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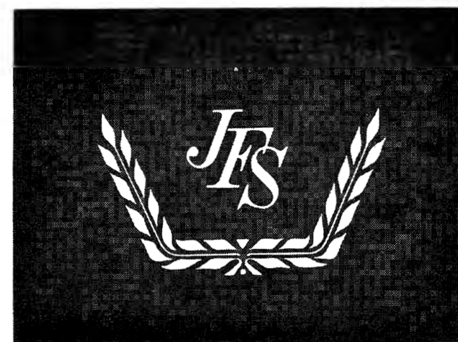

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(formerly Food Research)

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Institute of Food Technologists

The Institute of Food Technologists is a professional society of scientists, engineers, educators and executives in the field of food technology. Food technologists are professionals who apply science and engineering to the research, production, processing, packaging, distribution, preparation and utilization of foods. Individuals who are qualified by education, special training, or experience, are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are Institute members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation and opportunities for the individual and his business or profession.

OBJECTIVES

The Institute, as a non-profit, professional, educational society, has several major aims: to stimulate investigations into technological food problems; to present, discuss and publish the results of such investigations; to raise the educational standards of Food Technologists; and to promote recognition of the scientific approach to food and the basic role of the Food Technologist in industry. All of these activities have the ultimate objective of providing better and adequate foods for mankind.

ORGANIZATION AND PROGRESS

Organized July 1, 1939, at Cambridge, Mass., with a membership of less than 100, the Institute has grown to more than 9,500 (1968). It is world-wide in scope, with members in the Americas, Scandinavia, England, Holland, Germany, France, India, Australia, New Zealand, and Japan, among others.

QUALIFICATIONS FOR MEMBERSHIP

Professional Members. Any ethically qualified person who has had training and experience in food technology, or who in the opinion of the Council is recognized as distinguished in the contributing sciences as they apply to foods, shall be eligible to be a Professional Member of the Institute. The minimum training which shall qualify a candidate for such membership is, in general, graduation from a college, university or similar institution in which he has majored in one or more of the sciences or branches associated with food technology. The minimum experience shall be three years experience in food technology.

Members. Any ethically qualified person active in special or limited aspects of food technology, who is an Administrator, Director or Executive under whose jurisdiction operations in food technology are conducted; or those engaged in the dissemination of knowledge of food technology; or one whose work requires a general knowledge of the broad principles of food technology as it applies to the products, processes or equipment with which he or she is concerned; or a recent graduate in food technology, or applicable branches of science and engineering who has entered a career in food technology; shall be eligible to become a Member.

Student Members. Any ethically qualified person who is registered as a full-time student in an educational institution with at least Junior (third year) standing, who is a candidate for a Bachelor's or higher degree in one or more of the sciences or branches of engineering associated with food technology, shall be eligible for membership as a Student Member. That classification shall be his status only until the end of the calendar year in which he completes his schooling.

DUES

Professional Members and Members—$20.00 a year; Students—$2.50. Includes subscription to FOOD TECHNOLOGY, Annual Directory (and JOURNAL OF FOOD SCIENCE if requested).

PUBLICATIONS

The Institute publishes two journals. FOOD TECHNOLOGY, issued monthly, is the official journal of the Institute. Besides covering many fields of interest to food technologists throughout the world, it publishes the results of research in food technology and their practical application to industry. The JOURNAL OF FOOD SCIENCE, issued bimonthly, is devoted exclusively to papers presenting original investigations and basic research in fundamental food components and processes. In addition, an IFT WORLD DIRECTORY & GUIDE is published annually.

REGIONAL SECTIONS

Where 25 or more members live within commuting distance of a given point, a regional section may be established. Meetings can be held at more frequent intervals by such groups. Presently, there are 40 regional sections.

AFFILIATE ORGANIZATIONS

Affiliate certificates may be granted to food technology organizations outside the U.S.A. There are currently five chartered affiliate organizations.

ANNUAL MEETINGS

An Annual Meeting of the Institute provides a specially-organized technical program, awards banquet, and industrial exhibit of equipment, services, processes and ingredients. The program is designed to emphasize current trends and technological developments. Special guest speakers are invited.

AWARDS

The Institute administers the following awards:

NICHOLAS APPERT AWARD. Purpose of this award (Medal furnished by the Chicago Section, and $1,000) is to honor a person for pre-eminence in and contributions to Food Technology.

BABCOCK-HART AWARD. Purpose of this award ($1,000 and Plaque sponsored by The Nutrition Foundation) is to honor a person for contributions to Food Technology that have improved public health through some aspects of nutrition or more nutritious food.

IFT INTERNATIONAL AWARD. Purpose of this award (Silver Salver sponsored by Australian Institute of Food Science and Technology, and $1,000) is to recognize an IFT Member for promoting international exchange of ideas in Food Technology.

FOOD TECHNOLOGY INDUSTRIAL ACHIEVEMENT AWARD. Purpose of this award (Plaques to company and individuals) is to recognize and honor the developers of an outstanding new food process and/or product representing a significant advance in the application of Food Technology to food production, successfully applied in actual commercial operation.

IFT AWARD FOR RESEARCH. Purpose of this award ($1,000 and Plaque) is to recognize a research scientist 35 years of age or younger, who has demonstrated outstanding ability in Food Science or Technology research.

FELLOWSHIPS

- Florasynth—$1,000 and plaque
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- Nestlé—Two, each $1,000 and plaque
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- Samuel Cate Prescott—(donors: various industrial firms)—$1,000 and plaque

Purpose of IFT-administered Fellowships is to encourage graduate work in the field of Food Science and Technology directed to extending or improving knowledge in some phase of food conservation, food production, or food processing. Available to graduate students.

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- R. T. French (donor: R. T. French Co.)—Two, each $1,000; accompanied by plaque
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Purpose of IFT-administered Scholarships is to focus attention on the need for more young people in Food Science and Technology, and to encourage deserving and outstanding students to take undergraduate work leading to a Bachelor's Degree in Food Science, Food Engineering, or Food Technology. Available to incoming Juniors and Seniors who have completed at least one term of study at the institution from which they expect to earn a baccalaureate degree.

IFT SCHOLARSHIPS

- IFT Freshman/Sophomore Scholarships—Thirty, each valued at $500 and including a complimentary subscription to FOOD TECHNOLOGY during the tenure of the scholarship.

Purpose of the IFT Scholarships is to attract and encourage worthy students to enter the fields of Food Technology, Food Engineering or Food Science. Available to incoming college freshmen, and sophomores.

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AN OFFICIAL PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS

THEME:

DISCOVERY & DEVELOPMENT

ADD THE THIRD DIMENSION . . . .

. . . Discipline. It's at the root of growth in knowledge, in application, in personal stature. Food professionals continually ask themselves: how much do we really know about the substance and tools of our profession? We need to know more about the disciplines at the base of the processes we use. We need to rub gray matter with the high-caliber minds that roost in the speakers, the listeners at IFT's upcoming Annual Meeting. There the word will be BASIC, but for disciplined *use*—by you in reaching and keeping professional maturity and know-how; by your company or organization in gathering helpful information on accomplishments and, more important, trends in the food world. Add the third dimension of Discipline to your discipline—go, gather ye wisdom while ye may. It's disciplined sense to be present at the

IFT 28th ANNUAL MEETING

PHILADELPHIA, PA.

MAY 19–24, 1968

Philadelphia Civic Center

Memo from the Scientific Editor

☐ A YEAR AGO was published in this Journal my only previous Memo. Today, one year later, I feel even more strongly the sentiments expressed in that first Memo, namely, that a publication such as this comes into being only because of concerted efforts of a dedicated team of individuals. The experiences of 1967 amply demonstrated this.

The papers are the work of the authors and to them must go the primary recognition for what is published. But beyond the authors there are many who contribute. Without slighting in any way the value of the IFT Staff in the total team effort, I wish to acknowledge the enormous work of that large group of volunteers who assist the Scientific Editor in determining the suitability of a paper for publicaton . . . the reviewers. They and the Board of Editors are the ones who examine manuscripts from the many points of view that need to be considered. Generally each reviewer is selected because he is active in the field of the paper in question. His review, therefore, is that of one who can speak from personal knowledge on the significance of the work reported.

Several aspects of this volunteer effort are especially noteworthy: (1) Virtually all who are asked to review, do so; the few who refuse usually have other commitments rather than a lack of interest. (2) The reviews are carefully done, without bias, and often in great detail. (3) They are constructive and effectively provide the Scientific Editor the needed guidance for making a decision on a given paper.

Most authors, I find, are appreciative of the reviewer's efforts and frequently tell me this. They regard the review procedure as truly *judgment by one's peers* and not by a narrowly constituted group. This Memo allows me to join the authors in thanking the reviewers for their help and also to express the belief that our use of a large group of volunteer reviewers rather than a select small panel is not only a highly effective procedure but also is a good example of democracy in action.

We shall count on the large body of reviewers for continued help. We salute them for their willingness to serve.

WALTER M. URBAIN
Scientific Editor

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ABSTRACTS:

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THE INFLUENCE OF SUGAR CONCENTRATIONS ON THE VAPOR PRESSURE OF FOOD ODOR VOLATILES IN AQUEOUS SOLUTIONS. A. G. WIENTJES. *J. Food Sci.* **33**, 1–2 (1968)—The addition of fructose or invert sugar in very high concentrations to aqueous solutions containing strawberry volatiles or synthetic compounds respectively, results for some components in a decrease of peak heights in gas chromatograms of the vapors over the solutions; the phenomenon is thought to be useful for studying flavor retention in juice concentrates.

STUDIES IN MEAT TENDERNESS. 4. Changes in the Extractability of Myofibrillar Proteins during Meat Aging. C. L. DAVEY & K. V. GILBERT. *J. Food Sci.* **33**, 2–6 (1968)—Approximately 52% of the myofibrillar proteins of unaged meat is extracted in 40 min at 2°C whereas from aged meat as much as 78% is extracted. The rate and extent of these changes are determined largely by the ultimate pH value of the meat. Similar increases in protein extraction, displaying the same pH dependence, occur during the aging of well-washed myofibrillar preparations.

MOVING BOUNDARY ELECTROPHORESIS OF FOOD STABILIZERS. J. HIDALGO & P. M. T. HANSEN. *J. Food. Sci.* **33**, 7–11 (1968)—A high degree of homogeneity was observed for carboxymethylcellulose, arabic gum, alginate and λ-carrageenan. In contrast, κ-carrageenan was composed of a mixture of migrating components. The colloidal fractions of guar gum, locust bean gum, and corn syrup solids did not migrate in the electrical field. Mixtures of carboxymethylcellulose with guar gum or gum arabic could be separated at pH 7. Mixtures of carboxymethylcellulose with carrageenan could be effectively separated at pH 2. Electrophoretic separation at 23°C of different stabilizer mixtures was unsatisfactory and the patterns showed evidence of interactions.

STUDIES IN MEAT TENDERNESS. 5. The Effects on Tenderness of Carcass Cooling and Freezing Before the Completion of Rigor Mortis. B. B. MARSH, P. R. WOODHAMS & N. G. LEET. *J. Food Sci.* **33**, 12–18 (1968)—The tenderness of lamb loin is affected greatly by the time-temperature pattern imposed on the dressed carcass during the onset of rigor mortis. Very significant toughness develops in the longissimus dorsi muscle of carcasses exposed to low temperatures within about 16 hr of slaughter. This "processing toughness" is unrelated to the lack of aging. It appears to be due to muscle fiber shortening, earlier demonstrated to be responsible for massive toughening in excised muscles.

THE EFFECTS OF FREEZING, FROZEN STORAGE CONDITIONS AND DEGREE OF DONENESS ON LAMB PALATABILITY CHARACTERISTICS. G. C. SMITH, C. W. SPAETH, Z. L. CARPENTER, G. T. KING & K. E. HOKE. *J. Food Sci.* **33**, 19–24 (1968)—Freezing resulted in a highly significant increase in shear force values for loin chops and highly significant decreases in flavor, tenderness and overall satisfaction scores for leg roasts. The freezing of rib chops, in contrast to the results for loin chops and leg roasts, resulted in a highly significant decrease in shear force values indicating an increase in tenderness as the result of freezing. Several possible explanations concerning these contradictory findings are suggested and discussed.

DEGRADATION OF INOSINIC ACID IN CHICKEN MUSCLE DURING ASEPTIC STORAGE AND ITS POSSIBLE USE AS AN INDEX OF QUALITY. A. W. KHAN, J. DAVIDEK & C. P. LENTZ. *J. Food Sci.* **33**, 25–27 (1968)—Analysis of chicken breast and leg muscle stored under aseptic conditions at 0, 5, and 10°C showed over 75% loss of inosinic acid content of both breast and leg muscle occurred in 3–5 weeks at 0°, in 2–3 weeks at 5° and in about 1 week at 10°C. These storage periods correspond to the lengths of time after which quality deterioration has been detected by sensory evaluation. During the same periods of storage, the hypoxanthine content of these muscles increased gradually to a value of 200–400 μg/g of muscle.

5'-ADENYLIC ACID DEAMINASE IN PORCINE MUSCLE. E. D. ABERLE & R. A. MERKEL. *J. Food Sci.* **33**, 27–29 (1968)—There were no significant ($P > .05$) differences in adenylic acid deaminase activity within or between the longissimus dorsi, gluteus medius or rectus femoris muscles even though muscle morphology varied from dark, firm and dry to pale, soft and exudative. Simple correlation coefficients between muscle pH and adenylic acid deaminase activity indicated positive relationships between enzyme activity and muscle pH at 15 and 45 min post-mortem in all muscles studied.

DIELECTRIC PROPERTIES OF COMMERCIAL COOKING OILS. W. E. PACE, W. B. WESTPHAL & S. A. GOLDBLITH. *J. Food Sci.* **33**, 30–36 (1968)—The differences in dielectric properties among fats and oils appear to be attributable to the phase (solid *vs.* liquid) of the material and generally correspond to the degree of unsaturation as evidenced by iodine values. The differences in loss factors among these fats and oils at any given temperature and frequency (within the range at which the measurements were made) are too small to be of any practical importance in selecting any one of them for use in heating processes using microwaves.

IN THIS ISSUE

DIELECTRIC PROPERTIES OF POTATOES AND POTATO CHIPS. W. E. PACE, W. B. WESTPHAL, S. A. GOLDBLITH & D. VAN DYKE. *J. Food Sci.* **33**, 37–42 (1968)—Potato chips show a rapidly decreasing dielectric loss as moisture content is reduced; the loss values of the chips approach those of the oil used for frying them after moisture has been reduced to approximately 3% and the oil content has been increased accordingly. For finish drying of potato chips, a frequency of 3,000 megahertz (MHz) will result in 3–4.5 times greater power production in the chips than will the use of a frequency of 1,000 MHz (in the moisture and temperature range at which the data reported were obtained).

PHYSICAL AND BIOCHEMICAL PROPERTIES OF PORCINE MUSCLE AS AFFECTED BY EXOGENOUS EPINEPHRINE AND PREDNISOLONE. E. D. ABERLE & R. A. MERKEL. *J. Food Sci.* **33**, 43–47 (1968)—Rate of postmortem muscle pH decline, ultimate muscle pH, Munsell value and transmission value were not significantly ($P > .05$) altered by either epinephrine or prednisolone plus epinephrine injection. Significantly ($P < .05$) higher total phosphorylase activity and slightly greater phosphorylase *a* and % phosphorylase *a* (% of total phosphorylase activity) were observed in the muscles of epinephrine injected pigs. Neither total phosphorylase nor phosphorylase *a* was related to rate of pH decline or incidence of pale, soft, exudative muscle development.

A QUANTITATIVE AND MORPHOLOGICAL STUDY OF BOVINE LONGISSIMUS FAT CELLS. W. G. MOODY & R. G. CASSENS. *J. Food Sci.* **33**, 47–52 (1968)—The results indicate that traceable intramuscular fat is not a good measure of total intramuscular fat. There did not appear to be a consistent medial central and lateral marbling pattern among the three marbling groups, however, significant differences were apparent within groups. Fat cell size increased with increases in cell mass, marbling and total chemical fat of the muscle. Fat cells were observed to accumulate and grow in close proximity to portions of the circulatory system.

MEAT FLAVOR. 2. Procedures for the Separation of Water-Soluble Beef Aroma Precursors. L. L. ZAIKA, A. E. WASSERMAN, C. A. MONK, JR. & J. SALAY. *J. Food Sci.* **33**, 53–58 (1968)—Separations on the basis of gel filtration, adsorption, and anion exchange resulted in a number of fractions that developed roast beef aroma on pyrolysis, but differed in composition. Either sugar phosphates or free sugars are involved in aroma development. Tyrosine, phenylalanine, taurine and glutamic acid may be removed without affecting aroma. A number of amino acids were present in the aroma producing fractions in trace amounts, thus the requirement for their presence may be questionable.

CATHEPTIC ENZYMES IN MEAT TENDERIZATION. 1. Purification of Cathepsin D and Its Action on Actomyosin. C. B. MARTINS & J. R. WHITAKER. *J. Food Sci.* **33**, 59–64 (1968)—A cathepsin has been purified from chicken leg muscle by ammonium sulfate fractionation and by chromatography on carboxymethyl- and diethyl-aminoethylcellulose. With respect to the specific activity of the initial 2% potassium chloride extract the cathepsin was purified 580-fold. The purified cathepsin hydrolyzed urea-denatured hemoglobin readily at pH 4.40, but it had no activity on α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine, α-N-benzoyl-L-argininamide and α-N-acetyl-L-tyrosinamide. The data indicate the preparation was cathepsin D and that it did not contain cathepsins A, B and C. The cathepsin preparation had no activity on actomyosin at pH 4.95 and 5.90 as measured by viscosity and gel-filtration methods.

DIRECT SPECTROPHOTOMETRIC DETERMINATION OF FAT AND MOISTURE IN MEAT PRODUCTS. I. BEN-GERA & K. H. NORRIS. *J. Food Sci.* **33**, 64–68 (1968)—The near infra-red spectral absorption properties of 2-mm-thick samples of meat emulsions were measured by direct spectrophotometric techniques. The difference in optical density between 1.80 and 1.725 μ gave a high correlation with moisture content and the difference between 1.725 and 1.65 μ gave a high correlation with fat content. Direct spectrophotometric analysis predicted fat content within a standard error of $\pm$ 2.1% and moisture content within $\pm$ 1.4%.

ALTERATIONS OF BOVINE SARCOPLASMIC PROTEINS AS INFLUENCED BY HIGH TEMPERATURE AGING. G. B. THOMPSON, W. D. DAVIDSON, M. W. MONTGOMERY & A. F. ANGLEMIER. *J. Food Sci.* **33**, 68–72 (1968)—Up to three days storage, extractability of water soluble protein of the l. dorsi muscle appeared to be greatest in meat held at the elevated temperature. Thereafter, extractability was greater in muscles held at 3°C. Absorbance ratios (422/280 mμ) of extracts suggested higher extractable levels of oxymyoglobin associated with the high temperature treatment. Results of DEAE-cellulose ion exchange chromatography of the sarcoplasmic proteins showed only minor variations in profiles between aging treatments. Profile alterations appeared with time but did not appear related to anticipated increases in tenderness.

QUANTITATIVE METHODS FOR ANTHOCYANINS. 1. Extraction and Determination of Total Anthocyanin in Cranberries. T. FULEKI & F. J. FRANCIS. *J. Food Sci.* **33**, 72–77 (1968)—The method developed consists of extracting the anthocyanins with ethanol-1.5*N* hydrochloric acid (85:15) and measuring the O.D. of the extract, diluted with the extracting solvent, at 535 nm. The total anthocyanin content was calculated in absolute quantities with the aid of the extinction coefficients established for the four major cranberry anthocyanins dissolved in the alcoholic solvent system.

ABSTRACTS:
IN THIS ISSUE

QUANTITATIVE METHODS FOR ANTHOCYANINS. 2. Determination of Total Anthocyanin and Degradation Index for Cranberry Juice. T. FULEKI & F. J. FRANCIS. *J. Food Sci.* **33**, 78–83 (1968)—The method developed for total anthocyanin determination takes into consideration the interference caused by brownish degradation products which accumulate during storage. The method involves the measurement of absorbance at 510 nm on samples diluted with pH 1.0 and 4.5 buffers. The pigment content is calculated in absolute quantities with the aid of extinction coefficients established for the cranberry anthocyanins dissolved in the buffers.

A COMPARISON OF THE LIGHT AND DARK PORTIONS OF A STRIATED MUSCLE. G. R. BEECHER, L. L. KASTENSCHMIDT, R. G. CASSENS, W. G. HOEKSTRA & E. J. BRISKEY. *J. Food Sci.* **33**, 84–88 (1968)—The levels of physiologically related muscle constituents were determined in the light (white) and dark (red) portions of a striated muscle from the pig *(Sus domesticus)*. Myoglobin level, percent red fibers and succinic dehydrogenase activity were two-fold higher in the semitendinosus dark portion whereas ADP and inorganic phosphate levels were similar in both portions. Phosphorus levels were higher and sodium levels lower in the semitendinosus light portion than in the semitendinosus dark portion.

VOLATILE FATTY ACIDS IN SOME BRANDS OF WHISKY, COGNAC AND RUM. L. NYKÄNEN, E. PUPUTTI & H. SUOMALAINEN. *J. Food Sci.* **33**, 88–92 (1968)—Gas chromatography has been applied to eight different types of whisky, two of cognac, one of brandy, and four of rum, for determination of the relative proportions of volatile fatty acids, with the lower molecular acids as free acids, but upwards from caprylic acid as methyl esters. Rum contained the largest amount of volatile acids, 600 mg/L, while one of the brands of Scotch whisky contained the least, 90 mg/L. Acetic acid represented 40–95% of the total amount of volatile acids in the whisky; in cognac and brandy, the value was 50–75%, and in rum 75–90%.

ACTION OF MICROORGANISMS ON THE PEROXIDES AND CARBONYLS OF RANCID FAT. J. L. SMITH & J. A. ALFORD. *J. Food Sci.* **33**, 93–97 (1968)—The effects of 26 species of bacteria, molds, and yeasts on the hydroperoxides and monocarbonyls in rancid fat have been determined. All of the cultures were capable of decomposing the hydroperoxides. The activity of microorganisms on the monocarbonyl content of the rancid fat was quite varied and could be divided into: 1) microorganisms which produced large increases in at least two monocarbonyl classes; 2) microorganisms which removed 2,4-dienals; 3) microorganisms which removed 2,4-dienals and 2-enals, and 4) microorganisms which caused decreases in at least two classes of monocarbonyls (without destroying completely any class).

DIFFUSION OF AFLATOXINS IN FOODSTUFFS. H. K. FRANK. *J. Food Sci.* **33**, 98–100 (1968)—Apple juice, sliced and pre-packed bread, soft cheese and a model substrate with high water content were inoculated with toxic strains of *Aspergillus flavus*, isolated from Brazil nuts, and the migration of the aflatoxins into the substrate was determined. After 14 days apple juice showed the highest content of free aflatoxin B_1, which decreased remarkably in the next 12 days. In bread we could detect aflatoxins only in parts with visible mycelium; outside these parts toxins could not be estimated. In Tilsit cheese, however, aflatoxins were detectable after 6 days at 30°C at a depth of 10 mm below the fungus.

POST-MORTEM DEGRADATION OF ADENINE NUCLEOTIDES IN MUSCLE OF THE LOBSTER, *HOMARUS AMERICANUS*. J. R. DINGLE, J. A. HINES & D. I. FRASER. *J. Food Sci.* **33**, 100–103 (1968)—Thin-layer chromatography showed that post-mortem degradation of adenine nucleotides in the tail muscle of lobster *(Homarus americanus)* followed the route: adenosine 5′-triphosphate (ATP) → adenosine 5′diphosphate (ADP) → adenosine 5′monophosphate (AMP) → inosine 5′-monophosphate (IMP) → inosine → hypoxanthine. KCl extracts (0.6*M*) also degraded ATP by this route. Such extracts contained a weak AMP-aminohydrolase activity that was activated by ATP, but no adenosine aminohydrolase could be detected. Neither of these aminohydrolases were found in extracts made with water or 0.02*M* K-succinate.

EFFECT OF CHLOROPHENOXYACETIC ACID GROWTH—REGULATOR SPRAYS ON RESIDUES IN CANNED APRICOTS AND GRAPES. Y. N. LEE & B. S. LUH. *J. Food Sci.* **33**, 104–108 (1968)—A gas-liquid chromatographic method coupled with an electron capture detector has been developed for the analysis of chlorinated phenoxyacetic acid residues in canned fruits. The technique involves the conversion of the acid to its methyl ester with diazomethane, chromatography on a 5% silicone grease SE-30 column at 210°C, and subsequent detection of the compound by an electron capture detector. As low as 0.02 ppm of the residue could be detected. The method is superior to the colorimetric method because para-chlorophenoxyacetic acid, 2,4-D, and 2,4,5-T can be separated and quantitatively determined simultaneously.

ADAPTATION OF BIPHASIC CULTURE TECHNIQUE TO THE SPORULATION OF *CLOSTRIDIUM BOTULINUM* TYPE E. MARY K. BRUCH, C. W. BOHRER & C. B. DENNY. *J. Food Sci.* **33**, 108–109 (1968)—An improved method for producing high-yield spore crops of two strains of *Clostridium botulinum* type E was developed. Biphasic culture employing agar-water or agar-broth systems are described for two different types of culture vessels.

FACTORS AFFECTING THE STABILITY OF CHLORTETRACYCLINE, TYLOSIN AND FURYLFURAMIDE AGAINST LOW LEVEL OF IONIZING RADIATION. T. KAWABATA, T. KOZIMA, N. SHIMURA & E. YOSHIMURA. *J. Food Sci.* **33**, 110–113 (1968) Tylosin (Tl) in the phosphate buffer of pH 6–8 was very sensitive to low-level radiation, while either furylfuramide (FF) or chlortetracycline (CTC) exhibited fairly high resistance at the same levels. Removal of dissolved oxygen in the test solution by aerating with nitrogen gas enhanced the inactivation of Tl and FF at 0.05–0.1 Mrad of radiation, but it had an opposite effect on the inactivation of CTC. Much higher Tl and CTC activities were retained after irradiation at 0.1 or 0.2 Mrad when the drugs were added to albumin, gelatin, broth or minced meats of five species of fish, while the retention of FF did not change.

NOTICE: Page Charges for Publication of Research Papers

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The Influence of Sugar Concentrations on the Vapor Pressure of Food Odor Volatiles in Aqueous Solutions

SUMMARY—The addition of fructose or invert sugar in very high concentrations to aqueous solutions containing strawberry volatiles or synthetic compounds respectively, results for some components in a decrease of peak heights in gas chromatograms of the vapors over the solutions; the phenomenon is thought to be of interest for studying flavor retention in juice concentrates.

INTRODUCTION

THE ADDITION OF INORGANIC SALTS to increase the vapor pressure of volatile components in dilute aqueous solutions is often applied in headspace aroma analysis (Dobry-Duclaux, 1952; Bassette *et al.*, 1962; Kepner *et al.*, 1964). Nawar (1966) in a more detailed study of the matter, reported the same effect—if to a lesser extent—occurred on the addition of glucose. Nawar, for the first time, sounds a warning against the incorrect interpretation of results obtained when using the technique for quantitative studies.

Experiences in our laboratory confirmed Nawar's observations on increased vapor pressures after the addition of NaCl—in concentrations up to saturation—and of different sugars (glucose, sucrose, fructose, invert sugar)—in concentrations up to the 40% w/w level. However, experiments on the use of sugars at concentrations that were considerably in excess of these and of the ones used by Nawar, showed that with some types of volatile compounds no increase but a marked decrease of peak heights in gas chromatograms of vapors over aqueous solutions may be observed.

EXPERIMENTAL METHODS

Preparation of samples

From a batch of commercial strawberry juice about 10% was stripped off (Weurman, 1961) and to each of two samples of the distillate equal amounts by weight of water and of an oversaturated solution of invert sugar (Nulomoline, SuCred Corp., N. Y.) were added to produce an aqueous solution of strawberry volatiles and a solution of these volatiles at the same concentration (w/w) containing 73.1% w/w invert sugar.

By diluting a synthetic mixture two more solutions were prepared. One of them contained the compounds listed in Table 1 at the concentrations indicated. The other contained 79.1% w/w fructose (D (−) Fructose, Merck, Darmstadt) in addition to the same compounds at the same concentration by weight.

With an Abbe refractometer n_D^{20} was measured and sugar concentrations were calculated to percentages by

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Table 1. Composition of dilute aqueous synthetic mixture (Fig. 2).

No.	Component	ppm v/v
1	propanal	3
2	ethyl formate	2
3	ethanol	120
4	3-pentanone	4
5	ethyl butyrate	2
6	n-butanol	120
7	4-heptanone	5
8	3-heptanone	5
9	ethyl caproate	5
10	3-octanone	8

weight (ICUMSA, 1956). At 30°C invert sugar is saturated at 69.7% and fructose at 81.5% (Bates, 1942).

Gas chromatographic-measurement of headspace volatiles

Fifty ml samples of the above four solutions were brought under nitrogen in 130 ml infusion flasks, closed with a serum cap, and equilibrated in a 30°C waterbath for 40 min. Sugar-containing solutions were stirred slowly by magnetic stirrers. A 5 ml gas-tight syringe was used for direct headspace sampling. The vapor samples were analyzed on a Carlo Erba, model C, gas chromatograph, equipped with a hydrogen flame detector.

A 5 m × 4 mm i.d. Al column packed with 10% LAC I-R-296 on Chromosorb W and operated at 90°C, was used. N_2, H_2 and air flow rates of 20, 17 and 140 ml per min were used throughout.

RESULTS AND DISCUSSION

From the peakheights in the Figs. 1 and 2 the increase of the partial vapor pressure for a number of components in the solutions, and the decrease for others, is seen, when

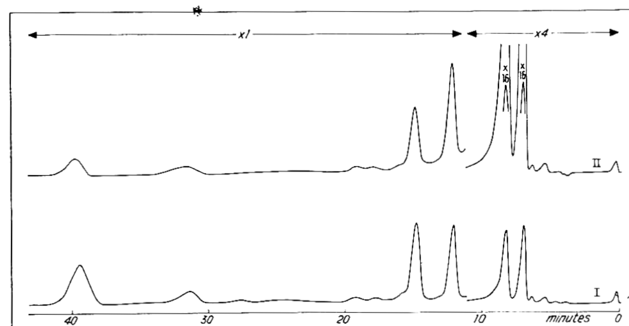

Fig. 1. Solution of strawberry aroma: I without invert sugar; II with 73.1% invert sugar.

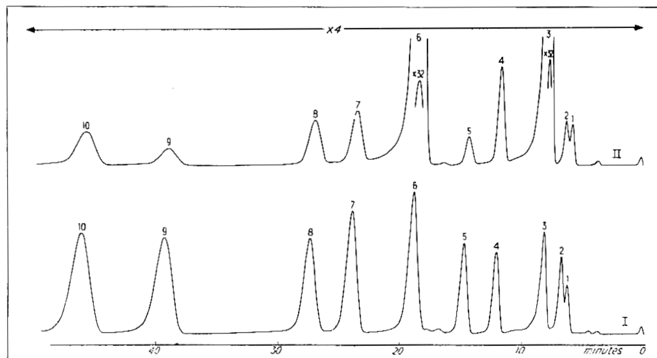

Fig. 2. Solution of synthetic mixture: I without fructose; II with 79.1% fructose.

oversaturating (invert sugar) or almost saturating (fructose) the solutions with sugars.

The results are thought to be of interest for the study of flavor retention in juice concentrates.

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Studies in Meat Tenderness. 4. Changes in the Extractability of Myofibrillar Proteins During Meat Aging

SUMMARY—Changes during aging in the extractability of the myofibrillar proteins of meat from beef and rabbit carcasses have been examined, using a buffer which dissociates the actomyosin complex of the muscle cell. Approximately 52% of the myofibrillar proteins of unaged meat is extracted in 40 min at 2°C whereas from aged meat as much as 78% is extracted.

The rate and extent of these changes are determined largely by the ultimate pH value of the meat. Similar increases in protein extraction, displaying the same pH dependence, occur during the aging of well-washed myofibrillar preparations.

The increase in the percentage of myofibrillar protein extracted during aging results from either a progressive weakening of the fibrous protein linkages with the insoluble stroma of the meat cell, or from a disintegration of the insoluble stroma itself.

INTRODUCTION

THE TENDERIZING OF MEAT occurring during storage at above freezing temperature—so-called aging—is usually considered to be a consequence of changes within the fibrous components of muscle. For this reason most studies of the aging mechanism have been concerned with changes during storage in the properties both of connective tissue and of myofibrils.

There is a convincing body of evidence, based on changes in the mechanical properties across and along the grain of meat, that aging is not related to alterations in the connective tissue (Steiner, 1939a, 1939b, 1939c). Although this conclusion is contrary to the work of Moran *et al.* (1929), it is substantially supported by the chemical studies of Prudent (1947), Ramsbottom *et al.* (1949), Winegarden *et al.* (1952), Wierbicki *et al.* (1954, 1955) and Bouton *et al.* (1958).

Changes during aging in the properties of the meat proteins, especially proteins of the myofibrils, have been extensively studied. Fujimaki *et al.* (1965) have shown that both the actomyosin-forming ability and adenosinetriphosphatase activity of myosin extracted from bovine muscle decrease during aging. They have proposed that aging is a result of a decrease in the interaction of actin and myosin, although many workers do not favor this view (Wierbicki *et al.*, 1956; Marsh, 1954).

Hamm (1960) and Fujimaki *et al.* (1958) have investigated the increase that occurs in the water-binding capacity of meat during aging. Similar studies of changes in metal-ion binding have been made by Arnold *et al.* (1956). It is to be expected that these changes occur largely in the myofibrillar component of meat, since extensive shifts

in metal-ion and water binding occur within the myofibrils with the onset of muscle activity (Needham, 1960).

As a guide to structural alterations in meat during aging, the pattern of extractability of myofibrillar proteins has been studied by a number of workers using solutions of high ionic strength ($I > 0.5$). Weinberg *et al.* (1960) have observed that the amount of protein extracted from chicken muscle is greater 24 hr post-mortem, than it is immediately after the death of the animal. The extra protein was considered to consist of actin and actomyosin.

As an extension of these studies, Kahn *et al.* (1964) have shown that the proportion of myofibrillar protein extracted from chicken muscle decreases rapidly to a minimum value 4–8 hr post-mortem, increasing thereafter to a maximum value after aging at 0° for 2–4 days. Aberle *et al.* (1966), using bovine muscle, have observed an increase of 10% approximately in the amount of myofibrillar protein extracted during a 14-day period of aging at 4°.

In contrast to these findings Fujimaki *et al.* (1965) have shown that there is no increased content of actin in the actomyosin fraction extracted from rabbit muscle aged for 7 days at 4°. Similarly no increase was observed in the amount of actomyosin extracted from turkey muscle aged for 48 hr at 5–10° (Scharpf *et al.,* 1964), or from bovine muscle aged for 312 hr at 4° (Goll *et al.,* 1964).

In this paper the chemical changes associated with aging occurring both in the myofibrillar component of meat *in situ* and in the isolated myofibrils from meat have been studied as a first step in elucidating the mechanism of the tenderizing process.

EXPERIMENTAL

Meat sampling

Meat was obtained from beef animals slaughtered at the local meat works, and from mature domestic rabbits. Sections of longissimus dorsi (LD) muscles adjacent to the 10th–14th ribs from a variety of beef animals were removed after the halved carcasses had been held for 24 hr at chiller temperature (2–4°).

Bovine sternomandibularis (SM) muscles obtained within 30 min of slaughter were trimmed and stored at 15°. Rabbit LD muscles, excised as soon as possible after decapitation, were stored at 2° for 16 hr in a moist nitrogen atmosphere. Zero time of aging for the muscles from beef and rabbit carcasses were taken to be 24 hr and 16 hr post-mortem respectively. In these times the ultimate pH values of the samples had been reached.

Composition of the extracting buffers

The buffer used to extract myosin selectively from the myofibrillar preparations (H-S buffer) had the composition $0.6M$ KCl, $0.1M$ phosphate, $1.0mM$ magnesium chloride, $10mM$ sodium pyrophosphate, pH 6.4 (Hasselbach *et al.,* 1951). Weber-Edsall (W-E) buffer (Haga *et al.,* 1965), used to extract actomyosin from the myofibrillar preparations, had the composition $0.6M$ KCl, $0.04M$ sodium bicarbonate, $0.01M$ sodium carbonate, pH 9.20.

Preparation of myofibrils

Well-washed myofibrils were prepared by a method closely similar to that described by Perry *et al.* (1956), all manipulations being carried out at 2°. Chilled, minced meat (200 g) was homogenized for 1 min with 5 vol $0.1M$ KCl, $0.039M$ boric acid pH 7.1 (KCl-borate) in a Waring blendor, and the homogenate centrifuged for 15 min at 600 G. The residue, resuspended in 5 vol KCl-borate, was poured through nylon mesh (mesh diameter 1.2 mm) to remove the major portion of connective tissue, and additional coarse material was removed by discarding the sediment produced on centrifuging the suspended myofibrils for 3 min at 400 G.

The myofibrils remaining in suspension were sedimented by centrifugation (15 min at 600 G) and were washed by a further suspension in KCl-borate (1.5 L). In some experiments samples containing large numbers of bacteria ($> 10^6$ organisms/g) were obtained by resuspending myofibrillar preparations in the KCl-borate supernatant of a previous preparation which had been stored for 10 days at 10° to allow build-up of micro-organisms. Contraction of the myofibrils did not occur during homogenization as the meat used in the present studies was in a state of rigor mortis.

Microscopical examination showed that satisfactory preparations were obtained in which the I and A bands of the sarcomeres were clearly visible. Apart from the discrete myofibrillar units present, the preparations contained a small proportion of fiber pieces consisting of laterally aligned myofibrils of up to 40 in number. In some experiments these coarser fiber pieces were separated from the fine material by settling them for 20 min from dilute suspensions of the myofibrillar preparations at 2° in KCl-borate.

The myofibrils, including the coarse and fine materials obtained by the settling procedure, were freed from excess washing solutions by centrifuging for 30 min at 2,000 G. All preparations were resuspended in $0.16M$ KCl, adjusted to the required pH with HCl ($0.01N$) and compacted by a further centrifugation for 30 min at 2,000 G. The myofibrils were aged at either 2° or 10°.

Extraction of myofibrils

A sample of the compacted myofibrils (8 g) containing approximately 10% protein was made into a thick slurry, free of lumps, with $0.16M$ KCl (10 ml), and 80 ml of either H-S buffer or W-E buffer was added. Samples of the gently stirred suspensions were removed for total protein determinations with a graduated pipette from which the end had been cut (Davey *et al.,* 1966). To determine the amount of protein extracted, portions of the suspensions were removed at intervals and centrifuged for 30 min at 10,000 G. The concentrations of extracted protein in the supernatants were expressed as percentages of the total protein concentration of the suspensions.

Protein and pH determinations

The total protein concentrations of myofibrillar suspensions were determined by a micro-Kjeldahl method (AOAC, 1955). The concentration of protein extracted from the myofibrillar suspensions was determined by a biuret method (Gornall *et al.,* 1949). Estimations of pH were made, using a glass electrode, on homogenates (Marsh *et al.* 1950) of meat samples (1–2 g) in 10 ml neutralized sodium iodoacetate solution ($2mM$).

Bacterial control

In order to limit bacterial spoilage, the meat samples were prepared under aseptic conditions, stored in a nitrogen atmosphere and sprayed with aureomycin (200 ppm) at intervals during the storage period (Davey *et al.*, 1966). The KCl-borate buffer used for washing myofibrillar preparations was boiled for 20 min. The extent of bacterial growth was determined on meat samples (1 g), 1 cm from exposed surfaces, and on 1 g samples of the compacted myofibrillar preparations.

After shaking the samples with sterile peptone water (10 ml, 0.1%), portions of the resulting suspensions were plated on yeast extract nutrient agar and incubated at 25° for 5 days. Strict adherance to these procedures ensured that bacterial numbers were maintained at the low level of $< 10^2$ organisms/g for meat aged 30 days at 2° and for myofibrillar preparations aged either 21 days at 2°, or 3 days at 10°.

RESULTS

FIG. 1 SHOWS THE TIME-COURSE OF PROTEIN extraction by W-E buffer of myofibrils prepared from bovine LD muscle at intervals of aging. Approximately 75% of the protein was released in 10, 30 and 60 min from myofibrils of meat aged 17, 10 and 2 days respectively. No relationship could be shown on prolonged extractions (> 10 hr) between the time of aging and the percentage of myofibrillar protein dissolved (78%–87%). Similar results were obtained using samples of the LD and SM muscles from six beef carcasses and the LD muscles from two rabbits.

Fig. 2, typical of 20 such experiments, shows the time-course of protein extraction with H-S buffer, from myofibrils of bovine LD muscles aged for different times. A rapid phase followed by a slow phase was observed in the time-course of extraction. In 6 min the degree of maximal extraction was 0.95, in 40 min it was 0.98, and in 200 min 1.00. At maximal extraction (200 min) 54%, 66% and 75% of myofibrillar protein had been released from meat aged 1, 10 and 17 days respectively.

An increase in the percentage of protein extracted by H-S buffer in 40 min at 2° (percentage extractability) is observed to occur during the aging, not only of myofibrils *in situ*, but also of well-washed isolated myofibrils.

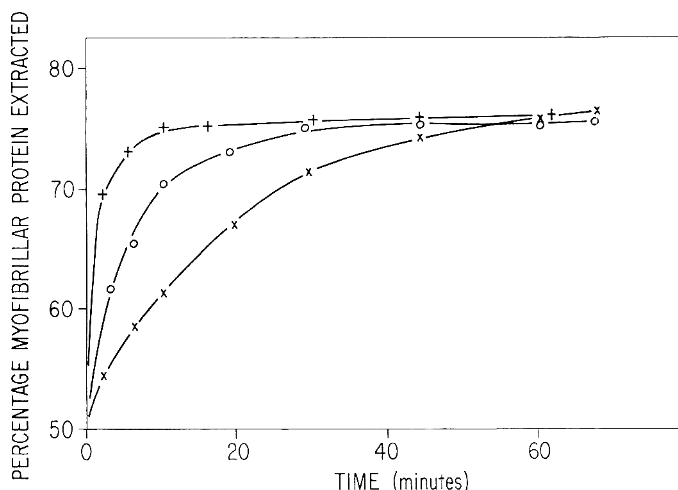

Fig. 1. The time-course of extraction with W-E buffer of myofibrils prepared from bovine LD muscle. Aged 2 days, X — X; aged 10 days, O — O; aged 17 days, + — +.

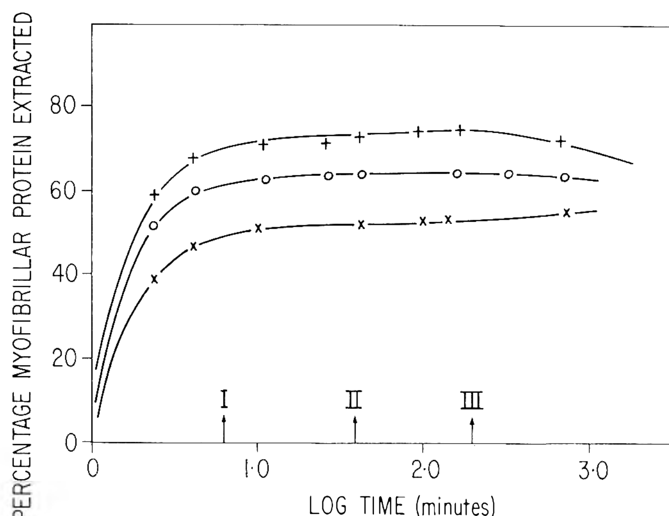

Fig. 2. The time-course of extraction wth H-S buffer of myofibrils prepared from bovine LD muscle. Aged 1 day, X — X; aged 10 days, O — O; aged 17 days, + — +. (Arrows, 1–3 on Log Time axis signify 6 min, 40 min and 200 min).

Fig. 3, characteristic of numerous such experiments, shows the change in the percentage extractability of myofibrils which were aged under isotonic conditions (I = 0.16) and at the ultimate pH of the meat from which they had been isolated.

Fig. 3 also illustrates the effect of storage time on the percentage extractability of myofibrils prepared at intervals during aging from bovine LD muscles. The meat from one carcass gave values which increased during aging from 53% to 60% in 4 days, reaching 72% in 10 days. In contrast percentage extractability values obtained for meat from the second carcass, increased very little during the 10-day period, from 53% to 56%.

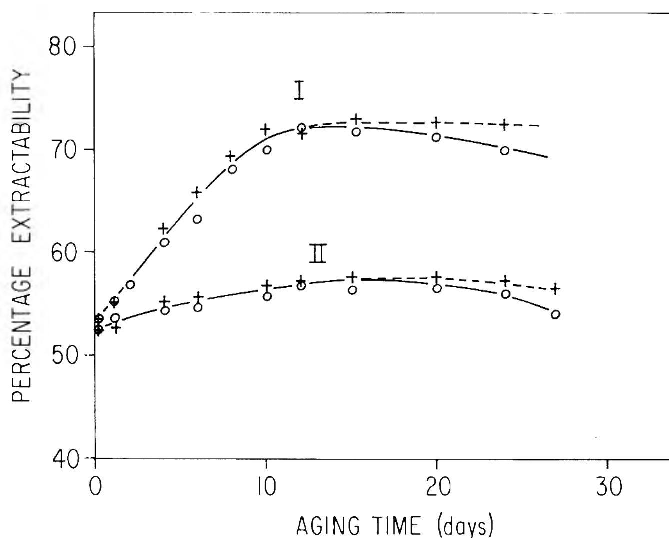

Fig. 3. The relationship between the percentage extractability and the aging time of the isolated myofibrils, + — +; and the whole meat, O — O; from two beef carcasses. Curve I, ultimate pH 6.03. Curve II, ultimate pH 5.50.

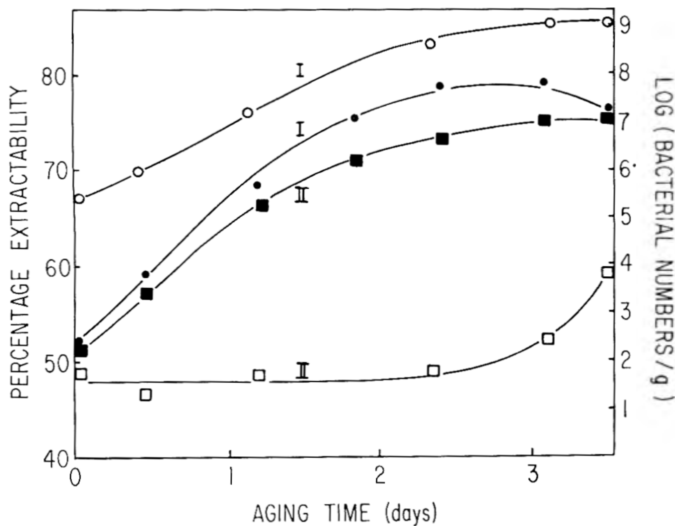

Fig. 4. The effect of bacteria on the relationship between the percentage extractability and the aging time at 10°, of isolated myofibrils from bovine LD muscles.
At high levels of bacterial contamination: *Log (bacterial numbers/g myofibrillar preparations),* ○ — ○. *Percentage extractability,* ● — ●. At low levels of bacterial contamination: *Log (bacterial numbers/g myofibrillar preparations),* □ — □. *Percentage extractability,* ■ — ■.

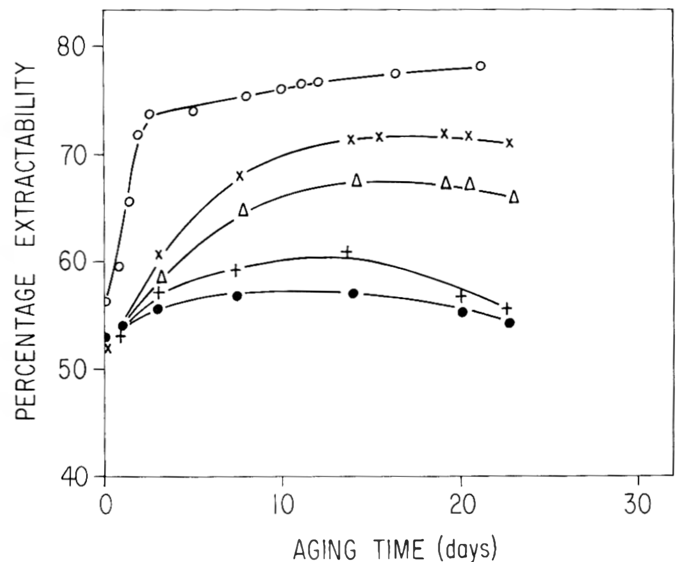

Fig. 5. The effect of ultimate pH values (in parentheses) on the relationship between the percentage extractability and aging time, of bovine LD muscles, × — × *(6.30),* Δ — Δ *(5.92),* + — + *(5.54),* ● — ● *(5.45), and of the LD muscle from one rabbit carcass* ○ — ○ *(5.65)*

Although the curves were similar, the percentage extractability at a given time of storage was usually 1–5% higher for the isolated myofibrils than for myofibrils aged *in situ.* This difference was further emphasized after 15 days aging by the onset of a more distinct drop in the values of the latter. No departure has been observed from this pattern of increased percentage extractabilities during the aging of myofibrils isolated from the SM and LD muscles of 50 beef carcasses.

It is important to show that the extractability changes observed to occur during aging are not due merely to bacterial action. This is illustrated in Fig. 4 which compares the effect of high and low levels of bacteria on extractability values. These values increase during aging in the virtual absence of bacteria ($< 10^2$ organisms/g), and initially at least the rate of increase is independent of the level of contamination. During the second half of the aging period, however, higher extractability values are associated with the higher levels of bacterial contamination ($> 10^7$ organisms/g).

It is apparent from Figs. 3 and 5 that ultimate pH values determine largely the rate of increase in the percentage extractabilities observed during the aging of LD muscles from beef carcasses. Values for maximum percentage extractability appear also to depend upon the ultimate pH of meat, rising from 57% for meat of ultimate pH 5.45 to 72% for meat of ultimate pH 6.30. A small decline from the maximum values obtained occurs on prolonged aging, especially at the lower values of ultimate pH.

The change in the percentage extractability values during the aging of the LD muscles from a rabbit carcass (ultimate pH 5.63) is also shown in Fig. 5. Higher values ($> 70\%$) were achieved in much shorter storage times than for bovine LD muscles.

It has been established that there is a progressive increase in the percentage extractability of myofibrils aged either *in situ* or as isolated myofibrillar components. It is important to demonstrate that this increase is not due merely to a greater permeability of the aged myofibrils to the extracting buffers. This is illustrated in Fig. 6, one of several experiments in which the effect of size of myofibrillar pieces on the time-course of protein extraction was determined.

Ninety percent of maximum extraction was achieved more quickly with fine myofibrils (4 min) than with coarse myofibrils (10 min). However, the percentage extractabilities of both preparations were the same, although permeability factors would be likely to be more significant when extracting the coarser myofibrils. It is concluded, therefore, that permeability factors do not determine values of percentage extractability.

The pattern of extraction of the myofibrillar preparations may be determined to some extent by changes during aging in the properties of the extracted proteins themselves. In this respect the viscous rather thixotropic proteins released from the myofibrillar preparations may act as a barrier limiting further ready extraction. However, in several experiments using both unaged and aged myofibrils no difference was observed in the time-course of percentage extraction at the myofibrillar concentrations of 2 mg/ml and 10 mg/ml. It is concluded, therefore, that the pattern of protein extraction obtained was not affected by the concentration either of the myofibrils or of the solubilized proteins.

DISCUSSION

THE RESULTS HAVE SHOWN that during aging, in the virtual absence of bacteria, an increase occurs in the percentage extractability of proteins from the myofibrillar components of meat. The properties of the extracting buffers determine whether such increases are observed. In this respect W-E buffer, which is used to extract actomyosin from muscle minces (Haga *et al.,* 1965), was shown to

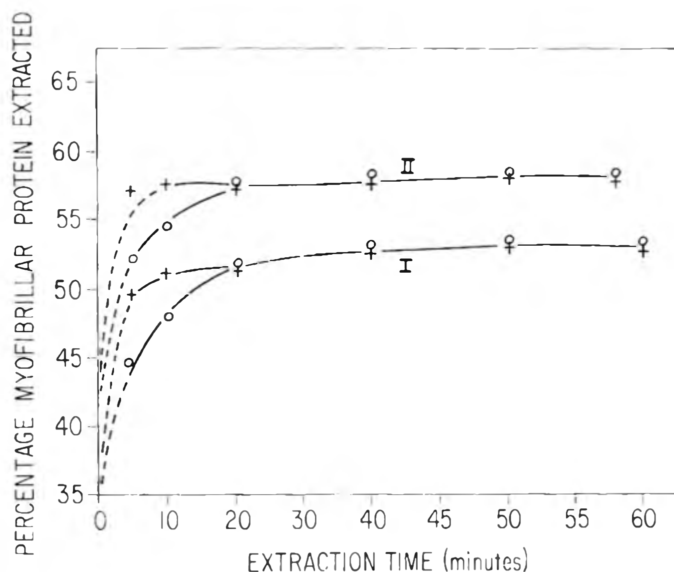

Fig. 6. The time-course of extraction with H-S buffer of myofibrils prepared from bovine LD muscles; coarse myofibrillar preparation, ○ — ○; fine myofibrillar preparation + — —. Curve I, unaged myofibrils; Curve II, myofibrils aged 5 days.

extract less myofibrillar protein from unaged meat than from aged meat during short periods of extraction (10 min). On longer extraction (10 hr), however, the percentages of protein extracted from both unaged and aged material were shown to be approximately the same.

On the other hand, H-S buffer has been used to extract specifically myosin from the myofibrillar components of the muscle cell. The present results have shown that 52% approximately of the protein from unaged myofibrils is extracted in 40 min at 2°. This buffer is ideal for observing changes in percentage extractabilities as there is a slow increase only (¼–½%/hr) in protein extracted over a further period of 50 hr. The ultimate pH values of meat and the pH values of myofibrils prepared therefrom determine not only the rates of increase in the percentage extractabilities but also the maximum values attained.

The small decline in extractability on prolonged aging ($>$ 17 days), especially pronounced at relatively low pH values (pH 5.4–5.8), is due probably to incipient denaturation of myofibrillar proteins during storage. It has been shown that if the pH values of meat and of the myofibrils ($I = 0.16$) prepared therefrom are the same, then the patterns of percentage extractability of both materials during aging are similar. The leaching out of a small amount of protein (1–5%) during the washing of myofibrils prepared from meat during aging is assumed to account for the differences encountered. However, it is emphasized that changes in percentage extractabilities are more usefully studied by aging myofibrillar preparations than by aging whole meat.

A more ready solubilization of the myofibrillar proteins might be due to an increased permeability of the myofibrils, which have disintegrated in some way during aging. This disintegration in whole meat would manifest itself as an increase in tenderness of the cooked meat. In this respect Hasselbach *et al.* (1951) claimed that the regularly aligned filaments of rabbit myofibrils prevent ready buffer penetration, thus making actin seemingly resistant to extraction. The present results have shown, however, that percentage extractabilities are independent of both the size and the concentration of myofibrillar pieces. The increases observed during meat aging are not due, therefore, to a higher permeability of the myofibrillar structures.

The more extensive and ready release of myofibrillar proteins into an extractable form during aging is consistent with the view that there is a progressive weakening of the linkages of these proteins with relatively insoluble components of the meat cell (Weinberg *et al.*, 1960). Alternatively, a relatively insoluble component such as Z-line material or the sarcoplasmic reticulum disintegrates in some way.

Future studies will be concerned with the nature of the proteins released from the myofibrils during aging. Changes in the insoluble components of the muscle cell will also be examined.

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Moving Boundary Electrophoresis of Food Stabilizers

SUMMARY—The electrophoretic characteristics of locust bean gum, guar gum, arabic gum, carboxymethylcellulose, κ- and λ-carrageenan, alginate and the non-dialyzable fraction of corn syrup solids were studied by moving boundary electrophoresis. The physical properties of these colloids require the use of modified techniques in order to eliminate boundary disturbances which otherwise might be interpreted as heterogeneity of composition.

A high degree of homogeneity was observed for carboxymethylcellulose, arabic gum, alginate and λ-carrageenan. In contrast, κ-carrageenan was composed of a mixture of migrating components. The colloidal fractions of guar gum, locust bean gum, and corn syrup solids did not migrate in the electrical field.

Mixtures of carboxymethylcellulose with guar gum or gum arabic could be separated at pH 7. Mixtures of carboxymethylcellulose with carrageenan could be effectively separated at pH 2. Electrophoretic separation at 23°C of different stabilizer mixtures was unsatisfactory and the patterns showed evidence of interactions.

INTRODUCTION

INTEREST IN THE CHARACTERISTICS and mechanism of stabilizer action prompted an examination of the electrophoretic purity and behavior of a number of hydrocolloids. Published findings indicate that zonal electrophoresis (Lewis *et al.*, 1967) as well as moving boundary electrophoresis (Asano, 1966; Colvin *et al.*, 1952; Northcote, 1954; Smith *et al.*, 1954; Smith *et al.*, 1955; Ward *et al.*, 1947; and Whistler *et al.*, 1965) can be used for this purpose. Lewis *et al.* (1967) employed electrophoresis on glass-fiber paper and reported that gum arabic, carrageenan, and agar are heterogeneous polysaccharides. Colvin *et al.* (1952) examined a number of polysaccharides dissolved in alkali by the moving boundary technique. They found inherent difficulties in the resolution of the patterns, but reported that this technique may be applicable for characterization and fractionation of the class of polysaccharides which are soluble close to neutrality. Whistler *et al.* (1965) suggested moving boundary electrophoresis as useful for the determination of polysaccharide homogeneity and for the determination of polysaccharide mixtures. Northcote (1954) measured the mobility of neutral polysaccharides, including starches and glycogens of different origins, in borate buffer. Apparently these colloids acquire a negative charge by interaction with the borate ion and, consequently, move in the electrical field. Excellent patterns were reported for this buffer system, whereas less satisfactory results were obtained with veronal and glycine buffers.

Smith *et al.* (1954) used free boundary electrophoresis in their study of carrageenan. Abnormalities associated with incipient gelation were encountered with concentrations higher than 0.2% for mixtures of the κ- and λ-fractions, but even at this low concentration they were unable to obtain a definite electrophoretic separation into components. Ward *et al.* (1947) have reported a detailed electrophoretic investigation of pectinates. Recently, Asano (1966) studied the interaction between milk protein and carboxymethylcellulose in fruit-flavored milk by moving boundary electrophoresis.

The present investigation was undertaken in order to study the electrophoretic behavior of common food stabilizers as single entities and as related mixtures.

PROCEDURE

Instruments

Electrophoresis was performed with a Perkin-Elmer Model 38-A electrophoresis apparatus employing a 2 ml

Tiselius cell of open design. The conductivity was measured with an L & N portable electrolytic resistance indicator using the Perkin-Elmer conductivity cell. The viscosity was measured with a Brookfield L.V.F. viscometer.

Sample preparation

The following materials were used: sodium alginate KGHV and KGLV (Kelco Company, San Diego, California); potassium salts of λ- and κ-carrageenan (Marine Colloids, Inc., Springfield, New Jersey); sodium carboxymethylcellulose, 7-HP and 7-LP, with a designated substitution range of 0.65 to 0.85 (Hercules Powder Company, Wilmington, Delaware); and commercial grades of gum arabic, guar gum, locust bean gum, and corn syrup solids type 36 DE (10% nondialyzable matter). The samples were prepared by dissolving at 60°C a weighed amount of dry stabilizer in buffer. The high viscosity of stabilizer solutions limited the sample concentration to approximately 0.5%.

After cooling, the solutions were dialyzed against 20 volumes of buffer for at least 24 hr at 5°C. Insoluble material, if present, was removed by centrifugation at 1000 G for one hour. The amount of insoluble material was estimated by weighing the sediment after drying at 110°C until constant weight (12 hr). Only locust bean gum and guar gum contained significant amounts of insoluble material (7 and 18% respectively).

Preliminary investigations revealed that the use of conditions similar to those commonly employed for the study of protein systems gave rise to considerable boundary disturbances. These consisted mainly in the appearance of a large number of peaks in the sample side of the Schlieren patterns which did not reproducibly reflect a heterogeneity of composition. Inspection of the patterns revealed the presence of both positive and negative refractive index gradients, similar to those observed if the system has not attained temperature equilibrium.

It is questionable, however, that unequal temperature arising from slow heat dissipation was the responsible factor, since the abnormal patterns did not occur when electrophoresis was performed on equally viscous solutions of neutral stabilizers (guar gum and locust bean gum) in which Joule heating would also be generated. The phenomenon appeared to be related to the rate of electrophoretic migration, and was particularly pronounced for stabilizers of high electrophoretic mobility. It was found that a reduction in the field strength to a value below 2 volts/cm effectively prevented these disturbances in most cases. These conditions were achieved by using an auxiliary power supply delivering 2 to 5 ma at 10 volts for a buffer of a specific resistance of 125 ohms.

Electrophoresis at 0–2°C

The electrophoresis cell was filled according to the usual procedures and then mounted in the tank of the instrument. The boundaries were moved into view at room temperature (23°C) at a slow, constant rate by injecting buffer into the descending-side buffer bottle. This was accomplished by means of a capillary tube connected to a closed glass bulb, equipped with platinum electrodes and filled with buffer, in which gas pressure was produced electrolytically.

The time requird to move the boundaries into view by this technique could be regulated conveniently by adjusting the flow of current between the electrodes, and a rate of movement of 1 cm/hr was sufficient to prevent initial boundary disturbances. After the boundaries had been shifted into position, the temperature was reduced to 0–2°C by ice water, and approximately 60 min were allowed for the system to reach temperature equilibrium.

The field strength used for electrophoresis was calculated from the relationship $\frac{I}{k \times a}$,

where I = current (amperes)
k = specific conductance of sample ($ohms^{-1}$ cm^{-1})
a = cross sectional area of cell (cm^2).

This value did not exceed 2 volts/cm.

Electrophoresis at 23°C

For a number of samples, which were either highly viscous or showed tendency towards gelation at 0–2°C, it was possible to perform electrophoresis at room temperature, if the field strength was reduced drastically. A similar procedure has been used successfully by Johnson *et al.* (1948) for analyzing colloids which were only sparingly soluble at lower temperatures. No special thermostat was necessary, since the instrument was located in a constant-temperature room at 23 ± 1°C, and the resulting temperature fluctuations of the water bath during electrophoresis were negligible. The field strength used did not exceed .2 volts/cm. Mobility measurements obtained at this temperature were corrected to 0°C by multiplying the observed values by the ratio of buffer viscosity at 23°C to buffer viscosity at 0°C (Johnson *et al.*, 1948; and Ward *et al.*, 1947).

The expected linear relationship between solute mobility and buffer viscosity was confirmed by the experimental

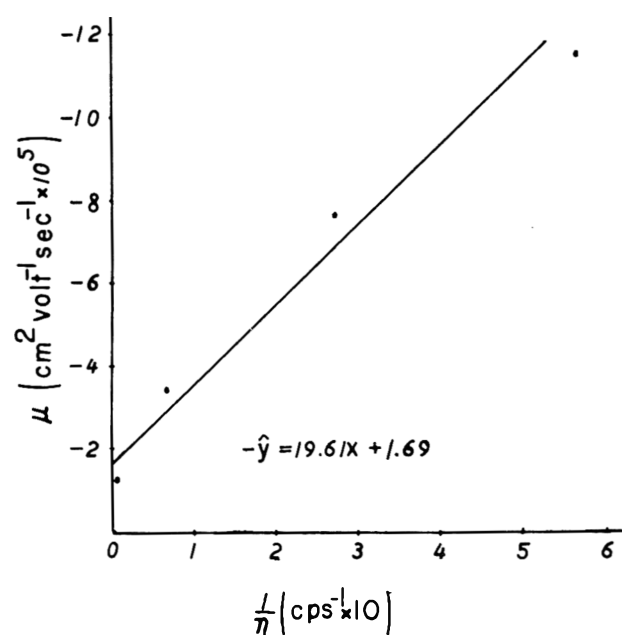

Fig. 1. Relationship between electrophoretic mobility of alginate and buffer viscosity at 0–2°C (sodium phosphate/sucrose buffer, pH 7, ionic strength 0.2).

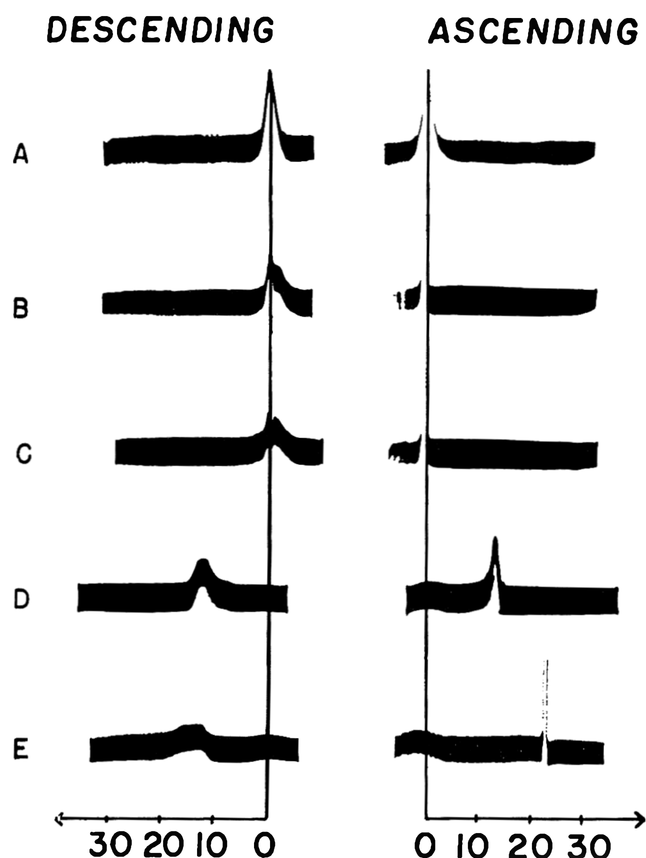

Fig. 2. Electrophoretic patterns of selected food stabilizers (sodium phosphate buffer, pH 7, ionic strenght 0.02. Mobility scale: — cm^2 $volt^{-1}$ sec^{-1} $\times$ 10^5).
A. Corn syrup solids (nondialyzable fraction: 10–15%)
B. Locust bean gum (0.3%)
C. Guar gum (0.3%)
D. Arabic gum (0.25%)
E. Carboxymethylcellulose (0.25%)

values in Fig. 1 which were obtained for alginate in sodium phosphate buffer containing different amounts of sucrose.

RESULTS

THE PATTERNS FOR A NUMBER of different stabilizers, including corn syrup solids, are shown in Fig. 2. At pH 7 the colloidal fraction of the corn syrup solids did not possess any net electrical charge since it did not move in the electrical field. Locust bean gum and guar gum did not contain components of appreciable net charge either, and the slight separation observed in the descending patterns for these neutral stabilizers was apparently caused by a displacement of the false boundary. No electrophoretic migration could be detected in their ascending patterns, which showed a hypersharp peak at the initial position.

