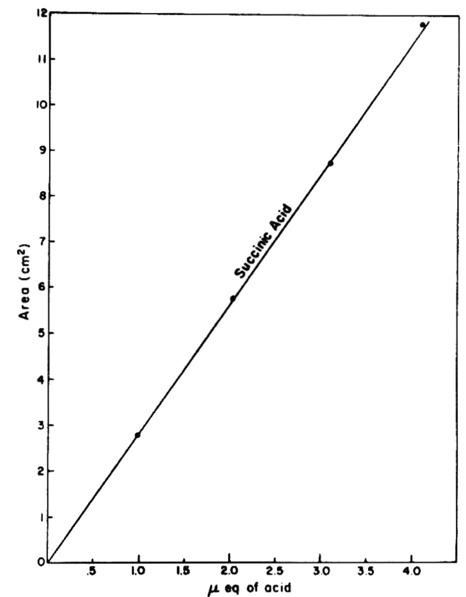

Fig. 3–Calibration curve for succinic acid.

method of Ramsey and Patterson, 1948). 50 ml of 0.1N H_2SO_4 were added to the oven-dried silica gel and the mixture stirred until lump free. 25g of this hydrated gel was then slurried with 40 ml of chloroform (Fisher, Certified ACS) and packed into a 0.9 × 50 cm column.

Samples were prepared prior to addition to the column by acidification with 6N H_2SO_4 (3 drops/g) followed by mixing with oven-dried silica gel until the mixture was free flowing.

The eluant was housed in a 5 chamber glass and Teflon Varigrad (Buchler Instruments, Fort Lee, N.J.) equipped with a heater on the final chamber to degas the solvent. The first chamber contained 200 ml of pure chloroform. Chambers 2 and 3 contained 7% (v/v) tert-amyl alcohol (Fisher, Reagent Grade)/chloroform, chamber 4 contained 30% (v/v) tert-amyl alcohol/chloroform and chamber 5 contained 50% (v/v) tert-amyl alcohol/chloroform. The volumes of solvents used in chambers 2 through 5 were determined by the density of their solvent mixtures. A volume equal in weight to the 200 ml of chloroform in chamber 1 was placed in each chamber.

The eluant was pumped through the sample and the column at the rate of 200 ml/hr by a Milton Roy Mini-Pump.

After the carboxylic acids have been separated on the column, the effluent stream is blended with a continuously flowing stream (50 ml/hr) containing an excess of indicator in the ionized salt form (1g of the sodium salt of o-nitrophenol (Eastman Organic Chemicals) in 21 of anhydrous methanol). This indicator is also the titrant. The optical density of this stream is then continuously recorded at 350 nm which corresponds to the maximum absorbance of the unionized (titrated) form of the indicator. When acid is not being eluted there is a minimum of absorbance and the photometer records a baseline. When an acid is eluted it is titrated by the indicator salt and produces the unionized form of the indicator which absorbs at 350 nm producing a curve, the area under which is proportional to the quantity of the acid eluted.

Calibration

Calibration of the instrument was undertaken once optimal conditions for separation were established in preliminary studies using both fresh and processed spinach purees.

Stock solutions of acids were prepared in a 50% (v/v) acetone-water mixture (Waters Associates AOAA Instruction Manual). From these stock solutions several mixtures of acids were prepared. To standardize each acid in the mixture, a sample equal to the amount transferred to the mixture–using the same pipette–was titrated. This minimized pipetting errors caused from poor draining which is characteristic of non-aqueous solutions. The acids were titrated to phenolphthalein end point with 0.01N sodium hydroxide using a stream of nitrogen for stirring.

The known mixtures of acids were analyzed on the AOAA in the concentration range of 0.05–5 μeq per acid. The area under the peaks was obtained by multiplying the net height of a peak by the width at half the peak height.

Identification

Tentative identification of all acids was based on retention times on the AOAA. A large number of standard acids were analyzed and their retention times noted. A known amount of standard acid was introduced into a puree which had been analyzed previously, and the increased concentration of the acid was noted. By subtraction of the amount of acid originally noted in the puree from this latter concentration, the amount of acid added was verified. By means of this type of internal standard, tentative identification of acids was accomplished.

Confirmatory identification

Further confirmation of the identification of organic acids was accomplished by means of paper chromatography. Fifteen samples of spinach puree were run on the AOAA. Each acid fraction was collected and concentrated under vacuum. Preliminary studies indicated that when the indicator was included in the eluant fraction collected, crystallization occurred upon concentration which interfered with subsequent paper chromatography. Therefore, the indicator flow was stopped, retention times re-

Table 1–R_f values of unknown versus known acids in butanol: 3N formic acid (50:50).

	R_f (× 100)	
Acid	Known	Unknown
Citric	45	47
Fumaric	84	84
α-Ketoglutaric	61	61
Lactic	82	82
Malic	51	53
PCA	55	56
Succinic	74	75

Table 2–Color and pH results in spinach puree over the temperature range 240°–300°F with an F_o = 4.9.

Process	pH	$tan^{-1}a/b$
Fresh	6.42	−37.9
240	6.12	− 5.9
250	6.29	−11.6
260	6.35	−15.1
270	6.40	−19.4
280	6.41	−26.6
290	6.41	−27.8
300	6.41	−29.0

calculated on the basis of a flow rate of 200 ml/hr rather than 250 ml/hr, and the acids were collected in solvent alone.

The concentrated fractions were redissolved in 50% methanol and spotted on Whatman No. 1 sheets. The chromatograms were run according to the method of Markakis et al. (1963) in which the spotted papers were irrigated descendingly by the upper phase of a mixture of 1 butanol: 3N formic acid 50:50 by volume. The lower phase of the mixture was used for vapor equilibration. After 12 hr the papers were dried in an air draft and sprayed with a 0.05% solution of bromphenol blue (Na salt) in 50% ethanol.

Oxalic acid analysis

With the experimental parameters described previously for the AOAA it was found that although a diffuse peak was obtained for oxalic acid, the resolution desired could not be achieved. Therefore, a chemical analysis based on the method of Baker (1952) was used. This method depends upon precipitation as calcium oxalate from a deproteinized extract and subsequent titration with potassium permanganate.

Color measurements

Instrumental color data were obtained from a HunterLab Model D25 Color Difference Meter (Hunter Associates Laboratory Inc., Fairfax, Va.). The data was reduced to the function $\tan^{-1}a/b$ as suggested by Clydesdale et al. (1969).

pH measurements

These were obtained with a Radiometer, model 25, pH meter.

Statistical evaluation

Statistical analysis of the organic acid data was done by the Range Method (Kramer and Twigg, 1966).

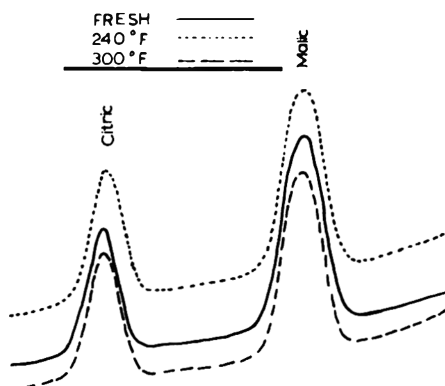

Fig. 4–Typical resolution of acids obtained with the AOAA on a sloping baseline.

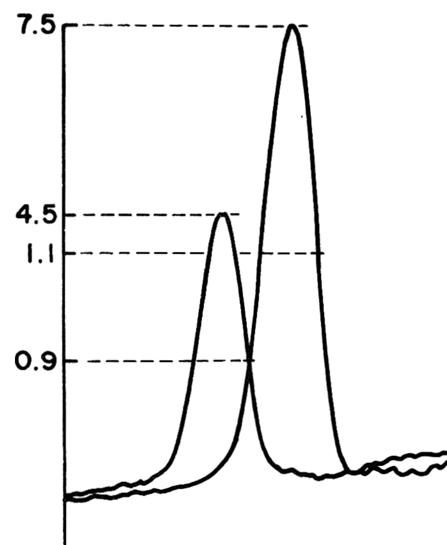

Fig. 5–Internal standard of acetic acid used with fresh spinach puree. Numerals indicate the peak height and half width.

RESULTS & DISCUSSION

CALIBRATION CURVES for the acids identified by the AOAA are shown in Figures 1, 2, and 3. From these curves the excellent relationship between peak area and concentration is evident. It should be noted that each curve has a different slope. In a static system containing uniform concentrations of chloroform, alcohol and indicator it is possible to equate changes in absorbance per microequivalent of acid regardless of whether the carboxylic group is contributed by a mono, di, or tricarboxylic acid. However, in a flowing system where there is a continuous change in the composition of the solvent, a uniform calibration constant in terms of units per microequivalent of acid is not possible. This is a result in part, of a change in the spectral characteristics of the indicator with changes in solvent composition. Consequently calibration constants are not uniform for all acids and are dependent upon the position of the curve at which they are eluted.

A second effect arising from this phenomenon is a sloping baseline. This may be seen in Figure 4 which also shows the typical resolution of peaks obtained in this work. However, as evidenced by the calibration curves, the sloping baselines do not introduce serious errors in the calculations and are relatively constant as long as the procedure is held constant.

Figure 5 shows the results of the internal standard check for acetic acid. This was done for all acids and results obtained were similar. In this figure the curves have been moved apart on the abscissa for clarity. The lines represent the peak height and half width in descending order on the ordinate. In this particular experiment 1g of fresh spinach was introduced into the column and an area response for acetic acid was obtained. This area response was equivalent to 2.22 μeq of acetic acid from the calibration curve. 1g of the same batch of fresh spinach plus 1.59 μeq of standard acetic acid was then added to the column. The response obtained in this case indicated 3.83 μeq of acetic acid. By difference the response obtained in the two cases for the spinach puree differed only by 0.02 μeq. The curves in both cases also showed identical retention times. These results indicated that the method was providing valid results in terms of quantitative identification of the acids within the spinach puree.

Table 1 shows the R_f values of the concentrated acid fractions obtained from the AOAA versus R_f values of known acids. This evidence was further confirmation of the identity of the acids.

Table 2 shows the changes which were noted in color and pH when the spinach puree underwent processing at different temperatures with the same F_0. The color data are reported in terms of $\tan^{-1}a/b$ which is an angular function of hue. The use of this function correlates very well

Table 3–Concentrations of organic acids in spinach puree over the temperature range 240°–300°F with an F_o = 4.9. (Concentrations are averages of duplicate analyses.)

	Acid concentration (μeq/g dry weight spinach)									
Process	Acetic	Formic	Fumaric	Lactic	Succinic	α-Keto glutaric	Pyrrolidone S-carboxylic	Malic	Citric	Oxalic
240	16.12	19.20	27.86	9.01	20.70	31.14	53.64	76.67	50.00	704.43
250	10.96*	18.07	29.15	9.60	21.09	31.10	36.67**	74.88	50.18	694.13
260	7.89**	14.33	29.65	10.29	18.01	29.24	29.36**	66.90	46.25	676.47
270	7.91**	18.65	38.37*	14.16	28.16*	34.00	33.59**	85.01	52.12	704.85
280	6.87**	16.82	38.62*	12.42	22.01	32.89	23.74**	77.79	48.68	712.93
290	8.05**	19.20	38.12*	14.64	24.27	34.54	28.58**	80.16	51.19	661.33
300	7.88**	18.60	37.68*	13.78	23.23	32.62	27.62**	81.11	51.74	782.79
Fresh	7.04**	16.90	36.08*	12.21	24.21	30.47	23.16**	75.38	43.80	795.20

*Significantly different from the concentration at 240°F at the 5% level.
**Significantly different from the concentration at 240°F at the 1% level.

with visual judgements of the color of processed spinach puree (Clydesdale and Francis, 1969). Larger negative values denote better color retention. As expected, HTST treatment provided the best color retention and processing at 240°F the worst. It should be noted that processing at temperatures greater than 280°F provided very little improvement in color retention.

As noted previously by Clydesdale (1966) HTST processing causes the least change in the pH of the spinach puree. Again temperatures of 280°F and greater had the same minor effect upon pH.

Table 3 shows the change in organic acid concentration caused by processing at different temperatures. The most notable changes occurred with acetic and pyrrolidone-carboxylic acids. In the case of acetic acid, processing at 240°F caused an increase of approximately 129% and approximately 56% at 250°F. However, at temperatures of 260°F or greater there was virtually no effect on the acetic acid concentration due to processing.

PCA showed the greatest increase of any of the acids analyzed. At 240°F there was an increase of about 132% and at 250°F an increase of about 57%. Increases were also seen at higher temperatures but the range was less–from about 3–45%.

Fumaric acid showed a decrease of about the same order up to 260°F. From 270°F–300°F there was virtually no change in concentration from the fresh sample. The other acids which were analyzed showed varying minor degrees of change or virtually no change.

It is evident that the acids which would contribute to a decrease in pH at lower process temperatures are acetic and PCA. However, although there is a large relative increase, the absolute increase of these acids is not large. Therefore the question arose as to whether the absolute increase in these acids could bring about a drop in pH of 0.3 (Table 2) or 0.65 as reported by Clydesdale and Francis (1968). To partially resolve this question, standard solutions of acetic acid and PCA were made up and appropriate amounts added to both fresh and processed (240°F) purees. The samples were then equilibrated with mixing for 1½ hr. The pH decreased from 6.41–5.55 in the fresh sample, and from 5.72–5.42 in the processed sample. This experiment did not take into consideration the decrease in fumaric acid but did show that the absolute increase in acids could account for the changes in pH noted at 240°F.

From pH, color and changes in acid concentration, the parameters evaluated in this study, it is apparent that HTST processing produces a better quality product which shows a minimum amount of change from the fresh material.

Many recent studies have shown chemical changes which occur at 240°F versus 300°F. However, a profile of temperatures has not been evaluated to determine if a plateau is reached at any given temperature in the HTST range. Recently, however, Teixeira et al. (1969) showed that thiamine retention associated with equivalent processes and a fixed Z value displayed an optimum retention. From the results presented in this paper an optimum plateau occurred at 270°F, in terms of the quality parameters measured. That is, little change in color, pH, and organic acids was noted above 270°F, and in certain cases processing above this temperature might be inappropriate.

It is interesting to note that PCA was found in fresh samples, since Markakis and Amon (1969) state that no PCA was found in fresh fruits and vegetables. However, Johnston and Hammill (1968) also found PCA in several vegetables. This leads to the conclusion that both type and variety of vegetable would be an important consideration in predicting the presence of PCA.

As mentioned previously there is a possibility of public health implications in HTST processed foods. In the temperature range of 270°F to 280°F very small changes in pH and increases in organic acids are noted. If at such temperatures, an F_0 value equivalent to that used at 240°F is employed, there might be a possibility of underprocessing if the food has a pH about 4.5. This class of food material has not been investigated in this manner but the implications from this work are such that an investigation of this type might prove invaluable.

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W. A. SISTRUNK and J. N. CASH
University of Arkansas, Agricultural Experiment Station, Fayetteville, Arkansas 72701

ASCORBIC ACID AND COLOR CHANGES IN SUMMER SQUASH AS INFLUENCED BY BLANCH, pH AND OTHER TREATMENTS

SUMMARY—The effect of time of blanch, pH, and other treatments on changes in ascorbic acid and color of summer squash puree was studied at 50°C to determine some of the chemical reactions that influence quality. Three types of changes occurred during holding at 50°C: (1) loss of yellow color; (2) browning reactions; and (3) loss of ascorbic acid. Ascorbic and dehydroascorbic acids were rapidly oxidized at 50°C. The reaction was slower at pH 5.0 than at pH 6.0 and 7.5. Color retention as measured by the CDM was greater at the higher pH levels. Time of blanch did not affect CDM a/b (hue) but did increase $\sqrt{a^2 + b^2}$ (chroma) and decreased the CDM L (lightness). Both color and ascorbic acid were lost very rapidly in unblanched squash puree under these conditions. Browning reactions had a major influence on CDM color values, even in blanched squash although the changes in carotenoid pigments were less pronounced.

INTRODUCTION

THE PRODUCTION OF summer squash for freezing has been increasing in recent years even though it is not especially desirable for freezing because of high water content. A good demand for sliced, frozen squash has prompted processors to continue to pack this item although they have become quite concerned about the loss of color and deterioration of eating quality during storage.

Ascorbic acid content has been recognized as a criterion for evaluating quality changes in frozen vegetables because of the ease with which it can be analyzed and speed of degradation under unfavorable conditions (Tressler and Evers, 1957). The recommended time of blanch for inactivation of enzymes in sliced summer squash is 3-1/2 min in boiling water and 4-1/2 min in steam. In most instances where vegetables were adequately blanched and packaged before freezing, ascorbic acid was fairly stable at temperatures of −18°C or lower.

Earlier studies on frozen summer squash indicated that there was a rapid loss of ascorbic acid during storage of sliced, packaged, frozen summer squash (Sistrunk and Cash, 1968). The change in ascorbic acid appeared to be associated with variety and inadequate blanching. Significant changes in color occurred during storage even in blanched squash. Maximum stability of ascorbic acid and color was attained by blanching 4 to 6 min in either boiling water or steam The rate of reduced ascorbic acid loss in frozen vegetables has been shown to be related to time-temperature history (Dietrich et al., 1957). They emphasized that there were differences between vegetables and varieties of the same vegetable in the loss of ascorbic acid and color, and that differences in stability may be due to pH.

The degradation of carotene and loss of color in carrots was studied in moist air at 62°C in the presence of oxygen in 24 hr (Weier, 1944). When diced carrots were blanched 5 min in water at 80–100°C there was very little loss of color during the incubation period. Weier (1947) demonstrated that color in carrot cylinders was more stable in neutral phosphate buffers than in acid or alkaline pH. Blanching seemed to release an inhibitor system that prevented degradation of color.

The present study was conducted on squash puree at an accelerated temperature of 50°C for a duration of 24 hr. The purpose was to further define: (1) the relationship of time of blanch to ascorbic acid breakdown; (2) the influence of ascorbic acid on color changes; and (3) the relationship of pH, holding at 50°C, time of blanch, catechol, and calcium content to color changes in summer squash.

EXPERIMENTAL

IN 1968, two varieties of summer squash, Seneca Prolific Hybrid and Yellow Crookneck, were washed and sliced in 3/8 inch slices. The sliced squash was placed into stainless steel baskets with covers and submerged in boiling water in a large steam kettle. Times of blanch were 0, 3, 4, 5, and 6 min. After blanching the squash was cooled in cold tap water, filled into heavy-duty plastic bags and frozen at −20°C. The

Table 1—The effect of blanching time, pH, and holding time on color and ascorbic acid of squash puree (Seneca Prolific).

Variable	Hunter CDM values 'L'	'-a'	'b'	'a'/'b' (hue)	Ascorbic[a] acid (mg/100g)	Dehydro-ascorbic acid (mg/100g)
Blanch time (min)						
0	56.6	5.65	18.8	.313	2.23	4.92
3	55.2	6.39	20.1	.318	2.06	2.93
4	54.2	6.46	21.1	.306	3.56	1.63
5	53.5	6.51	20.3	.321	3.68	1.77
6	54.9	6.66	22.1	.304	3.66	1.57
F value	18.556**	16.851**	18.788**	2.163	23.461**	31.895**
LSD @ 5% level	.8	.31	.8	NS	.49	.74
1% level	1.1	.42	1.11	NS	.67	1.00
pH						
4.2	58.1	6.18	20.2	.307	2.78	3.64
6.0	54.5	6.40	20.7	.307	3.18	2.39
7.5	52.2	6.69	20.6	.322	3.15	1.68
F value	183.679**	14.100**	1.500	5.020*	2.909	25.512**
LSD @ 5% level	.6	.20	NS	.011	NS	.57
1% level	.9	.27	NS	NS	NS	.78
Holding time (hr)						
0	55.7	7.05	21.4	.330	5.87	6.33
6	55.7	6.50	21.1	.308	3.03	2.17
12	55.3	6.32	20.3	.310	2.41	1.39
24	52.9	5.82	19.0	.302	0.83	0.37
F value	29.716**	42.280**	19.426**	7.588**	194.856**	132.840**
LSD @ 5% level	.7	.24	.7	.013	.44	.66
1% level	1.0	.31	1.0	.017	.59	.90

[a]No ascorbic acid added to puree.
*5% level of significance.
**1% level of significance.

Table 2–The effect of blanching time, pH, holding time, and calcium on the change in color and ascorbic acid in squash puree (Seneca Prolific).

Variable	Hunter CDM values 'L'	'-a'	'b'	$\sqrt{a^2 + b^2}$ (chroma)	Ascorbic acid[a] (mg/100g)	Dehydro-ascorbic acid[a] (mg/100g)
Blanch time (min)						
0	58.9	5.31	15.0	15.9	2.2	14.7
3	55.7	6.83	22.2	23.2	17.4	6.7
5	54.6	7.12	21.7	22.8	26.7	3.8
F value	107.771**	21.718**	747.340**	734.139**	1214.952**	200.176**
LSD @ 5% level	.6	.60	.4	.4	1.0	1.1
1% level	.8	.80	.6	.6	1.4	1.5
Holding time (hr)						
0	57.2	6.50	21.1	22.0	24.1	14.3
1	57.0	6.60	19.1	20.7	14.9	11.7
3	56.1	6.62	19.5	20.1	12.3	4.5
6	55.4	6.02	18.8	19.7	10.4	3.1
F value	10.627**	1.619	34.587**	33.267**	221.762**	139.926**
LSD @ 5% level	.7	NS	.5	.5	1.2	1.3
1% level	1.0	NS	.6	.7	1.6	1.8
pH						
5.0	56.5	6.08	19.2	20.1	16.5	9.6
6.0	56.7	6.34	19.8	20.8	15.1	9.0
7.5	56.0	6.85	19.9	21.1	14.6	6.6
F value	2.728	3.507*	6.980*	9.924**	7.593**	16.393**
LSD @ 5% level	NS	.60	.4	.4	1.0	1.1
1% level	NS	NS	.6	.6	1.4	1.5
Treatment						
Calcium	57.4	6.43	19.8	20.8	14.7	7.8
No calcium	55.4	6.41	19.4	20.5	16.1	9.0
F value	59.607**	.006 NS	4.871*	4.145*	12.435**	6.775*

[a]25 mg/100 g of ascorbic acid added.
*5% level of significance.
**1% level of significance.

frozen squash was stored until the various experiments were conducted.

Sufficient frozen squash of the different times of blanch was partially thawed in air until it could be chopped finely with a large knife. The squash was weighed and blended with .025M Na_2HPO_4 buffer (4:1) for 5 min. Then the entire batch was mixed thoroughly before dividing for the various treatments. Wherever an experiment involved different pH levels, the buffered squash was adjusted to the correct pH with either 25% Na_2HPO_4 or 25% H_3PO_4. The treatments were made up in buffer at 6.3 pH just prior to blending with the squash. After the treatments were applied, the samples were stirred 1 min with a malted milk mixer set at low speed. The puree was filled into five glass vials for each treatment to a fill of 30g. Two drops of toluol were placed in each vial to inhibit bacterial growth. The original set (0 time) was analyzed for color by the Hunter Color and Color Difference Meter (CDM) and placed immediately into the freezer at −20°C.

All other vials were placed in the water bath at 50°C. A plexiglass cover was placed over the entire lot of vials to prevent evaporation. Subsequent samples were removed and cooled before recording color as shown. Then the vials were capped tightly and frozen in the same manner as the original set. At each sampling the remaining vials in the water bath were shaken to mix the contents in the vials. The measurement of color on the CDM was greatly facilitated by using the small opening setting on the instrument and a small-holed plate to read the color directly in the glass vials. Preliminary studies indicated that this procedure gave accurate results. A white National Bureau of Standards plaque was used as a standard. The settings for the standard were as follows: L = 91.8; a = 1.6; and b = 1.6.

Analytical

Ascorbic acid was determined by the colorimetric method of Morell (1941) by thawing the vials of puree in cold tap water for 15 min before extracting. Dehydroascorbic acid was analyzed on the same extract by the 2,4-dinitrophenylhydrazine method (Roe and Oesterling, 1944). Total carotenoid pigments were determined by extracting 10g samples of the puree with 90 ml of Skellysolve B saturated with 95% ethanol (19:1). After blending the samples with an osterizer on low speed for 2 min, a 10 ml aliquot was added to 4g of anhydrous Na_2SO_4 in vials. The capped vials were shaken and after standing 30 min to allow clarification, the per cent transmission was recorded on a spectrophotometer set at 450 mμ. The data were calculated as beta carotene from a standard curve. Data were analyzed as factorial experiments by standard statistical procedures.

RESULTS & DISCUSSION

THE CHANGE IN color of unblanched squash was rapid after the blend was prepared. Nevertheless, it was included in each experiment to measure the effects of active enzymes on color and ascorbic acid when other variables were introduced.

Effect of pH on color

It was found in preliminary studies that squash that received no blanch and 3 min blanch was lower in pH than squash that was blanched for longer times (Sistrunk and Cash, 1968). The normal pH of blanched squash was 6.0–6.2. Earlier studies on carrots have shown that carotene was more stable at neutral pH (Weier, 1947). Actually there was an increase in CDM 'a' value by increasing the pH to 7.5 (Tables 1, 2, 3,). The a/b ratio (hue) shown in Table 1, and chroma (Table 2) were also higher at pH 7.5, a difference that was visible as a darker yellow color. There was a decrease in 'L' value (lightness) at pH 7.5 (Tables 1, 3). In the short term experiment higher pH did not affect 'L' value because of the influence of calcium (Table 2).

Effect of time of blanch on color

There was an increase in CDM 'a' value with an increase in time of blanch regardless of the other variables (Tables 1, 2, 3, 4). Time of blanch did not affect a/b ratio (hue) although the chroma was increased by longer blanching. Higher chroma values were visible as darker yellow color except where browning occurred.

The CDM 'L' value was decreased with longer blanching in the variety Seneca Prolific Hybrid (Tables 1, 2) and increased by blanching in Yellow Crookneck (Tables 3, 4). This difference was produced to some extent by the addition of catechol (Table 4) since the browning reaction was accelerated in unblanched squash. Also, the color of Yellow Crookneck squash was more stable in the blended, unblanched squash, which could have accounted for part of the difference in 'L' value.

Effect of holding time on color

There was a significant change in color during the first 6 hr of the experiments. Some of this change was due to the effect of pH, the rapid loss in the unblanched sample, and the acceleration of browning by catechol. However, the color was significantly changed during the first 6 hr of the experiment as demonstrated by the decrease in CDM 'a' and hue (Table 1). Chroma, CDM 'b' and 'L' values decreased rapidly in the short term experiment (Table 2). Calcium increased the

Table 3—The effect of blanch, pH, time of holding and ascorbic acid on color of squash puree (Yellow Crookneck).

Main effects	Hunter CDM values 'L'	'−a'	'b'	$\sqrt{a^2 + b^2}$ (chroma)	Ascorbic acid[a] (mg/100g)	Dehydro-ascorbic acid[a] (mg/100g)
Blanch (min)						
0	50.6	4.58	14.8	16.2	2.6	17.8
3	51.7	5.43	18.5	19.3	10.9	10.3
6	52.5	5.79	19.9	20.8	16.5	8.2
F value	17.292**	142.215**	266.922**	402.579**	18.364**	13.174**
LSD @ 5% level	.6	.15	.5	.3	1.6	4.2
1% level	.8	.20	.6	.4	2.2	5.6
pH						
5.0	53.0	5.05	17.2	18.4	12.7	15.7
6.0	52.5	5.19	17.6	18.6	9.4	11.3
7.5	49.2	5.57	18.4	19.3	8.0	9.3
F value	83.197**	30.010**	5.737**	15.152**	6.566**	10.377**
LSD @ 5% level	.6	.15	.5	.3	1.6	4.2
1% level	.8	.20	.6	.4	2.2	5.6
Holding time (hr)						
0	53.4	6.98	19.4	20.6	21.8	28.3
6	50.8	5.13	17.0	18.6	10.9	11.4
12	51.3	4.77	17.2	18.0	5.4	6.4
24	50.9	4.21	17.3	17.7	1.9	2.3
F value	21.988**	399.046**	35.308**	93.054**	42.970**	89.452**
LSD @ 5% level	.7	.17	.5	.4	2.1	.52
1% level	1.0	.23	.7	.5	2.9	6.9
Treatment						
Ascorbic	51.5	5.21	18.3	19.2	14.8	13.2
Dehydro-ascorbic	51.7	5.33	17.2	18.3	5.2	11.0
F value	.487 NS	4.138**	32.353**	41.855**	36.263**	2.865 NS

[a]Both ascorbic and dehydroascorbic added at rate of 25 mg/100 g blend.
*5% level of significance.
**1% level of significance.

Table 4—The effect of blanch, catechol, ascorbic acid and holding time on color of squash puree (Yellow Crookneck).

Main effects	Hunter CDM values 'L'	'−a'	'b'	$\sqrt{a^2 + b^2}$ (chroma)	Ascorbic acid (mg/100g)	Dehydro-ascorbic acid (mg/100g)
Blanch (min)						
0	44.3	2.65	13.8	14.1	4.5	25.0
4	45.7	4.97	19.2	19.8	22.7	10.1
6	47.5	5.36	20.9	21.5	25.6	8.3
F value	88.250**	635.534**	1326.795**	1557.083**	465.075**	15.495**
LSD @ 5% level	.5	.16	.3	.3	1.7	5.1
1% level	.7	.22	.4	.4	2.3	6.7
Catechol						
0	47.1	4.62	18.3	18.9	17.3	11.3
.005%	45.1	4.15	17.9	18.2	17.1	14.3
.01%	45.3	4.21	17.7	18.4	18.4	17.7
F value	43.506**	19.557**	8.797**	9.351**	0.718	3.393
LSD @ 5% level	.5	.16	.3	.3	NS	5.1
1% level	.7	.22	.4	.4	NS	NS
Holding time (hr)						
0	48.3	4.98	19.7	20.3	22.0	30.7
3	48.0	4.56	18.4	19.0	18.4	22.5
6	46.4	4.74	18.1	18.7	16.5	10.9
12	45.8	4.09	17.9	18.4	15.5	6.4
24	40.6	3.26	15.6	15.9	15.4	1.7
F value	189.646**	82.171**	133.935**	128.990**	22.348**	37.058**
LSD @ 5% level	.6	.21	.4	.4	2.2	6.6
1% level	.8	.28	.5	.5	3.0	8.7
Ascorbic acid "mg/100 g"						
0	45.5	4.14	17.0	17.5	1.5	8.3
25	45.7	4.29	18.2	18.7	15.6	13.7
50	46.2	4.55	18.7	19.3	35.7	19.7
F value	3.896*	12.247**	74.043**	64.080**	465.075**	8.281**
LSD @ 5% level	.5	.16	.3	.3	1.7	5.1
1% level	NS	22	.4	.4	2.3	6.7

*5% level of significance.
**1% level of significance.

Table 5–The effect of blanch, ascorbic acid, and holding time on carotene in squash puree (Yellow Crookneck)[a].

		Holding time (hr) at 50°C			
	Blanch	0	3	6	24
Treatment	(min)	(mg/100g)			
Ascorbic	0	1.92	.66	.62	.54
No ascorbic		1.92	.84	.72	.51
Ascorbic	4	1.89	1.26	1.02	1.08
No ascorbic		1.89	1.41	1.20	1.05
Ascorbic	6	2.09	1.83	1.23	1.11
No ascorbic		2.09	1.65	1.09	.99

[a]Data represents the mean of four replications.

reflectance (CDM 'L') and apparently had a stabilizing effect on the color since CDM 'L', 'b', and chroma were higher in the presence of Ca. A ready supply of available oxygen in the blend at the outset of the experiment produced browning reactions rapidly in the presence of active enzymes. Catechol resulted in more browning, even in blanched squash where enzymes were inactivated as demonstrated by a decrease in CDM 'L' (Table 4). The decrease in CDM 'L' during holding might have occurred partly from a dull browning due to change of chlorophyll to pheophytin. The dullness did not show up in samples where ascorbic acid was added until after the 12 hr holding time.

Effect of ascorbic and dehydroascorbic acid

Either ascorbic or dehydroascorbic acid was oxidized rapidly in squash. It was shown in previous studies (Sistrunk and Cash, 1968), that some varieties are better sources of ascorbic acid than others either raw or blanched.

The pH had a significant effect on the loss of ascorbic and dehydroascorbic acids as demonstrated by the higher retention at pH 5.0 (Table 2, 3). Dehydroascorbic acid was oxidized as rapidly as ascorbic acid as exemplified by the effect of holding time. Blanching influenced ascorbic acid retention, but there was no effect beyond a 3 min blanch where no ascorbic acid was added (Table 1). Differences in ascorbic and dehydroascorbic acid between 3 and 6 min time of blanch were more pronounced where ascorbic acid was added (Table 3). The rate of oxidation during holding was approximately the same in both the 2 acids since there was no significant difference in the dehydroascorbic acid when either ascorbic or dehydroascorbic acid was added to the blend (Table 3). The addition of ascorbic acid to squash puree resulted in higher CDM 'b' and chroma as compared to adding dehydroascorbic acid. There was no difference between the two acids on browning reactions since the 'L' values were comparable.

Calcium had an accelerating effect on oxidation of ascorbic acid since the values for both acids were lower when Ca was added (Table 2). There was a significant effect of concentration of ascorbic acid on the CDM 'a', 'b', and chroma (Table 4). The higher concentration retained more color. The increase in CDM 'L' with the acid resulted primarily from the inhibition of browning reactions produced by catechol.

Extracted pigments

The total pigments were extracted only on part of the samples in the various experiments. There did not appear to be as much change in extracted pigment during holding as there was in color as determined by the CDM. Nonenzymatic browning reactions had a major influence on color by the CDM in blanched squash. Ascorbic acid decreased the rate of pigment breakdown (Table 5). Also in unblanched squash, the pigment was lost rapidly following blending.

From the results of these studies it was concluded that both ascorbic and dehydroascorbic acids inhibited browning reactions in blanched squash and markedly influenced color. When summer squash was inadequately blanched, ascorbic acid was oxidized rapidly along with the carotenoid pigments. The most important factor contributing to complete loss of color and development of off-flavor was inadequate blanching, although non-enzymatic browning as well as loss of carotenoid pigments and ascorbic acid did occur in squash that was blanched properly. Further studies need to be conducted on the enzymes and other constituents that affect carotenoid pigment oxidation in squash as well as those that produce browning reactions.

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M. PELEG and C. H. MANNHEIM
Department of Food and Biotechnology, Technion-Israel Institute of Technology, Haifa, Israel

PRODUCTION OF FROZEN ORANGE-JUICE CONCENTRATE FROM CENTRIFUGALLY SEPARATED SERUM AND PULP

SUMMARY—An orange concentrate production process, based on centrifugal separation of the juice into pulp and serum, was evaluated. In this process, the serum alone was concentrated to a high degree (70–80°Brix) and recombined with the pulp before freezing, to produce a full-flavor concentrate of about 55°Brix. The effect of time and centrifuge acceleration on separation, as well as that of various heat treatments of the juice before separation on concentrate properties, was investigated. The pulp was found to be compressible. Most of the essential oil was found in the pulp fraction and the residual oil content in the serum fraction showed linearity with serum turbidity. Concentrates produced by the separation method in question were stable and had superior organoleptic properties compared with those produced by the conventional method using fresh cut-back juice. The essential oil content of the new concentrates was high enough to dispense with admixture of peel oil. It was found that stable concentrates can be produced without heat treatment of the feed juice.

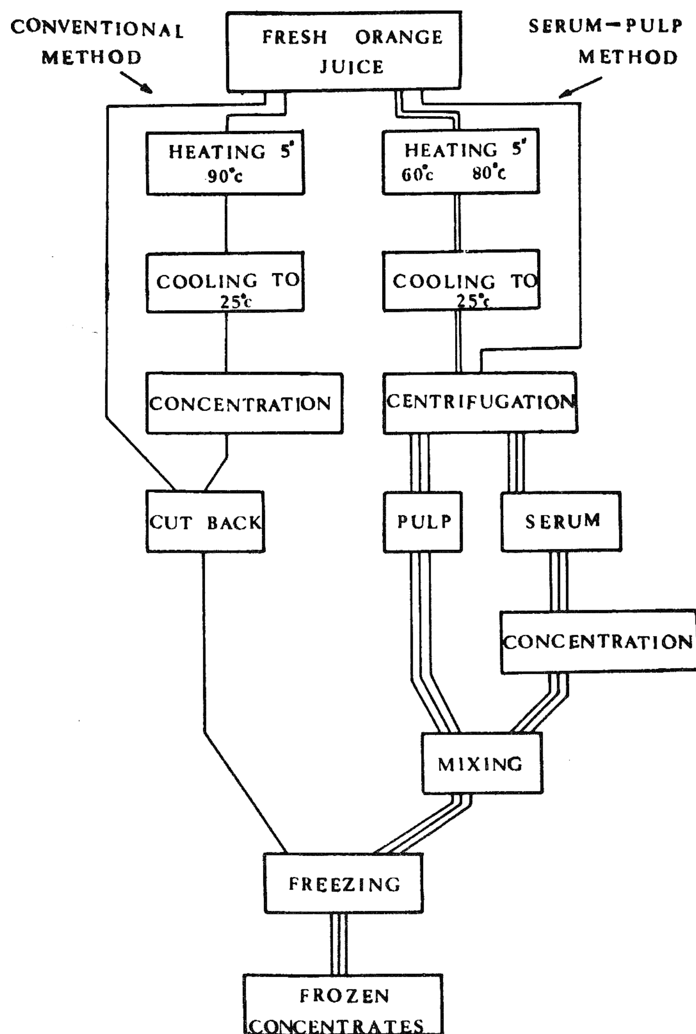

Fig. 1—Schematic diagram of processing frozen orange concentrates by conventional and pulp-serum method.

INTRODUCTION

THERE ARE several methods for flavor enhancement in citrus concentrates to compensate for losses during vacuum evaporation. The method most commonly used is that of "cutback" of fresh juice to concentrates with or without admixture of peel oil. Other methods are that of aroma recovery (Bomben et al., 1966; Mannheim et al., 1967), based on capture of the aromatic substances from the vapors and their concentration, and that of "juice emulsion," based on the addition of the oil fraction of the juice obtained by centrifugation (Lawler, 1964).

The production of tomato concentrate from centrifugally separated pulp and serum was described by Mannheim and Kopelman (1964). The main idea of this process is based on the low viscosity of the serum which substantially increases the heat transfer coefficients, facilitates concentration and reduces browning. Use of this process for citrus juice concentration was described originally by Meizner (1940), but no practical way of preserving the concentrate was suggested. Recently, several patents involving centrifugal separation in the production of citrus concentrates were issued (Brown, et al., 1966; Sargeant, 1968; Sperti, 1968).

If this process is to be used for high-flavor orange concentrate production, 2 major problems must be overcome. The first concerns heat treatment which is necessary for cloud stabilization (Guyer et al., 1956), but imparts a cooked flavor to the product. The other is the behavior of juice during centrifugation, especially the possibility of pulp compression which may affect the material balance and the final concentration. These 2 problems were investigated in this work.

MATERIALS & METHODS

RANDOM samples of Israeli Shamouti and Valencia oranges were taken at an industrial plant. After washing and sorting, the juice was extracted on a FMC In-line Juice Extractor model 718, screened in a Sweco-Vibro Separator Model S-18 (0.6-mm screen) and processed as described in Figure 1. The screened juice was heated to the required temperature in an Alfa-Laval plate heat exchanger Model PL-I-HB and, after holding for 5 sec, cooled in the same equipment. Separation was carried out in an Escher Wyss scraper-type centrifuge Model HS-400, with a basket diameter of 38 cm, using

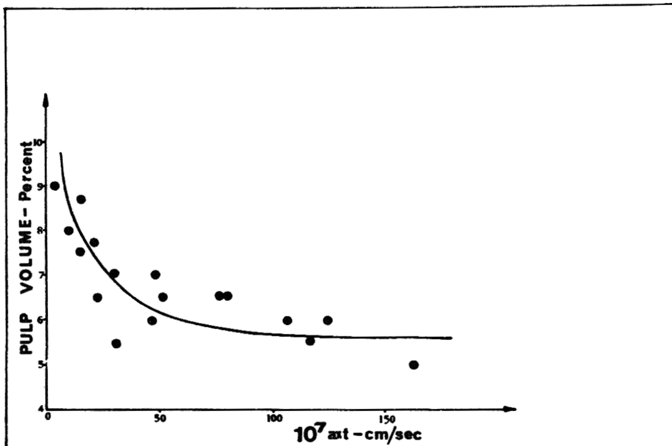

Fig. 2–Relationship between pulp volume and centrifugation conditions. a = acceleration (cm/sec²), t = time of centrifugation (sec).

