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FORMATION OF *N*-SUBSTITUTED PYRROLE-2-ALDEHYDES IN THE BROWNING REACTION BETWEEN D-XYLOSE AND AMINO COMPOUNDS. H. KATO & M. FUJIMAKI. *J. Food Sci.* **33**, 445–449 (1968)—From the reaction mixture of D-xylose and selected amino acid were isolated the following *N*-substituted pyrrole-2-aldehydes as methyl ester of 2,4-dinitrophenylhydrazone, respectively: (2-formylpyrrol-l-yl)acetic acid from glycine, 3-(2-formylpyrrol-1-yl)propionic acid from β-alanine, and 2-(2-formylpyrrol-1-yl)-4-methyl-valeric acid from L-leucine. The extent of pyrrolealdehyde formation from D-xylose and selected alkylamine or amino acid was in the following order which corresponded to the extent of melanoidin formation: *n*-butylamine > methylamine, β-alanine > glycine > DL-alanine.

RELATIONSHIPS AMONG SHEAR VALUES, SARCOMERE LENGTHS AND COOLING PROCEDURES IN TURKEYS. J. L. WELBOURN, R. B. HARRINGTON & W. J. STADELMAN. *J. Food Sci.* **33**, 450–452 (1968)—The chilling procedure treatments of 16°C, 16–8–0°C and 0°C exerted a significant effect only on the shear value of the turkey thigh muscle. The 0°C treatment resulted in the highest shear values. Significant differences in shear values were also observed between right and left thighs, and thigh muscles of hens and toms. Breast muscle of the toms was significantly higher. The outer slice of breast muscle also had a significantly higher shear value than other slices. Sarcomere lengths decreased with decreasing temperature, but not significantly. Correlations were not significant between shear values of breast and thigh muscles, or between shear values and sarcomere lengths.

ESSENTIAL AMINO ACID COMPOSITION OF CHICKEN MEAT AND DRIP AFTER 30 AND 90 DAYS OF FROZEN STORAGE. E. J. WLADYKA & L. E. DAWSON. *J. Food Sci.* **33**, 453–455 (1968)—Light and dark chicken meat from heavy hens was frozen, stored for 30 and 90 days, thawed, and drip was collected. Amino acid composition in dark and light meat drip from each was determined. Percentage of the essential amino acids in both dark and light meat decreased during frozen storage, and concentration of amino acids in the drip from each meat increased with storage time. Larger quantities of the essential amino acids were detected in the drip from light meat than from dark meat after both periods of storage. Concentration of each amino acid, as a percentage of the total amino acids, was similar in meat and drip.

COMPARISON OF SARCOMERE LENGTH TO OTHER PREDICTORS OF BEEF TENDERNESS. R. D. HOWARD & M. D. JUDGE. *J. Food Sci.* **33**, 456–460 (1968)—Sarcomere length in the longissimus dorsi muscles of 20 bovine animals was compared to other variables in predicting tenderness. The sarcomere length of a single muscle sample was of little value in predicting tenderness if certain carcass parameters were known. However, similar measurements taken at several locations across the cross section of the muscles showed that very small differences in contraction state were associated with marked differences in tenderness in restricted muscle areas. The study emphasized the importance of post-rigor contracture to tenderness but indicated that several estimates are necessary to characterize a single muscle.

ARBUTIN AND A RELATED GLUCOSIDE IN IMMATURE PEAR FRUIT. A. B. DURKEE, F. B. JOHNSTON, P. A. THIVIERGE & P. A. POAPST. *J. Food Sci.* **33**, 461–463 (1968)—Arbutin (p-hydroxyphenyl-β-D-glucoside) was separated from other phenols in an extract of immature fruit of *Pyrus* c.v. Kieffer by preparative paper chromatography and isolated as the penta-acetyl derivative. A monoacetylarbutin (p-hydroxyphenyl-6-0-acetyl-β-D-glucoside) was identified in the same pear extract. In addition, arbutin was found in both mature and immature pears, but at much higher levels in the immature fruit.

MICROSCOPIC CHARACTERISTICS OF COOKED MUSCLES SUBJECTED TO STRETCH-TENSION DURING RIGOR. E. M. BUCK & D. L. BLACK. *J. Food Sci.* **33**, 464–467 (1968)—Few consistent relationships between histological structure at the shearing site and shear force values were observed. Sections prepared from control samples exhibited significantly greater total amounts of perimysial connective tissue than sections prepared from stretched samples. Stretched sections tended to show greater amounts of perimysial tissue denaturation as indicated by degree of tissue granulation. A mechanical thinning of connective tissues due to stretching may account for a portion of the increased tenderness previously reported for stretched muscle samples.

PHOTOMICROGRAPHIC STUDIES OF DYNAMIC CHANGES IN MUSCLE FIBER FRAGMENTS. 1. Effect of Various Heat Treatments on Length, Width and Birefringence. R. L. HOSTETLER & W. A. LANDMANN. *J. Food Sci.*, **33**, 468–470 (1968)—Photomicrographs were made of muscle fiber fragments as the stage of the microscope was heated from room temperature to 80°C, or as fibers were held at 37, 45, 53, 61, 69, or 77°C on the heated stage for an hour. The possible relationship of changes in width, length and in birefringence brought about by heating to loss of moisture, water-holding properties, loss of acidic groups, and changes in tenderness are discussed. Changes in width appeared to be related to changes in water-holding capacity. Changes in length and loss of birefringence were related to loss of acidic groups, to coagulation of proteins, to volume change in cooked meat, and to changes in tenderness.