No gross electrophoretic heterogeneity was revealed by the remaining stabilizers, although abnormal spreading of the descending patterns always occurred and, in most cases, precluded accurate mobility measurements. The ascending boundaries remained sharp and appeared to be more suitable for mobility calculations except that, at low ionic strength, the ascending mobilities were considerably higher than those calculated from the descending side and were sensitive to small changes in the ionic strength.

The effect of variations in the ionic strength of the buffer on the electrophoretic patterns of sodium alginate at 0–2°C is illustrated in Fig. 3. At increasing ionic strength, the inequality of the mobilities from the ascending and descending patterns decreased. For both patterns, the higher ionic strength suppressed the electrophoretic mobility, but the effect was most pronounced for the ascending pattern. Apart from the influence of buffer salt upon the electrophoretic mobility, the shape of the peaks remained the same at all salt concentrations.

Electrophoresis of λ-carrageenan presented special problems which were manifested by gross disturbances in the descending pattern arising when the initial boundary was moved into view. Inspection of the cell image revealed a penetration of buffer into the sample solution during boundary displacement, causing a blurred Schlieren pattern with false peaks (Fig. 4A). In contrast, the ascending side displayed a sharp and well-defined initial boundary at all times.

Attempts to perform electrophoresis on λ-carrageenan without shifting the initial boundaries into view did not prevent the gradual deformation of the descending boundary; nor did the addition of a surface active agent (0.01% sodium lauryl sulfate). The disturbances were completely eliminated, however, by performing the analysis at elevated temperature (23°C) or by reducing the pH of the buffer to 2 at 0–2°C. Patterns obtained under these conditions are shown in Fig. 4B and 4C and show that clearly-defined patterns were resolved at sample concentrations as high at 0.5%. In both cases, the stabilizer appeared electrophoretically homogeneous. The mobilities observed at pH 2 were significantly lower than at pH 7.

Electrophoretic patterns of κ-carrageenan are shown in Fig. 5. For this stabilizer, consistent data could not be obtained in neutral buffer at 0–2°C, because at this pH and temperature the mobilities were found to be dependent upon the concentration of the sample. Thus, the κ-carra-

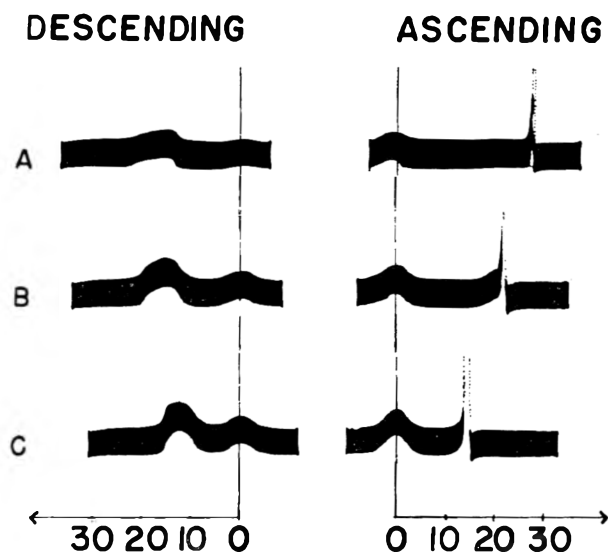

Fig. 3. Effect of buffer ionic strength on the electrophoretic mobility of 0.5% alginate (sodium phosphate buffer, pH 7. Mobility scale: — cm^2 $volt^{-1}$ sec^{-1}).
A. Ionic strength 0.02
B. Ionic strength 0.10
C. Ionic strength 0.20

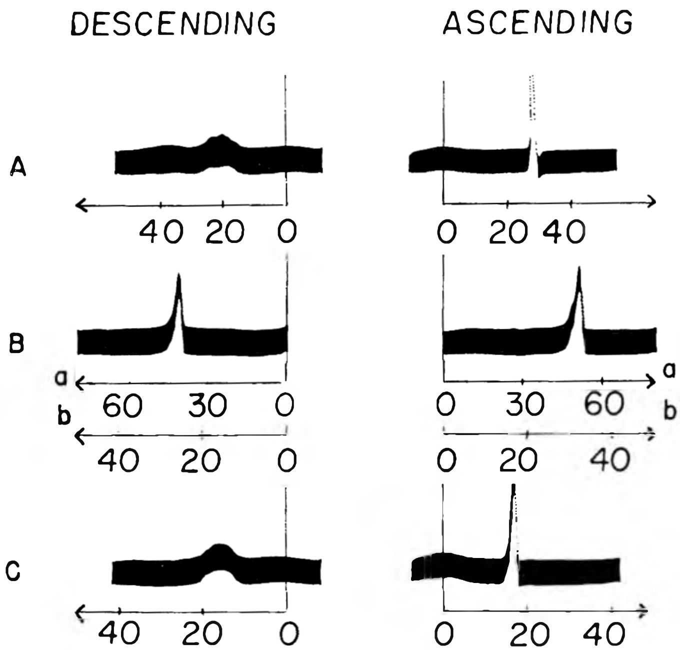

Fig. 4. Electrophoretic patterns of 0.5% λ-carrageenan (buffer ionic strength 0.2. Mobility scale: — cm² volt⁻¹ sec⁻¹ × 10⁵).
A. Electrophoresis at 0–2°C. Sodium phosphate buffer, pH 7
B. Electrophoresis at 23°C. Sodium phosphate buffer, pH 7 (a = mobility at 23°C; b = mobility at 23°C, corrected to 0°C for buffer viscosity change)
C. Electrophoresis at 0–2°C. Sodium chloride buffer, pH 2.

geenan exhibited a mobility of close to 15 electrophoretic units at low concentration (0.2%), but only 2 electrophoretic units at the concentration of 0.5% (Fig. 5A). A similar behavior was not found for the other stabilizers investigated and it is possible that incipient gelation at the higher concentration may have retarded the migration of κ-carrageenan.

Well-defined electrophoretic patterns which were not affected by the sample concentration within the range of 0.2–0.5% were obtained for κ-carrageenan in phosphate buffer of pH 7 at 23°C (Fig. 5B), and in sodium chloride buffer of pH 2 at 0–2°C (Fig. 5C). Multiple peaks in both patterns revealed this stabilizer fraction to be a heterogeneous mixture.

Solutions containing mixtures of stabilizers were subjected to electrophoresis to explore the conditions under which the individual components may be identified by this technique. The patterns for a mixture of carboxymethylcellulose and arabic gum in Fig. 6A revealed that a separation of the pair was achieved. A comparison of the mobilities calculated from this pattern with those obtained singly (Fig. 2), show some decrease in the mobility of carboxymethylcellulose with a corresponding increase in that of the arabic gum.

A similar effect was also observed for other pairs of stabilizers. Therefore, effective separation can be achieved only if the individual mobilities are sufficiently different. In the case of carboxymethylcellulose, alginate, and carrageenan, the individual mobilities at pH 7 did not permit a definite separation of their mixtures; however, as shown in Fig 6B and 6C, a complete separation of carrageenan from carboxymethylcellulose was possible at pH 2, because at this pH carboxymethylcellulose did not migrate.

The low concentration of individual components in mixtures of stabilizers made an accurate evaluation of their electrophoretic patterns difficult. An apparent increase in

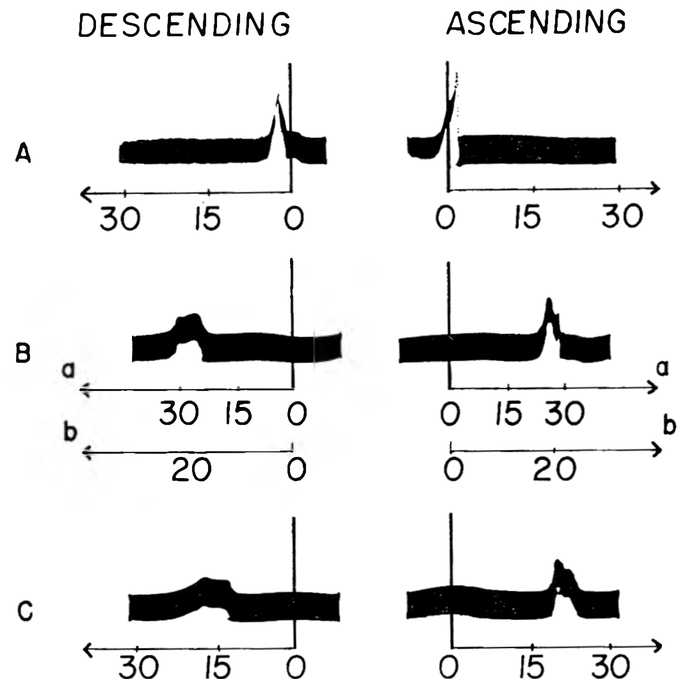

Fig. 5. Electrophoretic patterns of 0.5% κ-carrageenan. (Buffer ionic strength 0.2. Mobility scale: — cm² volt⁻¹ sec⁻¹ × 10⁵).
A. Electrophoresis at 0–2°C. Sodium phosphate buffer, pH 7
B. Electrophoresis at 23°C. Sodium phosphate buffer, pH 7 (a = mobility at 23°C; b = mobility at 23°C, corrected to 0°C for buffer viscosity change)
C. Electrophoresis at 0–2°C. Sodium chloride buffer, pH 2.

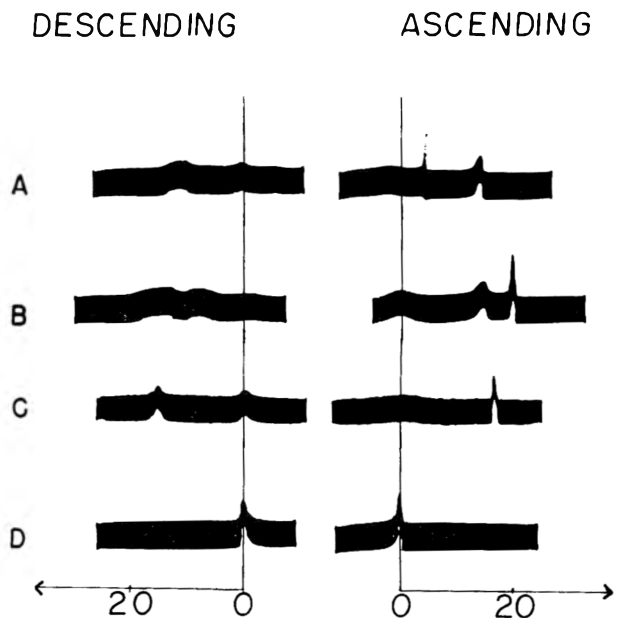

Fig. 6. Electrophoretic separation of mixtures of stabilizers at 0–2°C. (Mobility scale: — cm² volt⁻¹ sec⁻¹ × 10⁵).
A. Guar gum and alginate (concentration: 0.3%. Sodium phosphate buffer, pH 7, ionic strength 0.02)
B. Arabic gum and carboxymethylcellulose (concentration: 0.3%. Sodium phosphate buffer pH 7, ionic strength 0.02)
C. Carboxymethylcellulose and λ-carrageenan (concentration: 0.3%. Sodium chloride buffer, pH 2, ionic strength 0.1)
D. Carboxymethylcellulose (concentration: 0.15%. Sodium chloride buffer, pH 2, ionic strength 0.1).

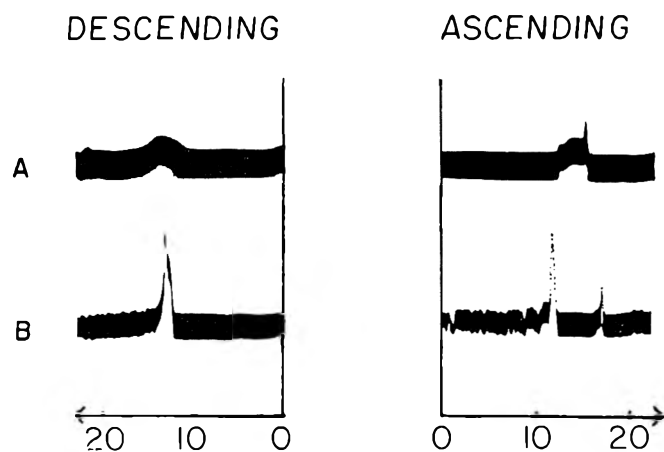

Fig. 7. Electrophoretic separation of mixtures of carboxymethylcellulose, carrageenan and guar gum at 23°C. (Sodium phosphate buffer, pH 7, ionic strength 0.2. Mobility scale: — cm^2 $volt^{-1}$ sec^{-1} $\times 10^5$).

A. κ-carrageenan, carboxymethylcellulose and arabic gum (concentration 0.3%)

B. *λ-carrageenan, carboxymethylcellulose and guar gum (concentration 0.6%)*

viscosity or tendency towards gelation in some of the combinations imposed a further limitation upon the maximum sample concentration for which electrophoretic patterns could be obtained at 0–2°C. Analysis of such mixtures at elevated temperature (23°C) permitted the use of higher sample concentration but gave evidence of interaction under these conditions.

The ascending patterns for the two mixtures in Fig. 7 show multiple peaks, while only one peak was apparent in the descending side. Such lack of enantiography may possibly be explained by a reversible binding of undissociated buffer acid to the colloids (Cann *et al.*, 1965) or by an interaction between the colloids in the mixture. The irregular patterns obtained in Fig. 7B illustrate the analytical difficulties encountered when gross interaction occurs.

DISCUSSION

THE RESULTS OBTAINED in this study suggest that free boundary electrophoresis may be useful in studies on food stabilizers. However, the method is of limited value for neutral stabilizers, except in analysis of their mixtures with colloids containing ionized groups.

The examination of corn syrup solids was included in order to compare this material with other stabilizers, since Wolfmeyer (1963) has pointed out that the higher polysaccharides present in these products may have attributes similar to some of the vegetable gums. According to our analyses, the particular sample investigated contained approximately 10% of non-dialyzable polysaccharides, which at the levels of corn sweetener used in many foods could exert an important influence on their texture. The electrophoretic neutrality of the material suggests its action would be similar to guar gum or locust bean gum.

The patterns obtained for single entities of stabilizers revealed these were, in general, homogeneous, except for κ-carrageenan. Such homogeneity is surprising, since a considerable range of molecular weights has been demonstrated for a number of stabilizers (Smith *et al.*, 1954; Ward *et al.*, 1947). Therefore, the observed electrophoretic homogeneity suggests that if different molecular sizes occur, these would either possess a uniform electrical charge per unit surface or would be complexed in a manner that would not permit electrophoretic separation. The electrophoretic analysis of mixtures of stabilizers gave evidence of some interaction, particularly at 23°C, but even for these patterns heterogeneity could be demonstrated on the ascending side.

The homogeneous patterns for λ-carrageenan obtained under different conditions (Fig. 4) are of interest because Smith *et al.* (1954) have reported heterogeneity of this fraction and assumed it occurred as the result of components possessing different hexose-sulfate ratios. Although heterogeneity cannot be discounted, the present study does not support that it would be due to components of varying ester sulfate content. There can be little doubt that the electrophoretic technique would detect differences in the degree of sulfation of carrageenan. Other studies showed that the heterogeneous patterns for κ-carrageenan reflected the presence of components of different ester sulfate content.

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Studies in Meat Tenderness. 5. The Effects on Tenderness of Carcass Cooling and Freezing Before the Completion of Rigor Mortis

SUMMARY—The tenderness of lamb loin is affected greatly by the time-temperature pattern imposed on the dressed carcass during the onset of rigor mortis. Very significant toughness develops in the longissimus dorsi muscles of carcasses exposed to low temperatures within about 16 hr of slaughter. This "processing toughness" is shown to be unrelated to the lack of aging. It appears to be due to muscle fiber shortening, earlier demonstrated to be responsible for massive toughening in excised muscles. Both cold shortening and thaw shortening are capable of toughness production, the latter type becoming prominent when meat, previously frozen before rigor completion, is cooked without a preliminary thawing.

INTRODUCTION

VERY APPRECIABLE SHORTENING may take place following the exposure to chilling or freezing temperatures of an excised muscle which is still in a pre-rigor condition. The length change may be due either to cold shortening or to thaw shortening; the former is the shortening observed when a pre-rigor muscle is cooled toward 0°C (Locker *et al.*, 1963), while the latter is the shortening which accompanies the rapid thawing of a muscle earlier frozen before rigor completion (Perry, 1950).

Recently, Marsh *et al.* (1966b) have demonstrated that very significant changes in tenderness accompany the length change, and have suggested that toughening of this shortening-induced type might occur even when the muscles are still attached to the skeleton.

It is the purpose of the present paper to extend these observations on excised muscles to the carcass. Until this has been done the earlier results are of little more than academic interest, for only rarely are muscles stripped from the skeleton before rigor onset. The previous study employed beef as its primary experimental material, but in the early stages of the present exercise it was suspected that the effects of muscle shortening on tenderness might be demonstrated more readily on the lamb carcass than on the side or quarter of beef.

The small size of a dressed carcass of lamb ensures a relatively rapid fall in its deep meat temperature toward that of the environment. Its longissimus dorsi muscle (LD) is large enough for tenderness evaluation both by taste panel and by tenderometer (Marsh *et al.*, 1966a). The LD is known to be capable of appreciable cold shortening (with accompanying toughening) when exposed to a cold environment after excision in a pre-rigor condition (Marsh *et al.*, 1966b). Furthermore, lamb as produced in New Zealand is relatively uniform in breed, nutrition and age at slaughter, these factors being of some importance when interanimal variability is to be minimized.

EXPERIMENTAL

LAMB CARCASSES were taken from the normal throughput of a large export meat-works. Whenever possible, all lambs for one experiment were taken from a single flock, but in a few cases (for instance in a series involving slaughter in both December and March) this was impractical. Lambs followed routine slaughter-house procedure to the time of weighing the dressed carcasses, 20–30 min post-mortem, but beyond this point several different experimental time-temperature patterns were imposed: these will be described later in the paper. Following freezing, the carcasses were stored at −12°C.

Cutting, pre-cooking and cooking procedures were described earlier by Marsh *et al.* (1966a). In addition, some experiments required the meat to be cooked without a preliminary thawing; in these cases the loins (pin-bone to ribs 5–6) were placed in the pre-heated ovens within 1 hr of their removal from the frozen store.

Sensory assessment of the LD was carried out as described by Marsh *et al.* (1966a), and objective assessment of tenderness was made on the mid-section of the cooked LD using the tenderometer of Macfarlane *et al.* (1966).

The pH attained by the muscle before the virtual arrest of glycolysis by freezing was measured by glass electrode on homogenates (Marsh *et al.*, 1950) of 1–2 g in 10–15 ml cold neutralized iodoacetate solution (2m*M*). The sample was removed from the frozen LD by a modified cork-borer, and was immersed in the iodoacetate solution before being cut finely with scissors and homogenized.

RESULTS

Plan of presentation of results

The first part of this report (experiments I, II) demonstrates the effect on lamb tenderness of a relatively rapid freezing of the carcass soon after slaughter and dressing. This is followed by an experiment (III) designed to establish that it is the time post-mortem at which freezing temperatures are applied, rather than the rate of freezing, which determines the tenderness of the meat. Two experiments (IV, V) are next described to show the effect on tenderness of increasing the delay between slaughter and the commencement of the freezing operation. Finally, it is demonstrated that both thaw shortening (experiments VI, VII) and cold shortening (experiments VIII, IX) are capable of producing excessive toughness in lamb carcasses.

Tenderness in relation to early rapid freezing and delayed slow freezing

The first major experiment (I) was designed to assess the relative effects on tenderness of weight, grade and

freezing pattern. Carcasses were selected for treatment at the time of weighing according to the following scheme.

Weight: 26 and 33 lb dressed weights, chosen for study because the average export lamb carcass weighs about 30 lb.

Grade: Prime and Y, defined respectively (Anon, 1965) as "good flesh content and finish," and "not graded as prime lambs because of conformation, less flesh or smaller fat coverage."

Freezing Pattern: Early rapid freezing (air at about −18°C with a velocity of about 500 ft/min, within 1 hr of weighing) and delayed slow freezing (24 hr at 15–20°C followed by exposure to relatively still air at −10°C).

All 8 (2^3) possible combinations of these variables were studied, 12 carcasses being allocated to each of the 8 blocks. The experiment was first undertaken on lambs killed in December (early summer), and was later repeated in its entirety on March-killed lambs; in all, then, 192 carcasses were involved. Following frozen storage of the carcasses, the left loins were removed by band-saw and were cooked after a standard thawing period of 24 hr at 5°C.

Within the assessment of the December-killed lambs (subjective only) and, later, of the March-killed lambs (subjective and objective), samples were randomized in order that panel members should not become aware of any developing trend or pattern. However, because of the desirability of storing the carcasses for comparable periods of time, December and March lambs could not be assessed together. No direct estimate can be made, therefore, of the effect of age, since we have earlier established the existence of minor fluctuations in the sensory assessment of tenderness (Marsh *et al.*, 1966a). In view of this element of uncertainty it would be unwise to compare sets of subjective evaluations made some months apart.

The results of the assessments of the 192 loins are shown in Table 1. Each score (± standard deviation, SD) is the mean of 72 evaluations (6 tasters × 12 loins) on a scale of 1 (very tough) to 9 (very tender).

The most obvious feature of the Table is the very great difference in tenderness between the lambs frozen rapidly soon after slaughter and those frozen slowly after a delay of 24 hr. For the 96 December-killed lambs the difference between mean tenderness scores for the two groups was 1.7 units, with a standard error (SE) of difference of 0.24. For the 96 March-killed lambs the corresponding values were 1.95 and 0.18. In each of the age-groups, therefore, the difference in post-mortem treatment caused a very highly significant difference in tenderness.

These differences due to post-mortem treatment were so great that possible effects of grade or weight might have been obscured. Consideration of only those groups which were frozen slowly after a delay of 24 hr does, in fact, indicate that these factors may have some effect on tenderness.

In the 48 December-killed slow- and delayed-frozen lambs, composed of equal numbers of Prime and Y grades, there was no significant difference between the scores of the two grades (Y 6.0, Prime 5.65, SE of difference 0.26). However, in these same lambs the mean tenderness of the 26-lb group (6.25) was significantly higher ($p < .01$) than that of the 33-lb group (5.4).

Later in the season, however, this situation was reversed. In the 48 March-killed slow- and delayed-frozen lambs no significant tenderness difference was found between the 26- and 33-lb groups (respectively 6.55 and 6.3; SE of difference 0.24). On the other hand a significant difference in tenderness ($p < .01$) due to grade was apparent in the same 48 carcasses, the Prime group scoring 6.75 and the Y group only 6.1.

The methods of cooking and assessment used in this study were quite capable, therefore, of detecting significant differences in tenderness in early lambs due to weight (or perhaps to rate of growth) and in later lambs due to grade (or possibly to fat content, since fat cover partly determines grade). Completely overshadowing these differences, significant though they were, was the effect of post-mortem treatment, the two variations of which produced a tenderness difference significant at far beyond the .001 probability level.

The magnitude of the effect is illustrated in Fig. 1 (combined December and March results, panel assessment of 192 lambs) and in Fig. 2 (March results only, tenderometer assessment of 96 lambs). It is clear from these histograms that the application of early rapid freezing has caused a mean fall in panel score of about 2 units relative to the values recorded for lambs frozen slowly after a delay. It has, in fact, introduced a degree of toughness (sensory scores 1–3, tenderometer values 40–80) not en-

Table 1. Effects on sensory tenderness score of grade, carcass weight and post-mortem treatment. Experiment I: 192 lambs.

December-killed								
Freezing	Early and rapid				Delayed and slow			
Grade	Prime		Y		Prime		Y	
Weight	26	33	26	33	26	33	26	33
Score ± SD	4.0 ± 1.0	4.6 ± 1.3	3.9 ± 0.9	4.1 ± 2.0	6.0 ± 0.8	5.3 ± 1.0	6.5 ± 0.5	5.5 ± 1.0

March-killed								
Freezing	Early and rapid				Delayed and slow			
Grade	Prime		Y		Prime		Y	
Weight	26	33	26	33	26	33	26	33
Score ± SD	5.1 ± 0.6	4.4 ± 1.2	4.4 ± 0.8	4.0 ± 0.9	6.9 ± 0.7	6.6 ± 1.0	6.2 ± 0.8	6.0 ± 0.7

countered at all among lambs frozen only after a delay of 24 hr.

In a small experiment (II) designed to confirm this effect of early rapid freezing with greater precision, 10 dressed lamb carcasses were halved longitudinally by band-

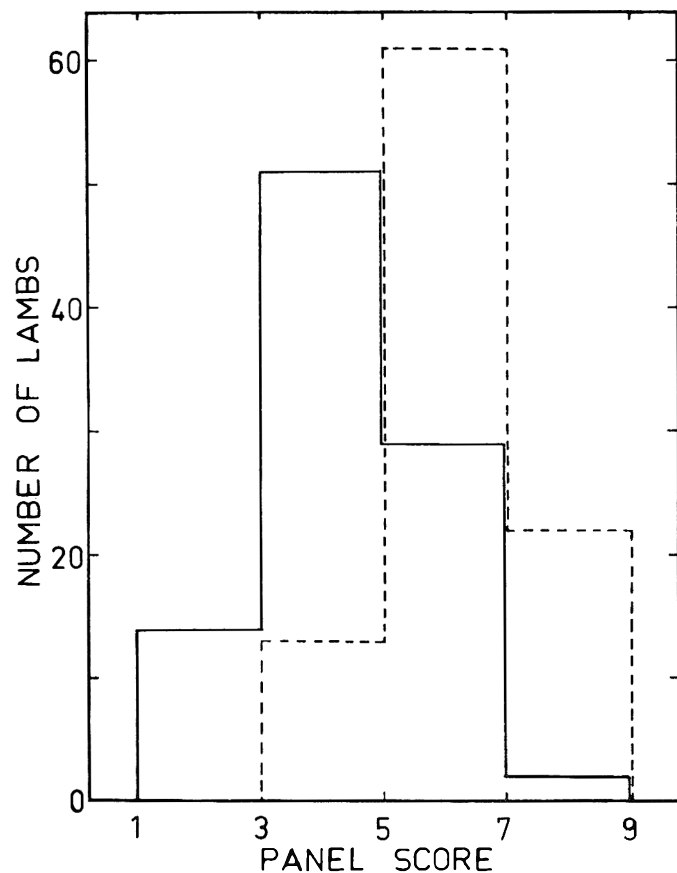

Fig. 1. The effect of post-mortem treatment on mean panel score for tenderness. ——— *Early rapid freezing;* - - - - - - - - - - *Delayed slow freezing; 96 lambs in each treatment.*

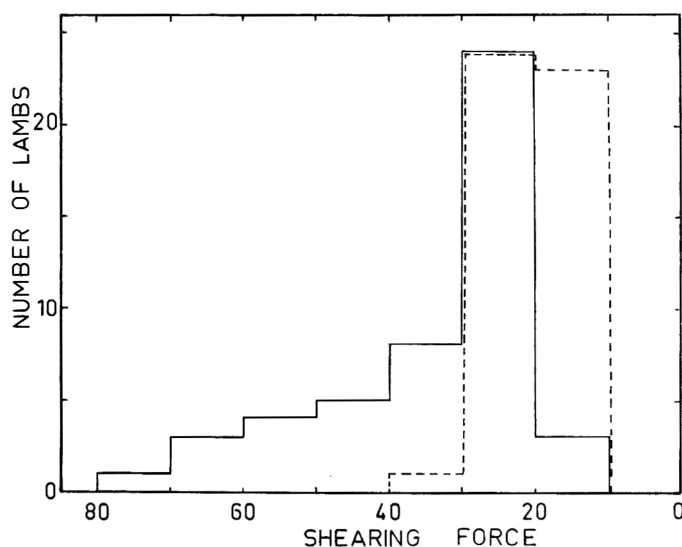

Fig. 2. The effect of post-mortem treatment on shearing-force requirement. ——— *Early rapid freezing;* - - - - - - - - - - *Delayed slow freezing; 48 lambs in each treatment.*

saw within an hour of slaughter. During and after sawing, considerable twitching of the musculature occurred. The effect was short-lived, however, the muscles returning to a relaxed condition within a few minutes of the treatment. In any case both sides of each carcass were stimulated to the same extent by the sawing operation which, therefore, could not have introduced a systematic difference between opposite sides.

The left sides were transferred immediately to a freezer (−18°C, air velocity 500 ft/min) and the right sides were held at about 20°C for 24 hr before being frozen in relatively still air at −10°C. After 3 months' frozen storage the loins of all 20 half-carcasses were removed, thawed (24 hr at 5°C), cooked and assessed by both panel and tenderometer. The mean difference (± SE) between paired loins in panel score was 2.0 ± 0.23, and in tenderometer value was 28 ± 5.0 units, the early-frozen sample being tougher in every case. These differences are very highly significant ($p < .001$).

Comparison of delayed rapid freezing and delayed slow freezing

The two post-mortem treatments applied in the above experiments differed both in length of delay before entry to the freezer (1 and 24 hr) and in rate of freezing (−18°C, 500 ft/min, and −10°C, still air). To ascertain which of these differences was responsible for the great tenderness difference between groups, a small experiment (III) involving 10 lambs was undertaken. The Y grade carcasses, all of 25–27 lb dressed weight, were left at about 20°C for 24 hr before being halved longitudinally. One side of each carcass was then frozen rapidly (−20°C, 500 ft/min) while the opposite side was frozen slowly in still air at −10°C.

After standard thawing and cooking the loins were assessed by panel in paired comparisons, and also by tenderometer. All loins from both groups were judged moderately tender to tender, the mean difference (± SE) in score between opposite sides being 0.0 ± 0.13 units. By tenderometer the mean difference in shearing force requirement between opposite sides was 3 ± 1.3 units, the rapidly frozen loins being more tender, though not significantly so ($p < .10$). It may be assumed that the large difference in tenderness caused in the first large experiment (I) by variations in post-mortem treatment was due to the difference in the delay period before application of freezing temperatures, and not to the difference in freezing rate.

Effect of varying delays before freezing

At this stage of the investigation two obvious explanations could be invoked to account for the great effect of post-mortem treatment on tenderness: aging and fiber shortening. A period of 24 hr at a temperature of about 20°C might well be expected to cause very considerable meat tenderizing, but there are serious objections to an aging hypothesis in the present work, as will be discussed later. The alternative explanation, fiber shortening (Locker, 1960), is a relatively new concept in meat tenderness research, but already much evidence has accumulated to show its relevance and importance (Marsh, 1964; Herring *et al.*, 1965; Marsh *et al.*, 1966b), particularly

when pre-rigor meat is exposed to temperatures capable of inducing cold shortening (Locker *et al.,* 1963).

To gain further information on this question of lack of aging or fiber shortening as the cause of toughness, two large experiments were carried out. In the first of these (IV), 49 lamb carcasses of 26–30 lb dressed weight were divided randomly into 7 groups of 7. Each group was held for a different period (½, 3, 6, 9, 12, 16 and 24 hr) at 18–24°C before being frozen fairly rapidly (−18 to −15°C, 500 ft/min). After 5–6 months' storage at −10°C one loin was removed from each carcass, cooked after a standard thaw at 5°C for 24 hr, and assessed by taste panel.

The results are illustrated in Fig. 3. The mean scores for the ½, 3 and 6 hr groups were virtually identical, and toward the tough end of the scale; delays of these lengths before entry to the freezer were equally ineffective, therefore, in tenderizing the meat. Carcasses delayed for 9 hr, however, had a distinctly higher tenderness rating, and this trend continued through the 12 hr group to the lambs delayed for 16 hr. No significant difference was detected between the groups delayed for 16 and 24 hr, all LD samples being at least acceptable to the panel even though several were still considered to be only moderately tender.

This pattern of a progressive improvement in tenderness was confirmed in another experiment (V) involving 3 groups each of 24 lambs (Y grade, 24–31 lb dressed weight). Mean tenderness scores for groups held 9, 12 and 16 hr at 18–21°C before entering the freezer were respectively 3.6, 4.5 and 5.1. The difference between the first 2 groups was very highly significant and between the last pair was highly significant (SE of differences 0.22).

It may be concluded from these two experiments that the insertion of a sufficient delay between slaughter and entry to the freezer causes a very real increase in tenderness, but the improvement does not occur gradually or steadily during the entire delay period. Rather, a three-phase picture is obvious, with little or no tenderizing during the first 6 hr of delay, a rapid and considerable change from then

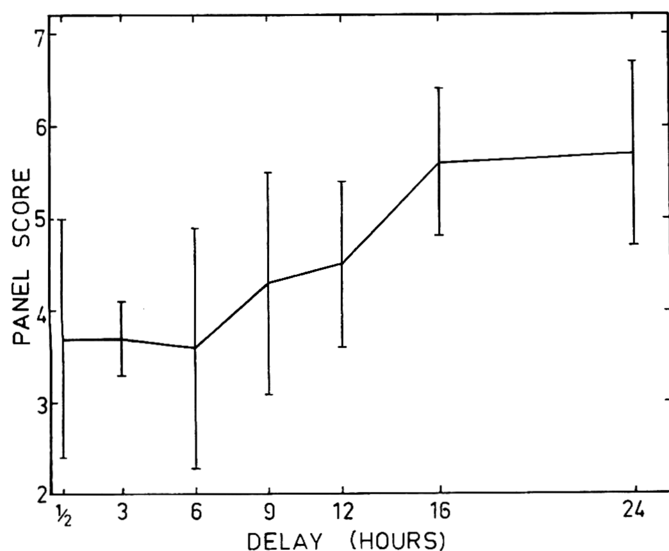

Fig. 3. The effect on panel tenderness assessment of delaying carcasses at 18–24°C before entry to freezer. Seven lambs per time-group. Vertical lines: Standard deviations.

till 16 hr or less, and finally a plateau extending to at least 24 hr. This pattern seems incompatible with any aging scheme which might be advanced. On the other hand it appears to run parallel to the physical changes of rigor mortis in lamb (Marsh *et al.,* 1958).

We suggest that lamb LD will be tough if cold-treated during the strictly pre-rigor phase, progressively less tough if exposed to cold with increasing delay into the rather prolonged rapid phase of rigor onset, and reasonably tender if the contractile muscle proteins are "locked" in full rigor before refrigeration is applied. This proposed relationship between tenderness and the rigor state at the time of cold exposure resembles that found in experiments with excised muscles (Fig. 6 of Marsh *et al.,* 1966b), with the difference that LD muscles on the carcass are unable to shorten to the high values attained very early post-mortem by isolated beef neck muscles.

Effect of thaw shortening

In the earlier study it was established that shortening and resultant toughening of excised muscles could be produced both by cold shortening and by thaw shortening. Attempts were made in the present investigation, therefore, to verify that the production of *either* type of shortening would cause toughening.

Experiment VI was designed to minimize or eliminate cold shortening in both LD muscles of a carcass and to permit thaw shortening to occur in only one of them. A suitable procedure was suggested by earlier observations on excised beef neck muscles, where it was shown that the shortening response to cold application becomes more delayed and more sluggish with the approach of rigor onset (Fig. 3 of Marsh *et al.,* 1966b). A rapid freezing applied at the right stage, therefore, should permit little or no cold shortening because of this diminishing response. At the same time it should arrest almost completely the glycolytic process at a point still far removed from full rigor, the muscle still being capable of appreciable shortening during later thawing. Thus in the first part of this experiment 8 lamb carcasses (Y grade, 24–31 lb dressed weight) were held at 20°C for 6 hr after slaughter and were then frozen rapidly (−21°C, 500 ft/min, no wraps). Both loins were then removed from each of the frozen carcasses.

The next phase of the treatment was based on the work of Marsh *et al.* (1958), who showed that, during a few days' storage of lamb LD (earlier frozen before rigor onset) at a temperature just below freezing point, normal glycolytic changes and rigor go to completion. The 8 left loins were held, therefore, at −3°C for 15 days before being returned to storage at −12°C, while the corresponding right cuts were stored at −12°C continuously. All loins were then cooked *without* a preliminary thaw in order that the rapid thawing during the first few minutes in the oven would accentuate any thaw-shortening tendency. No significant shortening would be expected in the cuts which had received treatment at −3°C since the chemical changes would have gone to completion during storage at that temperature. On the other hand, in the cuts held only at −12°C we would anticipate extensive shortening during the rapid oven thawing, since the chemical changes

leading to rigor had been prevented from reaching completion by the low storage temperature.

In each pair of cuts the left loin (treated at −3°C) was more tender than the right (untreated) loin, the mean differences (± SE) between pairs being 1.4 ± 0.21 panel units ($p < .001$) and 23 ± 6.0 tenderometer units ($p < .01$). Since the possibility of cold shortening was small, and in any case equal in the two groups, it is concluded that thaw shortening in one group only has been responsible for the large tenderness difference between treatments.

To confirm this finding, Experiment VII was carried out, using Y grade lamb carcasses of 25–27 lb dressed weight. Eleven of these were held at about 20°C for periods ranging from 1 to 9 hr (mean 5.3 hr) before being frozen fairly rapidly (−18°C, 500 ft/min). From the results of Experiments IV and V some degree of cold shortening would be expected in this group, and in addition the carcasses would have been frozen well before rigor completion, so appreciable thaw shortening might be anticipated with rapid thawing. In a second group, 7 carcasses were frozen at the same rate after a delay of 16 hr at 20°C; in these, cold shortening would not be expected at all (Experiment IV), but the possibility remains of freezing before the completion of rigor onset, so a small degree of thaw-shortening might occur with rapid thawing. In a third group 18 carcasses were frozen after a delay of 24 hr at 20°C. Neither cold shortening nor thaw shortening would be expected here, regardless of the rate of thawing, since the contractile proteins would be "locked" in full rigor before cold application.

Both loins were removed from all the frozen carcasses. The left cuts were thawed for 24 hr at 5°C before being cooked, and the right cuts were cooked with no preliminary thaw. By varying both the delay before freezing and the rate of thawing in this way, we would expect to produce wide variations in the extents of cold shortening and thaw shortening, with a consequent extensive range of tenderness values.

The results of this experiment are shown in Table 2, together with a summary of the shortening effects believed responsible for the evaluations in each treatment. In the 1–9 hr group, the mean difference (± SE) in shearing resistance between cuts capable of thaw shortening and those unable to thaw-shorten was 48 ± 6.3 units on paired cuts, a result of very high significance ($p < .001$). In the group delayed for 16 hr, the mean difference between paired samples—one thawed slowly and one rapidly—was 8 ± 2.6 units ($p < .05$). In the third group, all carcasses of which were delayed for 24 hr before freezing, the rate of thawing had no detectable effect; all cuts were satisfactorily tender, and obviously neither cold-shortening nor thaw-shortening had occurred at all.

Table 2. Effects of delay before freezing and rate of thawing on tenderometer evaluation of lamb LD (Experiment VII).

Delay (hr)	1–9	16	24
No. of cuts per thawing treatment	11	7	18
Slow thaw:			
Tenderometer (± SD)	41 ± 23	32 ± 6	34 ± 9
Type of shortening	cold	—	—
Rapid thaw:			
Tenderometer (± SD)	89 ± 16	40 ± 11	34 ± 9
Type of shortening	cold + thaw	slight thaw	—

This experiment therefore offers further evidence of the effect of shortening on tenderness, and in particular confirms the previous conclusion that thaw shortening alone can produce very appreciable toughening. At this point it is also appropriate to point out that, if the rapidly thawed 1–9 hr sub-group had been tough because of a lack of aging, we would be forced to postulate a remarkably rapid and peculiar aging pattern in the corresponding slowly thawed sub-group. A delay of up to 9 hr at 20°C would be virtually ineffectual in promoting tenderness; yet a thawing period of 24 hr, with internal temperatures mostly below freezing, would be very effective in the tenderizing process. Clearly the shortening-toughening hypothesis fits the facts much more readily than the aging-tenderizing theory.

Effect of cold shortening

The experiments just described have demonstrated that thaw shortening, uncomplicated by cold shortening, can produce tough meat. Two final experiments were undertaken to establish the converse: that cold shortening alone, with precautions taken to prevent thaw shortening, can also induce toughness.

In the first of these (VIII), 6 lamb carcasses (29 lb, Y grade) were used. To induce cold shortening, 3 of them were exposed to air at −18°C and 300 ft/min only 1 hr after slaughter, while the remaining 3 were frozen at the same time post-mortem but at a very fast rate obtained by applying crushed dry ice to both the internal and external surfaces. Thermocouples embedded deep in the LD muscles of this latter group established that the temperature fell through the zone of likely cold shortening (+ 10 to − 2°C) in only 20 min, a period known from unpublished experiments on excised muscles to be too brief to cause significant cold shortening.

The loins of all 6 carcasses were removed after temperature equilibration at −10°C. The possibility of thaw shortening was eliminated or minimized in both groups by cooking only after thawing for 24 hr at 5°C. Thus the two groups, equally lacking in aging and both thawed slowly before cooking to prevent thaw shortening, differed only in the likely extent of cold shortening.

The tenderness values of both LD muscles of all 6 carcasses were assessed by panel and tenderometer, and a large difference was found between the 2 groups. Those frozen extremely rapidly, and therefore without cold shortening, were scored 5.6 ± 0.6 (SD) by panel, and required a mean shearing force of 35 ± 4 tenderometer units, while the more slowly frozen (and therefore cold-shortened) were scored only 4.4 ± 0.7 and required a mean force application of 59 ± 9 units. The panel difference was significant at the .01 level and the tenderometer difference at the .001 level. Cold shortening alone, therefore, without the complication either of thaw shortening or of differential aging between the groups, caused a very appreciable toughening of meat still attached to the carcass.

In a related experiment (IX), 20 Y-grade carcasses of 25–30 lb dressed weight were held at about 24°C for 5 hr after slaughter to retard the tendency to cold-shorten. Ten of them were then placed, unwrapped, in a freezer (−21°C, 500 ft/min), while the remaining 10 were lightly insulated (a polyethylene bag sandwiched between 2 cloth bags) to lengthen the cooling phase, and were then transferred to the same freezer. One loin from each frozen carcass was thawed at 5°C for 24 hr before cooking to eliminate thaw shortening during the early stages of cooking.

The two groups were thus equally aged and equally incapable of thaw shortening. They differed only in their freezing rates, which would have determined the extent of cold shortening in their musculature; little or no shortening would be expected in the group frozen rapidly after a 5-hr delay (compare Experiment VI), but considerably more could take place in the slower-frozen group because of the longer exposure to temperatures in the cold-shortening zone before ice-crystal formation arrested further length changes.

To verify indirectly that different freezing rates had in fact been achieved, the pH values of samples of the frozen LD muscles from 5 carcasses of each group were measured. The faster-frozen group had attained a mean pH value (± SD) of only 6.00 ± 0.20, while the slower-frozen group had reached 5.67 ± 0.07.

The LD muscles of the more rapidly frozen lambs received mean tenderness ratings (± SD) of 5.1 ± 0.8 (panel) and 33 ± 7 (tenderometer), while the corresponding values for the slower-frozen group were 3.7 ± 1.0 and 54 ± 10. The respective differences are both highly significant (panel $p < .01$, tenderometer $p < .001$). Tenderness was affected very considerably, therefore, solely by the extent of cold shortening just before freezing, the other influences (aging and thaw shortening) having been equalized in the two groups.

DISCUSSION

THE BENEFICIAL EFFECT OF AGING has been established absolutely in many studies of meat tenderness, and it is logical therefore to seek an explanation of the results of the current investigation in terms of aging treatment. For a number of reasons, such attempts fail entirely to account for the observations.

If aging during the first few hours post-mortem were indeed responsible for the large effects found here, it would be necessary to assume that unaged lamb LD is very tough. These lambs were young, however, and had been raised on a fairly high plane of nutrition, so two important factors favoring tenderness—youth and rapid growth (Hammond, 1932)—had been met. Furthermore, many of the LD muscles of lambs frozen without delay were acceptably tender. Of the 48 early-frozen lambs used in preparing Fig. 2 for instance, 27 required shearing forces of less than 30 units, and forces of 40 or more were needed in only 13 cases. Thus in this group, frozen with no aging at all, more than half could be regarded as very acceptable, and only just over one-quarter as undesirable on the grounds of toughness. This result tells strongly against the suppositions that lamb is naturally very tough unless aged and that our observations can be interpreted in terms of aging or its lack.

The results of other experiments in this project give further support to the rejection of aging as a valid explanation of the observed effects. The pattern of increasing tenderness seen in Experiments IV and V is incompatible with any aging scheme which might be advanced. If, for instance, aging commenced at slaughter, a steady tenderizing would be expected from the start, perhaps with a decrease in rate as the internal temperature fell toward ambient and as the ultimate degree of tenderness was approached. If, on the other hand, aging commenced only with the completion of rigor, no tenderizing would be apparent for probably at least 12 hr, and the process would not be expected to cease abruptly at some time less than 16 hr post-mortem with a substantial degree of possible tenderizing still unachieved.

Evidence of a different kind is provided by Experiments VIII and IX, in both of which great differences in tenderness were found between groups which had received identical aging treatments. It is obvious, then, that aging is quite unable to explain the effects observed in this investigation, just as it failed completely to account for those detected in our earlier study on excised muscles.

The primary aim of this paper has been to extend our earlier work on tenderness from a scale using only excised muscles to one employing complete dressed carcasses. Although this object has been achieved, it is realized that the present report is not entirely self-supporting, for at several points it has been necessary to account for the observed effects in terms of the results of the previous laboratory-scale experiments. It was impractical to attempt the direct measurement of changes in LD fiber length produced by toughening treatments. We have chosen to link our observations on carcass lamb LD with the shortening-toughening relationship earlier established with some precision for excised beef neck muscle, and rather qualitatively, for excised lamb LD. In all cases the very great effects on tenderness which can be caused by variations in the normal time-temperature pattern are capable of ready interpretation by reference to the earlier study.

Only in one small respect—a failure to detect in lamb LD a second tender zone corresponding to beef neck shortening of 55–60%—do the two studies appear to diverge from parallel courses. We feel that this is due simply to the inability of LD muscles still attached skeletally to achieve shortening values of this magnitude, and presents no obstacle to the general supposition relating fiber shortening, meat toughening and the early post-mortem application of low temperatures.

Since cold shortening and thaw shortening can occur readily in the excised muscles of both sheep and beef animals, it might be supposed that the very appreciable toughening of meat on the carcass, as described in this work, could occur also in carcass beef. Before our results are extended from one species to another, however, consideration must be given to a most important relationship: that between the cooling rate of meat on the carcass and the time-course of rigor onset. The cooling rate is determined not only by ambient temperature, humidity and air velocity, but also by the size of the cooling body and the

depth below overlying tissue of the particular muscle being considered. The pattern of rigor onset is determined by the species (Lawrie, 1960), the characteristics of individual muscles within a single carcass (Lawrie, 1953), the ante-mortem treatment of the animal and the temperature treatment of the carcass after slaughter but before exposure to low temperatures (Bate-Smith *et al.,* 1949). The range of possible inter-relationships between cooling rate and rigor onset is therefore wide, and it would be unwise at this stage to assume the likelihood of toughness production in carcasses other than lamb.

Conversely, however, it would be equally unwise to assume that the phenomenon could occur *only* in lamb. Clearly, the introduction of earlier or faster chilling or freezing of the carcasses of any species (on the grounds, say, of speeding through-put or improving hygiene) should be preceded by an extensive and well-controlled study designed to detect any sign of the "processing toughness" described in this series of papers.

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The Effects of Freezing, Frozen Storage Conditions and Degree of Doneness on Lamb Palatability Characteristics

SUMMARY—Comparisons involving fresh versus frozen chops and roasts from 190 lamb carcasses indicated significant though nonconformable treatment effects on cooked-sample palatability characteristics. Freezing resulted in a highly significant increase in shear force values for loin chops and highly significant decreases in flavor, tenderness and overall satisfaction scores for leg roasts. Freezing rib chops, in contrast to the results for loin chops and leg roasts, resulted in a highly significant decrease in shear force values indicating an increase in tenderness as the result of freezing. Several possible explanations concerning these contradictory findings are suggested and discussed. In other paired-chop comparisons, higher final cooking temperatures resulted in increased shear force values for rib chops; wrapping samples prior to freezing appeared to have little effect on chop tenderness comparisons. In all comparisons, variance ratios between chops treated differently were not significantly altered.

INTRODUCTION

MODERN MEAT MERCHANDISING procedures employ freezing and frozen storage to preserve meat products in a condition which closely approaches that of the fresh product. Such preservation is accomplished by some degree of enzyme and/or microorganism inactivation and retardation of deterioration through the use of low storage temperatures. The effect of freezing on lamb palatablity has not been extensively studied, therefore, it is important from both industrial and research standpoints.

Recent consumer surveys by Lester *et al.* (1966) suggest that the housewife is increasingly aware of the advantages and disadvantages of frozen retail meats. Based on housewife interviews to determine product acceptance opinions, 44 to 50% of the homemakers preferred fresh lamb roasts, 27% frozen lamb roasts, and the remainder exhibited no preference. However, 82 to 88% of the interview group said they would purchase frozen lamb roasts if they were only available in that form.

Batcher *et al.* (1962) and Paul *et al.* (1964a, 1964b, 1964c) made important contributions to the knowledge of factors affecting lamb quality using frozen lamb samples. The use of frozen samples in such research emphasizes the need for greater understanding of the real effects of freezing meat on palatability traits and their measurement. Weir (1960) states that various investigators have reported no effect, increased tenderness, and decreased tenderness for freezer storage of beef. There is considerable variation in opinion regarding the tenderizing effects of freezing on the palatability of meat.

Unfortunately, much of what is now known concerning the effects of freezing applies to beef and pork and direct application to lamb cuts is not necessarily a direct consequence of their zoological relationship.

Hankins *et al.* (1940) reported significant differences in beef tenderness attributable to the temperature at which the meat was frozen. It seems probable that meat juiciness and cooking loss differences are also a consequence of the temperature at which freezing is accomplished. Birkner *et al.* (1960) concluded that the size, location and expansiveness of ice crystals is a consequence of the temperature at which freezing is accomplished. Yet, Child *et al.* (1937) reported that the overall palatability of meat was unaffected by freezing treatment.

Superimposed upon any objective or subjective attempt to measure the palatability traits of meat samples are differences in cooking technique. Weir (1960) reported differences in tenderness, juiciness, flavor and overall palatability ratings of beef steaks as a result of differences in the final degree of doneness to which the meat was cooked. The conclusion that increased degree of doneness decreases beef tenderness ratings is supported by the work of Cover *et al.* (1960).

In addition to the need for technical information of a practical nature applicable for lamb cuts, the advisability of storing research materials under frozen conditions for subsequent analysis needs to be ascertained. Many times in the process of performing routine organoleptic or shear force analyses for research or comparative purposes, insufficient attention is paid to the details of meat storage and cooking procedure. The actual effects of varying treatments upon meat samples are, as a whole, unknown. However, the implications drawn from previous investigations justify more thorough studies of sample handling and processing procedures.

This study was undertaken to investigate the effects of freezing lamb chops or leg roasts and the degree of doneness to which they are cooked upon palatability characteristics and their objective evaluation.

EXPERIMENTAL PROCEDURE

General procedures

Paired leg roasts from 47 lamb carcasses and either rib or loin chops from 190 lamb carcasses were utilized for sensory panel organoleptic analysis and Warner-Bratzler shear force determinations, respectively. A taste panel composed of eight semi-trained members was assembled and given preliminary training and instruction prior to the initiation of the actual testing sessions.

The technique referred to by Cover *et al.* (1960) as "oven-broiling" or "air cookery" was adapted for use in cooking the loin and rib chops in this study. Leg samples

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were prepared by standard oven-roasting procedures. The data were collected and subsequent phases of the study were contingent upon ideas developed as a result of previous stages of investigation.

Phase I

Forty-seven lamb carcasses ranging in weight from 25.8 to 67.9 lb and in grade from average choice to high prime (USDA standards) were stored for 6 days post-slaughter in a 1°C cooler. The carcasses were individually moved directly from the abattoir to the 1°C cooler for the ensuing 6-day storage period, so that the total time lapse from exsanguination to cooler entry was standard and not greater than 1 hr in length. Upon cutting, these carcasses provided 94 paired roasts (modified American leg roasts from the right and left sides) and 94 paired loin chops (1¼ in. thick chops from the anterior end of the right and left loins).

The leg roasts and the loin chops from the left sides of the carcasses were allotted to the frozen treatment group. Both cuts were double-wrapped in freezer paper and stored for 18 hr at −18°C (legs) or from 3 to 6 weeks at −23°C (loin chops). Following storage periods as described above, the legs and loin chops were thawed under 1°C cooler conditions to a uniform internal temperature of 1°C and the cooking and palatability tests performed.

The leg roasts and loin chops from the right sides stored for 18 hr after cutting at 1°C prior to cooking and subsequent analyses. Leg roasts representing fresh and frozen treatment sample pairs were roasted to an internal temperature of 70°C in a 177°C oven in groups of either four or six legs (two or three leg pairs) per cooking and tasting session. One-inch cores from the same anatomical position in each leg were presented to the sensory panel members for evaluation and scoring. A nine-point hedonic rating score ranging from 1 (dislike extremely) to 9 (like extremely), was assigned to each sample by the individual panel members for the following characteristics: flavor, juiciness, tenderness and overall satisfaction.

Each roast was then given a composite rating for each of the four palatability characteristics by averaging the eight independent scores assigned by the individual panel members. Each leg roast was weighed to the nearest gram prior to cooking (frozen legs were thawed at 1°C to a uniform internal temperature of 1°C and then weighed) and after cooking to facilitate measurement of evaporative and drip losses. After allowing the cooked roasts to cool to 22°C, three ½ in. diameter core samples taken from the *semimembranosus* muscle were used for shear force determinations.

The 94 paired loin chops were handled in a similar manner (i.e., weighed uncooked, oven-broiled to an internal temperature of 70°C in a 177°C oven, and weighed after cooking), but the tenderness evaluation consisted only of shear force determinations based on the average pounds of force necessary to shear three ½ in. diameter cores from the *longissimus dorsi* muscle.

These evaluations were conducted to achieve a comparison of: subjective flavor, juiciness, tenderness and overall satisfaction scores; proportionate cooking losses; and shear force value ratings for paired fresh versus frozen lamb cuts.

Phase II

Fifty-two additional lamb carcasses slaughtered and handled in the manner previously described for the phase I group of lambs and stored at 1°C for 6 days post-slaughter provided 208 chops which were used to compare their tenderness when handled by the following four methods: (a) fresh rib chops, unfrozen and stored at 1°C; (b) unwrapped chops frozen at −23°C (placed in a waxed cardboard container without having been wrapped); (c) wrapped chops frozen at −23°C (double-wrapped with freezer paper and placed in a waxed cardboard container); and (d) wrapped chops frozen at −34°C (double-wrapped with freezer paper and placed in a waxed cardboard container). The chops within cardboard containers were layered (two-deep) and spaced to insure as nearly uniform freezing rates for individual samples as possible.

Four rib chops taken from the 11th and 12th rib positions of the right and left sides of each carcass were randomly allotted (one chop from each carcass) to each of the four treatment groups. The fresh chops were stored for 11 to 14 days following cutting at the freezer temperatures required by the experimental design. Upon cooking to an internal temperature of 75°C in a 177°C oven an objective evaluation of muscle tenderness was achieved by shearing three ½ in. diameter core samples from each of the chops.

Phase III

To determine the effects on lamb tenderness of differing degrees of doneness as a result of final cooking temperatures, paired 11th and 12th rib chops from an additional group of 91 carcasses were obtained. These chops, from adjacent positions on the posterior end of the rack, were used to compare the tenderness of fresh rib chops oven-broiled to different final internal temperatures. The 12th rib chop from each lamb was cooked to an internal temperature of 75°C (well-done) and the 11th rib chop to an internal temperature of 65°C (medium-rare) in 177°C ovens. Three ½ in. diameter cores were used to objectively evaluate tenderness by utilization of the Warner Bratzler shear.

RESULTS AND DISCUSSION

Phase I

Statistical analysis of mean shear force values for fresh versus frozen loin chops indicates a significant ($P<.01$) decrease in tenderness as a result of freezing. Freezing did not affect cooking losses, however, and on the basis of the variance ratio test, freezing had no significant effect on the ratio of fresh versus frozen variances for either tenderness rating or proportionate cooking loss (Table 1). From these results one can conclude that although the actual value for shear force is affected by freezing, the differences between chops (i.e., the variation from chop to chop) is not significantly altered.

The 0.76 pound difference in shear force values and the 2.14 percent difference in cooking loss between treatments may be sufficiently large to warrant consideration from the

Table 1. Means, variances, and statistical analyses of shear force values and cooking loss data for lamb loin chops.

Trait	N	Mean	Variance	Statistical analysis
Fresh loin chop shear value (lb)	47	5.25	1.2844	$t = 4.17^{**}$
Frozen loin chop shear value (lb)	47	6.01	1.6161	$F = 1.2583$
Fresh loin chop cooking loss (%)	47	20.41	11.5320	$t = 1.36$
Frozen loin chop cooking loss (%)	47	18.72	17.4315	$F = 1.5116$

** $P < .01$.

standpoint of merchandising and industrial application. However, when considered from the standpoint of its application for research purposes, the lack of significant differences in variance between the two treatments implies that lamb chops maintain inherent structural differences and can be stored in a frozen state for subsequent analysis with little or no effect on the relative cooking loss percentages or tenderness scores.

The variance ratio test value for mean cooking loss percentage approached significance, and the values listed in Table 1 indicate a lower mean cooking loss percentage but an increase in cooking loss variance for frozen as opposed to fresh loin chops. In both cases, the results could be reasonably explained if moisture loss or dehydration occurred to a greater extent for some chops than for others in the frozen state. The degree to which such moisture loss occurred in this study is not known.

Statistical analyses of sensory panel scores, shear force values and cooking losses for fresh and frozen lamb leg roasts are reported in Table 2. In contrast to the results using loin chops, shear force values recorded for leg roast samples were not significantly affected by freezing. However, the mean shear value for frozen legs was 0.51 pounds greater than that for fresh leg roasts, indicating a trend in the same direction but of slightly less magnitude than that reported for frozen loin chops.

Cooking losses sustained by frozen legs were greater than those for fresh leg roasts, although the actual difference was again nonsignificant. When compared with loin chops, leg roasts have a much greater unit volume and much less proportionate surface area of exposed lean. As a result, it appears that if dehydration occurs during frozen storage it would not affect leg roasts to the extent that it would loin chops.

Many researchers believe that freezing affects meat juiciness in a negative manner, depending upon the degree of water crystallization and the size, location and expansiveness of the ice crystals formed (Birkner *et al.,* 1960). All of these factors are considered a direct consequence of the temperature at which freezing is accomplished.

Probably the most important factor influencing juiciness of cooked meat is the cooking procedure. Cooking procedures which result in the greatest retention of fluids and fat generally yield the juiciest meat. Cover *et al.* (1960) have reported that beef loin steaks broiled to the well-done stage (80°C internal temperature) are less tender as indicated by shear force values than are those cooked to the rare stage of doneness (61°C internally). The changes in tenderness and juiciness produced by cooking are closely related organoleptically since meat which becomes less juicy due to extended cooking and greater cooking shrinkage usually seems less tender.

In this study leg juiciness scores did not differ significantly between treatments. This evidence supports that elucidated from the cooking loss data. It would appear that little or no additional drip or evaporation loss resulted from the freezing-thawing procedure although

Table 2. Means, variances, and statistical analyses of sensory panel ratings, shear force values and cooking losses for lamb leg roasts.

Traits		Fresh[3]	Frozen[3]	Paired t value	F value[4]
	mean	6.23	5.96		
Flavor score[1]	variance	0.2516	0.2603	3.10**	1.0346
	mean	6.04	6.00		
Juiciness score[1]	variance	0.3103	0.3651	0.27	1.1766
	mean	6.27	5.72		
Tenderness score[1]	variance	0.6026	0.8728	3.08**	1.4484
Overall	mean	6.21	5.84		
Satisfaction score[1]	variance	0.3827	0.4358	3.16**	1.1388
Shear force	mean	5.16	5.67		
value[2] (lb)	variance	1.7609	1.4567	1.97	1.2088
	mean	20.91	22.07		
Cooking loss[2]	variance	25.1967	21.4147	1.46	1.1766

** $P < .01$.
[1] Higher numerical mean values indicate more desirable quality attribute.
[2] Lower numerical mean values indicate more desirable quality attribute.
[3] $N = 47$.
[4] All ratios computed as $\frac{\text{higher value}}{\text{lower value}}$ ratios.

such losses have been reported as a consequence of freezing by Birkner *et al.* (1960).

The nature and intensity of meat flavors depend in part on the type, length of time and temperature of cooking. Weir (1960) reported that lamb leg roasts cooked to an internal temperature of 65°C have an aroma and taste more distinctive of lamb than similar roasts cooked to an internal temperature of 75°C.

No explanation can be advanced concerning the highly significant differences in panel leg flavor scores in the present study since so little is presently known about this palatability trait. The release of volatile flavor components or fractions concurrent with some chemical or physical mechanism occurring during either the freezing or cooking processes might constitute a reasonable conjecture. Storage time appears to have been so brief that oxidative rancidity was not possible. At any rate, frozen legs were given significant ($P<.01$) lower ratings than were fresh leg roasts.

Subjective tenderness values for the leg roasts support those obtained from the analyses of shear force data for loin chops and the cooked *semimembranosus*. In this study, subjective and objective measurements of tenderness were apparently highly related. This is in agreement with the results of Ramsbottom *et al.* (1945), Cover *et al.* (1956), and Paul *et al.* (1956) in beef comparisons.

In our data, freezing significantly ($P<.01$) decreased leg tenderness scores but did not significantly alter the ratio of fresh to frozen leg roast variances. Since the variance ratio was not significantly affected by treatment, these data indicate that leg roasts can be stored in a frozen state, at least for brief periods of time prior to cooking, with reasonable confidence that variation between legs will not significantly change.

From a nonstatistical observation of overall satisfaction scores, they appear to lie midway between the values for flavor, juiciness and tenderness. The overall satisfaction score is no more closely related to tenderness than to flavor. Wider sample differences in any of these traits or the use of different panel members might result in overall ratings which were more closely associated with one or the other of these factors.

The highly significant difference in overall satisfaction scores in favor of fresh leg roasts does not agree with the findings of Child *et al.* (1937), who reported that palatability was unaffected by freezing treatment. It is apparent that differences in flavor and tenderness observed by the taste panel members are largely responsible for their preference for fresh as opposed to frozen leg roasts.

Lester *et al.* (1966) reported no differences in laboratory or consumer panel palatability ratings between fresh or frozen lamb roasts. Their results are not in agreement with those of the present investigation. While the range in weight of carcasses used in this phase of the study was rather wide, no attempt was made to relate carcass weight to tenderness values.

The reports of Marsh *et al.* (1966a and b) suggest that "cold shock" effects ascribable to differences in weight may affect palatability ratings. This plausible source of variation was not investigated in the present study, but no such trend appears in visual examination of these data.

Phase II

The divergence of opinion among researchers concerning the tenderizing effects of freezing may have resulted from the utilization of differential rates of freezing—that is, different speeds and/or temperatures employed for freezing. Hankins *et al.* (1940) reported significant differences in beef tenderness as a result of the temperature at which meat was frozen. Since rate of freezing has previously been implicated as an important factor in determining the effects of freezing on meat palatability, the data obtained from 208 lamb chops subjected to different freezing temperatures might help clarify the assumed relationship. The means and variances for shear force values by treatments are shown in Table 3. A complete randomized block design was used and the analysis of variance, employing orthogonal treatment contrasts, is shown in Table 4.

In this data, fresh lamb chops were significantly ($P<.01$) less tender upon cooking than chops subjected to frozen conditions. Nonsignificant differences were observed when wrapped chops frozen at −23°C or the mean of all chops frozen at −23°C (treatments B and C) were compared with wrapped chops frozen at −34°C. These data are in agreement with Hankins *et al.* (1940), who reported that frozen steaks were more tender than fresh (unfrozen) steaks and that freezing at −23°C and −40°C had significantly greater tenderizing affects than freezing at −7°C. However, they found no significant difference in tenderness between the steaks frozen at −23°C as opposed to those frozen at −40°C.

Table 4. Analysis of variance for rib chop shear force values.

Source of variation	df	Mean square	F values
Replications	51	14.85	
Treatments	3	37.47	22.17**
A versus B, C, and D	1	103.87	61.46**
C versus D	1	3.93	2.33
B and C versus D	1	4.61	2.73
Error	153	1.69	
Total	207		

** $P < .01$.

Table 3. Treatments, treatment means and variances for rib chop shear force values

	Treatment[1]			
	A Fresh (1° C)	B Frozen (−23°C) unwrapped	C Frozen (−23°C) wrapped	D Frozen (−34°C) wrapped
N	52	52	52	52
Mean	9.36	7.96	7.42	7.80
Variance	5.3274	5.6235	4.6614	4.3259

[1] Statistical analyses for means and variances are shown in Tables 4, 5, and 6.

Table 5. Variance ratio (F test) values by treatments for lamb rib chop shear force values.

Treatment[1]	Treatment[1] Frozen unwrapped (−23°C)	Frozen wrapped (−23°C)	Frozen wrapped (−34°C)
Fresh (1°C)	1.0556[2]	1.1429[2]	1.2315[2]
Frozen unwrapped (−23°C)		1.2064[2]	1.3000[2]
Frozen wrapped (−23°C)			1.0776[2]

[1] N = 52 for each treatment.
[2] All ratios computed as higher value / lower value ratios. All ratios were non-significant at the 95% probability level.

Variance ratio values for all possible treatment combinations indicated no significant change in variance as a result of treatments (Table 5). Duncan's multiple range test (Duncan, 1955) was used to compare treatments means at .05 and .01 significance levels (Table 6). Fresh chops were significantly (P<.01) less tender than chops from any of the frozen treatments. Differences between freezing treatments were small and although significantly different at the lower significance level (P<.05), displayed nonsignificant differences at the higher (P<.01) significance level.

Although it would appear that these results are in direct contrast to the data presented formerly (Phase I) in this report, the two groups of data are not necessarily contradictory. At least three variables must be considered in attempting to resolve the apparent disagreement. First, the time in frozen storage differed, being 21 to 42 days in the data from 94 chops and 11 to 14 days for the 208 chops utilized. Secondly, the temperatures used for storage and for cooking the two sets of chops differed. Until such time as the real effects of time and temperature of freezing are known, we can conclude only that tenderness appears to be altered by changes in one or both of these variables to an undetermined degree or extent. Finally, the two sets of chops were from different anatomical locations. The 94 chops were from the loin, while the 208 chops were from the rack. Since these two locations are adjacent anatomically (last rib chop-12th rib and the first loin chop-13th rib) and from the same muscle (*longissimus dorsi*), it is usually assumed that this variation would have negligible effect on the characteristics examined if the chops were taken from the same carcass. However, the magnitude of the difference observed (9.36 lb for rib chops and 5.25 lb for loin chops) for samples from different lambs suggests that the contrasting results may have been affected by sample location.