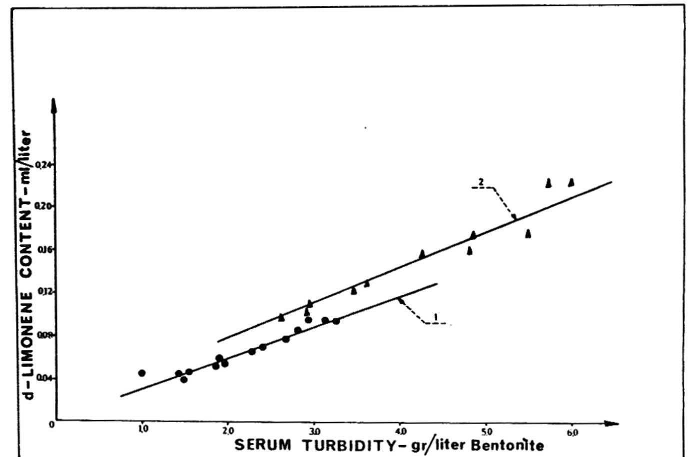

Fig. 3–Relationship between d-limonene content and serum turbidity. 1. Shamouti juice, 2. Valencia juice.

speeds of 2140 and 2940 rpm. The pulp was removed from centrifuge and kept under refrigeration (4°C) pending use. The serum was concentrated in an Alfa Laval Centritherm Evaporator Model CT-1. The concentrated serum was mixed with the pulp and packed in 211 × 108 (100-g) cans. The product was frozen in a blast freezer at about −35°C and stored at −18°C. Juice for the conventional concentrates was heated in a plate pasteurizer to 90°C for 5 sec, cooled and concentrated to 60-65°Brix. This concentrate was mixed with fresh juice to give a 45°Brix final product, which was also blast-frozen and stored at −18°C.

For the study of the juice behavior during centrifugation, juices were centrifuged in conical 50-ml tubes in a MSE laboratory centrifuge 39 cm in diameter. Speeds of 1300, 2050, 2500 and 3000 rpm and residence times of 2, 6, 10 and 14 min were used. The concentrates were diluted to 11.0°Brix before analysis.

Pulp content was measured by centrifuging reconstituted juice in conical 50-ml tubes in a MSE centrifuge at 1300 rpm for 10 min. The serum obtained in the pulp determination was examined for turbidity using a Klett-Colorimeter [with a red (No. 66) filter] calibrated with a bentonite suspension. Reconstituted juice was put in a 100-ml graduated cylinder for 30 min to determine the degree of serum separation. Essential oil, expressed as d-limonene, was measured by the bromate titration method (Scott and Veldhuis, 1966). The color of juice was tested in a Hunter Lab D-25 color difference meter, calibrated with a standard tile with values Rd = 69.2, a = −4.6.

The reconstituted juices were evaluated organoleptically, at room temperature, by a panel of at least 10 members in a testing room with dim lights to eliminate the effect of visual distinction. Samples were scored from 1 (worst) to 10 (best). Results were subjected to analysis of variance and wholly significant differences determined (Volk, 1958).

Table 1–Distribution of essential oil between serum and pulp in Shamouti orange juice.

Experiment No.	Serum (%)	dl in serum (ml/liter)	dl in serum (%)	Pulp (%)	dl in pulp (ml/kg)	dl in pulp (%)	Total dl in serum and pulp (ml/liter)	dl in original juice (ml/liter)
1	87.7	0.072	34.5	12.3	0.980	65.5	0.183	0.188
2	86.2	0.064	36.0	13.8	0.705	64.0	0.152	0.154
3	83.1	0.102	37.6	16.9	0.835	62.4	0.226	0.250
4	88.1	0.093	46.9	11.9	0.772	53.1	0.173	0.163

dl = Orange oil expressed as d-limonene.

Table 2–Relationship between serum turbidity and d-limonene content.

Experiment No.	Heat treatment °C	Correlation between d-limonene (dl) and turbidity (T)	Degree of freedom	r
1	60	dl = 0.0213T + 0.0201	14	0.891***
1	80	dl = 0.0256T + 0.0083	13	0.968***
1	90[1]	dl = 0.0166T + 0.0248	14	0.923***
2	60	dl = 0.0257T + 0.0082	14	0.955***
2	80	dl = 0.0245T + 0.0163	9	0.998***
2	90[1]	dl = 0.0222T + 0.0164	15	0.989***

[1] Hot-filled and cooled in container.
***Significant at 99.9% confidence level.

RESULTS & DISCUSSION

Juice centrifugation

Pulp determinations after centrifugation at different times and speeds showed (Fig. 2) that the pulp was compressible to such an extent that further sedimentation of fine particles from the cloud did not suffice to prevent a decrease in measurable pulp volume.

Table 1 shows that most of the essential oil, expressed as d-limonene, was found in the pulp fraction. The material balance showed that no oil was lost during centrifugation. The residual oil content in the serum fraction had a significant linear relationship with the serum turbidity (Table 2), irrespective of the heat treatment of juice. This turbidity is due to the presence of fine insoluble pulp particles on which microscopic oil droplets are absorbed. Figure 3 shows the linearity between turbidity and d-limonene content in Shamouti and Valencia juices.

Serum concentrates

The characteristics of concentrates prepared by pulp-serum separation, compared with those of conventional concentrates from the same raw material, are given in Table 3. The new concentrates, with full pulp admixture, had a concentration of about 55°Brix, much higher than that of commercial frozen concentrates, with cutback, on the market now.

The final Brix of the pulp-serum concentrates was a function of the degree of separation and the final serum concentration is given by the following relationship:

$$B_f = \frac{100B_j}{x + (100-x)B_j/B_s}$$

Table 3—Analyses of Shamouti and Valencia concentrates made by pulp-serum and conventional method with different heat treatments.

			Serum - pulp concentrate						Conventional concentrate					
Exp. No.	Variety	Heat treatment (°C)	Serum conc (°Brix)	Final conc (°Brix)	Pulp (%)	Essential oil (ml/liter)	Hunter a	Color Rd	Juice conc (°Brix)	Conc after cutback (°Brix)	Pulp (%)	Essential oil (ml/liter)	Color a	Hunter Rd
1	Shamouti	None	68	56.0	7.0	0.112	−3.4	33.5	62	47.0	7.7	0.076	−0.9	43.1
		60	63	53.5	7.0	0.136	−2.5	31.9						
		80	69	56.7	7.9	0.161	−2.3	33.2						
2	Shamouti	None	76	55.0	10.0	0.097	0.6	31.7	60	45.4	7.0	0.042	2.1	31.0
		60	76	56.1	9.8	0.102	0.9	30.9						
		80	80	54.0	14.0	0.152	3.9	34.9						
3	Valencia	None	80	61.2	10.0	0.120	5.7	26.4	68	46.1	7.0	0.056	5.1	26.7
4	Valencia	None	72	54.1	12.5	0.148	5.4	27.6	66	45.2	7.0	0.050	5.2	26.5

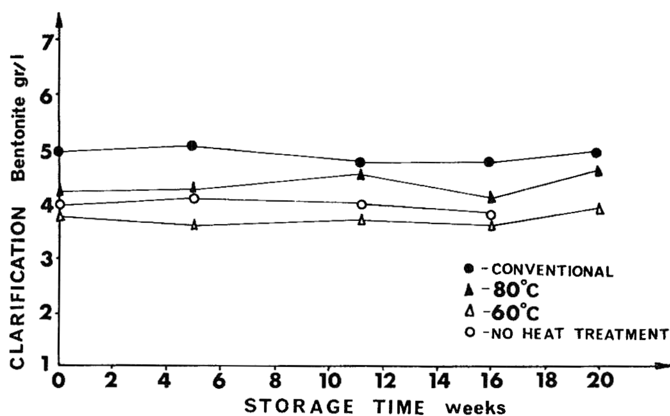

Fig. 4—Cloud stability of orange concentrates with various heat treatments during storage at −18°C.

Table 4—Organoleptic mean scores of Shamouti and Valencia frozen concentrates prepared by 2 methods.

		Pulp-serum concentrates		Conventional		
Exp. No.	Variety	Heat treatment °C	Mean score	conc Mean score	F	W.S.D.
1	Shamouti	None	6.67	5.60	3.18*	1.39
		60	7.80			
		80	7.42			
2	Shamouti	None	8.64	6.09	9.89*	1.31
		60	7.45			
		80	6.82			
3	Valencia	None	7.93	6.64	19.94**	0.87
4	Valencia	None	8.67	6.75	8.18**	1.08

*Significant difference at 95% confidence level.
**Significant difference at 99% confidence level.
W.S.D., wholly significant difference at 95% confidence level.

B_f = final product concentration (°Brix).
B_s = final serum concentration (°Brix).
B_j = °Brix of feed juice.
x = percent pulp separated

The low viscosity of the serum permitted a concentration as high as 80° Brix (Table 3). A 7% pulp separation prior to concentration yielded a final product of 55°Brix. No significant differences in color and appearance were found between the pulp-serum and conventional concentrates. Observed differences were due to variations in pulp (volumetric) content. The volumetric pulp content depends on degree of compression of pulp and will be discussed elsewhere.

Clarification of the reconstituted concentrates was independent of heat treatment and depended mainly on the amount of pulp. There was no change in clarification during storage for 20 weeks at −18°C (Fig. 4) and no separation in any of the concentrates throughout the storage period. These results lead to the conclusion that the heat treatment was not necessary for production of the pulp-serum concentrates. The stability of the new concentrates without enzyme inactivation is explained by the higher concentration (55° Brix) of the final product. As shown by Joslyn and Pilnik (1961), maximum clarification takes place at about 45°Brix, while a higher concentration is more stable as regards cloud.

The amount of essential oil in the pulp-serum concentrates was 2 to 3 times that of the conventional concentrates, because most of the essential oil was in the separated pulp which did not undergo evaporation (Table 1). The final oil content itself was influenced by the pulp content.

Organoleptic scores for the Shamouti and Valencia concentrates are given in Table 4. The pulp-serum concentrates were preferred throughout to the conventional concentrates. This was probably due to the higher oil content in the former concentrates. In addition, the pulp-serum concentrates were found to have a very pleasant bouquet resembling the aroma of fresh juice, which was absent in the conventional concentrates. No correlation was found between heat treatment and organoleptic scores. Apparently, uncontrolled factors (such as the holding time during the process and the time of evaporation) outweighed the influence of the heat treatment. No difference in pH, acidity and ascorbic acid content was found between the 2 types of concentrates.

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D. I. MURDOCK
The Coca-Cola Company Foods Div., Plymouth, Florida 32768
and G. L. K. HUNTER
The Coca-Cola Company, Atlanta, Georgia 30301

BACTERIOLOGICAL CONTAMINATION OF SOME CITRUS OILS DURING PROCESSING

SUMMARY Tangerine, lemon and grapefruit oil emulsions have been shown to undergo undesirable modification as a result of microbial growth. These emulsions contain microflora capable of producing alpha-terpineol by the hydrolysis of d-limonene. The greatest microbial activity occurred in tangerine and lemon, and the least in grapefruit. Lemon oil emulsions containing less than 1% oil produced the highest concentration of alpha-terpineol. d-Limonene concentration in dilute lemon oil slurries decreased with a corresponding increase in alpha-terpineol. A survey of seven plants producing grapefruit oil showed no correlation between sanitary conditions and the microflora concentration. Rapid microbial growth in the citrus peel oil recovery operation requires the maintenance of good sanitary practices in order to control contamination.

INTRODUCTION

MURDOCK et al. (1967, 1969) reported at the 1967 Florida State Horticultural Society meeting that orange oil emulsion was an excellent medium for microbial growth, the consequence of which yielded an undesirable oil. In addition, it was shown that this microbial process resulted in the biological formation of alpha-terpineol.

Good sanitary practices were found to play an important role in the production of high quality orange peel oil. Heretofore, cleanliness during the oil recovery process was believed to be unimportant; therefore, the citrus industry paid little or no attention to sanitation during this phase of the operation.

This paper is extended to include tangerine, lemon and grapefruit cold-pressed oils which are also produced in Florida. It concerns the oil recovery processes, with particular emphasis on the microbiological aspects and the development of alpha-terpineol.

PROCEDURE

THE FMC OIL recovery process was used to obtain lemon and grapefruit oils. Details were previously discussed by Murdock et al. (1969). Brown equipment was used to extract tangerine oil. Except for the method of juice extraction, the Brown oil recovery process was similar to that of FMC.

Bacteriological

Samples of tangerine, lemon and grapefruit oil slurries and emulsions were obtained from various stages in the manufacture of cold-pressed oil during the 1967 and 1968 processing seasons. The samples were stored at 30°C (86°F) for an extended period. They were plated in duplicate on orange serum agar and the plates counted after 3–5 days of incubation at 30°C. Some of the product was also analyzed periodically by gas chromatography (GC).

Analytical

The analytical procedure was the same as previously described by Murdock et al. (1967, 1969). Briefly it consisted of extracting the oil from the emulsion with methylene chloride and separating the extract from the emulsion by centrifuging, removing the solvent and analyzing the residue by gas chromatography. The oil constituents represented by the peaks in the chromatogram were identified by infrared and mass spectroscopy.

A Perkin-Elmer 226 Flame Ionization Gas Chromatograph was used to monitor the oil. The instrument contained a 0.01 in. × 300 ft Carbowax 20 M column which was temperature programmed at 2°/min from 75°–180°C. In addition, after 10 min of operation, the helium pressure was programmed from 40–180 psi over a period of 1 hr (2.3 psi/min). A 4 ml sample was injected and split 100/1.

The alpha-terpineol fold in the oil was calculated from its chromatographic peak height. Any deviations, because of the sample size or delivery loss of the sample into the gas chromatograph, were corrected using the natural n-decanal concentration in the oil as an internal standard. It was necessary to use caryophyllene as the internal standard in the lemon peel slurry oil analysis when the concentration of alpha-terpineol was close to its maximum. It can be seen in Figure 1 that the concentration of aliphatic and terpenic aldehydes decreased when d-limonene was present in relatively low concentrations.

The citrus oils used in this study has the following analysis:

	% Oil w/w*
Peel grit slurry	< 1
Finisher oil emulsion	< 1
Desludger oil emulsion	70–82
pH	4.3–5.4

*Modified Scott Method (Scott, 1966)

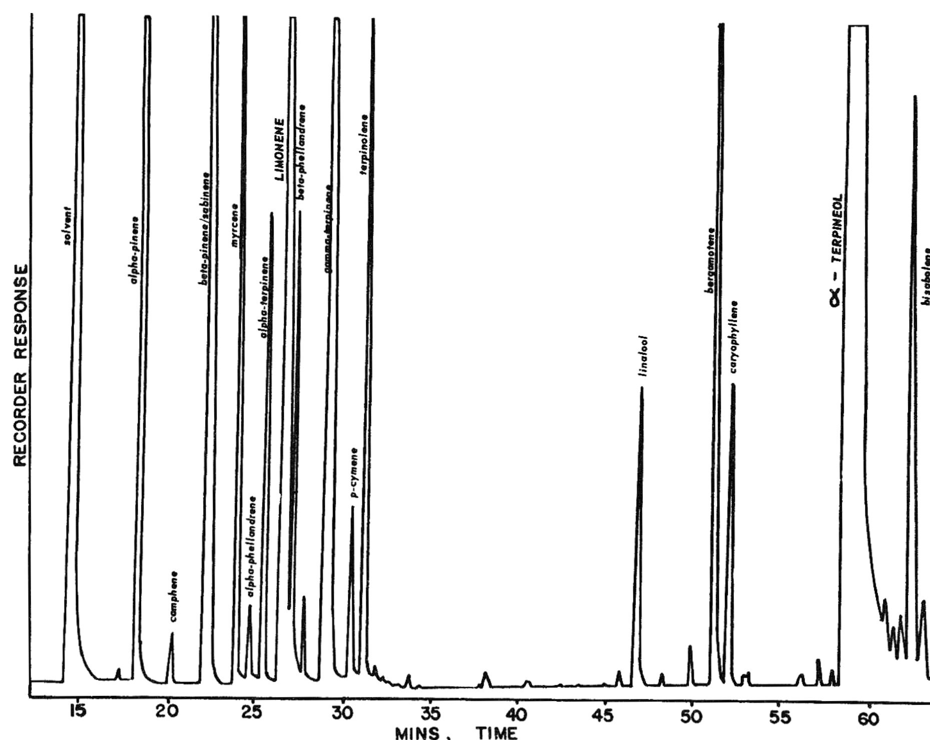

Fig. 1—Chromatogram of oil from lemon peel slurry 52 days after sampling.

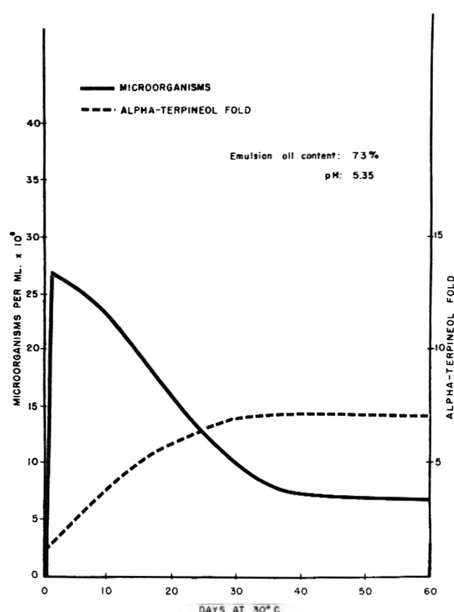

Fig. 2–Growth of microflora in tangerine oil emulsion and increase of alpha-terpineol in tangerine oil.

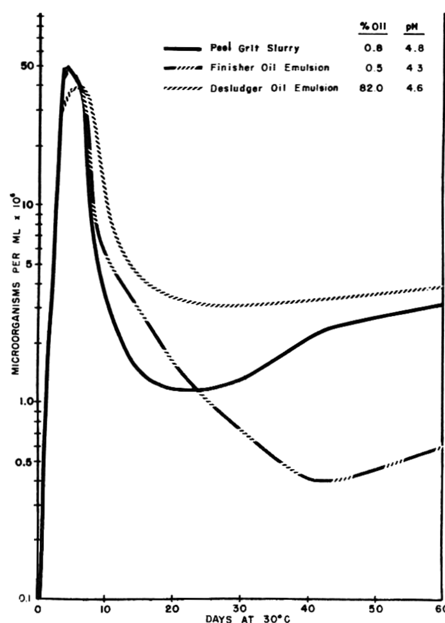

Fig. 3–Growth of microorganisms in lemon oil emulsions having less than 1.0% and more than 80% oil content.

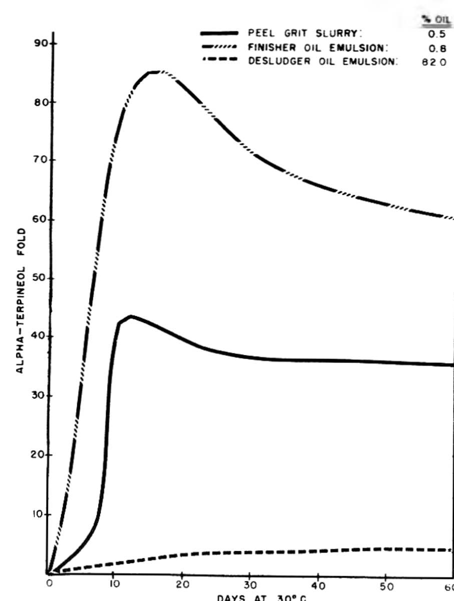

Fig. 4–Development of alpha-terpineol in lemon oil emulsions containing less than 1.0% and more than 80% oil content.

RESULTS & DISCUSSION

Tangerine oil

A sample of tangerine oil emulsion from the desludger was held at 30°C and plated at periodic intervals over a 60-day period. The results in Figure 2 show microbial growth occurred at an extremely rapid rate during the initial storage period, increasing in 48 hr from less than 100,000 org./ml to over 25 million. After reaching this peak, a gradual decrease in number occurred. Alpha-terpineol was also produced; however, the concentration was considerably less than that reported in orange oil, where over 80-fold developed in 60 days (Murdock et al., 1967)

Lemon oil

Three different sets of samples were obtained at one plant during the production of cold-pressed lemon oil. Each set consisted of peel grit slurry from the extractors, oil emulsion from the finishers, and oil emulsion from the desludger. All samples were stored at 30°C.

The first set, which was obtained in Sept. 1967 and represented oil from diphenyl-treated fruit, was held at 30°C for 150 days. Diphenyl is a fungicide used to control spoilage in a fresh fruit packing house. It penetrates the oil cells of the fruit and can be detected in the finished oil by GC.

The second and third sets were collected in Oct. and Nov. 1967 respectively, and represented oil recovered when grove-run fruit was being processed. Set 2 was held 94 days and set 3 for 85 days. For sake of space, only the results obtained during the first 60 days will be reported.

It was noted that higher plate counts occurred in oil extracted from diphenyl-treated fruit than from untreated (grove-run) fruit. This relationship is shown in data presented in Table 1. These data are from sets 1 and 2; because 3 was similar to set 2, it is not shown.

A further examination of the results from set 2 (Fig. 3) showed that microbial growth was very rapid at the start of the storage period. A corresponding rapid decrease in population then occurred, followed by another increase. This same growth pattern occurred when all 3 sets of lemon oil components were held beyond the 60 days reported. This subsequent increase in population was believed to be due to another group of microorganisms which started to take over, as was evidenced by different colony formations which began to appear on the pour plates.

There was also a decided pH increase in the low oil content samples throughout the storage period. For example, the pH of peel grit slurry in one test increased after 60 days from 4.0–7.3. On the other hand, there was no change in the desludger oil emulsion pH during this same period (4.6–4.5).

Alpha-terpineol produced throughout this test run is shown in Figure 4. The highest concentration occurred in emulsions containing less than 1% oil, and the least amount in the desludger oil emulsion. Bacterial growth in the emulsions of these oils also followed a somewhat similar pattern. In the low oil content sam-

Table 1–Growth of microorganisms in lemon oil emulsions from processing diphenyl-treated and untreated fruit.

	Diphenyl			Grove-run		
Days 30°C	Peel grit slurry[a]	Finisher oil emulsion[a]	Desludger oil emulsion[b]	Peel grit slurry[a]	Finisher oil emulsion[a]	Desludger oil emulsion[b]
	Organisms per ml × 10^6					
0	0.001	0.01	0.008	0.047	0.026	0.059
5	660.0	7.8	39.5	50.0	49.0	37.7
10	59.5	25.0	89.5	3.8	5.7	20.8
15	7.2	24.3	42.0	1.2	1.9	4.6
20	10.5	26.0	24.8		3.0	3.6
30	193.5	12.8	35.0	1.5	2.1	3.5
40	36.0	29.0	39.5	2.2	0.4	
60	17.5	34.5	53.5	3.0	0.6	3.3

[a]Peel grit slurry and finisher oil emulsion less than 1% oil.
[b]Desludger oil emulsion 80–82%.

Fig. 5–Chromatogram of oil from lemon peel slurry after sampling.

Fig. 6 Growth of microorganisms in citrus oil emulsions.

ples, the concentration of alpha-terpineol first increased and then, for some unexplained reason, decreased. This phenomenon was never observed in any of our previous studies.

It was noted that when bacteria were allowed to multiply in dilute solutions, such as lemon oil slurry, the d-limonene concentration (a major component of citrus oils) diminishes in direct proportion to the increase in concentration of alpha-terpineol (Fig. 5 and 1).

Figure 5 is a gas chromatogram obtained from fresh peel grit slurry oil, and Figure 1 shows the same material 52 days later. Note the reduction in concentration of d-limonene. Furthermore, when d-limonene was added to the desludger waste water (oil content 0.03%), to simulate peel grit slurry, over 90% of the d-limonene was converted to alpha-terpineol.

Table 2–Microorganisms found in grapefruit oil emulsions produced in Florida during 1968 processing season. (Samples obtained from desludger.)

Plant no.	Date 1968	Org. per ml
1	01–10	1,100,000
	02–29	61,000
2	01–09	1,000,000
	02–29	78,000
3	01–12	230,000
4	01–12	1,800,000
	02–29	1,000,000
5	01–12	23,000
	02–29	270,000
6	02–07	6,250,000
	02–29	22,000
	04–10	90,000,000
7	02–15	2,800,000

Grapefruit oil

Samples of desludger grapefruit oil emulsion were obtained from seven different processing plants during the 1968 citrus season. They were pour plated as previously described. Table 2 shows a wide range in microbial counts; e.g., in one plant the counts ranged from 22,000 to 90 million org/ml. No definite correlation could be established between bacterial counts and the sanitation of the operation.

Of the citrus oil emulsions investigated, grapefruit appeared to be the poorest medium for microbial growth. Also of interest, alpha-terpineol was not produced in any significant quantities in grapefruit oil emulsion.

Microbial growth and development of alpha-terpineol in citrus oils

Murdock et al. (1967) found that during the manufacturing of c.p. orange oil microbial growth occurred throughout the processing line. In this investigation, samples of tangerine and lemon oil emulsions from the desludger were also found to support very rapid microbial growth.

Fig. 7–Development of alpha-terpineol in orange, tangerine, lemon and grapefruit oil emulsions as a result of microbial growth.

On the other hand, two different lots of grapefruit oil emulsion did not consistently support as high a microbial population as the other emulsions investigated.

Growth curves, obtained after holding orange (Murdock et al., 1967), tangerine, lemon and grapefruit oil emulsions at 30°C for 60 days, are shown in Figure 6. The orange oil emulsion supported the highest microbial population followed by

tangerine, lemon and grapefruit. Alpha-terpineol produced in these citrus oils during this test period is shown in Figure 7. The curves show that approximately 11 times more alpha-terpineol is produced in orange oil emulsion in 60 days than in tangerine, and 20 times more than in lemon oil emulsion.

Sterilized control samples of each oil emulsion studied showed no increase in alpha-terpineol during the test period.

Sanitary aspects

Data have been presented to show that microorganisms grow at an extremely rapid rate in lemon oil emulsions, especially when they contain less than 1% oil, with a correspondingly rapid increase in concentration of alpha-terpineol. This same relationship occurs with orange and tangerine oil recovery processes, and to a lesser degree with grapefruit. This would indicate the need for maintaining an efficient sanitation program in the initial phases of the citrus oil recovery process to prevent rapid microbial growth and the corresponding development of alpha-terpineol.

Holding of slurries prior to desludging for any period of time would not be advocated. Also, most processors use equipment consisting of screw conveyors to convey peel grit slurry to the finishers. This equipment is extremely difficult to keep in a sanitary condition. Pumping of this slurry from the extractors to the finishers would greatly alleviate this problem.

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Ms. received 4/25/69; revised 6/10/70; accepted 7/3/70.

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H. SUGIMOTO and J. P. VAN BUREN
New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456

REMOVAL OF OLIGOSACCHARIDES FROM SOY MILK BY AN ENZYME FROM ASPERGILLUS SAITOI

SUMMARY—As part of a program to reduce the flatulence-inducing tendency of soy milk, a method for the enzymatic removal of galacto-oligosaccharides by means of an enzyme preparation from Aspergillus saitoi *was investigated. It was found that a partially purified preparation possessing both α-galactosidase and invertase, yet free from protease, could be obtained easily from a commercial* A. saitoi *acid-protease product by means of a simple molecular sieving procedure. The α-galactosidase exhibited its optimum pH between 5.0 and 5.5, and seemed to be stable between pH 4.0 and 8.0. The optimum temperature was found at about 55°C; however, the enzyme itself was inactivated by maintaining it at 70°C for 30 min. These properties appeared suitable for the enzymatic treatment of soy milk. p-Chloromercuribenzoate, N-bromosuccinimide, $HgCl_2$, $AgNO_3$ or $CuCl_2$ showed strong inhibitory effects on the enzyme. The presence of $1 \times 10^{-2}M$ galactose caused only slight inhibition. Km value of the enzyme with melibiose as a substrate was found to be $3.11 \times 10^{-3}M$ and the molecular weight of the enzyme estimated to be about 290,000 on the basis of a gel filtration technique. Investigations by means of thin-layer chromatography indicated that the addition of small amounts of this enzyme preparation to soy milk resulted in complete hydrolysis of galacto-oligosaccharides. The practicability of the present method was also discussed from an economic viewpoint.*

INTRODUCTION

SOY MILK has been given considerable attention as an economical high-protein beverage that can help overcome widespread protein deficiencies. However, there remain a number of qualitative problems which must be solved for this product to obtain a wider acceptance.

One of these problems is its tendency to induce flatulence, often accompanied by an uncomfortable feeling of fullness and intestinal activity. Recently, it has been suggested by some investigators that the flatulence caused by soy products could be due, at least in part, to their relatively high contents of galacto-oligosaccharides, especially stachyose and raffinose (Murphy, 1963; 1964 a; 1964 b; Burr, 1967; Rackis et al., 1967; Steggerda, 1967). On the basis of this hypothesis, flatulence in soy milk should be reduced by removal or decomposition of these oligosaccharides.

In preliminary studies by one of the authors (H.S.), it was found that considerable numbers of fungal strains belonging to the genus *Aspergillus* exhibited powerful abilities to produce galacto-oligosaccharide decomposing enzymes such as α-galactosidase (E.C.3.2.1.22) or invertase (E.C.3.2.1.26). Furthermore, some of the commercial enzyme products prepared from the same fungi contained considerable activities of both enzymes (manuscript in preparation).

This paper demonstrates that the oligosaccharides in soy milk can be hydrolyzed almost quantitatively by an enzyme partially purified from a commercial product. Some characteristics of the α-galactosidase which play a main part in the hydrolysis of the oligosaccharides, the qualitative changes of the oligosaccharides during the enzyme treatment, and the economical practicability of the present method are also described and discussed.

MATERIALS & METHODS

Soy milk

Freeze-dried raw soy milk prepared from Harosoy 63 variety was supplied by Dr. D. Fukushima, Kikkoman Shoyu Co., Ltd., Noda, Chiba, Japan. The chemical analysis of this material indicated the following composition: moisture 2.79%, crude protein (Kjeldahl N × 5.71) 41.90% and crude fat 24.8%.

Source of enzyme

A commercial acid-protease product, Molsin (Lot No. M19, 193) from *A. saitoi*, obtained from Seishin Pharmaceutical Co., Ltd., Tokyo, was used as a source of enzyme. 1 g of the enzyme showed 620×10^3 units of α-galactosidase activity.

Assays of enzyme activities

The reaction mixture for the assay of α-galactosidase (E.C.3.2.1.22) activity was composed as follows: 1 ml of 1% (w/v) melibiose dihydrate, 2 ml of 0.1 M acetate buffer, pH 5.0 and 1 ml of the enzyme solution. The reaction mixture was incubated at 40°C for 1 hr, then placed into a boiling water bath for 10 min. Glucose was estimated by a glucose oxidase reagent, Glucostat, according to the directions of the manufacturer, Worthington Biochemical Corp., Freehold, N.J. 1 unit of enzyme activity was defined as the amount of activity which liberated 1 mg of glucose under the conditions just mentioned. The activities of β-galactosidase (E.C.3.2.1.23), α-glucosidase

Table 1–Partial purification of enzyme preparation from Aspergillus saitoi.

	α-Galactosidase			Invertase			Protease		
	Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Dialysate	298×10^5	3,260	100.0	114×10^6	12,500	100.0	155×10^{-2}	17.1×10^{-5}	100.0
Acetone ppt.	298×10^5	4,980	100.0	61×10^6	10,100	53.0	87×10^{-2}	14.7×10^{-5}	56.4
Ultrafiltrate	284×10^5	69,300	95.4	21×10^6	50,700	18.2	3×10^{-2}	6.6×10^{-5}	2.0
Gel filtrate	226×10^5	181,000	75.9	9×10^6	72,800	8.0	0	0	0.0

(E.C.3.2.1.20), β-glucosidase (E.C.3.2.1.21) and invertase (E.C.3.2.1.26) were assayed in the same manner using 1% (w/v) solutions of lactose monohydrate, maltose monohydrate, cellobiose and sucrose, respectively, as substrates. In both cases of α-, and β-glucosidases, 1 unit of enzyme activity corresponded to 2 mg of glucose liberated. In investigating the effects of inhibitors or activators on the α-galactosidase activity, p-nitrophenyl-α-D-galactopyranoside was employed as a substrate, since it was necessary to avoid inhibitor or activator effects on glucose oxidase. In this case, the reaction mixture consisted of 1 ml of 0.5% (w/v) p-nitrophenyl-α-D-galactopyranoside in 0.1 M acetate buffer, pH 5.0, 1 ml of inhibitor or activator and 1 ml of the enzyme solution. The reaction was carried out at 40°C for 15 min and stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid. After addition of 9 ml of 0.3 N NaOH to the reaction mixture, the increase of the optical density of the mixture was estimated at 420 mμ. Protease activity was assayed at pH 6.2 according to the improved Hagihara's method B (1954). In this method, 1 unit of protease corresponded to 1 meq of tyrosine liberated from milk casein per min at 30°C.

Estimation of enzyme protein

Lowry's method (Lowry et al., 1951) was employed for the estimation of protein in the enzyme preparation and bovine hemoglobin (2× cryst.) was used as a standard protein.

Purification procedures of enzyme

The partially purified α-galactosidase, which had some other glycosidases such as invertase, although it was free from protease, was prepared from Molsin by the following procedures: All operations were carried out at 2°C. 50 g of Molsin were dissolved in 250 ml of water and dialyzed overnight with a fishskin tubing against running tap-water. After removal of insoluble material by centrifugation, the enzyme solution was brought up to a volume of 500 ml with distilled water. The same volume of cold acetone was then poured into the resultant solution, the precipitate formed collected, washed twice with 50% (v/v) aqueous acetone and pure acetone successively, then dried in vacuo. After the acetone precipitation, recovery of α-galactosidase activity was almost quantitative. The acetone powder was dissolved in 1,000 ml of 0.05 M acetate buffer, pH 5.5, and the solution concentrated to a volume of 100 ml by use of an Amicon ultrafiltration cell (Amicon Corp., Cambridge, Mass.) A Diaflo XM-100 membrane was employed under an air pressure of 1.5 kg per sq cm.

After addition of another 1,000 ml of 0.05 M acetate buffer, pH 5.5, the solution was concentrated again to a final volume of 20 ml. A 10-ml aliquot of the above enzyme solution was applied to a 2.0- by 140-cm column ot Sephadex G-200 equilibrated with 0.05 M acetate buffer containing 0.1 M KCl, pH 5.5. The column was eluted with the same buffer at a flow rate of 15 ml per hr and 10-ml fractions collected.

Figure 1 shows the elution profile of α-galactosidase from the column. The fractions showing intense α-galactosidase activity were pooled, concentrated and desalted by means of a collodion bag assembly. The concentrate was then freeze dried and employed in subsequent experiments as a partially purified enzyme

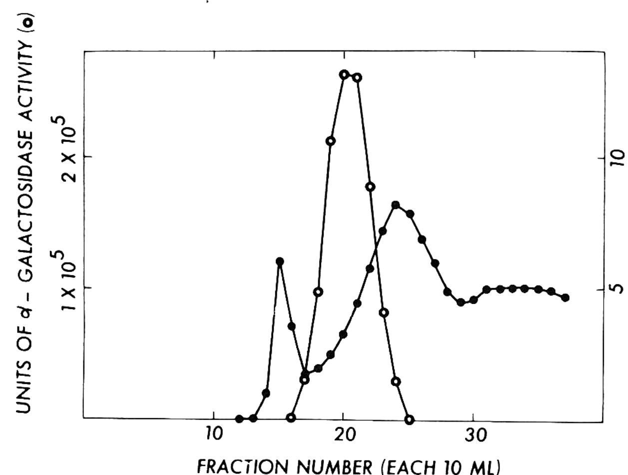

Fig. 1–Gel filtration of α-galactosidase on a Sephadex G-200 column.

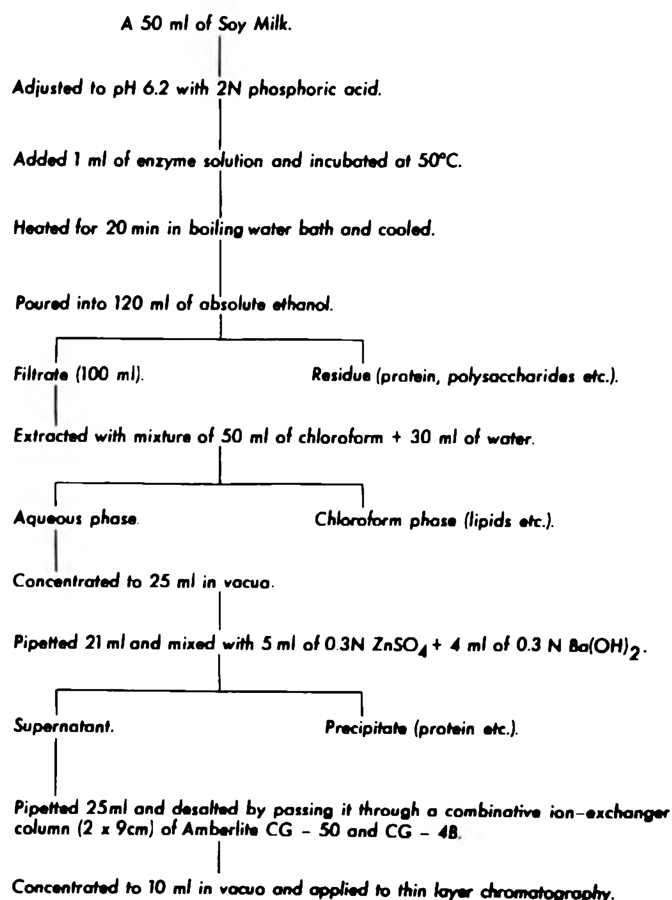

Fig. 2–Procedures for the isolation of oligosaccharides from soy milk.

preparation. Differing from the enzyme from *Aspergillus niger* (Bahl and Agrawal, 1969), the α-galactosidase in this freeze-dried preparation was stable during a storage of 2 months at room temperature. The results of enzyme assays, after each step of the purification procedures, are summarized in Table 1. The partially purified enzyme also contained β-galactosidase, α-, and β-glucosidases with specific activities (units per mg protein) of 1,800, 220,000 and 228,000, respectively. The yields of these 3 enzymes were 5.4, 7.0 and 18.2%, respectively. The enzyme preparation was completely free from protease activity.

Treatment of soy milk by enzyme

The enzymatic treatments of soy milk were performed by the following procedures: The freeze-dried soy milk powder was dissolved in distilled water containing a few drops of Antifoam A and concentration adjusted to 15% (w/v). It was then heated for 20 min in a boiling-water bath with a reflux and gentle stirring. After cooling, the pH value of the soy milk was adjusted to 6.2 from its initial 6.5 by addition of a few drops of 2 N phosphoric acid; then the final concentration of the solid material was brought to 12.0% (w/v) with distilled water.

50 ml of soy milk and 1 ml of the enzyme solution were mixed in a 100-ml Erlenmeyer flask and incubated at 50°C with a gentle reciprocal shaking for the indicated times. The enzyme reaction was stopped by placing the mixture in a boiling-water bath for 10 min. In the experiments employing pure oligosaccharides instead of soy milk as substrates, the reaction mixture was composed as follows: 10 ml of 1% (w/v) sugar solution, 9 ml of 0.04 M phosphate buffer, pH 6.2 and 1 ml of the enzyme solution.

Isolation of sugars from soy milk

50 ml of the boiled reaction mixture were poured into 120 ml of absolute ethanol and filtered. The filtrate was extracted with a tertiary solvent system according to the method of Chan and Cain (1966). The aqueous phase was concentrated in vacuo and, after removal of contaminating proteins by Somogyi's method (1945), desalted by passing it through a combination ion-exchange resin column. The neutralized eluate was concentrated and used for thin-layer chromatography. These procedures are summarized in Figure 2. In the experiments with pure oligosaccharides, the extraction procedure with the tertiary solvent system was omitted.

Thin-layer chromatography

20 μliters of the purified sugar solutions were applied to Eastman Chromagram sheets (6064, cellulose) and developed by ascending-partition chromatography using the solvent system, n-butanol-pyridine–0.1 N HCl (5:3:2 by vol) (Li et al., 1964a). When the sucrose content in soy milk was being examined, another solvent system, water-saturated n-butanol-95% ethanol-trichloroethylene (6:2:2 by vol) was employed (Chan and Cain, 1966). The latter solvent system gave a better separation of sucrose from galactose. In both cases, resolutions of sugars were enhanced by using a multiple development technique, with 4 successive developments.

To locate sugar spots on the chromatograms, 3 kinds of detectors were employed. Fructose-containing sugars were detected by dipping the chromatogram into the modified α-naphthol reagent (Albon and Gross, 1950) in which the solvent, ethanol, was replaced by acetone. Alkaline silver nitrate reagent was used to locate reducing sugars (Trevelyan et al., 1950), and diphenylamine-aniline-phosphate was applied to detect both reducing and non-reducing sugars (Buchan and Savage, 1952; Bailey and Bourne, 1960).

Estimation of oligosaccharides

The quantitative estimations of stachyose, raffinose and sucrose in soy milk were carried out by means of a so-called guide-strip technique. After the development of the sugars on the cellulose thin layer in the way described above, the chromatogram was cut into strips. The sugar spots on the guide strip were detected by the modified α-naphthol reagent. The sites of sugars on the other strips were located by reference to the guide strip. The squares containing sugars were cut out and eluted by 5 ml of distilled water in a test tube at 70°C for 2 hr with occasional shakings. After removal of cellulose powder by centrifugation, the sugar in the supernatant was estimated according to the phenol-sulfuric acid method (Dubois et al., 1956). The concentration of the sugar was calculated from standard graphs. Among standard sugar references, stachyose tetrahydrate was provided by Mr. A. Yasuda, Kikkoman Shoyu Co., Ltd., Noda, Chiba, Japan, and manninotriose by Dr. C. Y. Lee of this station.

RESULTS

Properties of α-galactosidase

Since α-galactosidase can play a most important part in the hydrolysis of soy milk oligosaccharides, some properties of the α-galactosidase in the partially purified enzyme preparation were investigated. The preparation exhibited its optimum pH between 5.0 and 5.5, but it was almost inactive below pH 2.0 and above pH 8.0 (Fig. 3). The enzyme seemed to be stable from pH 4.0–8.0; however, it

Table 2—Effect of inhibitors, metal ions, sugars and organic acids on α-galactosidase activity.

Inhibitors	Final concentration (M)	Relative activity (%)
None	–	100
KCN	1×10^{-3}	100
EDTA	1×10^{-3}	100
o-Phenanthroline	1×10^{-3}	100
8-Hydroxyquinoline	1×10^{-3}	100
Sodium thioglycolate	1×10^{-3}	100
Mercaptoethanol	1×10^{-3}	100
Cysteine	1×10^{-3}	100
p-Chloromercuribenzoate	2×10^{-5}	3
Sodium sulfite	1×10^{-3}	100
Iodine	5×10^{-4}	0
N-Bromosuccinimide	1×10^{-3}	0
Sodium arsenate	1×10^{-3}	100
Sodium fluoride	1×10^{-3}	100
Sodium pyrophosphate	4×10^{-2}	5
Metal ions		
KCl	1×10^{-3}	100
$MgCl_2$	1×10^{-3}	100
$CaCl_2$	1×10^{-3}	100
$BaCl_2$	1×10^{-3}	100
$SrCl_2$	1×10^{-3}	100
$ZnCl_2$	1×10^{-3}	35
$MnCl_2$	1×10^{-3}	91
$FeCl_3$	1×10^{-3}	49
$NiCl_2$	1×10^{-3}	100
$CoCl_2$	1×10^{-3}	100
$CuCl_2$	1×10^{-3}	3
$CdCl_2$	1×10^{-3}	91
$AgNO_3$	1×10^{-3}	0
$HgCl_2$	1×10^{-3}	0
$Pb(CH_3COO)_2$	1×10^{-3}	42
Sugars and organic acids		
D-Glucose	1×10^{-2}	85
D-Galactose	1×10^{-2}	97
D-Fructose	1×10^{-2}	100
Sucrose	1×10^{-2}	100
Sodium citrate	1×10^{-2}	62
Sodium maleate	1×10^{-2}	62
Sodium oxalate	1×10^{-2}	100
Sodium ascorbate	1×10^{-2}	100

was sensitive under more acidic conditions. During 18 hr of storage in 0.1 M glycine buffer, pH 2.5, at 30°C, about 95% of the activity disappeared.

As shown in Figure 4, the optimum temperature for the hydrolysis of melibiose was observed near 55°C. However, the enzyme itself was unstable at high temperatures. For instance, 45% of the enzyme activity was lost after the treatment at 60°C for 30 min in 0.1 M acetate buffer, pH 4.5, and it was completely inactivated by maintaining it at 70°C under the same conditions.

The effects of inhibitors, metal ions, sugars and organic acids on the reaction of the enzyme were examined using p-nitrophenyl-α-D-galactopyranoside as a substrate (Table 2). p-Chloromercuribenzoate, at a final concentration of 2×10^{-5} M caused 97% of inhibition. Therefore, the enzyme seemed to be a SH-enzyme similar in this respect to the enzymes from other origins (Hogness and Battley, 1957; Li et al., 1963; Pridham and Walter, 1964; Suzuki et al., 1966). But, from this point of view, it appeared to be somewhat different from the α-galactosidase of a higher fungus, *Calvatia cyathiformis* which is not inhibited by sulfhydryl blocking agents (Li and Shetlar, 1964b). The enzyme was also completely inhibited by the presence of 5×10^{-4} M iodine, 1×10^{-3} M N-bromosuccinimide or 4×10^{-2} M sodium pyrophosphate in the reaction mixture. Among metal ions, 1×10^{-3} M $AgNO_3$, $HgCl_2$ or $CuCl_2$ caused almost complete inhibition. Glucose at a concentration of 1×10^{-2} M caused 15% inhibition to the enzyme activity whereas galactose, at the same concentration, caused only slight inhibition. Certain organic salts such as sodium citrate or sodium maleate also caused considerable enzyme inhibition.

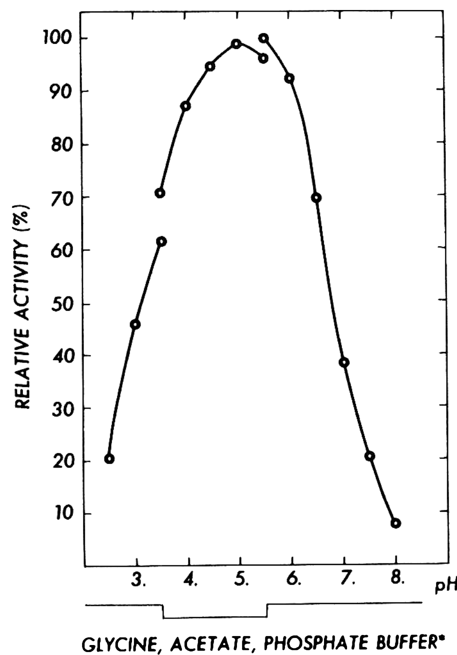

Fig. 3–Effect of pH on α-galactosidase activity. Ionic strength of all buffers in the reaction mixture is 0.1.

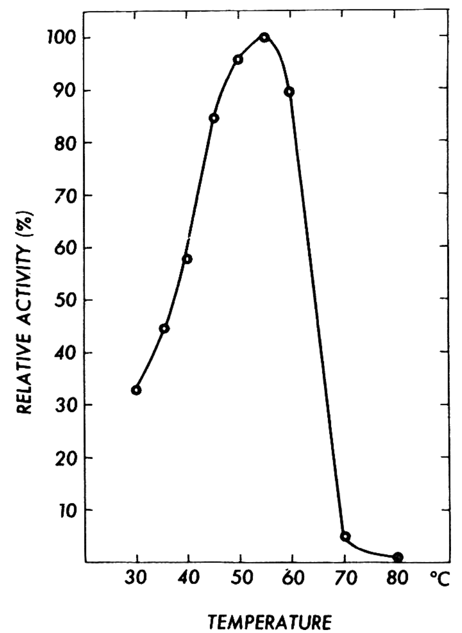

Fig. 4–Effect of temperature on α-galactosidase activity.

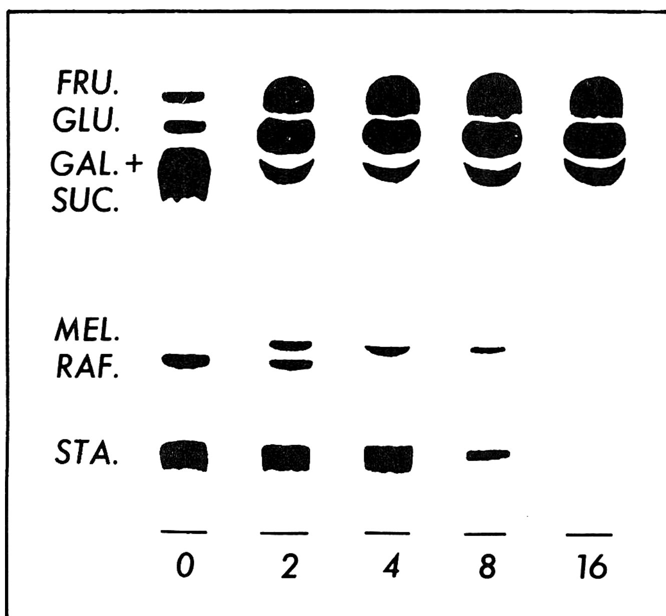

Fig. 5–Effect of the concentration of enzyme on the decomposition of oligosaccharides in soy milk. Numerals indicate the α-galactosidase activity × 10^{-3} per gram of solid material in soy milk.

Abbreviations: FRU. = fructose, GLU. = glucose, GAL. = galactose, SUC. = Sucrose, MEL. = melibiose, RAF. = raffinose, STA. = stachyose.

Detector: diphenylamine-aniline-phosphate reagent.

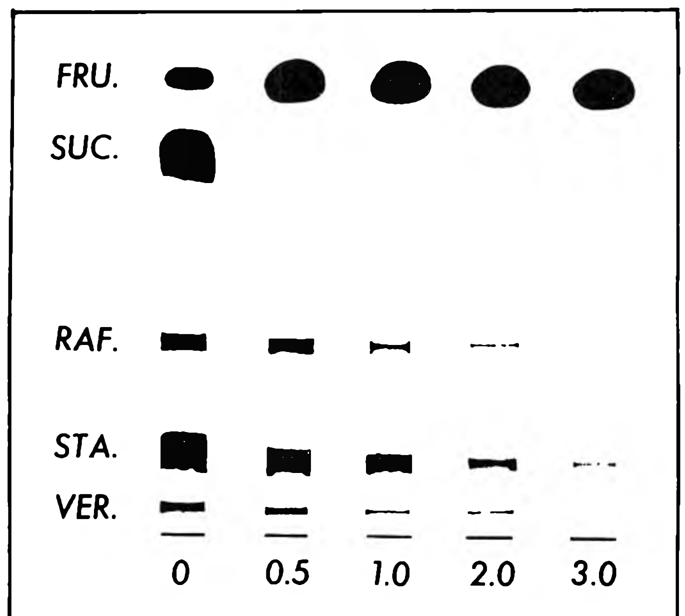

Fig. 6–Effect of incubation period of enzyme reaction on ketoses in soy milk. Numerals indicate the incubation period (hr). Abbreviations are the same as in Figure 5, except VER. = verbascose.

Detector: improved α-naphthol reagent.

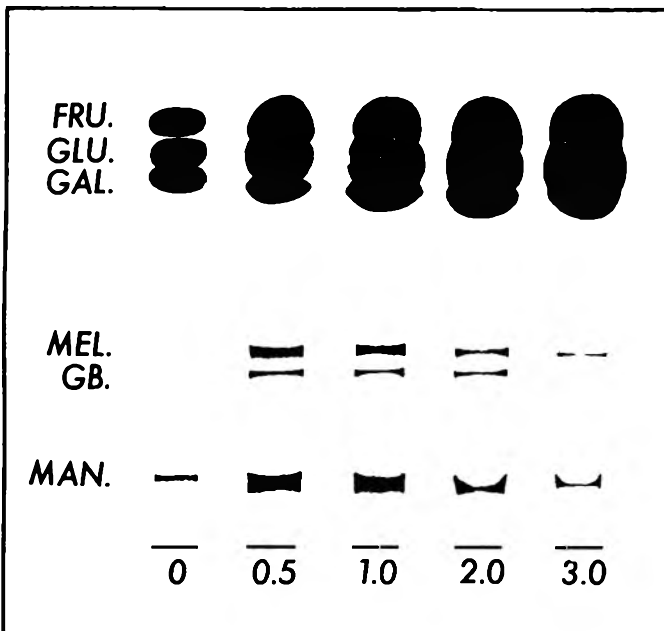

Fig. 7—Effect of incubation period of enzyme reaction on reducing sugars in soy milk. Numerals indicate the incubation period (hr). Abbreviations are the same as in Figure 5, except: GB. = galactobiose and MAN. = manninotriose.

Detector: alkaline silver nitrate reagent.

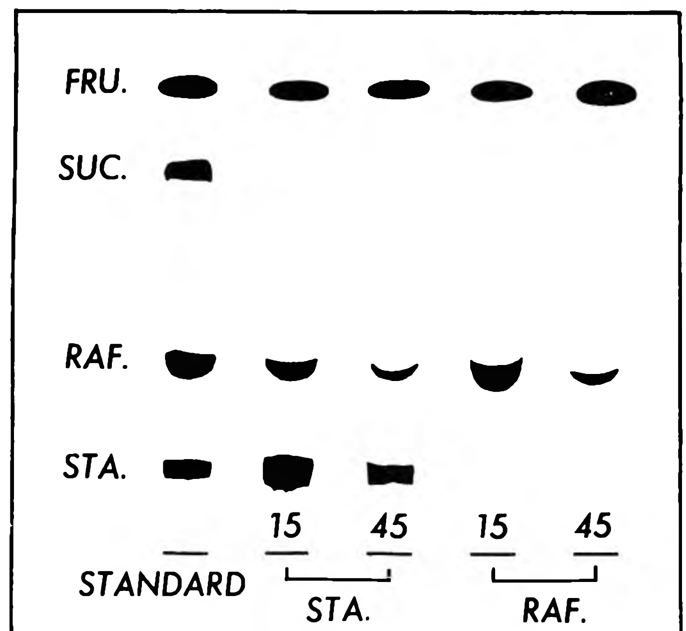

Fig. 8—Ketose-containing products of the enzymatic hydrolysis of pure stachyose and raffinose. Numerals indicate the incubation period (min). Abbreviations are the same as in Figure 5.

Detector: improved α-naphthol reagent.

Enzyme treatment was carried out as described in the text.

Lineweaver-Burk (1934) plots of the enzyme activity against the concentration of melibiose indicated a value of Km = 3.11×10^{-3}M.

The molecular weight of the α-galactosidase was determined roughly by means of a gel-filtration technique (Andrews, 1962; 1964) on a Sepharose 6B column (1.5 by 78 cm) equilibrated with a 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M KCl. Bovine tyroglobulin, horse apo-ferritin, human γ-globulin (Fr. II), bovine serum albumin (Fr.V), egg albumen (5× cryst.) and beef chymotrypsinogen A were employed for the establishment of a standard curve for globular proteins. The elution site of the α-galactosidase was detected by its activity on melibiose. It was observed between γ-globulin and apo-ferritin; consequently, the molecular weight of the α-galactosidase was assumed to be around 290,000.

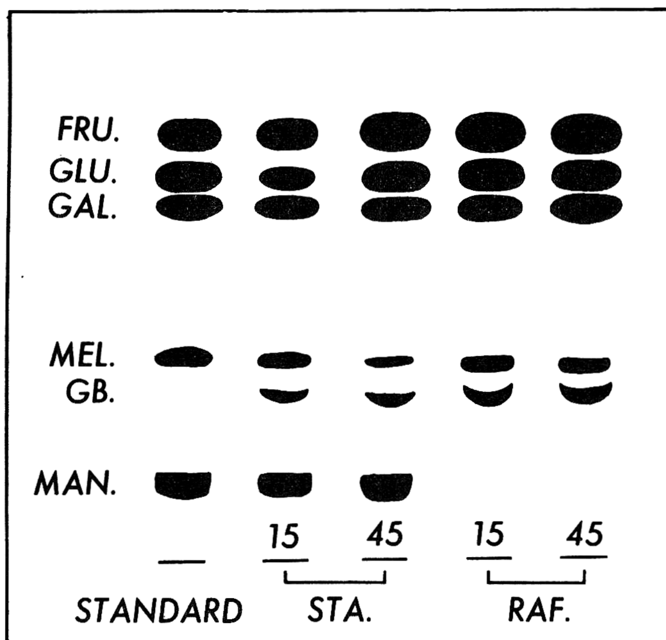

Fig. 9—Reducing sugar products of the enzymatic hydrolysis of pure stachyose and raffinose. Numerals indicate the incubation period (min). Abbreviations are the same as in Figures 5 and 7.

Detector: alkaline silver nitrate reagent.

Enzymatic hydrolysis of oligosaccharides in soy milk

Kawamura (1954) determined the contents of oligosaccharides in soybeans by means of paper chromatography and reported the following composition: sucrose 3.7%, raffinose 1.0%, stachyose 3.2% and total 7.9%. In the present experiment, as shown in Figure 5 and 6, all of these oligosaccharides could be recognized on the thin-layer chromatograms, and indicated the following contents on the dry basis of soy milk sample: sucrose 4.6%, raffinose 0.8% and stachyose 4.0% in terms of anhydrous material. Kawamura and Tada (1967) succeeded in detecting only a small amount of verbascose from Harosoy variety by means of column chromatography, but reported that pentasaccharides were not recognized on paper chromatograms. In our work, using soy milk prepared from the same variety, a sugar spot, supposedly verbascose, could be detected by the improved α-naphthol reagent on a cellulose thin-layer chromatogram (Fig. 6).

Kawamura and Tada also reported that

Harosoy variety contained free arabinose as a constitutive monosaccharide, but in our investigation no arabinose, which gives a greenish color with the diphenylamine-aniline-phosphate reagent, could be detected. However, a small amount of free fructose was observed as a constitutive monosaccharide of our soy milk sample.

As shown in Figure 5, treating the soy milk with 16×10^{-3} units of the α-galactosidase preparation per gram of the solid material completely decomposed all of these oligosaccharides to their constitutive monosaccharides after 3 hr of incubation.

Figures 6 and 7 show the time course of the hydrolysis of these oligosaccharides with the same activity of the enzyme preparation. In particular, during the earlier stage of the incubation, sucrose was decomposed very rapidly by the action of invertase, and parts of both raffinose and stachyose appeared to be hydrolyzed by the same enzyme to melibiose and manninotriose, respectively.

These facts were also demonstrated in experiments using pure substrates, authentic raffinose and stachyose, as shown in Figures 8 and 9. Manninotriose, raffinose and melibiose were seen on chromatograms to be intermediates in the hydrolysis of stachyose to monosaccharides, whereas sucrose did not accumulate in detectable quantities. With regard to other possible intermediates of hydrolysis, some slight indication of the presence of α-1,6-galactobiose was present on these chromatograms.

DISCUSSION

RECENTLY, an industrial application of α-galactosidase for the improvement of crystalline sucrose recovery from beet molasses has been reported by Suzuki et al. (1963; 1964; 1969). Their research is apparently the first and the only example concerned with an application of α-galactosidase for a food manufacture. For their purpose, it was necessary to avoid contamination with invertase in the enzyme preparation. However, in removing the oligosaccharides from soy milk, the presence of invertase in the enzyme preparation can be rather useful because, on the assumption of no serious problem of accelerated browning reaction, it can promote the hydrolysis of the oligosaccharides and increase the organoleptic sweetness of the soy milk.

On the other hand, contamination with protease activity causes many undesirable effects on soy milk such as coagulation, precipitation of protein, formation of bitterness and so on; consequently, the enzyme preparation for the present purpose must be strictly free from protease activity. Fortunately, the α-galactosidase from *A. saitoi* has an especially high molecular weight. Therefore, the separation of it from protease(s) can be easily attained by means of a simple molecular sieving method. This indicates that the enzyme can be obtained very economically as a by-product of purified protease production. In addition, the particular α-galactosidase from *A. saitoi* has a few advantages. For instance, since the optimum pH for enzyme activity is in the natural pH range of soy milk, there is almost no need to change the original pH value of soy milk. Furthermore, the enzyme itself is very stable in the same pH range, and it is not necessary to take any special care in its storage. Also, differing from some of the α-galactosidases previously reported (Li and Shetlar, 1964b; Suzuki et al., 1966), the activity of the enzyme from *A. saitoi* is relatively insensitive to the presence of an end product, α-D-galactose, in the reaction mixture. The enzyme has an undesirable feature in that it is inhibited by some organic acids present in soybeans in considerable amounts (Moriguchi et al., 1961).

Separate from the results described above, there is a fundamental question; namely, whether stachyose or raffinose is actually the main cause of flatulence of soy milk (Hellendoorn, 1969). Future physiological experiments with the oligosaccharide-free soy milk will be useful in testing this hypothesis.

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M. R. HART, R. P. GRAHAM, M. GEE and A. I. MORGAN JR.
USDA, Western Utilization R&D Div., ARS, Albany, California 94710

BREAD FROM SORGHUM AND BARLEY FLOURS

SUMMARY—Various additives were examined as possible aids in making bread from sorghum and barley flours. Good rise was achieved with doughs containing 45% solids. Several gums, especially 4000 centipoise methylcellulose, increased gas retention in sorghum bread and improved texture of both sorghum and barley breads. Several starches improved texture and loaf volume of sorghum bread. Glyceryl monostearate (GMS) improved the texture of sorghum bread but caused it to crumble badly. GMS improved softness in barley bread. Shortenings also softened sorghum and barley breads. The effect of several processing variables on sorghum bread was studied along with 2 methods of imparting a sour fermented flavor to sorghum bread.

INTRODUCTION

BOTH SORGHUM and barley are widely used in America as feed grains, but in many other areas of the world they are a staple part of the human diet. Barley is grown over much of the world in relatively cool climates where its short growing season often makes it more dependable than wheat or oats. In contrast, sorghum does well in hot semiarid regions. Its ability to resist droughts makes it especially valuable in many areas subject to chronic food shortages.

Sorghum is not easily utilized as a food. The grain remains tough even after extended parboiling. In addition, the flavor is rather unappetizing. When used as a staple it is commonly fermented into a form of beer or ground into a flour (Rooney and Clark, 1968). The flour is eaten as porridge or flat bread.

Barley is more easily utilized as a food, especially in malting and malt products. Lesser amounts are used for supplements in baby foods and soups. Barley flour can be incorporated into predominantly wheat breads and has been used to dilute wheat bread during wartime (Horder et al., 1954).

Barley flour can be used to make a yeast-raised bread (Landenberger and Morse, 1918), but it bakes into a coarse, heavy loaf that compares poorly to wheat bread.

Considerable effort has already been expended in making bread from non-wheat or gluten-free wheat flours. Jongh (1961) made bread from wheat starch and glycerol monostearate (GMS). Later studies were made by Jongh et al. (1968) on wheat and cassava starch breads using GMS. Kim and De Ruiter (1968) investigated various gums, starches and proteins along with GMS, as aids in making bread from combinations of cassava flour and soya or peanut flour. McGreer (1967) developed a bread from gluten-free wheat flour using cellulose gum.

It was thought that similar breads could be made from barley and sorghum through the use of suitable additives. Bread properties might also be improved through processing variations. With these objectives in mind, selected additives were evaluated for their effects on sorghum and barley bread, and certain processing variations were examined for their effect on sorghum bread.

Bread from sorghum and barley flours without additives

Samples of red milo sorghum and Firlback barley were obtained locally and ground into a flour by a Brabender Quadrumat Senior mill (C. W. Brabender Instruments, Inc., South Hackensack, N.J.). A yield of 65% was obtained for sorghum and 52% for barley.

Sorghum. When a dough of 35 to 45% moisture (percent based on dough weight) was prepared it lacked the consistency and elasticity associated with a wheat flour dough. It broke apart easily and failed to develop any elastic properties when worked or kneaded. Increasing the amount of water to 50–60% resulted in a more liquid dough or batter. It was assumed that a dough or batter must at least double in volume during mixing and proofing, to produce an acceptable bread. When 2% compressed yeast (percent based on flour weight) was added in making a sorghum dough (with 35 to 45% moisture) insufficient rise was obtained. When the same amount of yeast was added to a batter (with 50–60% moisture) sufficient rise was obtained, but the loaves collapsed during baking.

Barley. Unlike the sorghum flour, barley formed a dough that was sticky and held together well. Unfortunately, the barley dough also failed to rise properly. Although the barley dough rose higher than sorghum dough at comparable moisture contents it was still necessary to use a batter of 50–60% moisture to achieve a sufficient proof height.

Once sufficient rise was obtained the barley batter could be successfully baked without collapsing. During baking no further rise or oven spring occurred; consequently, the resultant bread lacked the domed shape associated with good quality. The top surface of the loaves was rough and lumpy and the texture very coarse.

The effect of bread additives

Evaluation procedure. Preliminary

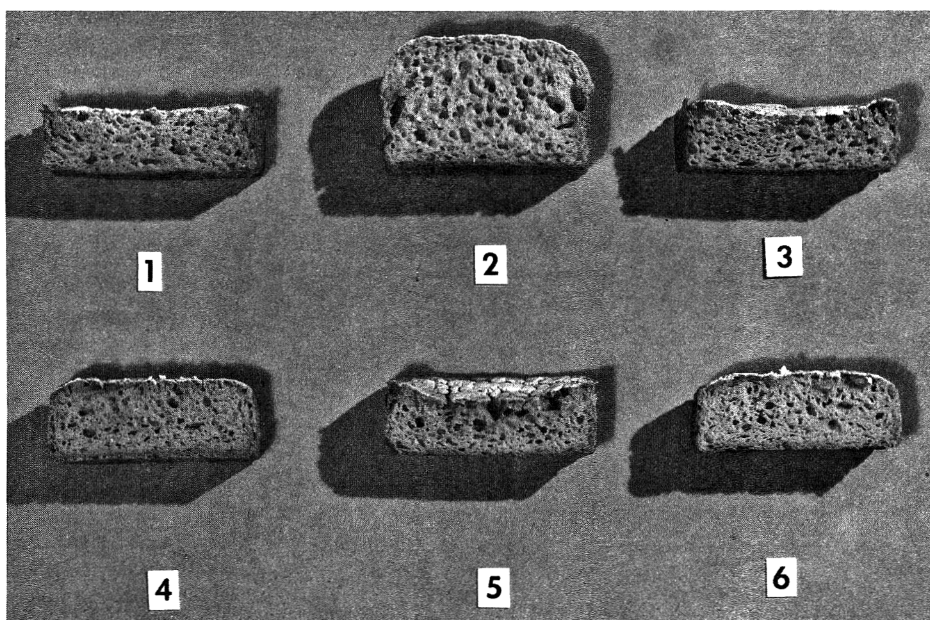

Fig. 1—Effect of different gums in sorghum bread. Loaf 1: no additive; 2: 2% 4000 cps Methocel; 3: 2% gum arabic; 4: 2% Kraystay type S; 5: 2% sodium CMC; 6: 2% Jaguar J2S-1.

tests were made to determine a suitable recipe for comparison purposes. Several gums thickened and strengthened the sorghum and barley doughs to where they more closely resembled conventional bread dough. However, the doughs still would not rise properly, and it became necessary to incorporate all additives into a batter rather than a dough.

When the additive was a gum or pectin, a moisture content of 55% or more was necessary, depending on the amount of additive. In contrast, viscosity was lowered by starches and shortenings, permitting a lower moisture content if desired. A moisture content of 55% was used for testing most of the additives.

The following basic recipe was used:

Table 1—The qualitative effect of various additives on sorghum and barley breads.

Additive	Source	Effect on sorghum bread	Effect on barley bread
I. Gums and pectins			
1. Arabic	---	None	None
2. Carrageenin with vegetable mono and diglycerides	Kraystay type S	Slight increase in gas retention. Prevents loaf from collapsing	Slight improvement in structure
3. Guar derivative	Jaguar J2S-1	Slight increase in gas retention. Prevents loaf from collapsing	Slight improvement in structure
4. Hydroxypropyl cellulose	Klucel GF	None	None
5. Methylcellulose and derivatives	Methocel:		
	10 cps MC	None	---
	50 cps MC	None	None
	400 cps 65HG	Increased gas retention. Prevents loaf from collapsing	Slight improvement in structure
	4000 cps 65HG 90HG	Increased gas retention. Prevents loaf from collapsing	Finer structure
	8000 cps MC	None	None
	15000 cps 90HG	Increased gas retention. Prevents loaf from collapsing	Slight improvement in structure
6. Sodium carboxy methylcellulose	Hercules 7LP	None	Slight improvement in structure
	Hercules 7HOP	None	---
	Dupont p-75-XH	None	Slight improvement in structure
7. Tragacanth	---	Slight increase in gas retention. Prevents loaf from collapsing	---
8. Rapid-set pectin	X Change citrus 3436, 150 grade	None	None
9. Slow-set pectin	X Change citrus 451, 150 grade	None	None
10. Low methoxyl pectin and calcium	X Change citrus 3466	Hinders rise but prevents collapse	Hinders rise but prevents collapse
II. Starches			
1. Sorghum	Laboratory-extracted	All starches produced similar results. When combined with 4000 cps Methocel all produced a finer structure and better rise	Slightly coarser structure increased oven spring when combined with gum
2. Sorghum, phosphate derivatized, cross bonded, waxy	Corn products 4832		---
3. Corn	Kingsford		---
4. Tapioca (cassava)	South Pacific Ltd.		---
5. Arrowroot	El Molino Mills		---
6. Potato	Mallinckrodt		---
III. Enzymes			
1. Protease	Wheateze	Increased adhesive quality of batter, slight increase in rise, weakened crumb structure	None
2. α-Amylase	Amflex	Weakened crumb structure	None
3. Combined protease and α-amylase	Proflex	Weakened crumb structure	None
IV. Emulsifier and shortenings			
1. Glycerol monostearate	Myverol 1800	When combined with 4000 cps Methocel produced finer structure, greatly weakened crumb structure	Softened crumb structure
2. Vegetable shortening	Crisco	Softened crumb structure when combined with 4000 cps Methocel	Softened crumb structure
3. Mono and diglycerides of fat-forming fatty acids	Atmul 500	Softened crumb structure when combined with 4000 cps Methocel	Softened crumb structure

50 parts flour, 1 part baker's yeast, 1 part sugar, 1 part salt, variable amounts of additive and 60 parts water.

Sample batters of 180 g each were mixed by hand until smooth and then placed into standard pup-loaf pans. Proofing was done at 85°F until sample volume had doubled or until no further rise was noted in samples that failed to double in volume. The samples were then baked at 400°F.

RESULTS

TABLE 1 lists the additives examined along with the qualitative effect of each on the loaf. No attempt was made to examine all additives available; those selected are examples of 4 common types.

Gum and pectins

The gums were tested by incorporating 1 part additive into the basic recipe and mixing as a dry component with the flour.

As listed in Table 1 only gum tragacanth, Jaguar J2S-1, Kraystay type S and certain Methocels had any beneficial effect on the sorghum bread. Most gums failed to develop gas retention properties or to prevent the loaves from collapsing during baking. Gum tragacanth, Jaguar J2S-1 and Kraystay type S developed some gas retention but not enough for the loaves to rise properly. However, they did prevent loaves from collapsing. Both results were achieved in loaves containing 400, 1500, 4000 or 15000 centipoise (cps) Methocel, which produced a suitable rise and prevented collapse during baking.

Figure 1 illustrates the effects of various gums at an additive level of 2% in each loaf. Loaves containing either no additive, gum arabic or sodium CMC collapsed while loaves containing either 4000 cps Methocel, Kraystay S or Jaguar J2S-1 were stable during baking. The height of these latter loaves was achieved during proofing. At this additive level no further rise occurred during baking, although some rise occurred in samples with a higher additive level. Only the loaf containing 4000 cps Methocel achieved enough rise to be considered acceptable. Figure 2 illustrates the differences between several different viscosity Methocels. While several different viscosities are good, 4000 cps Methocel produced the best rise and, therefore, was examined more extensively. Figure 3 compares sorghum bread containing increasing amounts of 4000 cps Methocel. Each sample was allowed to rise equally during proofing. The greater height of loaves containing 4 and 6% added Methocel was due to a further rise during baking. Increasing the amount of Methocel produced both a finer structure and a better rise. However, there was a corresponding weakening of loaf strength. Because of this an additive level of 2% seemed to be the best compromise between loaf strength and loaf texture.

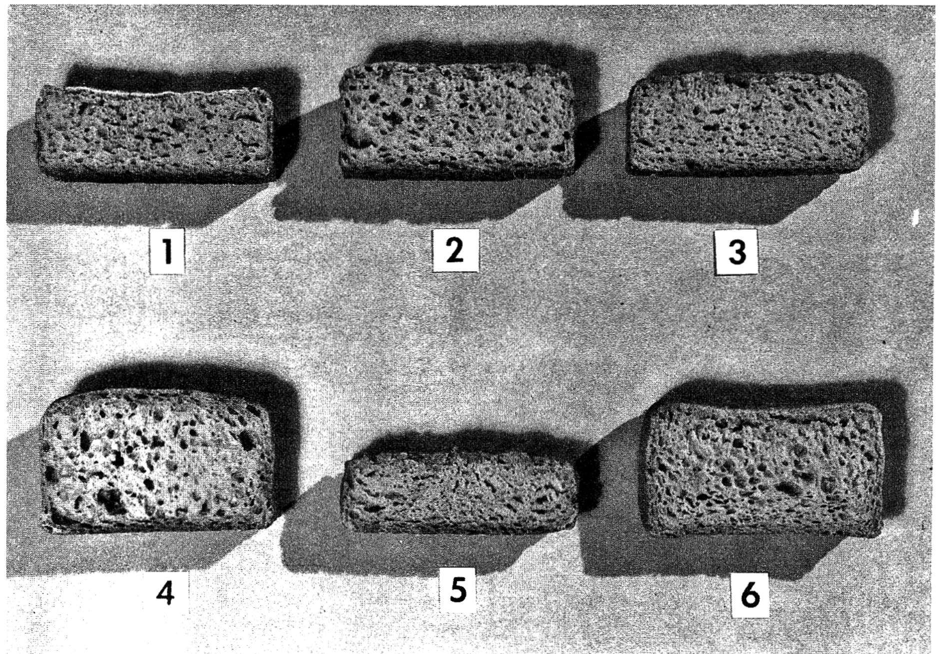

Fig. 2—Effect of different viscosity Methocels in sorghum bread. Loaf 1: no additive; 2: 2% 400 cps; 3: 2% 1500 cps; 4: 2% 4000 cps; 5: 2% 8000 cps; 6: 2% 15000 cps.

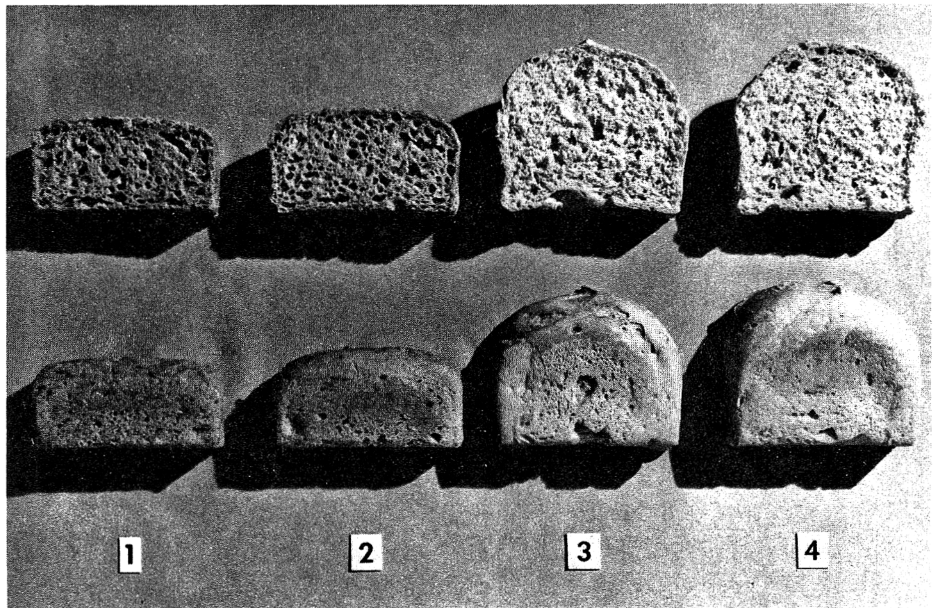

Fig. 3—Effect of increasing amounts of 4000 cps Methocel in sorghum bread. Loaf 1: 1%; 2: 2%; 3: 4%; 4: 6%.

Barley bread was also improved by the addition of certain gums. Again, 4000 cps Methocel produced the best bread as illustrated by Figure 4. Other gums that produced some improvement in structure are 15,000 cps Methocel and Jaguar J2S-1, shown in Figure 4, and Kraystay type S, 400 cps Methocel, Hercules 7LP and Du Pont CMC p-75-XH.

The addition of 4000 cps Methocel also partially improved the top crust of barley bread. By holding rise during proofing to no more than double the initial volume of bread batter, some oven

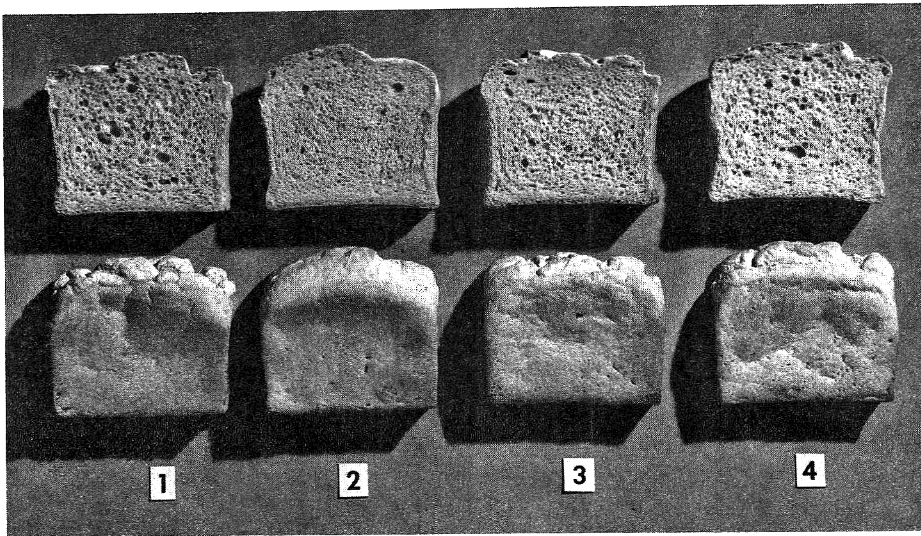

Fig. 4–Effect of different gums in barley bread. Loaf 1: no additive; 2: 2% 4000 cps Methocel; 3: 2% 15000 cps Methocel; 4: 2% Jaguar J2S-1.

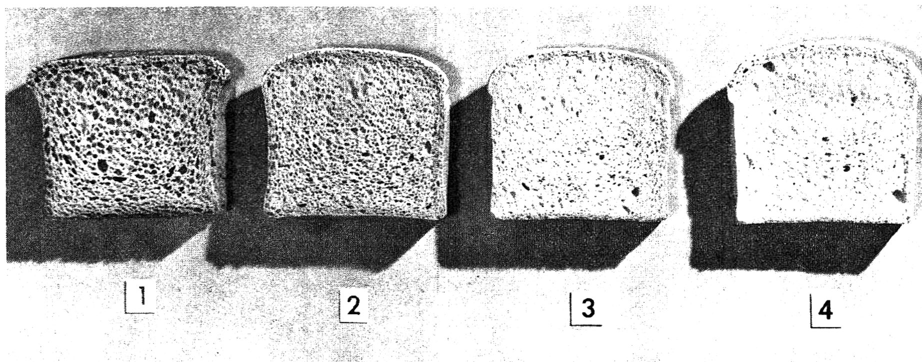

Fig. 5–Effect of increasing amounts of sorghum starch in sorghum bread containing 2% 4000 cps Methocel. Loaf 1: 0%; 2: 10%; 3: 20%; 4: 40%.

spring was achieved producing a domed-shaped loaf along with a smoother crust.

The pectins were tested by incorporating 2.5 parts additive into the basic recipe and mixing as a dry component with the flour. All sorghum loaves containing rapid or slow set pectin collapsed during baking. Similar loaves of barley bread did not collapse but did not improve in texture over loaves containing no additive. When low methoxyl pectin was used with a calcium source, there was insufficient rise in both sorghum and barley loaves although no collapse occurred.

Starches

Starches were initially tested by omitting any additive and replacing 10% of the flour in the basic recipe with the various starches listed in Table 1. Each starch caused sorghum bread to rise faster, but would not hold enough gas during baking to prevent collapse.

However, when 1 part of 4000 cps Methocel was added along with the starch, structure of the sorghum bread was finer. Various amounts of flour were then replaced by starch in sorghum bread containing 1 part added 4000 cps Methocel. Increased starch content caused an added rise during baking and a finer structure in the finished loaf. These results are illustrated by Figure 5.