Table 6. Multiple range test for lamb rib chop shear force values.

Significance level	Treatment[1] C Frozen wrapped (−23°C)	D Frozen wrapped (−34°C)	B Frozen unwrapped (−23°C)	A Fresh (unfrozen) (1°C)
P < .05	7.42[2]	7.80[3]	7.96[3]	9.36[4]
P < .01	2.42[5]	7.80[5]	7.96[5]	9.36[6]

[1] N = 52 chops per treatment.
[2,3,4] Mean values followed by the same superscript are not significantly different at P < .05.
[5,6] Mean values followed by the same superscript are not significantly different at P < .01.

Phase III

The data collected from the 91 pairs of rib chops were analyzed using the paired-t test and the variance ratio test in order to compare degree of doneness effects on shear force measurements. The means and variances by treatment and the analyses are included in Table 7. Lamb rib chops cooked to 75°C internal temperature required 2.14 pounds of additional force to accomplish shearing than did those cooked to 65°C. The magnitude and direction of this difference compares favorably with that reported for beef loin steaks cooked under similar conditions by Cover *et al.* (1960).

A significant (P<.001) decrease in tenderness was observed when chops were cooked to the well-done stage (75°C internally). A nonsignificant increase in chop-to-chop variation occurred when chops were cooked to a higher degree of doneness. It seems apparent that a 10°C increase in final cooking temperature adversely affects shear force values. The effect of a smaller increase in final internal temperature is not presently known, but one can assume that even small temperature differences are important when comparing data from chops cooked at different times or under different cooking and temperature conditions.

In a recent study comparing fresh versus frozen lamb roasts, using both sensory and consumer panel analyses Lester *et al.* (1966) found no significant difference between overall palatability scores for fresh as opposed to frozen leg or shoulder roasts. They concluded that freezing had no significant effect on lamb roast palatability differences but that cooking method significantly affected palatability ratings.

These data also lend credence to the widely held theory that differences in cooking techniques or conditions may be as important in explaining meat tenderness differences as are handling, storage, quality or heritability. In comparing palatability characteristics of samples derived from different sources, extreme care must be exercised to eliminate obvious errors in analysis such as differences in cooking technique.

CONCLUSIONS

The increased utilization of frozen storage for the preservation of meat justifies attainment of a more com-

Table 7. Means, variance and statistical analyses of shear force values for rib chops cooked to different degrees of doneness.

	Treatment[1] 65°C	75°C	Statistical analysis
N	91	91	
Mean (lb)	7.70	9.84	t = 10.24***
Variance	4.2776	5.4113	F = 1.2653

[1] Final internal temperature achieved during cooking.
*** (P < .001).

plete and comprehensive understanding of the effects of freezing on muscle tissue palatability characteristics. Because of time and labor requirements, meat cuts are often stored in a frozen state for analyses at some future date. Because such storage includes a multitude of procedural differences, faulty or even grossly incorrect conclusions could be made, inadvertantly, as a result of tissue changes occurring as a result of only minor differences in procedure. It is hoped that this study will promote further and more complete studies into meat handling technology.

On the basis of these studies it is concluded that:

a. Highly significant changes in meat palatability characteristics occur as a direct result of differences in handling procedure.

b. Freezing lamb loin chops resulted in a highly significant increase in loin chop shear force value. Freezing leg roasts resulted in highly significant decreases in flavor, tenderness and overall satisfaction scores. Leg roast shear force values, though nonsignificant, support the conclusions reached from loin chop data and the sensory panel; that freezing decreases lamb palatability.

c. Cooking loss differences favor fresh leg roasts but frozen loin chops result in lesser drip and evaporation losses during cooking. In both instances, however, the difference between treatments was nonsignificant.

d. Freezing lamb rib chops, in contrast to the results from leg roasts and loin chops, resulted in a highly significant decrease in shear force values, indicating an increase in tenderness as a result of freezing. Differences in anatomical location, time in frozen storage, temperatures employed in freezing, and cooking temperatures may have contributed to these contrasting results.

e. Wrapping meat samples does not appear to be essential to achieving accuracy in shear force determinations; but it is believed necessary if other palatability characteristics (such as juiciness or cooking losses) are to be evaluated. Similarly, whether meat is frozen at −23°C or −34°C does not appear to affect tenderness comparisons to the extent that freezing at any temperature affects comparisons with unfrozen samples. However, based on treatment mean comparisons at the .05 level of significance both wrapping as opposed to storing meat unwrapped and the temperature at which freezing is accomplished (−23°C versus −34°C) display significant differences in resulting tenderness measurements and are important sources of variation in chop comparisons.

f. A difference of 10°C in the degree of doneness to which meat samples were cooked resulted in highly significant ($P<.001$) differences in shear force values. Such results make it imperative that this source of variation be precisely controlled in intra- or inter-animal or carcass comparisons.

Rather large differences in shear force variances values are evident between groups of carcasses utilized in the three phases of this study indicating that the lambs in phase I were a much more uniform group than those used in phases II and III. Throughout the course of these studies, the ratio of the variances between treatments has been evaluated. Such analysis was considered necessary because from a research standpoint, the investigator is usually more interested in the differences between samples than in the actual magnitude of the values *per se.*

If the sample-to-sample variation is not increased or decreased by some procedure difference, the actual values become of secondary importance and can be sacrificed when circumstances necessitate the use of a given procedure. In every instance in this study, the variance ratio (F test) values indicate nonsignificant changes in variance as a result of the treatment employed. Therefore, meat samples can be handled under a variety of conditions with little danger of significantly affecting sample-to-sample variation.

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Degradation of Inosinic Acid in Chicken Muscle During Aseptic Storage and Its Possible Use as an Index of Quality.

SUMMARY—Analysis of chicken breast and leg muscle stored under aseptic conditions at 0, 5, and 10°C showed that the breakdown of inosinic acid and formation of hypoxanthine depended on storage temperature and time. Over 75% loss of inosinic acid content of both breast and leg muscle occurred in 3–5 weeks at 0°, in 2–3 weeks at 5° and in about 1 week at 10°C. These storage periods correspond to the lengths of time after which quality deterioration has been detected by sensory evaluation. During the same periods of storage, the hypoxanthine content of these muscles increased gradually to a value of 200–400 μg/g of muscle.

Since the level of inosinic acid in fresh muscle is fairly constant and related to flavor, the results indicate that an objective method of quality assessment of chicken meat based on inosinic acid content is feasible. A similar test based on the hypoxanthine content is also possible, but not likely to be as satisfactory, because losses occurring as a result of "weeping" and leaching in hypoxanthine content would show an apparent quality that is higher than it should be. On the other hand, an index based on inosinic acid would reflect similar losses by lowering of quality.

INTRODUCTION

WORK ON TASTE-PRODUCING SUBSTANCES has shown that ribonucleotides exert a major influence on the flavor of flesh foods (Kazeniac, 1961). The most abundant ribonucleotide in chicken muscle is inosinic acid which after the death of the bird quickly accumulates as a result of degradation and deamination of adenosine triphosphate (Davidek *et al.*, 1967; Terasaki *et al.*, 1965). Inosinic acid is then slowly degraded to inosine and hypoxanthine, mainly as a result of action of intrinsic muscle enzymes.

Since the degradation of inosinic acid to hypoxanthine has been associated with a loss of desirable flavor and with the development of bitter "off" flavor (Jones, 1963; Kazeniac, 1961; Spinelli, 1965), the measurement of inosinic acid and its breakdown products could form the basis of a useful objective test for the determination of meat quality. This paper describes changes in inosinic acid, inosine and hypoxanthine content of chicken muscle during storage at 0°, 5° and 10°C under aseptic conditions.

EXPERIMENTAL

SAMPLES WERE OBTAINED from one flock of chickens (broilers, Ottawa Meat Control Strain) that were raised and processed in the laboratory (Khan *et al.*, 1964). After aging for 24 hr in drained crushed ice, the carcasses were cut into halves. These halves were dipped in aqueous chlortetracycline solution (10 ppm) for 10 sec and then stored in sterile plastic bags at 0, 5 or 10°C for up to 6 weeks. Microbiological examination of samples (van den Berg *et al.*, 1963) showed no bacterial contamination in most cases. Results from experiments with samples found to be contaminated were discarded.

Analyses were made on pectoralis major and biceps femoris. Preparation of samples for analysis, and the extraction, separation and estimation techniques found suitable for the routine determination of inosinic acid, inosine and hypoxanthine have been described in detail elsewhere (Davidek *et al.*, 1967).

In brief, 20 g of minced sample was extracted with perchloric acid, and the inosinic acid, inosine and hypoxanthine in the extract were separated by paper chromatography and estimated by ultraviolet spectroscopy. A solvent system containing isopropanol : ammonia : water (7 : 1 : 2, v/v) was used for separating inosinic acid; and a solvent system containing n-butanol : acetic acid : water (4 : 1 : 5, v/v) was used for separating inosine and hypoxanthine. All analyses were made in duplicate and four samples were analyzed at a time.

RESULTS

THE AMOUNT OF INOSINIC ACID in both breast and leg muscle decreased with storage time and temperature (Fig. 1). Over 90% loss of the initial inosinic acid content of both breast and leg muscle occurred in 1–2 weeks at 10°C, in 3–4 weeks at 5°C and in 5–6 weeks at 0°C.

The inosine content of both breast and leg muscle increased rapidly during the initial 2–3 weeks of storage at 0, 5 and 10°C, and decreased thereafter in muscle stored

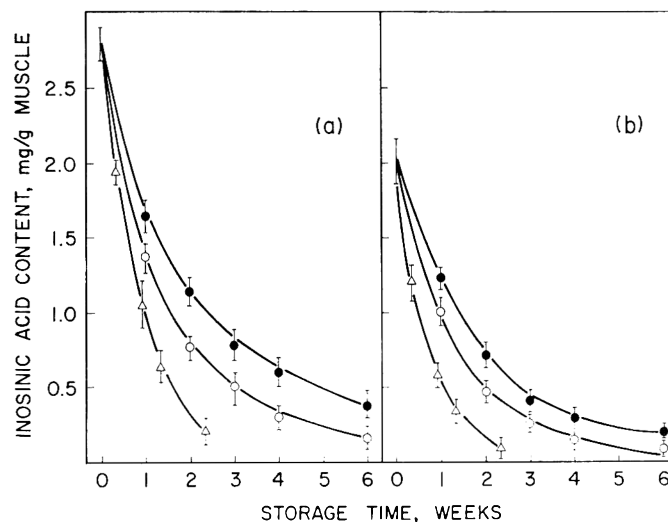

Fig. 1. Effect of storage on inosinic acid content of chicken muscle. a, pectoralis major; b, biceps femoris; ●, stored at 0°C; ○, stored at 5°C; △, stored at 10°C.

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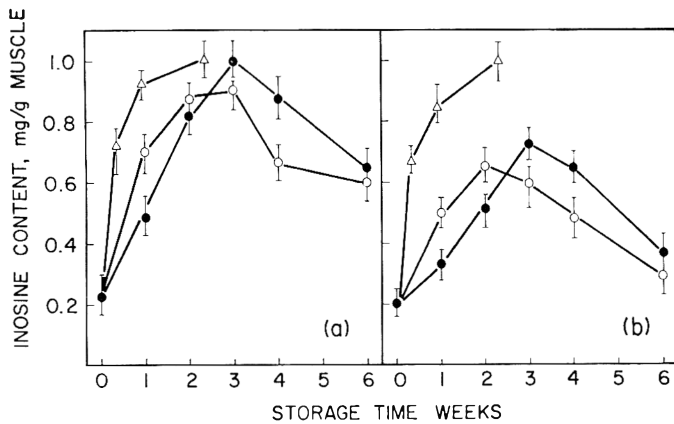

Fig. 2. Effect of storage on inosine content of chicken muscle. a, pectoralis major; b, biceps femoris; ●, stored at 0°C; ○, stored at 5°C; △, stored at 10°C.

at 0 and 5°C (Fig. 2.) Inosine is an intermediate product formed during the degradation of inosinic acid to hypoxanthine, and its concentration in muscle, therefore, depends on the rate of breakdown of inosinic acid to inosine and on the rate of formation of hypoxanthine from inosine.

The rates of formation and breakdown of inosine appear to vary with storage time. Terasaki *et al.* (1965) have shown that there is no apparent relation between the inosine content of muscle and the flavor of meat.

The amount of hypoxanthine increased with storage time and temperature in both breast and leg muscle (Fig. 3). Hypoxanthine is known to impart a bitter "off" flavor in fish (Jones, 1963; Kazeniac, 1961; Terasaki *et al.*, 1965), and the hypoxanthine content of fish muscle tissue has been used in an index of quality (Jones *et al.*, 1964; Spinelli *et al.*, 1965). It appears that accumulation of hypoxanthine is common in fish as well as in chicken muscle.

DISCUSSION

THE RESULTS SHOW that measurements of the amounts of inosinic acid and hypoxanthine present in chicken muscle can serve as useful indications of freshness and flavor, and

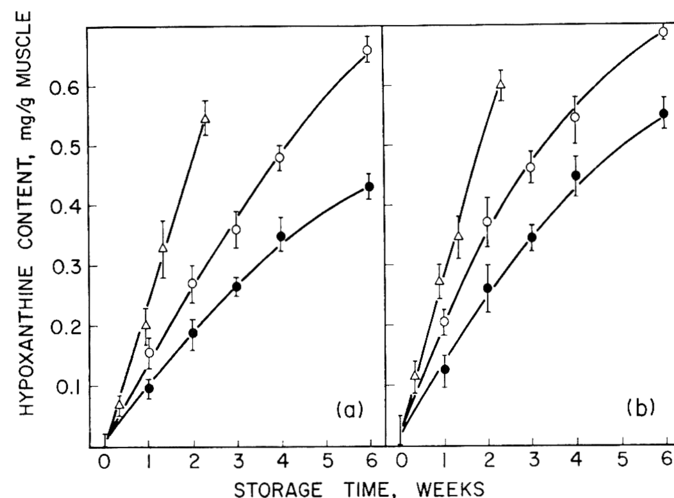

Fig. 3. Effect of storage on hypoxanthine content of chicken muscle. a, pectoralis major; b, biceps femoris; ●, stored at 0°C; ○, stored at 5°C; △, stored at 10°C.

hence of quality, of poultry meat. Measurement of inosine, however, cannot be used as an index of these attributes. The inosinic acid content is highest in fresh muscle and varies little with age of the bird, but decreases steadily during storage. Comparisons should be made between same muscle from birds slaughtered and aged under similar conditions (Davidek *et al.*, 1967).

The hypoxanthine content, on the other hand, was negligible in fresh chicken meat but increased gradually during storage as a result of degradation of inosinic acid. Reaction rate studies indicated that the reactions causing the disappearance of inosinic acid, and the accumulation of hypoxanthine during storage at above-freezing temperatures are of the first order, and that both of these parameters depend directly on storage temperature.

Most of the available chemical indices essentially measure bacterial spoilage; but the assay of inosinic acid and hypoxanthine would monitor both autolytic deterioration, as indicated by the present study, and bacterial deterioration as suggested by Kassemsarn *et al.* (1963).

A comparison of the results reported here with those obtained by organoleptic tests on chicken muscle stored under similar conditions (van den Berg *et al.*, 1964) showed that quality deterioration first detected by sensory evaluation after about one week of storage at 10°C, 2–3 weeks of storage at 5°C and 3–5 weeks of storage at 0°C, corresponds to the length of time required for loss of 75% or more of the initial inosinic acid concentration. This illustrates the relation between inosinic acid disappearance and quality as determined by the taste-panel method. During the useful storage life of poultry, based on this criterion, a 0.20–0.40 mg of hypoxanthine accumulates per g of muscle.

The value of an objective method based on the detection and measurement of degradation compounds produced by autolysis or microbiological growth is often limited by the fact that when significant amounts of these compounds have been produced, the meat is already in an incipient stage of spoilage. Therefore, an objecitve method based on the inosinic acid content of muscle, which is directly related to the flavor of meat, would appear to be more useful for evaluating quality in chicken meat than one based on the hypoxanthine content which depends on the extent of degradation of inosine.

Also, hypoxanthine is degraded further to uric acid and allantoin in avian muscle, and is subjected to loss by leaching and "weeping." The measurement of hypoxanthine in chicken muscle, therefore, would be subject to many errors and would show an apparent quality that is higher than it should be. The measurement of inosinic acid, on the other hand, would furnish direct information on freshness and flavor, and would reflect losses occurring as a result of leaching and "weeping."

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5′-Adenylic Acid Deaminase in Porcine Muscle

SUMMARY—The 5′-adenylic deaminase activity and AMP and IMP levels of several porcine skeletal muscles were determined on samples from 14 Poland China pigs excised immediately following exsanguination. There were no significant ($P > .05$) differences in adenylic acid deaminase activity within or between the longissimus dorsi, gluteus medius or rectus femoris muscles even though muscle morphology varied from dark, firm and dry to pale, soft and exudative. Likewise no significant ($P > .05$) differences in AMP or IMP levels were observed in the longissimus dorsi and gluteus medius muscles. Simple correlation coefficients between muscle pH and adenylic acid deaminase activity indicated positive relationships between enzyme activity and muscle pH at 15 and 45 min post-mortem in all muscles studied. IMP concentration was negatively related to pH of the muscle.

INTRODUCTION

Rate of post-mortem anaerobic glycolysis has been shown to have a profound effect upon the physical and biochemical properties of porcine muscle (Briskey *et al.*, 1961; Sayre *et al.*, 1963a). A rapid rate of glycogenolysis and low pH-high temperature conditions in the muscle have been implicated in the development of pale, soft, and exudative (PSE) porcine muscle (Briskey *et al.*, 1961; Bendall *et al.*, 1962). However, the factors responsible for the rapid glycolytic rate have not been elucidated.

It is recognized that AMP concentration influences the activity of certain glycolytic enzymes. Notably, phosphorylase *b* is able to degrade glycogen to glucose-1-phosphate in the presence of sufficient quantities of AMP (Cori *et al.*, 1943). Passonneau *et al.* (1962) reported that phosphofructokinase activity is increased by AMP.

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Although the post-mortem anaerobic glycolytic reactions catalyzed by the enzymes phosphorylase and phosphofructokinase have been shown to be rate limiting (Cori, 1956; Cori *et al.*, 1956; Passonneau *et al.*, 1962; Mansour *et al.*, 1962; Karpatkin *et al.*, 1964), Sayre *et al.* (1963 b, c and d) reported no significant relationship between activity *per se* of these two enzymes and the ultimate muscle morphology. Stetten *et al.* (1960) reported that phosphorylase *b* is active in living muscle only in the presence of AMP.

Sayre *et al.* (1963a) found most of the phosphorylase activity in porcine muscle was in the *b* form at 10 min post-mortem, and Morgan *et al.* (1963) suggested that AMP activation of the *b* form appears to be an important factor in the rapid rate of of glycogenolysis under anaerobic conditions. Additionally, adenylic acid deaminase is responsible for the degradation of AMP to IMP in muscle and activity of this enzyme could thus indirectly affect the rate of post-mortem anaerobic glycolysis. Abnormal adenylic acid deaminase activity has been observed in skeletal muscle of dystrophic mice (Pennington, 1961) and in humans afflicted with periodic hypokalemic paralysis (Engel *et al.*, 1964).

This investigation was initiated to study the adenylic acid deaminase activity of porcine muscle and to determine if the adenylic acid deaminase atcivity was related to rate of post-mortem glycolysis and ultimate muscle properties.

MATERIALS AND METHODS

Muscle samples from 14 market-weight (200–220 lb) Poland China pigs of similar breeding were used in this study. Samples of the longissimus dorsi, gluteus medius and rectus femoris muscles were excised within 10 min

following exsanguination and frozen immediately in liquid nitrogen. Muscle pH was determined at 15 and 45 min post-mortem, at 1 hr and at 30 min intervals thereafter until 3 hr post-mortem. Ultimate pH was determined at 24 hr post-mortem. Approximately 5 g of muscle were homogenized with 25 ml of 0.005*M* sodium iodoacetate and pH of the homogenate was determined. Values obtained with this procedure compared favorably with those obtained using the procedure of Marsh (1952).

Adenylic acid deaminase assay

Muscle samples were stored a maximum of 7 days at −20°C before being assayed for adenylic acid deaminase. Frozen storage for this length of time did not affect activity compared to that of fresh muscle. The enzyme was extracted from frozen muscle tissue as described by Lee (1963). The muscle was homogenized with 3.5 volumes of buffer (0.3*M* KCl, 0.09*M* KH_2PO_4 and 0.06*M* K_2HPO_4) at pH 6.5. The deaminase was extracted by stirring at 3°C for 1 hr. Assay of enzymatic activity was determined by the method of Lee (1963) with modifications suggested by Smiley *et al.* (1967).

The reaction was carried out in a total volume of 3 ml in the presence of 0.1*M* succinate and $1 \times 10^{-4}M$ AMP, pH 6.5. Rate of disappearance of AMP was determined by following the rate of decrease in absorbance at 265 mμ in a Beckman DU spectrophotometer equipped with a Gilford Model 20 absorbance indicator. Protein concentration was determined by the 280/260 mμ absorbance ratio. Specific activity of the deaminase (μ*M* AMP deaminated/min/mg protein) was determined by the formula (Smiley *et al.*, 1967):

$$\text{Specific activity} = \frac{\text{absorbance units decrease/min}}{(8.86)\ (\text{protein content per assay in mg})}$$

AMP and IMP determinations

Nucleotides were extracted from frozen muscle as described by Dannert (1966) with slight modification. Ten g of frozen muscle were homogenized with 50–60 ml of hot water in a VirTis homogenizer at maximum speed for 2 min. The homogenate was diluted to 100 ml, then centrifuged and the supernatant adjusted to pH 8.0. Ten ml of the supernatant were placed on an anion exchange column and the nucleotides separated as described by Macy *et al.* (1966).

The eluted peaks were quantitatively evaluated by the 2,4-dinitrophenylhydrazine (2,4-DNPH) derivative method of Lento *et al.* (1964). The quantity of 5′-mononucleotide present was calculated from a standard curve prepared for pure 5′-AMP rather than by use of extinction coefficients of the 2,4-DNPH derivatives.

Statistical analyses

Analysis of variance, standard error of the means and correlation coefficients were calculated according to the methods of Snedecor (1956).

RESULTS AND DISCUSSION

THE ADENYLIC ACID DEAMINASE ACTIVITY of the longissimus dorsi, gluteus medius and rectus femoris as well as the AMP and IMP levels of the longissimus dorsi and gluteus medius muscles are shown in Table 1. There were no significant (P > .05) differences in adenylic acid deaminase activity within or between any of the three muscles studied, even though ultimate muscle morphology varied from dark, firm and dry (DFD) to PSE (subjective score, as described by Briskey, 1964).

Table 1. Means and standard error of the means for adenylic acid deaminase activity, AMP, and IMP in porcine muscle.

Muscle	Adenylic acid deaminase[1]		AMP[2]		IMP[2]	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Longissimus dorsi	0.031	0.001	0.60	0.11	6.15	0.42
Gluteus medius	0.031	0.002	0.50	0.06	6.03	0.14
Rectus femoris	0.027	0.002				

[1] Adenylic acid deaminase activity = μM AMP deaminated/min/mg protein.
[2] μM/g fresh muscle.

However, the mean activity (μ*M* AMP deaminated/min/mg protein) in the rectus femoris muscle (0.027) was slightly lower than that observed in the longissimus dorsi (0.031) or gluteus medius muscles (0.031). These three muscles were specifically chosen because of their varying susceptibility to development of PSE morphology. The gluteus medius is most susceptible to development of PSE morphology, while the longissimus dorsi is intermediate and the rectus femoris is quite resistant.

The levels of AMP and IMP at 15 min post-mortem were not significantly (P > .05) different within or between longissimus dorsi and gluteus medius muscles (Table 1). The AMP concentrations in the longissimus dorsi and gluteus medius muscles were 0.60 and 0.50 μ*M*/g, respectively and the corresponding IMP concentrations were 6.15 and 6.03 μ*M*/g, respectively.

Few data concerning porcine muscle nucleotide levels are available in the literature and those values which have been reported were for muscle which had been held for varying lengths of time post-mortem. Nucleotide levels in such muscle would undoubtedly vary greatly due to enzyme degradation. For example, Dannert (1966) found 48 hr post-mortem IMP levels of 3.12, 3.23 and 2.98 μ*M*/g in porcine biceps femoris, longissimus dorsi and semimembranosus muscles, respectively, and corresponding AMP levels of 0.90, 0.33 and 0.33 μ*M*/g for those same muscles.

Simple correlation coefficients were calculated between adenylic acid deaminase activity and muscle pH at 15 and 45 min post-mortem to evaluate the relationship between enzyme activity and rate of pH fall (Table 2). Positive correlation coefficients were obtained between adenylic acid deaminase activity and muscle pH at either 15 or 45 min post-mortem in all three muscles studied. The correlations coefficients between deaminase activity and pH at 45 min ($r = 0.71$) in the gluteus medius and between enzyme activity and pH at 15 min ($r = 0.56$) in the rectus femoris muscle were statistically significant ($P < .01$ and $P < .05$, respectively).

These relationships indicate that muscles with more rapid post-mortem pH decline had lower 5′-adenylic acid deaminase activity and suggest the implication of this enzyme in conditions which favor rapid post-mortem gly-

Table 2. Simple correlation coefficients[1] between pH, adenylic acid deaminase activity, and AMP and IMP levels in porcine muscle.

	pH 15 min	pH 45 min
Longissimus dorsi		
Adenylic acid deaminase	0.29	0.41
AMP	0.23	0.00
IMP	0.11	—.41
Gluteus medius		
Adenylic acid deaminase	0.48	0.71
AMP	0.17	0.12
IMP	—.64	—.84
Rectus femoris		
Adenylic acid deaminase	0 56	0.43

[1] Correlation coefficients > 0.53 are significant at 5% level and those > 0.66 are significant at 1% level.

colysis. In some cases, pH had decreased measurably at 10 min post-mortem when the muscle sample was obtained. Whether this decline in pH had any effect on activity cannot be evaluated from these data. It should be noted, however, that the assay was carried out at pH 6.5, substantially below physiological levels.

Simple correlation coefficients between post-mortem muscle pH and AMP levels revealed no apparent relationship since all of the values were low and nonsignificant ($P > .05$). This observation seems to be contradictory to the relationship found between deaminase activity and post-mortem pH decline. The reason for these differences is not apparent from these data. IMP concentration was negatively correlated with post-mortem pH except for the low positive correlation ($r = 0.11$) obtained between IMP and 15 min pH in the longissimus dorsi muscle. The negative relationship was most pronounced in the gluteus medius muscle with the correlation between IMP and 45 min pH being significant ($r = -.84$, $P < .01$). This relationship would be expected since Briskey *et al.* (1961) reported that pH decline in porcine muscle was concomitant with the decrease in labile phosphate compounds.

Deficiencies in adenylic acid deaminase activity in skeletal muscle have been reported in certain pathological conditions. Pennington (1961) reported that 5′-adenylic acid deaminase was nearly 3 times lower in skeletal muscles of dystrophic mice than in muscles from normal mice. Engel *et al.* (1964) found a markedly lower activity of this enzyme in human skeletal muscles of patients suffering from hypokalemic paralysis.

Adenylic acid deaminase activity differences of this magnitude were not observed in this study with porcine muscles, even though a wide range of muscle morphology (DFD to PSE) and rates of post mortem pH decline existed among the pigs. The absence of consistently high positive relationships between adenylic acid deaminase activity and post-mortem pH values in porcine skeletal muscle supports the conclusion that activity of this enzyme is not highly related to the etiology of pale, soft and exudative porcine musculature.

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Dielectric Properties of Commercial Cooking Oils

SUMMARY—The finished drying of potato chips represents the first large-scale use of microwaves in the food industry on a continuous basis. Hence it was deemed desirable to study the dielectric properties of various types of commercial cooking oils.

The dielectric constant (ϵ'_r) and loss tangent ($\tan\delta$) of 11 commercial fats and oils have been determined at three different temperatures and at three different frequencies in the microwave range. The differences in dielectric properties among these fats and oils appear to be attributable to the phase (solid *vs.* liquid) of the material and generally correspond to the degree of unsaturation as evidenced by iodine values. The differences in loss factors among these fats and oils at any given temperature and frequency (within the range at which the measurements were made) are too small to be of any practical importance in selecting any one of them for use in heating processes using microwaves or in choosing an optimal frequency (300, 1000 or 3000 megahertz, MHz).

Data for one of the oils (number 9) were obtained over a wider range of frequencies and indicate that the loss factor peak(s) is/are found in the frequency range of 100 to 1000 MHz. Furthermore, from the data obtained for the other oils, it should be safe to assume that this oil is representative of dielectric properties of the entire group. The dielectric properties of bacon fat rendered by microwaves are almost identical to those of bacon fat rendered by conventional means.

INTRODUCTION

IN ANY PROBLEM related to heating, one of the first items of concern is the amount of energy needed. Regardless of the type of energy used, the basic answer can be simplified to

$$\text{ENERGY NEEDED} = \text{POWER} \times \text{TIME}$$

in which energy needed is in watt-hours and 1 BTU = 0.293 watt-hours.

Power, therefore, becomes the real determining factor, and the power which can be generated in a non-conductor placed in an electromagnetic field is shown by the following equation (Goldblith, 1967):

$$P = E^2 f \epsilon'_r \tan\delta \times 55.61 \times 10^{-14} \quad \text{[Eq. 1]}$$

in which P = power in watts per cubic meter
E = field intensity in volts per square meter
f = frequency in hertz
ϵ'_r = relative dielectric constant of the material and
$\tan\delta$ = loss tangent of the material.

It is now evident that the power which can be generated in a dielectric is dependent upon the voltage (E) applied, the frequency (f) used, the relative dielectric constant (ϵ'_r) and dielectric loss tangent ($\tan\delta$) of the material being heated. The product ($\epsilon'_r \times \tan\delta$) of these latter two factors is the dielectric loss factor (ϵ''_r).

A complicating factor in predicting heat generation by the use of microwave energy, however, is that both the ϵ'_r and $\tan\delta$ (hence ϵ''_r) for any material are functions of the operating frequency and of the ambient temperature. It now becomes clear that the frequency and temperature dependence of the dielectric loss factor of any given material must be known in order to predict its actual behavior as a dielectric. While there is a considerable amount of data available on the dielectric properties of food materials, very little is on frequencies in the microwave range.

A major use of microwave heating in the food industry is for finish drying of potato chips. This method has the tremendous advantage (over more conventional heating methods) of resulting in no additional darkening of the chips while they are being dried by the microwave energy. The work reported in this paper was undertaken in an effort to determine the dielectric properties of nine commercial fats and oils which are representative of the types used in the industry and to determine whether any of these possessed dielectric properties which would, *per se,* make its use advantageous for frying potato chips which were to be finish dried by microwaves.

EXPERIMENTAL MATERIALS

THE 11 FATS AND OILS FOR which dielectric properties are reported were supplied by the Food Research Division of Armour and Company, Oak Brook, Illinois. A description of the samples is presented in Table 1. Nine of the samples are commercial fats and oils; the other two are samples of bacon fat rendered by conventional means and by the use of microwaves.

EXPERIMENTAL METHODS AND EQUIPMENT

Dielectric measurements

Dielectric measurements on the 11 samples were made using the precision slotted line technique described by Roberts *et al.* (1946), Dakin *et al.* (1947), anonymous (1950), von Hippel (1954), Ginzton (1957), Soderman (1958), Westphal (1963), Beatty (1965), Gilmore (1966), and Westphal (1966). Details of the procedure and of the calculations have been described by Pace (1967). This procedure makes use of a coaxial transmission line, limiting the electromagnetic field to the inclosure of the line and resulting in the production of a standing wave when the transmitted wave is reflected back to the source by some terminating surface such as a metallic barrier (Fig. 1).

The field strength at any given point in the transmission

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Table 1. List and descriptions of samples of 11 commercial fats and oils.

Sample		Description
1	Soybean salad oil	Refined, bleached and deodorized
2	Corn oil	Refined, bleached and deodorized
3	Cottonseed cooking oil	Refined, bleached and deodorized
4	Lard	Deodorized
5	Tallow	Deodorized tallow used for deep-fat frying
6	Tex 425	Blend of meat and vegetable fats Especially suitable for deep-fat frying
7	Kremit	Hydrogenated all-vegetable shortening The standard all-purpose vegetable shortening
8	Specification 522	Partially hydrogenated cottonseed oil. Designed for deep-fat frying
9	Kremax	Selectively hydrogenated all-vegetable shortening. Designed especially for deep-fat frying
10	Conventionally rendered bacon fat	
11	Microwave-rendered bacon fat	

line is simply the vector sum of the strengths of the incident (A) and reflected (B) waves at the particular point. A point in the line can be found where this vector sum is at a maximum and likewise, where it is at a minimum. These two phenomena give rise to the standing wave ratio (SWR):

$$\mathrm{SWR} = \frac{E_{max}}{E_{min}} = \frac{|A| + |B|}{|A| - |B|} \qquad \text{[Eq. 2]}$$

in which E_{max} = voltage at maximum, and
E_{min} = voltage at minimum.

The insertion of a dielectric into the transmission line will result in a change in the SWR due to impedance to the wave and evidenced by changes in the position and the width of the standing wave nodes (Fig. 2). These changes are the basis for calculating ϵ'_r and tanδ.

Dielectrometers

To make the measurements (node locations, node widths, etc.) upon which the calculation of the dielectric properties of a material is based, a dielectrometer is used (Fig. 3). The complete unit consists of:

1. *Power supply.* The power supply draws electric power from the line and, if necessary, converts it to the voltage needed by the microwave generators.

2. *Microwave generators.* Oscillators or klystron tubes are used to convert the supplied power into microwave energy at the specific frequencies for which they are designed.

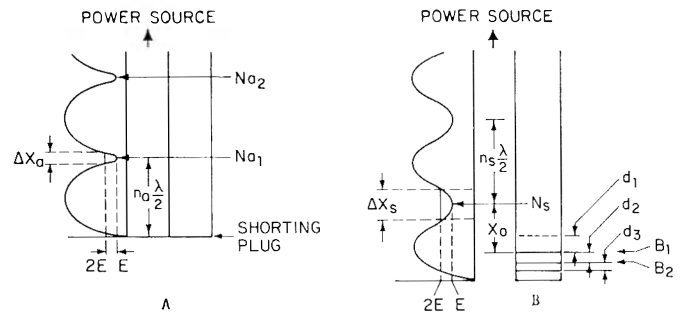


Fig. 2. Change in wave length and node width of the standing wave as a result of inserting a dielectric sample (with loss) into the line.

A. Air-filled line (closed off by shorting plug)

N_{o1}—first minimum above the terminus

N_{o2}—second minimum above the terminus

$N_o\frac{\lambda}{2}$—half wave length

E—minimum power point

E—twice minimum power points

ΔX_o—width of minimum as measured at the twice minimum power points

B. Line with sample holder (containing sample) attached.

N_s—first minimum above the terminus

$N_s\frac{\lambda}{2}$—half wave length

X_o—distance (cm) of the first minimum above the front face of the first dielectric

E—minimum power point

2E—twice minimum power points

ΔX_s—width of minimum as measured at the twice minimum power points

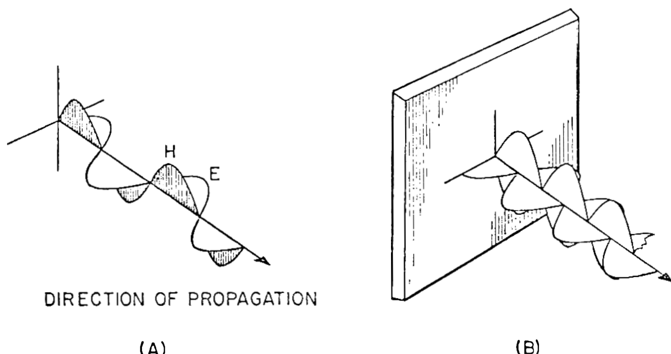
d_1—distance (cm) from top of sample holder to front face of first dielectric (sample cover)

d_2—thickness (cm) of the first dielectric

d_3—thickness (cm) of the second dielectric (sample)

B_1—boundary between air-filled portion of line and front face of first dielectric

B_2—boundary between bottom face of first dielectric and front face of second dielectric.



(A) (B)

Fig. 1. TEM mode as a traveling wave (A) and as a standing wave (B). E is the electric field strength and H is the magnetic field strength.

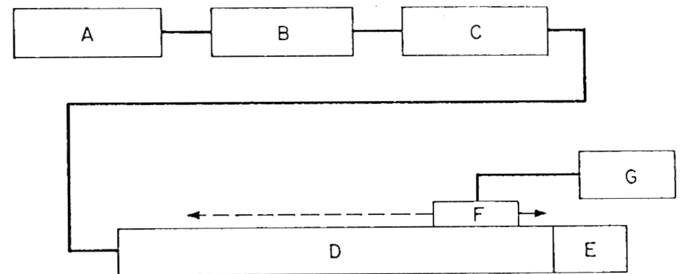


Fig. 3. Block diagram of a complete standing wave measuring system. A—power supply; B—microwave generator; C—attenuator; D—precision slotted line; E—sample holder; F—carriage for traveling probe; and G—detector.

3. *Attenuator.* An attenuator is used to regulate the power level of the signal picked up by the probe.

4. *Precision slotted line.* The precision slotted line gives the name to the technique in which it is used and consists of a precision-milled coaxial line with a slot along one side of the outer conductor running parallel to the axis.

5. *Sample holder(s).* The sample holders are precision devices which hold the sample whose properties are to be determined. They attach to the microwave transmission line in such a way as to become an integral part of the line.

6. *Traveling probe.* The probe is a fine micrometer driven wire which protrudes through the slot in the outer wave guide. It is used to sample the wave so that its relative strength can be measured at any given point, supplying data needed to determine the standing wave ratio and the positions and widths of the wave nodes.

7. *Detector.* The detector, an ampmeter attached to the probe, affords the operator a knowledge of the relative wave strength at the site of the probe.

In the work reported in this paper, dielectric measurements at 1000 and 3000 MHz (megahertz) were made on a Model 1 Microwave Dielectrometer (Fig. 4) manufactured by Central Research Laboratories, Inc., Red Wing, Minnesota. Measurements at 300 MHz were made on a similar piece of equipment constructed by personnel of the Laboratory for Insulation Research of the Massachusetts Institute of Technology.

Sample holder

The sample holder (Fig. 5) used with these fats and oils is a sealed unit. It is filled by liquefying the oils and then introducing them through a 15 gauge hypodermic needle made into the top of the holder at one edge of the sample cavity. The sample cavity has a volume of 30 cm^3. A second hypodermic needle, similarly positioned at the opposite edge of the top, allows escape of air as the sample cavity is filled. The wall of the holder is jacketed to allow passage of water from a constant temperature bath so that the temperature of the sample can be changed as desired. A thermocouple in the bottom of the holder allows a cross check of sample temperature against the temperatures of the water entering and leaving the jacket. The top of the holder, above the limits of the sample, is surrounded by coils for the passage of cooling water to prevent heating of the air-filled portion of the microwave transmission line.

The major problem encountered in using this holder is in assuring that air pockets do not form over the upper surface of the sample due to shrinkage of the sample when it is cooled to temperatures below that at which it was introduced into the holder (especially when such cooling results in partial-total solidification of the sample). The best way to prevent this is to cool the sample quite slowly while maintaining a positive pressure on the entry line (needle) and assuring that the air exit line (needle) remains open.

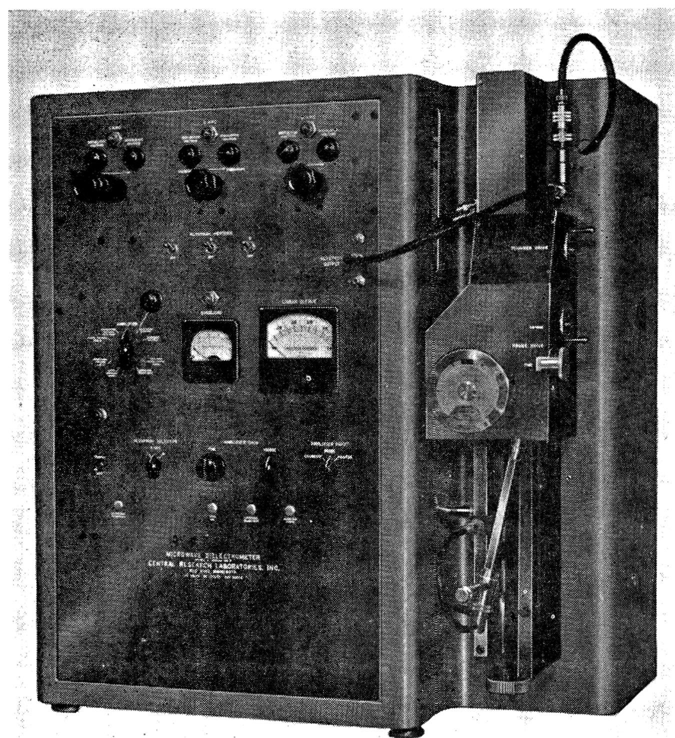


Fig. 4. Photograph of a Model 1 Microwave Dielectrometer (Courtesy of Central Research Laboratories, Inc.)

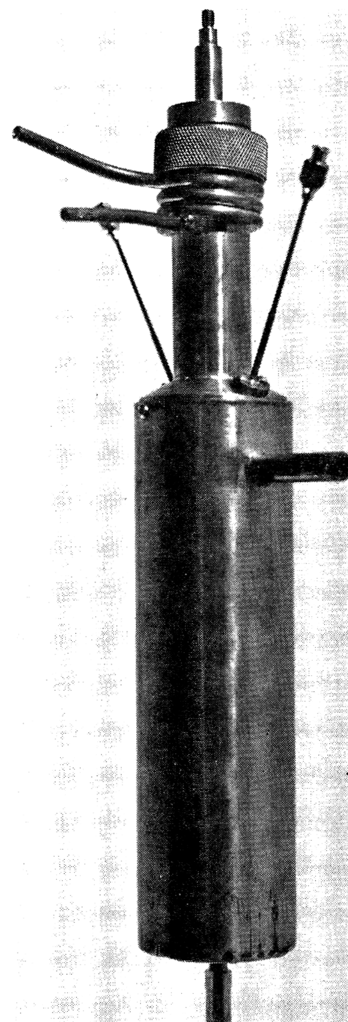


Fig. 5. Sample holder used for dielectric measurements on oils at 300 MHz (holders used at other frequencies were similar except for length).

Table 2. Dielectric data on 11 commercial fats and oils.[1]

Sample No.		300 MHz			1,000 MHz			3,000 MHz		
		77°F[2]	120°F[3]	180°F[4]	77°F[5]	120°F[6]	180°F[7]	77°F[5]	120°F[8]	180°F[9]
1	ϵ'_r	2.853	2.879	2.862	2.612	2.705	2.715	2.506	2.590	2.594
	$\tan\delta$	0.0559	0.0480	0.0322	0.0644	0.0645	0.0517	0.0551	0.0648	0.0617
	ϵ''_r	0.159	0.138	0.092	0.168	0.174	0.140	0.138	0.168	0.160
2	ϵ'_r	2.829	2.868	2.861	2.638	2.703	2.713	2.526	2.567	2.587
	$\tan\delta$	0.0615	0.0468	0.0359	0.0664	0.0643	0.0538	0.0566	0.0645	0.0630
	ϵ''_r	0.174	0.134	0.103	0.175	0.174	0.146	0.143	0.166	0.163
3	ϵ'_r	2.825	2.859	2.834	2.629	2.669	2.673	2.515	2.536	2.554
	$\tan\delta$	0.0606	0.0463	0.0363	0.0660	0.0641	0.0547	0.0568	0.0651	0.0627
	ϵ''_r	0.171	0.132	0.103	0.174	0.171	0.146	0.143	0.165	0.160
4	ϵ'_r	2.718	2.779	2.770	2.584	2.651	2.656	2.486	2.527	2.541
	$\tan\delta$	0.0564	0.0493	0.0394	0.0612	0.0600	0.0516	0.0509	0.0608	0.0583
	ϵ''_r	0.153	0.137	0.109	0.158	0.159	0.137	0.127	0.154	0.148
5	ϵ'_r	2.603	2.772	2.765	2.531	2.568	2.610	2.430	2.454	2.492
	$\tan\delta$	0.0485	0.0509	0.0381	0.0582	0.0567	0.0512	0.0487	0.0582	0.0576
	ϵ''_r	0.126	0.141	0.105	0.147	0.146	0.134	0.118	0.143	0.144
6	ϵ'_r	2.681	2.723	2.765	2.596	2.650	2.662	2.491	2.529	2.546
	$\tan\delta$	0.0543	0.0525	0.0402	0.0578	0.0575	0.0519	0.0497	0.0583	0.0573
	ϵ''_r	0.146	0.143	0.111	0.150	0.152	0.138	0.124	0.147	0.146
7	ϵ'_r	2.683	2.777	2.772	2.530	2.654	2.665	2.420	2.534	2.550
	$\tan\delta$	0.0524	0.0505	0.0371	0.0582	0.0576	0.0514	0.0482	0.0578	0.0571
	ϵ''_r	0.141	0.140	0.103	0.147	0.153	0.137	0.117	0.146	0.146
8	ϵ'_r	2.755	2.804	2.793	2.622	2.620	2.628	2.497	2.499	2.515
	$\tan\delta$	0.0573	0.0497	0.0391	0.0625	0.0588	0.0522	0.0533	0.0600	0.0587
	ϵ''_r	0.157	0.141	0.109	0.164	0.154	0.137	0.133	0.150	0.148
9	ϵ'_r	2.693	2.765	2.760	2.550	2.649	2.657	2.466	2.521	2.546
	$\tan\delta$	0.0523	0.0512	0.0392	0.0616	0.0557	0.0507	0.0511	0.0568	0.0570
	ϵ''_r	0.141	0.142	0.108	0.157	0.148	0.135	0.126	0.143	0.145
10	ϵ'_r	2.753	2.799	2.767	2.615	2.655	2.637	2.498	2.539	2.526
	$\tan\delta$	0.0622	0.0533	0.0356	0.0625	0.0606	0.0547	0.0532	0.0597	0.0588
	ϵ''_r	0.172	0.149	0.099	0.163	0.161	0.144	0.133	0.152	0.148
11	ϵ'_r	2.742	2.796	2.772	2.601	2.655	2.660	2.487	2.536	2.546
	$\tan\delta$	0.0576	0.0460	0.0354	0.0624	0.0607	0.0549	0.0507	0.0599	0.0590
	ϵ''_r	0.158	0.129	0.098	0.162	0.161	0.143	0.126	0.152	0.150

[1] Descriptions of these oils may be found in Table 1 and chemical data in Table 3.
[2] Temperature range 77°F–79°F.
[3] Temperature range 118.4°F–123°F.
[4] Temperature range 169.6°F–179.4°F.
[5] Temperature range 77°F–80.6°F.
[6] Temperature range 121°F–127.4°F.
[7] Temperature range 172.9°F–184°F.
[8] Temperature range 121°F–127.4°F.
[9] Temperature range 172.9°F–184°F.

Chemical methods

The determination of moisture and volatiles was made by the Vacuum Oven Method as described in Section 26.002 of AOAC (1960).

RESULTS AND DISCUSSION

DIELECTRIC DATA for the 11 fats and oils are presented in Table 2. Chemical data (iodine values, solids-fat index values, gas-liquid chromatography determinations of straight chain length of fatty acids, and moisture and volatiles content) are presented in Table 3.

Each oil is made up of a series of various fatty acids differing in relative quantities depending on the origin and type of oil (and/or fat) as shown in Table 1. Thus a logical analysis that one might make based on data for pure fats and fatty acids is not possible. However, the differences in magnitude of either the dielectric constant or loss tangent between the maximum or minimum values observed under any condition of frequency or temperature are not very great.

Buchanan (1954) measured the loss characteristics of typical fatty acids (capric, palmitic, and stearic acids). He also measured the dielectric constant and loss factor over a frequency range from about 600 to 23,800 MHz and found both ϵ'_r and ϵ''_r to be low and constant. When liquid palmitic and lauric acids were measured, the results were similar but showed a higher loss and a "suggestion of a region of relaxation loss (or peak in the ϵ''_r plot as a function of frequency) between 1 and 3 cm" (23,800 and 9,440 MHz, respectively).

Morgan (1934) measured the dielectric constant and loss of glycerol as a function of frequency and of temperature and showed that for pure glycerol (over the range 0.03 to 98,000 KHz), there is a peak in the dielectric loss

Table 3. Chemical data on 11 commercial fats and oils.[1,2]

	1	2	3	4	5	6	7	8	9	10	11
Iodine value (WIJS)	129.8	129.5	113.6	66.8	42.7	58.2	78.5	81.2	76.2	68.4	67.6
SFI[3] 50°F	None	None	None	25.3	37.2	NA[4]	30.0	19.2	NA	23.6	25.4
70°F	None	None	None	17.5	26.3	NA	20.0	9.6	NA	17.1	18.6
92°F	None	None	None	4.3	19.1	NA	12.5	1.6	NA	3.0	3.3
104°F	None	None	None	1.8	10.7	NA	6.5	Nil	NA	1.3	1.5
GLC[5] C-14	......	......	1.2	1.2	2.6	1.9	0.4	0.7	......	0.9	1.2
C-15	......	......	......	......	0.9	0.2	......	......	......	......	......
C-16	10.1	11.0	22.7	23.9	24.9	21.9	14.9	22.4	12.6	24.9	25.8
C-16′	......	......	0.6	2.6	2.0	2.2	......	0.5	......	2.3	2.2
C-17	......	......	......	0.2	1.3	0.5	......	......	......	......	......
C-18	4.3	1.9	2.1	12.3	24.5	18.3	11.8	5.1	11.1	12.0	11.4
C-18′	24.6	26.6	18.9	46.3	41.2	46.5	63.9	50.1	70.8	47.8	47.9
C-18″	52.8	59.6	54.5	11.9	1.8	7.2	8.1	20.0	4.8	10.6	10.1
C-18′″	8.2	0.9	TR[6]	1.6	0.8	1.3	0.9	1.2	0.7	1.5	1.4
Moisture and volatiles (%)	0.040	0.32	0.053	0.093	0.075	0.410	0.018	0.207	0.032	0.06	0.04

[1] Descriptions of these oils may be found in Table 1.
[2] Data in this table (other than moisture content) supplied by Food Research Division, Armour and Company, Oak Brook, Illinois.
[3] SFI—Solids-Fat Index, the amount of solids in relation to liquid at the given temperature.
[4] NA—Not available.
[5] GLC—Gas-Liquid Chromatography determination of straight chain length of fatty acids.
[6] Trace.

factor which decreases slightly in value as the temperature and the frequency increase. In addition, the increase in frequency seems to result in a broadening of the peak of the curve, with a maximum value showing up at higher temperature levels as the frequency increases.

Although not on pure compounds, the studies carried out in the work cited herein do suggest certain trends. First of all, the differences measured in loss factors (ϵ''_r) among these fats and oils are greatest in measurements made at the lowest temperature (~77°F) and at the lowest frequency (300 MHz) (Table 4). This is due to the greatest difference in phase of the components of the individual samples occurring at the lowest temperature. For instance, at 175°F most, if not all, of the material of all the samples, e.g., the samples of lard, tallow and Kremit, are in the solid phase. The losses are generally greater in the more liquid samples, e.g., corn oil and cottonseed oil (samples 2 and 3) *vs.* lard, tallow and Kremit (samples 4, 5 and 7). This is confirmed if one ranks the loss factors as a function of iodine values of the samples. These data confirm the work of Buchanan (1954) with fatty acids.

At constant temperatures, the dielectric constants decreased as frequency was increased. This change is in accord with the Debye equations for dependence of the dielectric constant on frequency which show that dielectric constants decrease from ϵ_0 to ϵ_∞ as frequency is increased, with most of the decrease occurring within a 2 log cycle

Table 4. Upper and lower limit of loss factor values (ϵ''_r) for the 11 samples.

Temperature	Frequency 300 MHz	1000 MHz	3000 MHz
~ 77°F	0.126–0.174	0.147–0.175	0.117–0.143
~120°F	0.129–0.149	0.146–0.174	0.143–0.168
~180°F	0.092–0.111	0.134–0.146	0.144–0.163

change in frequency (Hennelly *et al.*, 1948; Smyth, 1955).

ϵ_0, the static dielectric constant, is the maximum dielectric constant and exists when there is equilibrium between the orientation of the molecule and the electrical field at all times during a given cycle (Fuoss, 1943). ϵ_∞, the optical dielectric constant, is the square of the index of refraction and is a measure of the electronic displacement current; the electronic displacement current, present at all frequencies in all materials, is superimposed on any other current-carrying mechanism (Fuoss, 1943).

The change referred to is in accord with the statement of Singleton (1960) that generally, as frequency is increased, it becomes more difficult for the molecules to reorient against the viscosity of the medium, and the dielectric constant decreases.

The changes in loss factors (at constant temperature) which were observed are partially explained by the Debye equations which require that loss factors change from a small value through a maximum and then to a small value again as frequency is increased and temperature is maintained constant (Hennelly *et al.*, 1948). Schmidt *et al.* (1948) state that if dielectric absorption is due to dipole orientation, the value of the loss factor decreases slightly as temperature is increased. Their own work with Halowax produced the opposite effect which they explained, "Such a behavior can be accounted for in heterogenous dielectrics either by a change in the conduction of one component relative to that of the other or by a change in the relative proportions of the compounds."

This might also account for the changes in loss factor observed in this work when temperatures were varied while frequency was maintained constant at 3000 MHz. White *et al.* (1933) reported that the loss factor begins to increase with temperature at about the same temperature at which the dielectric constant begins to increase; that the loss factor passes through a maximum and then de-

creases to a low value at temperatures where no further rotation of dipoles is possible. They further stated that a second increase in the loss factor might be noted at still higher temperatures but that here it would be due to an increase in ionic conduction.

At a constant frequency of 1000 or 3000 MHz, the dielectric constants increased as temperatures were increased over the range used. At 300 MHz, however, dielectric constants showed a slight increase as temperature was increased from ~77° to ~120°F and then decreased slightly as temperatures were further increased to ~180°F. Fuoss (1943) stated that as temperatures increase, the dielectric constant goes through a sigmoidal increase (at any given frequency) as follows.

At low temperatures, high internal viscosity produces long relaxation times and, hence, little dipole orientation and low values for the dielectric constant and the loss factor. As temperature is increased, viscosity decreases and relaxation time decreases with it. When relaxation times get to the order of milliseconds, partial orientation within a cycle is possible and the dielectric constant and loss factor start to increase.

When the temperature has been further increased to a point where the relaxation time is small compared to the period of the field, polarization approaches synchrony with the field and one finds a relatively high dielectric constant but a decreasing loss factor. If the values reported in this paper are in this range, they could be explained on this basis. Lack of values over a wide enough frequency range, however, fails to give knowledge of specific locations on the over-all frequency curve.

In an effort to gain a clearer view of the changes occurring in the dielectric values with changes in frequency, a more detailed examination of one oil (9) was made. Dielectric values at 77°F were determined at 10 frequencies ranging from 100 Hz to 8000 MHz (8 GHz). The results of these determinations are shown in Figs. 6 and 7. Over

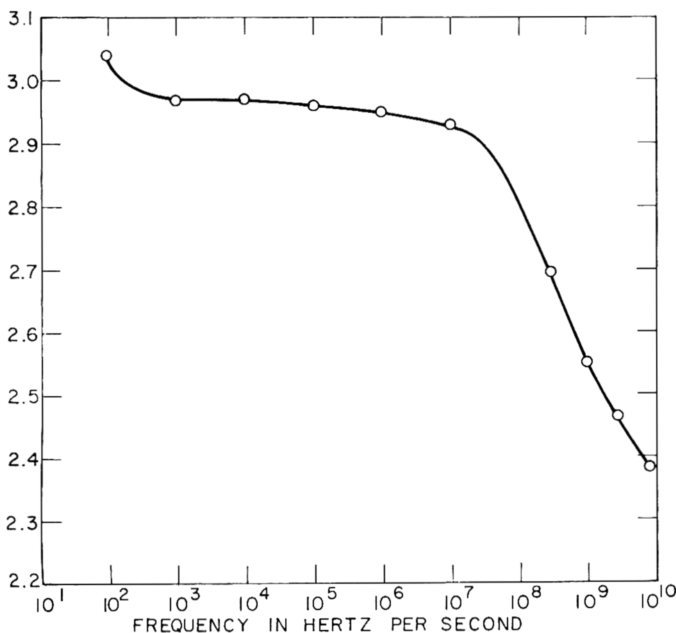


Fig. 6. Dielectric constant curve for oil No. 9 at 77°F.

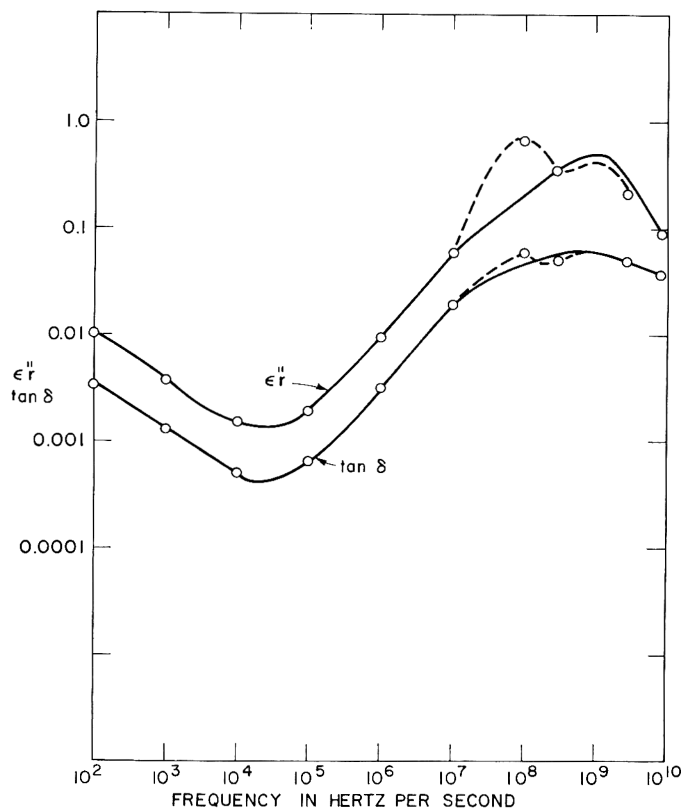


Fig. 7. Loss factor and loss tangent curves for oil No. 9 at 77°F.

this frequency range, the dielectric constant shows a general plateau from 1 KHz to 10 MHz and then a sharp decrease as frequencies are increased further. The loss tangent (Fig. 7) shows a decrease as frequency is increased from 100 Hz to 10 KHz, reaches a minimum, and then increases as frequencies are further increased to ~1000 MHz (1 GHz).

The curve of the loss factor closely follows that of the loss tangent. Between 10 MHz and ~1000 MHz, a double curve is shown for both the loss tangent and the loss factor. This has been done because the value for the loss tangent at 100 MHz is either slightly in error or else there is a double peak in this area. The heterogeneity of the oil makes it entirely possible that a double peak does actually exist. Again, the changes noted are in accord with the explanation of Fuoss (1943).

At constant temperatures of ~77°F, loss tangents increased with an increase in frequency from 300 to 1000 MHz and then decreased as frequency was further increased to 3000 MHz. At constant temperatures of ~120 and ~180°F loss tangents increased over the entire frequency range covered. It is possible that at ~77°F, the internal viscosity in the molecular system is too great to allow complete reorientation at frequencies as high as 3000 MHz.

At constant frequencies of 300 and 1000 MHz, loss tangents generally decreased as temperatures were increased. At a constant frequency of 3000 MHz, however, loss tangents increased as temperatures were increased from ~77 to ~120°F and then decreased as temperatures were further increased to ~180°F. This finding seems to agree with the earlier statement of Fuoss (1943) regarding de-

creases in loss factors after a temperature has been reached where the relaxation time is short compared with the period of the field and polarization approaches synchrony with the field.

Moisture content of the various samples (included in Table 3) ranged from a low of 0.018% to a high of 0.410%, but there was no apparent correlation between moisture content (at these low levels) and dielectric values.

It is difficult to draw further conclusions from a theoretical analysis of these data, because the samples are made up of a mixture of fatty acids of different types and proportions.

Nevertheless, from a practical point of view in terms of power absorption, there is little difference between an unsaturated vegetable oil, such as cottonseed, and an animal fat such as lard or tallow. Moreover, as one increases the temperature of the sample (as during heating of a fatty food material in a microwave oven), although there is a tendency for the loss factor to increase slightly at the higher frequencies, the magnitude of the increases is too small to be of any practical significance at all in terms of power absorption by the fat.

The samples of bacon fat rendered by microwaves *vs.* conventional means show little difference in dielectric loss characteristics or in chemical properties.

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Dielectric Properties of Potatoes and Potato Chips

SUMMARY—Dielectric properties (dielectric constant, ϵ'_r; loss tangent, tanδ; and dielectric loss factor, ϵ''_r) were determined for raw potatoes and for potato chips, using the precision slotted line technique. Measurements on raw potatoes were made at 77°F (25°C) and at frequencies of 300, 1,000, and 3,000 MHz (megahertz). Measurements on potato chips were made at three moisture levels; at 77°F (25°C), 125°F (51.6°C), and 180°F (82.2°C); and at frequencies of 300, 1,000, and 3,000 MHz. Moisture, total lipids, and nitrogen contents are reported for both the raw potatoes and the potato chips.

As would be expected from their high moisture content and the presence of various dissolved salts, the raw potatoes possess extremely high dielectric values.

Potato chips show a rapidly decreasing dielectric loss as moisture content is reduced; the loss values of the chips approach those of the oil used for frying them after moisture has been reduced to approximately 3% and the oil content has been increased accordingly.

For finish drying of potato chips, a frequency of 3,000 MHz will result in 3–4.5 times greater power production in the chips than will the use of a frequency of 1,000 MHz (in the moisture and temperature range at which the data were obtained). However, this difference in power production is due almost entirely to the difference in frequency since the difference in dielectric loss values at the two frequencies is quite small.

INTRODUCTION

COLOR IS ONE OF THE IMPORTANT FACTORS in processing and marketing potato chips. In potato chips processed by conventional frying, color formation is determined primarily by the reducing sugar content of the raw potatoes (Davis *et al.*, 1965). Successful "chipping" by conventional means requires potatoes with a glucose content of 0.1% or less (O'Meara, 1966). Darkening of the chips occurs at the terminal stage of frying, after the moisture level has been reduced to 6–8%; it is accelerated if reducing sugars are present.

When microwave heating is used to accomplish finish drying, the potatoes can be removed from the fryer while 6–10% of the moisture is still present and browning has not proceeded to a point where it would be objectionable (Davis *et al.*, 1965).

A knowledge of the dielectric properties of raw potatoes and of potato chips at different frequencies and at different moisture contents was deemed essential to select the optimum frequency for use and to determine the range of moisture content in which microwave energy can be utilized most efficiently. Measurements were made at 1,000 and 3,000 megahertz (MHz) as most of the data available in the literature on dielectric properties of various materials have been made at these frequencies. These two frequencies are quite close to the values which have been assigned by the Federal Communications Commission for industrial, scientific, and medical purposes, namely 915 and 2,450 MHz.

EXPERIMENTAL MATERIALS

THE RAW POTATOES with dielectric properties reported in this paper were "street purchases" of California, Maine, and Idaho white potatoes of unknown variety.

The potato chips with dielectric properties reported were prepared from a single "street purchase" of Idaho white potatoes of unknown variety. The potatoes were thinly sliced to a uniform thickness and were then fried in a commercial soybean oil at 350°F. Color development, previously correlated to approximate moisture content, was used as a guide in determining when to remove the chips from the oil.

When the slices had developed the desired level of color, they were removed from the oil and were finely crumbled. The crumbled chips were thoroughly mixed to give the best possible distribution of any variation in moisture and lipid content and were then tightly packed into the sample holder.

EXPERIMENTAL METHODS AND EQUIPMENT

Dielectric measurements

Dielectric measurements on both the raw potatoes and the potato chips were made by the precision slotted line technique. This technique has been described by several authors (Roberts *et al.*, 1946; Dakin *et al.*, 1947; Anon., 1950; von Hippel, 1954; Ginzton, 1957; Soderman, 1958; Westphal, 1963; Beatty, 1965; Gilmore, 1966; and Westphal, 1966) and is the same as used in determining the dielectric properties of certain commercial fats and oils (Pace *et al.*, 1968). Details of the procedure and of the calculations have been described by Pace (1967).

Dielectrometers

Dielectric measuring equipment was the same as was used in work previously reported on fats and oils (Pace *et al.*, 1968). Measurements at 1,000 and 3,000 MHz were made on a Model 1 Microwave Dielectrometer (manufactured by Central Research Laboratories, Inc., Red Wing, Minnesota). Measurements at 300 MHz were made on a similar piece of equipment (constructed by

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Sample holder for raw potatoes

The sample holder used for measurements on samples of raw potatoes is shown in Figs. 1 and 2. This holder consists of two separate sections (top and bottom), and the sample is positioned between them. The extension of the center conductor is permanently attached to the bottom section of the holder. The sample is positioned on the bottom section of the holder by slipping it down over the center conductor. The top section of the holder is then added on top of the sample in a like manner. The two sections of the holder are fastened together by a Whitley clamping ring.

The center conductor of the holder is solid copper with a 3/8-in. outside diameter. The outer conductor is milled from solid copper to an inside diameter of 1 in. Polystyrene, a material with dielectric properties constant over the temperature and frequency range used, is used as a base for the sample and as a sample cover.

This holder allows the use of extremely thin (0.050 in., 50 mils) samples. The use of such thin samples is desirable

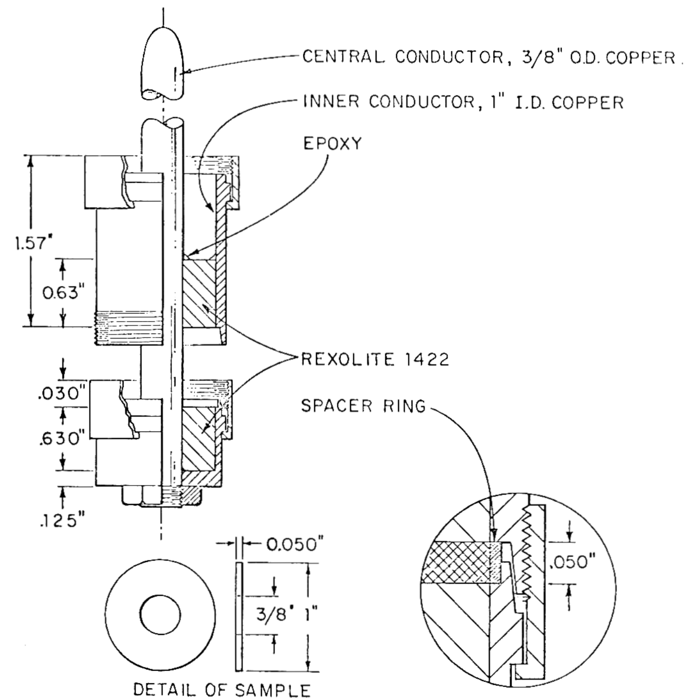

Fig. 2. Construction detail of sample holder used for dielectric measurements on raw potatoes. (Lower section shown here is for use at 3,000 MHz; longer sections were used at 1,000 MHz and at 300 MHz.)