Addition of starch to barley bread produced little effect. When 10 to 20% of the barley flour was replaced by sorghum starch a slightly poorer loaf structure resulted. Higher amounts of starch had no effect on loaf structure but did produce a slight oven spring during baking. When 4000 cps Methocel was added with starch, as illustrated by Figure 6, oven spring increased with increasing amounts of starch but the structure of each sample remained similar. Each starch tested produced similar results, but differences might show up in more critical tests.

Pregelatinized sorghum starch was tried in sorghum bread. To maintain a batter, only 10% of the sorghum flour was replaced by sorghum starch in the basic recipe. When larger amounts were tried, the solids content of the batter had to be reduced to compensate for the starch thickening. No combination of pregelatinized starch produced any beneficial results.

Enzymes

3 commercial baking enzymes, listed in Table 1, were tried. 50 mg of enzyme were incorporated in the basic recipe for each 10 g of flour. Protease increased the adhesive property of the sorghum batter somewhat and caused slightly more rise than did samples without enzyme, but sufficient rise in sorghum bread was not obtained from any enzyme and all weakened crumb structure. Enzyme additives had no noticeable effect on barley bread, probably due to a lack of suitable substrate.

Emulsifiers and shortenings

Again, initial testing was done by incorporating 1 part additive into the basic recipe. A 10% emulsion of glyceryl monostearate (GMS) in 140°F water was added while the other shortenings were dispersed by mixing. Neither GMS nor the other shortenings increased gas retention in sorghum or barley loaves.

It was necessary to incorporate a gum such as 4000 cps Methocel along with the GMS or shortenings to prevent collapse of the sorghum bread. The result was a fine-structured loaf. Unfortunately, the loaf strength was weakened so much by the GMS that it crumbled badly during slicing. The shortenings, when combined with Methocel, had little effect on structure but did soften the loaves.

GMS or shortenings had little effect on the structure of barley bread whether combined with a gum or added alone. However, the loaves were softened, as would be expected.

Effect of processing variations on sorghum bread

Several steps and variations in processing were examined. The effects of varying flour yield from milling, solids content of the bread batter and baking temperature of the loaves were determined. Flavor modification of sorghum bread by sour fermentation also was examined. All processing variations were tried using the basic recipe previously mentioned, with an added 2% of 4000 cps Methocel.

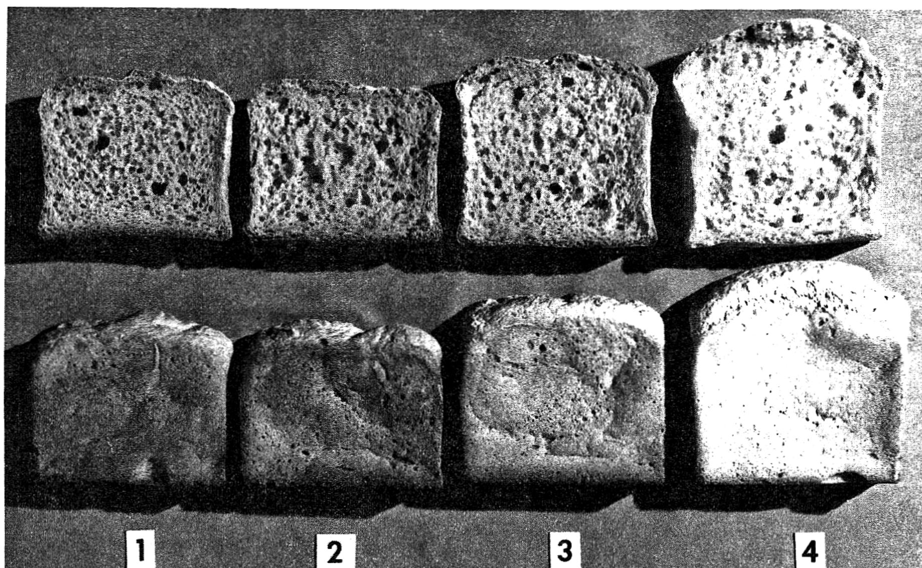

Fig. 6–Effect of increasing amounts of sorghum starch in barley bread containing 2% 4000 cps Methocel. Loaf 1: 0%; 2: 10%; 3: 20%; 4: 40%.

Using a Brabender Quadrumat mill, we obtained a yield of approximately 65% from grain containing 13.4% moisture. This produced a good baking flour when Methocel was added. This same flour was then sifted through a No. 80 mesh screen and a No. 100 mesh screen to produce flours of 51 and 37% extraction, respectively. Both of these higher extraction flours were slightly lighter in color and baked into bread of a slightly finer texture than the 65% extraction flour. However, this was a small gain when compared to the greatly increased milling loss. By drying grain to 7.0% moisture a yield of 86% was obtained. This produced a flour much darker in color with specks of bran in it. Bread from this flour was always very coarse in texture and rose poorly.

Moisture content of the bread batter was raised as high as 60%. Increasing the moisture content allowed a quicker rise during proofing. However, the batter became increasingly fluid, making it difficult to handle and producing bread with weakened structure. The best results were obtained with a batter moisture content of 55%. This produced batter that rose well and yet baked into bread of good strength.

Baking at 350°F or lower caused a white crust of ungelatinized flour on top of the loaf. Increasing the baking temperature to 400°F brought about proper browning.

2 methods of flavor modification by sour fermentation were tried. In one, the fermentation process was a modification of a traditional process used in Africa as described by Hulse (1968). Sorghum was decorticated, then soaked for 24 hr until the pH dropped to about 4. The grain was then dried and milled. The resultant flour was slightly darker in color than conventional sorghum flour. Bread made from it rose faster and retained a sour fermented flavor, but otherwise was similar to conventional raised sorghum bread.

In the other process, a natural sour dough was prepared by holding a dough, containing 50% moisture, overnight at 85°F. Yeast, water and other ingredients of the basic testing recipe were added after souring to make a batter containing 55% moisture. The resultant bread again resembled conventional sorghum bread except for its flavor.

As with conventional sorghum bread, texture of the sour bread could be improved by additional amounts of Methocel or starch.

CONCLUSIONS

VERY ACCEPTABLE pan breads can be made from milo sorghum flour by including a gum, 4000 cps Methocel, and any one of several starches. The amount of Methocel and starch added would depend both on the economics involved and on the desired loaf characteristics. However, a proportion of 1 part Methocel, 10 parts starch and 40 parts sorghum flour produced a loaf of good structure and oven spring.

Sorghum starch or certain locally available starch could be used. Crops such as corn, cassava, potato or arrowroot are often grown in the same regions as sorghum. Starch from these plants could readily be used in sorghum bread.

Where desired, a sour fermented flavor can be imparted to sorghum bread by either of 2 methods. The sorghum grain may be fermented before milling or a sour dough fermentation may be used.

Like sorghum, barley bread can also be improved by addition of gums. Several gums do very well in improving the texture of barley bread but again 4000 cps Methocel gives the best results. A proportion of 1 part gum to 50 parts barley flour would produce a loaf of good texture. Starch has virtually no effect in barley bread. If desired, it could be added as a diluent for flavor modification or because of economic considerations.

Both sorghum and barley bread have to be baked from a batter to achieve sufficient rise. A batter with a moisture content of 55% will rise sufficiently, yet is stiff enough to be handled conveniently. Since it is necessary to use a batter, pans of sufficient height must be used to contain any further rise during baking while the batter is still relatively fluid.

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O. H. MILLER and E. E. BURNS
Adriance Laboratory, Soil and Crop Sciences Dept., Texas A&M University, College Station, Texas 77843

STARCH CHARACTERISTICS OF SELECTED GRAIN SORGHUMS AS RELATED TO HUMAN FOODS

SUMMARY—Seventeen varieties and hybrids of grain sorghum were analyzed for content of total starch, amylose, amylopectin, starch density, and starch granule diameter. Correlations among these attributes and organoleptic quality were made. Starches of regular-starch type varieties had higher densities than waxy starch varieties. Starch density was related directly to amylose content and inversely to total starch and amylopectin content. Amylose content was directly related to total starch content, starch density, and inversely related to amylopectin content. The varieties with high amylopectin content were preferred for sorghum bread. Organoleptic quality was directly related to amylopectin content and inversely related to total starch and amylose content.

INTRODUCTION

PRODUCTION OF grain sorghum in the United States increased from 37.6 to 743.1 million bushels during the period 1930–1969 (Long, 1967; U.S.D.A., 1969). The United States now produces over 50% of the world's supply (U.S.D.A., 1964).

On a world-wide basis, grain sorghum ranks third among cereals for human consumption and is a staple food in Africa, China, and India. However, of the total U.S. production, it is estimated that only 2–3% is used for human food, the remainder being utilized industrially and as animal feed (U.S.D.A., 1970).

The goal of grain sorghum research in the past has been the development of varieties with desirable agronomic characteristics but with limited regard for attributes of the grain. There is a need for information concerning measurable characteristics which are closely related to the utilization of grain sorghum. The most efficient utilization of grain sorghum as a human food can be accomplished by objectively establishing attributes and matching these with appropriate food formulas. Suitability of grain sorghum for specific purposes depends primarily on starch characteristics.

Utilization

Starch, the most abundant constituent of grain sorghum, is utilized to the greatest extent. Grain sorghum starch is used for food products, adhesives, sizings, drilling muds, and other purposes (Ward and Browder, 1960). Sorghum starches are particularly favored in bland foods because they render less "cereal flavor" (Watson, 1959). Waxy sorghum starch can be useful where extraordinary stability is required such as in fruit pie fillings, soups, and canned foods (Boren, 1962). Pregelatinized sorghum starches are useful when their addition results in immediate thickening, such as in instant puddings (Waldt, 1960).

Previous Evaluations

Horan and Heider (1946) analyzed 101 different varieties of grain and forage sorghums for protein, starch, and crude fat content. They found that all the non-waxy sorghum samples contained approximately 25–30% amylose. Small quantities of amylose were found in the waxy varieties. Barham et al. (1946) studied 14 different varieties grown under the same conditions and found the chemical and physical attributes of the varieties to be distinguishable. Deatherage et al. (1955) surveyed 210 varieties from a world collection of sorghums in search of a high amylose variety but with no success. The amylose ranged from 21–28%.

Francis and Smith (1916) reported that grain sorghum starch granules ranged from 14–21 μ in diameter; corn measured 14 μ and potato starch granules 40 μ.

Very little work has been reported on the evaluation of grain sorghum varieties for human foods. Boren (1962) concluded that yellow endosperm varieties

Table 1—Classification of the grain sorghums used for analysis.

Sample number	Supplier's code	Endosperm characteristics
1	B607[a]	Waxy
2	Texioca 54[a]	Waxy
3	F_1 Hybrid[b]	Waxy
4	B3197[a]	Regular
5	Tx2520[a]	Waxy
6	F_1 Hybrid[b]	Intermediate
7	B398[a]	Regular
8	Tx2536[a]	Yellow
9	F_1 Hybrid[b]	Intermediate
10	–[c]	Yellow
11	–[c]	Yellow
12	C-600[b,d]	Yellow
13	B378[a]	Regular
14	Tx415[a]	Regular
15	RS671[a,b]	Regular
16	Popsorghum	Regular
17	SA5023-9-1-2[a]	Sugary

[a]Texas Agricultural Experiment Station number
[b]Hybrid of the two preceding samples
[c]Not Available
[d]DeKalb Seed Company number

Table 2—Mean values of chemical, physical, and organoleptic attributes of selected grain sorghum.

Sample No.	Total Starch (%)	Amylose Percentage of Total Starch (%)	Starch Granule Density	Starch Granule Diameter (microns) Range	Mean	Organoleptic[a]
1	64.20	4.80	1.39	10–20	15.00	1.70
2	69.98	4.76	1.16	5–20	12.50	2.80
3	65.88	0.79	1.30	7–20	13.25	1.80
4	69.74	27.72	1.14	10–20	15.00	3.20
5	66.37	5.74	1.39	5–25	15.00	2.40
6	70.61	27.22	1.50	5–20	12.75	2.40
7	67.71	29.15	1.51	10–20	15.00	2.50
8	66.52	24.82	1.48	4–14	9.00	2.40
9	68.73	23.64	1.56	15–20	17.50	2.50
10	69.44	28.08	1.65	7.5–20	13.25	3.00
11	67.52	27.13	1.61	7.5–20	13.25	2.50
12	70.60	34.87	1.90	10–20	15.00	2.40
13	69.12	25.23	1.63	7–18	13.00	2.40
14	69.84	25.93	1.75	10–20	15.00	2.70
15	70.57	25.97	1.94	4–20	11.75	3.10
16	65.26	30.66	2.01	5–18	8.25	2.40
17	57.39	26.80	2.21	10–20	15.00	2.30

[a]Score of 1 = Excellent–Delightful product, little room for improvement.
Score of 2 = Good–Would consume more of product without reluctance.
Score of 3 = Fair–Would consume product but with some reluctance.
Score of 4 = Poor–Would consume product but with great reluctance.

were preferable over common commercial varieties because prepared products (cookies and pancakes) had a more pleasing appearance due to the lack of the grey-blue colors of the pigmented varieties. It was also noted that the yellow endosperm varieties had less of a "feed-store" smell than the common commercial varieties (Boren, 1962). As Wilkens (1966) has indicated, the measurement of chemical and physical attributes of food constituents is meaningless until correlated with organoleptic evaluations. In this study organoleptic evaluations were correlated with the chemical and physical attributes.

EXPERIMENTAL

A NUMBER OF commercially available grain sorghum varieties and hybrids were analyzed in an attempt to evaluate the starch and organoleptic attributes of sorghums presently available and to determine differences and similarities. A list of varieties and hybrids analyzed is presented in Table 1.

Chemical

Total starch determinations were carried out on whole kernel meal prepared with a 0.003 in. mill.

The sugars were extracted from weighed samples with cold water. The starches, extracted by the method of Watson (1964), were hydrolyzed and then neutralized to the phenolphthalein end point. The neutralized solution was then analyzed by the Munson-Walker General Method and the dextrose equivalent multiplied by 0.90 was taken as the percent starch present in the sample (A.O.A.C., 1965). The method of Gilbert and Spragg (1964) was employed to determine amylose content.

Physical

Densities of the purified starch were determined by utilizing the Beckman Air Comparison Pycnometer (Joyce, 1961; Smith, 1964).

Average diameters of starch granules were determined following the method of Mac Masters (1964).

Organoleptic

A modified cornbread formula was used in preparing bread for organoleptic evaluation. A panel, consisting of seven experienced personnel, evaluated the sorghum bread made from 17 varieties and hybrids for texture, color, and flavor attributes and overall product acceptance.

Statistical

The percent total starch, amylose, starch density, diameter of starch granules, and organoleptic scores were each treated with an analysis of variance test. Simple correlations among all attributes were determined.

RESULTS & DISCUSSION

Chemical

Total starch, with one exception, ranged from 65–70% as shown in Table 2. This agrees with Watson (1959). The exception, No. 17, was the sugary-endosperm Kafir type, SA5023-0-1-2. It contained 58% total starch and was significantly different from the others.

The amylose percentages were significantly different. The waxy types, Nos. 1, 2, 3, and 5, contained very little amylose. This agrees with Watson (1959). The highest amylose content was found in the G-600 hybrid, No. 12, which has been available only recently. It is possible that the high amylose content may be one of its favorable characteristics.

The starch component of the waxy grain sorghums, Nos. 1, 2, 3, and 5, approached 100% amylopectin. This high amylopectin content would be most desirable for the production of thickeners, adhesives, and sizings.

Physical

There were significant varietal differences in starch density (Table 2.). Varieties high in amylose had higher starch densities. Density ranged from 2.22 g/cc in the sugary-endosperm, No. 17, to 1.14 g/cc in the waxy type, No. 4. Watson (1964) indicates that cereal starch has a density of 1.5 g/cc. It appears that high amylose content contributes positively to starch density.

During the starch purification procedures it was noted there were characteristic settling patterns and the high amyl-

Table 3–Simple correlation coefficients among chemical, physical, and organoleptic attributes of selected grain sorghum samples.

Attributes	% Values	Probability
Percent total starch × Percent amylose	0.293*	0.015
Percent total starch × Starch density	−0.339**	0.005
Percent total starch × Starch granule diameter	−0.007	0.953
Percent total starch × Organoleptic evaluation	0.521**	0.000
Percent amylose × Starch density	0.519**	0.000
Percent amylose × Starch granule diameter	−0.087	0.479
Percent amylose × Organoleptic evaluation	0.499**	0.000
Starch density × Starch granule diameter	−0.124	0.316
Starch density × Organoleptic evaluation	0.014	0.908
Starch granule diameter × Organoleptic evaluation	−0.040	0.746

*Significant at 5% level
**Significant at 1% level

Fig. 1–Grain sorghum bread.

ose starches settled more rapidly than the others.

Diameters of the starch granules ranged from 4–25 μ with the majority being 15–20 μ. This agrees with the findings of MacMasters (1964). The starch granules of popsorghum, No. 16, had the smallest mean diameter. Apparently the amylose content is not related to starch granule size.

Organoleptic

Data presented in Table 2 indicate that percentage amylopectin and organoleptic attributes are related. Some members of the panel detected a slight astringency in the popsorghum, No. 16, which is a Shallu type.

A representative loaf of grain sorghum bread is shown in Figure 1.

Statistical

A difference existed in total starch, amylose, amylopectin content, starch density, and starch grain size at the 1% level of significance, indicating these characters to be varietal attributes.

Organoleptic evaluation revealed that texture and crumb color of the sorghum bread affected the scoring of the evaluation panel significantly. Analysis of variance of product acceptance again indicated varietal significance at the 1% level.

Correlation coefficients for all attributes are presented in Table 3. Significant correlations were obtained at the 1% level for percent total starch × starch density; percent total × organoleptic evaluation; and percent amylose × organoleptic evaluation.

The correlation between percent total starch × starch density indicates an inverse relationship. This implies that the starch of the small hard-seeded regular-starch varieties is less dense than that of the larger softer-seeded regular starch varieties.

The correlation between percent total starch × organoleptic evaluation is negative. This indicates that varieties having lower total starch content are preferable for sorghum bread. This is probably because the varieties with lower total starch content were high in amylopectin content and were white seeded varieties. The white seeds contributed to a lighter colored bread which was more acceptable organoleptically.

The direct correlation of percent total starch × percent amylose indicates that amylose content directly affects total starch content.

The direct relationship of percent amylose × starch density is apparently caused by higher density of amylose.

The negative correlation of percent amylose × organoleptic evaluation indicates that higher amylose content decreases sorghum bread acceptability.

These observations indicate that physical and chemical attributes of grain sorghum varieties and organoleptic qualities are related.

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I. C. CHO[a] and L. J. BRATZLER
Department of Food Science, Michigan State University, East Lansing, Michigan 48823

EFFECT OF SODIUM NITRITE ON FLAVOR OF CURED PORK

SUMMARY—The effect of sodium nitrite in the curing pickle upon the flavor of the resulting cured and cooked pork roasts was investigated. Paired pork longissimus dorsi roasts were cured with varying amounts of salt, and with or without sodium nitrite. The effect of smoke was also studied. Taste panelists were able to select correctly (P <0.05 or 0.01) the different sample in triangle tests and indicated that the pork roasts cured with sodium nitrite had more cured pork flavor. Smoke did not mask this flavor, which was different from that attributable to the salt used in the curing pickle.

INTRODUCTION

THE CURING of meat is fundamentally a process of salting and was used originally as a method of preservation. Saltpeter (KNO_3) also has long been used to protect meat from spoilage and discoloration (Hoagland, 1908). The original preserving function of meat curing has been changed to one of flavor and color development, to satisfy present-day consumer tastes.

The characteristic heat-stable red color of cured meat is due to the interaction of nitrite with the myoglobin of meat to form the pigment, nitroso-myoglobin. When nitrate was used in the curing process, bacteria were necessary to reduce the nitrate to nitrite for color fixative purposes. Since 1929 the use of either sodium or potassium nitrite has been approved by the U.S. Meat Inspection

[a]Present address: Food Science Department, North Carolina State University, Raleigh, North Carolina.

Table 1–Composition of the curing pickles, residual nitrite, sugar, sodium chloride, phenol content of cooked roasts and taste panel results.

Trial	Pickle NaCl (%)	Pickle Sugar (%)	Pickle Nitrite (ppm)[1]	Residual nitrite (ppm)[2]	Cooked roasts Sugar[6] (%)	Cooked roasts NaCl[6] (%)	Phenols (mg/100 g)	Triangle test	Two-sample test
1	4.70	1.20	300	37	0.21	2.15	–	9/18[3] NS	
2	4.70	1.20	300	32	0.21	2.17	–	7/18 NS	
3	4.70	1.20	300	41	0.25	2.05	–	12/18**	
4	4.70	1.20	300	30	0.09	1.79	–	10/18*	
5	4.70	1.20	300	30	0.10	1.81	–	12/18**	
6	2.35	1.20	300	39	0.23	1.04	–	11/18*	15/18[4]**
7	2.35	1.20	300	42	0.29	1.14	–	10/18*	14/18*
8	2.35	1.20	300	19	0.28	1.01	–	21/36**	32/36***
R[5]	2.35	1.20	300	39	0.16	1.11	–	–	21/36 NS
9L	4.70	1.20	–	–	0.19	2.20	–	–	
R	4.70	1.20	–	–	0.14	2.11	–	–	
10L	2.35	1.20	300	35	0.16	1.00	–	–	23/36 NS
R	2.35	1.20	300	38	0.15	1.21	–	–	20/36 NS
11L	4.70	1.20	–	–	0.14	2.14	–	–	
12	–	–	300	23	–	–	–	14/23**	19/23**
13	–	–	300	22	–	–	–	11/19*	16/19**
14	–	–	300	22	–	–	–	11/18*	14/18*
15	2.35	1.20	300	32	0.33	0.97	3.21	17/26***	22/26***
16	2.35	1.20	300	19	0.32	1.15	9.22	14/22**	18/22**
17	2.35	1.20	300	26	0.25	1.02	6.20	13/18***	17/18***

[1] Either right or left sample of the pair.
[2] In sample cured with $NaNO_2$.
[3] Number of correct selections/number of panelists.
[4] Number of "more cured flavor" selections of $NaNO_2$ cured samples/number of panelists.
[5] R = Right, L = Left.
[6] Average of paired roasts.
NS = not significant, * significant at $P<0.05$; ** significant at $P<0.01$; *** significant at $P<0.001$.

Division. The amount is limited, so that the finished products contain no more than 200 ppm of sodium nitrite.

The distinctive flavor of cured meat may be due to the salt, sugar, nitrite or smoke, or both, used or applied during the curing process. Brooks et al. (1940) studied the function of nitrite in the curing of bacon and hams and reported that "the characteristic 'cured' flavour of bacon is due primarily to the action of nitrite on the flesh, and a satisfactory bacon can be made by using only sodium chloride and sodium nitrite." Taste panel data were not presented.

The present study was undertaken to determine if taste panelists could detect differences in pork cured with nitrite from pork cured without nitrite.

EXPERIMENTAL

PAIRED (right and left) sections of pork longissimus dorsi muscles were removed from 68.2–75.0-kg pork carcasses. The sections were from either the 10th to last thoracic vertebrae or the first to last lumbar vertebrae. The boneless roasts ranged from 0.45–1.08 kg in weight. After removal, they were packaged in Cryovac bags under partial vacuum, frozen and stored at –17.0°C until used (3–4 weeks).

Prior to curing, paired roasts were defrosted for 12 hr at 3°C, weighed and placed in the curing pickle in plastic bags and cured for 3 days at 2.0–3.0°C. Ratio of pickle to meat was always 2:1 (w/w). In every trial one of the paired samples was cured with $NaNO_2$ in the pickle and the other roast without $NaNO_2$. The curing pickles contained either 0.00, 2.35 or 4.7% NaCl; 0.00 or 1.20% sugar and 0 or 300 ppm of sodium nitrite.

The cured roasts were cooked in a 170°C oven to an internal temperature of 85.6°C. The cooked roasts were sliced into 5-mm-thick slices with a meat slicer. When the effect of smoke was studied, the slices were smoked (hardwood sawdust smoke) at 53.3°C and 34% R.H. for 10 min in an air-conditioned smokehouse. Some of the center slices of each roast were used for chemical analyses and the remainder for presentation to a nontrained flavor panel.

The triangle taste test was used to determine if a difference could be detected between the samples cured with $NaNO_2$ and those cured without nitrite. Red lights were used to mask sample color differences, but this procedure was discontinued in favor of a test in which the panelists were blindfolded and served the samples. In the triangle test the different sample was served first 1/3 of the time, second 1/3 of the time, and last 1/3 of the time. The samples were at room temperature when presented to panel members. The 2-sample taste test followed the triangle test. The panelists were asked, "Which of the samples had more 'cured flavor'"?

The samples cured without nitrite were served to the blindfolded panelists in an alternate order, i.e., panelist A received the nitrite-cured sample first and panelist B the no-nitrite-cured sample first, etc. All panelists used for the triangle tests were given the 2-sample tests, regardless of whether they had selected correctly the different samples. Statistical significance was determined by reference to Ellis (1961) for the triangle test and to Roessler et al. (1956) for the 2-sample test.

Chemical analyses for residual sodium nitrite, sodium chloride, sugar and phenols (smoked samples) were made on the center portions of the cooked roasts. Sodium nitrite and sodium chloride were determined by recommended methods (A.O.A.C., 1965). The method of Folin and Wu (1920) was used for determination of glucose. The sucrose in the sample was inverted by the method described by Harrow et al. (1955). The amount of sugar in the sample was the difference between glucose values before and after inversion. Phenols were estimated by the colorimetric method of Tucker (1942). Results are reported as mg phenols per 100 g sample.

RESULTS & DISCUSSION

COMPOSITION of the curing pickles used, data of some chemical analyses of the cooked roasts and results of the triangle and 2-sample taste tests are presented in Table 1. In each of the 17 trials listed, paired right and left roasts were used. Samples cured with $NaNO_2$ were equally divided between right and left roasts.

The taste panel tests of trials 1–5 were performed in booths employing red illumination to mask the normal visual difference between the roasts cured with nitrite and those cured without nitrite. In trials 1 and 2 no statistical difference in the triangle taste test was obtained. The next 3 trials (3, 4 and 5) resulted in correct selection of the different sample at a significant ($P < 0.05$ or 0.01) level. In all subsequent trials, samples were served to blindfolded panelists to eliminate the possibility of color difference being used rather than flavor difference.

The sodium chloride concentrations used did not appear to interfere with the panelist's ability to select the correct different sample in the triangle test when the results of trials 3, 4 and 5 are compared with trials 6, 7 and 8. The effect of 1.0 and 2.0% sodium chloride concentration combined with or without the nitrite cure, respectively, was studied in trials 9, 10 and 11. In each trial the lower salt concentration was in the samples cured with nitrite. While the 2-sample test did not demonstrate statistical significance, more panelists (64) selected the lower salt-nitrite-cured samples than those (44) who chose the higher salt-no-nitrite-cured samples as having "more cured flavor."

3 trials (12, 13, 14) were conducted in which neither salt nor sugar was added to the curing pickle. 1 of the roast pairs was

cured in a nitrite-containing pickle, whereas the pairmate was placed in distilled water. Both the triangle and the 2-sample tests showed that the panelists selected correctly the different sample and chose that sample with nitrite ($P < 0.05$ or 0.01) as having "more cured flavor."

Smoking is a process often applied to cured pork. Trials 15, 16 and 17 were conducted to determine if the smoke flavor would mask or dilute the cured meat flavor that the panelists indicated was present in the nitrite-cured sample and not present in the sample cured without nitrite. In spite of the heavy smoke concentration as shown by the phenol data, the panelists chose the correct different sample in the triangle test and indicated that the nitrite-cured smoked sample had "more cured flavor" than the smoked samples cured without nitrite ($P < 0.01$ and 0.001).

Results reported are in agreement with those of Brooks et al. (1940), who stated that a sodium chloride and sodium nitrite cure would produce a bacon satisfactory to English consumers. Wasserman and Talley (1969) reported that statistical significance in triangle tests was obtained only when the different sample was a smoked sample and not when the different sample was an unsmoked sample. In the present study the no-nitrite-cured and nitrite-cured samples were used equally as often as the different sample. Statistical significance was noted in 12 of the 14 triangle tests.

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ERNEST M. BUCK and BRUCE A. PETERS[a]
Department of Food Science and Technology, University of Massachusetts, Amherst, Masschusetts 01002

WEIGHT AND COLOR CHANGES DURING STORAGE OF BEEF STEAKS PACKAGED IN CLEAR PLASTIC, FOAM AND PULP TRAYS

SUMMARY–4 trials were conducted using 144 U.S. Choice-grade beef round steaks packaged in pulp, foam and clear plastic trays overwrapped with PVC film. Percent weight losses, at the end of 2 and 4 days of storage, were significantly greater for those steaks packaged in pulp trays as compared to clear or foam trays ($P < .01$). Evaporation losses from the various types of trays employed in this study were relatively uniform and differences in steak weight losses were primarily accounted for by absorbed or free juices in the tray. The color acceptability of all steaks declined during storage and there was no particular color advantage associated with any tray type. Visibility of steak undersides through clear plastic trays remained at an acceptable level throughout the storage period. A stacking effect was observed as top packages of stacks lost more weight and showed greater color discoloration than bottom packages.

INTRODUCTION

THE TRANSPARENT plastic tray used in some retail outlets at the present time was designed to assist the shopper in her decision-making by making more of the product visible, especially the underside. Relatively little information is available concerning the merits of the transparent plastic tray with respect to consumer acceptance, quality maintenance and economics of use.

This experiment was designed to investigate the effects of pulp, foam and clear plastic trays on certain physical characteristics of beef stored in an open, refrigerated display case for 4 days. Moisture losses, color changes and odor development of steaks were studied in all trays. Changes in the visibility of steaks through the underside of clear plastic trays were also studied.

EXPERIMENTAL

Sample source

4 trials were conducted using USDA Choice-grade beef rounds purchased locally. The prior history of the beef rounds was unknown; however, care was taken to select rounds showing no evidence of having been improperly handled or stored. Prior to purchase, rumps and sirloin tips were removed from the rounds which were otherwise intact to minimize contamination. Rounds were then transported under refrigeration to the University Meats Laboratory where they were held at 1–2°C.

Table 1–Steak assignment and package arrangement during storage. Trial I.

Stack	Top round steaks			Bottom round steaks			Eye round steaks		
position	Pulp	Foam	Clear	Pulp	Foam	Clear	Pulp	Foam	Clear
Top package	A[1]	B	C	A	B	C	A	B	C
Top – 1	D	E	F	D	E	F	D	E	F
Bottom + 1	G	H	I	G	H	I	G	H	I
Bottom package	J	K	L	J	K	L	J	K	L

[1] A = 1st experimental slice removed, B = 2nd slice, etc. The above configuration represents Trial I only. Tray assignment and stack position of the various slices were alternated in the other trials.

Tray types: Pulp–Molded pulp, internally sized to control absorption. Foam–Polystyrene foamed plastic tray. Clear–Clear plastic styrene with ridged bottom.

[a] Present address: Marketing Research Department, Carnation Company, Van Nuys, California 91412.

Table 2—Scale used in evaluating color acceptability of all packaged steaks and visibility of steaks packaged in clear plastic.

Score[1]	Color description	Percent of steak clearly visible through bottom of clear packages
5	Blackish–unacceptable	0
4	Brownish red–questionable	25
3	Dull red–acceptable	50
2	Bright red (bloomed)	75
1	Purplish red (prebloom)	100

[1] Packages were scored to the nearest one-half unit using a + or – system.

Table 3—Mean percent weight losses after 2 and 4 days storage of steaks packed in pulp, foam and clear trays.

Tray type	Pulp		Foam		Clear	
Days storage	2	4	2	4	2	4
Trial 1, $\bar{x}$	4.0	5.6	1.1	1.9	1.6	2.4
Trial 2, $\bar{x}$	3.4	4.6	1.3	1.5	2.0	2.6
Trial 3, $\bar{x}$	3.1	4.4	1.6	2.2	2.1	2.8
Trial 4, $\bar{x}$	3.3	5.2	1.2	2.5	1.6	3.4
Overall $\bar{x}$	3.5[1]	5.0[1]	1.3	2.0	1.8	2.8

[1] Significantly different from corresponding foam or clear mean ($P < .01$).

Table 4—Effect of stack position on percent weight losses of steaks packaged in pulp, foam and clear plastic trays.

Tray type	Pulp				Foam				Clear			
Stack position	Top		Bottom		Top		Bottom		Top		Bottom	
Days storage	2	4	2	4	2	4	2	4	2	4	2	4
Trial 1	4.4	7.0	3.6	4.7	1.5	3.2	1.0	1.3	1.9	3.4	1.5	2.2
2	4.1	5.8	3.4	4.6	1.6	2.4	0.9	1.2	3.5	4.4	1.6	1.9
3	4.4	5.9	2.2	4.2	2.4	4.1	1.6	1.9	3.1	3.6	1.9	3.1
4	4.0	6.7	3.1	4.7	1.9	3.7	0.8	1.9	2.2	4.7	1.1	2.4
$\bar{x}$	4.2	6.5	3.1	4.6	1.9	3.4	1.1	1.6	2.7	4.0	1.5	2.4

Preparation of samples

All samples were prepared, bloomed, packaged and stored under constant refrigeration of 1–2°C. The semimembranosus with adductor, semitendinosus and biceps femoris (known commercially as top, eye and bottom round muscles, respectively) were separated from the beef rounds at their natural connective tissue seams and sliced into 12-mm-thick steaks on a Toledo Meat Slicer. Use of a slicer ensured uniform thickness of steaks and smooth, even cut-surfaces. The first and last slices from each muscle were discarded to minimize the effects of light and contamination. 12 consecutive slices, A through L, were each weighed to the nearest 0.1 g on a laboratory scale and assigned to previously weighed pulp, foam and clear plastic trays (see Table 1). Weight of steaks was approximately 452, 226 and 113 g for top, bottom and eye of round, respectively. The trayed cuts were allowed to bloom in the cold for 45 min before being overwrapped with standard 75-gauge PVC film which was in contact with the meat surface after wrapping. Each trial consisted of 36 individual packages stacked 4-high in a single row across the front of a Hill open-topped display case. Tray assignment, slice location within a stack and tray positions in the case were all alternated for each trial, to minimize differences due to position. Case air inlet temperature was –2 to –3°C. Average temperatures of packaged steaks as determined at the end of the storage period were 5, 3, 2 and 0°C, respectively, for top, 2nd, 3rd and bottom positions in a stack.

Top packages were exposed alternately to 100 f.c. of fluorescent light for 12 hr and darkness for 12 hr throughout the storage period. All packages were subjectively scored for color by a panel of 3 judges, initially and every 24 hr thereafter to the end of the 4-day storage. Steaks packaged in clear plastic were also scored daily for visibility of steak through the package bottom. The latter determination was accomplished without disturbing released juices, by holding the package over a mirror placed in the bottom of the case and observing the reflection. Scoring procedures are outlined in Table 2.

At the end of 48 hr of storage, each package was opened and the weight of the steak it contained determined to the nearest 0.1 g. It was then returned to its original package and re-overwrapped. At the end of 96 hr of storage (4 days), all packages were given a final scoring, steaks and trays were weighed separately and the volume of free expressed juices, if any, determined.

The data were treated by analysis of variance according to Snedecor (1955).

RESULTS & DISCUSSION

Steak shrinkage

The mean percent weight losses occurring in all steaks after 2 and 4 days of display-case storage may be seen in Table 3. Shrinkage after either 2 or 4 days of storage was significantly greater for steaks packed in pulp than for those in foam or clear plastic ($P < .01$). Although mean foam-packed steak weight losses were less than those packed in clear plastic, these differences did not prove to be statistically significant. It is of interest that after 4 days of storage, steaks packed in clear or foam trays had lower average weight losses than steaks packed in pulp trays and stored only 2 days. It would seem clear that the blotter effect of the more absorbent pulp trays accounted for the greater shrinkage of steaks packed in pulp trays; however, the greater shrinkage of clear-packed steaks over foam-packed is not as easily explained. It is possible that the channels or ridges in the bottom of the clear plastic trays could have exerted increased pressure at points of tray-steak contact, resulting in the expression of greater amounts of liquid. The shrinkages reported in Table 3 are in line with those reported by other workers (Kraft and Ayres, 1952; Marriott et al., 1967) when allowance is made for differences in packaging and storage conditions.

Effect of stack position

It is obvious from the data in Table 4 that the steak in the top package of a stack loses greater amounts of weight throughout storage as compared to that in the bottom package of a stack. Since it was suspected that a considerable part of the weight loss of the steak in the top package was due to evaporation of moisture from the package surface, evaporation losses were determined by subtracting the weight of accumulated tray juices plus increase in tray weight from total steak weight loss. The unaccounted-for portion of the weight loss was then presumed to have been lost through evaporation. The average over-all percent loss due to evaporation after 4 days of storage was relatively uniform, with pulp, foam and clear trays showing losses of 1.8, 1.5 and 1.7%, respectively. However, when stack position is considered top packages, regardless of tray type, lost approximately 3% of weight through evaporation as compared to 1% for bottom packages.

The increased evaporation losses of top packages were not due solely to the increased exposed surface area. Undoubtedly, the variation in temperature due to stack position also played a role. Internal steak temperatures were determined for all packages at the end of each trial and,

Table 5–Color acceptability score of steaks after 2 and 4 days storage in pulp, foam and clear plastic trays.

Tray	Pulp		Foam		Clear	
Storage	2 Day	4 Day	2 Day	4 Day	2 Day	4 Day
Trial 1	2.9	4.1	3.2	4.1	2.5	4.0
2	2.8	3.5	2.8	3.6	2.8	3.5
3	3.2	4.1	3.1	4.1	3.0	4.3
4	3.5	4.2	3.5	4.3	3.3	3.8
$\bar{x}$	3.1	4.0	3.2	4.0	2.9	3.9

Table 7–Percent losses in weight of bottom round, eye round and top round steaks after 2 and 4 days of storage.

Trial	Bottom round		Eye round		Top round	
	2 Day	4 Day	2 Day	4 Day	2 Day	4 Day
1	1.8	2.9	3.1	4.3	1.7	2.8
2	2.0	2.8	2.8	3.5	1.7	2.3
3	2.1	2.7	2.7	3.7	2.1	3.1
4	1.7	3.3	2.1	3.8	2.4	4.0
$\bar{x}$	1.9	2.9	2.7	3.8	2.0	3.1

Table 6–Effect of stack position on color. Comparison of color scores of top and bottom trays in a stack.

Stack position	Top tray		Bottom tray	
Storage	2 Day	4 Day	2 Day	4 Day
Pulp, $\bar{x}$	4.5	4.8	2.8	3.4
Foam, $\bar{x}$	3.8	4.8	2.8	3.4
Clear, $\bar{x}$	3.4	4.5	2.7	3.5
$\bar{x}$	3.9	4.7	2.8	3.4

as was reported earlier, it was found that temperatures of steaks in top packages averaged 5°C, as compared to 3, 2, and 0°C, respectively, for steaks in 2nd, 3rd and bottom positions. There was also a tendency for some steaks in bottom packages to be partially frozen which would, of course, reduce shrinkage and evaporation in these packages.

Color and odor acceptability

The average color acceptability of prepackaged steaks, as reflected by the subjective scoring technique employed in this study, is shown in Table 5. As might be expected, the color acceptability of all steaks decreased with time. There was no color advantage associated with any particular tray type. On the average, all steaks were acceptable, color-wise, after 2 days of storage, but were of questionable acceptability after 4 days of storage. These results are similar to those reported by Dean and Ball (1960).

A comparison of top and bottom packages in a stack is shown in Table 6 and, as would be expected, the color acceptability of top packages deteriorated much more rapidly than that of bottom packages. This was probably due to the combined effects of light and temperature and is consistent with results reported by Brissey (1963) and Marriott et al. (1967).

The bottom surfaces of steaks in contact with the tray were also scored for color acceptability at the end of the 4-day storage and, although there was a trend for pulp-packed steaks to score in a more acceptable range, all undersides were of questionable acceptability after 4 days of storage. Mean scores for pulp, foam and clear trays were 3.7, 4.4 and 4.2, respectively.

There was no evidence of objectionable odor or slime development in any of the packages when examined at the end of the 4-day storage.

Visibility of product in clear packages

At the end of the 4-day storage, free liquids in all trays were poured off and the volume determined. The incidence of free liquid was low in pulp trays due to the comparative absorbent nature of the tray; it was greater in foam trays, and greatest in clear trays. Approximately 1.0% of all pulp packages had measurable free liquid; 5.5% of foam trays and 21% of clear packages had measurable free liquid. The volume of liquid varied, depending upon the weight of the steak and the muscle used. The largest volume of liquid, 9.6 ml, was removed from a clear tray containing a top round steak. The average volume of liquid in foam and clear trays was 3–4 ml. The free liquid in the clear trays did not seriously impair the visibility of steaks as viewed through the bottom of the package. The average visibility score of all clear trays after 2 and 4 days of storage was 1.2 and 1.3, respectively, indicating that 75% or more of the steak was visible through the bottom of the package.