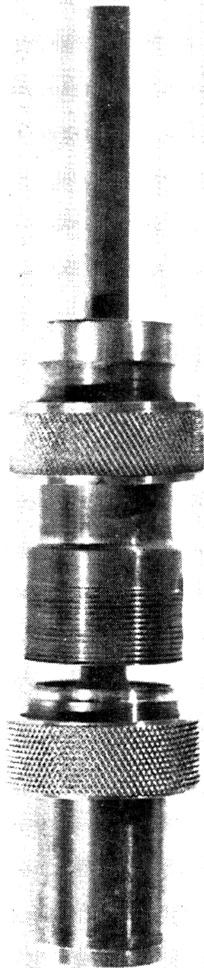

Fig. 1. Sample holder used for dielectric measurements on raw potatoes at 1,000 MHz.

when dealing with materials as lossy as raw potato. Samples of any appreciable thickness could result in a node of the standing wave actualy being present in the sample itself; measurements of the "apparent" first node above the sample would then be totally in error. A disadvantage of using such thin samples is that very minute variations in sample thickness result in sizable errors in the final values (a variation of ± 0.020 in. resulted in errors as great as 20% when making some of the initial measurements on raw potatoes).

While in the holder, the sample is surrounded (circumferentially) by a brass spacing ring 0.050 in. in thickness (same thickness as the sample). The ring prevents any compression of the sample by the holder when the top and bottom sections of the holder are fastened together. The sample must be cut so as to exactly fit the cavity provided for it in the holder. If the sample is too thin, air gaps will be present above and/or below the sample (dielectric constant of air = 1). If the sample is too thick, it will be compressed by the holder and errors in measurement will result from the increase in sample density. Because of the importance of sample preparation with respect to the accuracy of the data, this is gone into in some detail.

Samples for measurement in this holder are prepared by slicing the potatoes to the desired thickness and then punching the samples from the slices.

Initially, the raw potatoes were sliced on an electric food slicer. This method was abandoned when it was discovered that individual slices varied in thickness by as much as ± 0.050 in. An attempt was then made to slice the potatoes with stainless steel razor blades. To hold these parallel, they were fastened to opposite sides of a 0.045-in. thick strip of brass. This method was also abandoned because

the double bevel of the cutting edge resulted in an increase of 0.020 in. over a cutting distance of 1 in. Finally, a sample slicer was fabricated by soldering two shop-made blades (beveled only on one side) to a 0.050-in.-thick brass frame.

After the potato has been sliced, the samples are punched out with a "doughnut cutter" which has the same dimensions as those of the inner and outer conductors. As with the slicer, the cutting edges of the "doughnut cutter" must be beveled on only one side (the side away from the sample) in order to produce a sample whose opposite sides are parallel.

Extreme care must be taken in handling the raw potato slices and the samples to prevent changes in moisture content. The high surface-to-volume ratio allows rapid loss of moisture from the sample if environmental relative humidity is much different from the water activity of the sample.

As noted in the legend for Fig. 2, different bottom sections for the sample holder are used for making measurements at different frequencies. Changing the length of the line below the face of the sample is necessary in order to keep exact multiples of $\lambda/4$ distance between the face of the sample and the reflecting surface.

A better method than changing the lower section of the holder, however, would be to have the lower section made in two parts so that only the part not adjacent to the sample would have to be changed when changing frequencies. This design would have the advantage of allowing measurements at all frequencies within the range of the dielectrometer without disturbing the sample.

Sample holder for potato chips

The sample holder used for measurements on potato chips (Fig. 3) is a one-piece unit with an open top. This holder is heated electrically and is fitted with a thermocouple for determining temperature of the sample. Coils around the top of the holder, above the limits of the sample, allow passage of cooling water to prevent heating the air-filled portion of the line.

The sample is put into the holder in finely crumbled form. A length of polystyrene tubing, with diameters closely matching those of the conductors, is used to continuously pack the sample as it is being added. After the sample is in place, it is topped with a thin teflon washer whose diameters match those of the conductors. The washer is pressed tightly against the surface of the chips to give them the smoothest possible top surface.

This means of sample preparation and of filling the sample holder has two significant shortcomings:

1. Lack of a perfect fit (a) between the sample and the conductors, (b) between the sample and the bottom of the holder, and (c) between the sample and the sample cover presents a possible source of error in measurement. This deficiency is not a major problem in measuring low loss samples but could not be tolerated in working with samples having a very high loss. It is minimized by assuring that the sample is firmly packed as it is added into the holder.

2. Samples vary in density. This deficiency is minimized (a) by using the same weight of sample in all cases and packing it as nearly as possible to the same thickness and,

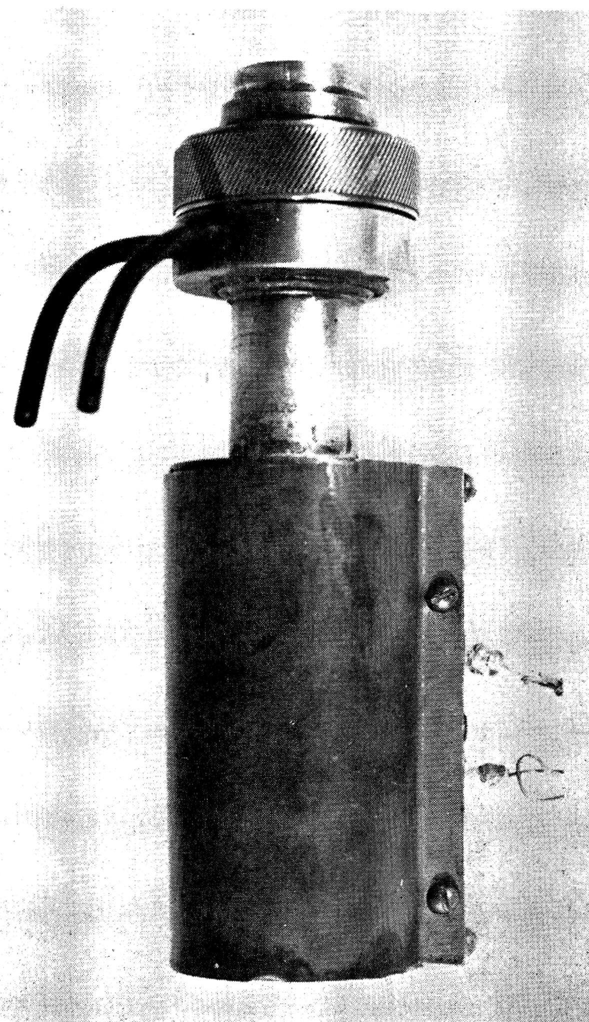

Fig. 3. Sample holder used for dielectric measurements on potato chips at 1,000 and 3,000 MHz.

more important, (b) by making all measurements on the same sample (of a given moisture content) without removing it from the holder. There is also a variation in density of any given sample as its temperature is changed, but this is a relative matter and so far we have found no manner of overcoming it. Since sample thickness changes with change in temperature, measurements of the sample thickness must be made each time the temperature is changed.

Sample thickness, while in the holder, is measured as follows:

1. The sample holder is removed from the dielectrometer.

2. A section of aluminum tubing, having an inner diameter slightly larger than the outer diameter of the center conductor, is slipped down over the center conductor until the aluminum tubing is in contact with the sample cover.

3. A solid piece of aluminum in the shape of a very thick (1–1½ in.) washer is slipped down over the aluminum tubing until it is in contact with the top of the sample holder.

4. The "washer" is locked in place on the aluminum tubing by use of a set screw.

5. The aluminum tubing and the "washer" (secured

in place by the set screw) are removed from the center conductor, and the distance from the bottom of the "washer" to the tip of the aluminum tubing is carefully measured.

6. The measurement determined in "5" is the distance from the top of the sample holder to the top of the sample cover. Knowledge of this distance, plus knowledge of the thickness of the sample cover and of the total depth of the sample holder, allows easy determination of the sample thickness.

Chemical methods

Moisture and volatiles, mineral content (ash), and nitrogen content were determined in accordance with Sections 26.002, 29.012, and 38.009–38.011, respectively, of the Official Methods of Analysis (AOAC, 1960). Total lipids were determined by the Chloroform-Methanol Extraction Method of Bligh *et al.* (1959). Values reported are the average of duplicate analyses in all cases.

RESULTS AND DISCUSSION

THE DIELECTRIC PROPERTIES of a number of samples of raw potato were determined in accordance with the methods previously described. All measurements were made at 77°F and at 300, 1,000, and 3,000 MHz. The results of the measurements, together with certain chemical data, are reported in Table 1.

Dielectric constants, loss tangents, and loss factors all decreased as frequency was increased. This is in accord with the statements of Singleton (1960) and with the Debye equations pertaining to dependence of dielectric constant on frequency (Hennelly *et al.*, 1948). The values obtained closely approximate those given by von Hippel (1954) for 0.1 Molal aqueous sodium chloride solutions.

Attempts were made to determine the dielectric properties of raw potatoes at lower moisture contents but results were not satisfactory. The measuring technique used requires a "perfect" fit of the sample in the sample holder and we have not yet been able to obtain such a fit with samples whose moisture content has been markedly reduced.

Data reported are the average of four determinations made at each frequency, each determination being made on a different sample. Initially, an attempt was made to measure a single sample at all frequencies, but this procedure was abandoned when it was discovered that loss of moisture from the sample during transfer from one sample holder to another resulted in changes which rendered the measurements meaningless. Use of a holder designed as previously described would circumvent this problem because one could then change the lower sections of the holder without exposing the sample.

The dielectric properties of 13 samples of potato chips, having various moisture and lipid properties, were made at ~77, ~122, and ~178°F and at 1,000 and 3,000 MHz. The results, together with certain chemical data, are shown in Table 2.

With the sample holder used, it was possible to make measurements at both frequencies and at different temperatures without disturbing the sample in any way. All data shown for a given moisture content are, therefore, from measurements of the same sample, thus providing precise knowledge of the changes occurring strictly as a result of change in frequency and/or temperature. However, this precise interpretation is not possible for changes occurring as a result of change in moisture content. In this case, the data are from measurements of different samples and are influenced to some unknown degree by other variables such as lipid content and packing density in the sample holder.

The data show that the loss factor of chips with 15.3% moisture content is somewhat higher at 3,000 MHz than at 1,000 MHz and increases as a function of temperature. This temperature phenomenon decreases as the moisture content becomes less and it becomes nil at ~3% moisture content. No further conclusions appear possible. This is probably due to the opposing effects of moisture content and lipid content; one decreases as the other increases. As the moisture content is reduced, the loss values of the chips approach those of the oil used for frying them after the moisture has been reduced to approximately 3% and the

Table 1. Dielectric and chemical data on raw potatoes.[1]

Sample[2]		300 MHz	1,000 MHz	3,000 MHz	Moisture and volatiles (%)	Total lipids (%)	Nitrogen content (%)	Ash (%)
620-C	ϵ'_r	80.0	65.1	57.3	79.5	0.192	0.340	0.116
	$\tan\delta$	0.597	0.301	0.274				
	ϵ''_r	47.8	19.6	15.7				
620-M	ϵ'_r	82.6	74.7	69.1	83.3	0.239	0.357	0.113
	$\tan\delta$	0.736	0.351	0.247				
	ϵ''_r	60.9	26.1	17.0				
620-I	ϵ'_r	77.4	70.3	62.9	81.2	0.211	0.348	0.113
	$\tan\delta$	0.756	0.368	0.274				
	ϵ''_r	58.5	24.8	17.2				

[1] Dielectric measurements were made at 77°F; data reported are the average of four determinations at each frequency (total of 36 samples). Initially, an attempt was made to measure a single sample at all frequencies but loss of moisture from the sample during the procedure (as a result of handling the sample when changing it from one sample holder to another) made the results unreliable, and this procedure had to be abandoned.

[2] Identification of samples:
620-C—California white potatoes, "street purchase"
620-M—Maine white potatoes, "street purchase"
620-I—Idaho white potatoes, "street purchase."

Table 2. Dielectric and chemical data on potato chips.[1]

Sample		1,000 MHz	3,000 MHz	Temperature (°F)	Moisture and volatiles (%)	Total lipids (%)	Nitrogen content (%)	Ash (%)
10-19-1	ϵ'_r	5.76	5.18	77	15.3	19.1	1.24	3.35
	tanδ	0.357	0.55					
	ϵ''_r	2.06	2.85					
10-19-1	ϵ'_r	6.74	5.74	122	15.3	19.1	1.24	3.35
	tanδ	0.373	0.66					
	ϵ''_r	2.52	3.78					
10-19-1	ϵ'_r	7.30	6.48	178	15.3	19.1	1.24	3.35
	tanδ	0.425	0.72					
	ϵ''_r	3.10	4.66					
10-4-1	ϵ'_r	7.90	5.86	77	13.2	30.5	1.30	3.36
	tanδ	0.202	0.257					
	ϵ''_r	1.59	1.51					
10-26-1	ϵ'_r	......	3.88	77	11.8	16.1	1.89	3.86
	tanδ	......	0.299					
	ϵ''_r	......	1.162					
10-17-2	ϵ'_r	3.90	3.80	71.6	8.3	27.0	1.39	3.12
	tanδ	0.156	0.188					
	ϵ''_r	0.61	0.71					
10-17-2	ϵ'_r	4.39	4.04	122	8.3	27.0	1.39	3.12
	tanδ	0.257	0.184					
	ϵ''_r	1.13	0.74					
10-17-2	ϵ'_r	4.61	4.23	178	8.3	27.0	1.39	3.12
	tanδ	0.287	0.195					
	ϵ''_r	1.33	0.83					
10-4-2	ϵ'_r	3.26	2.88	77	6.25	27.0	1.29	3.54
	tanδ	0.208	0.102					
	ϵ''_r	0.68	0.29					
10-17-3	ϵ'_r	1.888	1.860	70.7	2.6	35.9	1.35	3.43
	tanδ	0.0344	0.0367					
	ϵ''_r	0.065	0.068					
10-17-3	ϵ'_r	1.894	1.860	122	2.6	35.9	1.35	3.43
	tanδ	0.0405	0.0440					
	ϵ''_r	0.077	0.082					
10-17-3	ϵ'_r	1.980	1.910	176	2.6	35.9	1.35	3.43
	tanδ	0.0458	0.0515					
	ϵ''_r	0.090	0.098					
10-4-3	ϵ'_r	2.04	1.775	77	1.25	33.7	1.27	4.06
	tanδ	0.073	0.0383					
	ϵ''_r	0.15	0.068					

[1] Dielectric data reported here are the averages of duplicate measurements made on samples of chips prepared from "street purchases" of Idaho white potatoes fried in a commercial soybean oil.

oil content has been increased accordingly. For example, from Table 2, the dielectric constant, the loss tangent, and the loss factor of Sample 10-4-3 (finished potato chips) at 1,000 MHz are 2.04, 0.073, and 0.15 respectively. At the same temperature of measurement, for soybean oil the respective values are 2.612, 0.0644, and 0.168 (Pace *et al.*, 1968).

The dielectric loss factors of the 8.3% moisture chips are higher at 1,000 MHz than at 3,000 MHz at 122°F and at 178°F. This is not in accord with accepted theory and has not been explained. As can be seen from the data in Table 2, it is brought about by a more rapid decrease in the loss tangent at the higher frequency.

The selection of a better frequency (915 or 2,450 MHz) for finish drying of potato chips has been a subject of considerable controversy in the microwave industry. By using the data in Table 2 and the formula for power developed, some calculations are possible. The formula (Goldblith, 1967) is as follows:

$$P = E^2 f \epsilon'_r \tan\delta \times 55.61 \times 10^{-14}$$

in which

P is the power dissipated, in watts/cm³,
E is the electric field strength, in volts/cm,
f is the frequency in hertz/sec,
ϵ'_r is the dielectric constant of the material, and
tanδ is the loss tangent of the material.
($\epsilon'_r \tan\delta$ = loss factor = ϵ''_r).

In comparing the data available at 1,000 and 3,000 MHz, the only factors which change are the frequency and the loss factor. Therefore, the data in Table 3 will be sufficient to compare the relative effectiveness of the two frequencies. From these figures, it is readily seen that more

Table 3. Relative effectiveness of two frequencies.

Moisture content (%)	Temperature		$f\epsilon'_r \tan\delta$	
	°C	°F	@ 1,000 MHz	@ 3,000 MHz
15.3	25	77	2,060	8,550
	50	122	2,520	11,340
	81	178	3,100	13,980
8.3	22	71.6	610	2,130
	50	122	1,130	2,220
	81	178	1,330	2,490
2.6	21.5	70.7	65	204
	50	122	77	246
	80	176	90	294

heat per unit time per unit volume would be produced at 3,000 MHz than at 1,000 MHz, particularly in chips at the higher temperature and higher moisture content. Also, higher input temperatures give more rapid heat production at both frequencies; thus, this may be a pragmatic consideration in the design of the conveyor from the fryer to the microwave tunnel.

At the lower moisture contents (8.3% or less) the dielectric properties are closer at the two frequencies and thus the geometry of the tunnels, rather than the product characteristics, may become the limiting factor in determining which frequency to use. The 915 MHz frequency would permit larger entry and exit ports, important from the point of view of industrial practice. The data herein show relatively little to be lost from the standpoint of loss factor.

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Physical and Biochemical Properties of Porcine Muscle as Affected by Exogenous Epinephrine and Prednisolone

SUMMARY—The effects of intramuscular ante-mortem epinephrine of prednisolone plus epinephrine injection upon some physical and biochemical properties of porcine longissimus dorsi muscles were studied. Rate of post-mortem muscle pH decline, ultimate muscle pH, Munsell value and transmission value were not significantly ($P > .05$) altered by epinephrine or prednisolone plus epinephrine injection. Significantly ($P < .05$) higher total phosphorylase activity and slightly greater phosphorylase *a* and % phosphorylase *a* (% of total phosphorylase activity) were observed in the muscles of epinephrine injected pigs.

Neither total phosphorylase nor phosphorylase *a* was related to rate of pH decline or incidence of pale, soft, exudative muscle development. Muscle concentrations of AMP and IMP were not significantly ($P > .05$) different between treatments. IMP concentration was highly related to rate of pH fall. Correlation coefficients between longissimus dorsi muscle pH and IMP levels were —.93 and —.94 at 15 and 45 min post-mortem, respectively. Prednisolone treatment resulted in higher initial and ultimate muscle glycogen levels than those from the control or epinephrine injected pigs.

INTRODUCTION

RECENT RESEARCH HAS INDICATED that the physical and biochemical properties of porcine muscle may be deleteriously affected by conditions of low pH and high temperature during the initial post-mortem period (Briskey *et al.*, 1961; Bendall *et al.*, 1962b) with the subsequent development of pale, soft, exudative (PSE) muscle (Sayre *et al.*, 1963a). It has been shown that rapid post-mortem glycolysis is responsible for the low pH values. However, the factors responsible for the rapid glycolytic rate have not been established.

In skeletal muscle, glycolytic rate apparently can be regulated at several discrete metabolic steps. Phosphorylase can be converted from the less active *b* form to the more active *a* form (Cori, 1956). Epinephrine affects glycolysis by promoting the *b* to *a* conversion of phosphorylase (Cori *et al.*, 1956; Danforth *et al.*, 1962). It has been suggested that alterations in the activity of phosphofructokinase may be important in regulating the rate at which hexose monophosphate is converted to lactic acid (Passonneau *et al.*, 1962; Karpatkin *et al.*, 1964).

The activity of phosphofructokinase is strongly influenced *in vitro* by changes in concentration of a variety of cellular constituents such as AMP, ADP, ATP, P_i, NH_4^+, K^+, and fructose-6-phosphate. Whether these factors are operative in the development of PSE porcine muscle has not been established.

Ante-mortem stress and excitement (Briskey, 1964) as well as depressed secretion of adrenocorticosteroids (Ludvigsen, 1957; Judge *et al.*, 1966; Topel *et al.*, 1967b) have been implicated in the development of PSE porcine musculature. Topel *et al.* (1967a) reported lower adrenal gland weights and suppressed 17-hydroxycorticosteroid levels in the plasma of pigs subjected to prolonged prednisolone injection.

The objective of this experiment was to study the effects of ante-mortem epinephrine or prednisolone plus epinephrine injection upon post-mortem chemical events, muscle properties and phosphorylase activity of porcine skeletal muscle.

EXPERIMENTAL

Sources of animals and preslaughter treatment

Twenty-two market weight (200–220 lb) Poland China pigs were purchased from a local breeder. The pigs were divided randomly among three treatment groups. Group 1, composed of eight pigs, served as controls. Each pig in group 2 (eight pigs) was injected intramuscularly (biceps femoris) with 5 mg of epinephrine (1:1000 solution) per 100 lb body weight 10 min prior to exsanguination.

Group 3 (six pigs) each received a daily intramuscular (biceps femoris) injection of 100 mg of prednisolone (delta-1-hydrocortisone) for 10 days prior to slaughter. The pigs were slaughtered 24 hr after the last prednisolone injection. Ten min prior to slaughter, the pigs in group 3 also received an intramuscular epinephrine injection as described for group 2.

Sampling and muscle pH

A 50 g sample was removed from the left longissimus dorsi muscle 10 min after exsanguination and frozen immediately in liquid nitrogen. This frozen sample was powdered in a Waring blendor and stored at −20°C for future analysis. Muscle pH was determined on samples removed serially from the longissimus dorsi muscle at 15 min, 45 min and 1 hr post-mortem and at 30 min intervals thereafter until 3 hr post-mortem. Approximately 5 g of muscle were homogenized for 1 min in 25 ml of 0.005*M* sodium iodoacetate. The pH of the homogenate was determined with a Corning Model 12 expanded scale pH meter.

Munsell value and transmission value

Lightness or darkness of the longissimus dorsi muscle at 24-hr post-mortem was objectively determined using a Photovolt Photoelectric Reflection meter, Model 610. The instrument was standardized against magnesium oxide. Percentage reflectance was measured at three positions in the cross-section of the longissimus dorsi muscle; the three readings were averaged and the average converted to the

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equivalent Munsell value according to the conversion values of Nickerson (1946).

The transmission value described by Hart (1962) was determined as an objective measurement of muscle quality at 24 hr post-mortem. Ten g of finely ground muscle were placed in a calibrated tube and cold distilled water added to a total volume of 40 ml. The contents were thoroughly mixed and held at 2°C for 20 hr, after which the mixture was centrifuged and the supernatant filtered through S & S No. 588 filter paper. One ml of the clear filtrate was mixed with 5 ml of citric acid-sodium phosphate buffer (9.35 ml of 0.2*M* Na_2HPO_4 plus 10.65 ml of 0.1*M* citric acid), pH 4.6.

This mixture was held at 20°C for 30 min. Following this incubation, the transmission value (% transmission) of this mixture was read in a Bausch and Lomb Spectronic 20 colorimeter with the instrument standardized against a blank of 1 ml of the muscle extract in 5 ml of distilled water. Normal muscle will give a transmission value of 0–20 while severe PSE muscle will have values of 80–100 (Hart, 1962).

Phosphorylase assay

The frozen muscle powder was extracted and assayed for phosphorylase within 12 hr post-mortem using the methods described by Posner *et al.* (1965). This method is designed to measure the ratio of phosphorylase *a* to *b* present in muscle. The assay is conducted in the presence of 0.001*M* AMP to determine total phosphorylase activity and in the absence of AMP for phosphorylase *a* activity. Phosphorylase *a* activity was also expressed as a percentage of total phosphorylase activity. Phosphorylase *b* could be obtained by difference.

AMP and IMP determinations

AMP and IMP were extracted by placing 10 g of frozen powdered muscle in 50–60 ml of boiling deionized water and allowing the mixture to boil for 10 min. The slurry was then cooled and brought to a total volume of 100 ml, after which it was centrifuged, the supernatant filtered and the filtrate adjusted to pH 8.0 with 1*N* NaOH. A 10 ml aliquot was placed on an anion exchange column and the nucleotides were eluted and quantitatively evaluated using the methods previously described (Aberle *et al.*, 1968).

Glycogen

Glycogen was extracted from the frozen muscle powder using the method of Hansen *et al.* (1952). The only modification was to redissolve and reprecipitate the isolated glycogen. The phenol-sulfuric acid method described by Dubois *et al.* (1956) was employed to assay for glycogen.

RESULTS AND DISCUSSION

Muscle pH

Ultimate muscle pH (24 hr post-mortem) was not significantly ($P > .05$) affected by either epinephrine or prednisolone plus epinephrine injection (Table 1). Epinephrine injection at 24 and 12 hr ante-mortem in the bovine (Hedrick *et al.*, 1957) and at a 4 hr ante-mortem in the rabbit (Bendall *et al.*, 1962a) has been shown to significantly raise the ultimate muscle pH.

Radouco-Thomas *et al.* (1959) found that subcutaneous

Table 1. Means[1] and standard error of the means for pH, Munsell value and transmission value in control, epinephrine, and prednisolone plus epinephrine injected pigs.

	Control	Epinephrine	Prednisolone + epinephrine	Standard error
pH-15 min	6.35	6.25	6.29	0.09
pH-45 min	5.93	5.85	5.98	0.17
pH-24 hr	5.45	5.48	5.42	0.02
Munsell value[2]	5.58	5.70	5.43	0.14
Transmission value[3]	50.8	53.4	41.4	8.7

[1] No significant ($P > .05$) differences were observed between treatments for any of the data in this table.
[2] Higher values indicate lighter colored muscles.
[3] Higher values indicate poor protein solubility.

administration of 250 μg of epinephrine per kg body weight at 4 hr ante-mortem resulted in higher ultimate pH in porcine muscle. These same authors also reported that epinephrine administered at levels of 100 μg/kg and 250 μg/kg had almost identifical effects upon ultimate pH of rabbit muscle. The 100 μg/kg dose compares favorably with that used in the present study.

The higher ultimate pH values reported by other authors to result from ante-mortem epinephrine injection reflect the depletion of muscle glycogen prior to slaughter, thus decreasing the amount of lactic acid formed during anaerobic post-mortem glycolysis. In the present study, there apparently was insufficient time for significant glycogen depletion to occur between epinephrine administration and slaughter of the animal. This is substantiated by the nearly identical ultimate pH values for the various treatment groups (Table 1) and the fact that muscle glycogen was not significantly lowered at 15 min post-mortem by epinephrine treatment (Table 4 and Fig. 1).

Rate of post-mortem pH decline during the first hour was not affected by epinephrine injection since muscle pH values did not differ between treatment groups at either 15 or 45 min after exsanguination (Table 1). In contrast,

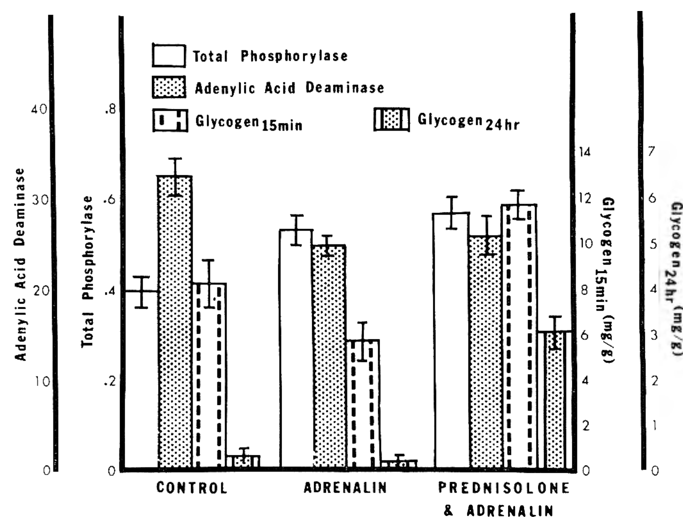

Fig. 1. Total phosphorylase, adenylic acid deaminase and 15 min and 24 hr glycogen levels in porcine longissimus dorsi muscle of control pigs and those injected with epinephrine and prednisolone plus epinephrine. Phosphorylase activity = μM P_i released/min/mg protein. Adenylic acid deaminase activity = μM AMP deaminated/min/mg protein.

Bendall *et al.* (1962a) found faster rates of pH fall in rabbits injected with epinephrine 4 hr ante-mortem. However, these same authors reported that cortisone administration 20 hr prior to epinephrine injection protected against the glycogen wasting effect of exogenous epinephrine and restored a normal rate of post-mortem pH fall.

Topel *et al.* (1967a) have shown that pigs subjected to prednisolone treatment (50 mg/100 lb/day) for 10 days had lower plasma levels of 17-hydroxycorticosteroids (17-OHCS); these glucocorticoids are known to play an important role in an animal's response to stress. Therefore, it was thought that the effect of exogenous epinephrine upon glycolytic rate in muscle from prednisolone treated pigs (low plasma 17-OHCS levels) might be more susceptible to the development of OSE musculature. The fact that more rapid glycolysis was not observed does not, however, preclude adrenal involvement in other aspects of the etiology of PSE musculature.

Munsell value and transmission value

Munsell value did not differ significantly ($P > .05$) between control, epinephrine or prednisolone plus epinephrine injected pigs (Table 1). Much greater variation was found within than between treatment groups. Munsell value was negatively correlated with pH at either 15 or 45 min, $r = -.69$ and $r = -.70$ ($P < .01$) respectively (Table 3). These data indicate that light colored muscles were associated with faster rates of pH fall and is in agreement with the results of other workers (Briskey *et al.*, 1961; Sayre *et al.*, 1963b). Muscle color intensity was also positively related to solubility of water extractable proteins as shown by the correlation ($r = 0.78$, $P < .01$) between Munsell value and transmission value.

Transmission value was not significantly ($P > .05$) affected by epinephrine or prednisolone plus epinephrine treatment (high transmission value indicates muscle with low protein extractability, Table 1). As expected, transmission value was negatively correlated with pH at 15 and 45 min post-mortem ($-.76$ and $-.88$, $P < .01$ respectively). Thus, more rapid rates of pH fall resulted in lower protein extractability. These results illustrate the importance of the rate of post-mortem pH decline to the ultimate porcine muscle quality.

Phosphorylase

Total phosphorylase activity was significantly ($P < .05$) increased by epinephrine injection as shown in Table 2 and Fig. 1. Enzyme activity in muscles from control pigs was 0.40 μM P_i released/min/mg protein compared to 0.53 and 0.57 for epinephrine and prednisolone plus epinephrine injected pigs, respectively. However, the increased total phosphorylase activity was not entirely accounted for by higher phosphorylase *a* activity.

This may have been due to the fact that phosphorylase *a* is more active in the presence of AMP (used for total phosphorylase assay) than in the absence of AMP as explained by Cori (1956), thus causing total phosphorylase to appear greater. Also, since approximately 10 min elapsed between exsanguination and excision of the muscle sample, considerable breakdown of phosphorylase *a* by phosphorylase phosphates could have occurred.

Posner *et al.* (1965) reported a 2 to 4 fold increase in phosphorylase *a* to *b* ratios in frog and rat muscles 12 min after epinephrine injection. In the present study, mean phosphorylase *a* activity was higher in epinephrine treated pigs and the increase approached significance ($P < .10$).

Low, non-significant correlation coefficients ($P > .05$) were obtained between total phosphorylase activity and pH at 15 min and 45 min post-mortem (Table 3). This is in disagreement with Wismer-Pedersen (1959) but concurs with the results of Sayre *et al.* (1963c); the latter authors found no significant relationship between total phosphorylase activity and rate of pH decline. The low correlation coefficients found between pH and phosphorylase *a* activity or % phosphorylase *a* and between Munsell value and transmission value and the various measures of phosphorylase activity tend to negate any postulation that some abnormality in the phosphorylase system is responsible for development of PSE porcine muscle.

AMP, IMP and glycogen

AMP level in the muscle was not highly correlated ($P > .05$) with muscle pH at either 15 or 45 min post-mortem, but IMP concentration was negatively correlated ($P < .01$) with pH ($-.94$ at 15 min and $-.93$ at 45 min post-mortem, respectively, Table 3). This almost linear, inverse relationship between pH and IMP indicates that in porcine muscle with rapid post-mortem glycolysis, a concomitant rapid liberation of ammonia occurs, 1 mole of ammonia being released for each mole of IMP formed. Briskey *et al.* (1961) reported very rapid ammonia liberation in muscle which ultimately became pale, soft and exudative.

The mean AMP concentrations observed were 1.82, 1.72 and 2.10 $\mu M/g$ of fresh muscle in control, epinephrine and prednisolone plus epinephrine treated pigs, respec-

Table 2. Means[1] and standard error of the means for total phosphorylase, phosphorylase *a*, and % phosphorylase *a* in muscle of control, epinephrine, and prednisolone plus epinephrine injected pigs.

	Control	Epinephrine	Prednisolone + epinephrine	Standard error
Total phosphorylase activity[2]	0.40[a]	0.53[b]	0.57[b]	0.03
Phosphorylase *a* activity[2]	0.03	0.05	0.07	0.01
% Phosphorylase *a*	7.7	10.2	12.0	2.4

[1] Means with different superscripts are significant ($P < .05$).
[2] Phosphorylase activity = μM P_i released/min/mg/protein.

Table 3. Simple correlation coefficients[1] between various observations in porcine muscle.

	pH 15 min	pH 45 min	Munsell value	Transmission value
Munsell value	−.69	−.70	....	....
Transmission value	−.76	−.88	0.78	....
Total phosphorylase	−.05	0.05	−.05	−.05
Phosphorylase *a*	0.20	0.28	−.22	−.31
% Phosphorylase *a*	0.22	0.30	−.22	−.33
AMP	0.24	0.13	−.35	−.19
IMP	−.94	−.93	0.75	0.83

[1] Correlation coefficients > 0.42 are significant at 5% level and those > 0.54 are significant at the 1% level.

tively. It has been reported that rabbit muscle phosphorylase *b* is activated ½ maximally in the presence of $1 \times 10^{-5} M$ AMP (Cori, 1956). The AMP concentrations reported above for porcine muscle are substantially greater than this level.

Morgan *et al.* (1963) observed a 20 fold increase in the rate of glycogen breakdown by rat muscle phosphorylase *b* when the reaction was carried out in the presence of anaerobic levels of AMP, ATP and P_i as opposed to conducting the reaction in aerobic levels of these substances. They stated that phosphorylase *b* activation would appear to be an important factor in the rapid rate of glycogenolysis under anaerobic conditions.

Based upon the observations in rat and rabbit muscle, the AMP levels found in porcine muscle would be sufficient for phosphorylase *b* to be active during post-mortem anaerobic glycolysis. However, the AMP requirements of porcine muscle phosphorylase *b* activity may possibly be quite different than those reported for this enzyme in rabbit or rat muscle.

Prednisolone plus epinephrine treated pigs had significantly ($P < .05$) greater amounts of muscle glycogen at 15 min and 24 hr post-mortem than either control or epinephrine injected pigs (Table 4 and Fig. 1). Mean 15 min glycogen was lower in epinephrine injected pigs than in controls but the difference was not significant ($P > .05$). Lower initial muscle glycogen in the epinephrine injected pigs was probably a reflection of the higher total phosphorylase activity, yet the rate of pH fall was not different from controls, indicating that the more rapid glycogen breakdown did not result in faster lactic acid production.

These data suggest the possibility of intermediate rate limiting reactions in post-mortem glycolysis affecting lactic acid production. Further work is needed to determine the effects of and possible synergism between the other glycolytic enzymes upon the ultimate porcine muscle morphology.

While glucocorticoids are important in promoting gluconeogenesis they are also believed to interfere with glucose utilization at the cellular level (Zarrow *et al.,* 1964). Concomitant with increased gluconeogenesis, they cause accumulation of liver glycogen. Prednisolone, a synthetic steroid, has 10 to 20 times the gluconeogenic potency of the naturally occurring glucocorticoid, cortisone. Therefore, the higher glycogen levels observed in the muscles from pigs subjected to prolonged prednisolone injection (10 days) would be expected.

Even though the prednisolone plus epinephrine treatment produced higher glycogen reserves and greater phosphorylase activity, the rate of pH decline, Munsell value (muscle lightness or darkness) and protein solubility (transmission value) were not indicative of PSE musculature. These data indicate the need for further study of the implication of hypoglucocorticoidism and physiological stress upon the etiology of PSE muscle morphology.

Table 4. Means[1] and standard error of the means for AMP, IMP and glycogen in control, epinephrine and prednisolone plus epinephrine treated pigs.

	Control	Epinephrine	Prednisolone + epinephrine	Standard error
AMP[2]	1.82	1.72	2.10	0.16
IMP[2]	2.53	2.74	2.54	0.46
Glycogen-15 min[3]	8.27[a]	5.85[a]	11.66[b]	0.87
Glycogen-24 hr[3]	0.34[a]	0.21[a]	3.14[b]	0.18

[1] Means with different superscripts are significant ($P < .05$).
[2] μM/g fresh muscle.
[3] mg/g fresh muscle.

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A Quantitative and Morphological Study of Bovine Longissimus Fat Cells

SUMMARY—A quantitative and morphological study was made of bovine fat cells from three marbling groups of longissimus muscle. Tracings on acetate paper were made of the visual marbling depots and the area measured at three positions with an ocular grid for both size and distribution of fat cells. Comparisons were also made of subcutaneous, intermuscular and intramuscular fat cell size. Intrafiber lipid accumulation was observed and the average number of red fibers per bundle determined. These results indicate that traceable intramuscular fat is not a good measure of total intramuscular fat. There did not appear to be a consistent medial, central and lateral marbling pattern among the three marbling groups; however, significant differences were apparent within groups.

Fat cell size increased with increases in cell mass, marbling and total chemical fat of the muscle. Fat cells accumulated and grew in close proximity to portions of the circulatory system. Lipid deposits adjacent to the muscle contained larger fat cells than was evident in the extrafascicular spaces within the muscle. Intrafiber lipid was readily apparent in approximately 35% of the muscle fibers and probably represented either mitochondria or triglyceride. The interrelationship of subjective marbling scores, chemically determined fat, fat cell size, fat cell distribution and intrafiber lipid characteristics are complex and require complete investigation before the association of muscle fat and meat quality can be resolved.

INTRODUCTION

THE ACCUMULATION OF INTRAMUSCULAR FAT in meat animals is an area of concern for many researchers. Intramuscular fat has been studied in regard to meat quality (Tuma *et al.*, 1963; Romans *et al.*, 1965; and Pearson, 1966), and the quantity of intramuscular fat has been an important factor in determining carcass grade (Briskey *et al.*, 1964; Romans *et al.*, 1965; Walter *et al.*, 1965). Consequently, several objective methods for determining intramuscular fat have been developed (Schoonover *et al.*, 1957; Orme *et al.*, 1958; Blumer *et al.*, 1959; Cook *et al.*, 1961), but the variations in quantity and distribution of intramuscular fat within a muscle give rise to a number of problems. Such variations have been well documented for the longissimus (Weir, 1953; Wang *et al.*, 1954; Lawrie, 1961; Doty *et al.*, 1961; Blumer *et al.*, 1962; Cook *et al.*, 1964), and there is also evidence that specie and breed differences exist (Hammond, 1932; McMeekan, 1940; Carpenter *et al.*, 1961).

The elucidation of fat synthesis, deposition and metabolism in muscle is a more fundamental problem and is an area which must be fully explored before the association of intramuscular fat with meat quality can be well understood. Kauffman *et al.* (1967) have proposed a theory regarding the influence of muscle architecture on pattern of fat deposition, and other workers (Bell, 1909; Waters, 1909; Hammond, 1932) have measured fat cell diameter in ovine and bovine muscle during various stages of growth. Allen *et al.* (1967b) have studied some enzymes involved in lipid synthesis and degradation in muscle while other workers have investigated the role of intramuscular lipids in energy production (Masoro *et al.*, 1966).

The research reported here was designed to study fat cell morphology. Specific objectives were: 1) to compare histological estimates of intramuscular fat content with subjective and chemical determinations, 2) to study the influence of intramuscular fat quantity and distribution on fat cell size and 3) to make additional observations on intermuscular and subcutaneous fat cell morphology and on lipid accumulation within muscle fibers.

EXPERIMENTAL

FORTY-FIVE 2.5 CM THICK STEAKS from the 13th rib portion of bovine loins of the same approximate physiological maturity were selected from a local packing plant

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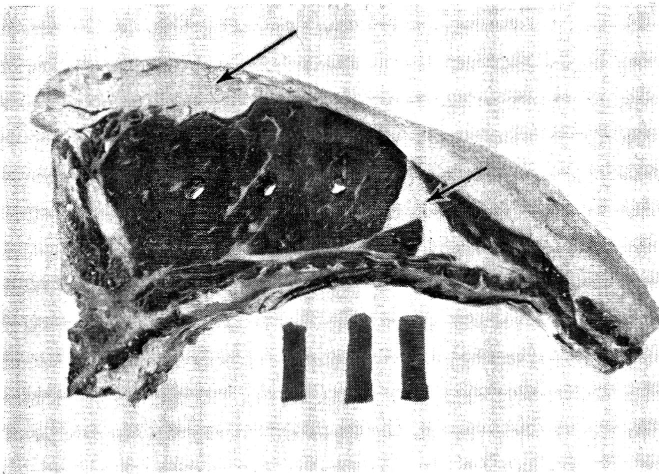

Fig. 1. Steak from the 13th rib section showing the medial, central and lateral core locations. The arrows show sampling locations for subcutaneous and intermuscular fat.

to represent equal numbers of traces, small and moderate degrees of marbling. Tracings on acetate paper were made of the visual marbling deposits, muscle perimeter and histological core locations on the cut surface of each steak. Subsequently, the area of intramuscular fat (large depots) and muscle area were determined with a compensating polar planimeter. Three 1.27 cm cores from the longissimus were selected at medial, central and lateral positions similar to the procedure described by Venable *et al.* (1962) and illustrated in Fig. 1. The remainder of the longissimus from the steak was frozen in liquid nitrogen and powdered according to the procedure of Borchert *et al.* (1965) and used for ether extract determinations according to the AOAC (1959) method.

About 0.6 cm of tissue was removed (anterior portion of longissimus steak) from each of the 1.27 cm thick cores and was frozen on dry ice blocks so that sections could be cut perpendicular to the long axis of the core. After samples were completely frozen they were mounted on a cryostat spindle, allowed to equilibrate at $-20°C$, and sectioned 16 μ thick. One section from each of the three positional locations (medial, central, lateral) was mounted on a precooled, gelatin coated slide. The three tissue sections were allowed to air dry for 45 sec and then placed in a coplin jar containing 30% formalin for 30 min. Sections were stained with oil red O and hematoxylin according to the procedure of Lillie (1965).

An ocular grid method (Lewis *et al.*, 1958; Herring *et al.*, 1967) was used to quantitate the amount of fat present in sections as fat cells. This fat measure was expressed as the percent of grid squares occupied by fat in a tissue area 1.27 cm in diameter (Magnification 25×). Three areas of fat were chosen to represent three degrees of fat cell mass accumulation on the basis of the number of cells present in each mass. The mass with the least number of cells contained from 5 to 10 cells, the intermediate 11 to 20 cells and the largest contained above 20 cells.

Fat cell accumulations representative of the three groups are illustrated in Fig. 4. Fat cell size was determined in the central core only and 15 cells per muscle in each of the mass areas were measured by counting the number of grid squares (if the square was one-half or more filled, it was counted as one) present in each cell at a magnification of 430×.

Five additional steaks, all with the same degree of marbling (moderate) and similar subcutaneous fat thickness (1.27 to 1.77 cm), were processed histologically as previously described and used to compare subcutaneous, intermuscular and intramuscular fat cell size. The subcutaneous fat was taken from the outer layer and the intermuscular fat was sampled between the longissimus and the longissimus costarum (see Fig. 1). In addition some muscle sections were stained with Sudan black B in Propylene Glycol (Chiffelle *et al.*, 1951) in order to study the intracellular fat in muscle fibers.

Photomicrographs were taken with a Zeiss Photomicroscope using Ansco Versapan (ASA 100) 35 mm film.

Means and standard deviations were determined on each group and analyzed statistically by analysis of variance, simple correlations and Duncan's new multiple range test (Steel *et al.*, 1960).

RESULTS AND DISCUSSION

Quantitative aspects of intramuscular fat

Fig. 2 illustrates representative sections from traces, small and moderate marbling groups. Muscle bundle structure is not clearly distinguishable because sections were cut directly from the surface of the cores (Fig. 1) with no attempt to orient fiber direction. This procedure was adopted to give a more direct comparison of the histological picture with subjective estimates of marbling than if fibers had been oriented for more exact cross sections. An increase in area occupied by fat cells is clearly shown with higher marbling scores.

Quantitative data for histologically determined fat at each of the three positional locations in the three different marbling groups are shown in Fig 3. The mean fat value was lowest in the traces group (11.4 ± 4.7) and highest in the moderate group (18.2 ± 8.0), being significantly different ($P < .01$). The histological fat content followed the same trends as the mean values for the ether extracts in each of the three marbling groups but was higher than the ether extract values. This observation agrees with the work of Venable (1963) who reported that intramuscular fat expressed as a percent area was higher than when expressed by weight.

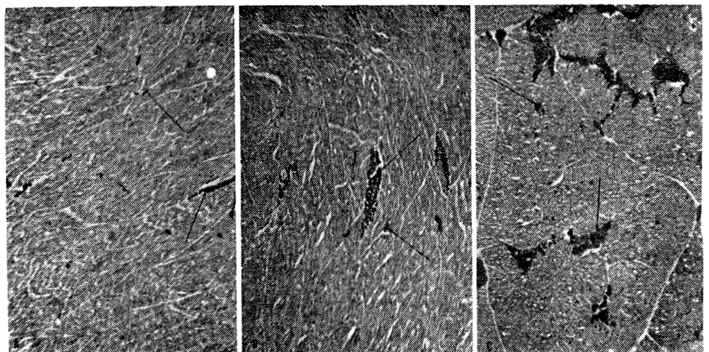

Fig. 2. Photomicrograph showing typical intramuscular fat pattern for traces (A), small (B) and moderate (C) degrees of marbling. Arrows indicate fat cells which have a positive contrast following oil red O staining (oil red O, Hematoxylin; 5.1X).

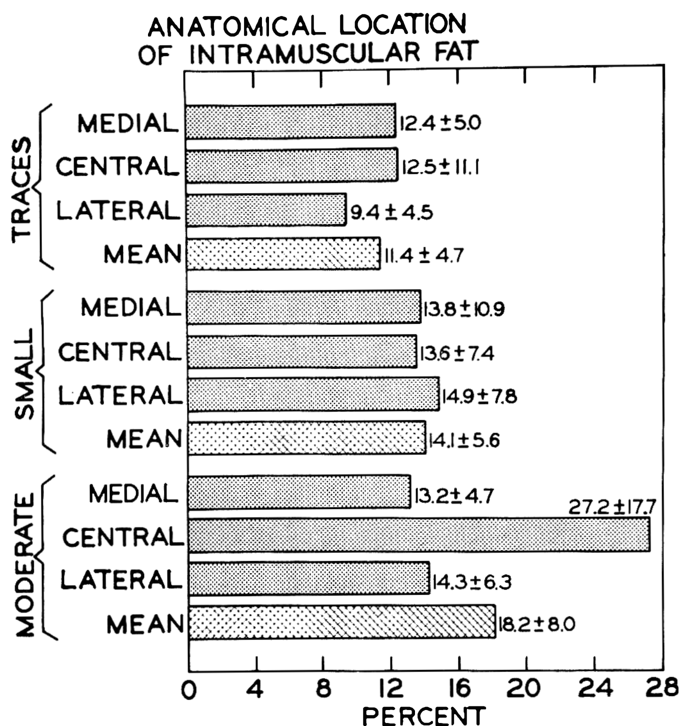

Fig. 3. Bar graph illustrating fat content at three locations in each of three marbling groups. Fat was expressed as the percent of grid squares occupied by fat in a tissue area 1.27 cm in diameter.

There was no significant difference in histologically determined fat in the medial position for the traces, small and moderate marbling groups (12.4, 13.8 and 13.2, respectively). Likewise, there was no appreciable difference between the traces and small group (12.5 vs 13.6) for the central position, but for moderate marbling the central position was extremely high. A review of some of the tracings made of the visual marbling depots verified the large tree-like marbling deposits in the central portion of the longissimus as previously described by Hammond (1932), and Venable (1963). In a few instances these fat deposits were attached to the epimysium and opposite the rib bone as reported by Blumer *et al.* (1962).

The traces group had significantly ($P < .01$) less fat in the lateral core position than did either of the other two groups. No significant difference was noted between the mean values for the small and moderate groups at this location. From these results there does not appear to be a consistent medial, central or lateral marbling pattern in bovine longissimus representative of the three marbling groups. However, on a within marbling group basis the traces group had the least amount of fat in the lateral position, whereas the moderate group had the most fat in the central position with about equal amounts in each position for the small group.

The large standard deviations at some of the positional locations give further evidence to the variation in fat distribution of the longissimus. This variation resulted when fat was measured at a precise anatomical location among steaks with the same subjective marbling scores. The variation in anterior-posterior marbling pattern of bovine longissimus has been clearly demonstrated (Wellington *et al.*, 1959; Blumer *et al.*, 1962) and the present results point out the medial to lateral variation of marbling.

Simple correlation coefficients between histologically determined fat and ether extractable fat are given in Table 1. The negative correlations for the traces group may have resulted from the fact that smaller fat cells were present (Table 2) than in other marbling groups and consequently the grid method was biased. Because a grid area was counted as one if it was half or more filled by fat, the small cells may have been given equal ranking compared to slightly larger cells.

Correlations were nonsignificant in the small group but significant in the moderate group. It appears that the histological method for estimating muscle fat is more accurate when more fat is present. However, the method is probably more useful for studying fat cell morphology and distribution than for estimating total muscle fat.

Correlations were also calculated between ether extractable material and visual fat determinations from the tracings made on acetate paper (Table 1). The over-all

Table 1. Simple correlations of intramuscular fat parameters with ether extractable material.[1]

Fat cell masses	Marbling score			
	Traces	Small	Moderate	Over-all
Histological core locations				
Medial core	—.47	.03	.53*	.13
Central core	—.33	.12	.61**	.47
Lateral core	—.11	.41	.72**	.49*
Visual fat[2]				
Total fat area (cm)[2]	.28	.40	—.21	.56*
Total fat area as % of L. dorsi area	.02	.26	—.20	.57*
Area of largest single fat mass	.19	.27	.08	.40
Fat cell masses				
5–10 cells	.09	.01	.44	.52*
11–20 cells	.08	.32	.51*	.42
> 20 cells	.21	.35	.63**	.67**

[1] Correlations expressed on fresh weight basis.
[2] Large visual marbling deposits traced on acetate paper and measured with a compensating planimeter.
* $P < .05$.
** $P < .01$.

Table 2. Relative size[1] of fat cells from three marbling groups of bovine longissimus.

Fat cell masses	Marbling groups: Traces (Mean[2] ± S.D.)	Small (Mean ± S.D.)	Moderate (Mean ± S.D.)
5–10 cells	7.0[3] ± 1.4	8.3 ± 2.3	9.9 ± 2.6
11–20 cells	9.3 ± 3.6	11.4 ± 2.2	11.7 ± 2.1
> 20 cells	12.4 ± 4.5	17.6 ± 4.7	20.5 ± 5.1
Mean	9.9 ± 2.2	12.4 ± 2.2	14.0 ± 3.0

[1] Means represent the number of grid squares required to cover the area of one fat cell. Each grid square covered an area of .004 mm^2.
[2] Any two means not underscored by the same line are significantly different ($P < .05$).
[3] Mean of 15 cells.

correlations were considerably larger than within group correlations, but the comparisons indicated that the tracing procedure was not sufficiently associated with ether extractable material to be of any practical importance.

Fat cell size

Table 2 contains means and standard deviations for fat cell size from the three marbling groups and from the three fat cell mass classifications. Fat cell size increased as marbling score increased, being significantly ($P < .01$) smaller in the traces group than in the moderate group for each of the fat cell mass classifications. A distinct increase in fat cell size was also found as the number of fat cells per mass increased. This observation is clearly illustrated in Fig. 4 where an increase in fat cell size is seen as the number of fat cells per mass increases.

The finding that fat cell size increases both with increased marbling and increased number of cells per fat cell mass may be important. It could be reasoned, that in order for a fat cell to attain a large size it has to be present in a group of cells rather than as an isolated one or few cells. Furthermore the higher marbling classifications must be a reflection of increased fat cell size regardless of the number of cells in a given fat cell mass.

Apparently, once a muscle is committed to increase fat content, both the size and number of fat cells increase. This process could be initiated by turning on or off an enzyme or hormone system (see Allen *et al.*, 1967b). Fat cell size is positively correlated with ether extract values in all three marbling groups (Table 1). All three fat cell mass classifications had higher correlations in the moderate marbling group than in the other marbling groups and it is apparent that fat cells associated with the largest fat mass gave the highest correlation with chemical fat. These fat cells are the largest and this provides further evidence that the cell size may be important when the grid method is employed.

If these large fat cell masses are indicative of coarse marbling patterns, then these data would agree with earlier work by Blumer *et al.* (1962) who found in general that bovine steaks which appeared to have coarse marbling also had a relatively large amount of chemical fat. Conversely, Carpenter *et al.* (1961) found a significant negative correlation between subjective fat cell size and chemical fat in the longissimus from light weight porcine animals.

Table 3 shows the comparison of intramuscular, subcutaneous and intermuscular fat cell diameter. It can be seen that the size of fat cell depends on location with subcutaneous fat having larger fat cells than intermuscular fat, and that both subcutaneous and intermuscular fat cells are much larger than intramuscular fat. These results agree with earlier work of Waters (1909) who reported that the size of fat cells within a muscle is small compared with that of the kidney fat or even the subcutaneous or intermuscular fat. Waters (1909) suggested that the relatively small size of intramuscular fat cells may be due to mechanical pressure and he drew an analogy to the small fat cells distributed in the tough white connective tissue of the brisket.

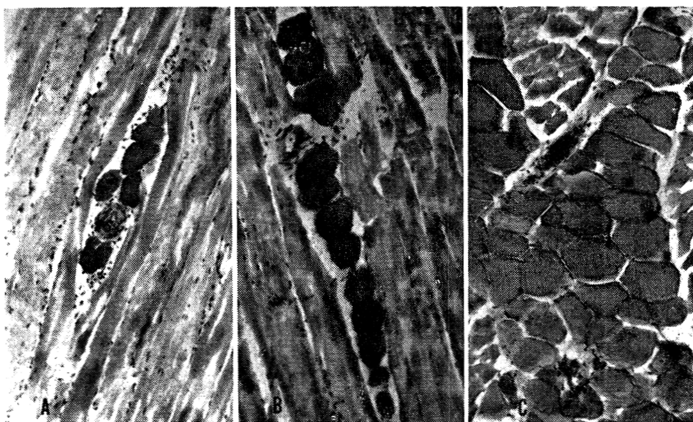

Fig. 4. Photomicrograph showing typical fat cell mass classifications. A represents 5–10 cells, B represents 11–20 cells and C represents above 20 cells. The increase in fat cell size as the number of fat cells per mass increases is obvious (oil red O, Hematoxylin; 55.2X).

Intrafiber lipid

Intramuscular lipid is found in the form of fat cells situated in the perimysial planes, as intracellular free lipid droplets and as an integral constituent of membranes. The fat cells accumulate and grow in the extrafascicular spaces in near proximity to portions of the circulatory system.

Fig. 5 illustrates the appearance of a bovine muscle section reacted with the lipid dye Sudan black B; it shows clearly that the fibers can be differentiated into two types on the basis of the histochemical reaction. The unreacted fibers are the classical white muscle fibers while the positively reacted fibers (dark) are the classical red fibers.

Muscles are composed of a mixture of red and white fibers with the predominant fiber type giving rise to the

Table 3. Comparison of fat cell diameter in the subcutaneous, intermuscular and intramuscular bovine fat depots.

Fat depots	Mean[1] μ	S.D. μ
Subcutaneous[2]	115.5	12.6
Intermuscular[2]	109.5	14.7
Intramuscular		
5–10 cell mass	46.0	8.9
11–20 cell mass	66.2	13.7
> 20 cell mass	87.0	15.3

[1] Mean of 15 cells.
[2] See Fig. 1 for location.

gross classification of the muscle as red or white. In the present study, approximately 35% of the fibers per muscle bundle were Sudanophilic or red fibers, compared with about 25% for porcine longissimus (Beecher *et al.,* 1965). The longissimus is considered a typical white muscle—most red muscles are composed of greater than 50% red fibers.

Red muscle maintains a more prolonged tonic function compared to white muscle which is used for short bursts of activity. Beatty *et al.* (1963) have described striking differences in the relative distribution of various metabolic pathways in red and white muscle and Moody *et al.* (1967) have described in detail the reciprocal relationship between reduced diphosphopyridine nucleotide tetrazolium reductase and amylophosphorylase in porcine muscle.

The red fibers in bovine longissimus are arranged in clusters throughout the bundle (Fig. 5), and the fine appearance of a typical red fiber is shown in high magnification in Fig. 6. The positively reacted sites most probably represent either mitochondria or triglyceride (Padykula *et al.,* 1963), and the lipid or lipid containing organelles are responsible for the positive reaction of red fibers with Sudan black B. The adjacent white fibers give a weak or negative reaction.

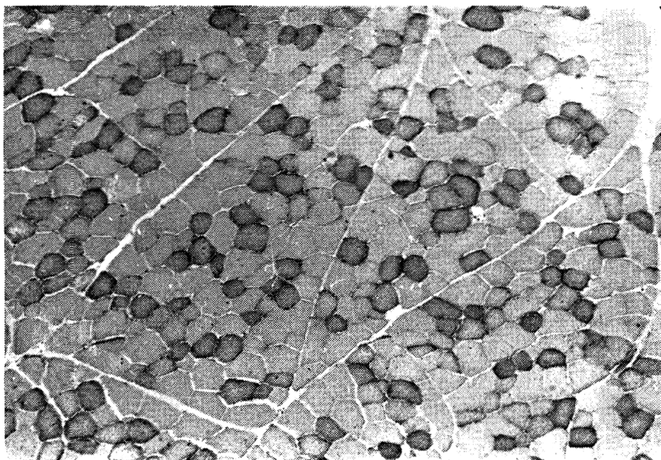

Fig. 5. Photomicrograph showing red and white fiber distribution in bovine longissimus. Sudanophilic fibers are classified as red and nonreactive fibers as white (Sudan black B; 64×).

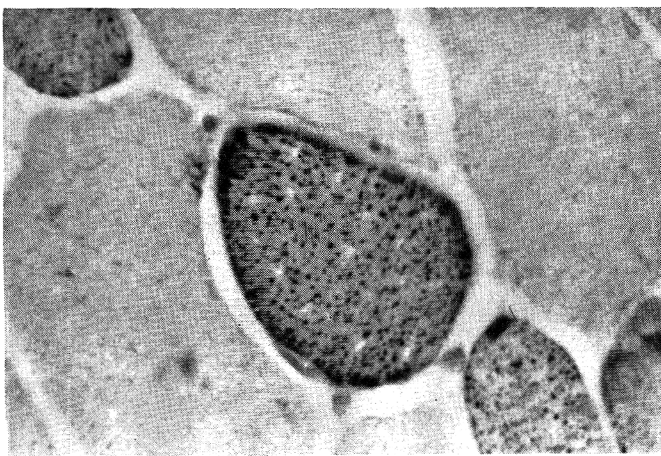

Fig. 6. Photomicrograph showing intracellular detail of a red fiber. Positive reaction sites are clearly visible and probably represent mitochondria or triglyceride (Sudan black B, 640.2×).

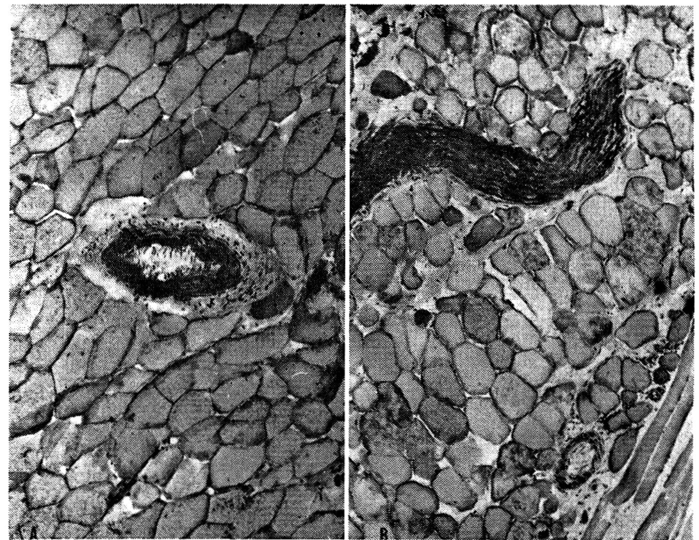

Fig. 7. Photomicrograph of bovine intramuscular fat deposits. Portion A shows an arteriole surrounded by fat cells and portion B shows a nerve coursing through fat cells (oil Red O, Hematoxylin; 52.7X).

This discussion is to emphasize the presence and potential importance of such intracellular lipids. Most certainly a portion of this intracellular lipid is detected in chemical determinations of fat and the association of such lipids with problems of keeping quality, role in post-mortem change and contribution to palatability should not be overlooked. Allen *et al.* (1967a) have examined the lipid composition of three different muscles and reported some differences in fatty acid composition.

Adipose tissue is richly supplied with a vascular system (Bloom *et al.,* 1964) and it has been clearly demonstrated (Blumer *et al.,* 1962) that the main marbling deposits are close to or within a heavy vascular network. In the present study, blood vessels of some size were always apparent in or near masses of fat cells. An arteriole in the center of a large mass of fat cells is shown in Fig. 7A and smaller vessels are noticeable in Fig. 4A. Figure 7B illustrates the appearance of a nerve coursing through a fat deposit.

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Meat Flavor. 2. Procedures for the Separation of Water-Soluble Beef Aroma Precursors

SUMMARY—Water-soluble, low molecular weight beef aroma precursors have been separated by column chromatography on Bio-Gel P-2, Amberlite XAD-2 and DEAE-Sephadex. Separations on the basis of gel filtration, adsorption, and anion exchange resulted in a number of fractions that developed roast beef aroma on pyrolysis, but differed in composition. Either sugar phosphates or free sugars are involved in aroma development. Tyrosine, phenylalanine, taurine and glutamic acid may be removed without affecting aroma. A number of amino acids were present in the aorma producing fractions in trace amounts, thus the requirement for their presence may be questionable. Creatine, creatinine and the purine derivatives (inosinic acid, inosine, and hypoxanthine) may also be removed without affecting aroma development.

INTRODUCTION

PRECURSORS OF THE CHARACTERISTIC AROMA of meat are low molecular weight, water-soluble compounds that are easily extracted from muscle tissue. So many individual components are present, however, that it has not been possible to identify the compounds most directly associated with aroma development. Separations on ion exchange resins have given promising results (Batzer *et al.*, 1960, 1962; Wasserman *et al.*, 1965).

Since our previous study on the fractionation of flavor precursors on strong ion exchange resins (Wasserman *et al.*, 1965), we felt that the strongly active resins, as well as acid and alkali solutions needed to elute compounds from chromatographic columns, might induce alterations in the structure of some molecules, thus introducing artifacts into the study. To investigate milder separation techniques we turned toward gel filtration, which involves separation of molecules on the basis of molecular weight (Porath *et al.*, 1959; Gelotte, 1960). Small molecules which diffuse into the gel particles are retarded, while large molecules which are completely excluded from the gel phase will migrate through the column without retention in the interstitial fluid.

Gel filtration on crosslinked dextran, Sephadex G-25, has been mentioned previously in connection with fractionation of beef muscle components (Batzer *et al.*, 1960; Wasserman *et al.*, 1965). However, we employed a crosslinked polyacrylamide, Bio-Gel P-2, a comparatively new gel filtration material of smaller pore size than previously available, which suggested that separation of lower molecular weight compounds may be possible.

While our studies involving separation of sugars, amino acids and purine derivatives were in progress, three papers on the use of Bio-Gel P-2 appeared. Schwartz *et al.* (1966) reported on the desalting of peptides and amino acids. From their data possible separation of several amino acids can be inferred. Schwartz *et al.* (1965), as well as Uziel *et al.* (1965) reported on the use of P-2 gel for desalting and separating nucleic acid components on the basis of molecular weight.

A new adsorbent has recently become available for separating water-soluble organic compounds. The crosslinked polystyrene resin, Amberlite XAD-2, contains no functional groups; separations on this resin are based primarily on hydrophobicity. Molecules containing nonpolar groups or aromatic nuclei are adsorbed on the resin and are eluted at a slower rate than highly polar molecules. To our knowledge there are no publications dealing with the uses of Amberlite XAD-2. However, on the basis of information in the manufacturer's technical bulletin we felt this resin could give a series of useful separations of the meat components.

The weak anion exchange material, DEAE-Sephadex, is useful for selective removal of anions. It has been employed to separate basic peptides from the pituitary (Porath *et al.*, 1961), amino acids and peptides (Carnegie, 1961) as well as glycogen, sugars, sugar phosphates and adenine nucleotides from blood cells (Öckerman, 1963).

Results reported in this paper describe the use of Bio-Gel P-2, Amberlite XAD-2, and DEAE-Sephadex in the separation of water-soluble compounds in a continuing study on the isolation and characterization of meat aroma precursors.

EXPERIMENTAL

Preparation of beef diffusate

One kilogram of frozen beef muscle was partially thawed, cut into small pieces and homogenized with 4 L of cold deionized water for 2 min in a blendor. The homogenate was allowed to stand for 2 hr and the solids were then separated by centrifugation. The supernatant was dialyzed against deionized water for 72 hr with three changes of water of 3 L each, using Visking dialysis tubing, prepared by exhaustive washing with water to remove glycerin. The entire operation was carried out at 4°C. The combined diffusates were lyophilized, dissolved in deionized water to a final volume of 250 ml and stored at −18°C until used.

The dry weight of the diffusate (96 mg/ml) constitutes 2.4% by weight of the raw beef muscle.

Chromatography on Bio-Gel P-2

Bio-Gel P-2 (100–200 mesh), purchased from Bio-Rad Laboratories, was washed exhaustively with ethanol to remove ultraviolet absorbing impurities. The gel was hydrated with water and fines were removed by decantation.

A chromatographic column (2.4 × 75 cm), pretreated with 1% trimethylchlorosilane in carbon tetrachloride to reduce "wall effects," was packed with hydrated gel to a height of 68 cm. Beef diffusate (1.7 ml) was diluted to 5 ml with water and applied to the top of the gel bed. Components were eluted with water at a flow rate of 27 ml/hr and 6.9 ml fractions were collected.