The greater amounts of free liquid associated with the clear plastic trays might have been due to the differences in heat capacity or insulating effect, or both, which would tend to balance temperature fluctuations in the pulp or foam trays. The greater temperature fluctuations in the clear plastic trays might possibly have set up a "moisture vapor pump" situation within the package, thus increasing release of fluid. This phenomenon is worthy of further investigation.

The color acceptability of the underside of steaks after 4 days in clear trays tended to be better when viewed through the plastic than when removed and examined. A mottled appearance apparently caused by the ridges in the tray was not obvious through the bottom of the package.

Increase in tray weight after 4 days of storage was insignificant in foam and clear trays. The slight increase in weight which did occur was due to droplets of moisture which clung to the tray surface. Wiping with an absorbent towel would return pulp and foam trays to their original weights. Steak weight losses were, for the most part, accounted for by free liquid and evaporation loss.

It will be recalled that steaks packed in pulp trays lost, on the average, 5.0% of their original weight. The increase in pulp tray weight due to absorption of liquids accounted for 3.2% of the 5% loss after 4 days of storage.

Shrinkage by type of steak

The mean percent losses in weight by type of steak used in this experiment are shown in Table 7. After 2 days of storage, eye of round steaks showed the greatest percent losses (2.7%), followed by top round (2.0%) and bottom round steaks (1.9%). This relationship was unchanged after 4 days of storage.

It may be concluded from this experiment that of the variables studied, only shrinkage is significantly affected by tray type, and that the visibility of steaks through the bottoms of clear packages is maintained at an acceptable level throughout a 4-day storage period. Also, a lack of free juices in pulp trays tended to preserve the surface of the steak in contact with the tray in a more acceptable condition during display-storage.

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C. L. KASTNER, R. L. HENRICKSON and B. L. CLARY
Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma 74074

COOLING OF PORCINE HAM BY OIL IMMERSION

SUMMARY—12 swine (8 Hampshire and 4 Yorkshire barrows) were selected for this study. Each animal was slaughtered and the hams removed from the "hot" carcass. Individual paired hams from the same animal were placed in 1 of 2 cooling systems (immersion in refrigerated light mineral oil or a forced-air system). Both systems were operated at 40°F and were equated on the basis of the heat-transfer coefficient. Cooling periods to reach a given temperature were recorded in order that evaluations could be made on how well the heat-transfer coefficients were equated and to assess economies of cooling times. Total moisture loss from the hams during cooling was recorded to compare oil immersion with air chilling. Cooling curves and the F-test in conjunction with the analysis of variance indicated that the difference in cooling time between the 2 systems was nonsignificant when the heat-transfer coefficients were equal. The objective to equate the heat-transfer coefficients was, therefore, accomplished. Chilling by oil immersion was 1.57 times faster than chilling in the forced-air system when both the air and oil velocities were 10.0 ft/sec. Moisture loss in the air chilling method was from 4.34 to 2.42 times greater than that in the oil immersion system when the air velocity was approximately 176 times greater than the oil velocity.

INTRODUCTION

DEMAND by the consumer for economical cuts of meat has led investigators to seek new methods of processing. New, rapid, more efficient cooling methods for meat have received limited study.

Thermal conductivity of meat was found to be greatest when the heat flow was parallel to the muscle fibers (Hill et al., 1967). The same authors stated that the greater the moisture content of lean tissue, the greater was the thermal conductivity.

The efficiency of a fluid as an immersion coolant can be evaluated using the Prandtl number as the criterion (Clary et al., 1970). Each cooling medium has a Prandtl number, which is a relative measure of the rate at which heat is transported from the surface of the object being cooled to the moving cooling medium. The larger the Prandtl number, the more efficient the cooling medium is as an immersion coolant.

During water-immersion chilling, the greater the immersion time and agitation of poultry carcasses, the greater the water absorption (Brant, 1963). Birds immersed in water-ice solution (33°F) had less total bacterial contamination than carcasses chilled in circulating air at 40°F (Casale et al., 1965).

Cellulose acetate and polyethylene films were permeable to microorganisms, whereas cellophane, Pliofilm and Cry-O-Vac were not significantly permeable (Hartman et al., 1963). Dunker and Hankins (1953) reported the time required for freezing of beef samples by air blast (2°F) was increased by 53% when the product was placed in Cry-O-Rap.

When several thermocouples were used to measure the mass center temperature, the thermocouple point that required the longest cooling time was considered the most accurate. This thermocouple point was judged to give the most representative record of the mass center temperature (May et al., 1961). Thermocouple placement in biological material can be accomplished by threading the wire (copper-constantan) into the meat with a needle. Lentz and van den Berg (1957) reported that while recording temperature changed in immersion-chilled poultry, a special jig with suitably spaced parallel needles was used to thread the thermocouples (30-gauge copper-constantan) through the flesh. The leads were extended through the end of the bag and the bag sealed by twisting and tying.

Associated with thermocouple placement was the geometric configuration of the product being cooled. As the geometry or shape of a biological material changed, so did the cooling time (Smith et al., 1967).

Meat, packaged hot immediately after slaughter has been described as emitting an off-odor once the package is opened. Clauss et al. (1957) vacuum sealed fresh, raw beef samples in Cry-O-Vac packages and found upon opening that a faint odor was present. At times, raw odor was almost imperceptible. The purpose of our investigation was to develop a more rapid method of chilling than using conventional air cooling, that could be used in conjunction with "hot processing" of pork.

MATERIALS & METHODS

12 MARKET-weight swine (8 Hampshire, 4 Yorkshire barrows) of similar management were used. The animals ranged in weight from 182 to 250 lb. The bled animals were skinned and rapidly eviscerated. Both hams were removed from the hot carcasses in the conventional manner, trimmed of excess fat and boned. To ensure that both hams were the same weight, they were weighed on a gram balance and the heavier of the 2 trimmed until their weights were the same. The hot weights were recorded and the hams inserted into cellulose casings using a ham stuffing horn. (Casings were used to prevent contamination and to ensure similar ham shapes.) The packaged, boneless, "hot" hams were then assigned at random to 1 of 2 treatments: cooling in air (40°F) or immersion chilling in light mineral oil (40°F). The packaged hams, sealed on 1 end, were forced into brackets made of metal rod to ensure that their dimensions were the same. 4 thermocouples were inserted through the open end of the bag (20-gauge copper-constantan) into the geometric center of each ham by using a threading needle (Lentz and van den Berg, 1957). It was assumed that the geometric center of the ham was the most difficult part to cool. Therefore, the thermocouple point(s) requiring the longest to reach 50°F were the most representative of the mass center temperature (May et al., 1961). Temperature recordings were initiated as soon as the packaged product was placed in the assigned cooling system. Each system's ambient temperature was 40°F (± 2°F).

Equating heat-transfer coefficients

Both the oil and air-cooling systems were designed and constructed so as to equate the heat-transfer coefficients, using fluid velocity as a basis. Air velocities of 100, 350, 600 and 750 ft/min were selected. The following general equation was reported by Clary et al., (1968) and is the basis for equating heat-transfer coefficients for boneless hams of the same dimensions with a geometry index of 0.45.

$$Nu = 0.367\,(Pr)^{0.333}\,(Re)^{0.564}$$

$$Nu = \frac{hl}{K}$$

Therefore:

$$h = \frac{K}{l}\,(0.367)\,(Re)^{0.564}\,(Pr)^{0.333}$$

Symbol	Quantity	Units
Nu	Nusselt Number $\frac{hl}{K}$	---
Pr	Prandtl Number $\frac{\mu C_p}{KNe}$	---
Re	Reynolds Number $\frac{Vd\rho Ne}{\mu}$	---
C_p	Specific heat of the cooling medium at constant pressure	Btu/lb$_m$ °F
d	Diameter 2 (1)	ft
h	Average heat-transfer coefficient	Btu/hr ft^2 °F

Table 1–Interpolated economies of cooling times at selected velocities.

Velocity[1] (Fig. 2) ft/sec	Cooling time[2] (min) (Fig. 3) Air	Oil	Economies of time (air)/(oil)
0.06	420	307	1.37
0.10	409	295	1.39
1.00	359	270	1.33
10.00	308	196	1.57

[1] Velocity determined from Figure 2.
[2] Interpolated from Figure 3.

K	Thermal conductivity of the cooling medium	Btu/hr ft°F
l	Characteristic dimension	ft
V	Velocity of the cooling medium	ft/sec
μ	Viscosity of the cooling medium	lb_f-sec/ft^2
ρ	Density of the cooling medium	lb_m/ft^3
Ne	Newton's Second Law Coefficient (0.0311)	lb_f-sec^2/lb_m-ft

The properties of the oil and air at 40°F are:

Air

K_a = 0.015 Btu/hr ft°F
μ_a = 1.2×10^{-5} lb_f-sec/ft^2
ρ_a = 0.0788 lb_m/ft^3
C_{pa} = 0.24 Btu/lb_m°F

Oil

K_o = 0.076 Btu/hr ft°F
μ_o = 0.0862 lb_f-sec/ft^2
ρ_o = 53.5 lb_m/ft^3
C_{po} = 0.46 Btu/lb_m°F

When the heat-transfer coefficient of the oil was set equal to the heat-transfer coefficient of the air, the oil velocity was solved for in terms of the velocity of the air, thus:

Velocity of oil = (.00568) Velocity of air

The hams from 3 animals were assigned to each of the 4 air velocities (100, 350, 600 and 750 ft/min) with corresponding oil velocities. All hams were chilled to 50°F; and theoretically the chilling times should be identical for both hams from the same animal because the heat-transfer coefficients were equated.

The reason for equating heat-transfer coefficients was to give a basis for evaluating economies of cooling times between both chilling methods.

Evaluation of equating heat-transfer coefficients

The F-test in conjunction with the analysis of variance and graphical analysis was used to evaluate how well the heat-transfer coefficients of both systems could be equated.

Cooling times to reach selected temperatures versus the cooling temperature were plotted graphically, where temperature was dimensionless as defined by:

$$T = \frac{Tc - To}{Ti - To}$$

T = Dimensionless temperature
Tc = Temperature increments during cooling (100, 90, 80, 70, 60, 50°F)
To = Average environmental temperature during chilling
Ti = Internal starting temperature of the ham

The reason for using a dimensionless temperature was to adjust for differences in environmental and internal starting temperatures of the hams.

The analysis of variance and F-test were utilized to evaluate statistical differences between cooling times to reach 50°F for each repetition at each velocity selection.

Economies of cooling times

2 graphs were constructed so that cooling times by 1 method could be directly compared with time saved by the other. Time in these comparisons was velocity dependent. From the general formula $h = \frac{K}{l}(0.367)(Re)^{0.564}(Pr)^{0.333}$ (Clary et al., 1968), for any given velocity a cooling medium heat-transfer coefficient can be calculated. Velocity versus heat-transfer coefficient was plotted for both the oil and air-cooling methods.

The companion graph to the heat-transfer coefficient versus velocity graph was the heat-transfer coefficient plotted against cooling time determined by the dimensionless temperature formula:

$$T = \frac{Tc1 - To}{Ti - To} = 0.2$$

T = Dimensionless temperature (constant)
Tc_1 = Adjusted temperature representative of 50°F
To = Average environmental temperature during chilling
Ti = Internal starting temperature of the ham

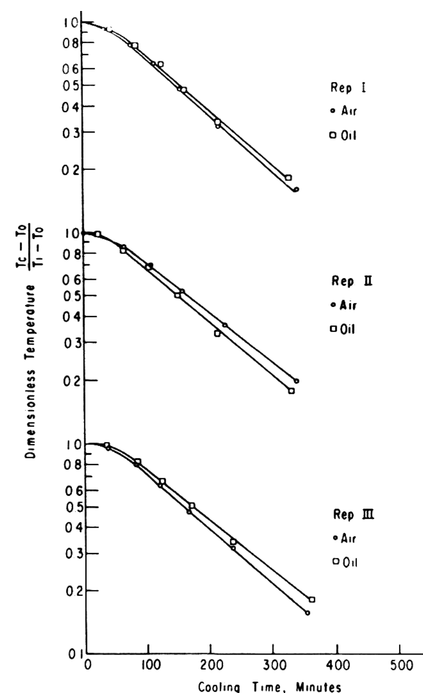

Fig. 1–Cooling curves for hams chilled at an ambient temperature of 40°F (heat transfer coefficient = 2.68 Btu/hr ft^2°F).

The dimensionless temperature formula was used to adjust for differences in the average environmental temperature during chilling and the internal starting temperature of the ham. The time required to chill to a temperature representative of 50°F was plotted against the corresponding heat-transfer coefficient. For each velocity on the velocity versus heat-transfer coefficient graph, a corresponding heat-transfer value was read for both oil and air. By entering the heat-transfer coefficient versus cooling time graph, differences in cooling time for both oil immersion and air chilling were determined for a given velocity.

Percent moisture loss

The sample calculations for percent moisture loss are:

$$\frac{\text{Before chill weight} - \text{after chill weight}}{\text{Before chill weight}} \times 100 = \text{Percent moisture loss}$$

Odor evaluation

As soon as the hams chilled to 50°F, the bag was removed from each ham and an organoleptic evaluation made of odor. If any off or undesirable odor was present, this was recorded.

RESULTS & DISCUSSION

Equating heat-transfer coefficients

The heat-transfer coefficients of both cooling systems were equated to provide a basis for evaluating economies of cooling times. Theoretically, when both heat-transfer coefficients are equal, identical hams should chill to the same temperature in the same length of time.

Cooling times to reach 50°F for each repetition with each velocity selection

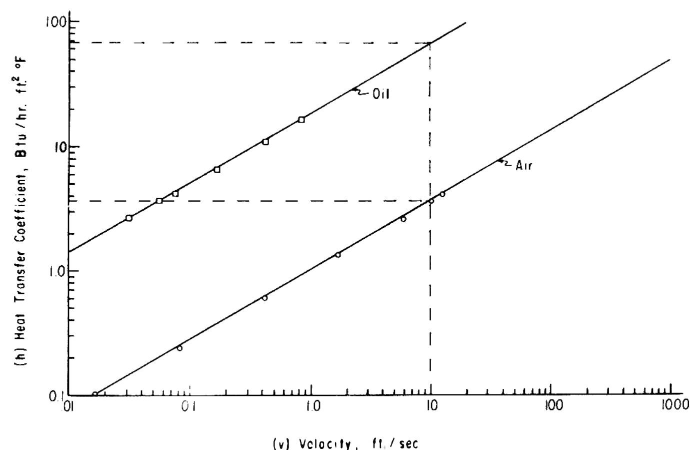

Fig. 2–Heat transfer coefficients at selected velocities for air and mineral oil.

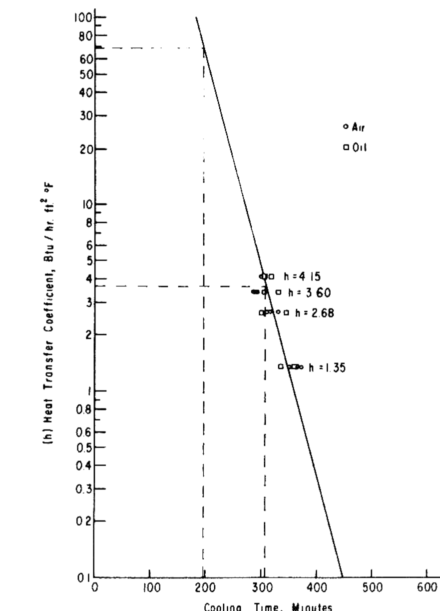

Fig. 3–Cooling times for corresponding heat transfer coefficients.

(100, 350, 600 and 750 ft/min) proved nonsignificant. Analyses were calculated on each velocity selection independent of the 3 other velocities.

Sample cooling curves plotted for each velocity selection are shown in Figure 1. The nonsignificance of cooling times to reach 50°F and the similarity of all cooling curves for each repetition at each velocity selection indicated that a satisfactory job of equating the heat-transfer coefficient was accomplished.

By equating the heat-transfer coefficients, it was expected that no difference in cooling times would result. Any differences in the cooling period could have been due to 1) errors in adjustment and measurement of velocities, 2) heterogeneous ham shapes, 3) differences in ham composition (lean-to-fat ratio), 4) ambient temperature differences, 5) differences in initial internal ham temperature, 6) error in thermocouple placement and 7) air pockets trapped beneath the cellulose casing which would retard cooling.

Economies of cooling times

Large differences in heat-transfer coefficients resulted between the oil immersion and air systems at any given velocity (Fig. 2). At a given velocity (ft/sec), the corresponding heat-transfer coefficient for oil and air can be read from Figure 2. By taking the resulting heat-transfer coefficients and entering Figure 3, differences in cooling times can be read directly. For example, at a velocity of 10 ft/sec (Fig. 2), the heat-transfer coefficient of oil would be 69.0 and for air, 3.7. The corresponding cooling times (Fig. 3) were 195 min (oil) and 308 min (air), giving a cooling time in oil approximately 1.6 times faster than cooling in the forced-air chamber. Selected economies of cooling time for oil immersion and air chilling are shown in Table 1.

The data presented in Figures 2 and 3 are dependent on the characteristic shapes of the hams and the properties of the air and mineral oil at 40°F. Response lines on Figures 2 and 3 may be extrapolated beyond the points plotted from the observed data.

Percent moisture loss

Moisture loss within the air system was 4.34–2.42 times greater than that experienced in the oil-immersion system when the corresponding air velocities were approximately 176 times greater than the oil velocities (Fig. 4).

Packaged product odor

No objective odor values were recorded; the subjective organoleptic tests revealed only characteristic odors that would probably be acceptable to the consumer.

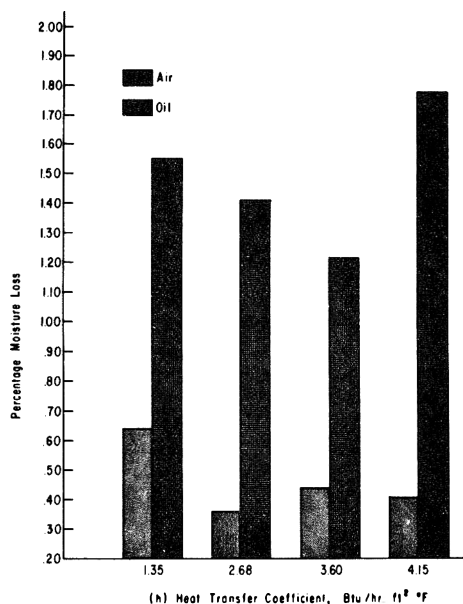

Fig. 4–Moisture loss from hams during air and oil immersion cooling.

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NORA YU-ANG TANG and DORIS M. HILKER
Department of Food and Nutritional Sciences, University of Hawaii, Honolulu, Hawaii 96822

EFFECT OF HEATING AND COLD STORAGE ON ANTITHIAMINE ACTIVITY IN SKIPJACK TUNA

SUMMARY—The stability of antithiamine factor(s) in skipjack tuna (Katsuwonus pelanus) *was tested after boiling by 2 methods, baking and cold storage at 10 and −10°C. The various heat treatments reduced the antithiamine activity from 55–86% of the control. Both frozen and refrigerated tuna, respectively, lost the antithiamine activity by 50–60% of the original level in 4 days.*

INTRODUCTION

THE PRESENCE of antithiamine substances in fish has been known since Chastek-paralysis, an acute dietary deficiency disease, was induced by inclusion of raw carp in the fox's diet (Green et al., 1942). Since then, similar antithiamine activity has been reported in the tissues of many fishes throughout the world. Yudkin (1949), Fujita (1954), Deutsch and Hasler (1943), Sealock and Goodland (1944), as well as others (Wooley, 1941; Krampitz and Wooley, 1944), reported thiaminase in various species of fish. Recently, Kundig and Somogyi (1967) found a thermostable antithiamine factor in carp viscera, later shown to be hemin or a related compound. Also, Hilker and Peter (1968) reported that the antithiamine activity in skipjack tuna was not enzymatic. The factor(s) was thermostable and contained protein and nonprotein moieties. The antithiamine activity was associated with the nonprotein moiety but modified by protein.

This investigation was conducted to examine the stability of antithiamine factor(s) in tuna under cooking and cold storage conditions. The conditions examined were: boiling by 2 different methods, baking, refrigeration and frozen storage.

EXPERIMENTAL

Fish extract preparation

The fish were purchased from Honolulu markets on the date of testing. The deep red muscle, usually not eaten, was removed. Since the antithiamine activity of the cross section of the fish muscle was found to be more uniform than longitudinal slices, cross-section slices were used in all studies. Approximately 2-in.-wide cross sections weighing 50 g were homogenized with 250 ml distilled water, centrifuged, then filtered to remove fat if present. The resulting supernatant was used as the fish extract.

Antithiamine activity determination

In antithiamine activity determinations, 2-ml aliquots of fish extract were added to 25-ml volumetric flasks containing 12 μg thiamine chloride. Appropriate dilutions were made with 0.001 N HCl and the remaining thiamine determined by the Somogyi (1966) modification of the thiochrome reaction. Thiochrome was determined using a Turner Model 110 Fluorometer with a 360-mμ primary filter and a 415-mμ secondary filter. Controls from the same fish were run simultaneously with the treated groups. The antithiamine activity was expressed as μg thiamine destroyed per mg extract (dry weight).

Heat treatments

The effect of boiling on the antithiamine activity of the fish section was tested in 2 ways: I. The fish section was placed in cold water, brought to boiling and boiled for 5 min, and II. The fish section was placed in boiling water and boiled for 5 min. The sections were cooled, homogenized and the antithiamine activity determined as described above. The effect of baking was studied by placing fish sections in an oven preheated to 149° C and baked for 30 min. The fish sections were cooled, homogenized and the antithiamine activity determined as described.

Cold storage

5 to 6 sections from a fish were wrapped separately with aluminum foil and placed in a freezer at −10° C or refrigerated at 10° C. Sections were removed daily and allowed to come to room temperature, then analyzed for antithiamine activity.

RESULTS & DISCUSSION

THE AMOUNT of antithiamine substance(s) obtained from the average of 28 fish extracts was 0.24 ± 0.05 μg thiamine destroyed per mg dry weight of the extract. When the control activity was less than 50% thiamine destruction, the data from the set were not used. The effect of heat treatments on the antithiamine activity is shown in Table 1. The values are the averages of 7 tests.

After various heat treatments, more than 50% antithiamine activity remained. Boiling (II) showed less antithiamine activity remaining than with boiling (I) and baking; but since there was a wide range of variations among these groups, particularly in boiling II, the difference was not significant.

Deutsch and Hasler (1943), Sealock et al. (1943), Melnick et al. (1945) and Gnaedinger and Krzeczkowski (personal communication) studied antithiamine substance(s) (thiaminase) and reported a complete heat inactivation under their experimental conditions. It appears that different antithiamine factors exist in different species of fish and also in the same fish. Studies in our laboratory indicated that more than 1 antithiamine factor is present in the tuna (Hilker and Peter, 1968).

The effect of cold storage on antithiamine activity is shown in Figure 1. The data show a marked decrease in both frozen and refrigerated fish. A sharp decrease in the first 2 days of frozen storage was noted, after which the decrease was more gradual. Tests had been extended to 3 weeks but no further inactivation occurred after 1 week. On the other hand, storage by refrigeration resulted in a linear decrease in antithiamine activity over a 5-day period. A fish sample frozen and thawed immedi-

Table 1—Residual antithiamine activity in tuna after 3 heat treatments.

	Treatment		
	Boiling I	Boiling II	Baking
Percent of control antithiamine activity	74.4 ± 9.7	54.4 ± 13.5	85.7 ± 5.4

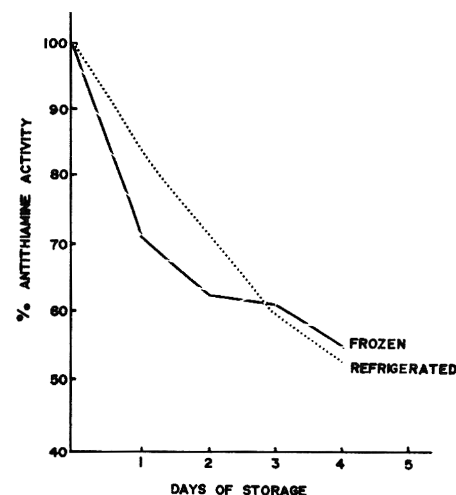

Fig. 1—Effect of cold temperature treatments on antithiamine activity. Values are given as relative percentages of the antithiamine activity of the fresh controls.

ately showed an antithiamine activity level similar to that of the fresh control. It appears that the length of storage is an important factor that could influence the stability of the antithiamine factor. The antithiamine activity was usually greater when the extract was more deeply colored; however, no linear relationship was observed between the antithiamine activity and the color intensity. Further investigations on the nature of the antithiamine factor in fish are now in progress.

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DAVID L. DUBROW and BRUCE R. STILLINGS

National Center for Fish Protein Concentrate, Bureau of Commercial Fisheries, College Park, Md. 20740

EFFECT OF HEAT ON THE CHEMICAL AND NUTRITIVE STABILITY OF FISH PROTEIN CONCENTRATE (FPC)

SUMMARY–Experiments were conducted to determine the heat stability of FPC (fish protein concentrate). FPC was subjected to one of two forms of heat: (1) dry air (oven) and (2) moist (autoclave). Temperatures of dry heat were 100°C, 120°C, or 150°C. Temperatures of 100°C or 120°C were used for autoclaving. Exposure times for both treatments were 0, 30, 60, 120, or 240 min. Proximate composition, amino acids and pH were determined. NPN was also determined in autoclaved samples. PER values were obtained for protein quality. Results of dry heat showed little change to occur in samples heated to 120°C for up to 4 hr. Samples heated to 150°C for 60 min or longer showed a decrease in lysine, arginine, available lysine and PER. Moist heating produced an increase in NPN, a decrease in pH and little change in amino acid concentration. The protein quality was decreased after 4 hr at 120°C.

INTRODUCTION

FISH PROTEIN CONCENTRATE (FPC) is one of several protein concentrates recognized as being capable of increasing the nutritive quality of foods low in protein quality. Its primary use therefore will be as a protein supplement to improve diets deficient in high-quality protein. FPC prepared by solvent extraction of whole hake with isopropyl alcohol (Bureau of Commercial Fisheries, 1966) has been shown to contain a high-quality protein similar to casein (Stillings, 1967).

The most effective use of FPC is to incorporate it in foods. However, when FPC is included in foods, changes may occur during processing that alter the quality of the protein. For example, others have shown that prolonged exposure to heat can adversely affect the quality of the protein in foods (Ellinger and Boyne, 1965; Donoso et al., 1962; Rice and Beuk, 1953). We therefore conducted two experiments to determine the effect of temperature of heating and time of exposure on the chemical and nutritional properties of FPC. Effects of both dry heat and moist heat on FPC were evaluated.

EXPERIMENTAL

Dry heat experiments

Materials. FPC was prepared from whole hake (*Urophycis chuss*) by solvent extraction with isopropyl alcohol (Bureau of Commercial Fisheries, 1966). About 27 kg was thoroughly mixed in a tumbling dryer. The material was then divided into 16 samples each weighing 1 kg. Each sample was next treated according to the procedures outlined below.

Treatments. 1 kg of FPC was placed on a stainless-steel tray, spread to a thickness of about ¼ in., and placed in a forced-air dry-heat oven. Thermocouples were inserted into the layer of FPC for continuously recording the temperature. The sample was then subjected to a temperature of 100°, 120°, or 150°C for 0, 30, 60, 120, or 240 min. Immediately after being treated, the sample was placed in a container and stored at −20°C. Timing of the heat treatment was started when the product reached the desired temperature.

Chemical evaluation. Samples of heat-treated FPC were analyzed for crude protein (N × 6.25) in accordance with approved methods (AOAC, 1965). Amino acids were analyzed by ion-exchange chromatography according to Moore et al. (1958). Available lysine was analyzed as described by Carpenter (1960), and tryptophan was analyzed by the method of Spies and Chambers (1949). Only samples heated for 0 and 240 min at each temperature were analyzed for all amino acids; however, basic amino acids were analyzed in all samples of FPC.

As an indication of possible changes in protein caused by heat, pH was determined in a 5% slurry of FPC and distilled water.

Nutritive evaluation. The nutritive quality of the samples was determined by animal feeding. Diets were prepared that contained 10% protein from the FPC samples. A diet containing 10% protein from casein was used as a standard. Stillings et al. (1969) have reported on the composition of the basal diet.

Male weanling rats of the Sprague Dawley strain were obtained when 22 days old, and fed a diet containing 15% casein for 2 days. Rats were then assigned to groups of 10 on the basis

Table 1–Crude protein, volatile content and pH values of FPC after heating in dry air at various times and temperatures.

Treatment				
Temp. °C	Time min	Crude protein (dry weight) %	Volatiles %	pH
100	0	87.4	1.9	7.56
	30	87.9	1.4	7.57
	60	87.8	1.1	7.54
	120	87.4	2.3	7.55
	240	87.7	1.3	7.56
120	0	87.6	1.4	7.52
	30	87.3	2.3	7.53
	60	87.6	2.4	7.51
	120	87.6	1.9	7.50
	240	87.2	1.4	7.52
150	0	88.0	2.6	7.47
	30	88.0	2.0	7.50
	60	88.0	1.8	7.52
	120	88.5	1.8	7.49
	240	88.5	1.5	7.49
Unheated		88.0	5.7	7.68

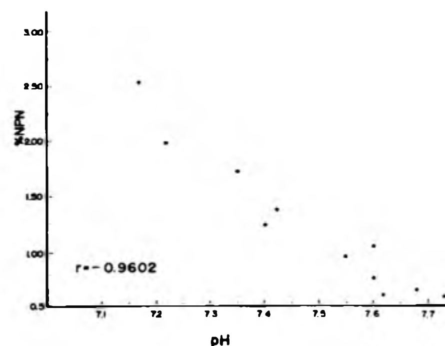

Fig. 1–Scatter diagram of the relation of pH to the percent NPN in FPC after it was autoclaved at 100°C or 120°C for up to 240 min.

Table 2–Amino acid contents of FPC after heating in dry air at various times and temperatures.

Treatment		Amino Acid					
Temp. °C	Time min	Arginine	Histidine	Tryptophan	Lysine	Available lysine	Methionine
		% of protein (N × 6.25)					
100	0	6.9	2.0	0.9	8.7	7.8	3.22
	30	6.7	1.9	1.0	8.3	7.7	
	60	6.6	2.0	1.0	8.2	7.8	
	120	7.1	2.0	1.0	8.9	7.8	
	240	6.4	1.9	1.1	8.2	7.7	3.15
120	0	6.9	1.9	1.0	8.7	7.4	3.32
	30	6.9	2.0	1.0	8.6	7.7	
	60	6.9	2.1	0.9	8.5	7.3	
	120	7.0	2.1	1.0	8.8	7.5	
	240	6.9	2.0	1.0	8.6	7.7	3.40
150	0	6.6	1.9	0.9	8.3	7.4	3.08
	30	6.9	2.1	0.9	8.5	6.6	
	60	6.7	1.9	0.8	8.1	6.3	
	120	6.5	1.9	0.8	7.9	6.3	
	240	6.3	1.7	0.9	7.7	5.9	3.18
Unheated		7.0	2.0	0.9	8.5	9.0	3.06

of body weight, and the groups randomly assigned to the experimental diets. The rats were fed *ad libitum* for 4 wk. Feed consumption and weight gains were recorded weekly, and the PER calculated by dividing the gain in weight by the amount of protein consumed.

Data were evaluated statistically by an analysis of variance, and differences between means determined by Tukey's procedure (Steel and Torrie, 1960).

Moist heat experiments

Materials. FPC used in this experiment was from the same lot as that used in the dry-heat experiment, with 1 kg samples used per treatment.

Treatments. FPC samples were placed in trays lined with aluminum foil, and thermocouples inserted at three different locations. Upon completion of the heating, the product was immediately placed in glass jars, sealed, and held at –20°C until needed.

Chemical evaluation. Analytical methods were the same as those described for dry heat; however, in addition NPN was determined by the method of Guitton (1964).

Nutritive evaluation. Procedures used to determine the nutritional quality of the samples were the same as those described for dry heat experiments.

RESULTS & DISCUSSION

Effect of dry heat

Table 1 presents results of the analyses for crude protein, volatiles, and pH. Crude protein levels of the FPC's heated at 100°, 120°, or 150°C changed little, ranging from 87.2–88.5%; unheated FPC had a protein level of 88.0%. The pH of the samples (Table 1) changed only slightly and was not significantly related to the heat treatments. Differences between the unheated and heated samples may have been due to volatilization of residual amines (Wick et al., 1967).

Table 2 shows amino acid values. Arginine concentration fluctuated between 7.1–6.4% of the protein in samples heated up to 120°C, but at 150°C, decreased from 6.9% at 30 min to 6.3% at 240 min. Histidine level ranged between 1.9–2.1% of the protein in samples heated to 120°C; however, at 150°C the level decreased from 2.1% at 30 min to 1.7% at 240 min: about a 19% decrease. Lysine also showed similar losses. Lysine concentration in samples heated at 100°C and 120°C ranged between 8.9–8.2% of the protein. The lysine levels in the samples heated to 150°C decreased after 30 min from 8.5–7.7% of the protein, or about a 9.5% decrease. The data appeared to reflect some change also in the tryptophan level; however, the control sample of non-heated FPC showed values within these ranges (Table 2).

The major change was in available lysine, with the greatest decrease occurring at a heating temperature of 150°C. At 100°C, available lysine ranged from 7.8–7.7% of the protein, whereas at 120°C, it ranged from 7.7–7.3%. At 150°C, available lysine fell from 7.4% after 0 min exposure to 5.9% after 240 min, representing about a 20% decrease in concentration.

The methionine content of the samples varied, but the variation did not appear to be related to the treatments. Other neutral and acidic amino acids were also not affected by the treatments, and values for these are therefore not reported.

Table 3 shows data from the animal-

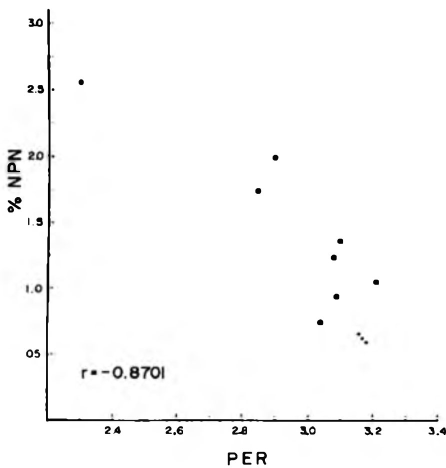

Fig. 2–Scatter diagram comparing the relation of the PER to the percent NPN of autoclaved heat treated FPCs.

Table 3–Effect of dry heat on the nutritive value of fish protein concentrate (FPC) prepared from hake.[a]

Treatment		Avg daily weight gain	Avg daily food intake	Protein efficiency
Temp. °C	Time min	g	g	ratio
100	0	4.97 ± 0.14[b]	15.15 ± 0.27	3.21 ± 0.05
	30	4.68 ± 0.13	14.45 ± 0.30	3.22 ± 0.04
	60	4.82 ± 0.16	14.76 ± 0.36	3.24 ± 0.05
	120	4.75 ± 0.14	14.95 ± 0.34	3.13 ± 0.04
	240	4.87 ± 0.14	14.99 ± 0.30	3.22 ± 0.09
120	0	4.81 ± 0.06	14.71 ± 0.27	3.24 ± 0.04
	30	4.84 ± 0.14	14.96 ± 0.35	3.15 ± 0.04
	60	4.85 ± 0.08	14.78 ± 0.31	3.24 ± 0.03
	120	4.88 ± 0.09	15.01 ± 0.23	3.20 ± 0.06
	240	4.72 ± 0.16	14.60 ± 0.37	3.17 ± 0.05
150	0	4.54 ± 0.16	14.37 ± 0.33	3.12 ± 0.10
	30	4.56 ± 0.13	14.42 ± 0.43	3.12 ± 0.04
	60	3.84 ± 0.13	14.00 ± 0.42	2.68 ± 0.04
	120	1.53 ± 0.08	10.98 ± 0.46	1.42 ± 0.05
	240	1.34 ± 0.08	10.58 ± 0.34	1.25 ± 0.06
Control – FPC		4.74 ± 0.28	15.09 ± 0.41	3.08 ± 0.12
Casein		4.20 ± 0.09	13.81 ± 0.27	3.09 ± 0.04
Tukey's W	$P<0.05$	.69	2.10	.30
	$P<0.01$	.79	2.40	.35

[a] All values are averages from 9–10 Sprague Dawley rats.
[b] SE.

Table 4–Percent of crude protein, volatile and non-protein nitrogen (NPN) content and pH of FPC after autoclaving at various times and temperatures.

Treatment		Crude protein (dry wt)	Volatiles	NPN (as is basis)	
Temp. °C	Time min	%	%	%	pH
100	0	86.6	6.4	0.60	7.74
	30	88.1	8.0	0.62	7.62
	60	88.7	9.7	1.06	7.60
	120	88.9	11.9	0.94	7.55
	240	88.3	13.1	1.37	7.42
120	0	88.5	7.1	0.75	7.60
	30	88.2	9.0	1.23	7.40
	60	88.4	10.5	1.74	7.35
	120	88.4	13.2	2.00	7.22
	240	87.5	15.2	2.56	7.17
Unheated		88.0	5.7	0.65	7.68

feeding study. No significant differences in nutritive quality were found between the unheated control and samples heated at 100°C and 120°C for 0–240 min, nor between the control and samples heated at 150°C for 0 and 30 min. However, the nutritive quality of the sample heated at 150°C for 60 min was significantly lower than that of the control, as measured by weight gain and PER. Severe depressions in weight gain, food intake, and PER resulted with samples heated for 120 and 240 min at 150°C. The major damage to the quality of the protein occurred between 60 and 120 min of heating; only a small further decrease in quality occurred between 120 and 240 min of heating at 150°C.

The effects of dry heat on the nutritive value of FPC are similar to those reported for casein (Block et al., 1934; Greaves et al., 1938; Eldred and Rodney, 1945). These investigators found that when casein was heated for 65 min or longer at 150°C, the nutritive value was lowered, although the retention of amino acids was high.

Effect of moist heat

Table 4 shows results of the chemical analyses. The volatile content of the FPC's increased slightly more than twofold as the time and temperature of autoclaving increased. The crude protein level, on a dry-weight basis, remained relatively constant at around 87–88% regardless of treatment.

The NPN content also increased as the time and temperature were increased. At 100°C, the values increased from 0.60% at 0 min to 1.37% at 240 min. The NPN level in samples heated to 120°C increased from 0.75%–2.56% after 240 min. Hughes (1963) reported an increase in non-coagulable protein in autoclaved herring, ascribing it to the conversion of collagen to gelatin and the formation of ammonia from unknown sources. He did not find an increase in the free amino acid level. However, this factor must be taken into consideration in the present study.

The pH values of the samples decreased as the temperature and time of heating increased. At 100°C, the pH decreased from 7.74 at 0 min to 7.42 at 240 min. Also, at 120°C, the pH dropped from 7.60 at 0 min to 7.17 at 240 min. A similar effect was found by Almquist and Maurer (1953) with autoclaved soybean meal.

A significant negative correlation (r = –0.96) was found between the NPN and the pH values. Figure 1 illustrates this relationship.

Table 5 shows the amounts of the basic amino acids, methionine and cystine in the samples. The amounts of arginine, histidine, tryptophan, and lysine were not significantly affected by the treatments. The available lysine values were lower in most of the heated samples, especially those heated at 120°C. Methionine was not affected by the treatments. The concentration of cystine in samples of FPC heated at 120°C for 0 min was equal to that in the unheated FPC. However, 30% of the cystine in the sample of FPC heated for 240 min at 120°C was lost. No

Table 5–Amino acid contents of FPC after autoclaving at various times and temperatures.

Treatment								
Temp. °C	Time min	Arginine	Histidine	Tryptophan	Lysine	Available lysine	Methionine	Cystine
		% of protein (N × 6.25)						
100	0	6.9	2.0	0.9	8.4	8.9	3.26	–
	30	6.6	1.9	0.8	8.0	8.2	–	–
	60	6.7	2.0	0.9	8.4	8.0	–	–
	120	7.2	2.0	0.9	9.0	7.4	–	–
	240	7.3	2.1	0.9	9.1	8.2	3.32	–
120	0	6.9	2.0	0.8	8.6	7.7	3.16	0.79
	30	6.8	2.0	0.9	8.4	7.7	–	–
	60	6.8	1.9	0.9	8.5	7.9	–	–
	120	6.6	1.9	1.0	8.0	7.9	–	–
	240	6.8	1.9	1.0	8.2	7.1	3.20	0.54
Unheated		7.0	2.0	0.9	8.5	9.0	3.06	0.78

Table 6–Effect of moist heat on the nutritive value of fish protein concentrate (FPC) prepared from hake.[a]

Treatment				
Temp. °C	Time min	Avg daily wt gain g	Avg daily food intake g	Protein efficiency ratio
100	0	4.93 ± 0.15[b]	15.20 ± 0.35	3.18 ± 0.04
	30	4.93 ± 0.10	15.25 ± 0.26	3.17 ± 0.05
	60	4.63 ± 0.21	14.54 ± 0.54	3.21 ± 0.03
	120	4.87 ± 0.13	15.34 ± 0.25	3.09 ± 0.05
	240	4.64 ± 0.24	14.77 ± 0.53	3.10 ± 0.08
120	0	4.41 ± 0.14	14.17 ± 0.40	3.04 ± 0.07
	30	4.46 ± 0.11	14.32 ± 0.26	3.08 ± 0.06
	60	4.70 ± 0.12	15.38 ± 0.27	2.85 ± 0.04
	120	4.22 ± 0.11	14.85 ± 0.26	2.92 ± 0.05
	240	3.11 ± 0.13	13.82 ± 0.55	2.30 ± 0.04
Control FPC		4.72 ± 0.15	14.60 ± 0.43	3.16 ± 0.03
Casein		4.43 ± 0.11	13.95 ± 0.38	3.15 ± 0.06
Tukey's W	$P < 0.05$	0.69	1.84	0.24
	$P < 0.01$	0.77	2.12	0.28

[a] All values are averages from 9–10 Sprague Dawley rats.
[b] SE.

changes in the other acidic and neutral amino acids were found, so the values are not reported.

Baldwin et al. (1950) reported 90–100% retention of these amino acids when casein was autoclaved for 120 min at 15 psi. Histidine, however, was retained to the extent of 83% of the concentration found in the unheated casein. Losses of lysine and cystine were reported by Evans and McGinnis (1948) who autoclaved soybean protein for 60 min at 130°C. In the present study, cystine was the only amino acid that appeared to be significantly affected by the extreme heat treatment.

Table 6 shows the data obtained from the animal study. Heating FPC samples at 100°C for 0–240 min or at 120°C for 0–120 min did not affect their nutritional quality. However, heating the samples at 120°C for 240 min significantly lowered their quality as evidenced by lower weight gain and PER than obtained with the other heated samples and unheated controls.

Results of the feeding studies suggested a relation between percent NPN and PER. This relation is shown in Figure 2 as a scatter diagram with a coefficient of correlation equal to −0.8701, which is statistically significant ($P < .01$). Further investigation into the cause of lowered protein quality is required to determine whether it is due to biologically unavailable amino acids, slower release of amino acids by digestion, or other causes.

A comparison of the data from the two experiments, using comparable times and temperatures, shows that dry heating of the FPC produced less damage to the quality of the protein than moist heating did.

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N. E. BENGTSSON and W. GREEN
Swedish Institute for Food Preservation Research, Göteborg, Sweden
and F. R. DEL VALLE
School of Food Technology and Marine Sciences, Guaymas, Mexico

RADIO-FREQUENCY PASTEURIZATION OF CURED HAMS

SUMMARY—Radio-frequency heating at 60 and 2450 MHz in conveyorized equipment was compared with conventional hot water treatment for pasteurization of small cured hams packed in Cryovac casings inside moulds. For lean 2-lb hams, treatment time to reach the desired central temperature could be reduced to 1/3 by heating in a condenser field tunnel operating at 60 MHz, with substantial reduction of juice losses and a tendency to improve sensory quality compared with that obtained with hot water processing. Treatment time could be further reduced by roughly the same extent by processing at 2450 MHz, but product thickness had to be reduced to secure sufficient heat penetration. Temperature gradients and juice losses were comparable to those in hot water treatment. At 2450 MHz in particular, but also at 60 MHz, bacterial surface counts were considerably higher than in the controls, suggesting the need for higher final surface temperature or supplementary heat treatment, which will lower the differences in processing time and juice loss between methods.

INTRODUCTION

THE SWEDISH MEAT industry produces small pasteurized cured hams (1–2 lb), "cooked" inside cans or plastic casings in hot water of about 85°C to a final central temperature of 65–70°C. The product has a limited shelf life and requires cold storage. Processing time is about 1 hr and juice loss is of the order of 25%. The juice lost is jelled around the ham with gelatin. This jelly is usually discarded by the consumer and implies both a quality negative and an economic loss. Since the use of water binding agents such as polyphosphates is forbidden in Sweden, we decided to find out whether any substantial reduction in juice losses and treatment time could be obtained by using radio-frequency (RF) heating. Under ideal conditions RF-heating might allow rapid heating throughout a food sample without any temperature gradients, provided field and sample are sufficiently homogenous. In practice such a process would have to allow continuous operation without requiring any very special electrode assemblies or sample holders differing widely in construction from the moulds commonly used in the processing of large hams. The processed ham also should be adequately protected against recontamination after processing and not require transfer to another container for supplementary heat treatment.

LITERATURE REVIEW

Processing methods

Pircon et al. (1953) and Pircon (1954) describe a process for diathermal sterilization of boned hams at 9 MHz. Based on fundamental studies of capacitance and conductivity of cured luncheon meats they designed test cells, in which hams were placed in pyrex tubes between steel electrodes, using an expansion follower plate for the "hot" electrode to take up pressure caused by expansion of the meat. Owing to the high conductivity of the meat, the tubes had to be made long and narrow. Experiments were also conducted with plastic-walled cans with concentrically corrugated metal caps, as the electrodes. They report 6-lb pieces of ham could be heated to sterilizing temperatures in about 10 min. Cooling of the perifery of the sample from the container walls was reduced by the use of external heat. Temperature differences along the meat cylinder ranged from 30–40°F. Cold regions were observed near the bottom of the cylinder and in melted fat pockets, and current control and agitation of the cell were used to equalize the temperatures.

Taste panel results showed a preference for the diathermal sterilization compared with conventional pasteurization. Shelf life of the hams was satisfactory. No mention is made of the amount of juice lost during processing. Despite these results the process has not been used commercially, probably because of high costs and problems in designing processing cells or cans suitable for large-scale operation. After processing, the ham had to be transferred under aseptic conditions to a separate container for supplementary surface heat treatment.

While the above process, with direct contact between product and electrodes, can be considered a combination of dielectric and resistance heating, Luijerink (1962) describes a process for the heat pasteurization of cured hams by pure conduction heating at mains frequency viz. line frequency or 220V a.c. Heating from room temperature to 80°C requires 8 min after which the ham is transferred to a can and dipped in boiling water for 5 min as supplementary surface heat treatment. Temperature differentials after conduction heating were given as about 30°C in 6-lb hams. Conventional processing with water heating required 5–6 hr.

A search of the literature failed to reveal any investigation where cured hams had been heat processed continuously by dielectric heating with an air gap between the sample and the electrodes. However, several have reported on dielectric thawing of foods in the frequency range 27–35 MHz by such processes. Jason and Sanders (1962), Bengtsson (1963) and Sanders (1966) described equipment for continuous dielectric thawing in laboratory and production scale equipment, and reported results obtained with fish and various meat products. The significance of the use of an air gap between sample and electrodes to reduce tendencies towards run-away heating are discussed by Bengtsson and Remi (1963) and Sanders (1966) on the basis of dielectric data, sample-electrode configuration and experimental results. Two important factors in influencing temperature distribution were conveyor speed and power density in the sample.

At higher frequencies, the only report found is a patent procedure by Williams (1965) on diathermal treatment in the curing and smoking of meats. He claims that the temperature in cured meats could be raised uniformly to 140°F in 1–5 min by microwave heating in the frequency range 450–900 MHz. Internal heating was slightly higher than the surface heating. Higher frequencies than 900 MHz could be used, but tended to produce more surface heating. The objective of the process was not ham pasteurization but to shorten the time of after-treatment in the smokehouse. No details are given concerning the actual processing equipment or procedure. Otherwise, there are numerous reports in the literature on the application of microwave heating for cooking purposes, mostly in small, domestic ovens. More recent work has been reported on industrial cooking of foods in large scale, conveyorized microwave equipment (Anon. 1967).

Fundamental and theoretical

When a material is heated in a dielectric field, whether by capacitive heating or by microwave heating, the heat generated in the material is directly proportional to its dielectric constant and loss tangent, to frequency and to the square

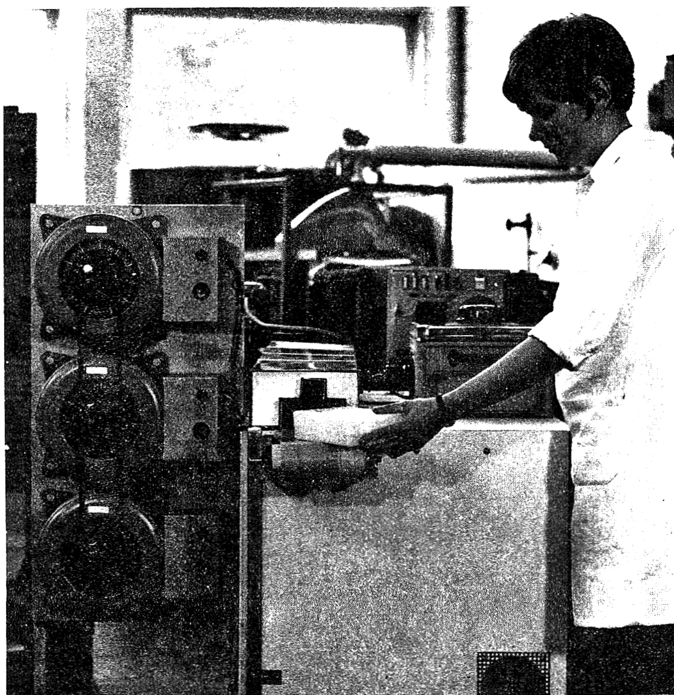

Fig. 1–5 kW microwave tunnel.

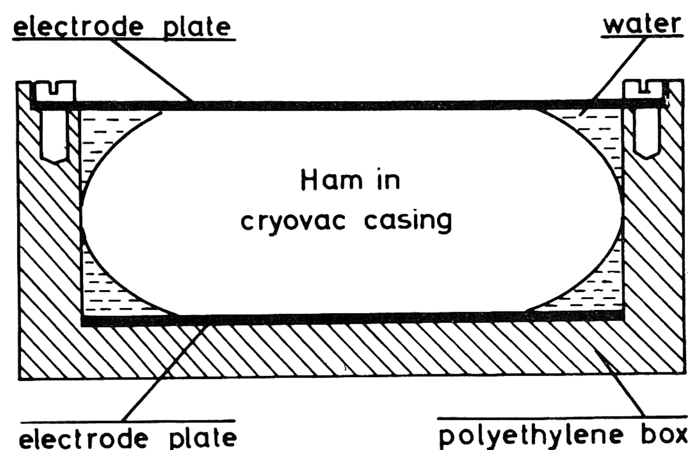

Fig. 2–Mould-electrode arrangement for RF-processing at 60 MHz; (top) cross section; (bottom) actual view.

of the field strength in the material. The degree of energy penetration into the material, generally expressed as the depth at which the power of the incident wave is reduced to 1/2 or to 1/e (1/2.71), decreases with increasing dielectric constant, loss tangent and frequency. Since the dielectric properties thus determine both the rate of heating and the degree of power penetration, knowledge of such data is important in all dielectric heating applications.

Pircon (1954) determined permittivity and resistivity of cured hams at 7 and 29 MHz and 70°F and obtained values corresponding to a conductivity of 0.009 $ohm^{-1} \cdot cm^{-1}$ and a dielectric constant of 132. Ede and Haddow (1951) and Hartshorn and Rushton (1946) report conductivities of 0.02 $ohm^{-1} \cdot cm^{-1}$ at 20°C and 10–20 MHz for bacon and lean ham. For ham fat they report a conductivity of 0.001 $ohm^{-1} \cdot cm^{-1}$ Otherwise, no dielectric data on salty meats could be found in the literature.

Differential effects in dielectric heating of layered or embedded material are important in the heating of hams, which usually have a layer of fat as well as embedded streaks or particles of fat. These factors have been discussed by Morris-Thomas (1951) and Schwan et al. (1953) in relation to dielectric properties and layer thickness. According to Morris-Thomas, in capacitance dielectric heating of rectangular slabs of layered material, a layer of higher conductivity will heat less than one of lower conductivity below a certain critical frequency, while the opposite will be true above that frequency. Schwan et al. (1953) studied diathermic heating of fat-muscle layers in the human body, and conclude the fat will be overheated in capacitive dielectric heating up to 100 MHz. This tendency decreases with increased frequency, while in radiation diathermy heating of the fat layer increases with the frequency.

The fundamental equations for circuit calculation in capacitive dielectric heating are discussed by Riegel (1952, 1953). It should be possible then, by calculating equivalent parallel resistance and capacitance for an electrode-sample combination, to determine the proper changes in dimensions of a different material to maintain unchanged load matching if the dielectric properties are known.

Microbiological considerations

It is obviously important that microbiological factors be taken into account when considering application of RF-heating to reduce treatment time in the pasteurization or sterilization of cured hams. The possible reasons for the observed safety of canned, cured meats are discussed by Spencer (1966). Factors considered other than heat treatment are: content of salt and nitrite, pH, and incidence of clostridial spores. He concludes that there are no quantitative data on the combined effects of heat process and curing ingredients on the ability of *Clostridium botulinum* to survive and grow. Perigo et al. (1967) report inhibitory activity on vegetative growth of *Clostridium sporogenes* from an unknown substance formed when heating a laboratory medium containing nitrite, and they discuss the finding in the context of sublethally processed cured meats.

The problem of green discoloration sometimes occurring in cooked hams is discussed by Gardner (1967) who attributes the fault to either improper distribution of curing salts or bacterial activity from survival of lactobacilli.

EXPERIMENTAL

Equipment

The experiments at 35–60 MHz were made with a generator of 1 kW output and a conveyor for feeding the material to be heated between the electrodes of the load condensor. Power input from the mains, electrode spacing and conveyor speed could be varied continuously, and by a simple modification of the circuit the frequency could be shifted between 35 and 60 MHz. The equipment has been described previously (Bengtsson, 1963).

At 2450 MHz two conveyorized units of different design were used, one of which has been described previously (Bengtsson, 1963). This was a 2 kW experimental tunnel of the Philip's parabolic design. The second unit was a modified commercial Husqvarna tunnel module, developed for heating precooked frozen or refrigerated meals. It consists of a 5.5 × 12 cm tunnel and 1.5m in length into which the waveguides from three 1.5 kW magnetrons are coupled by the so-called magic T-principle. The equipment is shown in Figure 1. For both microwave tunnels power input and conveyor speed could be varied continuously.

Power efficiency in the experiments was calculated as the power absorbed in the form of heat in the load in percent of the input from the mains to the generators, as estimated from instrument readings, temperature rise and available enthalpy data. In the experiments, efficiencies of about 25% were obtained at 60 MHz and 25–35% at 2450 MHz.

Table 1–Pasteurization of ham at 60 and 2450 MHz. Processing conditions for most satisfactory results.

	60 MHz	2450 MHz parabolic tunnel	2450 MHz wave guide tunnel
Ham thickness, cm	6	3	3
weight, g	900	400	400
packaging	Cryovac	Cryovac	Cryovac
Water immersion, cm^3	100–200	–	–
temp. initial, °C	50	–	–
Electrode length, cm	25	–	–
Air gap, cm	5–6	–	–
Power input to generator P_1 kW/kg load	1000	4000	2800
Power output in load P_2 kW/kg load	250	1000	1000
Power efficiency P_2/P_1 %	25	25	35
Conveyor speed, cm/min	6	20	45
Number of passages	6–8	8	2
Effective heat treatment time, (min)	16–20	4	4

Raw material and methods

Hams were obtained from three different processing plants using slightly different curing methods, giving differences in salt content and uniformity of salt distribution. For comparative experiments between cooking methods, pairs from the same plant and production lot were always used. Normally hams had been pumped to 6–10% content of 18° Bé brine (NaCl with 0.6% $NaNO_2$) and stored in brine for 1–2 wk. They were then cut down, trimmed to 2-lb size and shipped to the SIK Institute under refrigeration. The trimmed hams were usually taken from the inside round. Average salt content varied from 3–5% between hams and sometimes by as much within hams. Average water content was 68–70% and the total fat content 10–15%. Hams were usually trimmed to less than 5 mm surface fat layer, but experiments were made also with hams with a 1 cm fat layer and thick embedded streaks and masses of fat tissue. All together some 250 hams were used in the experiments.

In preliminary work the processing methods summarized in Table 1 were developed. For treatment at 60 MHz hams were trimmed to about 2-lb weight and rounded rectangular shape and packed in 1.5 mil × 6 in. Cryovac tubing, sealed with plastic clips or nylon string after the ends had been folded over to reduce risks for reinfection after processing. A vacuum was pulled before closing the top opening completely, and the casing was shrunk around the ham by a hot water dip.

The trimmed and encased ham was compressed into a polyethylene mould (9.8 × 18 × 5.5 cm inner dimensions) between two flat aluminum electrode plates by a simple compression device, exerting a pressure of approximately 15 kg on the ham surface. The top electrode was secured to the mould by polyethylene screws, and empty spaces around the ham in the mould was filled with 100–200 cc warm water through holes in the top electrode. The principle of the mould-electrode arrangement is shown in Figure 2. Compression between electrode plates and the use of immersion water serve to level out field and temperature distribution in the ham. Compression is necessary to give the ham correct shape and to make it hold together after heat treatment.

The hams were fed repeatedly between the primary electrodes of the load condensor, until a center temperature above 65°C was reached, and left in the mould for 10 min before being cooled in a refrigerator. Processing time was usually 15–20 min and final water temperature between 65–75°C when using a well-insulated mould.

In the experiments at 2450 MHz, sample thickness was reduced to 3 cm and hams were turned upside down between successive passages through the tunnels to obtain sufficient penetration depth. A polyethylene mould (inner dimensions 19 × 9 × 3 cm) was used. It consisted of two halves which were screwed together after compression. Immersion in water was not used partly for practical reasons and partly because previous experience from microwave thawing of meat had indicated water immersion would not be helpful. Because of the rapid heating at 2450 MHz, a small opening in one of the closures of the Cryovac tubing was used during processing to prevent bursting pressures from developing. This channel was closed as near aseptically as possible after processing, and the ham dipped in boiling water for 5 min. Hams were passed through the tunnels repeatedly to reach a final center temperature above 65°C.

Pasteurization in water at 85°C was used for reference. The ham was trimmed, packed and shrunk in Cryovac casing and compressed between metal plates in a mould. The plates were perforated and in direct contact with the processing water, which was agitated with a stirrer to improve heat transfer. For comparisons with RF heating at 60 MHz 2 lb hams were used and for the comparisons with microwave processing 1 lb hams, the water processing times being approximately 1 hr and 30 min respectively, to reach a center temperature of 65°C.

Temperature was measured with spear-type thermocouples. For the hams to be analyzed without storage, temperature was measured at 18 or 27 symmetrically situated points in the hams processed by RF, and at the surface and center for water processed hams. For hams intended for storage testing temperature was measured only at the center and surface, using a sterile sensor or surface temperature sensed with a special thermocouple applicator without penetrating the Cryovac casing.

Analytical methods

Juice losses were determined by weighing the hams before processing and after adhering juice and jelly had been scraped off. Water retention ability was determined by centrifugation using a modification of a method of Aitken (1962). Salt content was measured at 5 different locations in the ham, using potentiometric titration with silver nitrate.

Sensory evaluation of appearance comprised intensity and evenness of color, lightness and general appearance of ham surface and cut slices using 5-point scales. Intensity of ham-meat flavor, juiciness and saltiness and texture and taste preference were evaluated by 5-point scales in the early comparison. Then direct paired comparisons were made for taste, juiciness, tenderness and saltiness. Bacteriological examination was made by surface sampling, using a cork-bore technique, plating on ATP-agar and incubating at 30°C for 3–4 days. A limited study of the bacterial flora present in raw hams was made, but otherwise only total counts were made.

Preliminary dielectric data were obtained for cured ham and fat tissue in the temperature range of 20-70°C and frequency range of 35–100 MHz using a Boonton RX-meter and a test cell design described in a previous publication (Bengtsson et al., 1963). At 2450 MHz a few measurements were made with a perturbation technique described by Labuda and LeGraw (1961).

Table 2–Difference between RF-pasteurization of hams at 60 MHz and hot water processing. Grand averages for 28 runs evaluated after 3 days and 9 runs after 3 months' storage at +10°C.

Processing conditions and evaluations	Evaluation after 3 days' storage			Evaluation after 3 months' storage		
	RF-processing	Hot water processing	Difference	RF-processing	Hot water processing	Difference
Processing time, min.	28	70	RF much shorter	16	65	RF much shorter
Final center temp. °C	72	68	small	72	69	small
Temp. range in single hams, °C	12	15	RF slightly better	15	15	none
Juice loss, %	12.4	19.8	RF much better	13.1	19.7	RF much better
Centrifugation loss, %	15.7	14.8	none	16.0	11.3	RF significantly higher
Sensory evaluation score general appearance	3.5	3.2	RF tended to be better	3.5	3.5	none
				Paired comparisons		
taste	3.8	3.6	none	taste		none
texture	3.6	3.5	none	tenderness		none
juiciness	5.6	5.2	RF tended to be better	juiciness		RF significantly better
Microbial total plate count, surface	–	–	–	$8.5 \cdot 10^5$	$0.4 \cdot 10^5$	RF worse

Determination of suitable processing conditions

At 35–60 MHz empirical experimentation with 2-lb hams and model system work with ground ham were made in parallel to determine most suitable processing conditions. In the model system work, the influence of sample and electrode geometry, and water immersion on load matching was studied, along with heating patterns in layered or embedded fat using Plexiglass cylinders or rectangular polyethylene boxes for sample holders and sample weights from 50–1000g. In the testing with 2-lb hams, these were trimmed to 4–7 cm thickness, packed in Cryovac casing in plastic moulds with or without compression between secondary electrodes and with or without immersion in water of different volume, salt content and initial temperature. Other variables investigated were salt and fat content of the ham, conveyor speed, power density and generator frequency. The following observations of interest were made in the roughly 100 preliminary heating experiments.

Heat generation was considerably higher in unsalted pork than in ham with 3% salt content, while the difference was only slight between 3 and 6% salt content. Power efficiency was higher at 60 MHz than at 35 MHz. It increased with compression of the ham but at the expense of temperature distribution and juice loss. When water immersion was not used, the size of the air gap was critical to power efficiency and had to be small, while water immersion required larger airgaps and permitted more variation. Instead, water immersion slightly lowered power efficiency while salt addition to the water raised it.

By improving the tightness of fit of the ham in the mould, water volume could be decreased to a little over 100 ml resulting in a rise in final water temperature from 50 to 70°C, and more even temperature distribution between surface and interior of the processed ham. The salt content of the ham influenced the optimal ratio between air gap and ham thickness, so that different sources of hams required noticeably different air gaps.

In the model experiments, use of equations by Riegel (1952, 1953) permitted scale-up from 50g to 1000g with unchanged power efficiency, in that sample thickness, air gap and surface area were always changed in the same proportions. The relationships did not hold when using water immersion of the ham.

Temperature distribution in the ham was improved by using lean hams of uniform salt content, by increasing the frequency from 35 to 60 MHz, by compressing the ham between secondary electrodes at moderate pressure, by water immersion in a small volume of water and high final water temperature and by good thermal insulation of the moulds or a short dip in hot water after RF processing.

Increasing conveyor speed and number of oven passages also improved temperature distribution, especially when the hams were turned 180° between passages to compensate for the disturbance caused by the feed strip to the top (hot) electrode. Temperature spread in the ham appeared to increase with sample weight, heating rate, compression, and when contact with the secondary electrodes were poor. The average temperature range in a lean 2-lb ham cooked by RF heating at 60 MHz was 10°C, mainly as higher temperature in the interior than at the surface. In the water cooking at 85°C the surface temperature was about 15°C higher than the final desired central temperature. Horizontal layers of fat were always overheated, while vertical layers (perpendicular to the electrode) and embedded balls or cylinders of fat were not.

At 2450 MHz similar preliminary heating experiments with ground and whole hams were made. To obtain sufficient power penetration, product thickness had to be reduced to

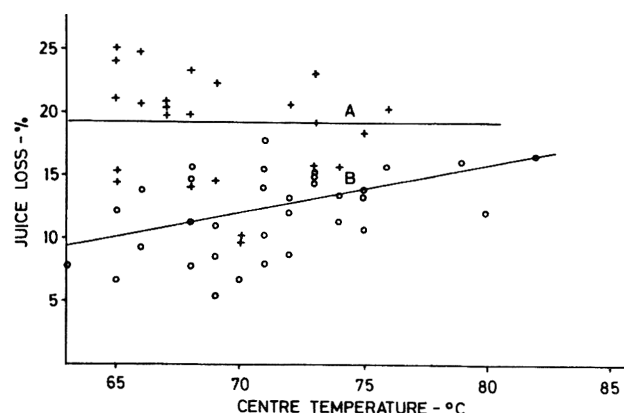

Fig. 3–Juice loss as a function of final central temperature in hams: A = hot water processing; B = RF-processing at 60 MHz.

Table 3—Difference between RF pasteurization at 2450 MHz and hot water processing. Averages for 8 runs and two storage times.

Processing conditions and evaluations	RF processing	Hot water processing	Degree of difference	
Processing time, min.	5	35	RF much shorter	
Center temp., °C	66	68	small	
Temperature range in single hams, °C	20	15	RF slightly poorer	
Juice loss, %	16.7	18.1	none	
Centrifugation loss, %	12.6	13.3	none	
Microbiological evaluation after 3 weeks storage, surface total plate count	10^7	3.10^2	RF much worse	
Sensory evaluation by paired comparison			3 days storage	3 weeks storage
General appearance, surface			RF tended to be poorer	RF significantly poorer
General appearance, interior			none	RF tended to be poorer
Taste			none	RF sign. poorer
Juiciness			none	none

about 3 cm. Temperature distribution was improved by cutting the ham to rectangular shape and compressing in plastic moulds, but water immersion did not seem to produce any improvement. Relatively high conveyor speeds had to be used. To compensate for variations in field distribution across the tunnel sections, hams had to be turned upside down and 180° sideways between passes. This procedure would at the same time simulate in-line processing with several parabolas or wave-guide tunnels after each other with power fed alternately from above and below the product.

Considerably higher power density and shorter heat treatment time (3–5 min) could be used compared with the lower frequencies, but only at the expense of less even temperature distribution. Power efficiencies were in the order of 30%. The two tunnels gave comparable heating efficiency and temperature distribution with temperature spreads of about 15–20°C, or about the same as in conventional hot water processing. The center of the ham was heated least.

Continuous movement through the tunnels was found essential at both frequencies if results obtained were to be acceptable.

Experimental plan

On the basis of the preliminary work, a series of more well defined experiments were planned to compare RF heating at each of the frequencies 60 and 2450 MHz with processing in hot water. For practical reasons, direct paired comparisons between the two frequencies could not be included.

About 40 runs were made comparing processing at 60 MHz and hot water processing and 10 runs comparing 2450 MHz and water processing. A planned series at 915 MHz had to be postponed owing to lack of suitable equipment.

Dielectric properties of hams at 60 and 2450 MHz were determined in an effort to correlate the results of heating experiments with basic data.

RESULTS

RF-pasteurization of hams at 60 MHz

Two series of experiments were made with lean hams and one with fat hams. An attempt was made to reach the same final center temperature with both heating methods for each pair of hams in order to make direct paired comparison more valid.

In the first series, with short time storage of 30 pairs, lower power density and larger volume of immersion water were used in the RF processing than for the second series, which comprised 10 pairs and 3 months' storage. The results of these experiments are summarized in Table 2.

Quite significantly lower juice losses were obtained with RF-processing than in hot water processing and treatment time was less than half. Temperature spread was less than in hot water processing in the first series and essentially equal in the second series, where higher power density and smaller water volume were used in the RF-heating. Temperature spread tended to increase with final temperature in the ham and to decrease with increasing final temperature of the immersion water. There was no consistent temperature gradient in the ham during RF-processing. Sensory evaluation showed a general tendency towards better quality of RF-processed hams, particularly in juiciness. Higher centrifugation loss of RF-processed ham is in accord with higher degree of juiciness.

In Figure 3 juice loss is shown as a function of final center temperature of the ham for the first series of 30 pairs. Juice loss shows no correlation with final central temperature in hot water processing. RF-heating showed a positive correlation with final temperature ($r = 0.36$), juice loss being about half that for water processing at a center temperature of 65°C. In the second series the results were similar.

Microbiological examination after prolonged storage showed considerably higher total counts for RF-processed hams, indicating need for higher final temperatures or supplementary heat treatment. Total counts decreased with increasing salt content and final water temperature, and in one of the runs, where salt content was 5% and final temperature above 75°C in the immersion water, bacterial counts were very low. Variation between runs was considerable, probably to a larger extent because of variation in the raw material available.

Fat hams with a surface fat layer of about 12–15 mm were used in a series of four comparisons. Because preliminary experiments had shown overheating of the fat layer when placed parallel to the electrodes, the fat layer was now positioned perpendicular to the electrodes. To decrease microbial surface contamination hams were dipped for 5 min in boiling water after processing as a supplementary treatment, and gelatin was added to bind the juice released. Heat processing time was 16 min by RF and final center temperature as high as 75°C, temperatures in the fat layer being lower than in the meat. Evaluation after 3 wk storage at +10°C indicated slightly lower quality of RF-processed fat hams, and only 1–2% lower juice loss than in hot water cooking. The fat layer had a more "raw" consistency in the RF-treated ham. Microbial counts were about 10 times higher than in hot water processing.

The effect of adding gelatin to bind released juice was studied separately, and juice losses appeared to be much smaller when no gelatin was used. This appeared to confirm observations that RF-processed hams have a higher tendency to reabsorb released juices when processed inside shrink-packed Cryovac casings without gelling agent.

Table 4–Dielectric data obtained using a modified test cell.

Material	temp. °C	35 MHz dielectric constant ϵ'	35 MHz loss tangent	60 MHz dielectric constant ϵ'	60 MHz loss tangent
Lean pork, unsalted	20	77	5	73	3.2
Ham, 1% salt	20	90	9	80	7.0
Lean ham, 3% salt	20	125	12	115	8.5
Lean ham, 3% salt	40	125	14	120	11
Fat tissue from salted ham (0.9% salt)	20	28.5	2.1	22	2.0

RF-pasteurization of hams at 2450 MHz

Since the preliminary work at this frequency had proved less rewarding, more well defined experiments were limited to 8 runs comparing microwave processing with hot water processing for 1-lb hams. Four of the pairs were evaluated after 3 days' storage at 5°C and the rest after 3 wk at 10°C. The results are given in Table 3.

On examination immediately after processing the hams differed but little in quality with the processing method used. Also temperature spread and juice losses were roughly equal. Color development and eating characteristics were surprisingly close for the two methods considering that processing time by microwave heating was only 5 min. On the other hand, the temperature remained high for some time after processing.

After 3 wk storage the appearance and general quality of the microwave processed ham had deteriorated. The microbial counts were very high with noticeable green discoloration of the surface.

Dielectric measurements and their significance

To explain the experimental heating results and to obtain basic data for future choice of equipment and processing parameters, measurements of dielectric properties were made at the frequencies of 35, 60 and 2450 MHz.

For the measurement at the lower frequencies a previously developed test cell had to be modified because of the high conductivity of the salted ham. Precision became rather low with a range of ± 25% between replicates in some cases. The data obtained are given in Table 4.

For comparison it may be mentioned that Pircon (1954) reported a dielectric constant of 132 for cured ham at 29 MHz and room temperature. For lean bacon Ede and Haddow (1951) report a conductivity of 0.040 $ohm^{-1} \cdot cm^{-1}$ at 40°C and 20 MHz, which compares well with a value of 0.034 calculated from our own measurements at 35 MHz and one of 0.025 $ohm^{-1} \cdot cm^{-1}$ reported by Hartshorn and Rushton (1946) for lean ham at 20°C and 10 MHz. They also found the conductivity for ham fat at the same temperature and frequency as 0.001 $ohm^{-1} \cdot cm^{-1}$, compared with 0.0012 calculated from our own data at 35 MHz.

Applying these data to the ham heating experiments, we first consider the case where a constant electrode voltage is used and direct contact between electrodes and sample. Power density in the ham defined as generated heat will then be proportional to $\epsilon' \times \tan\delta \times$ frequency. Using the tabulated data, power density is found to increase with salt content and temperature in the ham and with frequency. However, when an airgap is introduced between sample and electrodes, voltage gradients in air and sample will also depend on the dielectric properties of the sample, as demonstrated by Bengtsson and Remi (1963) and by Sanders (1966). For a 1:1 ratio between air gap and sample thickness and a constant voltage over the electodes, the following relative values for power density are calculated:

	35 MHz	60 MHz
unsalted pork, 20°C	2.4	6.4
ham, 1% salt 20°C	1.2	2.9
ham, 3% salt 20°C	0.6	1.7
ham, 3% salt 40°C	0.4	1.2

In agreement with the heating experiments made, the dielectric data suggest that the rate of heating increases with frequency and decreases with salt content and temperature.

For differential heating in plane parallel layers of fat and meat and in embedded fat, calculations based on the formulae given by Morris-Thomas (1951) and the above dielectric data result in a ratio of power density between meat and fat of about 1:20. This occurs when the layers are parallel to the electrodes, while the ratio will be about 20:1 when the layers are turned perpendicular to the electrodes and 10:1 when a spherical lump of fat is embedded in the ham.

The calculations seem to agree with observations in our heating experiments with hams and with Sanders' (1966) observations in dielectric thawing of fat and meat layers.

An attempt also was made to use the dielectric data measured for ham in the equations of Riegel (1952, 1953) to calculate sample dimensions for optimal load matching. It was known that the power efficiency of the generator combination used was as high as 50% when heating wood of known dielectric properties. The attempt was, however, not successful.

2450 MHz

Preliminary measurements made in a TMO12 mode cavity by a perturbation technique indicated that, at the same field strength, heat generated in the ham will be about twice as high at 2450 MHz as at 60 MHz for cured ham. Penetration depth, defined as the depth where the energy of the wave is reduced to 1/e (37%) of the original value was calculated as 5 mm at 20°C. This is unexpectedly low considering the fact that rapid heating was obtained for 3 cm thick samples, and we have no ready explanation to offer.

DISCUSSION

DIELECTRIC pasteurization of lean hams at 60 MHz resulted in acceptable temperature distribution and substantially reduced heat treatment time and juice losses with indications of an advantage in sensory quality. On the other hand, the shortened heat treatment, in combination with a lower surface temperature than in conventional hot water processing, gave a higher surface infection. The reduced juice losses found in the dielectric process are probably due both to reduced surface heat treatment, with less release of juice, and to a higher ability of the surface of the ham to reabsorb juice during cooling under the reduced pressure inside the cryovac casing. Tests showed the use of a jelling agent interfered with this reabsorption of juice.

The higher surface infection of RF-processed hams is probably a direct result of the lower and shorter surface heat treatment. A limited study of the microflora in raw hams revealed no presence of pathogenic bacteria. Cocci, yeast and Gram-positive rods seemed to dominate. After processing, only spore-forming bacteria were found indicating that heat treatment was sufficient to kill vegetative cells. It must be remembered, however, that RF-pasteurization involves reduced surface heat treatment in a process where, according to Spencer (1966) the actual reasons for apparent hygienic safety are not clear.

In the experiments made, surface infection in the raw material as well as salt content were quite variable. Improved raw material control would have to be combined with higher final central tem-

perature than in the hot water process and final temperature of the immersion water around 75°C. Or the RF-process could be combined with some supplementary surface treatment, such as steam, smoke, IR, ionizing radiation or antibacterial agents. Such supplementary heating may be most effective if done before RF-processing.

The results indicated that increasing brine content (and improving distribution) reduced surface counts. It is possible that the corresponding increase in nitrite level contributed to this effect, judging from the observations of Perigo et al. (1967).

The highest power efficiency that could be obtained with salted ham in the generator-electrode combination used was 25%, while 50–60% would be required in an industrial application. It is believed that with knowledge of the dielectric properties of the material to be heated, this could be obtained by proper design of the generator.

While Pircon et al. (1953) in their RF-sterilization work with hams had to use batchwise heating in a special processing cell with direct contact between meat and electrodes and aseptic transfer to a container after processing, our pasteurization technique allows continuous heat processing without such transfer from the Cryovac casing after treatment. Lowering the goal from a sterilizing treatment to pasteurization eliminates problems encountered when working above atmospheric pressure. The use of an airgap and continuous treatment simplifies process control, as discussed by Sanders (1966) with regard to so-called runaway heating.

A problem present in all dielectric heating applications is that of temperature measurement, since temperature sensors can generally not be used in RF-fields. By using treatment in several successive RF fields after each other, temperature could be controlled during the passage in between heating zones; for example, by pressing a surface temperature sensor against the outside of the ham. Unless weight, fat content and salt content of the hams are closely controlled, there will be some variation in heating rate between hams. In our experiments the final temperature achieved by the same treatment time varied by about 10°C.

At 2450 MHz with 1-lb hams considerably higher power density in the ham and shorter heat treatment time could be used than at 60 MHz. Power efficiency was higher. At the same time, surface temperatures reached were considerably higher than at the lower frequency, and juice loss was roughly comparable to that in hot water processing at 85°C. While initial sensory quality of the microwave processed ham was acceptable, surface infection after 3 wk storage was far too high and greenish discoloration of the surface made the product unsightly. According to Gardner (1967), green discoloration of hams is caused either by improper distribution of curing salts, or, by bacterial activity which was probably the cause in this case. Owing to the rapid heating and swelling of the ham during microwave heating the Cryovac casing could not be completely sealed until after processing, which may have increased the risk for reinfection in spite of precautions taken. At none of the frequencies was there any indication of a specific effect of RF-treatment on microorganisms.

Results have shown that "old-fashioned" capacitative dielectric heating at 60 MHz may be useful for the pasteurization of hams, even if it is not known whether surface infection can be controlled without appreciable loss of the advantages of reduced juice loss and processing time. The only advantage to heating at 2450 MHz, under the experimental conditions used, was shortening of the processing time. However, it is possible that a slower heating rate combined with periods of temperature equilibration and external heating with steam might have produced more positive results, at the expense of longer processing time. The combined results at 60 and 2450 MHz suggest that an intermediate frequency may very well be optimal for the purpose (Williams, 1965). However, pilot equipment at 450–915 MHz was not available for our investigations.

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G. L. HARGUS[a], G. W. FRONING, C. A. MEBUS, SANKARA NEELAKANTAN and T. E. HARTUNG
Department of Poultry Science and Department of Veterinary Science, University of Nebraska, Lincoln, Nebraska 68503

EFFECT OF PROCESSING VARIABLES ON STABILITY AND PROTEIN EXTRACTABILITY OF TURKEY MEAT EMULSIONS

SUMMARY—The effect of chopping temperature and time on the stability of white and dark turkey meat emulsions was determined. White meat emulsions remained stable over extended chopping times in a 1.5°C temperature environment. When chopped in room temperatures, the white meat sausages became very unstable after 18.2°C and were most stable at 12.8°C. Dark meat sausages were significantly more unstable than white meat emulsions under both chopping environments. Tensile strength of cooked white and dark meat emulsions declined with increases in chopping temperature. When prepared in 1.5°C surroundings, tensile strength changes were notable only after 10 min chopping for white meat. Dark meat sausage tensile strength seemed unaffected by chopping time. In contrast to earlier red meat emulsion work, protein denaturation was indicated to occur in turkey meat emulsions prepared under both cold and room temperature environments. Decreases in soluble proteins due to increased chopping occurred under both conditions. Protein denaturation is believed to be partially responsible for emulsion breakdown. Dark meat showed more protein denaturation than white meat emulsions. Photomicrographs of histologically prepared meat emulsions showed disruption of the protein-fat globule interface as a result of increased chopping or temperature, or both. Coalescence and emulsion breakdown occurred at end-point chopping temperatures similar to those reported by red-meat workers.

INTRODUCTION

SINCE the advent of economically mechanically deboned turkey meat, it may become feasible to utilize turkey meat in new emulsion convenience items. Formulation of a number of sausage-type prepared turkey meat items from various meat sources to satisfy variety and cost criteria may be the likely result. Meat sources may include mechanically deboned neck, back and frame meat in various combinations with hand deboned meat from other portions of the carcass.