Chromatography on DEAE-Sephadex

DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) was washed and converted to the formate form as suggested by the manufacturer. The gel was equilibrated with 0.1*N* formic acid and packed to give a column 1.5 × 87 cm. Beef diffusate (1 ml), diluted with 1 ml 0.1*N* formic acid, was applied to the column and eluted with 0.1*N* formic acid at a flow rate of 0.5 ml/min. Four ml fractions were collected. After analysis, appropriate fractions were combined, lyophilized and redissolved in a minimum quantity of water.

Chromatography on Amberlite XAD-2

Crosslinked polystyrene polymer Amberlite XAD-2 (20–50 mesh, Rohm and Haas Co.) was washed extensively with methylene chlorine-methanol (1:3 v/v), methanol, and finally water to remove soluble impurities. The resin was suspended in water and poured into a chromatographic column to give a resin bed 2.9 × 34.5 cm. Beef diffusate (5 ml) was applied to the column and eluted with water at a flow rate of 1 ml/min while 4 ml fractions were collected. After analysis, appropriate fractions were combined, lyophilized and redissolved in a minimum quantity of water.

Analytical methods

Chromatographic column effluents were collected with a Beckman Model 132 Fraction Collector maintained at 5–10°C. The ultraviolet absorbance of the effluents was monitored at 248 mμ with a Gilford Model 2000 Multiple Sample Absorbance Recorder. Fractions were analyzed for total amino acids by the ninhydrin method of Cocking *et al.* (1954) and for carbohydrates with the anthrone method used by Toennies *et al.* (1964). Ultraviolet spectra were recorded with a Bausch and Lomb Spectronic 505 and absorbance in the visible region was measured with a Beckman Model B Spectrophotometer. Amino acid analyses were performed according to Spackman *et al.* (1958) using a Phoenix automatic amino acid analyzer.

The phosphorus analysis was carried out by a modified molybdenum blue spectrophotometric method (AOAC, 1965).

Thin-layer chromatography

Qualitative analytical determinations of fraction components were carried out by thin-layer chromatography on plates of Silica Gel G (Merck), Cellulose MN 300 (Macheray and Nagel), and DEAE-Cellulose (Serva), and Eastman Chromagram sheets (type K301R2).

The following solvent systems were used:

A. n-propanol-ammonia (70:30 v/v)
B. 88% phenol (Mallinckrodt)-water (100:10 v/v)
C. i-propanol-ammonia (70:30 v/v)
D. n-butanol-acetic acid-water (74:19:20 v/v)
E. Formic acid-2-butanone-t-butanol-water (15:30:40:15 v/v)
F. i-propanol-pyridine-acetic acid-water (40:40:5:20 v/v)
G. diethyl ether-90% formic acid (7:1 v/v), saturated with water

Amino acids were separated on Silica Gel G plates or on Eastman Chromagram sheets with solvent systems A, B, C or D and detected with 0.2% ninhydrin in 95% ethanol.

Carnosine and histidine were detected with Pauly's diazo reagent (Rockland *et al.,* 1964) on chromatograms developed with solvent A or C. To detect creatine and creatinine the chromatogram, developed in solvent A or C, was sprayed with 1% aq. picric acid solution, heated at 110° for 1 hr, then sprayed with 4% sodium hydroxide solution (Block *et al.,* 1952).

Sugars, phosphates, and purine derivatives were chromatographed on Cellulose MN 300 with solvent E or F. Sugars were revealed with 0.1*M* p-anisidine phthalate in 95% ethanol (Randerath, 1963), and phosphates with Hanes-Isherwood reagent (Bandurski *et al.,* 1951).

Lactic acid was detected with a spray reagent consisting of 0.3% bromphenol blue and 0.1% methyl red in 95% ethanol after chromatography on Cellulose MN 300 developed with solvent E, or on Silica Gel G developed with solvent G. Lactic acid was also detected with Hanes-Isherwood reagent on Silica Gel G (Ting *et al.,* 1965).

Purine derivatives were also separated on DEAE-cellulose with 0.01*N* hydrochloric acid as solvent and detected as dark, absorbing spots under ultraviolet light at 257 mμ (Randerath, 1962). Quaternary ammonium compounds were separated on Silica Gel G with solvent A or C and detected by exposure to iodine vapor (Brante, 1949).

Aroma

Separation of the beef aroma precursors was followed by the development of meaty aroma on pyrolysis. Samples (0.25 ml) in 10 ml beakers were heated on a hot plate (surface temperature 150–160°) until completely dry. The aromas obtained were compared to the aroma produced on pyrolysis of the diffusate in a similar manner.

Four to six members of the Meat Laboratory evaluated the aromas informally. Since only major changes in odor were of interest to us at this time, formal, statistical taste panel procedures were not followed and the subjective, descriptive analyses were used. The panel responses were in agreement in differentiating meaty aromas resembling those of the diffusate control from others also produced on pyrolysis.

RESULTS AND DISCUSSION

OUR CHROMATOGRAPHIC EXPERIMENTS indicate that considerable separation of the low molecular weight components of beef muscle may be achieved by mild treatment. Fractions that gave desirable meat-like aromas on pyrolysis were obtained by separation of the diffusate on Bio-Gel P-2, Amberlite XAD-2, and DEAE-Sephadex, thus excluding a number of compounds from consideration as aroma precursors.

Table 1. Elution sequence of beef diffusate components from a Bio-Gel P-2 column.

1. Sugar phosphates, peptides, amino acids (most), lactic acid, quaternary amines
2. Free sugars, creatine, glycine, taurine, serine, urea
3. Inosinic acid, phenylalanine, inorganic salts
4. Creatinine
5. Tyrosine, unknown abs. max. 260 mμ
6. Inosine
7. Hypoxanthine
8. Unknown, abs. max. 265 mμ

The elution sequence of components of the diffusate from Bio-Gel P-2 is shown in Table 1 and Fig. 1.

Most of the amino acids were eluted together, but preliminary experiments indicated that some separation may be achieved within this group. Glycine, taurine and serine were eluted somewhat later followed by phenylalanine. Tyrosine, the last amino acid to be eluted, was well separated from the others. The sugar components were separated into two peaks containing sugar phosphates and free sugars, respectively. The amino acid and sugar peaks essentially overlapped, although the elution of sugars tended to be slightly retarded with respect to the amino acids. Inosinic acid, inosine and hypoxanthine were well separated, emerging from the column in that order.

The order of separation of components on Bio-Gel P-2 indicates that two major mechanisms are in operation: (1) exclusion from the gel on the basis of molecular weight, and (2) adsorption on the surface of the gel. In this respect the principles of separation on P-2 are similar to those on Sephadex (Gelotte, 1960). The elution order of purine derivatives was similar to that observed by Uziel *et al.* (1965) and Schwartz *et al.* (1965) who claimed that the movement of these compounds was governed by adsorption rather than by exclusion (or gel filtration).

Other factors affecting resolution on Bio-Gel P-2, besides column dimensions and flow rate, may be the pH of the eluting medium as well as the presence of other components in the sample. A mixture of six amino acids, carnosine, and sodium chloride was chromatographed using water as eluent.

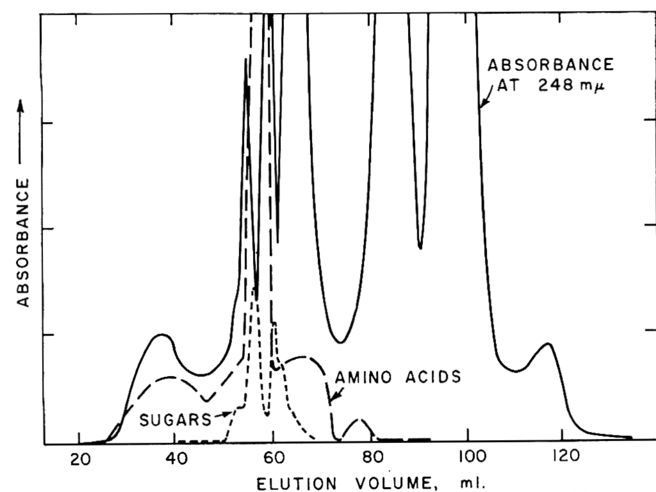

Fig. 1. Beef diffusate chromatographed on Bio-Gel P-2 (100–200 mesh).

Although the individual components were not completely resolved, the following elution sequence was clearly visible: (1) aspartic acid; (2) alanine, valine and leucine; (3) cysteic acid; (4) carnosine; (5) phenylalanine; (6) sodium chloride. However, during chromatography of the diffusate, carnosine was eluted together with alanine, valine and leucine. The presence of acids and bases in the diffusate may have caused this shift in the elution of carnosine. Schwartz *et al.* (1966) in their studies on desalting of amino acids on Bio-Gel P-2 reported that the elution volumes of glutamic acid and arginine were dependent on the pH of the eluting medium.

To obtain sufficient material for organoleptic evaluation, we chromatographed 25 ml of beef diffusate on a 5 × 75 cm column of Bio-Gel P-2 (200–400 mesh). The separation of components was similar to that shown in Fig. 1. The aromas obtained on pyrolysis of the eluted fractions were compared with the aroma of pyrolyzed beef diffusate. The most intense odors were obtained from fractions containing both amino acids and sugars. The composition of the fractions corresponding to the two sugar peaks (Fig. 1) are shown in Table 2.

The first fraction, containing glucose-6-phosphate, yielded a meaty aroma very similar to that of the diffusate. The odor from the second fraction, containing the free sugars—glucose, fructose and ribose—was also meat-like but had a pronounced plant-like or grassy character. Fraction 1, which yielded the most meat-like aroma thus could be regarded as containing aroma precursor compounds.

Among the components identified in this fraction are a number of amino acids, carnosine, anserine, phosphate sugars, quaternary amines and lactic acid. Trace amounts of creatine and inorganic salts were also found in this fraction, although the bulk of these compounds was found

Table 2. Composition of flavor fractions of beef diffusate separated on Bio-Gel P-2.

Fraction 1		Fraction 2
Aroma—meaty		Aroma—meat-like, grassy
1-methyl-histidine	Cysteic acid[1]	Taurine
Lysine	Taurine[1]	Urea
Histidine	Serine[1]	Aspartic acid
Anserine	Asparagine[1]	Glutamic acid
Carnosine	Glutamine[1]	Serine
Threonine	Arginine[1]	Threonine
Proline	Glucose 6-phosphate	Asparagine
Glutamic acid	Unknown sugar	Glutamine
Glycine	phosphate	Glycine
Alanine	Lactic acid	Methionine
Valine	Creatine[1]	Phenylalanine
Methionine	Choline	Alanine[1]
Isoleucine	Carnitine	Carnosine[1]
Leucine	Organic phosphates	Inosinic acid
	$PO_4\equiv$[1]	Creatine
		Creatinine
		$PO_4\equiv$
		Organic phosphates
		Glucose
		Fructose
		Ribose

[1] Trace amount.

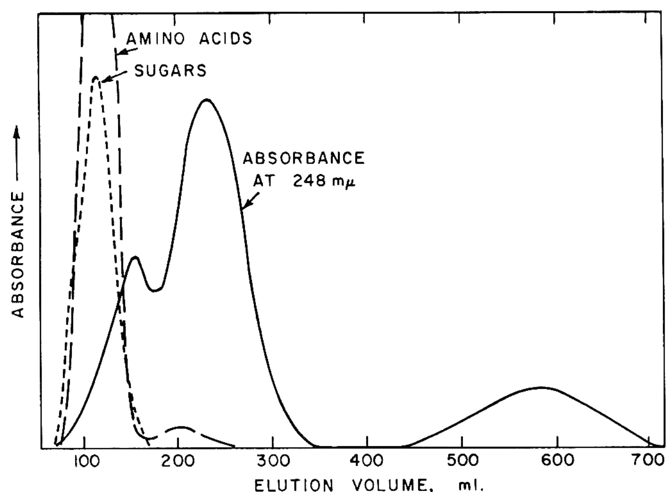

Fig. 2. Beef diffusate chromatographed on Amberlite XAD-2 (20–50 mesh).

in other fractions. The odor precursor fraction was free from purines and their derivatives as well as from other aromatic compounds.

Another type of separation of the components of beef diffusate was afforded by chromatography on crosslinked polystyrene polymer, Amberlite XAD-2. Three ultraviolet absorbing peaks were obtained when the diffusate was separated on an XAD-2 column (Fig. 2). The bulk of the material, listed in Fraction 1, Table 3, was located in the first UV-absorbing peak. This fraction contained most of the amino acids, all sugars, inosinic acid, and other polar compounds.

The subsequent fractions contained compounds which possess a high degree of aromaticity or nonpolar groups. The presence of a large hydrocarbon chain in leucine and isoleucine or of an aromatic ring in phenylalanine and tyrosine resulted in the retardation of these compounds on XAD-2 resin, making the separation of these amino acids possible.

The order of elution of hypoxanthine and inosine from the XAD-2 resin was the reverse of that observed from the P-2 gel. This may be due to the decreased ionic character of inosine as a result of substituting the sugar moiety for the N-9 hydrogen of the purine nucleus.

Table 3. Composition of fractions of beef diffusate separated on Amberlite XAD-2.

Fraction 1	Fraction 2	Fraction 3
Aroma: meaty	Aroma: pungent, grassy	Aroma: none
Amino acids (all except those in Fraction 2)	Leucine	Inosine
	Isoleucine	
Sugars	Phenylalanine	
Phosphates	Tyrosine	
Lactic acid	Hypoxanthine	
Creatine	Unknown, abs. max. 265 mμ	
Creatinine		
Inosinic acid	Unknown, abs. max. 283 mμ	
Inorganic salts		

On pyrolysis Fraction 1 gave a meaty aroma, similar to that of the diffusate. The odor of pyrolyzed Fraction 2 was pungent and plant-like; Fraction 3 gave little or no aroma.

Since the meaty fractions from the separations on P-2 gel and XAD-2 resin contained sugar phosphates or inosinic acid and these compounds were implicated in flavor development (Wood, 1961; Batzer *et al.,* 1962), it was of interest to observe the effect of the removal of these anions on aroma. The diffusate was washed through a column of DEAE-Sephadex [$HCOO^-$] with 0.1*N* formic acid. The total material eluted contained no phosphorus and gave on pyrolysis a meaty aroma quite similar to that of the diffusate.

In subsequent separations of the diffusate on DEAE-Sephadex, fractions of the eluate were collected. The absorbance of the eluted fractions monitored at 248 mμ is shown in Fig. 3. Of the four peaks obtained, the first had an absorption maximum at 260 mμ; the second peak, with a maximum at 247 mμ, contained hypoxanthine and inosine. Peaks 3 and 4 showed absorption maxima at 265 mμ and 283 mμ respectively. The compounds responsible for the ultraviolet absorption in peaks 1, 3 and 4 are at present unidentified. (The spectra referred to were measured in 0.1*N* formic acid.)

The amino acids were eluted from DEAE-Sephadex in the order expected from their isoelectric points (Cohn *et al.,* 1943). They were distributed into five groups: basic, neutral and phenylalanine, tyrosine, taurine, and glutamic acid in the order of their elution. On the basis of these separations, and the analysis of other components, the effluent was divided into seven fractions as shown in Table 4.

Factors other than ion exchange also influence the separation of compounds on DEAE-Sephadex. Adsorption and molecular sieving effects most likely govern the elution of aromatic and non-ionic substances. The retardation of purine derivatives and tyrosine is probably due to adsorption effects. Free sugars are considerably retarded on DEAE-Sephadex emerging together with tyrosine, probably as a result of gel permeation effects. Öckerman

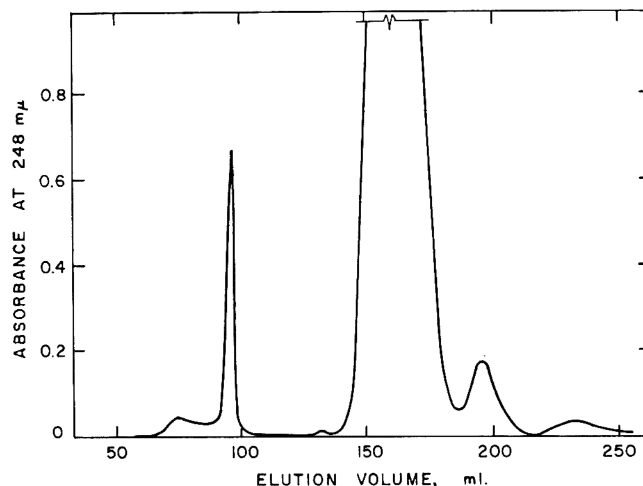

Fig. 3. Beef diffusate chromatographed on DEAE-Sephadex A-25 [$HCOO^-$].

Table 4. Composition of fractions of beef diffusate eluted from DEAE-Sephadex [$HCOO^-$] with 0.1*N* formic acid.

Fraction number						
1	2	3	4	5	6	7
Aroma		Aroma	Aroma			
Carnosine	Creatine	Threonine	Tyrosine	Taurine	Glutamic acid	Glutamic acid
Anserine	Creatinine	Serine	Glucose	Inosine	Unknown, abs. max. 265 $m\mu$	Unknown, abs. max. 283 $m\mu$
Lysine		Asparagine	Fructose	Hypoxanthine		
1-methyl-histidine		Glutamine	Ribose			
		Proline	Inosine			
Histidine		Glycine	Hypoxanthine			
Arginine		Alanine				
Ammonia		Valine				
Creatinine		Methionine				
Choline		Isoleucine				
Carnitine		Leucine				
Unknown, abs. max. 260 $m\mu$		Phenyl-alanine				
		Creatine				

(1963) reported that glucose emerges after glycogen (polysaccharide) from DEAE-Sephadex and attributes this separation to residual molecular sieving properties of unsubstituted Sephadex.

Three of the fractions yielded odors on pyrolysis. However, these odors did not closely resemble the aroma from the diffusate. Fraction 1, containing the basic compounds, yielded an amine-like odor reminiscent of lamb. The odor from the neutral amino acid Fraction 3 was pungent and potato-like. The sugar-containing Fraction 4 produced a fainter sweet, acrid odor. Combination of Fraction 4 with either Fraction 1 or 3 resulted in more meat-like aromas, while combination of Fractions 1, 3 and 4 resulted in an aroma very similar to that of beef diffusate. Apparently the components in Fractions 2, 5, 6 and 7 do not contribute significantly to the meat aroma.

CONCLUSION

THE SEPARATIONS OF BEEF DIFFUSATE on Bio-Gel P-2, Amberlite XAD-2 and DEAE-Sephadex yielded fractions that gave good meaty aromas on pyrolysis, each containing variations in the constituent components. While we still have not identified the precursors of the characteristic meaty aroma, it is possible to eliminate a number of meat components that do not play an important role in flavor development. Components separated from meat aroma-producing fractions were considered not to contribute significantly to the basic meaty aroma. However, these compounds could contribute to the overall odor of broiled or roasted meat.

Although amino acids and sugars are present in all flavor fractions, there do not appear to be any requirements for specific components. In the flavor fraction from P-2 gel only sugar phosphates were present, while the free sugars were equally effective in producing a meaty aroma in the DEAE-Sephadex fractions.

The separation of the amino acids in the flavor fractions is not sufficiently clear at present to evaluate their individual contributions to the meat flavor; but tyrosine, phenylalanine, taurine and glutamic acid may be removed without seriously affecting the aroma. Creatine and creatinine may not be involved in aroma development; they can be removed almost entirely from the flavor fractions by passage through the Bio-Gel P-2 or DEAE-Sephadex.

Purines and purine derivatives have been implicated in the development of meat flavor and aroma (Batzer *et al.*, 1962); however, we excluded inosinic acid, inosine and hypoxanthine from the aroma fraction by chromatography on Bio-Gel P-2. Hypoxanthine and inosine also can be removed by separation on XAD-2 while treatment with DEAE-Sephadex removes inosinic acid. It appears, therefore, that these purines are not involved in the development of meaty aroma.

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Catheptic Enzymes and Meat Tenderization. 1. Purification of Cathepsin D and Its Action on Actomyosin

SUMMARY—A cathepsin has been purified from chicken leg muscle by ammonium sulfate fractionation and by chromatography on carboxymethyl- and diethylaminoethylcellulose. With respect to the specific activity of the initial 2% potassium chloride extract the cathepsin was purified 580-fold. The purified cathepsin hydrolyzed urea-denatured hemoglobin readily at pH 4.40, but it had no activity on α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine, α-N-benzoyl-L-argininamide and α-N-acetyl-L-tyrosinamide.

The data indicate the preparation was cathepsin D and that it did not contain cathepsins A, B, and C. The cathepsin preparation had no activity on actomyosin at pH 4.95 and 5.90 as measured by viscosity and gel-filtration methods. On the other hand trypsin, with 0.014 the potential activity of the cathepsin used and at pH 7.9, hydrolyzed actomyosin readily. While the cathepsin prepared here, probably cathepsin D, did not hydrolyze actomyosin, the data do not exclude the possibility of hydrolysis of actomyosin by other cathepsins or by cathepsin D in combination with other cathepsins.

INTRODUCTION

ATTEMPTS TO OBTAIN EVIDENCE for the role of proteolytic enzymes in post-mortem tenderization of meat are numerous. The methods used have included changes in nonprotein nitrogen (Hoaglund *et al.*, 1917; Husaini *et al.*, 1950a,b; Wierbicki *et al.*, 1954; Davey *et al.*, 1966), degradation of the fine structure of muscle fiber (Carey, 1940; Paul *et al.*, 1944; Zender *et al.*, 1958; Sharp, 1963), solubility changes (Wierbicki *et al.*, 1956; Weinberg *et al.*, 1960; Scopes, 1964), end group analysis (Locker, 1960), electrophoretic changes (Zender *et al.*, 1958; Maier *et al.*, 1966) and determination and/or isolation of cathepsins from muscle (Balls, 1938; Sliwinski *et al.*, 1959; Bandack-Yuri *et al.*, 1961; Bodwell *et al.*, 1964; Doyle *et al.*, 1964).

While the majority of the data would indicate there is some change as measured by the parameter used, there is not complete agreement in this respect. Another difficulty has been finding a correlation between the measured parameters and changes in tenderness (for example, see recent report of Davey *et al.*, 1966).

These difficulties are the result of using methods too insensitive to measure the changes expected and/or being unable to interpret the observed changes in the complex system which muscle is. Whitaker (1959, 1964) has previously pointed out the difficulties in this connection and has suggested that the techniques of zone electrophoresis, gel filtration and ion-exchange chromatography would supply the sensitivity needed. The second difficulty is interpretation of results. This difficulty may be partially overcome by using isolated components of the muscle. Recent work by Bodwell *et al.* (1964) and Scharpf *et al.* (1966) have used such systems.

In the present investigation actomyosin and cathepsin were isolated from the muscle and recombined in a model system. The effect of cathepsin on actomyosin was measured by two very sensitive methods, viscosity and gel-filtration. Since the formation of actomyosin is responsible for the rigor state of muscle, it would appear most logical that changes in actomyosin might be responsible for changes in tenderness on aging. It is necessary to have the cathepsin as pure as possible so that the results can be interpreted unambiguously.

EXPERIMENTAL

Materials

Actomyosin. Actomyosin was prepared from the skeletal muscles of a New Zealand white rabbit as described below.

Cathepsin. The cathepsin was prepared as described below from Arbor Acre adult broiler-type chickens.

Other reagents. Carboxymethyl cellulose (CM-cellulose) was prepared in this laboratory by the method of Peterson *et al.* (1956). Diethylaminoethyl cellulose (DEAE-cellulose; Lot No. B1780, 0.76 meq exchange groups per g) was from Bio-Rad Laboratories, Richmond, California. Agarose (Lot No. 119, suspended in water and stabilized with NaN_3) was from Pharmacia, Uppsala, Sweden. Hemoglobin "substrate powder," α-N-benzoyl-L-argininamide, α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine, α-N-acetyl-L-tyrosinamide and twice crystallized trypsin (10,000 BAEE units of activity per mg) were from Mann Research Laboratories, Inc., New York, N. Y. Dithiothreitol (Cleland's reagent, Lot No. 50241) was from CalBiochem, Los Angeles, California. All other compounds were reagent grade. Deionized water was used throughout the investigation.

Methods

Protein determination. The protein content of fractions from columns was determined at 280 mμ in a Beckman DU spectrophotometer. Protein content of all other preparations was determined by the biuret method (Gornall *et al.*, 1949). Crystallized bovine plasma albumin from Armour Pharmaceutical Company, Kankakee, Illinois was used as the reference protein.

Enzymatic activity. The hydrolysis of hemoglobin by cathepsin and trypsin was performed by a modification of the method of Anson (1938). Sufficient hemoglobin was dissolved in water to give a 10% solution. The solution was exhaustively dialyzed against deionized water before

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being denatured in an alkaline solution with urea. The pH was then adjusted with glacial acetic acid to 7.85 for trypsin assay or 4.40 for cathepsin assay.

The reaction mixture consisted of 2.50 ml of 2% denatured hemoglobin adjusted to the appropriate pH and sufficient water to bring the total volume to 5.0 ml when the enzyme solution was added. Sixty seconds after adding the enzyme a 2-ml aliquot was removed into 3 ml of 5% trichloroacetic acid (TCA) to serve as the blank. At a later appropriate interval another 2-ml aliquot was also removed into TCA. After 1 hr the samples were centrifuged and the increase in absorbance of the supernatant liquid was determined at 280 mμ. One unit of activity is defined as that amount which causes a change in absorbance of 0.001 per hr.

The effect of cathepsin on α-N-benzoyl-L-argininamide, α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine and α-N-acetyl-L-tyrosinamide was determined at pH 4.40 and 35°C, a substrate concentration of 0.02*M* and in the presence and absence of $1 \times 10^{-3}M$ mercaptoethanol. The formation of products was determined by the ninhydrin method (Moore *et al.*, 1954).

The effect of trypsin at pH 7.90 and cathepsin at pH 5.90 on actomyosin was measured at 4°C in a 5 ml Ostwald viscosimeter. The effect of trypsin at pH 7.90 and cathepsin at pH 4.95 on actomyosin was also determined by gel filtration on a 1.70 × 110-cm column of agarose equilibrated against Weber-Edsall solution (0.60*M* KCl, 0.01*M* Na_2CO_3 and 0.04*M* $NaHCO_3$, pH 9.0).

Preparation of actomyosin. The first steps in preparation of actomyosin were identical to those of Szent-Györgyi (1951). A New Zealand white rabbit (obtained from Experimental Animal Resources, University of California, Davis) was stunned by a blow on the head, decapitated, skinned, and the muscle from the dorsal part removed.

The muscle was washed with cold water (0°C) and immediately transferred to a cold room (4°C). One hundred g of muscle, cleaned of visible fat and connective tissue, were ground in a chilled meat chopper. Three hundred ml of Weber-Edsall solution were added to the ground muscle and the suspension was permitted to stand overnight in a cold room. The next day the viscous suspension was diluted with 500 ml of 0.60*M* KCl and the suspension then was centrifuged in a Servall centrifuge at 13,200 G at 4°C for 1 hr.

The precipitate, mainly meat particles, was discarded. The white, viscous supernatant liquid was diluted with five volumes of cold water. On decreasing the concentration of KCl to 0.10*M* the actomyosin was precipitated as a white voluminous, flocculent precipitate. After centrifugation and decantation of the supernatant liquid, the precipitate was dissolved in 600 ml of Weber-Edsall solution. This process of precipitation and dissolution was repeated three times. The white, viscous solution was then centrifuged at 59,000 G at 0°C for 30 min. The clear, opalescent solution, in plastic containers, was frozen and stored at −20°C.

Cathepsins. The initial parts of the method for extraction and purification of the cathepsins was essentially that of Doyle *et al.* (1964). Unless otherwise noted, the purification was carried out at 4°C. The purification procedure is outlined in Table 1.

Five hundred g of leg muscles from chickens, cleaned of visible fat, was ground through a coarse plate of a food chopper and mixed with 1000 ml of cold 2% KCl. The mixture was homogenized for three min in a Waring blendor and then allowed to stand overnight. The thick, slimy homogenate was centrifuged at 13,200 G for 150 min and the precipitate was discarded. Solidified fat particles were removed by filtration through eight layers of cheese cloth. The supernatant liquid (Fraction I) was clear and slightly reddish in color.

The pH of Fraction I, initially 6.00, was adjusted to 5.10 with glacial acetic acid. The temperature of the solution was then raised rapidly to 35°C by placing it in a 50°C water bath. The solution was stirred continuously. After the temperature reached 35°C, the solution was removed, and placed in a 35°C water bath for 10 min. It was then cooled rapidly to below 10°C and centrifuged. The clear supernatant liquid (Fraction II) was saved.

The pH of Fraction II was adjusted to 4.20 with glacial acetic acid and sufficient solid $(NH_4)_2SO_4$ was added to make the solution 5% saturated at 4°C with respect to $(NH_4)_2SO_4$. After 1 hr, the solution was centrifuged at 13,200 G for 30 min. The precipitate was discarded. Sufficient solid $(NH_4)_2SO_4$ was added to the supernatant liquid (Fraction III) to make it 45% saturated at 4°C with respect to $(NH_4)_2SO_4$. After 1 hr, the solution was again centrifuged for 30 min at 13,200 G. The precipitate was discarded. The supernatant liquid (Fraction IV) was brought to 70% saturation at 4°C with solid $(NH_4)_2SO_4$. After 1 hr, the solution was centrifuged as above. The supernatant liquid (Fraction V) was discarded and the precipitate was dissolved in 100 ml of 2% KCl (Fraction VI).

After dialysis overnight against 0.01*M* sodium acetate buffer, pH 5.20, followed by centrifugation the clear, supernatant liquid (Fraction VII) was chromatographed on a column (1.2 × 25 cm) of CM-cellulose which had been prepared as described by Sgarbieri *et al.* (1964) and equilibrated against 0.01*M* sodium acetate buffer, pH 5.20. Separation on the column was carried out using a stepwise increase in the concentration of acetate buffer, pH 5.20. Better purification was obtained when chromatography was performed at room temperature. The flow rate was approximately 450 ml per hr. The results of this purification step are shown in Fig. 1. Peak E, eluted with 0.30*M* acetate buffer, was saved for further purification.

The solution comprising peak E was dialyzed against 0.01*M* phosphate buffer, pH 7.00. It was then chromatographed on a column (1.2 × 40 cm) of DEAE-cellulose prepared and equilibrated against 0.01*M* phosphate buffer, pH 7.00 (Kon *et al.*, 1965). Chromatography was performed at 4°C and at a flow rate of 50 ml per hr. The results are shown in Fig. 1. All the proteolytic activity was eluted from the column with 0.02*M* phosphate buffer, pH 7.0.

The majority of the protein, which had no proteolytic activity, was eluted from the column at a much higher buffer concentration (not shown in Fig. 1). The final material, representing 3% of the total original activity, was 580-fold purified with respect to the initial 2% KCl extract of the muscle homogenate. This purified material

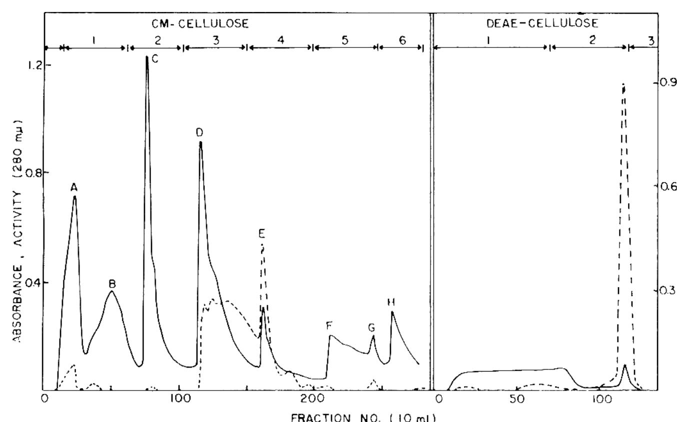

Fig. 1. Chromatographic purification of the cathepsin prepared by precipitation between 45 and 70% saturated ammonium sulfate on CM-cellulose and DEAE-cellulose columns. Elution from CM-cellulose was performed by a step-wise increase in acetate buffer concentration, all at pH 5.20. The acetate buffer concentrations were: 1, 0.01M; 2, 0.10M; 3, 0.20M; 4, 0.30M; 5, 0.40 M; 6, 0.50M. Peak E was dialyzed and rechromatographed on a DEAE-cellulose column. The column was equilibrated in 0.01M phosphate buffer, pH 7.0 and eluted in a step-wise manner with 1, 0.01M; 2, 0.02M; and 3, 0.03M phosphate buffer, pH 7.0. The protein, ———, was determined at 280 mμ and the activity, - - - -, on hemoglobin at pH 4.40.

with a specific activity of 10,700 units/mg protein was used in all subsequent work.

RESULTS AND DISCUSSION

Purification of cathepsin

The increase in purity due to a combination of pH adjustment, heat denaturation of unwanted proteins, and ammonium sulfate precipitation was less than five-fold (Table 1). There is an indication that some of this increase in specific activity was the result of removing inhibitors of catheptic activity. The majority of the catheptic activity was precipitated between 45 and 70% saturation of the solution with $(NH_4)_2SO_4$ and at pH 4.20. Chromatography of the fraction precipitated with 70% saturated $(NH_4)_2SO_4$ on a CM-cellulose column at pH 5.2 resulted in a 20- to 40-fold purification (Fig. 1, Table 1).

Table 1. Purification of cathepsin rom chicken leg muscle.

Purification step	Activity recovered (%)	Specific activity[1]	Purification[2] (fold)
Fraction I	100	16.4–48.2[3]	1.00
Fraction II	94.5–186[3,4]	46.6–81.2	1.86–2.84[3]
Fraction III	43.7–244[4]	59.2–122	0.795–2.98
Fraction IV	5.3–61	44.2–212	0.915–7.74
Fraction V	1.2–8.0	18.4–93.4	1.12–3.78
Fraction VI	3.92–82	20.9–197	0.465–5.98
Fraction VII	3.04–130[4]	38.2–200	0.790–12.2
CM-cellulose column	14.2	1660	40.5
DEAE-cellulose column	2.86	10,700	580

[1] The specific activity is defined as the increase in absorbance at 280 mμ times 1000 due to the trichloroacetic acid-soluble products produced from hemoglobin in one hour at 25.0° and pH 4.40 by 1 mg of enzyme protein per milliliter.

[2] The range of values for fold purification are calculated from the results on individual purifications and cannot be derived directly from the range of specific activities presented.

[3] A number of preparations were carried through these steps and the results indicate the lowest and highest values obtained.

[4] In a number of preparations more than 100% recovery of activity was obtained in these steps. This suggests the presence of inhibitory material which was removed during the purification.

A component with the highest specific activity was eluted with 0.30*M* acetate buffer, pH 5.2 (peak E). Appreciable catheptic activity was also eluted with 0.20*M* acetate buffer (peak D). The relationship between the catheptic enzymes in peaks D and E was not determined. Chromatography of the material from peak E on a DEAE-cellulose column at pH 7.0 gave an additional 14.5-fold increase in purity of the material. The catheptic activity was all contained in a peak eluted with 0.02*M* phosphate buffer, pH 7.0 (Fig. 1).

The purification procedure resulted in a 580-fold increase in specific activity as compared with the specific activity of the 2% KCl extract. However, the total fold purification is higher. The major part of the proteolytic activity of muscle is extracted with 2% KCl but only 0.1 to 0.2 of the total protein of muscle is extracted. Therefore, the over-all purification is some 2500 to 6000-fold with respect to the original muscle. Measured on hemoglobin at pH 4.40 and 35°C, the cathepsin preparation had a specific activity of 1.07×10^4 units/mg protein. Under comparable conditions but at pH 7.85, twice crystallized trypsin had a specific activity of 1.03×10^5 units/mg protein.

The classification of the cathepsins is based on their ability to hydrolyze certain synthetic substrates (Tallen *et al.*, 1952). The activity of the purified cathepsin preparation was determined on the three synthetic substrates, α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine (for cathepsin A), α-N-benzoyl-L-argininamide (for cathepsin B) and α-N-acetyl-L-tyrosinamide (for cathepsin C), in the presence and absence of $1 \times 10^{-3}M$ mercaptoethanol. No activity on any of these substrates was found even when the incubation time was increased 4-fold over that normally used when determining the activity of a crude preparation (for example, Fraction I).

Increasing the enzyme concentration to several hundred times that normally found in a crude preparation did not cause any hydrolysis of the substrates. We must conclude, therefore, that the purified cathepsin preparation did not contain cathepsins A, B and C. Cathepsin D has been shown not to hydrolyze the synthetic substrates used here (Press *et al.*, 1960). Therefore, our results, while not entirely conclusive since there are reports of other cathepsins, indicate that we have purified cathepsin D from chicken muscle.

Press *et al.*, (1960) concluded that the major catheptic enzyme of beef spleen is cathepsin D. Doyle *et al.* (1964) have previously found that cathepsins A (high activity) and B (low activity) appear to be present in crude extracts of chicken muscle while no cathepsin C activity was found.

Activity on actomyosin

Trypsin. Five ml of actomyosin solution, which contained 16 mg protein per ml and which had been adjusted to pH 7.90 with acetic acid, was treated with 0.20 ml of a trypsin solution (9.0×10^{-3} mg; 920 units of activity on hemoglobin) at 4°C. The reaction was carried out in an Ostwald viscosimeter. The decrease in relative viscosity as a function of incubation time is shown in Fig. 2. There

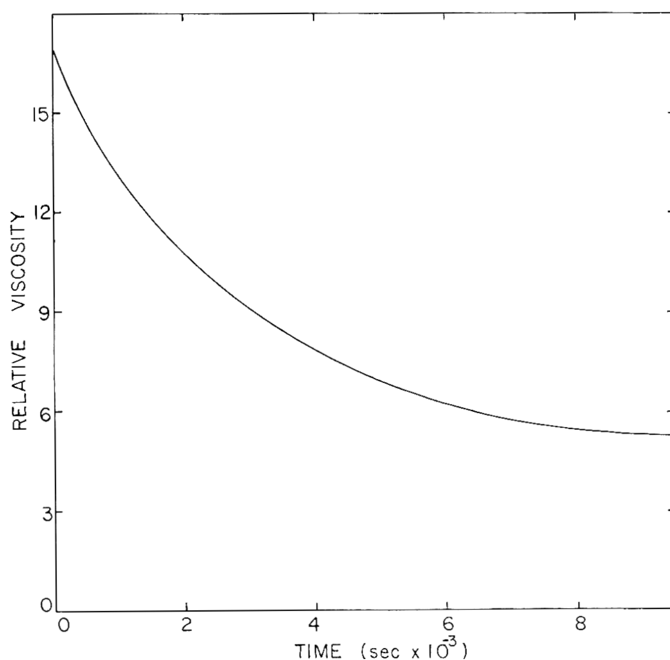

Fig. 2. Effect of trypsin on the viscosity of actomyosin. Five milliliters of actomyosin solution (16 mg protein/ml) at pH 7.90 was mixed with 0.20 ml of trypsin (9.0 × 10^{-3} mg trypsin; 920 units of activity on hemoglobin) at 4°C in an Ostwald viscosimeter. The outflow time was determined at various intervals.

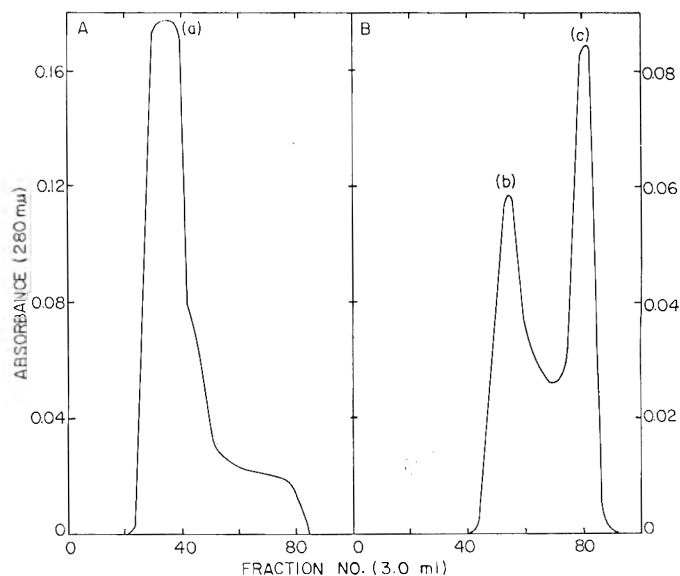

*Fig. 3. Chromatography of actomyosin on a 1.70 × 110-cm column of agarose before (A) and after (B) treatment with trypsin at pH 7.90. Two ml of actomyosin solution (16 mg protein/ml) were added to the column before trypsin treatment and one ml after trypsin treatment. The column was equilibrated with 0.60*M *KCl, 0.01*M *Na_2CO_3 and 0.04*M *$NaHCO_3$, pH 9.0 and the same solution was used for elution of the column at 4°C.*

was a rapid decrease in the viscosity of the actomyosin solution.

When there was no further change in the viscosity of the solution, a one-ml aliquot of the solution was placed on top of an agarose column equilibrated with Weber-Edsall solution. Columns packed with agarose will separate molecules with molecular weights of several million (Hjertén, 1962). Dextran Blue-2000, with an average molecular weight of 2 × 10^6 is separated into at least two components on the agarose column used here.

Chromatography of actomyosin on the agarose column before and after treatment with trypsin is shown in Fig. 3A and 3B, respectively. The major peak (a) of untreated actomyosin completely disappeared and there was the formation of two new peaks, (b) and (c). The center of the major peak of untreated actomyosin was at 102 ml of eluting fluid while the centers of the new peaks formed by trypsin hydrolysis were at 165 and 243 ml, respectively. Since a relationship between the elution volumes and known molecular weights of several solutes was not established for the agarose column, we do not know the molecular weights of these components.

However, we can say that they are considerably smaller than the original actomyosin. Therefore, chromatography on an agarose column, as well as decrease in viscosity, indicates there is a rapid hydrolysis of actomyosin by trypsin at pH 7.90. Hydrolysis of myosin [MW ~500,000 (Weber *et al.,* 1965)] by trypsin has been shown to result in the formation of two smaller units, heavy meromyosin (MW ~232,000) and light meromyosin (MW ~96,000) (Mihályi *et al.,* 1953; Szent-Györgyi, 1953).

Cathepsin. If the ionic strength of an actomyosin solution is maintained at 0.6 with KCl, the pH may be lowered to 4.4 with the formation of only a trace of precipitate. However, viscosity studies could not be carried out at this pH or at pH 4.95 because the actomyosin tended to form aggregates (long fibers) on flowing through the capillary tube of a glass viscosimeter. For this reason, viscosity studies were performed at pH 5.90, close to the pH of rigor muscle. Even after 72 hr no change in the viscosity of the solution could be detected. In these studies, 2.50 ml of purified cathepsin solution and 2.50 ml of actomyosin solution were combined and incubated at 4°C. The amount of cathepsin used had 2230 units of activity on hemoglobin at pH 4.40.

The cathepsin was maximally active on hemoglobin at 4.4 and had less than 10% maximal activity at pH 5.9. However, the combination of amount of enzyme and length of incubation should have resulted in some change in the viscosity of the actomyosin solution if the cathepsin were able to hydrolyze actomyosin. To further substantiate the inability of the purified cathepsin preparation to hydrolyze actomyosin, incubation of actomyosin with cathepsin, both in the presence and absence of dithiothreitol at pH 4.95, was carried out for 72 hr at 4°C.

Incubation was performed in plastic tubes because glass caused an aggregation of actomyosin at this pH. After 72 hr, an aliquot of the solution was chromatographed on an agarose column equilibrated with Weber-Edsall solution. The results are shown in Fig. 4. There is no evidence for the hydrolysis of actomyosin by the purified cathepsin preparation. In the presence of 1 × $10^{-3}M$ dithiothreitol the reaction mixtures remained clear throughout the incubation period. However, in the absence of dithiothreitol, approximately 60% of the actomyosin precipitated during the 72 hr incubation period.

The relative amounts of cathepsin and trypsin used in these experiments should be compared in order to present a true picture of what these results mean. As indicated

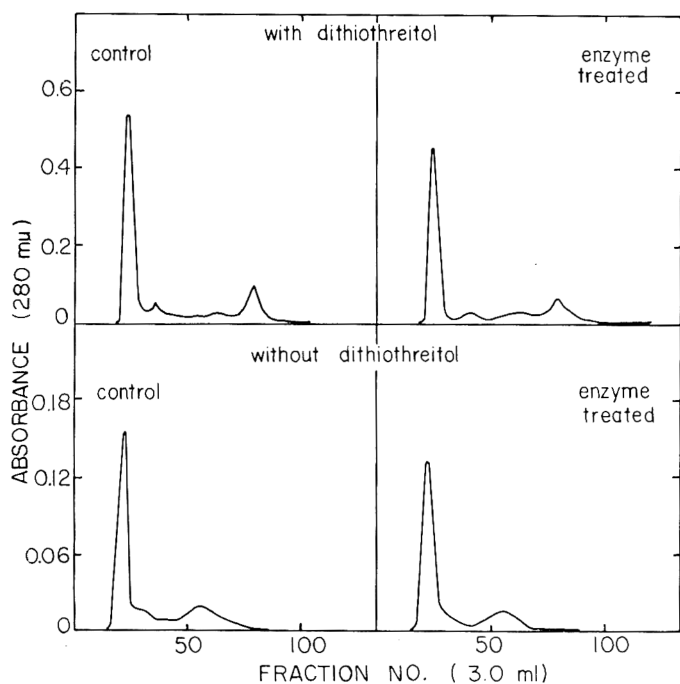

Fig. 4. Chromatography of actomyosin on a 1.70 × 110-cm column of agarose before (control) and after treatment with cathepsin for 72 hr at 4°C and pH 4.95 in the presence and absence of dithiothreitol. Chromatography was performed as described in Fig. 3.

above, 920 units of trypsin (as determined on hemoglobin at pH 7.85) or 2230 units of cathepsin (as determined on hemoglobin at pH 4.40) were added to the actomyosin solutions. Therefore, considering the difference in time of incubation of actomyosin with the two enzymes, the ratio of potential enzymatic activity was 50- to 70-fold in favor of the cathepsin. Yet, no hydrolysis of actomyosin by the purified cathepsin preparation could be detected.

The results presented here do not give a complete answer to whether some of the cathepsins may be involved in meat tenderization. Our data indicate that the purified cathepsin was probably cathepsin D and that it was free of cathepsins A, B and C. At least two other cathepsins, cathepsins A and B, are present in chicken muscle (Doyle *et al.*, 1964) and their ability to hydrolyze actomyosin was not determined in this work.

Bodwell *et al.* (1964) could not detect any activity of a crude cathepsin preparation (considered to be cathepsin A but cathepsin D could also have been present) on actin, myosin or actomyosin. They used as criteria of hydrolysis a change in absorbance at 280 mμ of TCA-soluble material. This technique is a much less sensitive assay to determine proteolysis than the two methods used in the present study.

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Direct Spectrophotometric Determination of Fat and Moisture in Meat Products

SUMMARY—The near-infrared spectral absorption properties of 2-mm-thick samples of meat emulsions were measured by direct spectrophotometric techniques. The resulting spectra are interpreted in terms of absorptions from O-H and C-H stretching vibrations combined with scatter losses. Optical-density differences are correlated with fat and moisture contents. The difference in optical density between 1.80 and 1.725 μ gave a high correlation with moisture content and the difference between 1.725 and 1.65 μ gave a high correlation with fat content. Direct spectrophotometric analysis predicted fat content within a standard error of $\pm$ 2.1% and moisture content within $\pm$ 1.4%. The possibilities of this technique are explored and the problems to be solved in developing a rapid, accurate method are discussed.

INTRODUCTION

FAT AND MOISTURE CONTENTS of meat products are important to both the consumer and the producer. In certain products, the upper limits of water and fat content are regulated by the Meat Inspection Division, Consumer and Marketing Service, U. S. Department of Agriculture (1965). With the industry's desire to maintain optimal level of fat and water in meat products and the control exercised by the Federal Government, the need for a rapid accurate analysis is emphasized.

The conventional methods for determining moisture (distillation and oven drying) and fat content (ether extraction) are too slow for many purposes. Many techniques for a rapid drying analysis have been explored (Everston *et al.*, 1965) but to date an adequate method has not been developed. Davis *et al.* (1966) recently reported on a rapid extraction for simultaneous determination of fat and moisture, but this still requires two hours.

Norris *et al.* (1965) have shown that moisture content of grains can be determined by direct spectrophotometry, so this technique was studied for application to meat products. Spectral absorption curves of different types of meat samples were recorded for the 1.5- to 1.85-μ region and the results were related to composition as determined by conventional techniques.

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MATERIALS AND METHODS

MODEL SAMPLES OF MEAT EMULSIONS were prepared in a simulated factory operation, from lean, fat, and water. Some of the samples were cooked in a hot-water bath to an internal temperature of 156°F, and some were kept raw. Samples of bacon, ham, all-meat franks, and bologna were of commercial origin. The samples were passed through a meat grinder twice and were refrigerated prior to the spectrophotometric studies. Information regarding fat, protein, and moisture content of all the samples was obtained according to AOAC methods (1960). For the spectrophotometric studies 2 g of the sample material were packed into a 2-mm-deep metal cell having a glass bottom.

Absorption measurements were made with a recording spectrophotometer designed for analysis of light-scattering samples (Norris *et al.*, 1961). Monochromatic radiation from a double-prism monochromator is reflected down to the sample which is mounted directly above a large-area lead sulfide cell as shown in Fig. 1. The energy transmitted through the sample is measured by the lead sulfide

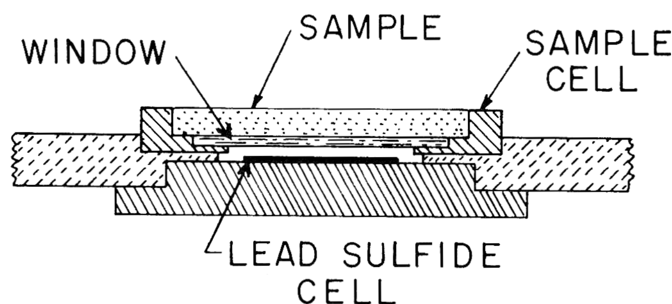

Fig. 1. Sample mounting for special spectrophotometer.

cell and, after suitable amplification and signal conditioning, is recorded on the Y-axis of an X-Y recorder. The X-axis records a signal proportional to the wavelength.

The Y-axis signal is recorded on a logarithmic scale so that absorption differences can be read directly from the recorded curve. The wavelength range from 1.5 to 1.85 μ was recorded for each sample four times with the sample rotated 90° between each measurement. The average differences in optical density between 1.725 μ and 1.65 μ and between 1.8 μ and 1.725 μ were computed and tabulated.

The absorption spectra for a thin layer of water, a thin layer of pure beef fat, and a thin layer of defatted beef were recorded with a special Cary 14 Spectrophotometer incorporating an integrating sphere for measurement of diffuse transmittance. The sample, of a thickness chosen to give a reasonable absorption, was mounted close to the entrance port of the sphere for measurement. The operating conditions provided a spectral bandpass of less than 0.005 μ.

RESULTS AND DISCUSSION

EXPLORATORY ABSORPTION MEASUREMENTS were made over a wide wavelength region and the region from 1.5 to 1.85 μ appeared to be the most promising. Measurements at longer wavelengths required such a thin sample that it was difficult to obtain a suitable preparation. The absorption spectrum of a sample of all-meat frankfurters shown in Fig. 2 is typical.

This curve was recorded with the Cary Spectrophotometer on a sample approximately 0.2-mm thick. The strong absorption bands at 1.93 and 1.45 μ are from O-H vibrations of water. The sharp bands at 1.725 and 1.76 μ are from C-H vibrations. The small group of bands at 1.2 μ include O-H and C-H vibrations. The radiant energy, scattered and reflected by the particles without being absorbed, introduces a general absorbance of approximately 0.3 on this sample.

For quantitative work a thicker sample must be used, but the absorption is too great for measurements with the

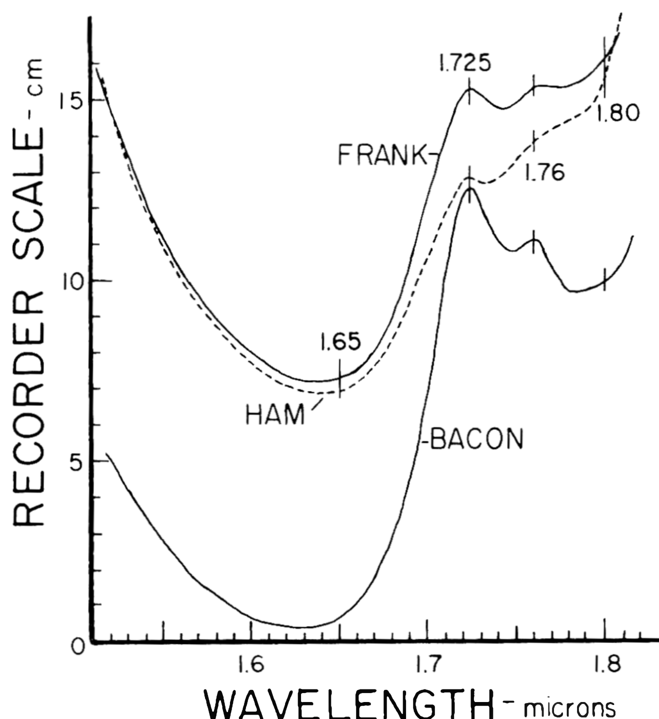

Fig. 3. Absorption spectra of ham, bacon, and all-meat frankfurter as recorded with special spectrophotometer. Sample thickness—2mm; spectral bandpass—0.020 μ. Recorded curves include system response. Absorbance at 1.65 μ on sample of bacon was 1.5.

Cary Spectrophotometer because of the low efficiency of the integrating sphere. Measurements with our special spectrophotometer on 2-mm-thick samples of different meat emulsions, as shown in Fig. 3, show the same absorption bands at 1.725 and 1.76 μ. The bands are not clearly defined because the spectral bandpass of the instrument is 0.020 μ.

The absorption curves for pure fat, defatted beef, and water for this limited wavelength region, as recorded with the Cary Spectrophotometer (Fig. 4), provide adequate description of the character of the curves for the meat emulsions. Other components of the meat contribute to the overall absorption, but the fat and the water appear to be the main components contributing to the differential absorption. The defatted meat shows weak absorption bands at 1.70 and 1.74 μ and the strong absorption of water as the wavelength approaches 1.9 μ.

Absolute absorbance is not suitable for compositional analysis on scattering samples, but absorbance differences can be used. The curves of Fig. 4 indicate that the best wavelengths for fat determination should be 1.725 and 1.65 μ. The water absorption band at 1.93 μ is too strong for measurement on a 2-mm sample, but a measurement of the difference in absorbance at 1.8 and 1.725 μ should be sensitive to water. Therefore, these wavelengths were chosen for study.

Compositional analyses and optical-density differences for 17 samples of commercial origin and 17 samples of meat emulsion models are presented in Table 1. The relationship between fat content and Δ O. D. (1.725–1.65) μ (Fig. 5) is nearly linear over the total range of samples. The same is true for water content and Δ O. D. (1.80–1.725) μ, as shown in Fig. 6.

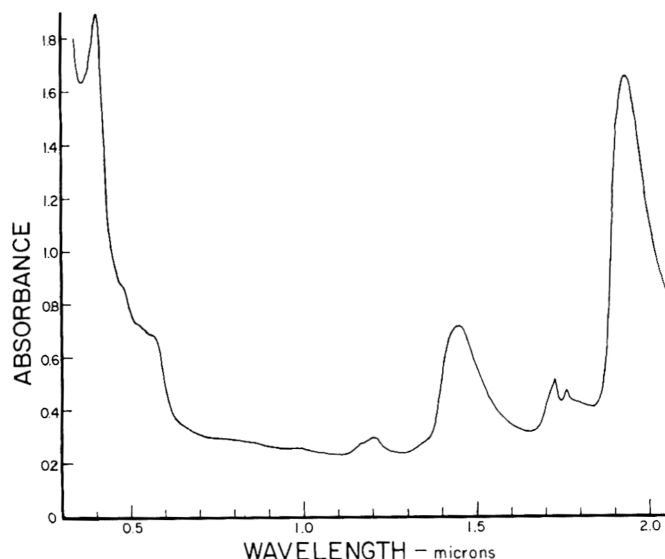

Fig. 2. Absorption spectra of a thin layer (0.2mm) of all-meat frankfurter as recorded with a Cary 14 Spectrophotometer incorporating our integrating sphere.

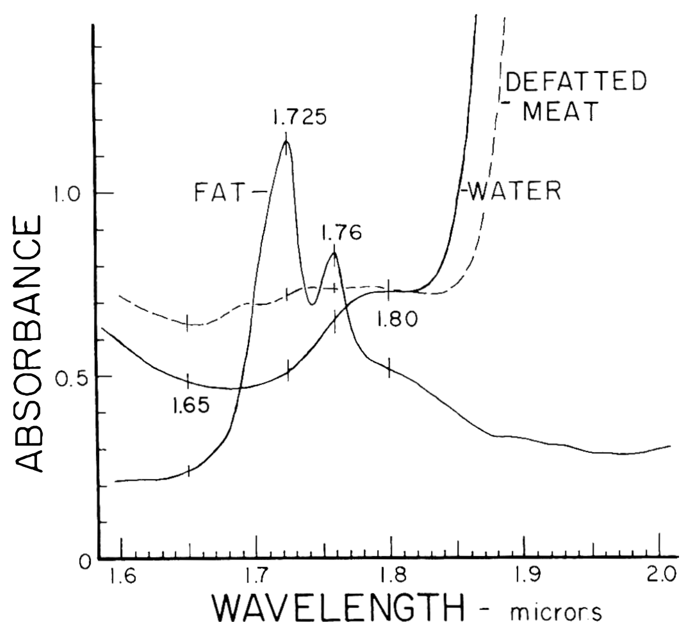

Fig. 4. Absorption spectra of pure fat, water, and defatted beef as recorded on Cary 14 Spectrophotometer. Sample thickness adjusted to provide a reasonable absorption on each sample.

An adequate treatment of absorbance vs. composition for scattering samples has not been developed, but the Kubelka-Munk analysis (1947) indicates that the relationship is not linear. Since the deviations from linearity are small, a linear regression analysis was made. Correlation coefficients of 0.974 between fat content and Δ O. D. (1.725–1.65) μ and of 0.977 between water content and Δ O. D. (1.80–1.725) μ were obtained. A multiple correlation using all three wavelengths improved the correlations to 0.976 and 0.978, respectively.

The relationship of the absorption measurements to protein content was also studied. A correlation coefficient of −0.751 was obtained between protein content and Δ O. D. (1.725–1.65) μ. Most of this relationship can be explained by the correlation between fat content and protein content (−0.745). Thus the C-H vibrations of the protein apparently do not contribute to the absorption at 1.725 μ in the same way as the fat. This agrees with the results on defatted beef shown in Fig. 4. The Δ O. D. values predicted the fat content and water content within a standard error of ± 2.1% and 1.4%, respectively, if the one sample of very high fat content is omitted. The standard error is reduced to ± 1.6% and 1.3%, respectively, if only the commercial samples are considered.

The major source of error in these measurements is

Table 1. Compositional analysis and absorbance—difference values for meat emulsions.

Sample No.	Sample type	% Fat	% Water	% Protein	% Residue	Δ O.D. (1.725–1.65)μ	Δ O.D. (1.80–1.725)μ
1	Commercial processed sausage	19.9	60.5	16.4	3.2	0.520	0.160
2		32.5	54.4	10.5	2.6	0.788	0.040
3		25.7	58.1	12.6	3.6	0.672	0.100
4		36.3	48.8	11.3	3.6	0.820	−0.012
5		33.7	51.9	11.2	3.6	0.828	0.036
6		30.2	55.0	11.7	3.1	0.756	0.064
7		33.9	50.4	12.6	3.1	0.848	0.016
8		28.8	55.8	11.8	3.6	0.692	0.092
9		32.4	52.7	11.3	3.6	0.768	0.076
10		35.6	49.5	10.8	4.1	0.832	0.024
11		32.5	51.0	13.3	3.2	0.764	0.048
12		35.3	49.9	10.7	4.1	0.828	0.000
13		33.0	53.1	11.1	2.8	0.744	0.056
14		29.4	53.9	13.6	3.1	0.704	0.068
15		33.6	52.6	10.6	3.2	0.752	0.068
16		33.7	50.5	12.0	3.8	0.736	0.036
17		33.2	51.2	12.1	3.5	0.728	0.056
18	Commercial ham	19.9	60.3	14.4	5.4	0.588	0.152
19	Commercial bacon	53.4	31.3	6.4	8.9	1.272	−0.284
20	Comm. all-meat franks	33.6	49.5	11.7	5.2	0.824	0.000
21	Model emulsion—raw	13.8	63.2	16.4	6.6	0.508	0.204
22		12.8	66.2	15.2	5.7	0.464	0.208
23		21.6	61.6	11.6	5.2	0.640	0.150
24		9.1	76.1	10.4	4.4	0.368	0.276
25		5.5	72.5	14.5	7.5	0.312	0.264
26		12.5	68.9	13.4	5.2	0.468	0.232
27		33.5	53.5	8.7	4.3	0.860	0.028
28	Model emulsion—cooked	21.4	62.6	13.4	2.6	0.600	0.152
29		22.0	61.1	14.3	2.6	0.656	0.132
30		22.5	60.1	15.4	2.0	0.648	0.128
31		9.7	70.2	17.7	2.1	0.372	0.240
32		15.9	65.3	15.9	2.9	0.504	0.188
33		23.7	59.3	14.1	2.9	0.652	0.120
34		30.4	53.9	12.8	2.9	0.784	0.040

found in a sample preparation. It is difficult to pack a cell with a 2-mm-thick sample in a reproducible, uniform layer. A second major source of error is the low signal-to-noise ratio obtained because of the low transmittance of the samples. The signal-to-noise ratio can be improved by use of a more efficient source of monochromatic energy.

It may also be possible to improve the detector and electronic amplifiers, although we believe that this improvement will be small unless the detector is cooled to dry-ice temperature or lower. It should be possible to construct an instrument using interference filters for these measurements and a four-filter instrument is under development in our laboratory.

We do not have a solution to the sample-preparation problem, but a sample cell in which the sample is compressed between two windows might be effective. Dilution of the sample with a non-absorbing liquid is another possibility. Norris *et al.* (1965) chose carbon tetrachloride for use with grain. The ideal material for dilution would be a non-absorbing liquid having an index of refraction matching that of the sample. Such a liquid would greatly reduce the scatter losses and permit the use of a larger sample.

We have shown that a direct spectrophotometric analysis for fat and moisture content is possible on meat emulsions. Packing of the sample cell is the only sample preparation required. Special instrumentation is required, but we believe that a direct-reading instrument and suitable sample-handling technique can be developed to provide an accuracy within ± 0.5% for both fat and moisture.

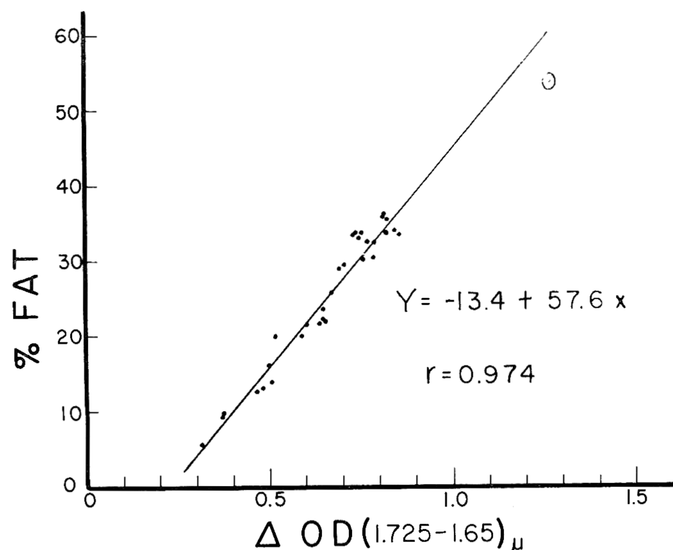

Fig. 5. Relationship between fat content and absorbance differences at 1.725 and 1.65 μ for different meat emulsions. Circled point not included in regression analysis.

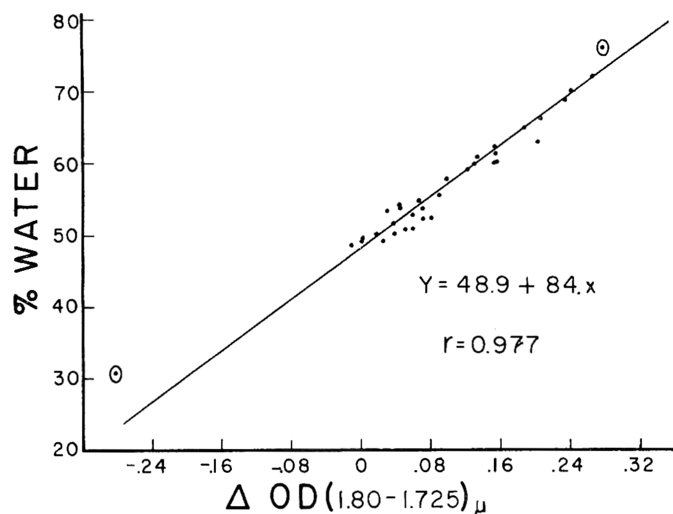

Fig. 6. Relationship between water content and absorbance difference between 1.80 and 1.725 μ for different meat emulsions. Circled points not included in regression analysis.

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Mention of specific instruments or trade names is made for identification purposes only and does not imply any endorsement by the United States Government.

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Alterations of Bovine Sarcoplasmic Proteins as Influenced by High Temperature Aging

SUMMARY—Effects of high temperature aging upon certain characteristics of bovine l. dorsi muscle were studied. Paired wholesale ribs of carcasses were obtained subsequent to slaughter. The left rib of each pair was held at 30°C for 24 hr, then stored at 3°C. Analogous right ribs were immediately stored at 3°C. A sampling schedule of 0, 1, 2, 3, 4, 7 and 10 days was followed.

There were minor variations in moisture, pH, tyrosine-tryptophan indices of non-protein nitrogenous compounds and expressible moisture ratios between treatments and with time. These differences were not statistically significant.

Up to three days storage, extractability of water soluble protein was greatest from muscles held at the elevated temperature. After the third day, however, extractability was greater for muscles held at 3°C.

Color differences between muscles treated via the two storage temperatures were marked. Absorbance ratios (422/280 mμ) of extracts showed that muscles held at the high temperature had higher extractable levels of oxymyoglobin than ribs held at 3°C. This difference remained apparent throughout the aging period.

Results of DEAE-cellulose ion exchange chromatography of the sarcoplasmic proteins showed only minor variations in profiles between the two aging treatments. Alterations did appear with time. Profile alterations did not appear related to anticipated increases in tenderness.

INTRODUCTION

TENDERNESS OF MEAT is generally believed to be influenced by the state of the myofibrillar proteins which form the contractile mechanism of muscle (Donnelly *et al.*, 1966). Sarcoplasmic proteins, however, have also been implicated in meat tenderness. Bendall *et al.* (1962) postulated that sarcoplasmic proteins can be adsorbed on the myofibrillar proteins during post-mortem changes in pork thus altering their functional properties. While sarcoplasmic proteins make up only 20–25% of total muscle protein, they do contain many muscle regulating enzymes which could contribute to their importance in tenderness (Kronman *et al.*, 1960). Fujimaki *et al.* (1964) also concluded that sarcoplasmic proteins may reflect some aspects of post-mortem changes in meat.

Cellulose ion exchange chromatography has provided a tool to fractionate the many proteins in the sarcoplasm. Fujimaki *et al.* (1964) and Rampton *et al.* (1965) studied the fractionation of sarcoplasmic proteins but presented data only at the beginning and the final stages of the aging process.

This study was intended to elucidate the changes that occur in sarcoplasmic extracts of beef animals from the pre-rigor state through a normal and high temperature aging process using diethyl-aminoethyl-cellulose (DEAE-cellulose) ion exchange chromatography.

EXPERIMENTAL PROCEDURE

RIB SECTIONS (6th through 13th ribs inclusive) were removed from both sides of three beef carcasses subsequent to slaughter. Carcasses were representative of three different quality grades (good, commercial and utility) according to U.S.D.A. beef grading standards. Rib sections from the right side of the carcasses served as controls and were aged at 3°C throughout the study. Rib sections from the left side of the carcasses were initially held in a tunnel dryer at 35°C for 45 min to dry partially the exterior surfaces to retard microbial growth. These sections were then held at 30°C for 24 hr before being stored at 3°C for the remainder of the aging period.

One-inch steaks were removed from rib sections at 2 hr and 1, 2, 3, 4, 7 and 10 days post-mortem. Samples from the l. dorsi muscle were removed for the determination of expressible and total moisture. Fifty g of tissue were homogenized with 50 ml of distilled water in a water-cooled Waring blendor for 90 sec. Homogenate pH was measured, adjusted to 7.0 with 1*N* NaOH, and homogenized for an additional 30 sec. After centrifugation of the homogenate at 30,000 × G for 20 min, the supernatant was filtered through Whatman No. 12 filter paper to remove fat particles.

One ml of each filtrate was removed for tyrosine-tryptophan index determinations. The remainder of the sarcoplasmic extract was dialyzed against a 160-fold volume of the chromatographic starting buffer (0.04*M* Tris adjusted to pH 9.0 with concentrated H_3PO_4) for 22 hr at 4°C and used for chromatographic analysis.

Tyrosine-tryptophan index determinations were made in duplicate on sarcoplasmic extracts (Zender *et al.*, 1958). One-half ml of the extract was placed in a 25 ml volumetric flask and brought to volume with 0.5 ml of 0.1*N* NaOH and distilled water. Absorbance was measured at 280 mμ with a Beckman DB spectrophotometer. Another 0.5 ml of the extract was placed in a test tube to which 9.5 ml of 15% TCA were added. After mixing, the extract was filtered through Whatman No. 42 filter paper and the absorbance of the filtrate was read at 280 mμ.

A spectrophotometric scan from 750 to 245 mμ was made on the diluted dialyzed extract with a Beckman DB recording spectrophotometer. The maximum absorbance of a characteristic peak at 422 mμ was used to indicate the amount of oxymyoglobin present (Mackinney *et al.*, 1962; Theorell, 1934). A ratio of absorbance at 422 to 280 mμ

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was used to express the amount of oxymyoglobin on a protein basis.

Filter paper moisture absorption determinations were made by the procedure of Briskey *et al.* (1960). Moisture was determined by drying samples for 24 hr at 80°C under partial vacuum (43 cm), and measuring loss in weight.

Chromatography

Four 2 × 50 cm DEAE-cellulose ion exchange columns (Schleicher and Schuell, Selectacel Type 20, 0.83 meq/g) were prepared, washed, re-equilibrated and operated according to procedures described by Rampton *et al.* (1965). The columns were prepared simultaneously to minimize column variation. Sarcoplasmic extracts of the 0, 2, 4, 7 and 10 day sampling times were chromatographed.

The elution procedure consisted of 130 ml of starting buffer followed by a gradient mixture at pH 9.0 (0.04*M* Tris-phosphate) and ending with a final buffer at pH 3.6 (0.5*M* Tris-phosphate). Elution was completed by placing 300 ml of the final buffer in the last chamber of the variable-gradient mixer followed by elution of 500 ml of 0.1*N* NaOH. Columns were washed and re-equilibrated after each run.

RESULTS AND DISCUSSION

ALTHOUGH THERE WERE MINOR VARIATIONS in moisture, pH, tyrosine-tryptophan indices of non-protein nitrogenous compounds and expressible moisture ratios between treatments and with time, these differences were not statistically significant. Hence, for the sake of brevity, these data are not presented and will not be discussed.

The tyrosine-tryptophan index obtained on the meat extracts prior to TCA precipitation served as a relative measure of the extractability of proteinaceous substances (Zender *et al.*, 1958). Results of this determination are shown in Fig. 1. The high temperature aged meat had a higher index level for the first three or four days post-mortem after which the normal aged meat showed higher index values. Statistical analysis failed to reveal any significant differences in the indices between the two treatments.

Sayre *et al.* (1963) found that the solubility of sarcoplasmic proteins was greatest immediately after death. Goll *et al.* (1964) reported similar findings and proposed that the protein solubilities were altered by post-mortem physiological conditions. The variation in protein solubility observed in this study was probably due to standardization of the homogenates to pH 7.0 during extraction. Thus the pH was constant for all extracts rather than being carried out at variable physiological pH's. This difference alone would greatly influence the extractability of the proteins.

Observed ratios (422/280 mμ) presented in Fig. 2 graphically depict the oxymyoglobin levels in relation to

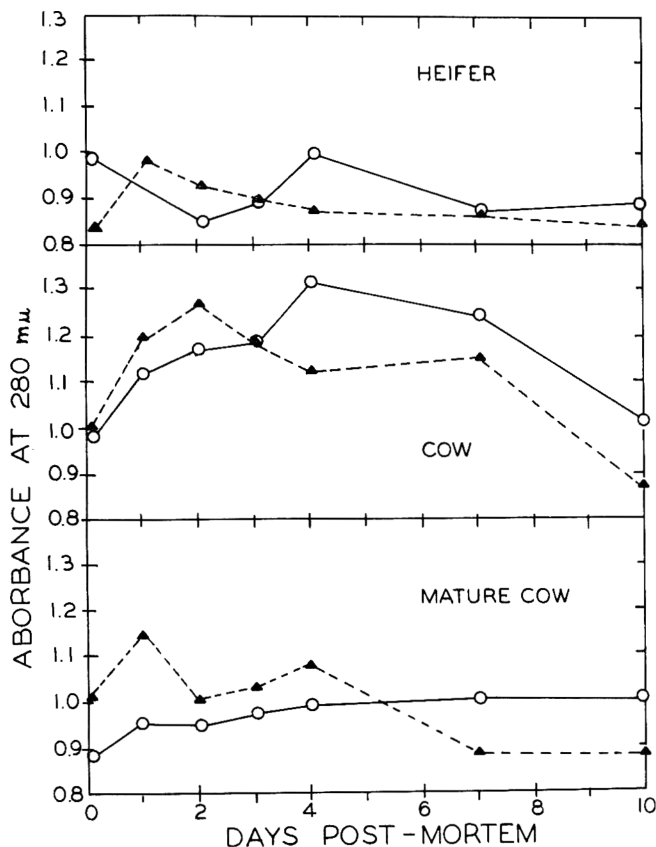

Fig. 1. Tyrosine-tryptophan index for protein (absorbance at 280 mμ) of undialyzed water extracts of l. dorsi muscles aged by normal and high temperature methods. Legend: High temperature aging - - - -; Normal aging ———.

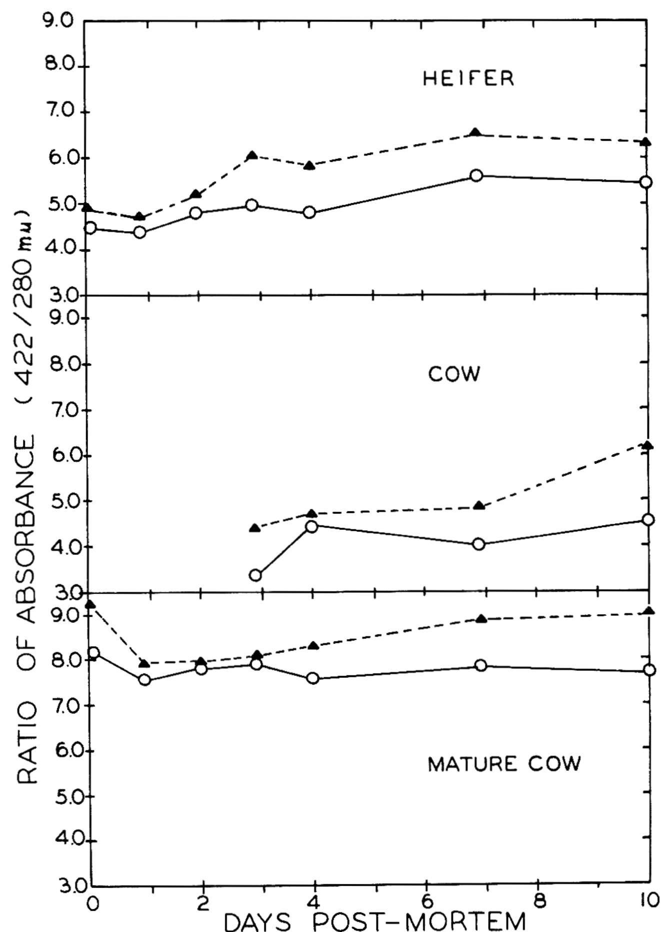

Fig. 2. Absorbance ratios (422/280 mμ) of water extracts of l. dorsi muscles aged by normal and high temperature methods. Legend: High temperature aging - - - -; Normal aging ———.

protein content. These ratios were used to verify color differences observed visually. After freshly cut muscle surfaces were exposed to the air for 20 min, high temperature aged meat showed a brighter cherry red color than the normally aged meat. There was little difference in the ratios for the two aging treatments during the first two days post-mortem. The heifer and cow extract ratios showed marked differences at the third day post-mortem and the mature cow on the fourth day post-mortem. The differences between the color-protein ratios appeared to remain constant thereafter.

Statistical analysis showed significant differences ($P < 0.01$) between the two aging treatments for the heifer and the cow samples and significant differences ($P < 0.01$) between the ratios with time for the heifer. Spectrophotometric data were initially collected for the cow samples at three days post-mortem when the color differences were first noted. Consequently, there were not sufficient data for statistical treatment of this sample although the difference was observed visually.

McCarthy *et al.* (1942) showed that the press juice of meat aged by the Tenderay® process (48 hr at 16°C) had a greater color density than meat aged at 2°C. However, they did not relate the color density to the protein concentration of the press juice. Color differences observed in the present study cannot be readily explained since the myoglobin concentrations should be similar in both l. dorsi muscles of the same animal.

Chromatography

Chromatographic profiles are presented with the elution volume shown in 10 ml fractions on the abscissa and the absorbance of the eluant read at 280 mμ on the ordinate. No attempt was made to quantitate the results by measuring the profile peak heights or areas under the profiles, although reference is made to peak heights in a relative sense. Chromatographic profile peaks are referred to as individual peaks with the understanding that a single peak is not necessarily a homogeneous protein fraction, but can consist of several distinct proteins that have similar elution times (Fujimaki *et al.,* 1964).

Results of the DEAE-cellulose ion exchange chromatographic separation of the sarcoplasmic proteins from the l. dorsi muscles of the cow are presented in Fig. 3. Differences between the elution profiles of the two treatments at the various sampling times appear to be quite minor. Marked changes, however, can be observed in the chromatograms as storage time increased.

Generally, the chromatograms were characterized by a large breakthrough peak followed by three or four small peaks, and then a large peak that was eluted between fractions 64 and 105. This large peak contained most of the muscle pigments, and was followed by 8 to 20 less-defined peaks. These small, often poorly-separated, peaks seemed to be eluted at about the same time for the two treatments, although the heights of the peaks were not constant with the different storage times.

Changes that occurred with increasing storage time were manifested by changes in the elution profile in the region of the breakthrough to pigment peaks. The most noticeable change in profiles of the sarcoplasmic proteins between zero day and two days post-mortem was the appearance of a double or split peak in place of the single pigment peak. In addition, a very small peak near fraction 35 became more distinct.

The four day post-mortem profile indicates that the breakthrough peak had decreased to about one-half of its initial height. Moreover, the small peak at fraction 35 had increased to form a large peak between fractions 30 and 46 while a small peak appeared near fraction 26. The pigment portion also had been altered so that a smaller peak was eluted before the main pigment peak.

The chromatograms for the seven and 10 day extracts both showed an increase in the height of the breakthrough peaks and a decrease in the peaks that appeared between fractions 30 and 46 at the four day sampling time. The seven day profile of the pigment proteins showed only a main peak with one or two shoulders which appeared to emerge as a distinct peak at 10 days post-mortem. The profiles of the samples held at the elevated aging temperature showed some loss of peaks at fraction 26 and between fractions 30 and 46.

Chromatographic profile patterns of the heifer were similar to those noted for the cow. Breakthrough and pigment peaks showed marked changes along with minor alterations in the latter part of the elution profile. The peak between fractions 30 and 46 that appeared at four days post-mortem was not evident at 7 days while the breakthrough peak disappeared completely by the 10 day post-mortem sampling. The chromatograms showed very small differences between the two temperature treatments.

Chromatograms of the sarcoplasmic proteins of the l. dorsi muscles of the mature cow showed less variation in the breakthrough and pigment peaks than was noted for the other two animals. The profiles did seem to show, however, more variation with the sampling times than between the two temperature treatments.

As mentioned previously, most of the changes in the chromatograms seem to have occurred in the first 120 fractions of the elution procedure. The changes in the breakthrough and other peaks up to the pigment peak might be due to changes in the extractability of the particular proteins involved. Such a change as dissociation might cause a shift of one peak to another position. Some differences in the elution times might be due to slight inherent differences between the four chromatographic columns, even though they were prepared as identically as possible.

Changes in the pigment peak, however, are not clear. There seemed to be a general shifting of this peak in some cases, or possibly even the appearance or increase of peaks that may have had similar elution times. Some change of this type occurred in all of the three series of chromatograms. In all of these cases the bulk of the pigmentation always appeared under the largest peak of this group. The reasons for these changes are not clear and merit further investigation.

The literature contains reports on the occurrence of variations in the chromatographic profiles of sarcoplasmic proteins of beef and chicken muscle during post-mortem aging. Fischer (1963) used DEAE-cellulose chromatography to study changes in the sarcoplasmic proteins of chicken

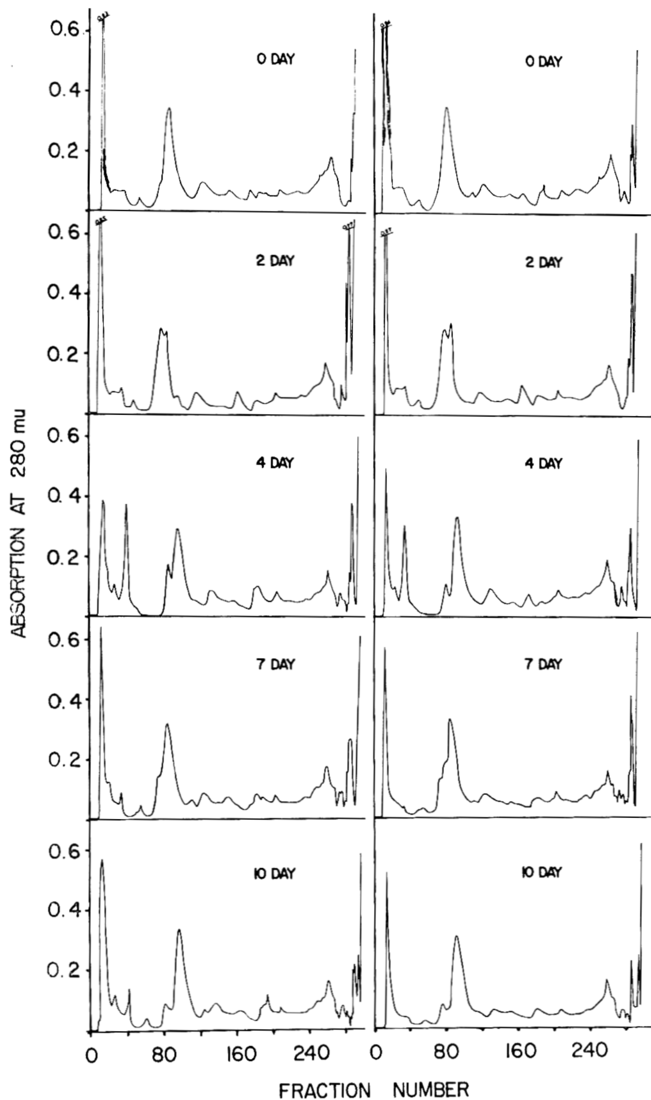

Fig. 3. Chromatographic profiles of sarcoplasmic proteins extracted from cow l. dorsi muscle at various aging periods. Legend: Normal aging method on left; high temperature aging on right.

breast muscle. He found seven distinct protein fractions, one of which increased with the aging time.

Fujimaki *et al.* (1964) used both cellulose-phosphate and DEAE-cellulose chromatography to study the differences in beef sarcoplasmic proteins at one and 12 days post-mortem. Using a stepwise elution scheme, they found that some of the eluted fractions disappeared while others decreased. Rampton *et al.* (1965) used DEAE-cellulose chromatography with a concave gradient of pH and salt concentration to show that some protein fractions of beef sarcoplasmic proteins disappeared; others diminished, while some new components appeared after 10 days of aging.

The series of chromatograms obtained in this study not only confirm the observations reported previously, but also show clearly that definite changes do occur in the sarcoplasmic proteins as measured by DEAE-cellulose chromatography. Although these changes can be followed from day to day during the aging period, they do not seem to be significant as a measure of tenderness due to the variation noted between the three animals.

Fujimaki *et al.* (1964) analyzed certain chromatographic fractions of beef sarcoplasmic proteins fractionated on cellulose-phosphate columns for enzyme activity. They found activities for aldolase, lactic dehydrogenase and myokinase in seven, eight and three fractions, respectively, of the 10 fractions studied. They concluded that the individual chromatographic peaks do not contain a particular homogeneous protein nor is all of any one protein found in a single specific fraction.

In view of the report by Fujimaki *et al.* (1964), aldolase, lactic dehydrogenase (band five) and myokinase were fractionated by the DEAE-chromatographic procedure to gain information about the elution profiles of these muscle enzymes. These enzymes (grade A purity) which had been extracted from rabbit muscle were purchased from Calbiochem, Los Angeles, California. Approximately 20 mg of aldolase and 10 mg of both myokinase and lactic dehydrogenase were chromatographed individually. In addition, a blank chromatogram was obtained by the chromatography of 9 ml of starting buffer in place of 9 ml of sample extract. The resulting chromatograms are presented in Fig. 4.

The chromatogram for the blank showed an absence of UV absorbing material up to fraction number 250 when a peak was observed followed by a very small peak at fraction number 280. These two peaks appeared after the elution gradient had been completed, and at the time when the pH became strongly alkaline due to the addition of 500 ml of 0.1*N* NaOH at the start of the clean-up procedure. The rapid increase in absorbancy at the end of the chromatogram was due to the Triton X-100 detergent used in the final wash solution.

The remaining chromatograms indicated that aldolase was eluted as a breakthrough peak while lactic dehydrogenase appeared as three peaks at the beginning of the elution scheme. Myokinase appeared as a single peak near fraction number 200, even though a stabilizer—albumin—had been added to the myokinase by the supplier during preparation of the purified enzyme. The peaks that appeared on the blank chromatogram are also quite evident on chromatograms for the enzymes. Although these enzymes were obtained from rabbit muscle, the results might be indicative for similar enzymes found in beef muscle.

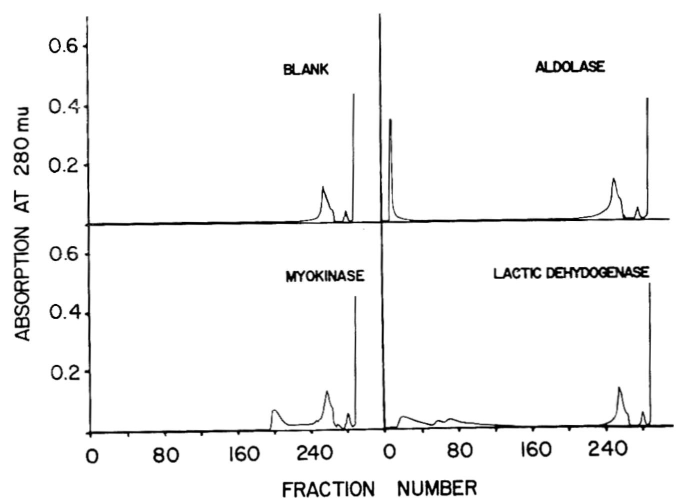

Fig. 4. Blank and enzyme chromatograms.

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Quantitative Methods for Anthocyanins. 1. Extraction and Determination of Total Anthocyanin in Cranberries.

SUMMARY—The method developed consists of extracting the anthocyanins with ethanol-1.5*N* hydrochloric acid (85:15) and measuring the O.D. of the extract, diluted with the extracting solvent, at 535 nm. The total anthocyanin content was calculated in absolute quantities with the aid of the extinction coefficients established for the four major cranberry anthocyanins dissolved in the alcoholic solvent system.

INTRODUCTION

CRANBERRIES (Cr) and many other fruits, as well as products made from them, owe their attractive color to the presence of anthocyanin (Acy) pigments. The total anthocyanin (T Acy) content of these fruits varies a great deal depending on factors such as species, variety, growth conditions, physiological state of the plant and fruit, size, position of the fruit on the plant, application of chemicals, etc. (Blank, 1958; Ribéreau-Gayon, 1959). This investigation was carried out as part of a project to develop methods for the quantitative determination of individual Acys in Cr.

LITERATURE REVIEW

THE EXTRACTION OF ANTHOCYANINS (Acys) is necessarily the first step in the determination of total as well as individual Acys in any type of plant tissue. The extraction method should be such that a maximum amount of Acy will be recovered with a minimum amount of adjuncts and the loss of Acy due to enzymatic and non-enzymatic changes will be kept at the minimum. This is usually accomplished by repeatedly extracting the macerated plant material with cold 1% hydrochloric acid in methanol.

The extraction procedure employed previously for cranberry anthocyanins (Cr Acys) was that developed by Francis (1957) and its slightly modified version (Servadio *et al.*, 1963). The modified process improved the sampling by blending the Cr with a small amount of water prior to sampling. An aliquot of the slurry was blended with ethanol–0.1*N* HCl (85:15) and after a short settling period a diluted aliquot at the supernatant was used for the O.D. measurement. This extraction procedure did not attempt to extract the Acy completely, but only to reach an equilibrium in pigment concentration throughout the slurry and the supernatant.

The T Acy can often be determined in crude extracts containing other naturally occurring phenolic compounds by measuring the absorptivity of the solution at a single wave length. This is possible because the Acys have absorption maximum in the 510–550 nm region (Harborne, 1958). This is far removed from other common phenolics,

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and the group of compounds with spectral maxima nearest this range is the flavenoids with absorption maximum in the 350–380 nm region (Sondheimer *et al.*, 1948a).

Such a simple, direct T Acy determination method was used in previous studies on Cr. A portion of the extract was diluted with the extracting solvent and the absorbance was measured at 535 nm. The pigment content was expressed as mg Congo Red per g of fresh berries by using the appropriate factors for the conversion.

The absorption maximum and molar absorptivity, used in simple spectrophotometric methods, are markedly affected by pH (Sondheimer, 1953; Ribéreau-Gayon, 1959), solvent (Harborne, 1958), presence of certain metals (Jurd, 1962), time of standing, and temperature. In addition to these, straight line relationships between concentration and absorptivity can be expected only at low concentrations, therefore, considerable dilution is usually necessary.

It is desirable to express the results of the T Acy determinations in terms which can be compared to results obtained by different workers. Sondheimer *et al.* (1948b) suggested that the results of the T Acy measurements could be expressed in terms of Congo Red equivalents. The shortcoming of this method is that the absorbance of the dye varies with each lot (Francis, 1957). Other authors have used commercially available Acy or anthocyanidin to express their results. This practice can be also misleading because of the gross impurities found in some of the commercial preparations (Fuleki *et al.,* 1967a).

The best way to express the results of T Acy determination is in terms of the absolute quantities of Acys present in the particular mixture. For this it is necessary to establish not only the identity of the pigments but also their extinction coefficient (E) in the solvent used for the T Acy determination and the ratio of each pigment. It is sufficient to establish the E value only once but the application of this method is hindered by the scarcity and lack of agreement between the E values reported in the literature (Table 1). This lack of uniformity is understandable if we consider the difficulty of preparing crystalline Acy, free from impurities, in sufficient quantities to allow several weighings under optimal conditions.

The Acys are rather unstable, particularly in the pure form, and are often contaminated with these polymeric

Table 1. Molecular extinction coefficients on anthocyanins and anthocyanidins reported in the literature.

Pigment[1]	Mol Wt[2]	Medium	Absorption max. nm	ε max. (× 10^4)	References
Pelargonidin	324.5	0.1% HCl in EtOH	504.5	1.78	Schou (1927)
Pl-3-Gl	486.5	1.0% HCl in water	496.0	2.73[3]	Jorgensen *et al.* (1955)
Cyanidin	340.5	0.1% HCl in EtOH	510.5	2.46	Schou (1927)
		0.1% HCl in EtOH	547.0	3.47	Ribéreau-Gayon (1959)
Cy-3-Ga	502.5	0.1% HCl in MeOH	530.0	3.43	Siegelman *et al.* (1958)
		0.1*N* HCl-EtOH (15:85)	535.0	4.49	Sakamura *et al.* (1961)
		0.1*N* HCl-EtOH (15:85)	535.0	4.62	Zapsalis *et al.* (1965)
Cy-3-Ar	472.5	0.1*N* HCl-EtOH (15:85)	538.0	4.44	Zapsalis *et al.* (1965)
Cy-3-RhGl	650.5	1.0% HCl in water	512.0	2.82[3]	Jorgensen *et al.* (1955)
Cy-3,5-Gl	664.5	0.1% HCl in EtOH	535.0	1.25	Ribéreau-Gayon (1959)
Peonidin	354.5	0.1% HCl in EtOH	511.0	3.72	Schou (1927)
		0.1*N* HCl-EtOH (15:85)	532.0	4.08	Sakamura *et al.* (1961)
Pn-3-Gl	516.5	0.1% HCl-in MeOH	536.0	1.13	Somers (1966)
Pn-3-Ga	516.5	0.1*N* HCl-EtOH (15:85)	532.0	4.84	Sakamura *et al.* (1961)
		0.1*N* HCl-HCl-EtOH (15:85)	532.0	4.84	Zapsalis *et al.* (1965)
Pn-3-Ar	486.5	0.1*N* HCl-EtOH (15:85)	532.0	4.61	Zapsalis *et al.* (1965)
Delphinidin	356.5	0.1% HCl in EtOH	522.5	3.47	Schou (1927)
		PPht	547.0	3.24[4]	Spaeth *et al.* (1950)
Dp-3-Gl	518.5	1%HCl in MeOH	543.0	2.90	Asen *et al.* (1959)
Petunidin	370.5	PPhT	549.0	3.46[4]	Spaeth *et al.* (1950)
Pt-3-Gl	532.5	0.1% HCl in MeOH	546.0	1.29	Somers (1966)
Malvidin	400.5	0.1% HCl in EtOH	520.0	3.72	Schou (1927)
		0.1 HCl in EtOH	557.0	3.62	Ribéreau-Gayon (1959)
		0.1% HCl in MeOH	547.0	3.16	Koeppen *et al.* (1966)
		PPhT	551.0	3.71[4]	Spaeth *et al.* (1950)
Mv-3-Gl	562.5	0.1% HCl in MeOH	546.0	1.39	Somers (1966)
		0.1% HCl in MeOH	538.0	2.95	Koeppen *et al.* (1966)
Mv-3,5-Gl	724.5	0.1% HCl in EtOH	519.0	1.07	Schou (1927)
		0.1% HCl in EtOH	545.0	1.03	Ribéreau-Gayon (1959)
Mv-3-p-coumaroyl-Gl	718.5	0.1% HCl in MeOH	536.0	3.02	Koeppen *et al.* (1966)

[1] Pl = pelargonidin, Cy = cyanidin, Pn = peonidin, Dp = delphinidin, Pt = petunidin, Mv = malvidin, Gl = glucoside, Ga = galactoside, Ar = arabinoside, Rh = rhamnoside.

[2] Since most of the determinations were carried out in a HCl-containing media, the molecular weights of the chlorides are given and they include one molecular of water of crystallization.

[3] The authors expressed doubts in regard to the purity of the anthocyanin used in the determination of extinction values.

[4] The authors used the organic phase of 10% phosphoric acid-phenol-toluene (PPhT) as solvent and the extinction coefficient was determined at 550 nm instead of the maximum.

degradation products. The presence of impurities, in most cases, would result in a lower E value. In addition to these factors, the absorbancy of the Acy molecule also depends on the pH and the nature of the media (Ribéreau-Gayon, 1959). Lowering the pH and/or the use of alcoholic media appreciably increases the E values of Acys.

The major Acys in Cr were identified by Sakamura *et al.* (1961) and Zapsalis *et al.* (1965) as cyanidin-3-galactoside (Cy-3-Ga), cyanidin-3-arabinoside (Cy-3-Ar), peonidin-3-galactoside (Pn-3-Ga) and peonidin-3-arabinoside (Pn-3-Ar). The extinction values were established by Zapsalis *et al.* (1965) on freshly prepared and highly purified pigments dissolved in ethanol–0.1*N* HCl (85:15).

A comparison of the values listed in Table 1 shows that the highest extinction values were obtained by these authors. This, and the fact that all Acys were obtained in the crystalline form, indicates that these values are probably highly reliable.

MATERIAL AND METHODS

THE CR USED IN THIS WORK (*Vaccinium macrocarpon* Ait., var. Howes) were grown at the University of Massachusetts Agricultural Experiment Station, Wareham, Massachusetts. The berries were stored at 0°F until used.

Solvents

The following solvents were used:

MAW	Methanol–glacial acetic acid–water (90:5:5)
Original Extracting Solvent	95% ethanol–0.1*N* HCl (85:15)
Extracting Solvent	95% ethanol–1.5*N* HCl (85:15) The pH of the solvent was adjusted as required to obtain a final pH of 1.0 in the Cr extract diluted for O.D. measurement.

Spectral measurements

The absorption maxima and E values were established using Cr extract and purified individual Cr Acys (Fuleki, 1967) diluted with the appropriate solvent. The measurements were carried out on a Turner "Spectro" Model 210 spectrofluorometer (G. K. Turner and Associates, Palo Alto, Calif.). A Hitachi Perkin-Elmer, Model 139 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) was used for the T Acy determinations.

The appropriate media were used as blanks in the determination of the E values. For routine analyses, distilled water was used as a blank after it was established that there was no difference in absorption in the 250–650 nm spectral region between distilled water and that of the medium used in the T Acy determination.

Selection of the extracting solvent

The acidity of 85:15 mixtures of 95% ethanol and aqueous solutions of hydrochloric acid in various strengths was measured with a pH meter. The selected solvent was checked by carrying out the standard extraction and dilution procedures with the solvent and measuring the pH of the diluted extract.

Extraction procedure

One hundred g frozen Cr were macerated with 100 ml extracting solvent in a Waring blendor at full speed. The sample was transferred quantitatively to a 400 ml beaker using approximately 50 ml of extracting solvent for washing the blendor jar. The beaker was covered with parafilm and stored overnight at 4°C. The sample was filtered on Whatman No. 1 paper through a No. 2 Buchner funnel. The volume of the "first run" filtrate was about 215 ml.

The beaker as well as the residue on the filter was washed repeatedly with the extracting solvent until approximately 450 ml of extract was collected. The extract was transferred to a 500 ml volumetric flask and made up to volume. To prepare the extract for spectrometric measurement, an aliquot of approximately 25 ml was filtered through a fine porosity sintered glass filter or a polyvinyl chloride (Polyvic) millipore filter (Millipore Filter Corp., Bedford, Mass.), and made up to appropriate volume.

Evaluation of the extraction procedure

To evaluate the efficiency of the extraction procedure, the extraction profile was determined. Six 100 g samples were extracted as described previously with the following exceptions:

a. The "first run" extract was collected separately and made up to 250 ml.
b. The washings were collected separately until almost 250 ml were obtained and then made up to 250 ml.
c. A third fraction called "second washings" was obtained by washing the residue with additional aliquots of the solvent until close to 100 ml were collected and then made up to 100 ml.

The amount of T Acy present in each fraction was determined using the method described for Cr extract.

Total anthocyanin determination

A small aliquot of filtered extract was diluted with the extracting solvent to yield O.D. measurements within the optimum range of the instrument. The diluted extract was stored in the dark for 2 hr and the O.D. was measured at the absorption maxima of Cr extract (535 nm). The T Acy content was calculated with the aid of the appropriate weight, volume, and dilution factors and E values.

Establishment of the extinction coefficients

The E values obtained by Zapsalis *et al.* (1965) were used as a basis for the establishment of E values for the Cr Acys dissolved in the media used in the T Acy determination. The purified individual Cr Acys were obtained in the form of a concentrated solution in MAW, from the final purification by paper chromatography (Fuleki, 1967). The stock solutions of individual Acys were further acidified with a few drops of approximately 4*N* HCl in ethanol, and one ml aliquots were diluted to 25 ml using calibrated glassware. After 2 hr standing in the dark the O.D. of the solutions in triplicate were measured at the absorption maxima.

Since the concentration of the pigment was the same in the original extracting solvent (standard) for which the extinction coefficients were available and in the medium with unknown extinction coefficient, (sample), the differ-

ence between their optical densities could be directly related to the difference in their absorbancies using the following equation:

E 1%/1 cm for sample = O.D. in sample/O.D. in standard × E 1%/1 cm for standard. Extinction coefficients at the visible and UV maxima as well as 535 nm, were established for the four pigments on triplicate samples.

RESULTS AND DISCUSSION

Extraction of anthocyanins

Extracting solvent. In previous work with Cr the Original Extracting Solvent was used for the extraction of Acy and also for the T Acy determination. However, to assure high accuracy and sensitivity in the T Acy determinations, it was essential that the acidity of the extracting solvent should be such that the pH of the extract, diluted for O.D. measurement was 1.0. The Original extracting solvent resulted in a considerably higher pH (2.6) where small changes in pH would introduce a large error. The relationship between pH and absorbancy was shown by Fuleki *et al.* (1967b).

It was desirable to retain the basic composition of the Original Extracting Solvent. Ethanol is non-toxic, more economical and its extracting power is almost as good as that of the generally used methanol (Deibner *et al.*, 1965). The inclusion of water helps the extraction of the more hydrophilic Acys (Deibner *et al.*, 1965) which are present as minor pigments in Cr (Fuleki, 1967) and it also allows the use of ethanol recovered by distillation. The hydrochloric acid stabilizes the pigments and lowers the pH to a level where the absorbance of the Acys is at their maximum. In view of the above considerations the original composition (EtOH-aqueous HCl) and proportions (85:15) were retained. The strength of the HCl solution was increased (1.5*N*) to attain the desired pH (1.0) in the diluted extract.

Extraction procedure. The procedure previously employed for Cr (Francis, 1957; Servadio *et al.*, 1963) did not completely extract the Acy but only equilibrated the pigment concentration throughout the macerated berries and the extracting solvent. It was necessary to develop a new procedure which would extract the pigments completely and provide a representative sample of the Acys in a concentrated form for their individual determination if desired.

The procedure developed was described in the "Materials and Methods" section. The volume of the extracting solution was kept low in the initial phase of the process in order to have high Acy concentration in the "first run" extract. The overnight submersion of the macerated berries in the extracting solvent allowed the Acy to diffuse through the cell membranes. Some additional extracting solvent was required to wash the residue free of Acys.

The new extraction procedure was evaluated by establishing an extraction profile (Table 2) which showed that the Acys were almost completely extracted in the first 250 ml. Washing of the residue with an additional 250 ml of Extracting Solvent removed essentially all the Acys.

Determination of the total anthocyanin

Absorbancy measurements. The O.D. measurements for the T Acy determination were carried out in the extracting

Table 2. Recovery of total anthocyanin from cranberries on extraction with EtOH-1.5*N* HCl (85:15).

Fraction			Recovered T Acy	
Code	Name	ml	mg/100 g	% of Total
A	First run	250	48.04 ± 0.59 [1]	86.02
B	First washings	250	7.81 ± 0.53	13.98
	Total A & B	500	55.85 ± 0.77	100.0
C	Second washings	100	0.11	0.20

[1] Standard error of the mean.

solvent. This ethanolic medium gave a dilute Acy solution with a pH of 1.0. The 2 hrs standing period was sufficient to attain equilibrium between the different forms of Acy present at pH 1.0. The O.D. measurements were carried out at 535 nm.

Extinction coefficients. Since E values for Cr Acys were available only for the original extracting solvent it was necessary to establish E values for the EtOH-1.5*N* HCl medium (Table 3). In ethanolic media the peak of absorption in the visible range was 535 and 536 nm for the cyanidins and at 532 nm for the peonidin glycosides. In the long UV region, the maximum was found at 281–282 nm for all four pigments. These values agree closely with those reported in the literature (Table 1) for these pigments.

The absorption of Acys in alcoholic media at the observed UV maximum was about 45% of that for the visible maximum. The ratios calculated from the values reported by Ribéreau-Gayon (1959) for Cyanidin-3,5-glucoside and Malvidin-3,5-glucoside were 40 and 52% respectively. The data of Koeppen *et al.* (1966) showed a similar ratio for malvidin-3-glucoside (46%) but the ratio for malvidin-3-p-coumaroyl-glucoside was quite different (approx. 88%). The greatly increased absorbance at the UV marima in the last case is probably due to the presence of p-coumaric acid in the molecule.

Most non-acylated pigments substituted in the 3-position show a ratio approx. 63% whereas those in the 3,5 or 5 position show a value of approx. 42% (Harborne, 1963). An increase in the pH greatly decreases the absorption at the visible maximum while the absorbance is only slightly affected at the UV maximum (Fuleki *et al.*, 1967b).

The difference in pH of the media used by these workers might be responsible for the discrepancies in the reported ratios. A third very intense maximum occurred between 209 and 212 nm. A similar peak was reported at 210 and 211 nm by Ribéreau-Gayon (1959) for cyanidin-3,5-glucoside and malvidin-3,5-glucoside respectively.

The E values at 535 nm were used in the calculation of T Acy content. Since the differences between the E values of the four Cr Acys were rather small and the ratio of individual Acys was not always known or determined, the av E of the four major Cr Acys (982) can be used to calculate the T Acy. The use of an average rather than a weighted E will result in an error less than 0.2% for the pigment ratios found in cranberries.

Calculation of the total anthocyanin content. The total O.D. (T O.D.) is calculated first so the O.D. measured on a small aliquot of the diluted extract will apply to a certain

quantity (100 g or other) of berries. The T O.D. obtained is transformed to mgs of Acy with the aid of the E value.

The T O.D. for 100 ml extract is calculated using the following equation:

(1) T O.D. = O.D. × DV × VF where

O.D. = the absorbancy reading on the diluted sample (1 cm cell).

DV = Diluted Volume or the volume in mls of the diluted extract prepared for the O.D. measurement.

VF = Volume Factor which corrects for the difference in size between 100 ml, the Original Volume (OV) for which the calculation is made and that of the Sample Volume (SV) or the volume of extract used for the absorbancy measurement.

$$VF = \frac{OV}{SV} = \frac{100}{SV}$$

Since the T O.D. for 100 g berries is desired the T O.D. calculated for 100 ml extract should be brought up to this level using the following equation:

(2) T O.D. per 100 g = T O.D. for 100 ml extract × TEV/CrW where

TEV = Total Extract Volume. The total volume of the extract in mls obtained from the Cr sample used for extraction.

CrW = Cranberry Weight or the weight of the Cr in gms used for extraction.

The T O.D. value gives the absorbance as if all Acy present in the berries was concentrated in 1 ml of solution. Since it is more convenient to calculate the Acy in mgs while the E values were given for a 1% (10 mg per 1 ml) solution, the E/10 values are used in the calculation of T Acy. The T Acy in mgs per 100 ml Cr extract is calculated from the calculated T O.D. value using the av $E^{1\%}_{1\,cm}$ 535 value for Cr Acys (982).

$$(3)\quad \text{T Acy, mgs per 100 g} = \frac{\text{T O.D.}}{\text{av } E^{1\%}_{1\,cm}\,535 \,/\, 10} = \frac{\text{T O.D.}}{98.2}$$

The above formulae reduce to:

(4) T Acy in mgs per 100 g = OD × DV × 100/SV × TEV/CrW × 1/98.2

Previous data calculated as mg C.R./g can be recalculated as mg Acy/100 g by multiplying the C.R. data by 0.5 (Fuleki *et al.*, 1967a).

Example

To facilitate the calculation of the T Acy content an example is given as follows:

The total volume of the extract obtained from 100 g (CrW) of fresh berries was 500 ml (TEW). A 2 ml (SV) sample of the extract was diluted with the Extracting Solvent to 100 ml (DV) and the O.D. at 535 nm was measured as 0.400 (O.D.). The T Acy content should be calculated in mgs for 100 g Cr.

The T O.D. for 100 ml extract is calculated:

(1) T O.D. = 0.400 × 100 × 100/2 = 2,000

The T O.D. for 100 g berries is calculated:

(2) T O.D. per 100 g Cr = 2,000 × 500/100 = 10,000

The T Acy content is calculated with the aid of the appropriate E value:

(3) T Acy = 10,000/98.2 = 101.7 mg per 100 g Cr.

The data presented in Table 3 show that there are differences in the E values of Cr Acys. Although these differences are negligible for general work they might be important in cases where the highest accuracy is desired as in studies on biosynthesis, etc. In such cases the quantity of each anthocyanin present should be determined using a calculation method employing successive approximation as described in a later paper of this series. The sum of the determined quantities for individual Acys will give the amount of T Acy. The large difference between the E values of Acys occurring in some plants (for example, mono- and diglycosides) might be minimized by calculating with molecular extinction coefficients (ϵ).

Evaluation of the total anthocyanidin method. The precision of the method was evaluated by analyzing 10 identical aliquots of the same Cr extract. The variation was found to be well within an acceptable level (1.4% Coefficient of Variability).

The extraction and T Acy determination method developed for Cr can be used for other plant material and solid or semisolid products as well. With samples containing chlorophyll the T Acy determination method can be applied after washing the extract with ethyl ether to remove the chlorophyll and pheophytin.

Plant materials for which the E values of the pigments are not available present a special problem. If the absorp-

Table 3. Extinction coefficients for cranberry anthocyanins in alcoholic media.

	EtOH-0.1*N* HCl (85:15)				EtOH-1.5*N* HCl (85:15)					
	max.1	max.2	max.1[1]	max.2	max.1[1]	max.2	max.1	max.2	535 nm	
Pigment	nm		$E^{1\%}_{1\,cm}$		nm		$E^{1\%}_{1\,cm}$			ϵ 535 ($\times 10^4$)[2]
Cy-3-Ga	535	281	920	422	536	282	958	484	958	4.81
Cy-3-Ar	535	281	941	428	535	282	1002	421	1002	4.73
Pn-3-Ga	531	281	936	427	532	282	987	450	985	5.09
Pn-3-Ar	532	281	947	450	532	282	981	470	981	4.77
Average									982	4.85
Weighted av.									981	

[1] As determined by Zapsalis *et al.* (1965).
[2] Molar extinction coefficient.

tion coefficients cannot be determined then the best approach is to calculate the T Acy content using one of the E values established for the Cr Acys. The error introduced by this substitution will vary according to the pigments present but it can be neglected when comparative studies are made.

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Quantitative Methods for Anthocyanins. 2. Determination of Total Anthocyanin and Degradation Index for Cranberry Juice

SUMMARY—The method developed for total anthocyanin determination involves the measurement of the absorbance at 510 nm on samples diluted with pH 1.0 and 4.5 buffers. The pigment content is calculated in absolute quantities with the aid of extinction coefficients established for the cranberry anthocyanins dissolved in the buffers. An index of anthocyanin degradation, based on a new concept, could be calculated from the measurements obtained for the total anthocyanin determinations.

INTRODUCTION

THE ANALYSIS of the total anthocyanin (T Acy) in juice or other products stored for an appreciable length of time introduces problems which prevent the use of the method developed for cranberries (Fuleki *et al.*, 1967). During storage a considerable amount of brownish degradation products accumulate. Although these degradation products have an absorption maxima around 415 nm (Ponting *et al.*, 1960) they also absorb to some extent at 510 nm. This would introduce an error if a simple direct T Acy determination method, similar to that developed for cranberry (Cr) was used.

This paper describes the development of an improved T Acy determination method which minimizes the interference due to these brownish colored degradation products.

LITERATURE REVIEW

FREQUENTLY A SIMPLE DIRECT T Acy determination method cannot be applied because of interference due to chlorophyll or its degradation products, as in ripening fruits (Swain *et al.*, 1959), or brown colored degradation products formed either from sugar-amino acid reactions (Dickinson *et al.*, 1956) or from the degradation of Anthocyanins (Acys). In such cases, indirect methods are used to determine the T Acy content.

The first method was developed by Sondheimer *et al.* (1948). Their method is based on the fact that the absorptivity of anthocyanin (Acy) is markedly dependent on pH. They used differential measurements between solutions of pH 2.0 and 3.4 to determine the concentration of Acy in strawberries and strawberry products. A solution of Congo Red was used as the standard of color intensity. Swain *et al.* (1959) suggested the use of differential measurements between solutions of pH 1.0 and 0.5.

If we call the above indirect determinations of T Acy pH differential methods, then other available techniques could be called subtractive methods. These methods are based on O.D. measurements taken before and after destroying the color due to Acy in the plant extract or removal of the degradation products before measuring the concentration of Acys.

Dickinson *et al.* (1956) bleached the color due to Acy with sodium sulfite while Swain *et al.* (1959) destroyed the Acy by oxidation with hydrogen peroxide. In both cases pure Acy was used to establish the standard curve. It was shown that the reagents used had no effect on the interfering background material. Lempka *et al.* (1966) developed a T Acy determination method for stored juices based on removal of the degradation products on an ion exchange column prior to the absorption measurement.

Ribéreau-Gayon (1964) compared the pH differential method (difference between pH 0.6 and 3.5) with the subtractive method using sodium bisulfite on red wines of different ages. According to his findings, the two methods gave comparable results. However, the sulfite bleaching method always gave higher values (10–25 mg/L) which could be significant in old wines where the Acy content was rather low. The results obtained using the two methods were expressed in mg grape Acy per L of wine with the aid of standard curves established using purified crystalline grape Acy.

The accumulation of brownish degradation products increases the absorption in the 400–440 nm region, while the loss of Acy is reflected in the decreasing O.D. at the Acy absorption maxima. This phenomenon can be utilized for the calculation of an index which is indicative of the proportion of degraded Acy in the sample. Such an index is quite useful when the original Acy content is not known and a measure of the state of Acy degradation is needed.

Absorption ratios such as 535/415 for cranberry sauce (Servadio *et al.*, 1963), 515/415 for cranberry juice (Francis *et al.*, 1963), 440/500 (Lukton *et al.*, 1956) for strawberry juice, 520/420 for grape and boysenberry juices (Ponting *et al.*, 1960), 420/520 for wine (Ribéreau-Gayon, 1964), 520/430 for blueberry wine (Fuleki, 1965) are widely employed. Another type of absorption ratio method uses the accumulation of degradation products in the UV range of the spectrum for comparison. Yang *et al.* (1950) used 515/340 ratio to measure the color and the quality of various berry wines.

MATERIAL AND METHODS

The "Ocean Spray" brand cranberry juice cocktail (CrJ) used was obtained in 1 pint bottles from the manufacturer (Ocean Spray Cranberries Inc., Hanson, Mass.) as well as from the market.

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Solvents

The following solvents were used:

pH 1.0 Buffer 0.2*N* KCl–0.2*N* HCl (25:67)
pH 4.5 Buffer *N* Sodium acetate—1*N* HCl-water (100:60:90)

The pH of the buffers was adjusted as required to obtain a final pH of 1.0 and 4.5 in the CrJ diluted with the respective buffers.

The preparation of the other solvents and the method for spectral measurements, including the establishment of extinction coefficients for the Acys, were described in a previous paper (Fuleki *et al.*, 1968).

Selection of the pH for the media

A stock solution was prepared by diluting 200 ml CrJ to 1 L with 0.1*N* HCl. Twenty-five ml aliquots of the stock solution were measured into 50 ml volumetric flasks. The samples were made up to volume with water and were allowed to stand in the dark at room temperature. After a 1 hr equilibration period, the pH and the O.D. were determined. The O.D. measurements were carried out on a Hitachi Perkin-Elmer Model 139 spectrophotometer at 510 nm.

The stability of Cr Acys at pH 4.5 was determined by diluting 5 ml CrJ to 50 ml with pH 4.5 Buffer. The O.D. at 510 nm was measured at 1 hr intervals. CrJ diluted to the same extent with pH 1.0 Buffer and stored for the same period of time was used for comparison.

Changes during equilibration

To determine the time required by the Acys to reach equilibrium, 5 ml aliquots of CrJ were diluted to 50 ml with each of the pH 1.0 and 4.5 Buffers. The O.D. at 510 nm was measured at short intervals on the Hitachi Perkin-Elmer spectrophotometer.

The O.D. change at 510 nm in the first 6 min after the addition of the pH 4.5 Buffer was recorded on a Beckman DB spectrophotometer connected to a Speedomax H (Leeds and Northrup Co., Philadelphia, Pa.) chart recorder operating at the speed of one-half inch per min.

The pH differential method for the determination of total anthocyanin

Ten ml aliquots of CrJ were diluted to 250 and 50 ml with the pH 1.0 and 4.5 Buffers respectively. The diluted samples were equilibrated in the dark at room temperature for 2 hr. The O.D. of the samples was measured at 510 nm, using distilled water as blank. The O.D. difference (ΔO.D.) was obtained by subtracting the total O.D. at pH 4.5 (T $O.D._{pH\ 4.5}$) from the total O.D. at pH 1.0 (T $O.D._{pH\ 1.0}$). Both values were calculated from the O.D. readings using the appropriate dilution and calculation factors. The T Acy in mg was calculated with the aid of the av delta E (ΔE) value established for the Cr Acys.

RESULTS AND DISCUSSION

Determination of total anthocyanin content

The method used for CrJ should be such that it could be used not only for fresh but also for stored products containing brownish compounds developed during storage. The various indirect T Acy determination methods were considered for use. The subtractive methods employing hydrogen peroxide or sodium sulfite were suspected to decrease the absorption of some degradation products as well, resulting in an error in the positive direction (Ribéreau-Gayon, 1964).

Altering the pH does not affect the absorption of the degradation products (Sondheimer *et al.*, 1948) while it changes the absorption of the Acys to a great extent. For these reasons, efforts were directed toward the development of a pH differential method for the determination of T Acy in CrJ.

Selection of the pH for the media. The absorption maximum was determined for CrJ to be at 510 nm. This value was used in the following as the wavelength of measurement for the T Acy determination. The effect of pH on O.D. at the wavelength of maximum absorption in aqueous and alcoholic media (535 nm) is presented in Fig. 1. The results show no appreciable difference between the two media. In both cases, the highest absorbance was observed around pH 1.0 and as the pH increased, a sharp decrease occurred in the O.D., leveling off around pH 4.5. These results are in general agreement with those given by Sondheimer *et al.* (1948), Dickinson *et al.* (1956), and Swain *et al.* (1959) for aqueous media.

In order to ensure the highest sensitivity and accuracy the pH values were selected for the pH differential method for the following reasons:

1. The difference in O.D. should be the greatest possible, between the two pH values where the measurements are made.
2. Small variations in pH around the values selected for the measurements should cause only slight changes in O.D.
3. The Acy should be stable at the pH where the measurements are made.

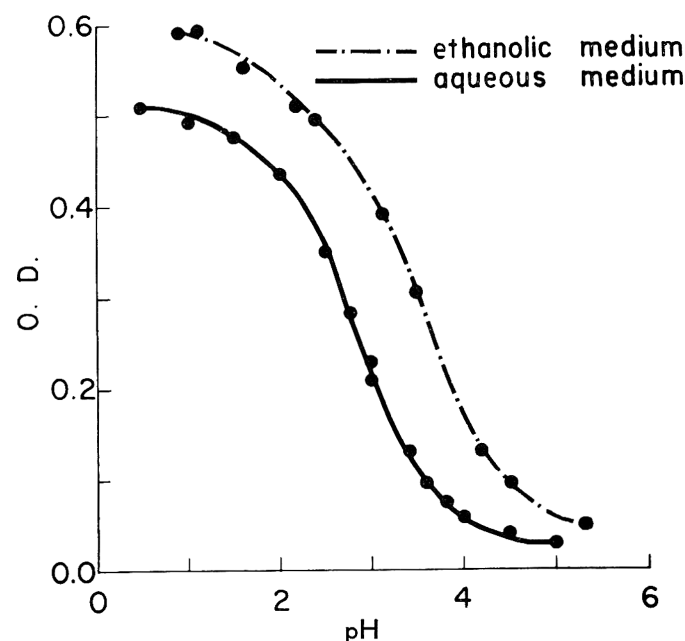

Fig. 1. Effect of pH on the O.D. of cranberry juice at the absorption maximum in aqueous (510 nm) and ethanolic medium (535 nm).

Considering the above requirements, pH 1.0 and 4.5 were selected for the pH differential T Acy method. The data presented in Fig. 1 show that the greatest difference in O.D. at the absorption maximum occurs between these two points, while the change in O.D. with slight variations in pH is negligible.

The highest pH described in the literature for T Acy determinations was 3.5 (Ribéreau-Gayon, 1964). This and the generally recognized instability of Acys at low acidity required a study of the stability of Acy at pH 4.5. The stability data obtained with triplicate samples of CrJ are presented in Table 1. The variation in O.D. at pH 4.5 was remarkably small, particularly if the comparison was made—as it would occur in the T Acy determinations—on the difference in O.D. between pH 1.0 and 4.5. In either case, the variation due to change in the pH 1.0 sample was greater than that at pH 4.5. The probable reason for this was not that the Acy was more stable at pH 4.5, but the much higher sensitivity of detection at pH 1.0, where the absorption of Acy was approximately seven times greater than that at pH 4.5. The results in Table 1 demonstrate that pH 4.5 can be used with confidence for the low-acid medium.

Other workers reported measurements at pH values which result in O.D. values on the steep part of the pH vs. O.D. curve. In such cases, slight variation of the pH results in a large error in the O.D. reading. For example, the classical pH differential method of Sondheimer *et al.* (1948) employed pH 2.0 and 3.4 for the measurements. Ribéreau-Gayon (1964) improved upon it by using a more acid medium (pH 0.6) but still retained pH 3.5 for the low-acid medium. Swain *et al.* (1959) noted that at the pH values used by Sondheimer *et al.* (1948), the change in color on slight variation of the acidity was still too large to ensure the highest accuracy.

To counter this problem, they proposed pH 0.5 and 1.0 as the high and low acidity levels. However, the O.D. difference (Fig. 1) between pH 0.5 and 1.0 for unit quantity of Acy is very small, therefore the application of the Swain *et al.* (1959) modification on the pH differential method would result in a greatly decreased sensitivity. pH values which were too high have also been employed in the simple, single pH T Acy determinations (Fuleki, 1967).

Equilibration period. It takes about one hour for the various forms of Acy to reach a state of equilibrium after the medium or the acidity of the medium has been changed. The data presented in Table 2, obtained on triplicate samples of CrJ diluted 5:45 with the buffers, support this conclusion. Since the time to reach equilibrium at pH 1.0 is somewhat longer, the extension of the equilibration period to two hours is desirable.

Table 1. Stability of cranberry anthocyanins in pH 1.0 and 4.5 buffers.

Time hrs	pH 1.0 Buffer		pH 4.5 Buffer		Difference in O.D.
	pH	O.D.	pH	O.D.	
1	1.05	0.387	4.49	0.055	0.332
2	1.02	0.392	4.47	0.055	0.337
3	1.00	0.391	4.49	0.054	0.337
4	1.00	0.396	4.49	0.052	0.344
6				0.053	
24	1.02	0.397	4.50	0.049	0.348

Table 2. Change in O.D. at 510 nm of cranberry anthocyanins in pH 1.0 and 4.5 buffers during the equilibration period.

Time min	pH 1.0 Buffer		pH 4.5 Buffer	
	pH	O.D.	pH	O.D.
1		0.370		0.080
1½		0.370		0.065
2		0.371		0.060
5	1.03	0.373	4.50	0.058
10	1.02	0.378	4.49	0.057
30	1.02	0.381	4.49	0.055
60	1.02	0.385	4.49	0.054
120	1.02	0.388	4.48	0.054

It was frequently observed that a sudden intensification of color, accompanied by a shift in hue toward the blue, occurred when the pH 4.5 Buffer was added to the CrJ. The color, however, faded almost completely within 4 min. This observation gives support to the theory (Jurd, 1963; Bentley, 1960) that the colorless carbinol base is formed through the violet anhydro base. To verify this phenomenon, the changes in transmittance at 510 nm of a diluted CrJ were recorded in the first 6 min after the addition of the pH 4.5 buffer (Fig. 2). This tracing of the changes in transmittance demonstrates the sudden intensification as well as the gradual fading of the color.

Establishment of extinction coefficients. It was necessary to establish E values for the Cr Acys in the media selected

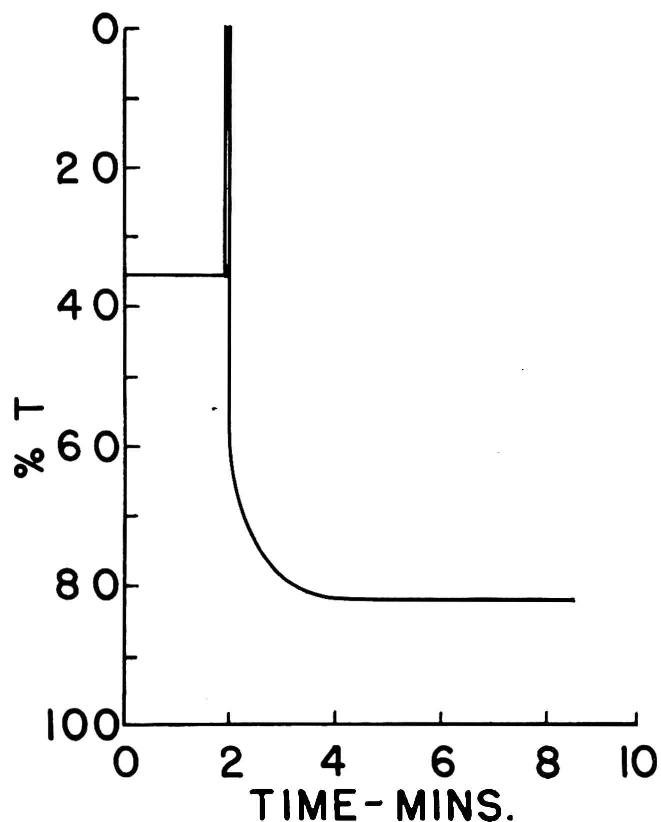

Fig. 2. Changes produced in the absorption of cranberry juice at 510 nm after the addition of pH 4.5 buffer (buffer added at 2 min).

Table 3. Extinction coefficients for cranberry anthocyanins in aqueous media.

Pigment	Buffer pH 1.0 max. 1 (nm)	Buffer pH 1.0 max. 2 (nm)	Buffer pH 1.0 max. 1 ($E^{1\%}_{1\,cm}$)	Buffer pH 1.0 max. 2 ($E^{1\%}_{1\,cm}$)	Buffer pH 1.0 510 nm ($E^{1\%}_{1\,cm}$)	Buffer pH 4.5 max. 1 (nm)	Buffer pH 4.5 max. 2 (nm)	Buffer pH 4.5 max. 1 ($E^{1\%}_{1\,cm}$)	Buffer pH 4.5 max. 2 ($E^{1\%}_{1\,cm}$)	Buffer pH 4.5 510 nm ($E^{1\%}_{1\,cm}$)	$\Delta E510^{1\%}_{1\,cm}$
Cy-3-Ga	512	279	851	553	851	517	279	86	460	86	765
Cy-3-Ar	516	279	903	1324	895	521	280	122	1224	122	773
Pn-3-Ga	512	278	887	604	886	517	277	88	418	88	798
Pn-3-Ar	512	277	858	702	858	512	277	95	606	95	763
Average					873					98	775

for the pH differential T Acy determination method. The difference in E values between the pH 1.0 and 4.5 Buffers (ΔE values) for the Cr Acys was used to calculate the T Acy content in absolute quantities from the O.D. measurements obtained for the CrJ sample.

The E and ΔE values for the aqueous buffers (Table 3) were established at the same time as those for the ethanolic extracting solvent (Fuleki *et al.,* 1968). A comparison of the E values for the aqueous and alcoholic media showed that the solvent had a considerable influence on the position of the wavelength of maximum absorption in the visible range of the spectra. The two comparable solvent systems, having the same pH (pH 1.0 buffer vs. Extracting Solvent), showed that a change from alcoholic to aqueous system resulted in a 19–24 nm hypsochromic shift in the visible maximum of the pigments. This agrees closely with the values reported in the literature (Harborne, 1958; Ribéreau-Gayon, 1959). A small hypsochromic shift (3 nm) occurred in the position of the UV maximum at 282 nm on a similar change of the solvent system.

The absorbancy of Acys was also affected by the change from an alcoholic to an aqueous system. With both media at pH 1.0, the E values were decreased approximately 10% at the visible maximum while a considerable increase was observed at the UV maximum. Ribéreau-Gayon (1959) reported for delphinidin, a 34% decrease of absorption at the visible maximum and a small decrease at the long UV maximum on change of media from 98% ethanol to water containing 10% ethanol, both containing the same concentration of HCl.

The change in pH had no effect on the position of the absorption maxima of Acys in the alcoholic solvent (pH 1.0 vs. 2.6) and it had very little if any influence in the aqueous media (pH 1.0 vs. 4.5). As was expected, a decrease in acidity reduced considerably the E values at the visible maximum. In the aqueous media where the pH difference was the largest, the change from pH 1.0 to 4.5 resulted in approximately a tenfold decrease in the extinction coefficients.

An increase in the pH of the media resulted in a somewhat smaller decrease in the absorbancy indices at the UV maxima. The decreased absorbancy at the visible maxima on increased pH was reported by several authors (Sondheimer *et al.,* 1948; Sondheimer, 1953; Dickinson *et al.,* 1956; Swain *et al.,* 1959; Ribéreau-Gayon, 1959, 1964). Less information is available on the effect of pH on the extinction values at the UV maxima. Ribéreau-Gayon (1959, 1964) reported a gradual but small decrease in the extinction coefficients at 280 nm as the pH was increased.

The av $\Delta E510^{1\%}_{1\,cm}$ value for the Cr Acys (775) was used in the pH differential method developed in this study. However, if a simplified method is desired and no interference is anticipated from various degradation products, then it is sufficient to measure the O.D. at pH 1.0 only and calculate the approximate quantity of total Acy using the av $E510^{1\%}_{1\,cm}$ obtained in the pH 1.0 Buffer (873) as described previously for cranberries (Fuleki *et al.,* 1968).

Calculation of the total anthocyanin content. The total O.D. (T O.D.) for 100 ml CrJ at pH 1.0 and pH 4.5 was calculated using the following equation:

(1) T O.D. = O.D. × DV × VF where

O.D. = the absorbancy reading on the diluted sample (1 cm cell).

DV = Diluted Volume or the volume in mls of the diluted sample prepared for the O.D. measurement.

VF = Volume Factor which corrects for the difference in size between the Original Volume (OV), usually 100 ml, for which the calculation is made and that of the Sample Volume (SV) or the volume of the CrJ used in preparing the diluted sample for the absorbancy measurement.
VF = OV/SV = 100/SV

The difference in T O.D. between the CrJ at pH 1.0 and and 4.5 (ΔO.D.) was calculated as follows:

(2) $\Delta O.D. = T\ O.D._{pH\ 1.0} - T\ O.D._{pH\ 4.5}$

The T Acy content in mg for 100 ml CrJ, or for the OV, is calculated from the Δ O.D. value with the aid of the av $\Delta E510^{1\%}_{1\,cm}$ value for Cr Acys (775).

$$(3)\ \text{T Acy, mg per 100 ml} = \frac{\Delta\ O.D.}{\text{av}\ \Delta\ E510^{1\%}_{1\,cm} / 10} = \frac{\Delta O.D.}{77.5}$$

Calculation of the total anthocyanin content in fresh cranberry juice. In juices with no degraded pigment, it is sufficient to use a single pH T Acy determination method similar to that described for Cr extract (Fuleki *et al.,*

Table 4. Results of the total anthocyanin determinations by the pH differential method on 10 identical cranberry juice samples.

Sample No.	pH 1.0 O.D.	pH 1.0 T O.D	T Acy[1] mg/100 ml	pH 4.5 O.D	pH 4.5 T O.D	ΔO.D.	T Acy mg/100 ml	DI	DI Simp.
1	.338	845	9.69	.153	77	768	9.90	0.98	1.10
2	.333	833	9.54	.148	74	759	9.79	0.97	1.10
3	.335	838	9.60	.145	73	765	9.87	0.97	1.10
4	.338	845	9.68	.152	76	769	9.92	0.98	1.10
5	.334	835	9.56	.156	78	757	9.77	0.98	1.10
6	.337	843	9.66	.148	74	769	9.92	0.97	1.10
7	.341	853	9.77	.150	75	778	10.03	0.97	1.10
8	.336	840	9.62	.152	76	764	9.85	0.98	1.10
9	.337	843	9.66	.153	77	766	9.88	0.98	1.10
10	.338	845	9.68	.154	77	768	9.90	0.98	1.10
Mean		842	9.65		75.5	766	9.88	0.98	1.10
Coef. var. %		0.69	0.69		2.25	0.76	0.76	0.64	0.00

[1] Calculated from the results of the O.D. measurements at pH 1.0 using the av $E^{1\%}_{510\ 1\ cm}$ value for Cr Acys in pH 1.0 buffer (873).

1968). The O.D. is measured at 510 nm on a sample diluted with the pH 1.0 buffer. The T O.D. is calculated using equation (1). The T Acy is calculated as follows:

$$(4)\quad \text{T Acy mg per 100 ml} = \frac{\text{T O.D.}}{\text{av } E^{1\%}_{510\ 1\ cm}/10} = \frac{\text{T O.D.}}{87.3}$$

The data presented in Table 3 show that the difference in both E and Δ E values between Cr Acys was negligible for general work. If a higher accuracy is required, then a method employing successive approximation could be applied (Fuleki, 1967).