Much of the previous work on poultry meat has involved the model system approach. Maurer and Baker (1966) studied various parts of different classes of poultry to emulsify fat. Their work indicated that collagen content is a detriment to emulsifying capacity and observed that voluntary muscle meats were superior to gizzard, heart or skin with respect to emulsification capacity. Hudspeth and May (1967) observed that emulsifying capacity of salt-soluble protein from dark meat was higher than that noted from white meat. This was true even though white meat had higher quantities of salt-soluble protein present. Franzen and May (1968) investigated phospholipids and found that they may be involved in improvement of emulsifying capabilities of meat from certain tissues. More recently Hudspeth and May (1969) reported that skin is the least desirable tissue with respect to emulsification properties, that heart tissue is intermediate and gizzard tissue the most desirable of these 3 tissue sources. None of these sources was as effective for emulsification as muscle tissue.

The effect of processing variables on actual poultry meat emulsion formulations has largely been unexplored. Guidelines are needed to establish production procedures for accurately controlling the quality of poultry emulsion products.

Chopping time and temperature interrelationships have been studied extensively utilizing red meat sources. Saffle (1968) indicated that it has been known for some time in the sausage industry that chopping temperatures exceeding 15–22°C will cause emulsion breakdown. Hansen (1960) found that chopping time must be sufficient to form a protein matrix enclosing the dispersed fat. The proteins, myosin and actomyosin, appeared to supply stabilizing membrane around the fat globule. Excessive chopping temperatures were observed to partially denature the protein matrix and thereby result in coalescence of fat globules.

Helmer and Saffle (1963) noted that emulsions were stable at chopping temperatures of 60°F and were also stable when emulsions were chopped to 90°F, chilled to 40°F and rechopped to 60°F. Chopping to 90°F caused emulsion breakdown. Although there was a slight drop in percent extractable protein at the higher chopping temperatures, the change in solubility was not significant. They concluded that protein denaturation was not a factor in emulsion instability.

Excessive periods of comminution can also cause emulsion instability. According to Wilson (1960), myosin is salt extracted from meat during chopping to form an interface between liquid-protein and fat phases of an emulsion. With continued chopping, semisolid fat is cut up into increasingly smaller fat globules, creating a larger total fat surface for the protein to cover. If insufficient myosin is available, fat globules not fully surrounded will coalesce, causing emulsion breakdown.

The purpose of this research was to investigate processing variables which influence turkey meat emulsion stability and to examine changes in protein solubility as a factor related to emulsion breakdown. The study was divided into 2 parts: Part 1 dealt primarily with the effect of comminution temperatures; Part 2 emphasized the effect of comminution time. In addition, white and dark meats were compared for efficiency as emulsifiers.

EXPERIMENTAL

Meat source

The meat used in this study was from 25-week-old Broad Breasted White tom turkeys grown under uniform management conditions. After slaughter in the University of Nebraska poultry laboratories, the birds were blast frozen and stored at −29°C. When ready for evaluation, the birds were thawed 48 hr at 6°C and hand deboned. The meat was separated into white and dark components and the muscles from all birds were mixed thoroughly together after grinding twice through 3-mm grinder plates. After weighing into formula portions, the meat was frozen at −29°C until utilized in the emulsions.

Emulsion preparation

The basic emulsion was as follows: 600 g meat, 250 g finely crushed ice, 135 g rendered chicken fat (with added antioxidant), 13.0 g NaCl, and 2.0 g pepper. White and dark emulsions were prepared identically in a Hobart silent cutter precooled to 1.5°C. Partially frozen meat was added to the chopping bowl with the salt and pepper. After 30 sec of chopping, the fat (1.5°C) was added and ice was added 30 sec after the fat addition. Comminution was then continued until the desired emulsion temperature or chopping time was reached.

Comminution in Part 1 was performed at room temperature. Temperature levels of 1.7, 7.2, 12.8 and 18.2°C were obtained without additional heating. The food cutter was placed before an open oven operating at 232°C to obtain 23.8 and 29.4°C levels. The time required to reach the various emulsion temperatures for the replications was controlled as closely as possible by maintenance of constant room and oven temperatures.

In Part 2, where emphasis was placed on the effect of comminution time, temperature increases were kept minimal by carrying out the emulsification procedure in a 1.5°C cold-room.

[a]Present address: Campbell Institute for Food Research, 100 Market Street, Camden, New Jersey 08101.

After the initial mixing of the ingredients, the silent cutter was stopped every 5 min to record temperature until chopping times of 10, 20, 30 and 40 min were reached. The temperature increased to a maximum of 10.5°C during the longest chopping period. These chopping times are in excess of those normally encountered; however, the objective was to establish extreme conditions for the variable.

In both Parts 1 and 2, the silent cutter bowl and top were periodically scraped to prevent any accumulation of nonemulsified materials.

After comminution, the emulsions were placed into a precooled sausage stuffer and were extruded into 50 by 350–400-mm No-Jax links. Raw emulsions were collected for histological fixing and for the stability test. The sausages were cooked for tensile strength tests in an electric rotating rack oven at 94°C for 1 hr, after which the oven controls were adjusted to 121°C until the internal sausage temperature reached 78°C.

Emulsion stability

The method of Townsend et al. (1968) was used to evaluate the effects of chopping time and temperature on the emulsions produced in this study. The amount of fat, water and solids released during cooking was used as an indicator of stability of the sausages. Fat release was considered the best indicator.

Tensile strength

This test was used to determine whether emulsion stability was related to tensile strength of the cooked emulsions. A device similar to that of Swift and Ellis (1957) was used to measure the amount of force necessary to break a 51- by 20- by 4-mm center slice of turkey sausage.

Protein solubility

Protein denaturation due to increased chopping temperatures and times was determined by measuring protein solubility at various levels of comminution.

At each level of chopping, a sample of the emulsion was subjected to an extraction similar to that used by Khan (1962). Cold (1.5°C) KCl-borate buffer (0.6 m KCl, .065 m $Na_2B_4O_7$, .0013 m H_3BO_3, pH 7.5, ionic strength of 1.0) was used to extract the proteins in a precooled mortar in a cold-room (1.5°C). 3 g of emulsion were ground 1 min with 5 g of sand in the mortar, followed by grinding 1 min with 50 ml of buffer. The resulting supernatant was poured off into a holding beaker and grinding was continued for 30 sec with 25 ml of buffer. After pouring off this supernatant, the remaining residue was washed into the holding beaker with 15 ml buffer. The extracting buffer and materials were thoroughly mixed and then centrifuged 10 min at 18,000 × g in an International Model HT in the cold-room. The resulting supernatant was analyzed for nitrogen by a micro-Kjeldahl technique (AOAC, 1965).

Histological methods

To examine the effects of processing variables on the emulsion matrix, a procedure of paraffin embedding (Gray, 1958) was used to prepare raw emulsion samples for differential staining. Prior to embedding, samples were placed in cellulose casings and fixed 4 days in a formal-saline (1.8% NaCl, 10% formaldehyde) solution. After embedding and blocking, slides were prepared by slicing sections 2.5-μ thick and mounting on to slides. Heidenhain's staining method (Clayden, 1962) was used to show the emulsion matrices of the samples. Photomicrographs of the prepared sections were taken at 400× magnification. The complete embedding method, as modified for emulsions, is that reported by Froning et al. (1970).

Table 1–Effect of temperature of emulsification on amounts of components released on cooking to 68.8°C. Volume of components released expressed in units per 100 g emulsion (Part 1).[1]

Maximum temp during chopping (°C)	Average chopping time (min)	Fat (ml)		Gel water (ml)		Solids (g)	
		White	Dark	White	Dark	White	Dark
1.7	6.7	.2b	.8a	8.4a	12.7a	.3b	.4a
7.2	9.8	.1a	2.0b	5.7b	18.6b	.3b	.6b
12.8	13.9	.0a	3.7c	4.1b	25.6c	.1a	1.3c
18.2	25.8	.4c	5.6d	8.8a	28.4c	.3b	1.5c
23.8	31.0	1.0d	6.1d	9.6a	27.0c	.5c	1.5c
29.4	42.8	1.9e	6.3d	7.3a	25.9c	.5c	2.0c

[1] Means within a column for different subscripts are significantly different at .05 level of probability.

Table 2–Effect of chopping time on amounts of components released on cooking to 68.8°C. Volume of components released expressed in units per 100 g emulsion (Part 2).[1]

Maximum chopping time (min)	Average chopping temp (°C)	Fat (ml)		Gel water (ml)		Solids (g)	
		White	Dark	White	Dark	White	Dark
10	-2.1	NF	1.4a	4.8a	6.6a	.3a	.3a
20	3.3	NF	2.1b	4.6a	6.0a	.2b	.2b
30	8.4	NF	2.7b	3.4b	8.6b	.2b	.3a
40	10.4	NF	3.9b	3.6b	13.7c	.2b	.4c

NF = No fat released at any length of chopping time.

[1] Means within a column for different letters are significantly different at .05 level of probability.

Statistical analysis

Analysis of variance was performed on data collected from 3 replications in Part 1 and 2 replications in Part 2 (Snedecor, 1956).

RESULTS & DISCUSSION

Stability test

Results of the stability test are shown in Tables 1 and 2.

Fat release of dark meat emulsions significantly increased up to 18.2°C chopping temperature and appeared to remain constant for the remaining advancing temperature periods. After reaching a minimum fat release at 12.8°C, white meat emulsions exhibited a step-wise significant increase in fat release with advancing temperature. Gel-water release did not increase appreciably in either white or dark meat emulsions after 12.8°C. Although solids released were somewhat variable, there was a trend upward in each of the meat sources associated with increasing chopping temperature. These results represent a combination of chopping time and temperature. Thus, the importance of Part 2 of this study is further exemplified.

When compared to results of Part 1, increasing comminution periods under controlled temperature conditions produced emulsions with much improved stability (Table 2). No fat release was observed from white meat emulsions at any of the chopping periods. Dark meat emulsions exhibited fat release at each of the chopping periods, but no significant increase was found after 10 min of chopping. Gel-water release of white meat sausage remained somewhat uniform at all chopping periods although the 10- and 20-min periods were significantly higher than the 2 other periods. Gel-water release from dark emulsions significantly increased with longer periods of chopping. Solids release from both white and dark emulsions showed no apparent trend with advanced periods of chopping.

The superiority of white over dark meat as a fat emulsifier could be due to higher levels of salt-soluble proteins in white meat. Khan (1962) showed significantly higher amounts of extractable myosin nitrogen/100 g tissue in white than in dark meat. The more coarse meat fiber bundles in dark meat also resist extraction more than white meat under commercial emulsification conditions. In addition, the higher fat content of the dark meat adds to the total amount of fat to be emulsified. Data previously reported by Hudspeth and May (1967)

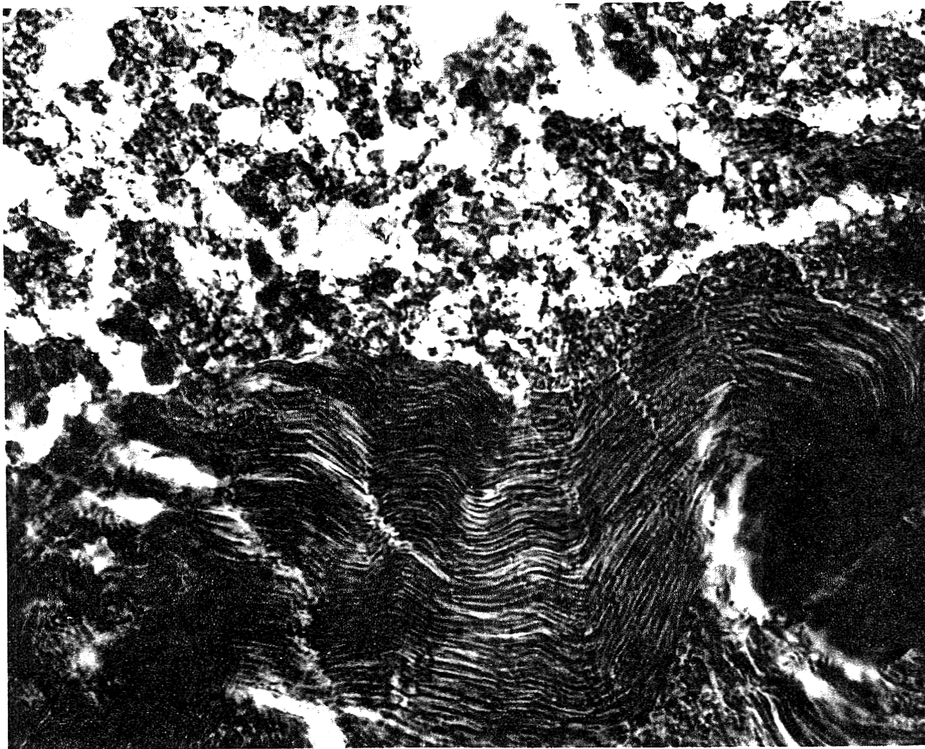

Fig. 1—Effect of chopping temperature of 1.7°C (chopping time 6.7 min) on photomicrographic appearance of raw white meat emulsions (200× magnification).

Table 3—Effect of chopping temperature on tensile strength of white and dark turkey emulsions (Part 1).[1]

Maximum temp of chopping (°C)	Average chopping time (min)	Tensile strength (g)	
		White	Dark
1.7	6.7	270a	203a
7.2	9.8	233b	145b
12.8	13.9	236b	129b
18.2	25.8	180c	142b
23.8	31.0	168d	136b
29.4	42.8	135e	127b

[1] Means within a column for different letters are significantly different at .05 level of probability.

Table 4—Effect of chopping time on tensile strength of turkey emulsions (Part 2).[1]

Maximum chopping time (min)	Average chopping temp (°C)	Tensile strength (g)	
		White	Dark
10	–2.1	280a	190a
20	3.3	221b	212a
30	8.4	186c	204a
40	10.4	194c	179a

[1] Means within a column for different letters are significantly different at .05 level of probability.

indicated that dark meat had superior emulsifying capacity when compared to white meat. It should be emphasized, therefore, that emulsifying capacity and stability are 2 measurements, each with different objectives. Thus, it appears both should be considered when studying emulsifying characteristics.

Tensile strength

Considering Part 1 and Part 2 simultaneously, tensile strength of white meat sausages tends to decrease with increasing amounts of chopping (Tables 3 and 4). In Part 1, the tensile strength of dark meat emulsions was more sensitive to temperature increases during chopping than white meat sausages. Tensile strength appears to be somewhat of an indicator of stability, although maximum tensile strength was always at the onset of chopping, and fat release in white meat was minimal at 12.8°C. White meat emulsions exhibited significantly higher tear strength than the less stable dark meat sausages.

The downward trend in tensile strength of white meat emulsions was significant ($P < 0.05$) in Part 1, whereas the tensile strength of dark meat emulsions remained fairly constant after reaching 7.2°C. There was no significant decline in tensile strength of dark meat emulsions at any of the chopping times (Part 2) observed. White meat emulsions from Part 2 did exhibit a significant decrease in tensile strength as chopping time advanced, although differences between the 30- and 40-min periods of chopping were not significant ($P < 0.05$).

Protein denaturation

Percent extractable protein was found to decrease significantly due to increases in chopping temperature and time (Tables 5 and 6). This indication of protein denaturation was coincidental to the increases of instability in white and dark

Fig. 2—Effect of chopping temperature of 12.8°C (chopping time 13.9 min) on photomicrographic appearance of raw white meat emulsions (200× magnification).

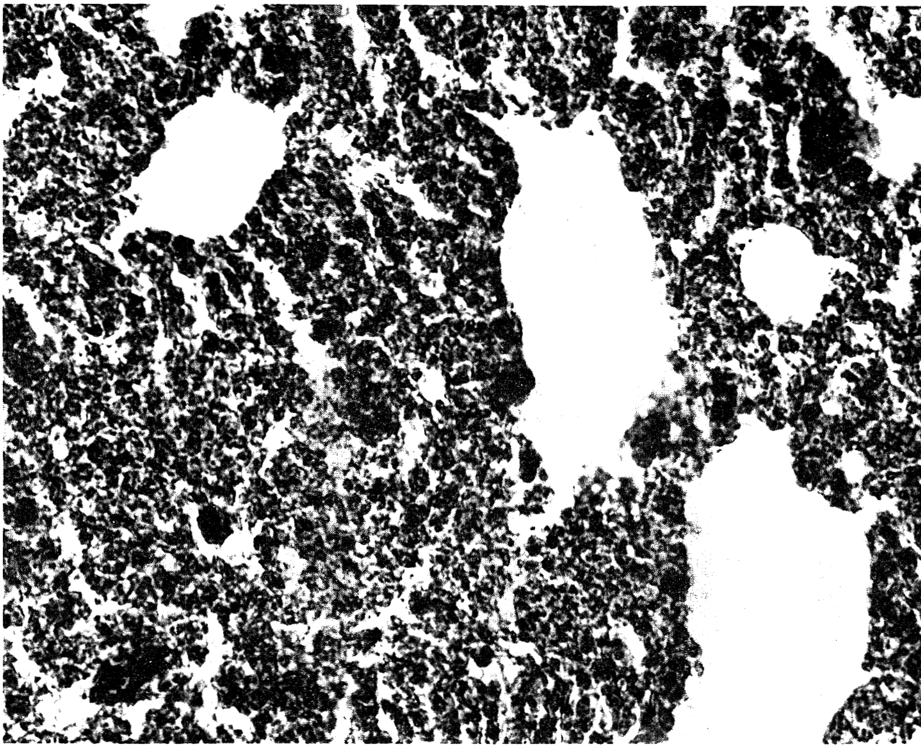

Fig. 3–Effect of chopping temperature of 29.4°C (chopping time 42.8 min) on photomicrographic appearance of raw white meat emulsions (200× magnification).

meat sausages in Part 1 and dark meat emulsions in Part 2. The decline in the amount of soluble proteins was slightly greater in Part 1 than in Part 2 presumably due to a temperature effect. Dark meat consistently exhibited lower protein extractability which may explain differences in stability between white and dark meat sources.

The decline in soluble proteins coincidental with decreased stability indicates that protein denaturation may be partially involved in emulsion breakdown. This research does not agree with the earlier work reported by Helmer and Saffle (1963). Perhaps differences in extraction procedures may in part explain results. This study utilized a different extraction buffer which extracted total proteins. Also, the use of sand in the extraction procedure may possibly improve the over-all efficiency of the extraction.

The apparent stability of white meat emulsions chopped at low temperature, even with a decrease in soluble proteins, indicated that more was involved in emulsion breakdown than protein denaturation. Perhaps physical effect produced denaturation from extended chopping periods. Townsend et al. (1968) showed emulsion breakdown occurring at chopping temperatures coinciding with the change of fat from semisolid to liquid. Perhaps the interaction of protein denaturation and fat transitional states may be contributing to instability of emulsions chopped to high (+20°C) temperatures.

The decline in protein solubility also may not materially affect the emulsification characteristics. This is especially true when the protein capacity to emulsify fat has probably not been reached in the formulations utilized in this study. Perhaps, the protein available for stabilizing the fat globule is so overwhelming that the loss in protein solubility is not a

Table 5–Effect of chopping temperature on percent protein extractable from white and dark turkey meat emulsions (Part 1).[1]

Maximum chopping temp (°C)	Average chopping time (min)	Percent extractable protein	
		White	Dark
1.7	6.7	54.8a	52.0a
12.8	13.9	53.4b	51.0b
23.8	31.0	51.4c	47.7c

[1] Means within a column for different letters are significantly different at .05 level of probability.

Table 6–Effect of chopping time on percent extractable protein of turkey emulsions (Part 2).[1]

Maximum chopping time (min)	Average chopping temp (°C)	Percent extractable protein	
		White meat	Dark meat
10	– 2.1	54.6a	47.8a
20	3.3	53.0b	45.1b
30	8.4	52.1c	44.0c

[1] Means within a column for different letters are significantly different at .05 level of probability.

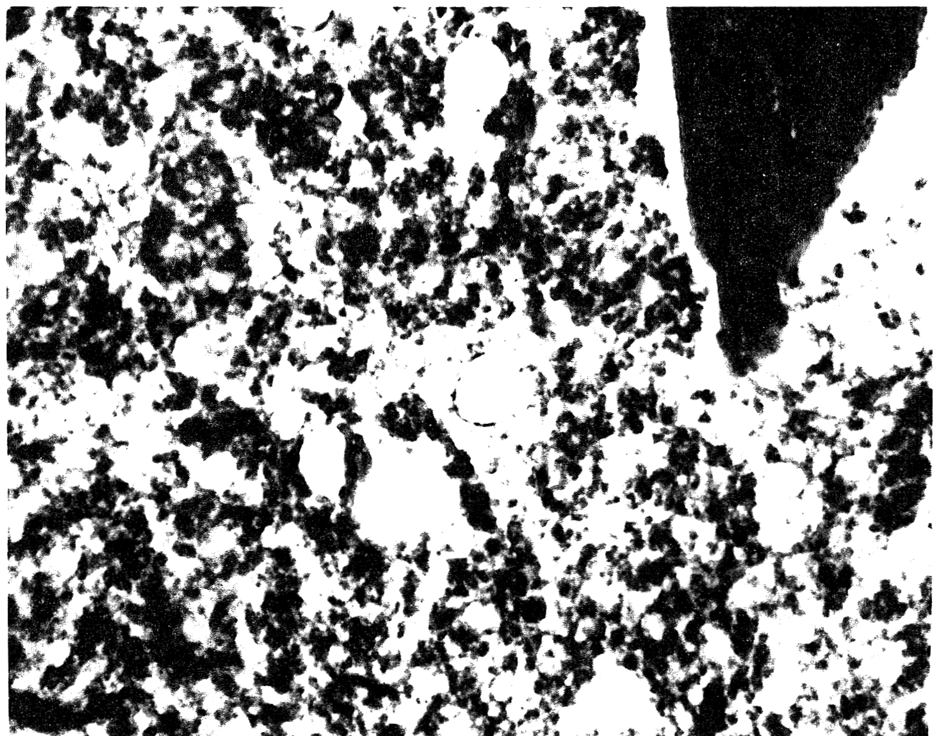

Fig. 4–Effect of chopping time of 10 min (chopping time–2.1°C) on photomicrographic appearance of raw white meat emulsions (200× magnification).

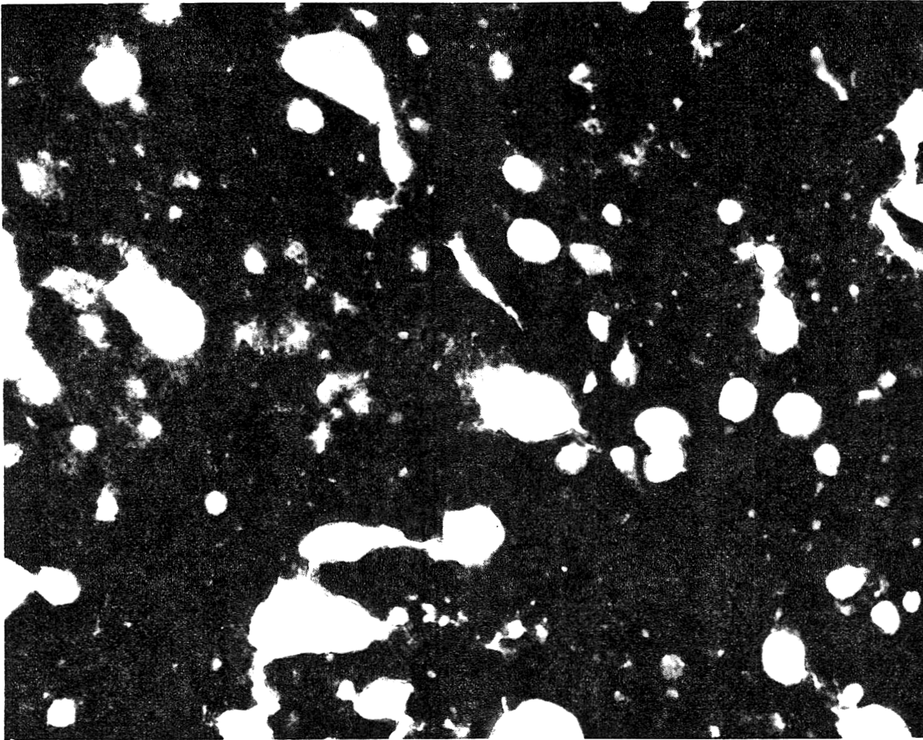

Fig. 5–Effect of chopping time of 40 min (chopping temperature 10.4°C) on photomicrographic appearance of raw white meat emulsions (200× magnification).

significant factor. This aspect would be an interesting investigation for future consideration, especially if higher fat levels were utilized in the emulsion formulation.

Histological survey

The photomicrographs presented in Figures 1, 2 and 3 show the effect of chopping temperature and time on the white meat emulsion matrices at 1.7, 12.8 and 29.4°C. At 400× magnification, a decrease in fat globule size occurs between 1.7 and 12.8°C. At 29.4°C, fat globules are even smaller in some instances, but large irregular holes apparently are the result of fat globule coalescence and emulsion breakdown. Amounts of unbroken muscle fibers were also shown to decrease with continued chopping.

Figures 4 and 5 show raw white meat emulsions at 10- to 40-min chopping time in Part 2 of this study. Intact tissue was noted to be present after 10 min of chopping and fat globules showed no apparent evidence of coalescence. 40 min of chopping, however, appeared to show fat globule breakdown, although the emulsion stability test indicated no fat release.

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R. C. BAKER, J. DARFLER and D. V. VADEHRA
Poultry Science Department, Cornell University, Ithaca, N.Y. 14850

EFFECT OF pH ON THE QUALITY OF CHICKEN FRANKFURTERS

SUMMARY—Chicken frankfurters were made with chicken meat adjusted to pH levels from 4.6 to 8.6. Finished frankfurters were tested subjectively for tenderness, juiciness, flavor and preference by an experienced taste panel. These were subjectively tested for compression values by Kramer shear press and for punch and deformation values by Instron. Results indicated that at normal pH (6.1) the chicken frankfurters were the most firm. Below pH 6.1 they rapidly became more tender, because of the instability of the emulsion. Above pH 6.1, they gradually became more tender, due to lower moisture loss. Juiciness was little affected by pH, except at pH 5.6, which produced a drier sample.

INTRODUCTION

ONE OF THE criticisms of frankfurters made from chicken meat is that their texture is too tender. Earlier attempts in this laboratory at increasing the firmness include addition of skin at various levels (Baker et al., 1968) and changes in level of the fat and protein in the formula (Baker et al., 1969). Results showed that the addition of skin at levels above natural proportions increased firmness, but the chewy skin pieces were somewhat objectionable. Increasing fat and decreasing water when protein was held constant made a somewhat firmer frankfurter. Raising the protein level was the most effective in increasing firmness. The effect of pH of the meat from which the emulsion was made on the finished product was investigated. Changes in pH level have been found (Hellerdorn, 1962; Swift and Sulzbacher, 1963) to affect both the water binding capacity and the emulsifying ability of meat when using meat slurries and several protein extracts. Levels above the normal pH of meat (about 6.0) resulted, in general, in an increase in both of these factors. In actual practice, higher pH values have been found to increase the water binding capacity and emulsifying ability of red meats used in sausage manufacture, while low pH tend to "short out" meat which reduces its emulsifying qualities (Sair, 1965). To what extent and in what manner increased water binding and/or emulsifying capacity affects tenderness in a frankfurter or other emulsion type meat products cannot be found in the literature.

MATERIALS & METHODS

CHICKEN MEAT from heavy fowl was procured from a commercial processing plant, hand deboned and frozen. Chicken fat was obtained from the same source. All meat and fat were stored at −20°C until needed.

Frankfurters were made from the formula given in Table 1. Ice was made by freezing distilled water mixed with citric acid or sodium bicarbonate to obtain the desired pH when mixed with the meat. In series 5, sodium hydroxide was used to adjust pH.

The emulsion was made by chopping the meat and ice for 1 min when a small sample was removed for a pH reading. The cure and seasonings were then added and chopping continued to 2–3°C. The fat was added and the emulsion chopped to a final end temperature of 12°C ± 0.5°C. The frankfurters were then stuffed, linked and cooked as described by Baker et al. (1968). After storage overnight at 2°C they were peeled and stored in sealed plastic bags until used for testing.

Frankfurters were made up in this manner in five series: (1) pH 5.4, 5.8, 6.1 (normal) and 6.6 (exploratory only); (2) 4.6, 5.1, 5.6, 6.1 (normal); (3) 6.1 (normal), 6.6, 7.1, and 7.6; (4) 5.6, 6.1 (normal), 6.6, 7.1 and 7.6 and (5) 6.1 (normal), 7.1, 7.6 and 8.6. pH readings on the meat and ice mixtures showed a variation from the expected pH of not more than ± 0.1. Each series was duplicated.

After being heated, the frankfurters were evaluated organoleptically by a panel of six members, all of whom had considerable experience in judging chicken frankfurters. The frankfurters were prepared for heating by removing the "skin" or outer proteinaceous layer (since only the interior was of interest), and cutting each into three sections. The pieces were then sealed in a boilable plastic bag, immersed in boiling water and boiled for 20 min. This method lessened the possibility of breaking up the more tender and mushy samples, which might have occurred had they been heated directly in the water. The frankfurters were scored for tenderness, juiciness and flavor, using a semi-structured scale from 9 to 1, with the higher numbers denoting the more tender, more juicy and better flavored products. Samples with the two lowest pH values of series (pH 4.5 and 5.1) were not presented to the panel as they were completely unacceptable with regard to appearance, and were too tender and mushy to be cut into sections for serving. All testing was done in individual booths in an air conditioned room under red lights.

All samples presented to the taste panel were tested objectively for tenderness by the Kramer shear press. Except for series 4, samples were heated before testing according to the procedure outlined by Baker et al. (1968). Maximum force and compression values were computed from each curve. Series 4 was also evaluated by the punch test and a compression test, using the Instron Universal Testing Machine, as described in an earlier paper (Baker et al., 1968). These tests were performed on cold samples as were the Kramer shear tests.

Samples were analyzed for fat and moisture. The O'Haus Moisture Determination balance was used for the moisture analyses. Fat was determined by the method of Salwin et al. (1955) with the substitution of sulfuric acid and water for acetic acid and perchloric acid. Results of the analyses are in Table 2.

The project extended over several months and two factors became evident: One was the week to week variability of the taste panel members, and the other was a variability among the "normal" (pH 6.1) samples in each series, as tested objectively. To eliminate these factors, an arbitrary value of 6.0 was given to the "normal" samples, and the scores of the other samples were adjusted arithmetically. In each series the scores were changed proportionately to their standard value. The taste panel data were then subjected to an analysis of variance as a whole. Duncan's new multiple range test (Steele and Torrie, 1960) was used to determine the significance among the treatment means. Correlation coefficients were run on a selected data.

Table 1—Formula for chicken frankfurters

Ingredient	Quantity
Chicken meat	4313g (9–½ lb)
Chicken fat	1135g (2–½ lb)
Chipped ice	1362g (3 lb)
Salt	109g
Seasoning (a commercial blend)	55g
Onion powder	10g
Sodium nitrate	10g
Sodium nitrite	1.1g
Dextrose	38g

Table 2—Effect of pH on the moisture and fat content of finished chicken frankfurters.[1]

pH	Moisture %	Fat %
4.6	58.6	17.6
5.1	58.9	21.0
5.6	57.3	27.9
6.1	58.0	26.1
6.6	58.6	25.5
7.1	58.0	26.3
7.6	58.2	26.2

[1] Each mean is the average of at least two batches.

RESULTS & DISCUSSION

Tenderness

Taste panel scores for tenderness (Table 3) showed a maximum tenderness at the lowest pH value tested (5.4) and a sharp and significant increase in firmness to a maximum at pH 6.1. Above this

Table 3–Effect of pH on taste panel scores for tenderness, juiciness and flavor of chicken frankfurters.[1]

pH	Tenderness[2]	Juiciness[2]	Flavor[2]
5.4	8.75	5.08	5.04
5.6	6.71	6.13	5.77
6.1	6.00	6.00	6.00
6.6	6.29	6.25	6.08
7.1	6.48	6.42	5.88
7.6	6.87	6.58	5.67
8.6	7.04	6.42	5.75

[1] Each value is an average of 48 scores.

[2] Score of 9 denotes most tender, most juicy, and least flavor. Means connected by the same vertical line are not significant from each other ($P < 0.05$).

Table 4–Effect of pH on compression values of chicken frankfurters, tested hot.[1]

pH	Compression values[2] (lb/g)
5.6	0.51_d
6.1	0.89_a
6.6	0.84_a
7.1	0.75_b
7.6	0.63_c
8.6	0.47_d

[1] Each mean is the average of at least 6 determinations.

[2] Means followed by the same subscript letter are not significantly different ($P < 0.05$).

level, samples were judged progressively less firm. Samples below pH 5.4 were not served to the taste panel; they were too soft and mushy to be cut up for serving and were extremely unappealing in appearance.

Maximum force values computed from the Kramer shear press curves showed that there was little difference in the frankfurters made from meat adjusted to pH levels below normal and those made from meat at normal pH and levels above normal. In appearance, however, as the pH was lowered, the frankfurters became progressively much softer and mushier. The lack of change in the maximum force values may be due to the presence of skin and connective tissue fragments in the emulsion, the shearing of which could contribute in large measure to the value for "maximum force." The quantity and toughness of these fragments was approximately the same for all frankfurters in each series. "Compression" values were calculated from the first peak on the curve. Such a peak appeared in nearly all cases below pH 6.1. These compression values seemed to be more reliable estimates of the relative tenderness of frankfurters. These compression values (Table 4) showed the same pattern as the taste panel's tenderness scores, although inversely: that is, a very low value for pH 5.6 climbing to a high at 6.1–6.6, denoting maximum firmness, and dropping again as the pH was raised above normal. A correlation coefficient of $r = -0.538$ shows a significant correlation between taste panel tenderness scores and Kramer shear compression values.

The Instron punch test, which was used on the samples in Series 4, showed the same trend as the compression values (Table 5) with a highly significant correlation of $r = 0.948$. Deformation test results appear to be unrelated to the tenderness of the frankfurters.

Juiciness

Juiciness, as determined by the taste panel scores, showed that there was little difference among the samples, except in the case of those at pH 5.4, which were significantly less juicy than the others. Since the analyses for fat and moisture (Table 2) at normal pH and above varied very slightly, the lack of difference in juiciness scores is to be expected. Both fat and moisture have been shown to be important in the apparent juiciness of frankfurters and bologna (Baker et al., 1969; Swift and Weir, 1954). The low juiciness score for the sample at pH 5.6 can be explained by the low water retention for those samples (Table 6). A considerable amount of water and fat cooked out of the samples on heating (10% more than at pH 6.1) resulting in a dried sample.

Flavor

Flavor scores appeared highest in the pH range 6.1–6.6 although these were not significant. Bouthilet (1949) found that when the pH of chicken meat was changed, there appeared a strong chicken flavor at pH 5.8. Below this level the chicken flavor was strong but distilled in a few minutes. Above this level (pH 6.2–6.8) the flavor became less "chickeny" and more "meaty" (and possibly more in keeping with the flavor of a frankfurter). At pH 7.0–7.2, the flavor became weaker. In general, taste panel results appear to agree with these findings.

Preference

Preference scores can be compared only within each series, and not over the range of pH values. On this basis, pH 6.1 was preferred over 5.6, but at levels above 6.1 there was no clear-cut preference. From these results, one may conclude that some judges preferred more tender franks than other judges, or that the flavor of some of the franks was preferred over those of the others, since preference is a personal matter. The small size of the taste panel precludes drawing any inference as to how the public in general may prefer one pH level over another and is of interest only.

General Appearance

General appearance of the finished frankfurters showed no striking differences due to pH except in the case of those with pH values below normal. In these instances, due to "shorting out" of the emulsion, both fat and moisture were lower than in the "normal" frankfurters, with much cook-out in the casings; the degree depended on the pH. The degree of "shorting out" or "breaking" of the emulsion, which was obvious after chopping was completed, also depended on the acidity of the meat. The greater the acidity the more apparent "breaking" and the poorer the appearance of the finished frankfurters. Above the normal pH of meat, there was little, if any, apparent difference in the appearance of the emulsions, and the frankfurters made from them all had no fat caps, a good "skin" and peeled well. Color in the casings, however, appeared to be pH dependent;

Table 5–Effect of pH on compression values and punch and deformation tests of chicken frankfurters (series 4) tested cold.

pH	Kramer compression[1] lb/g	Punch test[2] g	Deformation[3] cm
5.6	0.67	520	0.079[4]
6.1	1.02	1124	0.065
6.6	1.03	1185	0.070
7.1	0.80	966	0.078
7.6	0.81	907	0.083

[1] Average of 6 determinations.

[2] Using a #8 punch, with a crosshead speed of 20 cm/min; average of 80 determinations.

[3] Deformation under a 20g load (between 5 and 25g); average of 160 determinations.

[4] Means connected by the same line are not significantly different from each other ($P < 0.05$).

Table 6–Effect of pH on water retention by chicken frankfurters.[1]

pH	Weight loss[2] %
5.6	31.8
6.1	21.7
6.6	15.7[3]
7.1	12.2
7.6	11.7
8.6	11.1

[1] Each mean is the average of at least 6 determinations.

[2] Heated in a sealed plastic bag for 20 min in boiling water.

[3] Means connected by the same line are not significantly different from each other ($P < 0.05$).

as the pH of the frankfurter was raised the color became noticeably paler. This difference in color in no way influenced the taste panel as it could not be detected under the red lights used in the taste panel room.

Water Loss

Water loss was calculated by the following formula:

$$\frac{\text{original wt.} - \text{wt. after heating}}{\text{original wt.}} \times 100 = \%\ \text{water loss}$$

Retention increased as the pH was increased (Table 6). This is in agreement with others who have worked both on protein extracts and slurries (Hellerdorn, 1962; Swift and Sulzbacher, 1963) and on emulsion products (Sair, 1965). Increased pH has been found to increase both the water binding ability and fat emulsification qualities of meat, which would lead to a higher water retention value when heated.

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K. IJICHI, H. H. PALMER and H. LINEWEAVER
USDA Western Utilization R&D Div., ARS, Albany, California 94710

FROZEN WHOLE EGGS FOR SCRAMBLING

SUMMARY—Commercially blended, pasteurized liquid whole eggs were used for testing methods of preparing products that would be suitable for scrambling after frozen storage. The eggs were subjected to separate and combined effects of homogenizing, colloid milling, mixing and addition of various levels of liquid skim milk, salt, sugar or dextrose. After removal from frozen storage the products were thawed and evaluated for the effect of treatment on appearance, viscosity and sweetness. Products were developed having good appearance and low viscosity with a low enough level of sweetness to prevent detection.

INTRODUCTION

IN THE LAST few years the institutional food-service industry has increased its use of whole eggs frozen in 5- to 15-lb quantities for scrambling. Discussions with representatives of this industry revealed that the usual commercial frozen whole egg does not supply the desired uniform appearance, smooth consistency and low viscosity needed to facilitate mixing with milk and other scrambled-egg ingredients. This is true even though freezing increases the viscosity of whole eggs from about 0.1 to less than 30 poises and the eggs are fairly fluid; thawed commercial yolks have a viscosity of several thousand poises.

In addition to increased viscosity, thawed whole eggs tend to have a curdled appearance and exhibit separation of a darker liquid after thawing that is unattractive. Wertheim (1964) reported that defrosted liquid whole egg may be lumpy and may contain "gelled aggregates, sometimes separated by a thin orange to red liquid."

Treatments known to influence these quality factors include mechanical thinning and addition of substances such as salt, sugar and milk. Reduction of viscosity of thawed whole eggs by prefreezing mechanical treatments such as stirring, milling, homogenizing and pumping through pasteurizing equipment has been reported by numerous investigators (Thomas and Bailey, 1933; Pearce and Lavers, 1949; Farrand, 1956; Heller et al., 1962; Wertheim, 1964; Kline and Sugihara, 1966; Sugihara et al., 1966). These reports do not show that the mechanical treatments eliminate the unattractive appearance and nonuniform consistency of the thawed product. Keith (1914) and Thomas and Bailey (1933) showed that addition to whole egg of mixtures of sugar and salt ranging in concentration from a few tenths of 1% to less than 2% reduce the thickening effect of frozen storage by as much as 50%. This report extends those viscosity observations and includes observations on the effect of treatments on appearance and liquid separation. It also describes the effects obtainable by the separate and combined use of mechanical treatments and additives at concentrations acceptable in scrambled eggs. The results show how to prepare whole egg products that have varied physical properties after thawing and should help manufacturers meet buyers' specifications at minimum cost.