Evaluation of the total anthocyanin method. The precision of the developed method was evaluated by analyzing 10 identical CrJ samples (Table 4). The data show that only minor variations occurred and the precision of the method was very high. The methods developed for T Acy determination can be used for other fruit juices and wines without difficulty. In cases where degradation products are present the pH differential method (pH 1.0 and 4.5) should be used, otherwise the single pH method (pH 1.0) should be adequate.

When the Acy analyses are carried out on solid or semisolid plant material or products, extraction of Acys is necessarily the first step. The extraction procedure described elsewhere (Fuleki *et al.*, 1967) should be applicable in most cases.

Degradation index

A great advantage of the proposed pH differential T Acy determination method is that the date obtained can be also used for the calculation of an index, which is indicative of the proportion of degraded Acy in the sample. Such an index is quite useful, particularly in cases where the original Acy content is not known and a measure of the state of Acy degradation is needed.

Calculation of the degradation index. The T Acy content of the sample is determined using the pH differential method. Using the T $\text{O.D.}_{\text{pH 1.0}}$ value obtained in the above calculation, the T Acy content is also calculated by the single pH method (Equation No. 4). The Degradation Index (DI) is calculated as follows:

$$(5)\quad \text{DI} = \frac{\text{T Acy by the single pH method}}{\text{T Acy by the pH differential method}}$$

In most cases particularly where the Acys are unidentified or the E values are not known, a simplified DI (DI simp.) can be obtained using the following equation:

$$(6)\quad \text{DI simp.} = \text{T O.D.}_{\text{pH 1.0}}/\Delta\text{O.D.}$$

The proposed DI is based on the principle that the measurements obtained at pH 1.0 will include the absorption due to the degraded as well as the non-degraded Acy, while the difference in absorption between the pH 1.0 and 4.5 media will be due only to the non-degraded Acy. Samples not containing degraded pigment or other brownish colored compounds, should give almost identical results for T Acy with the single pH and pH differential methods. However, as the degradation proceeds, the amount of reddish brown pigments will increase. Consequently, the T Acy determined with the single pH method and the DI will be higher than that determined with the pH differential method and the DI will be higher than 1.0.

The simplified DI value will be greater than 1.0 even for fresh samples not containing degraded pigment because the Acys have a small absorption at pH 4.5 which is reflected by the fact that the $\Delta E^{1\%}_{510\ 1\ cm}$ values are smaller than the corresponding $E^{1\%}_{510\ 1\ cm}$ value in the pH 1.0 medium.

The DI and the simplified DI values were calculated for the samples used in establishment of the precision of the pH differential T Acy determination method (Table 4). The low DI values indicated that the CrJ used showed practically no degradation.

The DI should give a better measure of the actual color of the sample than either the T Acy content, the T O.D. or the ΔO.D. value. The principal use foreseen for the

proposed DI will be similar to that of absorption ratios. A limit value should be established for the DI above which the degraded color becomes visible and the products should be rejected. It may be possible to establish a direct correlation between DI and the per cent of the original pigment which is still present in the sample.

The proposed DI and/or simplified DI may be easily adopted for other fruit and berry juices and wines. The advantage of this new index of degradation is that it does not require any additional measurement. The calculation of the DI value is based on the absorbancy measurements taken for the determination of T Acy content by the pH differential method. The DI together with the T Acy content should give a measure of not only the pigment content and state of Acy degradation but that of the color of the sample as well.

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A Comparison of the Light and Dark Portions of a Striated Muscle

SUMMARY—The levels of physiologically related muscle constituents were determined in the light (white) and dark (red) portions of a striated muscle from the pig (*Sus domesticus*). Myoglobin level, percent red fibers and succinic dehydrogenase activity were two-fold higher in the semitendinosus dark portion whereas ADP and inorganic phosphate levels were similar in both portions. Phosphorus levels were higher and sodium levels lower in the semitendinosus light portion than in the semitendinosus dark portion. Zinc and iron contents were greater in the dark portion than in the light portion; calcium, nickel, boron and potassium levels were similar in both portions. The semitendinosus light portion also had more lipid and more sarcoplasmic nitrogen than did the semitendinosus dark portion. These data suggest that the light (white) and dark (red) portions within the semitendinosus have physio-chemical properties similar to uniformly white and uniformly red muscles, respectively.

INTRODUCTION

MUSCLES WHICH ARE UNIFORM in color usually have metabolism related to their color intensity; red muscles are equipped for aerobic metabolism whereas white muscles depend largely on anaerobic metabolism (Beatty *et al.*, 1963; Needham, 1926; Ogata, 1960). Work with the gastrocnemius of the cat and rat revealed that internal or deep parts contained a greater number of red fibers than external or superficial layers (Denny-Brown, 1929; Ogata, 1958; Ogata *et al.*, 1964; Sreter *et al.*, 1963). These observations imply that different parts of the same muscle may have distinctly different types of metabolism and may even possess different physiological functions (Henneman *et al.*, 1965).

The semitendinosus of the pig (*Sus domesticus*) is a unique muscle; it contains a large dark (red) portion in close proximity to the femur and a large light (white) portion near the subcutaneous lipid layer (Fig. 1a). Both the light and dark portions, however, constitute the same muscle (semitendinosus) and have the same origin and insertion. On this basis a study was designed to characterize the composition of distinctly light (white) and dark (red) portions within this muscle.

EXPERIMENTAL

Materials

The semitendinosus of *Sus domesticus* was used in this study. A 200–250 g cross-section of the semitendinosus was excised from each animal immediately after exsanguination. The muscle was freed of external lipid and connective tissue and divided longitudinally into light, intermediate and dark portions. The intermediate portion was discarded and the light and dark portions were immediately frozen in liquid nitrogen and powdered according to the procedures of Borchert *et al.* (1965) for later analyses.

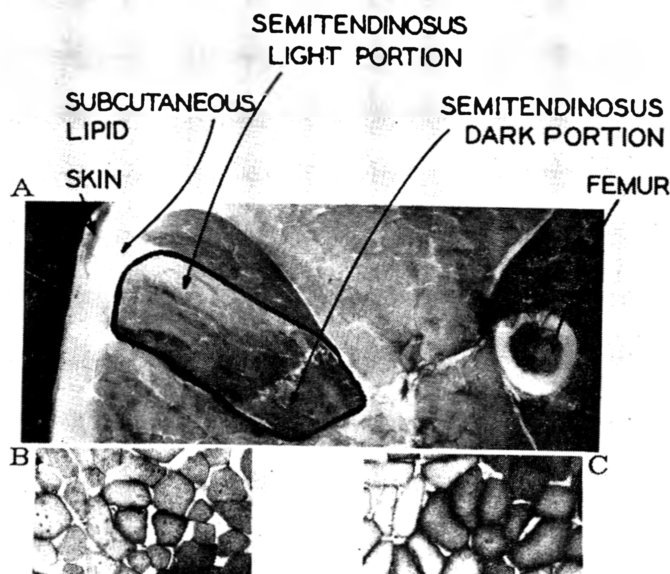

Fig. 1. Macroscopic and microscopic appearance of the semitendinosus light and dark portions. a. Cross-section of the semitendinosus showing the relation of the semitendinosus light and dark portions to the femur, subcutaneous lipid and skin. b. Photomicrograph of the semitendinosus light portion stained for red fibers with Sudan Black B. 24x. c. Photomicrograph of the semitendinosus dark portion stained for red fibers with Sudan Black B. 24x.

Analytical methods

The red and white fiber contents were determined in muscle samples which were fixed (10% calcium formol), freeze-sectioned (18 μ), and stained with Sudan Black B (Ogata, 1958). The results are reported as the percent red fibers of the total fiber content in 10 muscle bundles.

Adenosine triphosphate (ATP) (Lamprecht *et al.*, 1963b), adenosine diphosphate (ADP) (Adam, 1963), adenosine monophosphate (AMP) (Adam, 1963) and creatine phosphate (CrP) (Lamprecht *et al.*, 1963a) concentrations were determined from perchloric acid extracts of the semitendinosus light and dark portions. Inorganic phosphate (P_i) (Wahler *et al.*, 1958), glycogen (Dubois *et al.*, 1956), lactic acid (Barker *et al.*, 1941) and myoglobin (Poel, 1949) values were also determined.

All mineral analyses were conducted in collaboration with the Wisconsin Alumni Research Foundation with a Jarrell-Ash direct reading emission spectrometer. Muscle samples were analyzed for total moisture by weighing before and after drying for 24 hr at 105°C. Lipid (ether extract) was determined as the weight loss resulting from

24 hr continuous Soxhlet extraction of dried samples with diethyl ether. Myofibrillar and sarcoplasmic proteins were extracted according to the method of Helander (1957). Nitrogen content of the powdered tissue and of protein extracts was determined by the semimicro-Kjeldahl method (A.O.A.C., 1960). Myofibrillar and sarcoplasmic nitrogen are reported as percent of total tissue nitrogen.

Statistical analysis

Means, standard error of the mean (S.E.M) and paired difference analysis were calculated according to Steel *et al.* (1960).

RESULTS AND DISCUSSION

Association of myoglobin concentration, red fiber content and succinic dehydrogenase activity

Myoglobin concentrations, percent red fibers and succinic dehydrogenase activities were more than two-fold higher in the semitendinosus dark portion than the semitendinosus light portion (Table 1). Figs. 1b and 1c are photomicrographs of the semitendinosus light portion and semitendinosus dark portion, respectively, stained for red fibers with Sudan Black B.

The present observations on a distinctly light (white) and dark (red) portion within the same muscle compare with previous findings on uniformly red and uniformly white muscles (Briskey *et al.*, 1960; Cassens *et al.*, 1963a; Cassens *et al.*, 1963b; Sreter, 1964) and further substantiate the close relationship between the visual color (myoglobin content), red fiber content and succinic dehydrogenase activity of groups of muscle fibers (Dawson *et al.*, 1964; Ogata, 1958; Romanul, 1964). Internal parts of the gastrocnemius of the cat and rat have previously been observed to contain a greater concentration of red fibers than external or superficial layers (Denny-Brown, 1929; Ogata, 1958; Ogata *et al.*, 1964; Sreter *et al.*, 1963).

Henneman *et al.* (1965), working with cats, have indicated that two muscles (gastrocnemius and soleus) of the same muscle complex varying widely in red fiber content but functioning parallel and inserting into the same tendon may provide for economy of energy. The soleus, with a majority of red fibers, supports aerobic metabolism and contracts tonically whereas the gastrocnemius contracts tetanically under less efficient anaerobic conditions when bursts of speed are required by the animal. The semitendinosus of *Sus domesticus,* similar to the cat gastrocnemius, is also concerned with mobility of the animal and the existence of distinctly light (white) and dark (red) portions may economize energy.

Table 1. Characteristics of the semitendinosus light and dark portions.[1]

Characteristic	No. obs.	Semitendinosus portion: Light	Semitendinosus portion: Dark	P[2]
Myoglobin (mg per g muscle tissue)	5	1.49 ± 0.36	4.00 ± 0.43	.05
Red fibers (% of total fibers)	6	14.8 ± 2.1	42.7 ± 4.1	.01
Succinic dehydrogenase activity[3]	8	1.29 ± 0.14	2.81 ± 0.22	.01
Glycogen (mg per g muscle tissue)	5	3.40 ± 0.81	3.85 ± 0.97	.10
Lactic acid (mg per g muscle tissue)	8	4.66 ± 0.37	3.68 ± 0.19	.01

[1] Sample source was *Sus domesticus.* Values are means ± S.E.M.
[2] Statistical analysis based on paired observations.
[3] Units of activity per g muscle tissue (Berman, 1961).

Glycogen and lactic acid

Glycogen concentrations were similar in the semitendinosus light and dark portions (Table 1). Previous workers (Bär *et al.*, 1965; Beatty *et al.*, 1963; Bocek *et al.*, 1966a,b; Drahota *et al.*, 1963) have found the glycogen content of white muscles (small laboratory animals) to be higher than red muscles. Conversely, Beecher *et al.* (1965a,b) reported higher glycogen concentrations in the semitendinosus dark portion *(Sus domesticus)* than in the semitendinosus light portion and Bocek *et al.* (1966b) reported higher glycogen levels in rat red muscles than in white muscles.

Beecher *et al.* (1965a,b) and Bocek *et al.* (1966a) suggested that glycolysis, immediately post-mortem, in white muscle was accelerated to a greater degree than in the dark muscle by the physiological stresses at the time of death. These present findings suggest that environmental conditions, at the time of death, were not of sufficient stressor magnitude to deplete the glycogen stores in the semitendinosus light portion to values lower than in the semitendinosus dark portion.

Lactic acid concentrations were significantly ($P < .01$) higher in the semitendinosus light portion than in the semitendinosus dark portion (Table 2). These data indicate that the semitendinosus light portion may have been less able to metabolize pyruvate aerobically than the semitendinosus dark portion and as a result lactic acid accumulated.

Phosphate compounds

The concentrations of ATP and CrP tended to be higher in the semitendinosus light portion than in the dark portion but the differences were not statistically significant ($P > .05$); ADP and P_i concentrations were similar in both portions (Fig. 2). The AMP content was significantly ($P < .05$) higher in the semitendinosus dark portion than in the light portions. Higher concentrations of ATP (Ogata, 1960) and CrP (Beatty *et al.*, 1963;

Table 2. Composition of the semitendinosus light and dark portions.[1]

Characteristic	No. obs.	Semitendinosus portion: Light	Semitendinosus portion: Dark	P[2]
Moisture (%)	9	70.2 ± 1.4	75.7 ± 0.4	.01
Lipid (% of dry wt.)	9	31.2 ± 5.0	18.9 ± 1.9	.05
Myofibrillar (% of total N)	10	49.0 ± 2.5	52.5 ± 3.6	.10
Sarcoplasmic (% of total N)	10	24.7 ± 0.8	22.4 ± 0.9	.05

[1] Sample source was *Sus domesticus.* Values are means ± S.E.M.
[2] Statistical analysis based on paired observations.

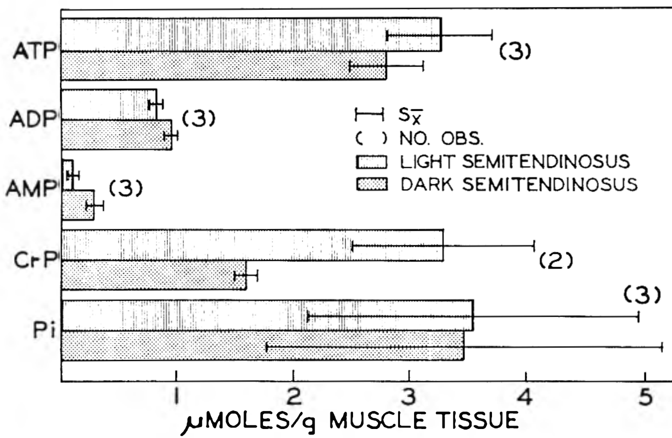

Fig. 2. Concentrations of several phosphate compounds in the semitendinosus light and dark portions.

Ogata, 1960) and lower concentrations of P_i (Ogata, 1960) were previously found in white muscles than in red muscles.

Lawrie (1952) indicated that myoglobin content and the resting level of energy-rich phosphate bear an inverse relationship in skeletal muscle. The present observations further substantiate that distinctly light (white) and dark (red) portions within the semitendinosus have the characteristics respectively of uniformly white muscles and uniformly red muscles.

Minerals

The phosphorus content of the semitendinosus light portion was significantly ($P < .05$) greater than that of the semitendinosus dark portion whereas the level of potassium was similar in both portions (Fig. 3). Sodium (Fig. 3) concentrations were significantly ($P < .01$) greater in the semitendinosus dark portion than in the semitendinosus light portion whereas the magnesium content was similar in both portions of the semitendinosus (Fig. 3). Several workers (Drahota, 1961; Sreter *et al.*, 1963; Swift *et al.*, 1959) have observed higher concentrations of potassium and lower concentrations of sodium in white than in red muscles.

Similar observations were made on the white portion of the gastrocnemius compared to the red portion of the same muscle (Sreter *et al.*, 1963). The intracellular concentration of potassium is higher and that of sodium is lower than the extracellular concentrations, and it has been suggested that red fibers have a slightly lower resting potential than white fibers (Sreter *et al.*, 1963). Phosphorus, potassium and magnesium (Baldwin *et al.*, 1952) and potassium, phosphorus and CrP (Kernan, 1965) are closely interrelated in the cell. These observations are further supported by the present study and emphasize that the semitendinosus light portion is physiologically oriented to carry on twitch activity compared to the tonic activity of the semitendinosus dark portion.

The concentrations of zinc and iron were significantly higher ($P < .01$ and $P < .05$, respectively) and the concentrations of copper were slightly but not significantly ($P > .05$) higher in the semitendinosus dark portion than in the semitendinosus light portion (Fig. 4). The calcium, nickel and boron contents were similar in both semitendinosus portions (Fig. 3). Swift *et al.* (1959), Berman (1961) and Cassens *et al.* (1963a, 1963b) have also noted higher concentrations of zinc in distinctly red muscles compared to distinctly white muscles.

Although zinc is known to be associated with several enzymes (Vallee, 1959), it is doubtful that the large differences in zinc content observed in the semitendinosus dark portion compared to the semitendinosus light portion could be explained on this basis. A more plausible explanation for the large difference in zinc content may be its ability to increase the duration of the active state of the red muscle fiber membrane (Edman *et al.*, 1961; Isaacson *et al.*, 1963; Mashima *et al.*, 1964). The action of zinc on the muscle membrane is apparently mediated by a slowing down of potassium conductance during the repolarization process (Isaacson *et al.*, 1963). Mashima *et al.* (1964) have also reported that nickel, although a divalent cation, had no effect on the muscle cell membrane.

The association of iron with myoglobin and copper with the cytochrome system of the mitochondria is probably responsible for the higher concentrations of these two elements in the semitendinosus dark portion than in the semitendinosus light portion. Although calcium concen-

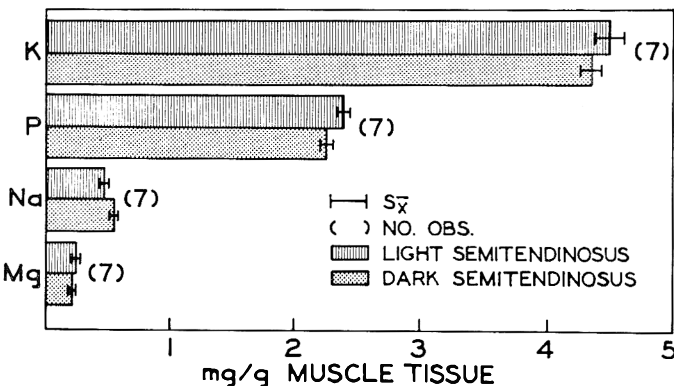

Fig. 3. Concentrations of major mineral elements in the semitendinosus light and dark portions.

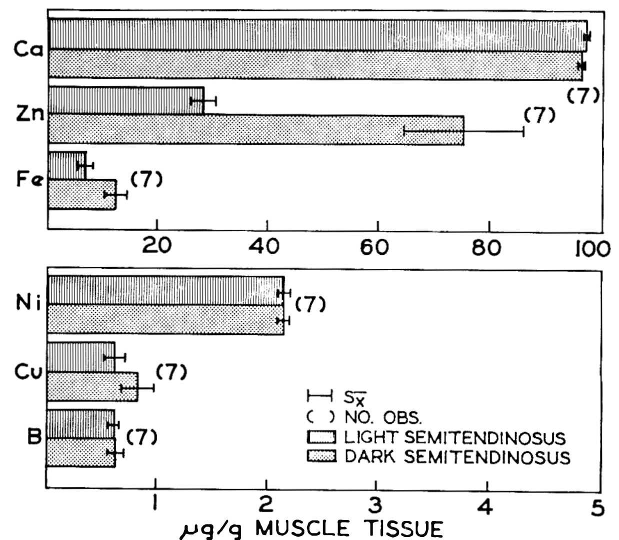

Fig. 4. Concentrations of minor mineral elements in the semitendinosus light and dark portions.

trations were similar in both portions of the semitendinosus, recent findings by Sreter (1964) indicated that isolated sarcotubular preparations from white muscles of rabbits had a calcium uptake activity 10-fold greater than similar preparations from red muscles of rabbits. These observations, along with the higher zinc concentrations, explain in part the tonic activity of red muscle fibers and the tetanic activity of white muscle fibers.

Moisture and lipid

The moisture content was significantly ($P < .01$) lower and the lipid content significantly ($P < .05$) higher in the semitendinosus light portion than in the semitendinosus dark portion (Table 2). Lawrie *et al.* (1964) also noted higher lipid levels and lower moisture values in distinctly white muscles than in distinctly red muscles of *Sus domesticus*. In contrast, previous workers (Swift *et al.*, 1959; George *et al.*, 1961) have noted higher lipid concentrations in the red muscles than in the white muscles of several species of laboratory animals. The high lipid content of *Sus domesticus* muscles also makes this study unique.

Myofibrillar and sarcoplasmic nitrogen

Statistically, myofibrillar nitrogen values were similar in both semitendinosus portions whereas sarcoplasmic nitrogen values were slightly but significantly ($P < .05$) higher in the semitendinosus light portion than in the semitendinosus dark portion (Table 4). These observations further support the work of Barany *et al.* (1965). Although myofibrillar nitrogen concentrations were similar in both semitendinosus portions, the calcium activated ATPase activity in homogenates from the semitendinosus light portion was considerably higher than in homogenates of the semitendinosus dark portion (Beecher, G. R., Unpublished data).

The calcium activated ATPase activity of myosin (Barany *et al.*, 1965) and of actomyosin (Seidel *et al.*, 1964) were also considerably higher in uniformly white muscles than in uniformly red muscles. These observations further elucidate some of the differences between red and white muscles.

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Volatile Fatty Acids in Some Brands of Whisky, Cognac and Rum

SUMMARY—Gas chromatography was applied to eight different types of whisky, two of cognac, one of brandy, and four of rum to determine the relative proportions of volatile fatty acids; with the lower molecular acids as free acids, but upwards from caprylic acid as methyl esters. Acetic acid and the total amount of volatile acids were measured quantitatively. Rum contained the largest amount of volatile acids, 600 mg/L, while one of the brands of Scotch whisky contained the least, 90 mg/L. Acetic acid represented 40–95% of the total amount of volatile acids in the whisky; for cognac and brandy, the value was 50–75%, and for rum 75–90%. The relative amounts have been reported for 21 acids, with acetic acid excluded. Capric, caprylic and lauric acid were the main components in whisky, cognac and brandy. Of the beverages analyzed, rum contained the largest quantity of lower fatty acids, particularly propionic and butyric acid; the main component of Jamaican rum was propionic acid. The main components of the group of long-chain fatty acids were myristic, palmitic and palmitoleic acids. Scotch whisky contained equal amounts of palmitic and palmitoleic acid; palmitoleic acid regularly appeared in smaller amounts in the other beverages.

INTRODUCTION

Several researchers have analyzed the aroma of whisky (Nykänen *et al.,* 1963; Sihto *et al.,* 1962; Suomalainen *et al.*, 1966a) of cognac and brandy (Baraud, 1961; Egorov *et al.,* 1964) and of rum (Baraud *et al.,* 1963; Maarse *et al.,* 1966; Maurel, 1964; Maurel *et al.,* 1965; Sihto *et al.*, 1962; Stevens *et al.,* 1965). The aroma here, as in other alcoholic beverages produced by fermentation (Lawrence, 1964; Stevens, 1960; Webb *et al.,* 1963), consists to a significant degree of fusel alcohols and esters of fatty acids. In fractions which contain only neutral components, the esters form the most numerous group of aroma components, with the ethyl esters of C_6–C_{12} acids as main components. Undistilled and distilled beverages contain not only alcohols and esters, but also free acids. Acetic acid is the acid found in greatest abundance in distilled beverages; moreover, grain spirits (Deckenbrock *et al.*, 1958) and wine distillates (Frey *et al.,* 1956) contain propionic, butyric, valeric and caproic acid; brandy (Egorov *et al.,* 1964) contains propionic, isobutyric, isovaleric and caproic acid; and whisky (Yamada *et al.,* 1963), butyric, caproic, caprylic, capric, lauric and myristic acid. On study of the formation of aroma compounds in nitrogen-free sugar fermentation, a total of 19 volatile fatty acids have been identified in alcohol distillates (Suomalainen *et al.,* 1966a).

The aroma of beverages such as whisky and brandy is very similar in composition, as far as fusel alcohols and esters are concerned, even if the beverages are produced from quite different raw material: whisky from grain, and brandy from grapes (Suomalainen *et al.,* 1964). This fact, coupled with the observation that in sugar fermentation the yeast produces the same aroma compounds as those found in these beverages, indicates that the metabolic reactions in yeast play a central role in the formation of aroma (Suomalainen *et al.,* 1966a,b).

Fatty acid synthesis, of which the mechanism has been elucidated particularly by Lynen (1967) and Wakil (1964), represents such a reaction. Consequently, interest was aroused in the proportions in which the volatile fatty acids appear in distilled beverages of different origin, especially in view of the volatile fatty acids being important aroma factors.

EXPERIMENTAL

Isolation of free fatty acids

To determine the minor components by gas chromatography, a sample of 500 ml was taken of the beverage to be examined. The solution was evaporated to 100 ml; 1*N* NaOH was added before evaporation to an amount such that after evaporation the pH was 8.5. Sulphuric acid was then added until the pH had fallen to 3, and the free fatty acids were separated by steam-distillation; 700 ml distillate was collected, made slightly alkaline (pH 8.5) and allowed to evaporate to 50 ml at 35°C *in vacuo*. From the acidic

remainder (pH 1), saturated with NaCl, the fatty acids were extracted three times with 50 ml of fresh distilled ether.

The compounds disturbing the chromatography were removed by three extractions of the acids back into the water phase with 50 ml 5% NaOH. The combined alkaline solution was washed with 50 ml ether. After the water phase had again been acidified to pH 1, and saturated with NaCl, the acids were finally extracted three times with 100 ml ether. The combined ether solution was dried overnight with anhydrous Na_2SO_4, and distilled using a Widmer column until 10 ml remained. The remainder was employed for the gas chromatographic determination.

Gas chromatography of free fatty acids

An injection was made of 5–10 μl of the ether solution dependent on the acid content. Gas chromatography was effected by an isothermal run at 152°C in a column 1.5 m, O.D. 6 mm, of 20% butanediol succinate and 2.5% phosphoric acid on 60/80 mesh Chromosorb W HMDS. Helium, which had been passed through water-free formic acid before the chromatography, was utilized as carrier gas, and the velocity was 240 ml/min. The apparatus was a Perkin-Elmer 116 E, equipped with a flame-ionization detector.

Gas chromatography of methyl esters of fatty acids

After part of the ether solution had been carefully evaporated in a test tube, the acids were esterified with diazomethane. An injection of 5–10 μl of the solution was made and the methyl esters were subjected to chromatography in a temperature-programmed run, first for 8 min at 65°C isothermally, then with an increase in temperature by 4°C/min, and finally isothermally at 210°C. A good separation of methyl esters was obtained with a 2.4 m long column, packed with 10% Carbowax 20*M* on 60/80 mesh Chromosorb W HMDS, and helium as carrier gas with a flow rate of 70 ml/min. The chromatograph was a F & M unit, model 810, equipped with a flame-ionization detector.

Table 1. Amounts of volatile fatty acids and acetic acid in some brands of whisky, rum, cognac and brandy.

Beverages	Total volatile acids mg/L calculated as acetic acid	Acetic acid % of volatile acids
Brands of whisky		
Scotch I	90	40
Scotch II	140	60
Irish I	120	95
Irish II	110	85
Japanese I	100	75
Japanese II	120	60
Bourbon	410	90
Spanish	120	50
Brands of cognac		
Cognac I ("Trois Etoiles")	170	50
Cognac II ("Trois Etoiles")	210	50
South African Brandy	360	75
Brands of rum		
Martinique I	600	85
Martinique II	240	85
Martinique III	300	75
Jamaican	300	90

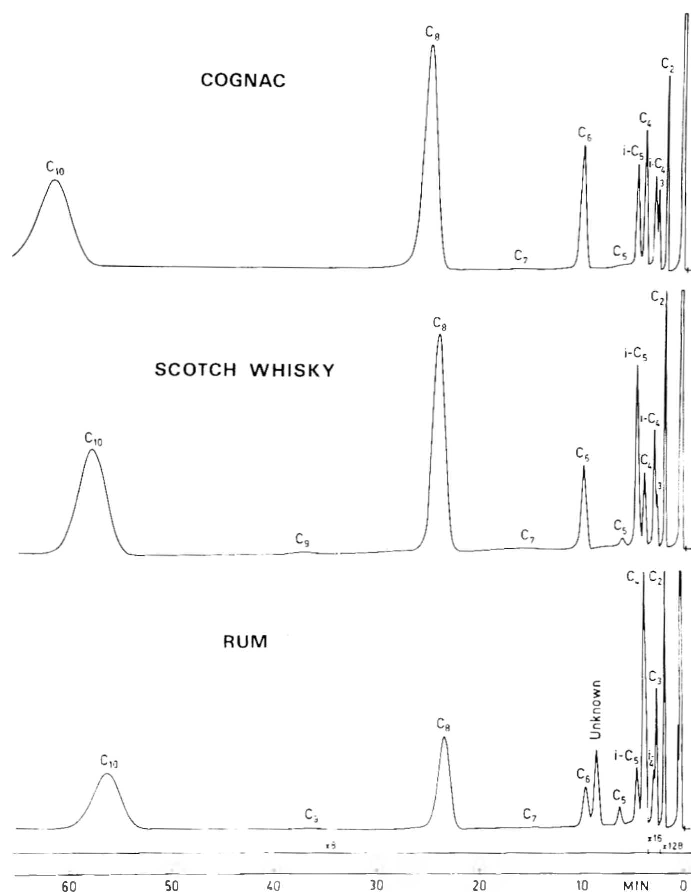

Fig. 1. Gas chromatograms of the free fatty acids of a brand of cognac, one of Scotch whisky and one of rum. Sample 6 μl, column 1.5 meters of 20% butanediol succinate and 2.5% phosphoric acid on Chromosorb W HMDS 60/80 mesh, temperature 152°C, helium flow 240 ml per min.

The technique presented has been applied previously in our laboratories by Arkima (1965) for determining free fatty acids in beer.

RESULTS AND DISCUSSION

THIS METHOD MAKES IT POSSIBLE to determine by gas chromatography the fatty acids, up to capric acid, as free fatty acids, except for formic acid. Thus, the first peak appearing in the chromatogram is that of acetic acid (Fig. 1), and according to the chromatograms obtained, acetic acid is clearly the main acid component of the beverages concerned. For this reason, the content of acetic acid was determined quantitatively by comparison with solutions of known concentrations. The results are presented in Table 1.

The titrimetric determinations indicated that the various brands of whisky examined contained 90–140 mg of volatile acids per L, with the exception of American Bourbon whisky, in which the amount of volatile acids was four times as great. The brands of cognac, brandy and rum contain volatile acids in greater abundance; the content in a Martinique rum was as much as 600 mg/L.

The proportion of acetic acid in the total amount of volatile acids is very large. In Scotch whisky and Spanish

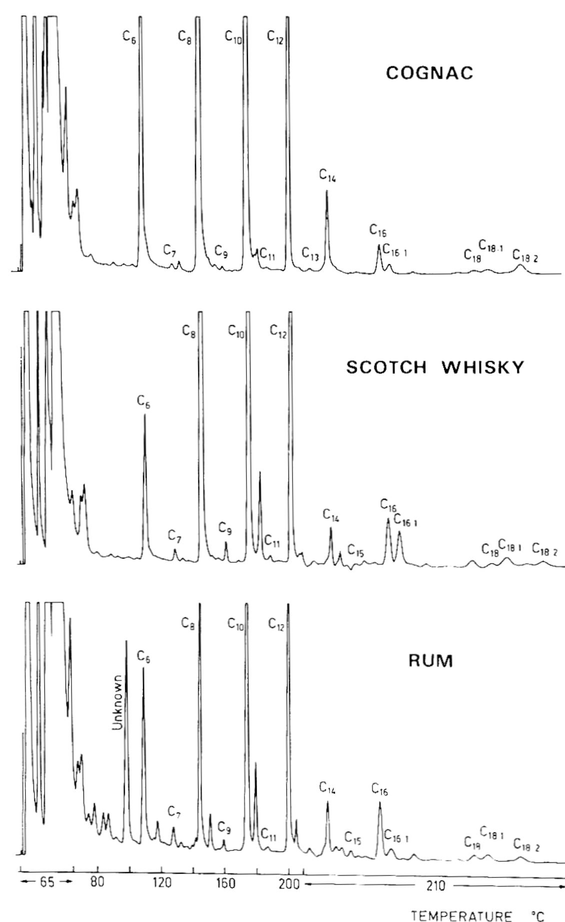

Fig. 2. Gas chromatograms of higher fatty acids of a brand of cognac, one of Scotch whisky and one of rum chromatographed as methyl esters. Sample 8 μl, column 2.4 meters stainless steel, packed with 10% Carbowax 20M on Chromosorb W HMDS 60/80 mesh, temperature program, isothermal run 8 min at 65°C, temperature upscale rate 4°/min to 210°C, isothermal run at 210°C, helium flow 70 ml/min, injection port 280°C, detectors 330°C, range 10⁸, attenuation 4.

whisky, acetic acid represents half of the total amount of volatile acids; in the other varieties of whisky, the proportion was even larger, 60–95%. In both brands of cognac, the proportion of acetic acid corresponded to that in Scotch whisky, while the brandy contained acetic acid up to 75% of the total acid amount. In the four brands of rum, the corresponding proportion was 75–85%.

The proportion of acetic acid will not be examined further here, and thus the acid complexes discussed hereafter comprise only fatty acids above acetic acid. The relative proportions of acids are calculated from the peak areas, the lower acids from the chromatograms of free fatty acids (Fig. 1), and the higher acids from the chromatograms obtained from the methyl esters of the acids (Fig. 2). The methyl ester of caproic acid is the first distinct component in the chromatograms of cognac and whisky (Fig. 2). In the corresponding chromatogram of rum, an unknown compound is apparent before the caproic acid ester; it is of about the same order of magnitude, possibly a branch-chained C_6-acid, although not isocaproic acid.

The acid content of each brand of whisky analyzed is indicated in Table 2. If acetic acid is disregarded, capric acid is the largest component, with a relative amount which varies between 20 and 45%. Then follow caprylic, lauric and caproic acid in descending order. Both varieties of Scotch whisky, and the first Irish whisky, contain isobutyric and isovaleric acid to approximately the same extent as caproic acid.

Propionic and butyric acid usually average 1–2.5%; but in some cases, such as in the first Irish and in the Bourbon whisky, these acids appear in markedly high amounts. The former beverage contains up to 10% of each of these acids, and the latter about 5% propionic acid and 13% butyric acid. Spanish whisky contains the lowest percentages of lower fatty acids. Myristic, palmitic and palmitoleic acid are the most abundant long-chain fatty acids in whisky.

Scotch whisky contains about equal amounts of palmitic

Table 2. Relative amounts of volatile fatty acids, excluding acetic acid, in brands of whisky.

Acids	Scotch I %	Scotch II %	Irish I %	Irish II %	Japanese I %	Japanese II %	Bourbon %	Spanish %
Propionic	1.5	1.7	9.4	2.4	2.2	1.0	5.3	0.7
Isobutyric	4.9	4.5	6.2	2.6	2.4	2.8	2.4	1.6
Butyric	1.5	1.7	10.1	2.4	2.6	1.3	13.2	0.8
Isovaleric	5.9	6.2	7.8	3.5	4.7	4.3	9.2	2.5
Valeric	0.1	0.3	2.4	0.6	0.6	0.6	0.8	0.2
Caproic	4.2	5.2	8.6	4.0	5.8	4.6	14.0	3.3
Enanthic	0.1	0.2	0.4	0.2	Tr	0.5	0.8	0.1
Caprylic	26.7	28.8	17.5	19.9	24.8	27.3	19.3	25.0
Pelargonic	0.2	0.8	....	0.8	1.5	0.7	0.4	0.2
Capric	31.6	31.8	21.1	45.0	40.8	35.8	20.0	43.2
Undecanoic	0.1	Tr	....	....	....	Tr	....	....
Lauric	16.2	13.7	7.0	13.8	12.0	16.2	7.0	17.3
Tridecanoic	0.1	....	....	....	....	Tr	....	....
Myristic	2.2	0.8	1.2	1.2	0.7	1.3	2.3	2.2
Pentadecanoic	0.1	Tr	....	....	....	Tr	0.1	....
Palmitic	1.7	1.7	3.4	1.8	0.7	1.1	3.2	0.8
Palmitoleic	2.0	1.5	1.2	0.6	0.2	1.0	0.7	1.4
Heptadecanoic	Tr	Tr	....	....	....	Tr	Tr	Tr
Stearic	0.3	0.2	2.4	0.6	0.4	0.5	0.4	0.1
Oleic	0.4	0.5	1.3	0.6	0.2	0.4	0.9	0.2
Linoleic	0.2	0.5	....	....	0.4	0.6	....	0.4

and palmitoleic acid. In their study of the fatty acid composition of yeast, Suomalainen *et al.* (1963a,b) have found that baker's yeast contains an abundance of palmitoleic acid. The fact that in Scotch whisky the content of palmitoleic acid attains the same level as palmitic acid does not relate solely to the free acids; the same proportion is also apparent between their ethyl esters (Nykänen *et al.*, 1963; Suomalainen *et al.*, 1966a), and, roughly speaking, this is observable in respect to the proportions of other acids and their ethyl esters. If a comparison is made between the acid compositions of whisky and of beer, analyzed by Arkima (1965), the most significant difference is the appearance of caprylic acid instead of capric acid as the main component in beer.

The acid compositions of the two brands of cognac, and of the brandy, are indicated in Table 3. Caprylic and capric acid are the main components of all three beverages; the caprylic acid predominates, whereas caproic and lauric acid appear in a proportion about one-third that of caprylic and capric acid. Brandy contains further isobutyric and isovaleric acid in abundance; in the cognac, the lower acids, C_3-i-C_5, are present in very similar proportions. The acids with a longer chain than C_{12} are found only in low percentages, and consequently no marked differences are apparent between the compositions of the beverages.

The acid composition of each of the kinds of rum is indicated in Table 4. In this group of beverages, the strength of flavor, determined by taste and smell, varied appreciably in the four samples. Martinique I with the strongest flavor had the highest content of volatile acids, 600 mg/L. However, strengthening of the flavor does not seem to exercise a significant influence upon the acid composition, and is thus not directly dependent upon an increase in the relative amounts of individual components.

Some differences are observable between the acid composition of rum and that of whisky, and cognac or brandy. It is noticeable that the lower acids, particularly propionic and butyric acid, are very plentiful in rum. The large proportion of propionic and butyric acid reduces the relative amounts of caprylic and capric acid, and thus it is impossible to make such a definite statement about a main component as in the whisky, cognac and brandy.

Table 3. Relative amounts of volatile fatty acids, excluding acetic acid, in two brands of cognac and one of brandy.

Acids	Cognac I %	Cognac II %	South African brandy %
Propionic	2.7	3.4	1.3
Isobutyric	3.6	2.4	7.0
Butyric	3.6	2.3	2.9
Isovaleric	3.3	2.0	6.4
Valeric	Tr	0.1	0.4
Caproic	8.2	7.7	8.6
Enanthic	Tr	Tr	2.1
Caprylic	35.0	36.8	28.6
Pelargonic	Tr	0.4	Tr
Capric	30.4	32.8	21.9
Undecanoic	Tr	.....	Tr
Lauric	8.6	9.4	10.2
Tridecanoic	0.1	Tr	.....
Myristic	1.6	1.4	2.6
Pentadecanoic	Tr	.....	.....
Palmitic	1.1	0.8	3.0
Palmitoleic	0.5	0.3	1.4
Heptadecanoic	.....	Tr	.....
Stearic	0.1	0.1	0.4
Oleic	0.5	0.1	2.2
Linoleic	0.7	.....	1.0

Table 4. Relative amounts of volatile fatty acids, excluding acetic acid, in brands of rum.

Acids	Martinique I %	Martinique II %	Martinique III %	Jamaican %
Propionic	15.7	14.5	7.4	30.2
Isobutyric	3.6	2.9	4.1	4.3
Butyric	15.3	8.5	12.8	8.0
Isovaleric	4.7	6.2	3.0	6.5
Valeric	6.5	1.7	1.5	1.8
Caproic	5.4	5.3	4.5	6.6
Ethanthic	Tr	2.4	0.3	0.3
Caprylic	14.5	13.5	24.1	8.9
Pelargonic	Tr	Tr	0.3	0.5
Capric	17.5	26.1	32.0	16.6
Undecanoic	0.3	0.7	.....	.....
Lauric	6.5	12.0	8.3	9.0
Tridecanoic	0.1	.....	.....	Tr
Myristic	1.1	1.5	0.4	1.7
Pentadecanoic	0.1	Tr	.....	0.1
Palmitic	4.0	3.2	0.5	2.9
Palmitoleic	1.0	0.6	0.2	0.9
Heptadecanoic	.....	.....	.....	Tr
Stearic	0.3	0.4	0.2	0.5
Oleic	1.2	0.5	0.2	0.5
Linoleic	2.2	.....	0.2	0.7

Rhum Martinique I contains almost similar amounts of propionic, butyric, caprylic and capric acid, whereas Martinique II and III contain more capric acid; the latter also contains caprylic acid in abundance. In Jamaican rum, the main component is propionic acid; moreover, the average proportion of valeric acid in the brands of rum examined exceeds that in the other two types of beverages. The acids higher than valeric acid, and with odd numbers of carbon atoms, appear in small amounts in rum, as in the other beverages. Lauric, palmitic, and myristic acid appear as quantitatively significant components in the rum.

The investigations described previously have proved that in different types of beverages certain differences in the acid composition appear. However, the limited material does not enable examination of the extent to which the variances are characteristic of each type of beverage. It may be stated, however, that the formation of acids in all cases occurs principally in the same way, and a change in the raw material does not exert a marked influence upon the composition of acids.

This confirms our previously expressed opinion (Suomalainen, 1965; Suomalainen *et al.*, 1964; Suomalainen *et al.*, 1966a,b) that the aroma compounds, including volatile acids, are formed in proportions which are prescribed by the yeast used and the fermentation conditions, and that the formation depends on the raw material to a minor extent only. In this case, it appears to be a question of fatty acids, synthesized by the yeast, as the same acids have been found as components of yeast fat (Suomalainen *et al.*, 1963a,b; Suomalainen *et al.*, 1967).

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Action of Microorganisms on the Peroxides and Carbonyls of Rancid Fat

SUMMARY—The effects of 26 species of bacteria, molds, and yeasts on the hydroperoxides and monocarbonyls in rancid fat have been determined. All of the cultures were capable of decomposing the hydroperoxides. The activity of microorganisms on the monocarbonyl content of the rancid fat was quite varied and could be divided into: 1) microorganisms which produced large increases in at least two monocarbonyl classes; 2) microorganisms which removed 2,4-dienals; 3) microorganisms which removed 2,4-dienals and 2-enals, and 4) microorganisms which caused decreases in at least two classes of monocarbonyls (without destroying completely any class).

Two microorganisms produced methyl ketones, a monocarbonyl class which does not appear in rancid lard. There appeared to be a relationship between the ability to decompose peroxides strongly and the ability to produce a great increase in the monocarbonyl content. There apparently is no relationship between the ability to decompose peroxides and lipolytic activity. The possible importance of microorganisms in controlling hydroperoxides and monocarbonyls in fats is discussed.

INTRODUCTION

MICROORGANISMS DECOMPOSE HYDROPEROXIDES (Updegraff *et al.*, 1958; Finnerty *et al.*, 1962) and can utilize aldehydes (Racker, 1950; Black, 1951; Seegmiller, 1953; DeMoss, 1954). However, there are no reports concerning the action of microorganisms on rancid fats which contain hydroperoxides and carbonyls. Since carbonyl compounds (formed by decomposition of hydroperoxides) have been found to cause flavor changes in certain foods (Hoffmann, 1962; Day, 1966), we decided to investigate the effect of microorganisms on the peroxides and monocarbonyl content of rancid lard.

METHODS AND MATERIALS

Preparation of rancid lard emulsion

Freshly rendered lard was spread in thin layers in baking dishes and exposed to ultraviolet light at 25°C until the peroxide value was 90–100 meq/kg fat (24–30 hr). Emulsions containing 20% rancid lard were prepared as described previously (Alford *et al.*, 1963), except that the nitrogen atmosphere was eliminated.

Microorganisms and media

The microorganisms utilized are listed in Table 1 along with the conditions of incubation. The cultures were obtained from a number of different sources and were assigned numbers in our collection for ease of identification. These strain designations are given in Table 1. The media used in this study were:

Medium 1—Case peptone, 1.0 g; 1*M* phosphate buffer (pH 7.0), 5 ml; water, 94 ml.

Medium 2—NH_4Cl, 1.0 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.01 g; Edamin S (Sheffield), 40.0 g; glucose, 2.5 g (25 ml of a 10% solution was sterilized separately); distilled water, 933 ml.

Medium 3—Bacto tryptose phosphate broth with additional 2% tryptose.

Medium 4—Case peptone, 2.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 7.0), 5 ml; distilled water, 93 ml.

Medium 5—Case peptone, 1.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 6.0), 5 ml; distilled water, 94 ml.

Medium 6—Difco veal infusion broth.

Medium 7—Case peptone, 1.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 7.0), 5 ml; distilled water 94 ml.

Medium 8—Difco nutrient broth plus 3% glucose (sterilized separately as a 10% solution).

Medium 9—Difco nutrient broth.

Medium 10—Difco tryptose phosphate broth.

Medium 11—BBL APT broth.

Wherever phosphate buffer was used it was equimolar Na:K. All media were sterilized by autoclaving at 121°C for 15 min. For static cultures, 500 ml of medium were dispensed into 3-L, low-form culture flasks. For shaken cultures, 125 ml of medium were dispensed into 1-L Erlenmeyer flasks; after growth had occurred, 500 ml of growth culture were dispensed into sterile 3-L, low-form culture flasks.

Action of microorganisms on rancid fat

When lipase production had reached its peak (or when cell growth was near the maximum if no lipase was produced), non-sterile rancid lard emulsion was added to the cultures so that the final concentration of lard was 3%. All culture-lard emulsion mixtures were incubated at the appropriate temperatures (Table 1) as stationary cultures until 80–100% of the peroxides had disappeared, or for five days. Uninoculated medium-lard emulsion mixtures were utilized as controls and were kept refrigerated at 4–5°C until needed. The rancid lard emulsion was not sterilized because heat changed the monocarbonyl pattern drastically (Gaddis *et al.*, 1959a).

Assay of lipase

Lipase assays were determined as previously described (Alford *et al.*, 1963) with incubation at 35°C for one hour. All cultures were assayed at pH 7.0 except *Candida lipolytica*, *Aspergillus niger*, *Rhizopus oligosporus*, and *Thamnidium elegans*, which were assayed at pH 6.0.

Table 1. Microorganisms employed and methods of producing cells for study of effect on rancid lard.

Microorganisms	Medium used[1]	Incubation of culture before the addition of lard emulsion
		Static cultures
Pseudomonas fragi (43)	1	20°C, 3 days
Pseudomonas species (92)	1	20°C, 4 days
Pseudomonas ovalis (36)	1	20°C, 3 days
Streptomyces species (280 & 281)	9	25°C, 10 days
Streptococcus lactis (263)	11	20°C, 24 hours
Streptococcus cremoris (264)	11	20°C, 24 hours
Lactobacillus casei (266)	11	25°C, 24 hours
Pediococcus cervisiae (270)	11	25°C, 24 hours
Leuconostoc citrovorum (267)	11	25°C, 24 hours
Leuconostoc dextranicum (268)	11	25°C, 24 hours
Leuconostoc mesenteroides (269)	11	25°C, 24 hours
Candida lipolytica (181)	5	20°C, 4 days
Aspergillus niger (172)	4	35°C, 3 days
Aspergillus flavus (88)	4	25°C, 5 days
Rhizopus oligosporus (173)	5	20°C, 5 days
Thamnidium elegans (195)	5	20°C, 4 days
Penicillium roqueforti (174)	7	30°C, 10 days
Geotrichum candidum (165)	2	20°C, 4 days
		Shaker cultures
Serratia marcescens (279)	6	25°C, 200 rpm, 24 hours
Escherichia coli (107)	6	25°C, 100 rpm, 24 hours
Staphylococcus aureus (63)	3	35°C, 400 rpm, 24 hours
Staphylococcus aureus (66)	10	35°C, 400 rpm, 24 hours
Micrococcus cryophilus (90)	10	20°C, 200 rpm, 24 hours
Micrococcus freudenreichii (115)	10	20°C, 200 rpm, 24 hours
Sarcina lutea (112)	10	25°C, 200 rpm, 24 hours
Bacillus cereus (283 & 284)	6	25°C, 100 rpm, 24 hours
Hansenula anomala (282)	8	25°C, 200 rpm, 24 hours

[1] The composition of the various media is given in the text.

Peroxide determination

The peroxide value was determined by the cold method of Lea (1952).

Monocarbonyl determination

At the end of the incubation period, the culture-lard emulsion mixtures were centrifuged and the fat was extracted from the supernatant with petroleum ether (40–60°C b.p. range). The monocarbonyl fraction was isolated by column chromatography as described by Schwartz *et al.* (1962, 1963), and monocarbonyls were further separated into classes by the chromatographic techniques of Gaddis *et al.* (1959b). The classes were identified by their R_f values on paper and by their absorption maxima utilizing a Cary recording spectrophotometer. To make it possible to calculate concentrations on a molar basis, an average molecular weight of 1000 was assumed for the lard.

RESULTS

THE MONOCARBONYL COMPOUNDS isolated from rancid lard consist of three classes: 2,4-dienals, 2-enals, and n-alkanals. Although the microorganisms studied in this investigation varied widely in the extent of their action on these monocarbonyls, the activity of any given culture was consistent and repeatable. Each value shown in Tables 2–6 is from a representative run. Every experiment was repeated at least once and with most organisms three to six times. Although initial values on the rancid lard varied among runs, the percentage variation among runs for the same microorganism was always within 10 to 20%. On the basis of this activity the microorganisms could be divided into five groups.

Group 1 produced relatively large increases in at least two of the monocarbonyl classes. The data for this group are recorded in Table 2. The activity of *Aspergillus flavus* in removing peroxides coupled with the sharp increases in 2,4-dienals, alkanals, and methyl ketones made it the most active culture in monocarbonyl production. *Streptococcus lactis* was the only culture of the group which caused a large increase in all three of the main monocarbonyl classes.

The complete destruction of the 2,4-dienal class of the monocarbonyl fraction of rancid lard was the most significant action of the microorganisms that belong to Group 2. At the present time, the products of the decomposition are unknown. The data for the group are presented in Table 3. Strong lipolytic and peroxide decomposing activity was shown by *Pseudomonas fragi*; other members of the group were non-lipolytic and possessed weak peroxide-destroying capacity. *P. fragi* also produced a large quantity of methyl ketones. It and *A. flavus* were the only two cultures of the 29 examined which produced this type of monocarbonyl.

The microorganisms that decomposed completely both the 2,4-dienals and 2-enals were placed together in Group 3, and the data for the group are presented in Table 4. Cultures of *Staphylococcus aureus, Rhizopus oligosporus,* and *Geotrichum candidum* were active lipase producers and a majority of the organisms in the group possessed a strong capacity to decompose peroxides. Eight of 10 orga-

Table 2. Microorganisms producing relatively large increases in at least two classes of monocarbonyls in rancid fat (Group 1).

Microorganisms	Lipase activity when fat added µeq FFA/hr	Peroxide value Start meq/kg fat	Peroxide value End meq/kg fat	Monocarbonyls[1] 2,4-Dienals	2-Enals	Alkanals	Methyl ketones
A. flavus (88)	153.2	75.5	0.0 (3 days)	6.1	3.9	48.1	30.9
Control	......	75.5	74.3	1.5	3.6	21.3	0.0
S. lactis (263)	6.4	81.4	45.1 (5 days)	6.8	8.8	50.6	0.0
Control	......	81.4	75.8	1.6	3.8	30.4	0.0
P. cerevisiae (270)	5.0	80.8	59.3 (5 days)	3.3	5.3	29.1	0.0
Control	......	80.8	74.7	1.4	2.9	26.2	0.0
L. dextranicum (268)	8.6	77.4	29.8 (5 days)	3.6	6.0	19.9	0.0
Control	......	77.4	73.0	1.6	3.5	20.2	0.0
M. freudenreichii (115)	0.0	75.0	50.5 (5 days)	9.8	7.7	19.9	0.0
Control	......	75.0	76.4	1.4	5.1	38.2	0.0

[1] Expressed as $\mu M/10^4\ \mu M$ fat.

Table 3. Microorganisms which completely remove the 2,4-dienals from rancid fat (Group 2).

Microorganisms	Lipase activity when fat added µeq FFA/hr	Peroxide value Start meq/kg fat	Peroxide value End meq/kg fat	Monocarbonyls[1] 2,4-Dienals	2-Enals	Alkanals	Methyl ketones
P. ovalis (36)	0.0	73.9	62.7 (5 days)	0.0	5.1	35.2	0.0
Control	......	73.9	76.8	2.2	5.2	39.2	0.0
Pseudomonas sp. (92)	10.4	78.2	65.7 (5 days)	0.0	2.5	23.2	0.0
Control	......	78.2	74.0	1.6	3.6	22.3	0.0
P. fragi (43)	111.1	82.1	25.6 (5 days)	0.0	7.7	45.4	36.5
Control	......	82.1	83.8	1.5	6.7	34.9	0.0
B. cereus (284)	9.0	77.6	57.2 (5 days)	0.0	2.0	16.9	0.0
Control	......	77.6	76.5	1.5	3.9	44.4	0.0
B. cereus (283)	10.0	77.6	55.6 (5 days)	0.0	2.5	23.7	0.0
Control	......	77.6	76.5	1.5	3.9	44.4	0.0

[1] Expressed as $\mu M/10^4\ \mu M$ fat.

Table 4. Microorganisms which completely removed 2,4-dienals and 2-enals from rancid lard (Group 3).

Microorganisms	Lipase activity when fat added µeq FFA/hr	Peroxide value Start meq/kg fat	Peroxide value End meq/kg fat	Monocarbonyls[1] 2,4-Dienals	2-Enals	Alkanals
E. coli (107)	0.0	82.8	11.7 (5 days)	0.0	0.0	22.4
Control	......	82.8	80.8	1.4	3.2	24.6
S. marcescens (279)	22.4	71.8	33.9 (5 days)	0.0	0.0	51.6
Control	......	71.8	64.5	1.2	3.4	20.1
M. cryophilus (90)	18.0	76.5	9.8 (5 days)	0.0	0.0	30.2
Control	......	76.5	75.1	1.5	3.5	40.0
S. aureus (63)	217.0	77.6	0.0 (3 days)	0.0	0.0	46.1
Control	......	77.6	66.9	1.1	2.7	26.4
S. lutea (112)	3.0	75.0	8.4 (5 days)	0.0	0.0	88.1
Control	......	75.0	76.5	1.4	5.1	38.2
S. aureus (66)	310.4	73.7	8.1 (3 days)	0.0	0.0	58.3
Control	......	73.7	75.1	0.9	2.3	23.4
P. roqueforti (174)	2.1	68.3	27.9 (5 days)	0.0	0.0	60.4
Control	......	68.3	68.3	2.5	5.8	40.8
A. niger (172)	5.1	110.0	20.7 (3 days)	0.0	0.0	41.1
Control	......	110.0	106.9	1.6	4.7	24.9
R. oligosporus (173)	96.8	118.1	23.4 (4 days)	0.0	0.0	71.4
Control	......	118.1	115.9	1.7	5.7	33.6
G. candidum (165)	115.1	105.4	0.0 (3 days)	0.0	0.0	58.3
Control	......	105.4	97.4	2.0	4.3	18.7

[1] Expressed as $\mu M/10^4\ \mu M$ fat.

Table 5. Microorganisms causing decreases in at least two classes of monocarbonyls but which do not completely remove any class (Group 4).

Microorganisms	Lipase activity when fat added μeq FFA/hr	Peroxide value		Monocarbonyls[1]		
		Start meq/kg fat	End meq/kg fat	2,4-Dienals	2-Enals	Alkanals
C. lipolytica (181)	51.9	95.1	11.8 (3 days)	0.8	2.2	32.3
Control	......	95.1	94.9	3.4	6.6	39.8
Streptomyces sp. (281)	2.2	83.4	65.5 (5 days)	0.6	1.5	12.2
Control	......	83.4	80.0	1.7	3.7	29.8
Streptomyces sp. (280)	2.2	83.4	53.7 (5 days)	0.5	1.5	13.9
Control	......	83.4	80.0	1.7	3.7	29.8
H. anomala (282)	0.0	64.7	40.3 (5 days)	1.9	2.6	12.8
Control	......	64.7	68.8	1.3	3.2	20.0
L. casei (266)	0.0	79.1	37.1 (5 days)	1.1	3.3	24.8
Control	......	79.1	77.9	1.9	4.6	34.1

[1] Expressed as $\mu M/10^4$ μM fat.

nisms produced a large increase in the alkanal content, thus leading to an increase in the total monocarbonyl concentration.

Those cultures which caused a decrease in at least two monocarbonyl classes, but were not sufficiently active to remove any one of them completely, are shown in Table 5.

The remaining cultures examined have been arbitrarily combined in a single group that is characterized by relatively weak ability to alter the monocarbonyl content of rancid lard (Table 6). Although the slight increases or decreases were consistent in repeated trials, they did not have a great effect on the total monocarbonyl content of the rancid fat, and the activity in reducing the peroxide level was moderate to weak.

It was obvious from observing the 2,4-dinitrophenylhydrazone bands that developed on the Seasorb and alumina columns that most of the microorganisms utilized in this study caused changes in several types of polar carbonyls present in the rancid fat in addition to the changes produced in the monocarbonyl fraction. Both strains of *S. aureus* and *A. flavus* destroyed ketoglycerides so that no ketoglyceride band was visible on the alumina columns. None of the other cultures had any discernible effect on the ketoglyceride band. With most of the microorganisms utilized, the odor and taste of the rancid lard were changed so that the typical organoleptic response to rancid lard was not obtained.

DISCUSSION

HYDROPEROXIDES ARE DECOMPOSED by both animal tissues and microbial cells. A variety of microorganisms and animal tissues decompose t-butyl, p-menthane, and cumeme hydroperozides; however, none of the products were identified (Updegraff *et al.*, 1958). *Micrococcus cerificans* metabolizes 1-alkyl hydroperoxides (C_{12}, C_{14}, C_{16}, C_{18}); 1-octyldecyl hydroperoxide was transformed into stearyl stearate (Finnerty *et al.*, 1962). Although the hydroperides present in rancid fat were decomposed by almost all of the microorganisms we investigated, the differences in the extent of attack ranged from a small percentage decrease by *Leuconostoc mesenteroides* to 85–100% destruction by *S. aureus, A. flavus,* and *Candida lipolytica.*

Corresponding increases in monocarbonyls were not found, however. *A. flavus* produced an increase in all monocarbonyl fractions including methyl ketones, while *C. lipolytica* decreased the three main classes of monocarbonyls. There does not appear to be any relationship between strong lipolytic activity and the ability to strongly decompose peroxides; for example, *Escherichia coli, Micrococcus cryophilus,* and *Aspergillus niger* had little lipase activity, yet 80% or more of the hydroperoxides were decomposed.

The development of a green flavor defect in mixed strain lactic starter cultures is a serious problem encountered in the manufacture of cultured buttermilk and sour cream,

Table 6. Microorganisms with weak activity relative to monocarbonyls (Group 5).

Microorganisms	Lipase activity when fat added μeq FFA/hr	Peroxide value		Monocarbonyls[1]		
		Start meq/kg fat	End meq/kg fat	2,4-Dienals	2-Enals	Alkanals
S. cremoris (264)	8.0	80.8	74.7 (5 days)	1.6	4.0	27.6
Control	......	80.8	74.7	1.4	2.9	26.2
T. elegans (195)	35.8	115.0	43.1 (5 days)	2.9	5.3	27.6
Control	......	115.0	121.0	2.2	6.8	27.1
L. mesenteroides (269)	5.4	79.1	76.0 (5 days)	0.7	4.1	33.9
Control	......	79.1	77.9	1.9	4.6	34.1
L. citrovorum (267)	5.6	79.1	58.0 (5 days)	0.8	4.0	30.2
Control	......	79.1	77.9	1.9	4.6	34.1

[1] Expressed as $\mu M/10^4$ μM fat.

and acetaldehyde has been shown to be responsible for the undesirable flavor (Lindsay *et al.*, 1965). Certain *Leuconostoc* species which can decompose acetaldehyde added to the starter eliminated this defect (Keenan *et al.*, 1966a; Keenan *et al.*, 1966b). Short chain aliphatic aldehydes are oxidized to the corresponding acids by dehydrogenases isolated from yeast (Black, 1951; Seegmiller, 1953), and the existence of alcohol dehydrogenases capable of reducing short chain aldehydes to the corresponding alcohols has been reported (Racker, 1950; DeMoss, 1954).

The ability of microorganisms to selectively attack specific types of compounds is supported by the data reported here. The apparently wide range of possible effects that can be obtained by judicious selection of cultures indicates that it might be feasible to selectively produce or remove volatile carbonyl compounds in fats or fat-containing foods.

The production of methyl ketones from fat by microorganisms has been well established (Hawke, 1966). Lipolytic fungi growing on fats containing low molecular weight triglycerides liberate fatty acids which are converted to β-keto acids and then decarboxylated to form the methyl ketones. Both of the cultures found to produce methyl ketones in our study were lipolytic, and a similar route to ketone production may have occurred.

It also has been shown that methyl ketones can be produced from alkanes by *Pseudomonas methanica* (Leadbetter *et al.*, 1960) and by *Myobacterium smegmatis* (Lukins *et al.*, 1963). Alkanes have been shown to be products of rancidity (Evans, 1961) and, thus, methyl ketone production by *P. fragi* and *A. flavus* may have been derived from alkanes rather than products of lipase activity.

The use of microorganisms to remove monocarbonyls and peroxides from rancid fat and to delay the onset of autoxidation in fresh lard and other fats is being investigated. Although no attempt was made to determine the similarity of activity within taxonomic groups, three species within the genus *Pseudomonas* were similar as were two strains of *S. aureus* and two of *B. cereus*. Further investigation of this activity may provide additional criteria for their differentiation.

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A Research Note
Diffusion of Aflatoxins in Foodstuffs

The common domestic foodstuffs that frequently turn moldy under household conditions in Germany are fruit juices, sliced pre-packed bread and cheese.

Since aflatoxins are excreted by the mycelium, we examined the diffusion of these toxins in the moldy foodstuffs. For this purpose, we followed the production of aflatoxins in apple juice stored at room temperature by assaying the aflatoxin content of the mycelium-free liquid as well as of the harvested mycelium. We also demonstrated the diffusion of toxins in whole-rye and whole-wheat bread, and in Tilsit cheese. Subsequently, we studied the kinetics of the diffusion of the toxins in a model substrate composed of solid "Cream of Wheat" (300 g) and sucrose (25 g) in tap-water (1000 ml). The matrix was cut into 3 cm cubes and autoclaved.

ESTIMATION OF AFLATOXINS

For estimating aflatoxins the fermented substrates were dialysed in methanol-water and the solution extracted with chloroform, dried over sodium-sulfate, and the highest dilutions of aflatoxin B_1 and G_1, respectively, under UV light on thin-layer silica gel plates determined (Frank, 1966).

Apple juice was filtered and extracted with chloroform. Mycelia were extracted after drying and weighing.

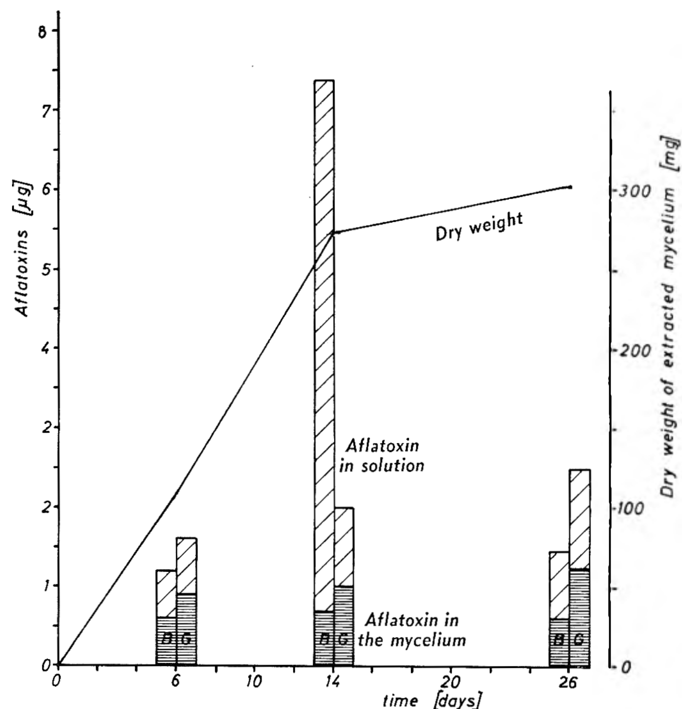

Fig. 1. Aflatoxins by Aspergillus flavus *strain No. 373 on 100 ml apple juice and dry weight of mycelium in relation to time of incubation at 22°C.*

RESULTS

Apple juice

One hundred ml juice in 500-ml Erlenmeyer flasks were inoculated with a suspension of conidia of *Aspergillus flavus* No. 373, and incubated at 22°C. After 6, 14 and 26 days, respectively, three flasks were examined independently. Fig. 1 shows a linear increase in dry weight of mycelium in the first 16 days. After this time, growth slows down.

Although the dry weight of mycelium increases, as indicated, the actual aflatoxin content of the whole mycelium remains fairly constant until the end of 26 days. However, the aflatoxin content of the mycelium per dry weight unit is at a maximum after 6 days, and at this time it is present in almost the same amount in the liquid. The concentration of the free aflatoxins in solution increases rapidly to a maximum at 14 days at which time 6.7 µg/100 ml B_1 is present. During the next 12 days B_1 disappears steadily, and at 26 days almost equals the 6-day level. The concentration of aflatoxin G_1 shows a steady increase to 2.5 µg/100 ml at 26 days.

The causes for the loss of B_1 are unknown. Perhaps it is destroyed photochemically or by a decrease in pH which dropped from 4.5 to 2.8. It should be added that we could not detect photoproducts of aflatoxins on thin-layer chromatograms as they were recently observed by Andrellos *et al.* (1967).

Using our strain 373 the content of aflatoxin in apple juice is small: 10 µg/100 ml. A different strain isolated at a later date from food produced a manifold increase in this compound: 120 µg/100 ml B_1. Infections with such strains might become dangerous for the consumer.

Bread

Sliced whole-rye and wheat bread was inoculated on one side in the opened package with *A. flavus* strain No. 373 and incubated for 6 days at 30°C. The single slices were covered by a dense mycelium mat with conidiophores on the crust of the bread, near to the point of inoculation. The mycelium grew over part of the surface as shown in Fig. 2. The slices were cut in pieces as shown and the aflatoxin content estimated in the numbered parts. A small edge along the inoculated side showed a remarkable light-blue fluorescence with ultraviolet light of 350 nm. Part 1 with luxuriant fungal growth had 30 µg of aflatoxin B_1 and 18 µg G_1 per g of bread. Part 2 contained more than twice the content, i.e. 61 µg/g and 60 µg/g, respectively. The proportion of G_1 to B_1 is increased further in part 3.

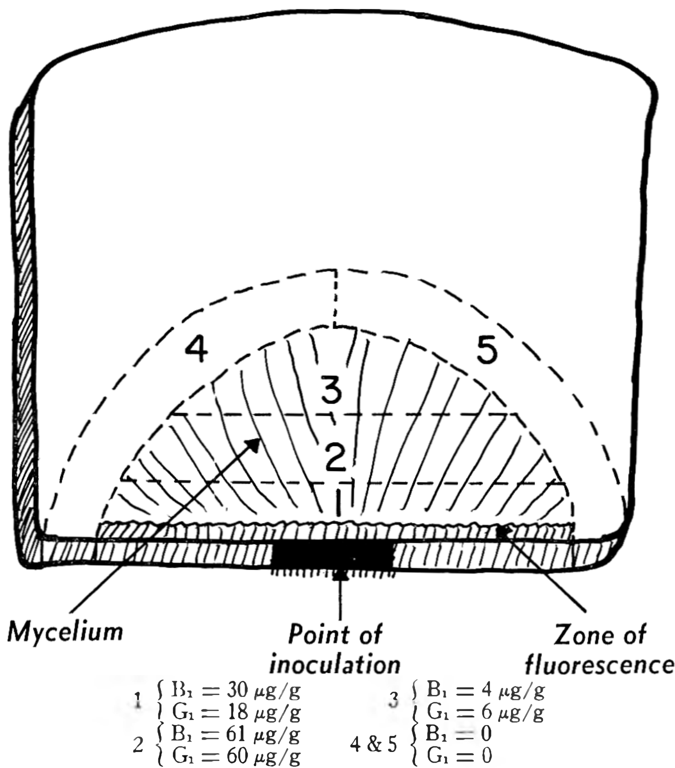

Fig. 2. Growth of Aspergillus flavus *strain No. 373 on bread and distribution of aflatoxins after 6 days at 30°C.*

Here we found 4 µg/g B_1 resp. 6 µg/g G_1. No aflatoxin was detectable in parts 4 and 5 which were always without mycelium.

Cheese

Cubes of Tilsit cheese, 5 × 5 × 5 cm, were inoculated with *A. flavus* strain 400 on one side and incubated at 30°C. The fungus grew well and formed a loose, yellowish "turf" of conidiophores, less dense, however, than on most other media. After 6 days the cubes were cut into 0.5 cm slices. The first slice with fungus contained 0.2 µg/g aflatoxin B_1 and 0.6 µg/g G_1. The second slice contained only 0.02 µg B_1 and G_1, and in the layers below no aflatoxin was detectable. The second slice was free of mycelium.

Experiments with other strains of *A. flavus* on cheese and other animal products showed a lower production of aflatoxins than on substrates with high carbohydrate contents. However, the results of W. van Walbeek show that this is not always the case (personal communication).

Kinetics of aflatoxin diffusion

Fifteen cubes of the model substrate were wrapped in aluminum foil with the exception of one side which was left exposed and was inoculated with *A. flavus* strain 400. Each day, three cubes were sliced into seven parts and the aflatoxin content was estimated in each. The results are shown in Table 1 and Fig. 3. The highest content of aflatoxin was found in the first slice containing the fungus. The maximum was found after 3 days at which time toxin had diffused down to layer No. 4. On the fourth day aflatoxin B_1 had reached slice No. 6, and on the fifth day was detectable in all parts of the cubes, i.e. at a distance of 30 mm from the fungus mat.

The consistency of substrate changed during the observation period. The whole cube was granular initially, but became more and more cheese-like. After 5 days of fermentation only the last slice was granular.

It is interesting to see the increase and later the decrease of aflatoxin in the whole system. There is a steep rise in the first 3 days to 248 µg/g B_1 and a decrease to 123 µg/g on the fifth day. A photochemical destruction therefore can be excluded.

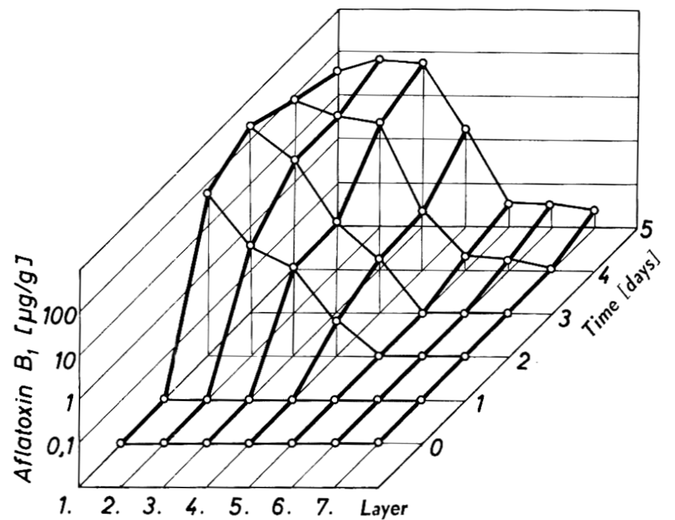

Fig. 3. Distribution in a model substrate of aflatoxins produced by Aspergillus flavus *strain No. 400 growing on one side of a cube of the substrate.*

Table 1. Distribution of µg/g of aflatoxin B_1 and G_1 from the site of growth of *Aspergillus flavus* No. 400 on a cube of a model substrate during five days at 30°C.

Slice No.	1-day		2-day		3-day		4-day		5-day	
	B	G	B	G	B	G	B	G	B	G
1			41	6	220	33	59	9	30	4.5
2			3.2	0.5	27	4	29	4.3	49	7
3			1.4	0.2	1.3	0.2	16	2.4	42	6.3
4			1.3	0.2	0.1	tr	0.1	tr	1.5	0.2
5							0.02	tr	0.15	tr
6							tr		0.03	
7									0.02	
Total			46.9	6.9	248.4	37.2	104.1	15.7	122.7	18

DISCUSSION

All these results show a possible danger for consumers who eat foodstuffs with closely cut-off moldy spots. The content of aflatoxin below the fungus mat in the depth of a food depends on the strain growing on the surface, the water-content of the food, its physical properties, and the storage time and temperature.

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Post-mortem Degradation of Adenine Nucleotides in Muscle of the Lobster, Homarus americanus

SUMMARY—Thin-layer chromatography showed that post-mortem degradation of adenine nucleotides in the tail muscle of lobster *(Homarus americanus)* followed the route: adenosine 5′-triphosphate (ATP) → adenosine 5′-diphosphate (ADP) → adenosine 5′monophosphate (AMP) → inosine 5′-monophosphate (IMP) → inosine (Ino) → hypoxanthine (Hx). KCl extracts (0.6*M*) also degraded ATP by this route. Such extracts contained a weak AMP-aminohydrolase activity that was activated by ATP, but no adenosine aminohydrolase could be detected. Neither of these aminohydrolases were found in extracts made with water or 0.02*M* K-succinate.

INTRODUCTION

Adenosine 5′-monophosphate aminohydrolase (E.C. 3.5.4.6.) has been found to occur in aqueous extracts of muscle of carp and mackerel (Hidaka *et al.,* 1960), lingcod (Tarr *et al.,* 1964), and pre-rigor cod (Dingle *et al.,* 1967). Several marine invertebrates, however, were reported to lack this enzyme (Kitagawa *et al.,* 1957; Saito *et al.,* 1958; Arai, 1966). We have found that post-mortem degradation of adenosine 5′-triphosphate (ATP) in lobster muscle proceeds by way of adenosine 5′-monophosphate (AMP) and inosine 5′-monophosphate (IMP), but that the extractability and apparent activity of the AMP-aminohydrolase involved in the degradation differ from those found in cod.

METHODS

Rested and feeding lobsters from the local fishery, held in running seawater at 15°C in laboratory aquaria, were semi-immobilized by slicing through the carapace just behind the eyes. The excised tail muscle was stored in polyethylene bags at 0°C. Extracts for enzyme assay were made by homogenizing samples of the muscle in a Waring blendor with 17 parts of a) water, b) 0.02*M* K-succinate (pH 6.8), or c) 0.6*M* KCl, and then centrifuging for 20 min at 13,000 G. All operations were carried out at 3°C in a cold room.

Enzyme reaction mixtures contained 1.2m*M* nucleotide, 1.5m*M* $MgCl_2$, and one-tenth volume of extract, and were incubated at 0–3°C. Continuous stirring was not used since even in the presence of precipitated protein, it had no significant effect on the results. Aliquants from the mixtures, after removal of protein with one-ninth volume of cold 30% perchloric acid and neutralization with KOH, were assayed for inosine-type compounds by ultraviolet absorption spectrophotometry, and for inorganic phosphate by the method of Rockstein *et al.* (1951).

Thin-layer chromatograms of nucleotides and derivatives were run on PEI-cellulose. Details of preparation of samples from muscle and of development of the chromatograms were described by Fraser *et al.* (1967). Neutralized perchloric acid supernates from reaction aliquants were spotted directly on the plates.

RESULTS

The changes in the nucleotides of lobster tail muscle during storage at 0°C were determined by thin-layer chromatography. The results, summarized in Table 1, show a pattern similar to that found for cod (Fraser *et al.,* 1967), namely, a gradual conversion of ATP through ADP and AMP to IMP, with only a low accumulation of the intermediates. The IMP was eventually further degraded to inosine and hypoxanthine. The combined amounts of IMP and inosine, estimated from the ultraviolet absorption spectra of the extracts and expressed as percent of the total nucleotides, are shown in Fig. 1. Corresponding data for relaxed cod, calculated from Fig. 2a of Fraser *et al.* (1967) are included for comparison.

The results of incubation of ATP, ADP, AMP, and adenosine with aqueous and 0.6*M* KCl extracts of pre-rigor lobster muscle are given in Table 2. The aqueous extract gave no significant reaction with any of the substrates up to 72 hr at 3°C. This finding was confirmed with other aqueous extracts, even in the presence of 0.1*M*

Table 1. Degradation of nucleotides in tail muscle of a lobster during storage at 0°C.

Days at 0°	ATP	ADP	AMP	IMP	Ino	Hx
0	+++++	+	–	–	–	–
1	+++++	+	?	–	–	–
2	+++	++	–	?	–	–
3	+++	+	++	++++	–	–
7	+	++	+	++++	+	–
9	?	++	+	++++	+++	++
14	–	–	–	–	–	+++

Perchloric acid extracts of muscle samples were fractionated by thin-layer chromatography on PEI-cellulose. The relative amounts of the components were estimated visually from the intensities of the spots revealed by ultraviolet illumination of the plate.

KCl which had been found to activate AMP-aminohydrolase in aqueous extracts of pre-rigor cod muscle (Dingle *et al.*, 1967).

The amounts of inorganic phosphate (P_i) formed from ATP and ADP by the 0.6*M* KCl extract approached the amounts to be expected from these substrates in the presence of ATPase (myosin) and adenylic kinase which presumably occur in such extracts. The amounts of IMP formed, however, showed that deamination was far from complete in most cases. The formation of IMP from ATP and from ADP was usually greater than from AMP in the presence of KCl extract; this was shown in other experiments to be due to an activation of the AMP-aminohydrolase by ATP (Fig. 3). P_i had no activating effect on the AMP reaction.

Centrifugation of 0.6*M* KCl extracts at 13,000 G usually yielded soft residues so poorly packed that only about two-thirds of the extracts could be decanted free of undispersed material. This residue was estimated to contain about 16% of the total protein nitrogen of the muscle in unextracted form. The undispersed material did not appear to contain an important amount of the muscle AMP-aminohydrolase, however, because when equal volumes of the residue and of the supernatant solution were incubated with AMP, the extents of reaction in the two cases did not differ significantly.

Ultracentrifugation of the KCl extracts (45 min at 140,000 G) removed most of the enzyme activities but the results were not reproducible between extracts.

The fates of the nucleotides in the presence of a 0.6*M* KCl extract were followed with time by thin-layer chromatography (Fig. 2). The stock solutions of ATP and ADP evidently contained some ADP and AMP as impurities, respectively. After 5 min of reaction, traces of IMP were discernible in the ATP, ADP and AMP + ATP reactions, but not in the reaction with AMP alone as substrate. The formation of ADP in the ATP and AMP + ATP reactions, and of ATP and AMP from ADP are also clearly shown. After 3 hr, only traces of ATP and ADP remained in any of the mixtures. It can further be seen that the extent of deamination of AMP was least in the reaction with AMP alone, in which neither ATP nor ADP was present at any time. These reactions were also followed by assays for P_i and inosine-type compounds, and the results (Fig. 3) confirm those already noted.

A KCl extract readily dephosphorylated ITP, but not IDP (Table 3). The activities of this extract with the adenine nucleotides are given for comparison.

Table 2. Enzymic activities of pre-rigor lobster tail muscle extracts with adenine derivatives.

Extracting solution	[KCl] in reaction	Substrate	Products, μ moles/ml P_i	Products, μ moles/ml Inosine-type compounds
0.6*M* KCl	0.06	ATP	2.26	0.65
		ADP	1.20	.93
		AMP	.061	.57
		Adenosine	.011	.10
0.6*M* KCl	0.6	ATP	2.00	0.33
		ADP	1.23	.63
		AMP	.11	.44
		Adenosine	.074	.10
Water	0	ATP	0.055	0
		ADP	.014	.02
		AMP	.044	.06
		Adenosine	.004	.08

Substrate concentration, 1.2m*M*; $MgCl_2$, 1.5m*M*; pH 7. Incubation for 72 hr at 3°C.

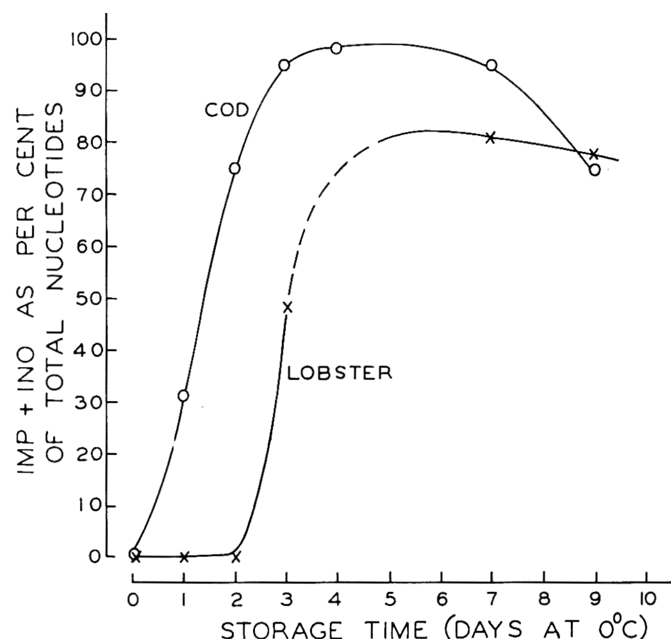

Fig. 1. Production of inosine-type compounds in muscle of a lobster tail and a relaxed cod during storage at 0°C. Data for the lobster were calculated from ultraviolet absorption spectra of perchloric acid extracts of the muscle. Data for the relaxed cod were calculated from Fig. 2a of Fraser et al. *(1967).*

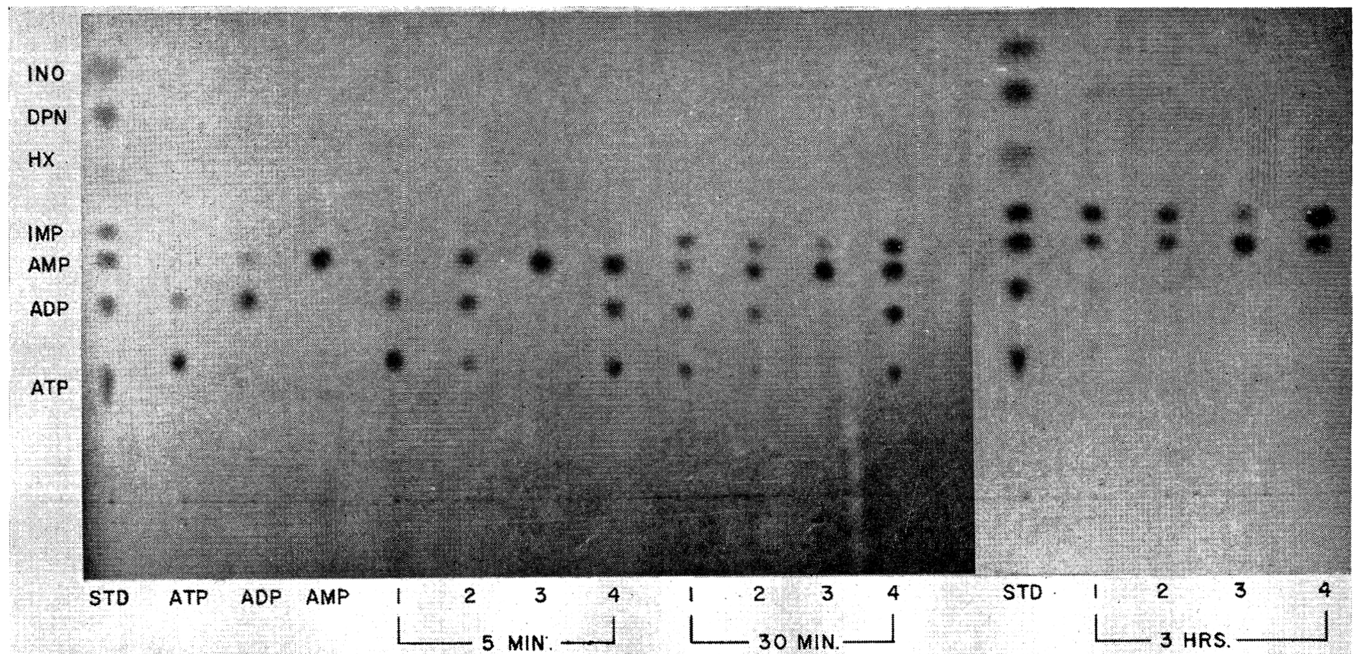

*Fig. 2. Degradation of adenine nucleotides by a 0.6*M *KCl extract of pre-rigor lobster tail muscle. Thin-layer chromatograms on PEI-cellulose. STD, standard mixture; ATP, ADP, and AMP, stock solutions of nucleotides; 1, 2, and 3, reaction mixtures with ATP, ADP, and AMP, respectively, each at 1.2m*M*; 4, reaction mixture with both ATP and AMP, each at 1.2m*M. *Incubation at 3°C. Samples withdrawn for analysis at the times shown.*

Table 3. Enzymic activity of a 0.6*M* KCl extract of lobster tail muscle with inosine and adenine nucleotides as substrates.

Substrate	Products, μM/ml: Inosine-type compounds	ΔP_i
ITP	......	0.933
IDP	......	.071
ATP	0.605	1.23
ADP	.687	.955
AMP	.351	.079

Mixtures contained 1.2m*M* nucleotides, 1.5m*M* $MgCl_2$, one-tenth volume of extract, and were incubated for 19 hr at 3°C.

DISCUSSION

THE THIN-LAYER CHROMATOGRAPHIC EVIDENCE indicates that the post-mortem degradation of nucleotides in lobster tail muscle follows the same pathway as in muscles of fishes and higher vertebrates. Possible alternate routes, involving the deamination of ATP or ADP for example, appear to be ruled out by the failure of the extracts to dephosphorylate IDP. Moreover, no significant amount of an adenosine aminohydrolase activity was found in the muscle extracts. In this respect the lobster differs from some other invertebrates which employ this enzyme rather than AMP-aminohydrolase (Arai, 1966).

There are, nevertheless, curious differences between the muscle of lobster and that of cod (Dingle *et al.*, 1967). Virtually no AMP-aminohydrolase activity could be extracted with water or 0.02*M* succinate from pre-rigor lobster muscle, whereas these extractants removed a large proportion of it from pre-rigor cod muscle. Moreover, the activity of the lobster enzyme appeared to be much lower than that of cod. A 0.02*M* succinate extract of cod muscle, for example, when diluted 50-fold, had an activity of 0.2 μM/ml/min at 2°C. A 0.6*M* KCl extract of lobster

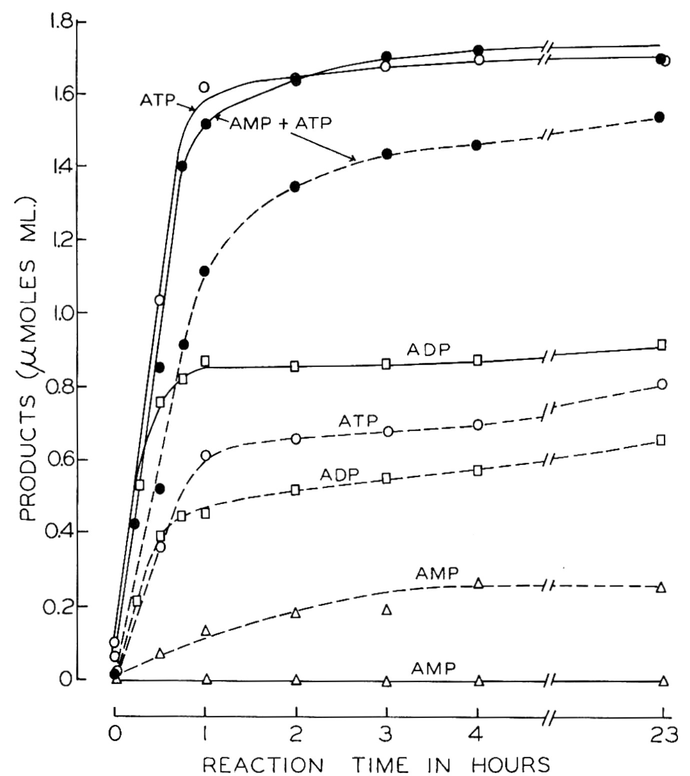

*Fig. 3. Production of inorganic phosphate and inosine-type compounds from adenine nucleotides in the presence of a 0.6*M *KCl extract of pre-rigor lobster tail muscle. The concentrations of each of the nucleotides was 1.2m*M *in all reactions; 1.5m*M *$MgCl_2$ was also present. Incubation at 3°C, pH 7. Solid lines, inorganic phosphate; broken lines, inosine-type compounds.* ○, *ATP;* □, *ADP;* △, *AMP;* ●, *AMP + ATP.*

muscle, which contained most of the available enzyme, had an activity of 0.0022 μM/ml/min at 3°C when diluted 10-fold, and only 0.027 μM/ml/min even when activated by ATP.

The data of Fig. 1, however, suggest that the formation of IMP in the two muscles occurs at rates of the same order of magnitude. It is possible that the apparent discrepancy may be due to an inactivation of the lobster enzyme by adsorption on some surface. We had previously suggested that extractants of low ionic strength might cause swelling and disruption of some membranous structure in cod and consequently release the enzyme in an active form. Evidently this does not occur with lobster muscle. A study of the effects of some other treatments, such as freezing and irradiation, known to be capable of disrupting mitochondria and other intracellular particles, could prove interesting.

Although IMP, an important flavor-enhancing substance (Kuninaka *et al.,* 1964), is produced in lobster muscle during post-mortem storage, it is doubtful whether full advantage is taken of this in the cooking methods now in use. Lobster is usually cooked while still alive, so that the enzymes degrading the nucleotides may be destroyed before much IMP is formed. This matter is now under study.

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Effect of Chlorophenoxyacetic Acid Growth-Regulator Sprays on Residues in Canned Apricots and Grapes

SUMMARY—A gas-liquid chromatographic method coupled with an electron capture detector has been developed for analyzing chlorinated phenoxyacetic acid residues in canned fruits. The technique involves converting the acid to its methyl ester with diazomethane, chromatography on a 5% silicone grease SE-30 column at 210°C, and subsequent detection of the compound by an electron capture detector. As low as 0.02 ppm of the residue could be detected. The method is superior to the colorimetric method because parachlorophenoxyacetic acid (PCPA), 2,4-D, and 2,4,5-T can be separated and quantitatively determined simultaneously. However, separate standard curves are needed for each, because they differ in chlorine content and in sensitivity toward the electron capture detector. Levels of 2,4-D and 2,4,5-T residues in canned apricots, and of PCPA in canned grapes are reported.

INTRODUCTION

CHLOROPHENOXYACETIC ACIDS have been used as growth regulators. Application of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) to apricot trees at the pit-hardening stage can prevent preharvest fruit drop, increase fruit size, and hasten maturity (Crane, 1955). Para-chlorophenoxyacetic acid (PCPA) was used to increase the size and yield of boysenberries (Bringhurst *et al.*, 1956). Widespread use of the chlorinated phenoxyacetic acids as growth regulators necessitates the development of methods for determining residues in the fruits.

Erickson *et al.* (1962) used gas chromatographic method for determination of 2,4-D residue in citrus fruit. Quantitative analysis of benzene hexachloride, aldrin, dieldrin, DDT, chlordane, endrin, toxaphane, and other chlorinated organic pesticides can be made in a single determination within one hour.

Bevenue *et al.* (1962) and Yip (1962) determined 2,4-D in dry crops and walnuts using a microcoulometric gas chromatograph. Gutenmann *et al.* (1963) worked on pesticide residue analysis by gas chromatography with an electron affinity detector. Daoud *et al.* (1962) applied the colorimetric method of Marquardt *et al.* (1955) to determine 2,4,5-T residue in canned apricot. Luh *et al.* (1964) used a gas-liquid chromatographic method to determine PCPA residue as a C^{14}-labeled methyl ester in boysenberries. A liquid scintillation counter was used to determine the radioactivity.

Hagin *et al.* (1965) developed a method for determining 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) and 2,4-D in forage plants by electron capture gas chromatography. Recovery of the herbicide exceeds 96%. Marquardt *et al.* (1964) reviewed the analytical methods for pesticides, growth regulators and food additives.

This study reports a procedure for extraction and purification of 2,4-D and 2,4,5-T residues in canned apricots, and of PCPA in canned Thompson seedless grapes which were sprayed with chlorophenoxyacetic acid as growth regulators.

EXPERIMENTAL MATERIALS AND METHODS

Apricots

Groups of three Blenheim apricot trees grown at Brentwood, Santa Clara, and Winters were sprayed between April 10 and 20 with 6 gal of 25 ppm 2,4-D (triethanolamine salt) solution per tree. Equal number of unsprayed trees were used as control. The fruits were harvested at canning ripeness 80 days after the spray. Six Blenheim apricot trees grown at Winters, California were fertilized with NH_4NO_3 at a rate of 0.5 lb N per tree, and three at 1.5 lb N per tree. Groups of three apricot trees were sprayed on April 10 with 6 gal of 2,4,5-T (triethanolamine salt) solution per tree. Three unsprayed trees (0.5 lb N per tree) were used as the control.

The apricots were harvested at canning ripeness on June 30. One hundred lb of apricots from each tree were washed with cold water, halved, and pitted in an apricot pitting machine. To each No. 2½ can was added 19.5 ± 0.5 oz of pitted fruit and 10.5 oz 40° Brix sucrose syrup. The cans were sealed under 16 in. of vacuum and heat processed at 212°F in a rotary cooker for 19 min and then cooled in a rotary water cooler. Each sample consisted of 48 No. 2½ cans.

Thompson seedless grapes

Twenty 10-year-old Thompson seedless vines were used for the investigation. Six plants were sprayed on June 8, 1965 with 15 ppm PCPA (ammonium salt) solution containing 0.1% "Tween 20" as a wetting agent. Seven plants were sprayed with 40 ppm gibberlin A3 (potassium salt) solution containing 0.1% "Tween 20" as a wetting agent. The vines were sprayed with the growth regulators to drip, equivalent to 200 gal per acre. Seven plants were left unsprayed to serve as a control.

The grapes were harvested on August 18 and again on September 8. Promptly after harvest, the grapes were weighed, stemmed and washed with cold water. To each No. 2½ can was added 20 oz grapes and 10 oz 20° Brix sucrose syrup. The cans were sealed under 16 in. of vacuum, processed at 210°F for 23 min in a continuous rotary cooker, and cooled in a rotary cooler.

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Ten lb of representative grape leaves and petioles were picked from sprayed and unsprayed vines on August 18 and September 8. The leaves and petioles were washed with water, separated, freeze dried, pulverized in a mortar, and kept in tightly covered bottles at 0°F until needed for analysis.

Determination of PCPA, 2,4-D and 2,4,5-T

A modification of the method described by Daoud *et al.* (1962) and Luh *et al.* (1964) was used. One hundred g of the drained apricots or grapes were macerated with 250 ml of redistilled chloroform and 20 ml 3*N* HCl for 5 min. The slurry was transferred to a 500-ml Erlenmeyer flask and shaken on a rotary shaker for one-half hour. The product was centrifuged. The chloroform layer was used for column chromatography.

Column chromatography. A chromatography column was packed with 40 g of Woelm grade number 1 basic aluminum oxide (Research Specialties Corp., Richmond, Calif.). The glass column (2.5 × 24-cm) was fitted with a fritted glass filter, a vacuum take-off, and a 24/40 standard taper male joint. A vacuum was applied to the column to facilitate close packing, and the Al_2O_3 was washed with 50 ml chloroform. The chloroform extract was concentrated in a flash evaporator, and then transferred to the Al_2O_3 column with chloroform.

The column was washed with 300 ml of chloroform, connected to a vacuum source for 20 min to remove chloroform. The chlorophenoxyacetic acids were eluted from the column with 40 ml of 1% $NaHCO_3$ followed by 20 ml of water. The eluate was acidified with 3*N* HCl to a pH less than 1, and the solution was extracted three times in a separatory funnel with 50 ml each of chloroform. The chloroform extracts were combined and concentrated to about 5 ml in a flash evaporator. The concentrate was transferred to a graduated hematocrit tube and concentrated further under an air stream to 1 ml. One ml of diazomethane solution was added to convert the acids into methyl esters (Luh *et al.,* 1964). The solution was evaporated to dryness and the residue dissolved in 20 ml of redistilled benzene.

Gas chromatography. An Aerograph, Model A-600-C Hy-Fi (Wilkens Instrument and Research, Inc.) equipped with an electron capture detector was used. Nitrogen at 10 psia was used as a carrier gas. The flow rate was 60 ml/min. The column contained 5% SE-30 silicone grease on 60/80 mesh firebrick in a 5 ft copper tubing of ⅛ in. diameter. It was allowed to equilibrate at 210°C for 24 hr with nitrogen gas flowing at 60 ml/min. The unit was operated at range 10, attenuation 1, and at a chart speed of ½ in. per min. Aliquots of 0.5, 1.0, and 2.0 μl were used. The area under the peak was measured with a planimeter. The quantities of PCPA, 2,4-D, and 2,4,5-T were calculated from the respective standard curves.

The detector may show diminished sensitivity due to fouling or adsorption. If this happens, the source may be cleaned with 10% methanolic KOH in an ultrasonic generator, and then rinsed five times with redistilled alcohol.

Determination of retention time

Five nanograms of PCPA, 2,4-D, and 2,4,5-T (as methyl esters) per μl of benzene were injected into the Aerograph with a Hamilton microsyringe. The retention time was measured on the recording chart rotating at a speed of 30 in. per hr. The area under each peak was measured with a planimeter.

Preparation of standard curves

Samples of 2.5, 5.0, 7.5, and 10.0 nanograms of the standard 2,4-D and 2,4,5-T methyl esters were chromatographed on the column. A standard curve was also constructed, using 0, 0.25, 0.50, 0.75, and 1.00 μg of PCPA methyl ester. The areas under the peaks were measured with a planimeter. A standard curve was plotted for each compound, with the area in square inches as the ordinate and nanograms of chlorophenoxyacetic acid as the abscissa. The standard curves were made on the same day as the unknown samples.

RESULTS

Gas chromatography of methyl esters of chlorophenoxyacetic acids

A gas-liquid chromatograph of the methyl esters of PCPA, 2,4-D and 2,4,5-T separated on a 5% silicone grease column (60/80 mesh firebrick) at 210°C is shown in Fig. 1. One microliter of the methyl ester solution containing 0.5 μg each of the three acids was injected onto the column with the response of the detector attenuated at 1X. A distinct separation of the three compounds was accomplished. The retention times for the PCPA, 2,4-D, and 2,4,5-T methyl esters were 1.2, 2.4, and 4.0 min respectively. The peak shown by the 2,4,5-T methyl ester was somewhat broader and shorter. The linear response of the recorder to varying quantities of PCPA is shown in Fig. 2.

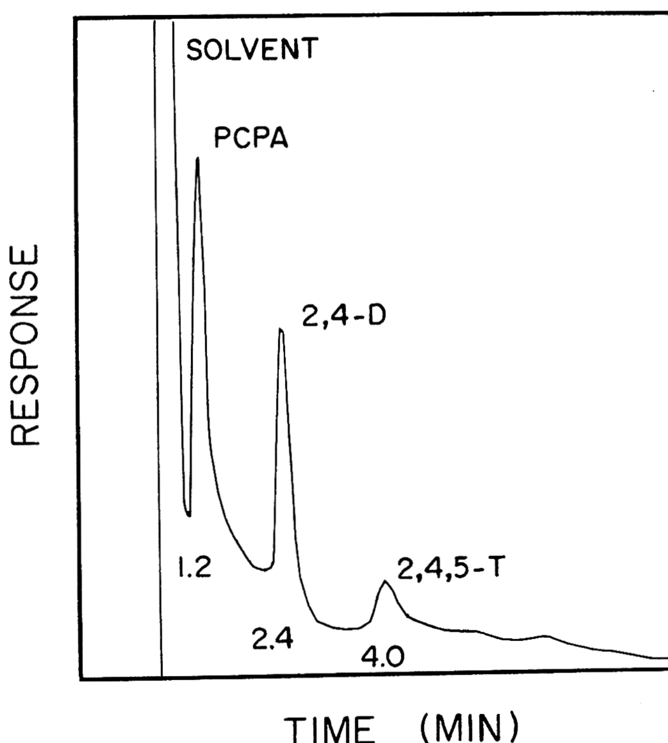

Fig. 1. Gas chromatography of PCPA, 2,4-D and 2,4,5-T as methyl esters on a 5% SE-30 silicone grease column (60/80 mesh firebrick) at 210°C.

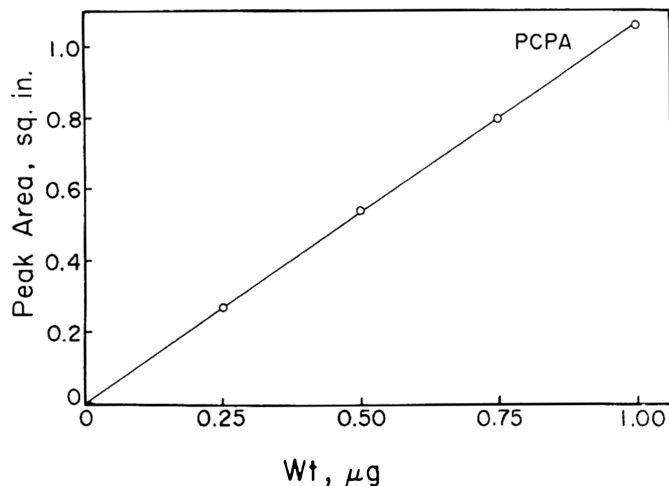

Fig. 2. Calibration curve for gas chromatography of PCPA as methyl ester on a 5% SE-30 silicone grease column (60/80 mesh firebrick) at 210°C.

Determination of 2,4-D in apricots

The residue in canned apricots which received 25 ppm 2,4-D sprays at the pit-hardening stage was determined. Apricot trees grown at Brentwood, Santa Clara, and Winters were used in this investigation. In each set, the control sample was fortified with 0.1 ppm 2,4-D in the laboratory to determine the percentage recovery. Table 1 shows the 2,4-D residues in the canned apricots. The control samples contained 0.10 to 0.23 ppm of 2,4-D. The average 2,4-D residue in the canned product ranged from 0.66 to 0.70 ppm. The recovery of 2,4-D from the extraction, purification, and esterification procedures was 90–94%.

Determination of 2,4,5-T in canned apricots

Three sets of apricot trees differing in nitrogen fertilizer treatments were sprayed with 6 gal of 75 ppm 2,4,5-T solution per tree at the pit-hardening stage. The 2,4,5-T residue in the canned product is shown in Table 2. The residue in the canned product ranged from 0.66 to 0.77 ppm. Nitrogen fertilizer was not a factor that affects the level of 2,4,5-T residue in the canned product. The recovery of the residue was 90%.

PCPA residue in canned grapes

Thompson seedless grape vines were sprayed to drip on June 8, 1965, with 15 ppm PCPA containing 0.1% "Tween 20" as a wetting agent. The grapes were harvested 72 and 92 days thereafter. Table 3 shows the levels of PCPA residue in the canned product. The sample harvested 72 days after spray contained 1.08 ppm PCPA residue, and that harvested 92 days after spray contained 0.98 ppm. The recovery of added PCPA was 83 to 87%.

PCPA residues in the freeze-dried leaves and petioles of the grape vines are shown in Table 4. The leaves harvested 72 days after spray contained 0.267 ppm PCPA, and that 92 days after spray contained 0.188 ppm. The corresponding petioles contained 0.523 and 0.408 ppm respectively.

It appears that the leaves contained less PCPA residue than the petioles, and the petioles contained less PCPA residue than the fruits. The difference may be explained by their positions on the vines and their structural differences. The leaves were thin, and received more sunlight which can cause photolysis of PCPA. The PCPA can penetrate the leaves and be relocated into the petioles and fruits, causing a higher residue in the latter.

From the above results, the chlorophenoxyacetic acid residue was present in the fruit. The quantity of residue in the product may vary with the type and quantities of growth regulators used and the harvest dates. Crops harvested at a later date contained smaller amounts of residue.

DISCUSSION

THE CHLOROPHENOXYACETIC ACIDS have been used either as growth regulators or weed killers. Their actions

Table 1. Gas chromatography of 2,4-D residue in canned apricots as methyl ester on a S.E. 30 silicone grease column (5% on 60–80-mesh fire brick) at 210°C.

Sample	Growing area	Growth regulator	2,4-D residue, ppm (80 days after spray) I	II	III	Ave.	Recovery %
1	Brentwood	Control	.26	.23	.20	.23	
2	Brentwood	Control + 0.1 ppm 2,4-D	.35	.32	.29	.32	90
3	Brentwood	Sprayed with 2,4-D, 25 ppm	.64	.71	.67	.67	
4	Santa Clara	Control	.11	.13	.10	.11	
5	Santa Clara	Control + 0.1 ppm 2,4-D	.20	.23	.19	.21	94
6	Santa Clara	Spray with 2,4-D, 25 ppm	.71	.72	.67	.70	
7	Winters	Control	.10	.12	.08	.10	
8	Winters	Control + 0.1 ppm 2,4-D	.19	.21	.17	.19	90
9	Winters	Sprayed with 2,4-D, 25 ppm	.66	.62	.70	.66	

Table 2. Gas chromatography of 2,4,5-T residue in canned apricots as methyl ester on a S.E. 30 silicone grease column (5% on 60–80-mesh fire brick) at 210°C.[1]

Sample	Growing area	Growth regulator	Nitrogen fertilizer lbs N/tree	2,4,5-T residue, ppm (80 days after spray) I	II	III	Ave.
1	Winters	Control	0.5	.11	.12	.09	.11
2	Winters	2,4,5-T, 75 ppm	0.5	.66	.70	.62	.66
3	Winters	2,4,5-T, 75 ppm	1.5	.78	.72	.81	.77

[1] The recovery was 90% when 0.1 ppm 2,4,5-T was added to the control sample.

Table 3. Gas chromatography of PCPA residue in canned Thompson seedless grapes as methyl ester on a S.E. 30 silicone grease column (5% on 60–80-mesh fire brick) at 210°C.

Sample	PCPA spray	Harvest dates	Days after spray	PCPA residue, ppm I	II	III	Ave.	Recovery %
1	Control	Aug. 18 (1st harvest)	72	0	0	0	0	
2	Control + .1 ppm PCPA	Aug. 18 (1st harvest)	72	.08	.08	.09	.083	83
3	Sprayed with 15 ppm PCPA + 0.1% Tween	Aug. 18 (1st harvest)	72	1.12	.98	1.14	1.08	
4	Control + .1 ppm PCPA	Sept. 8 (2nd harvest)	92	.09	.09	.08	0.087	87
5	Sprayed with 15 ppm PCPA + 0.1% Tween	Sept. 8 (2nd harvest)	92	.88	1.05	1.01	.98	

are specific to certain plants. When applied in right amounts and at the right time, they can increase the size and yield of the fruits (Bringhurst *et al.*, 1956; Crane, 1955; Luh *et al.*, 1964). Recent interests on spray residues and consumer safety have produced a pressing need for sensitive and reliable tests by which residues of these compounds can be identified and determined. The gas chromatographic method coupled with an electron capture detector appears to be a useful tool for analyzing chlorinated phenoxyacetic acid residues as methyl esters in canned fruits.

It is interesting that the control samples show small amount of spray residue. This may be caused by the presence of the residual growth regulators in the soil from previous applications, or by the presence of other electron affinitive constituents in the extract. This problem needs to be investigated.

The colorimetric method for determining chlorophenoxyacetic acids described by Marquardt *et al.* (1955 and 1961) and Daoud *et al.* (1962) could detect residues only in amounts greater than 1 ppm. The method described here is more sensitive and reliable. Furthermore, it can separate PCPA, 2,4-D, and 2,4,5-T as methyl esters quantitatively on the column and determine them simultaneously. However, separate standard curves are needed for each since they differ in chlorine content and in sensitivity toward the electron-capture detector.

Luh *et al.* (1964) pointed out that environmental conditions of the plant and the ripeness level of the fruit are important factors influencing the level of spray residue in the fruit. This was determined by weathering of the growth regulator, photolysis, and biological changes of the chemicals in the plants. Maxie *et al.* (1962) found accumulation of C^{14} in Tilton apricots sprayed with carboxyl labeled 2,4,5-T. This work indicates the translocation of 2,4,5-T from the leaves of the fruit.

Crane *et al.* (1965) reported on 2,4,5-T residue in apricot fruit by the gas chromatographic method. They found a gradual decrease in 2,4,5-T residue in the fruit during maturation. Their results are comparable to those reported here except that the levels of residue found were slightly different. The difference may be explained by differences in climatic and environmental conditions which affect the stability of the chlorophenoxyacetic acids toward weathering and biological transformations.

Table 4. Gas chromatography of PCPA residue in freeze-dried leaves and petioles of Thompson seedless grape vines.

Sample	PCPA spray	Harvest dates	Days after spray	PCPA residue ppm[1] Leaves	(Freeze-dried sample) Petioles
1	Control	Aug. 18 (1st harvest)	72	0.025	0.020
2	Control + 0.1 ppm PCPA	Aug. 18 (1st harvest)	72	0.079	0.086
3	Sprayed with 15 ppm PCPA + 0.1% "Tween 20"	Aug. 18 (1st harvest)	72	0.267	0.523
4	Control	Sept. 8 (2nd harvest)	92	0.010	0.025
5	Control + 0.1 ppm PCPA	Sept. 8 (2nd harvest)	92	0.075	0.096
6	Sprayed with 15 ppm PCPA + 0.1% "Tween 20"	Sept. 8 (2nd harvest)	92	0.188	0.408

[1] The samples were analyzed 7 days after harvest.

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Research Note

Adaptation of Biphasic Culture Technique to the Sporulation of Clostridium Botulinum Type E

INTRODUCTION

THE INCREASED NUMBER OF CELLS obtained in a given volume of fluid culture when it is incubated over a layer of agar medium containing nutrients was reported first by Hestrin *et al.* (1943). The term, biphasic culture technique, has been used to distinguish this system from the concentrated cell mass that can be obtained by dialysis sac culture (Tyrrell *et al.*, 1958; Gerhardt *et al.*, 1963; and Schneider *et al.*, 1963).

Some recent work by Tyrrell (1962) with biphasic systems has pointed up some of the factors that influence the yields of cells, e.g., variations in volume and composition of the agar and fluid layers. In an attempt to find a production method that would yield high numbers of clean clostridial spores, a biphasic culture system (liquid-agar) was adapted for the sporulation of *Clostridium botulinum* type E.

MATERIALS AND RESULTS

TWO DIFFERENT TYPES of flasks containing agar layers were satisfactory (Fig. 1). In the first system a large 3200 ml Fernbach-type culture flask (Arthur H. Thomas Cat. No. 4372 F) was used with a fluid layer of 500 ml overlaying an agar base of 1200 ml. In the alternate

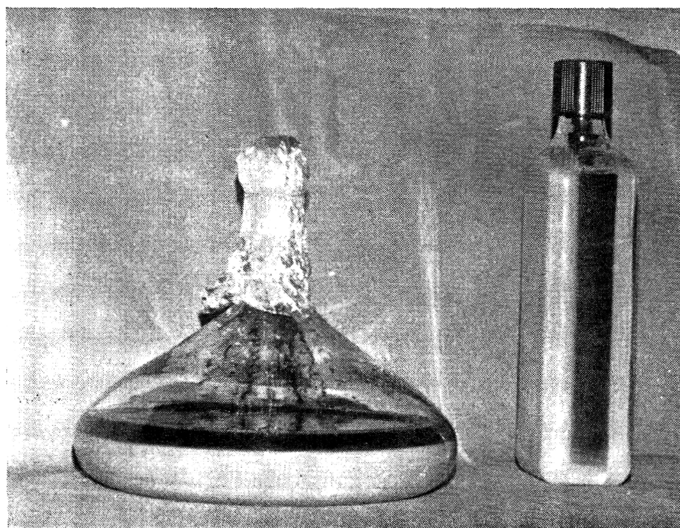

Fig. 1. Fernbach-type flask and screw cap Blake bottle used in biphasic culture system (liquid-agar).

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approach, a 1-L, screw-cap Blake bottle (Bellco Cat. No. 3204) with 250 ml of agar covering each of two opposite vertical surfaces of the bottle with 400–500 ml of fluid medium in between them was successful.

This latter system is more feasible for fastidious organisms that require very low levels of oxygen tension but is more laborious to execute. No precautions were taken to reduce oxygen tension other than to add sodium thioglycollate to the agar layers and tightly cover the cotton plugs on the Fernbach-type flasks with heavy aluminum foil.

The procedure finally developed for the sporulation of "Saratoga" and "Beluga" strains of *C. botulinum* type E was carried out as follows. Five hundred ml of distilled water was inoculated with a small volume (5 ml or 1%) of a 24 hr broth culture and placed over 1200 ml of TPG medium (trypticase, 5%; peptone, 0.5%; glucose, 0.4% and sodium thioglycollate, 0.2% at pH 7) solidified with 3% agar (Schmidt *et al.*, 1962).

TPG broth is a commonly used sporulation medium for *C. botulinum* type E. Incubation temperature was 30°C. Maximum sporulation occurred between 24 and 48 hr after inoculation depending on the strain being sporulated.

The development of the spores was followed using a Petroff-Hauser counting chamber with phase microscopy. A 95% or better sporulation was obtained with both strains. Counts of viable spores after heating an aliquot 5 min at 60°C ranged from 1×10^7 to 5×10^8 per ml of water culture (500 ml total) of the two strains.

As a comparison, spore crops were produced in TPG broth using deep culture technique with 1500 ml of TPG broth in 2-L Povitsky bottles. Spore development was followed as previously with phase microscopy. Maximum sporulation again occurred in from 24 to 48 hr but it never exceeded 60% of the total microscopic count. Counts of viable spores after heating an aliquot for 5 min at 60°C ranged from 1×10^6 to 5×10^6 per ml of the liquid TPG medium (1500 ml total). Assays for the number of viable spores after the heat treatment in both instances were made in TPG agar in Prickett tubes.

The flasks were refrigerated (2°C) at least 24 hr to allow for autolysis of vegetative cells before the spore crops were harvested finally by centrifugation and washed.

In sporulation studies with other organisms the choice of distilled water or nutrient broth as the liquid phase, as well as the choice of type of flask to be used must be determined by preliminary tests. Both systems produced good results with the *C. botulinum* strains tested in our laboratory. The highest percentage (95% or better) of sporulation as well as cleaner spore crops was produced when the inoculation was made into distilled water in the Fernbach-type flask. The often relied upon procedure of inoculum build-up of vegetative cells as outlined by Reed *et al.* (1951) and Collier (1957) terminating with 10–20% inoculum into the sporulation flask may be used for sporulation of organisms other than type E in a biphasic system.

An intriguing aspect of this method as applied to sporulation of anaerobic organisms is the possibility of including particulate material in the agar phase which will stimulate spore production, e.g., soil, vegetable matter, meat chunks, or in the case of type E, pieces of fish. Preliminary tests showed that a clean spore crop could be produced in the fluid layer when whole peas and soil were incorporated in the agar layers.

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Factors Affecting the Stability of Chlortetracycline, Tylosin and Furylfuramide Against Low Level of Ionizing Radiation

SUMMARY—Effect of low levels of ionizing radiation (0.01–0.2 Mrad) on the stability of chlortetracycline (CTC), furylfuramide (FF) and tylosin (Tl) were investigated. Tl in the phosphate buffer of pH 6–8 was very sensitive to low-level radiation, while either FF or CTC exhibited fairly high resistance at the same dose levels. Removal of dissolved oxygen in the test solution by aerating with nitrogen gas enhanced the inactivation of Tl and FF at 0.05–0.1 Mrad of radiation, but it had an opposite effect on the inactivation of CTC. Much higher Tl and CTC activities were retained after irradiation at 0.1 or 0.2 Mrad when the drugs were added to albumin, gelatin, broth or minced meats of five species of fish; the retention of FF did not change.

The remaining activity of Tl at 0.1 or 0.2 Mrad of radiation was more or less influenced by adding various sugars and amino acids. The presence of sugars (mono- or disaccharides) did not change retention of Tl markedly, but gave a weak protective effect. Tryptophane, histidine, phenylalanine, methionine and tyrosine exhibited a fairly high protective action on the inactivation of Tl after irradiation.

INTRODUCTION

FISH MEAT TREATED with an excess radiation dose may cause changes in odor, color and flavor and lower the acceptance of the treated fish. Kawabata *et al.* (1966a) have investigated the radiation threshold, i.e. the levels of irradiation which would not impart a significant change in quality to the 15 species of fish and six species of shellfish tested. In most of the fish, undesirable changes caused by irradiation were almost inevitable when fish were treated with more than 0.2 Mrad.

Kawabata *et al.* (1966a) also compared the storage life of fish fillets which had been treated with different doses of radiation, and the changes in the decomposition pattern between treated and untreated fish samples. A lowered preservative effect was noted at low levels of radiation which would not impart a significant discoloration, off odor or off flavor. These results strongly suggest that it would be difficult to extend the storage life of fish only by treating with a lower radiation dose. Such a treatment would not impart any appreciable change unless it is aided by other adequate means, e.g., low temperature freezing during irradiation to minimize the effect on quality, a combination of radiation with appropriate chemicals to increase the lethal effects of radiation, an increase in the storage life of irradiated food by use of a food preservative, etc.

A low level of radiation in combination with chlortetracycline has been reported to extend the storage life of shrimp markedly (Awad *et al.*, 1965). Cain *et al.* (1958) reported that the tetracycline antibiotics retained sufficient activity at radiopasteurization dosages of 0.18 to 0.72 Mrad to offer protection to meat during storage; however, before forming a general conclusion on the effectiveness of the complementary effect of a drug, further fundamental studies should be carried out to elucidate factors affecting the stability or inactivation of a drug against radiation treatment.

In the present study, the following three drugs, chlortetracycline (CTC), tylosin (Tl) and furylfuramide (FF) were employed, and the rates of inactivation of the three drugs were examined at low doses of gamma radiation under various conditions. In addition, the protective effects of certain food components on the inactivation of Tl were investigated.

CTC is permitted for preserving such perishable food as fish or chicken in several countries. The antibiotic is also approved in Japan for preserving raw fish for fish paste products as "Kamaboko," and for canned or salted salmon.

Furylfuramide, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide is a new preservative permitted in Japan since May 1965, and it has been substituted for nitrofurazone and nitrofuryl acrylamide. FF has a wide spectrum in antibacterial activity and is fairly stable to heat, its use is permitted in fish sausage at 20 ppm, "Kamaboko" at 2.5 ppm and in similar types of semi-preserved food.

Tylosin, an antibiotic of the macrolide class having a narrow range antibiotic spectrum with low toxicity, has not yet been approved for use in Japan. It does not inhibit the growth of Gram-negative bacteria, but it exhibits a fairly high antibiotic activity to Gram-positive organisms including *Bacilli* and *Clostridia* (McGuire *et al.*, 1961). Many trials have been undertaken in Japan using Tl in the preservation of such semi-preserved food as fish sausage (Shibasaki *et al.*, 1963; Shibasaki, 1964; Yokoseki, 1966), packaged bean curd cake in plastic film (Suzuki, 1966) as well as certain canned foods (Tanaka, 1966; Tanikawa, 1966). Obviously, Tl can not be a preservative for raw fish, since it is ineffective to *Pseudomonas,* the principal organisms causing spoilage in raw fish.

Masurovsky *et al.* (1963) reported that a decided change occurred in the constitution of the microbial population in haddock fillets and shucked soft-shelled clams before and after treatment with doses of from 0.05 to 0.8 Mrad, and the great majority of surviving microorganisms were micrococci, sporeforming bacilli, and certain yeasts, molds and actinomyces.

Similar experiments have been done by Kawabata *et al.* (1966a) showing that a marked change in the microflora in fish fillets (halibut and swordfish) occurred after irradi-

ation with doses of from 0.1 to 0.75 Mrad. Whereas radio-sensitive *Pseudomonas-Achromobacter* flora diminished markedly, comparatively radio-resistant Gram-positive cocci and sporeformers, and certain yeasts survived to become predominant in the treated fish meat during refrigerated storage. Both cocci and sporeforming bacilli are sensitive to the test drugs, but especially highly sensitive to Tl, which is one of the reasons Tl is included in the present study.

MATERIALS AND METHODS

Drugs to be tested

Tl (tylosin lactate) prepared by Eli Lilly & Co., FF manufactured by Ueno Pharmaceutical & Co., Osaka, and CTC (chlortetracycline-HCl) produced by Lederle Japan, Ltd. were used in this experiment. A 1,000 ppm aqueous solution of each test drug was prepared as the original solution, distributed in 5 ml portions in small polyethylene tubes with plugs and kept in a freezer before use.

Radiation source

The ^{60}Co source of 1,400 curies at the Tokyo University of Fisheries was employed throughout the present study. Samples were set on the turntable (1 rotation/5 min), and the dose-rate of the equipment was 6.6×10^4 r/hr at the distance of 6 cm from the source. The radiation dose actually exposed to the sample was controlled by distance.

Preparation and irradiation of samples

Each test solution to be irradiated consisted of 1 ml of a test drug solution (1,000 ppm) and 9 ml (g) of one of the various substrates placed in a test tube of 16 mm inside diameter.

The temperature in the radiation chamber was kept at $20° \pm 1°$C during the time when all samples were exposed to radiation except in one case of samples irradiated at 0°C.

Assay procedure for the antibiotic activities of the drugs

Potencies of the drugs were determined microbiologically by the cylinder-agar diffusion plate assay technique (cylinder-plate method). Tl activity was assayed with *Sarcina lutea* ATCC 9341 (Kawabata *et al.*, 1966b), CTC was assayed with *B. cereus* var. *mycoides* ATCC 9634 (Tomiyama *et al.*, 1957), and FF with *B. natto* (Matsuda *et al.*, 1965). The initial concentration of the drug in each test solution was 100 ppm, therefore, figures of residual activities indicate directly the percent retention.

RESULTS AND DISCUSSION

Effect of pH and gaseous environment on the inactivation of the drugs after irradiation

A 1 ml aliquot of each drug solution (1,000 ppm) was added to 9 ml of $M/15$ phosphate buffer of pH 6.0, 7.0 or 8.0. The test solutions of different pH were aerated for 5 min, then were irradiated at 0.01, 0.05 or 0.1 Mrad, and the residual drug activities were measured microbiologically. At the same time, changes in the potencies of the test solutions, which had been bubbled with nitrogen gas for 15 min and tightly sealed, were determined in the same manner as described above. Results obtained are shown in Table 1.

Table 1. Percent retention of tylosin, furylfuramide and chlortetracycline in the phosphate buffer of different pH after irradiation. (Initial concentration of test drug: 100 ppm.)

Drug	pH	Environment	Remaining activity, %		
			0.01 Mrad	0.05 Mrad	0.1 Mrad
Tl	6.0	Aerobic	72	17	1.0
		Anoxic	53	4	0.6
	7.0	Aerobic	63	25	1.3
		Anoxic	79	13	0.5
	8.0	Aerobic	75	16	0.5
		Anoxic	76	16	0.3
FF	6.0	Aerobic	85	60	19
		Anoxic	61	51	16
	7.0	Aerobic	78	48	36
		Anoxic	63	44	12
	8.0	Aerobic	76	60	31
		Anoxic	71	43	16
CTC	6.0	Aerobic	90	60	34
		Anoxic	100	65	36
	7.0	Aerobic	80	48	24
		Anoxic	90	40	28
	8.0	Aerobic	50	20	10
		Anoxic	80	36	15

Tl in the phosphate buffer was very sensitive to radiation. At a pH of 6 or 7, the retention decreased to about 1% after irradiation at 0.1 Mrad, and in test solution of higher pH values, less residue remained. After irradiation at 0.1 Mrad, it was noted that the inactivation of Tl in the buffer solution was enhanced under anoxic conditions, and this tendency was much more conspicuous in acidic than in neutral solutions. However, the reason the removal of free oxygen enhances the destruction of Tl during irradiation remains to be solved in future studies.

FF in the phosphate buffer was more stable against radiation than Tl, and after irradiation at 0.1 Mrad, the stability of FF was lowered by aerating with nitrogen to coincide with the results observed for the Tl solution.

CTC was more stable against radiation than the other two drugs. The stability of CTC, however, depends much on the pH, and after irradiation at 0.1 Mrad, the higher the pH of the test solution the less residue remaining; probably due mainly to the nature of CTC itself. The antibiotic has been reported to be quite unstable at an alkaline pH, especially at high temperatures (Kawabata *et al.*, 1960).

Effect of gelatin, albumin or nutrient broth on the stability of test drugs

One ml aliquot of the original test drug was added to 9 ml of $M/15$ phosphate buffer of pH 7.0, 10% albumin, 10% gelatin or nutrient broth (Difco, 10/9 concentration), and each test solution (100 ppm equivalent of test drug) was irradiated at 0.01, 0.05 or 0.1 Mrad. The pH values of gelatin, albumin and nutrient broth were 6.3, 5.3 and 7.0 respectively. Results obtained are shown in Table 2.

As mentioned in the previous experiment, Tl in the phosphate buffer is quite labile to radiation, however, its stability increased markedly when added to gelatin, albumin or nutrient broth. Tl activity was retained markedly

Table 2. Percent retention of the test drugs in different media after irradiation. (Initial concentration of test drug: 100 ppm.)

Test drug	Medium	Remaining activity, %		
		0.01 Mrad	0.05 Mrad	0.1 Mrad
Tl	Buffer, pH 7.0	66	18	1
	Albumin, 10%	88	80	60
	Broth, pH 7.0	92	90	80
	Gelatin, 10%	100	70	70
FF	Buffer, pH 7.0	88	74	38
	Albumin, 10%	72	40	19
	Broth, pH 7.0	94	65	30
	Gelatin, 10%	95	69	33
CTC	Buffer, pH 7.0	80	46	18
	Albumin, 10%	72	72	65
	Broth, pH 7.0	98	78	55
	Gelatin, 10%	100	95	70

in the nutrient broth consisting of comparatively low molecular weight substances. No marked difference in the remaining activity of FF was observed among the four kinds of test solutions. The residual activity of CTC increased apparently in albumin and gelatin solution and even in the nutrient broth.

Inactivation of the drugs in fish meats after irradiation

One ml aliquot of each original drug solution was added to 9 g of well minced and homogenized meat from horse mackerel, big-eyed tuna, mackerel, halibut and skipjack; and the meats treated with doses of 0.05, 0.1 or 0.2 Mrad. The residual drug activities were measured microbiologically. Results obtained are shown in Table 3.

As shown in Table 3, fairly high levels of Tl activities were retained in the fish meats after irradiation at 0.1 Mrad which almost coincided with that obtained in albumin solution. In addition, about 60 to 70% of Tl activities were retained in the fish meats even after irradiation at 0.2 Mrad. Considering the error in the microbiological assay of the drug, there was no marked difference in the protective action for retaining Tl activity among the fish species so far tested.

Table 3. Percent retention of the test drugs in minced fish meats after irradation. (Initial concentration of test drug: 100 ppm.)

Drug	Name of fish	Remaining activity, %		
		0.05 Mrad	0.1 Mrad	0.2 Mrad
Tl	Horse mackerel	88	82	74
	Big-eyed tuna	76	75	68
	Mackerel	88	73	55
	Halibut	93	82	55
	Skipjack		81	70
FF	Horse mackerel	71	45	3
	Big-eyed tuna	52	30	9
	Mackerel	42	28	7
	Halibut	62	44	7
	Skipjack	90	68	15
CTC	Horse mackerel	85	85	50
	Big-eyed tuna	68	55	42
	Mackerel	60	47	37
	Halibut	86	86	66
	Skipjack	70	66	43

As to the stability of FF, almost no appreciable difference was observed in the FF residue between that remaining in the phosphate buffer and that in the fish meats, except for the case in which higher FF residue was detected in the muscle of skipjack. A marked inactivation occurred in the fish meats which had been exposed to 0.2 Mrad. CTC added to the fish meats remained at fairly high levels, and about 50% of the activity was retained even after receiving 0.2 Mrad.

Effect of temperature on the inactivation of the drugs during irradiation

Previous experiments have been conducted at 20° ± 1°C, and in this experiment, the effect of temperature on the rates of inactivation of test drugs were examined. One ml of each drug solution was added to 9 ml of $M/15$ phosphate buffer of 7.0, 10% gelatin or the nutrient broth of pH 7.0 (Difco, 10/9 concentration), and these test solutions were subjected to 0.1 Mrad of radiation at either 19.2° or 0°C. The remaining drug activities were compared with each other. The results obtained are shown in Table 4.

The velocity of a chemical reaction depends much on temperature. However, as far as the present experiment is concerned, no marked difference was observed in the residual levels of test drugs irradiated at 0° and 19.2°C, except the one case with Tl in the phosphate buffer. Tl in the phosphate buffer of pH 7.0 after being irradiated at 0°C remained 4 times as much as that at 19.2°C, while in the other media, slightly higher residues were measured in the samples which had been irradiated at 0°C. Concerning the remaining activities of both FF and CTC, only slightly higher residues were detected in the samples which had been irradiated at 0°C.

Effect of sugars or amino acids on the inactivation of tylosin by radiation treatment

It has become clear that Tl in the phosphate buffer is rather sensitive to radiation, while the same antibiotic is fairly stable when added to gelatin, albumin, nutrient broth or fish meats. The protecting effect on the inactivation of Tl was observed not only in such protein substances as

Table 4. Effect of temperature on the retention of test drugs in different media after irradiation at 0.1 Mrad. (Initial concentration of test drug: 100 ppm.)

Test drug	Medium	Remaining activity, %	
		0°C	19.2°C
Tl	Buffer, pH 7.0	4	1
	Albumin, 10%	75	60
	Broth, pH 7.0	95	80
	Gelatin, 10%	85	70
FF	Buffer, pH 7.0	42	35
	Albumin, 10%	36	32
	Broth, pH 7.0	49	39
	Gelatin, 10%	39	32
CTC	Buffer, pH 7.0	17	15
	Albumin, 10%	55	48
	Broth, pH 7.0	55	50
	Gelatin, 10%	70	68

Table 5. Effect of various sugars and amino acids on the retention of tylosin after irradiation at 0.1 Mrad. (Initial concentration of Tl: 100 ppm.)

Sugar	Remaining activity, %	Amino acid	Remaining activity, %
Control (without sugar or amino acid)	2.0	Glycine	1.2
		DL-alanine	2.8
		DL-valine	8.0
Glucose	9.4	Leucine	6.9
Fructose	10.0	Serine	8.5
Galactose	10.3	DL-threonine	2.2
Arabinose	7.8	L-phenylalanine	21.1
Xylose	8.1	L-tyrosine	22.5
Sucrose	12.1	DL-tryptophane	39.7
Maltose	8.6	Cystine	10.6
Lactose	11.1	Cysteine	3.8
Starch	1.1	DL-methionine	21.0
Dextrin	1.1	L-proline	5.5
		L-hydroxy proline	4.1
		Aspartic acid	2.6
		Glutamic acid	4.8
		Histidine	29.3
		Arginine	3.9
		Lysine	7.2

albumin, fish meats or gelatin but also in the broth medium consisting of comparatively low molecular weight substances such as amino acids, peptides and sugars, etc.

In this experiment, the effects of various sugars or amino acids in the test solution on the inactivation of Tl during irradiation were examined. All amino acids and sugars (mono- and disaccharides) were prepared at 1 mM concentration in $M/15$ phosphate buffer of pH 7.0. And the concentration of starch and dextrin were at 300 ppm with the same buffer solution, respectively.

A 1 ml of Tl solution (1,000 ppm) was added to 9 ml of each sugar or amino acid solution, and the test solutions thus prepared were subjected to 0.1 Mrad of radiation. The temperature during irradiation was fixed at 21°C. A mixture consisting of 1 ml Tl solution and 9 ml of phosphate buffer of pH 7.0 was used as a control which was treated in the same manner as the test solutions. The results obtained are shown in Table 5.

Either mono- or disaccharides in the test solutions resulted in an increase in Tl residue to 10% and corresponded to 5 times as much as that remaining in the control buffer solution. Adding starch or dextrin did not affect the residual level.

Among amino acids belonging to mono-amino monocarboxylic acid, valine and serine increased the Tl residue from 2 to 7–8%, but seemingly the protective effect of these amino acids are comparatively weak. However, neither glycine nor DL-threonine exhibited significant increase in Tl residue. Aromatic amino acids, such as phenylalanine, tryptophan and tyrosine showed a fairly high protective effect on the inactivation of Tl, especially tryptophan gave the highest retention of Tl to 40%.

Cystine and methionine in the test solution gave Tl residues to 11% and 21%, respectively; a very weak effect was seen in the solution containing cystine. Also weak protective effect was observed in the solution containing such imino group compounds as proline or hydroxyproline. Mono-amino dicarboxylic acids, such as aspartic acid and glutamic acid, exhibited a weak protective effect. A residual Tl as high as 29% was retained in the presence of histidine; arginine and lysine showed a rather weak effect, and residual values of 4% and 7%, respectively.

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