MATERIALS & METHODS

LIQUID whole eggs were obtained from a local egg processor after they had been machine broken, churned, pasteurized at 60–61°C (141–142°F) for 3.5 min and chilled. Temperatures of the material being treated were between 1 and 3°C (34 and 37°F).

The eggs were homogenized in a Creamery Package Multi-Flo Model 3DD13 homogenizer at 500, 1000 and 2000 psi. The temperatures of the products leaving the homogenizer were 4, 5 and 9°C (40, 41 and 48°F) after homogenization at 500, 1000 and 2000 psi, respectively. The eggs were colloid milled in a Tri-Homo Model 2 mill with serrated stainless steel rotor and stator. Heating was prevented by circulating ice water through the stator jacket. The rotor speed was 4,480 rpm. The mill was fed at a rate equivalent to 1038 g of water per min by a Moyno pump driven by a motor equipped with a Reeves Vari-Speed transmission. The Varikinetic mixer Model VK-1 1/2 (Manton-Gaulin) was operated at 1740 rpm with the vanes opened to approximately 30 degrees. 20 lb of liquid whole egg were stirred in an open container 9.5 in. in diameter. By having the mixer off-center and within 1 in. of the bottom, it was possible to avoid vortexing and air incorporation.

For comparison of effects of homogenization pressure, 6-lb samples of unhomogenized and homogenized eggs were frozen for 24 hr in 10-lb cans in circulating air at −23°C (−10°F) and stored for 1, 2 and 4 months at −29 and −18°C (−20 and 0°F). After storage, the samples were partially thawed overnight at ambient temperature and then placed in a water bath at 32°C (90°F) until they reached 21°C (70°F). They were given a minimum stirring during the latter period.

For comparison of homogenization and

Table 1–Influence of homogenization and freezing on viscosity of whole eggs.

	Viscosity			
		Homogenization treatment		
Storage temperature[1]	Unhomogenized	500 psi	1000 psi	2000 psi
	Brookfield viscometer (cp)[2]			
Unfrozen	9	7	6	6
−18 or −29°C	1600	800	650	500
	Corn Industries viscometer (g cm)			
	Initial viscosity			
−18 or −29°C	350–430	80–100	70–80	60–70
	Final viscosity[3]			
−18 or −29°C	240	30–50	30–40	20–40

[1] Samples were frozen 3 days at −23°C (−10°F) before storage. Values obtained at 1, 2 and 4 months' storage and 2 storage temperatures were averaged, since no further viscosity changes were apparent after 1 month's storage.

[2] Thawed samples were measured on Brookfield viscometer, Model RVT at a shear rate 10 rpm. Values were calculated for a shear rate of 30 rpm (see Methods).

[3] After 15 min stirring with Corn Industries Impellers.

Table 2–Effect of freezing rate, colloid milling and homogenization on viscosity of whole eggs.

	Viscosity (cp)[1]		
Freezing and storage conditions	Untreated	Colloid milled[2]	Homogenized[3]
Unfrozen	10	9	7
−29°C slow airflow, 5 days; −18°C, 1 month	1940	1650	1100
−29°C blast freeze; −18°C, 1 month	1430	1100	650

[1] Thawed samples were measured with Brookfield viscometer Model RVF at a shear rate 2.5 rpm and the results converted to values for a shear rate of 30 rpm (see Methods). Values are averages of duplicate samples.

[2] Tri-Homo colloid mill with clearance set at 0.003 in.

[3] Creamery Package Company, Multi-Flo Homogenizer Model 3DD13 at 1000 psi.

milling treatments, approximately 500-g samples were frozen in 32-oz polyethylene tubs in a −29°C (−20°F) room with circulating air. Under these conditions, samples reached the temperature of the room within 12 hr. Samples were also frozen in a blast freezer at −29°C. Samples were held at this temperature for 5 days before transferring them to storage at other temperatures. After storage, samples were thawed overnight at 2°C and then held at 21–23°C for 6–7 hr.

Samples with and without additives were stirred equally before they were frozen in 32-oz polyethylene tubs, 10-lb cans and 10-lb plastic bags packed 4 per carton. Samples were frozen and stored at −12, −18, −23 and −29°C in still air or frozen in a blast freezer at −29°C and stored at −18°C. They were thawed overnight in air at 2°C or in 21°C water and held at 21–23°C for 6–7 hr before photographing and measurement of viscosity.

Viscosity was measured at product temperature of 21–23°C where values for thawed whole eggs are approximately 2/3 the value at 10°C (50°F). 3 measures of viscosity were used. The Corn Industry viscometer was used to measure initial viscosity and viscosity after 15 min of stirring. Viscosity was also measured with a Zahn No. 2 cup and reported as flow time in seconds. Most of the data reported were obtained using Brookfield viscometers, Models LVF and RVT, at shear rates (measurement speeds), from 2.5–30 rpm. Unfrozen samples were measured with Model LVF and UL adapter. Since liquid whole eggs, as well as other yolk-containing liquid eggs behave as pseudoplastic materials after freezing (Palmer et al., 1969a; 1969b), their viscosity increases with decrease in shear rate. Information obtained from measurements at several shear rates permitted conversion of viscosity data obtained at one shear rate to predicted values at other shear rates, as indicated in Figure 1. The following equations define the lines in Figure 1:

$$\text{Viscosity at 5 rpm} = -46.27 + 0.7095\, y^a$$

$$\text{Viscosity at 10 rpm} = -121.21 + 0.5442\, y^a$$

$$\text{Viscosity at 30 rpm} = -119.66 + 0.3222\, y^a$$

^{a}y = viscosity at 2.5 rpm.

Within the limits of the data, the relationship appears to be linear and is useful for interpolation. However, because of the possibility of a slight upward curve, caution should be used in extrapolation.

Triangle tests were conducted to determine levels of added sucrose or dextrose not readily distinguishable in scrambled eggs. The eggs were prepared with liquid nonfat milk; for a few of the tests a bland oil was added to the mixture. The egg mixture was cooked in a water bath and coded samples were served to a panel of 6–8 judges. Samples with additives were compared with samples containing no additives. Judges were asked to identify the odd sample and comment on the basis for identification. Comparisons were replicated to provide about 15 judgments for each.

RESULTS & DISCUSSION

Freezing and storage

Viscosity of commercially broken, churned, pasteurized whole eggs increased from about 9–13 cp before freezing to a range of 1400–2500 cp after thawing (Tables 1, 2 and 3). The thawed eggs had a curdled appearance and a darker liquid portion separated from the egg mass (Fig. 2). Storage in the −23 to −18°C (−10–0°F) range produced maximum viscosity increases (Fig. 3). Storage for 4 months produced no obvious changes beyond those evident after 1 month. This

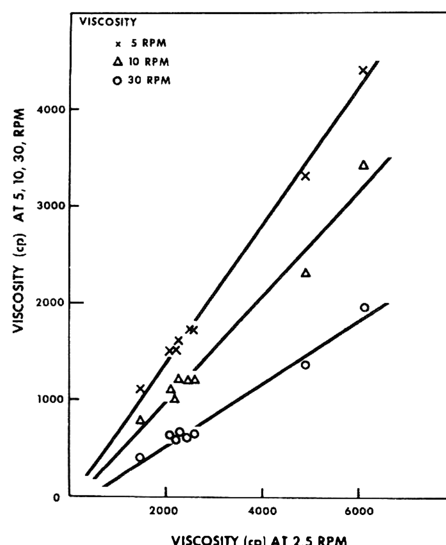

Fig. 1–Comparison of viscosity of thawed egg samples measured at 2.5 rpm and at 5–30 rpm.

Fig. 2–Effect of freezing on appearance of liquid whole eggs thawed after 1 month at −18°C.

result is similar to observations on yolk (Palmer et al., 1969a).

Freezing rate influenced viscosity. Blast freezing at –29°C (–20°F) limited viscosity increase to about 3/4 of the viscosity of whole eggs frozen in a slow airflow at –29°C but was not effective in preventing appearance defects. The effect of freezing rate on whole egg and yolks was reported in previous studies, but was not isolated from effects of freezing temperature (Pearce and Lavers, 1949; Thomas and Bailey, 1933; Lopez et al., 1954).

Mechanical thinning

Homogenization, colloid milling or stirring with a mixer before freezing limited viscosity increase in liquid whole eggs during frozen storage, but none of these mechanical treatments was effective in preventing liquid separation and a curdled appearance in eggs thawed after storage at –18 to –29°C (0 to –20°F).

Of the 3 treatments tested, homogenization was most effective in limiting the viscosity increase caused by frozen storage. Viscosities of the thawed unhomogenized samples ranged from 1400–1900 cp; the viscosities of homogenized samples ranged from 500–1100 cp, 30 to 60% of the viscosity of unhomogenized samples (Tables 1 and 2). Homogenization treatments at 1000 and 2000 psi were of comparable value in limiting viscosity and were more effective than homogenization at 500 psi. Kline and Sugihara (1966) reported a reduction in viscosity from 1342–430 cp due to homogenization of whole eggs at 500 psi, and comparable reductions were reported by Sugihara et al. (1966).

Table 3–Effects of colloid milling and mixing treatments and freezing on viscosity of whole eggs.

Milling treatment	Viscosity test: Time (sec) (Zahn No 2) Unfrozen samples	Viscosity (cp)[2] (Brookfield viscometer) Unfrozen samples	Frozen –29°C, 5 days; –18°C or –7°C, 1 month
Untreated	22	13	2490
Tri-Homo colloid mill 0.003, 0.010 and 0.030 in. clearance	18–19	8–9	1810–1940
Varikinetic Mixer,[1] 2, 6 and 10 min	16–17	8	1620–1750

[1] Varikinetic Mixer Model VK-1 1/2.
[2] Thawed samples were measured with Brookfield Viscometer Model RVT at a shear rate 2.5 rpm; values reported are for 30 rpm (calculated) (see Methods).

The viscosity of colloid-milled samples (clearance, 0.003 in.) averaged about 75% of the viscosity of the unmilled samples (Tables 2 and 3). Milling at a clearance of 0.003 in. lowered the viscosity only slightly more than milling at a clearance of 0.030 or 0.010 in. The viscosity of samples mixed in the Varikinetic mixer for 2–10 min was about 70% of the viscosity of the unstirred samples (Table 3). Stirring 10 min lowered the viscosity only slightly more than stirring 2 min.

Additives

Viscosity increase, a curdled appearance and liquid separation after thawing can all be prevented by addition of low levels of salt, salt and sugar, or salt and liquid nonfat milk to whole eggs before freezing. It is feasible to add concentrations of sugar low enough to avoid organoleptic detection but still effective in preventing undesirable viscosity and appearance changes.

A. Sample size: 2 lb or less.

The effects of added salt and sugars on viscosity of thawed samples after 1–2 months at –18°C (0°F) are shown in Figure 4. A salt concentration of 0.8% limits the viscosity increase to less than 100 cp compared to about 1500 for no salt; 0.6% salt limits viscosity to 200–300 cp. Lower levels of salt are associated with progressively higher viscosities in the thawed products.

Sucrose and dextrose added at levels of 0.8% have practically no effect in limiting viscosity increase in the eggs during frozen storage. Yet lower levels of added sugar are effective in further reducing the viscosity of samples containing added salt. Mixtures of 0.4 or 0.5% salt and 0.1 to 0.4% sucrose or dextrose yield viscosities of thawed samples of 300 cp or less compared to 600–800 cp for salt alone. Increase in the sugar concentration above 0.4% has little additional effect on viscosity. Addition of 25% liquid skim milk to liquid eggs before freezing limits viscosity of the thawed product to less than 200 cp; a combination of 25% skim milk and 0.4% salt limits viscosity to less than 50 cp.

The curdled appearance in thawed whole eggs is effectively prevented by suitable concentrations of additives (Fig. 5). A concentration of 0.6% salt is effective; concentrations of 0.4 or 0.5% are

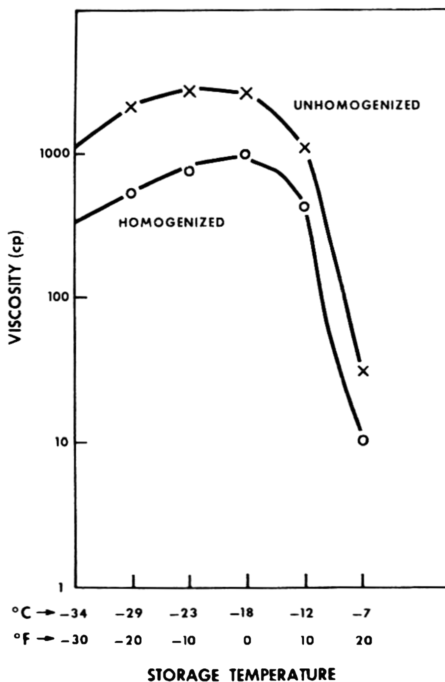

Fig. 3–Effect of storage temperature and homogenization (1000 psi) on viscosity of whole egg thawed after 1 month (Brookfield viscometer, 30 rpm).

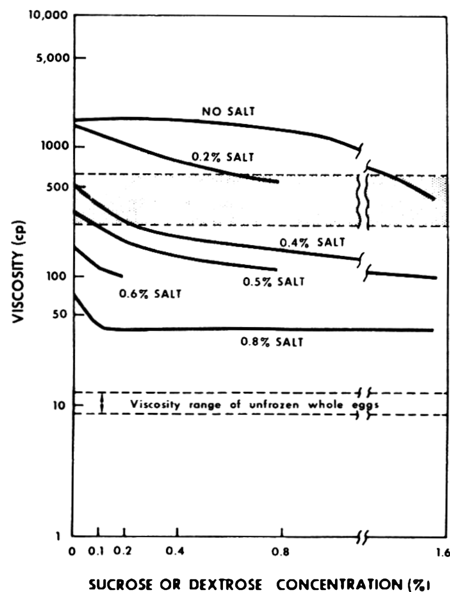

Fig. 4–Effect of salt and sugar on viscosity of whole eggs thawed after storage at –18°C (Brookfield viscometer, 30 rpm; 1 and 2 months of storage. Products in the shaded area have appearance defects; those above the area have major defects; those below the area have a satisfactory appearance).

Fig. 5–Effect of suitable concentrations of additives on appearance of whole eggs thawed after 1 month at –18°C.

not always effective. Dextrose or sucrose alone is ineffective at concentrations ranging up to 1.6%. The combination of 0.4% salt and 0.2% dextrose prevents the curdled appearance after thawing. At this same salt concentration, addition of 1.6% sucrose is required to equal the effect of the 0.2% dextrose on appearance. A salt concentration of 0.2% is not effective even in combination with 0.8% dextrose. With concentrations of salt higher than 0.4%, correspondingly lower amounts of sugar can be used. In general, viscosities below 250 cp after thawing were associated with absence of curdled appearance in samples containing salt and dextrose (represented in Figure 4 by the area below the shaded section). Addition of 25% skim milk to liquid eggs before freezing does not prevent development of a curdled appearance in the thawed product, but addition of 0.4% salt to the milk-containing product eliminates the appearance defects.

Homogenization can limit the viscosity increase and eliminate liquid separation and curdling of samples that exhibit borderline defects due to use of low concentrations of additives. The necessary improvement can be made for samples containing 0.4% salt, 0.4% salt + 0.2% sucrose or 0.4% salt + 0.1% dextrose. Samples in the viscosity range of 200–600 cp without homogenization are limited to viscosities of 100 cp or less by homogenization before freezing (samples within the shaded area of Fig. 4).

B. Sample size: 10 lb.

Treatments that limit viscosity and prevent a curdled appearance in small amounts of eggs (less than 2 lb) are inadequate for larger quantities if such quantities require longer freezing or thawing times. Amounts of 2 lb or less freeze in 8–12 hr at –29°C in still air; this freezing rate can be achieved in 10-lb cans or in 4, 10-lb packages in a carton by use of a blast freezer at –29°C. Amounts of 2 lb or less thaw within 30 hr in still air at 2°C; 50–70 hr are required for 10-lb quantities, and the slower thaw increases the viscosity and curdled appearance. Desired viscosity and appearance can be achieved under these conditions by increases in additive concentration. Addition of a mixture of 0.8% salt, 0.4% dextrose and 25% liquid skim milk, for example, is adequate without homogenization. The following are adequate combinations with homogenization: 1. 0.6% salt, 0.4% dextrose, 25% milk. 2. 0.8% salt, 0.4% dextrose. Alternate approaches involve a decrease in the thawing time by thawing in running water or by use of a riving machine and heat exchanger. Eggs in 10-lb cans thaw in 21°C water in about 8 hr; 10-lb packages removed from the container thaw in about 6 hr. The viscosity is less than 100 cp and the thawed eggs have a smooth appearance.

Flavor

Since sweetness is not associated with the flavor of scrambled eggs, additives used in frozen whole eggs intended for scrambling should not contribute an obvious sweet flavor. Triangle test comparisons were made to determine levels of added sugar that could reasonably be expected to escape detection in scrambled eggs.

Samples containing 0.4% salt were compared with samples containing 0.4% salt plus 0.4% dextrose; no flavor difference was detected. In similar tests substituting sucrose for dextrose, a difference between samples was detected, but it was not recognized as a difference in sweetness. The sweetness was recognized, however, in comparison of samples containing 0.4% salt and samples containing 0.4% salt plus 0.8% sucrose. Sweetness recognized in a direct comparison might not be detected in a single sample test or if samples were served with additional seasonings. However, use of dextrose rather than sucrose is favored by evidence that the intensity of sweetness of dextrose is about 2/3 that of sucrose (Schultz and Pilgrim, 1957).

CONCLUSIONS

VARIOUS combinations of low levels of salt, sucrose, dextrose or liquid skim milk and homogenization before freezing can be used to produce satisfactory frozen whole eggs for scrambling. These products have a low enough level of sweetness to avoid detection, a low level of viscosity and the absence of appearance defects. The information obtained in these studies makes it possible to select the most economical method of achieving an acceptable product.

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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.

KAREN J. MORGAN, KAYE FUNK and MARY E. ZABIK
Department of Food Science & Human Nutrition, Michigan State University, East Lansing 48823

COMPARISON OF FROZEN, FOAM-SPRAY-DRIED, FREEZE-DRIED, AND SPRAY-DRIED EGGS
7. Soft Meringues Prepared With a Carrageenan Stabilizer

SUMMARY—This study compared the quality characteristics of soft meringues prepared from frozen, foam-spray-dried, freeze-dried and spray-dried albumen each from a common lot. All meringues contained the same amount of carrageenan stabilizer. Ranked in order of increased average whipping times needed to obtain specific gravities of 0.24 to 0.22 were meringues prepared from frozen, freeze-, foam-spray- and spray-dried albumen. Percentages of drainage and evaporation did not differ significantly among types of albumen; however, the data suggest drainage decreased and evaporation increased during refrigerated holding. No significant differences were found in objective measurements of tenderness, color and height. Sensory evaluations showed no significant differences for color and appearance, slippage and drainage, texture, tenderness, flavor and general acceptability. However, evaluations of shrinkage varied significantly. All meringues prepared under the conditions of this study from the four types of albumen and with added stabilizer were judged acceptable. This study also compared soft meringues prepared from spray-dried albumen with and without the added carrageenan stabilizer. Meringues without added stabilizer had a significantly higher ($P < 0.01$) percentage of drainage and were significantly tougher ($P < 0.05$), as indicated by Allo-Kramer shear press measurements expressed as area-under-the-curve, than meringues with added stabilizer. The results of this investigation suggest the quality characteristics of soft meringues prepared with spray-dried albumen may be improved with the addition of a carrageenan stabilizer.

INTRODUCTION

USE OF ALBUMEN processed by foam-spray-, freeze- and spray-drying offers convenience such as improved keeping quality, time economy and reduced storage space. However, concomittant to their use is the quality of a prepared product such as soft meringue.

The effect of filling temperature as well as baking times and temperatures on the quality of soft meringues has been investigated (Felt et al., 1956; Hester and Personius, 1949; Gillis and Fitch, 1956). Also, amounts of sugar as well as the best time for adding sugar have been studied (Gillis and Fitch, 1956). Investigating whipping times, Briant et al. (1954) recommended use of high speeds throughout whipping for optimum quality meringues. In contradiction, Godston (1950) reported medium speed yielded better meringues characterized by fine cell structure.

Franks et al. (1969) studied the performance and palatability of frozen, foam-spray-, freeze- and spray-dried albumen in angel cakes and reported satisfactory cakes could be made with the four types of albumen only when low whipping speeds and hence, increased whipping times, were used to produce foams of equal specific gravity. Zabik and Brown (1969) had previously reported decreased foam stability for spray-dried albumen when constant high-speed whipping times were used for the same four types of albumen.

According to Felt et al. (1956), Godston (1950) and Glabau (1948), the use of a vegetable gum stabilizer in a meringue decreased the amount of liquid collected between the filling and the meringue. Glabau (1948) concluded the rigidity of meringues as shown by the resistance to shearing with a modified MacMichael apparatus was directly related to the amount of added stabilizer.

The purpose of this study was to compare quality characteristics of soft meringues prepared with frozen, foam-spray, freeze- and spray-dried albumen. For this purpose, three lemon meringue pies were prepared for each of the six replications of each albumen process. A carrageenan stabilizer was added to all meringues. Because foods may be held in many food service systems for varying periods of time between preparation and service, the quality characteristics of one pie from each replication were objectively measured after 20–22 hr of refrigerated holding. Meringues evaluated 2–3 hr after preparation were designated as fresh and those evaluated after refrigeration as held. A second purpose of this study was to determine the effect of stabilizer on quality characteristics as determined by objective measurements and for this purpose meringues were prepared from spray-dried albumen with and without added stabilizer.

EXPERIMENTAL METHODS

PROCUREMENT processing, packaging and storage of all types of albumen were as outlined by Franks et al. (1969). All ingredients were purchased in common lots. Unbaked pie crusts in 9-inch aluminum foil pans and lemon-flavored filling mix were purchased commercially. Sugar and albumen were preweighed into appropriate amounts before storage while other ingredients were weighed or measured just prior to preparation. Pie crusts and albumen were stored at −23°C while sugar was stored at room temperature.

Preparation and baking

Thawed, pricked crusts were baked in a 219°C Hotpoint oven, model HJ225, for approximately 10 min. Six 4-oz packages of filling mix, 12 egg yolks, 400g sugar and 3548 ml tap water were placed in the top part of a 12-qt double boiler, blended and cooked to a temperature of 82–84°C. After cooling to a temperature of 42–45°C, the filling was poured into the crusts to a depth of 2.0 cm from the bottom of the pan as measured with a vernier caliper. Filled crusts were covered with inverted aluminum foil pans and allowed to cool to room temperature or 24–32°C.

To provide sufficient meringue for covering the pies and objective measurements, the formula contained 244g sugar, 1.87g salt and 0.2930g stabilizer (Viscarin brand, Marine Colloids, Inc., Springfield, N.J.) along with 293g frozen albumen or 43.6, 42.6 and 43.7g of foam-spray-, freeze- and spray-dried albumen, respectively, reconstituted with appropriate quantities of distilled water to equal 293g. Amounts of dried albumen were based on the solids content of frozen albumen corrected for variance in powder moisture among processes (A.O.A.C., 1955).

Defrosting and reconstitution procedures were as outlined by Franks et al. (1969). After the albumen was weighed into a 5-qt bowl of a KitchenAid mixer, model K5-A, the temperature was adjusted to 25°C ± 1°. Using the whip attachment, the albumen was whipped for 30 sec at speed 2 (67 rpm) before the salt was added and whipping was continued for 45 sec. The sugar and stabilizer, previously blended with a KitchenAid mixer, model K4-B, for 3 min at speed 1 (34 rpm), were added over a 1-min interval during which the mixer was operating at speed 2. Finally, the mixture was whipped at speed 4 (98 rpm) for the additional time needed to obtain a specific gravity between 0.24 and 0.22. Thus, whipping speeds and times, determined in preliminary investigations, were based on the recommendations of Franks et al. (1969).

Meringuing was as described by Mallman et al. (1963). The meringue was leveled to a depth of 1.91 cm using a 12-in. spatula. Each lot of three pies was placed in a 163°C ± 2° oven regulated with a Versatronik controller. The lightly browned pies were removed from the oven following 34–36 min of baking, set on racks and allowed to cool for approximately 2 hr. Baking time and oven temperature were selected on the basis of the study of Mallman et al. (1963). One pie from each lot designated for objective measurements after holding was then refrigerated at 5°C.

Objective measurements

Specific gravity was determined in duplicate

Table 1–Averages and standard deviations for objective measurements of soft meringues prepared from albumen processed by four methods.

Objective measurement	Albumen process: Frozen	Spray-dried	Foam-spray-dried	Freeze-dried
Whipping time (min)	13.5 ± 0.4	16.1 ± 1.5	16.0 ± 2.5	13.9 ± 0.7
Specific gravity	0.23 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.23 ± 0.01
pH	6.3	6.7–6.8	6.2–6.3	6.2–6.3
Evaporation, fresh (%)	20.9 ± 2.1	20.6 ± 1.9	20.5 ± 1.3	21.2 ± 1.5
Evaporation, held (%)	21.8 ± 2.4	21.2 ± 2.6	21.3 ± 1.9	22.3 ± 1.4
Drainage, fresh (%)	12.9 ± 5.0	11.8 ± 5.4	10.4 ± 4.0	9.9 ± 2.6
Drainage, held (%)	10.3 ± 6.5	10.1 ± 4.0	9.0 ± 3.6	6.1 ± 2.9
Height, fresh (cm)	1.85 ± 0.03	1.87 ± 0.08	1.87 ± 0.05	1.87 ± 0.08
Height, held (cm)	1.78 ± 0.13	1.74 ± 0.09	1.78 ± 0.07	1.76 ± 0.05
Maximum force, fresh (lb force/g)	0.23 ± 0.04	0.23 ± 0.03	0.23 ± 0.06	0.23 ± 0.02
Maximum force, held (lb force/g)	0.21 ± 0.05	0.21 ± 0.03	0.18 ± 0.04	0.19 ± 0.02
Area-under-the-curve Fresh (cm^2)	2.991 ± 0.612	3.183 ± 0.453	3.279 ± 0.626	3.409 ± 0.588
Area-under-the-curve Held (cm^2)	2.726 ± 0.324	2.754 ± 0.447	2.730 ± 0.374	2.794 ± 0.289
Hunter L, fresh	82.09 ± 2.33	81.54 ± 3.85	81.51 ± 2.45	80.44 ± 2.46
Hunter a_L, fresh	3.37 ± 1.04	3.48 ± 1.79	3.47 ± 1.56	4.61 ± 1.15
Hunter b_L, fresh	19.92 ± 1.78	19.53 ± 2.44	19.50 ± 1.53	20.31 ± 1.73

as outlined by Platt and Kratz (1933). Losses due to evaporation and drainage were determined using techniques similar to Funk et al. (1966). Percentages of evaporation were calculated from the weight differences of unbaked and baked meringued pies divided by the weight of unbaked meringue. The amount of drainage was determined by weighing the pan, crust and filling before the pie was meringued and after the meringue had been carefully removed from the pie. The difference between these two weights was divided by that of the unbaked meringue and the answer converted to percentage.

Height measurements, as an indication of shrinkage, were made after one-half of the meringue had been carefully removed from the pie. Using a vernier caliper, the height of the meringue remaining on the pie was measured at the center.

Tenderness was measured with an Allo-Kramer shear press, model SP12. Using a 5.39 cm square cutter, two samples were cut from each meringue previously removed from a pie. Each sample was weighed to the nearest 0.01g before it was placed in the center of the standard shear compression cell. Using a 30 sec downstroke, a 10-lb range, 25-lb pressure with a 100-lb proving ring, the sample was sheared. The pounds of force required for shearing were recorded on a time-force-curve by a Varian electronic recorder, model E2EZ. Calculations of tenderness values, expressed as maximum force and area-under-the-curve, were as outlined by Franks et al. (1969); however, a conversion factor of 187.2 was used.

Color was measured by a Hunter color-difference meter, model D-25, standardized with an optical lens-covered white tile, (L 93.0; a_L, -0.6; b_L, -0.1) in preparation for determining L (lightness), a_L (redness) and b_L (yellowness) values of meringues. A cut piece of meringue was placed on a flat piece of glass and covered with an optical lens. Two sets of values were derived from each sample by moving the glass supporting the meringue under the viewing area one quarter of a turn after the first reading. The two sets of values were averaged before the mean was determined from duplicate measurements of each replication.

Sensory evaluation

Seven judges subjectively scored meringue samples for color and appearance, shrinkage, slippage and drainage, texture, tenderness, flavor and general acceptability using a seven-point scale with a score of seven showing excellent quality and a score of one indicating unacceptable quality. Descriptive terms aided the judges in their evaluations. A glass of lemon water (2 tsp/qt of water) at room temperature was provided for judges to use between evaluations of three samples served at each session. Pieces of pie were served according to a predetermined rotation plan; hence, each judge evaluated pieces which were baked in six different positions. To minimize variance due to judges, all scores for each palatability characteristic were averaged for each replication.

Statistical analyses

Data were analyzed for variance due to albumen process and replication. Duncan's (1957) multiple range test was used to pinpoint further the sources of significant differences.

RESULTS & DISCUSSION

Unbaked meringues

Ranked in order of increasing average whipping times of 13.5, 13.9, 16.0 and 16.1 min were meringues prepared from frozen, freeze-, foam-spray- and spray-dried albumen. Analysis showed freeze-dried albumen required less whipping time than spray-dried albumen ($P < 0.01$) and foam-spray-dried albumen ($P < 0.05$). In a study of angel cakes, Franks et al. (1969) ranked processed albumens from the same common lot in the same order for whipping times. Investigating the whipping properties of pasteurized albumen, Garibaldi et al. (1968) suggested heat denaturation of the ovomucin-lysozyme complex of the albumen was responsible for increased whipping times.

Meringues prepared with spray-dried albumen had a significantly higher ($P < 0.01$) pH of 6.7 6.8 than the pH of 6.2–6.3 for meringues prepared with the other three types of processed albumen. Zabik and Brown (1969) reported similar results for pH values of processed albumen.

Baked meringues

Meringues prepared from frozen, foam-spray-, freeze- and spray-dried albumen lost an average of 20.9, 20.5, 21.2 and 20.6% moisture, respectively, due to evaporation during baking and subsequent cooling. These differences were not significant. Evaporation increased during holding for meringues prepared from frozen, foam-spray-, freeze- and spray-dried

Table 2–Average scores and standard deviations for quality characteristics of soft meringues prepared from albumen processed by four methods.

Quality characteristics	Albumen process: Frozen	Spray-dried	Foam-spray-dried	Freeze-dried
Color and appearance	4.9 ± 0.6	4.5 ± 0.9	4.9 ± 0.6	5.3 ± 0.4
Shrinkage	5.1 ± 0.7	5.7 ± 0.5	5.9 ± 0.5	6.1 ± 0.3
Slippage and drainage	5.4 ± 0.5	5.3 ± 1.0	5.6 ± 0.5	5.9 ± 0.3
Texture	5.4 ± 0.4	5.1 ± 0.5	5.0 ± 0.5	5.5 ± 0.5
Tenderness	4.8 ± 0.6	5.1 ± 0.4	4.9 ± 0.7	5.1 ± 0.4
Flavor	5.2 ± 0.6	5.0 ± 0.5	5.0 ± 0.5	5.1 ± 0.3
General acceptability	5.0 ± 0.5	4.8 ± 0.5	4.9 ± 0.4	5.2 ± 0.2

albumen showing averages of 21.8, 21.3, 22.3 and 21.2%, respectively. Again, no significant differences attributable to processing were found. However, significant differences ($P < 0.05$) existed among replications. Standard deviations indicate most variance among replications of meringues prepared with spray-dried and frozen albumens (Table 1).

No significant differences attributable to process or replication existed for percentages of drainage from fresh or held meringues. As indicated by the standard deviations, considerable day-to-day variance was noted (Table 1).

No significant differences were found in height measurements of either fresh or held meringues (Table 1). However, a comparison of averages revealed meringues prepared with the three types of dried albumen decreased significantly ($P < 0.05$) in height during holding. Such a decrease was not found in meringues prepared from frozen albumen.

Allo-Kramer shear press measurements of tenderness, expressed as maximum force and area-under-the-curve, showed no significant differences in either fresh or held meringues due to egg processing; however, maximum force values of held meringues differed significantly ($P < 0.01$) among replications. Data suggest tenderness increased during holding as indicated by lower maximum force and area-under-the-curve values (Table 1).

Hunter color-difference meter values showed meringues prepared from frozen albumen to be lightest in color followed by meringues prepared from spray-, foam-spray- and freeze-dried albumen although differences were slight. For Hunter a_L+ values, meringues prepared from freeze-dried albumen had highest mean redness values followed by spray-, foam-spray-dried and frozen albumen. Meringues prepared from freeze-dried albumen were most yellow as indicated by Hunter b_L+ values (Table 1).

Sensory evaluations showed no significant differences among processes or replications for color and appearance, slippage and drainage, texture, tenderness, flavor and general acceptability (Table 2). However, the standard deviations suggest day-to-day variance among the meringues and/or judges. Shrinkage scores differed significantly ($P < 0.05$) with most shrinkage indicated for meringues prepared from frozen albumen followed by spray-, foam-spray- and freeze-dried albumen.

Meringues prepared from freeze-dried albumen were scored as being most acceptable followed by those prepared from frozen, foam-spray- and spray-dried albumen. However, all were judged acceptable. These data agree with those of Franks et al. (1969) who found angel cakes prepared from the same types of albumen to be acceptable.

Meringues without stabilizers

To demonstrate the effect of stabilizer on quality characteristics of soft meringues, six replications of meringues prepared from spray-dried albumen with no added carrageenan were subjected to objective measurements as outlined. Percentages of drainage decreased significantly ($P < 0.01$) in fresh meringues when stabilizer was used although the standard deviations indicate considerable variance from day-to-day. Meringues prepared with and without stabilizer had percentage drainage losses of 11.8 ± 5.4 and 20.1 ± 2.5, respectively. This finding is in general agreement with those of Glicksman (1962), Felt et al. (1956), Godston (1950) and Glabau (1948). When the meringues were held for 20–22 hr, percentage drainage losses of 10.4 ± 4.0 and 14.6 ± 5.5 were found for meringues with and without stabilizer, respectively. The percentages for held meringues did not differ significantly. Perhaps the lower drainage values shown for held meringues can be attributed to evaporation during holding. In a discussion of foams, Glabau (1948) indicated sugar may be present in a meringue as a syrup or as dry sugar within the cellular walls. Perhaps during baking, the sugar in solution separated from the meringue and then during the holding period, dry sugar absorbed liquid which had previously drained from the meringue.

Fresh and held meringues prepared with and without stabilizer had similar average shear press values when expressed as maximum force. Comparison of means for tenderness measurements, expressed as area-under-the-curve, showed fresh meringues prepared with and without stabilizer were not significantly different; however, held meringues prepared with stabilizer received significantly ($P < 0.05$) lower area-under-the-curve values than held meringues prepared without stabilizer thus indicating more tender meringues. These findings suggest disagreement with Glabau (1948) who concluded added stabilizer caused more rigid meringues as shown by resistance to shearing; however, Glabau (1948) measured the shear resistance of unbaked meringues with varying quantities of added stabilizer. Also, the meringues in his study were whipped for a constant time rather than to a constant specific gravity as was done in the present experiment.

No significant differences were found for any of the three color measurements as determined with a Hunter color difference meter for fresh meringues prepared with and without added stabilizer. Color of meringues which were held was not determined.

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R. O. B. WIJESEKERA[1] and C O. CHICHESTER
Department of Food Science and Technology, University of California, Davis, California 95616

A Research Note

A METHOD FOR THE DETERMINATION OF 5-HYDROXYMETHYL-2-FURALDEHYDE IN THE PRESENCE OF 2-FURALDEHYDE (FURFURAL)

INTRODUCTION

MANY METHODS are available (Linko, 1961; Moye, 1964) for the determination of 5-hydroxymethyl-2-furaldehyde (5-HMF); however, in many foods the occurrence of 2-furaldehyde interferes with the spectrophotometric measurements. Linko (1961) overcame this interference by employing two different color reactions: one with a reagent to which both compounds responded and another which was specific for 2-furaldehyde. Recently Jacin et al. (1968) determined 5-HMF by gas chromatographic analysis of its trimethylsilyl derivative.

During the course of our studies on the carbonyl intermediates of the Maillard reaction we developed a simple technique for the simultaneous assay of both 2-furaldehyde and 5-HMF. The two aldehydes are first converted to their 2,4-dinitrophenylhydrazones, which are then extracted and chromatographed on silica gel plates. The respective spot areas are measured and the concentrations determined by the method of Purdy and Truter (1962).

METHODS

PRODUCTS of the Maillard reaction include both 2-furaldehyde and 5-HMF. The reaction mixture was extracted with cyclohexanone which was then removed under reduced pressure at 40°C. The extract was treated with an excess of 2,4-dinitrophenylhydrazine reagent in aqueous hydrochloric acid (400 mg in 500 ml distilled water and 40 ml concentrated HCl). After standing for 2 hr the hydrazones were extracted into ethyl acetate and the solvent removed under reduced pressure. The extract was dissolved in a measured volume of a mixture of tetrahydrofuran/ethyl acetate (1:2, v/v) and spotted onto a precoated silica gel plate (E. Merck A. G.) by means of a micro-pipette (Camag Inc.). Standard solutions of the 2,4-DNP's of the two aldehydes are chromatographed on the same plate. The developing solvent was toluene/ethyl acetate/butyl acetate (5:4:2 v/v). The mean R_f values of 2-furaldehyde and 5-HMF were 0.67 and 0.24 respectively when the distance traveled by the solvent front was 15 cm. Precautions outlined previously for quantitative TLC based on spot area measurements (Purdy and Truter, 1962; Senanayake and Wijesekera, 1968) were observed. The developed plate was placed on a light-box and the illuminated outline of each spot was traced onto millimeter graph paper. The squares within each outline were counted with the aid of a flash-magnifier.

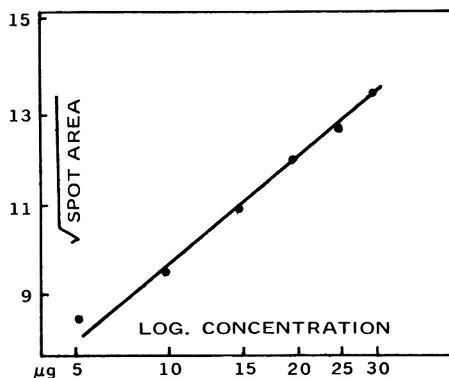

Fig. 1—Variation of concentration of 5-HMF with the square root of the spot area for the range 5–30 µg, using precoated analytical (250 µ) plates.

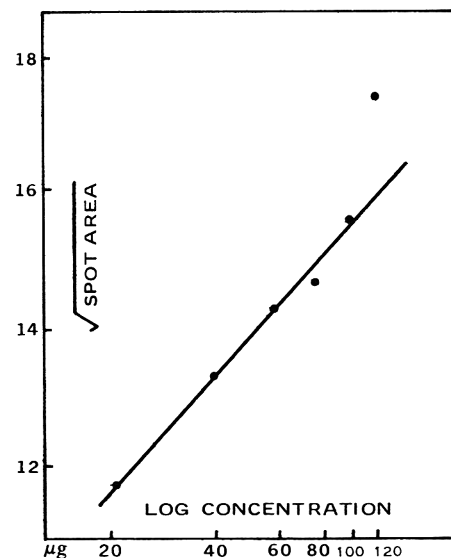

Fig. 2—Variation of concentration of 5-HMF with the square root of the spot area for the range 20–120 µg, using precoated preparative (500 µ) plates.

RESULTS & DISCUSSION

QUANTITIES WITHIN the range 5–30 µg of both 2-furaldehyde and 5-HMF could be assayed on precoated analytical plates of 250µ thickness (Fig. 1) while larger amounts (30–80 µg) were best assayed on the thicker (500µ) preparative plates (Fig. 2). Plates prepared in the laboratory could also be used but the results were not as consistent. Both aldehydes could be assayed on a single plate. However, it is preferable that standards containing equal quantities of each aldehyde be chromatographed together. A minimum of 3 or 4 standards within the appropriate range were chromatographed together with duplicates of the unknown solutions. Good recoveries (83–94%) were obtained when known amounts of 5-HMF were added to the products of a Maillard reaction prior to extraction with cyclohexanone. The method assumes that the conversion of the aldehydes to their 2,4-DNP's occurs stoichiometrically. The method is simple, requires no elaborate equipment and is suitable for multiple assays.

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[1] Permanent address: Ceylon Institute of Scientific and Industrial Research, (CISIR) Colombo 7, Ceylon.

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