QUANTITATIVE METHODS FOR ANTHOCYANINS. 4. Determination of Individual Anthocyanins in Cranberry and Cranberry Products. T. FULEKI & F. J. FRANCIS. *J. Food Sci.* **33**, 471–478 (1968)—The ratio of individual anthocyanins was determined by separating the purified pigments on paper and measuring their relative quantities densitometrically. The densitometric response followed Beer's law and the difference in attenuancy between the four major cranberry anthocyanins was negligible. The amount of each anthocyanin was calculated from the total anthocyanin content and from the ratio of individual anthocyanins. The reproducibility of the method (coefficient of variation) was established as 6% for the arabinosides and 4 to 5% for the galactosides.

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FOOD VOLATILES: GAS CHROMATOGRAPHIC DETERMINATION OF PART TION COEFFICIENTS IN WATER-LIPID SYSTEMS. P. E. NELSON & J. E HOFF. *J. Food Sci.* **33**, 479–482 (1968)—the distribution of volatile solutes between two immiscible solvents was determined gas chromatographically. The procedure takes advantage of the relationship that exists between the partition coefficient and Henry's constants of the solute in the two separate solvent systems. Effects of salting-out and of temperature on the partial pressure of volatiles in dilute solutions were studied. The results may be utilized to quantify food volatiles in operations involving extraction and gas stripping prior to analysis.

INTENSITY-CONCENTRATION RELATIONSHIPS FOR SUGAR AND SALT SOLUTIONS. JOAN GORDON. *J. Food Sci.* **33**, 483–487 (1968)—Intensity of sweetness or saltiness was examined as a function of concentration and logarithm of concentration in solutions of sugar and salt in water and orange or tomato juice. No clear-cut basis for choosing between functions emerged, and the sources of such equivocalness in experimental situations are discussed.

LIMONIN MONOLACTONE, THE NONBITTER PRECURSOR RESPONSIBLE FOR DELAYED BITTERNESS IN CERTAIN CITRUS JUICES. V. P. MAIER & G. D. BEVERLY. *J. Food Sci.* **33**, 488–492 (1968)—Tissues of early-season navel oranges and grapefruit were found by paper electrophoretic procedures to contain a nonbitter precursor of limonin, but no significant amounts of limonin. Limonin is the intensely bitter triterpenoid dilactone responsible for the bitterness which develops in certain navel orange juices on standing. The nonbitter precursor was identified as limonin monolactone by comparison with the authentic compound prepared by partial hydrolysis of limonin and by acid-catalyzed conversion of the natural compound into limonin. Limonin monolactone is stable in the tissues of intact fruit (which are not bitter) because it is apparently not in direct contact with the acidic juice. It slowly converts into limonin (and the juice becomes bitter) when fruit tissues come in contact with the juice, after the juice is expressed from the fruit.

CALCIUM AND OXALATE IONS EFFECT ON THE TEXTURE OF CANNED APRICOTS. A. A. MOHAMMADZADEH-KHAYAT & B. S. LUH. *J. Food Sci.* **33**, 493–498 (1968)—Softening of canned apricots was accompanied by increase in soluble pectin and syrup viscosity. Calcium ions have the ability to decrease, to some extent, the movement of pectic material from the fruit to the syrup. Added oxalate ions removed calcium from pectin in the cell wall, causing an increase in water-soluble pectin in the syrup and softening of texture. Low potassium in the fruit seems to be related to low pH in the cell sap, causing hydrolysis of pectic materials through hydronium ion catalysis and softening during heat processing and storage.

SALTING AND DRYING FISH. 3. Diffusion of Water. F. R. DEL VALLE & J. T. R. NICKERSON. *J. Food Sci.* **33**, 499–503 (1968)—Two distinct phases, each with a Fickian diffusion coefficient, were found in the falling rate period. The diffusion coefficient for the first phase was greater than that for the second in all cases. It increased, passed through a maximum and then decreased with degree of salting, and was directly correlated with degree of muscle hydration. Variations in diffusion coefficient due to degree of salting and addition of acid, base, and phosphate are explained by the hydration-dependence of the coefficient. Temperature variation of both coefficients was not great.

PENETRATION GRADIENTS OF SODIUM NITRITE AND SODIUM TRIPOLYPHOSPHATE IN HADDOCK FILLETS. P. G. SCHEURER. *J. Food Sci.* **33**, 504–506 (1968)—Studies were carried out to determine the penetration gradients of sodium nitrite, containing radioactive sodium 24, and sodium tripolyphosphate, containing mostly phosphorus 32 in addition to sodium 24. In a parallel experiment, the penetration of sodium nitrite was also determined colorimetrically. Results on this technique compare closely with those obtained from the radiological method.

CO-DRIED CARBOHYDRATES EFFECT ON THE PERFORMANCE OF EGG YOLK SOLIDS. J. R. SCHULTZ, H. E. SNYDER & R. H. FORSYTHE. *J. Food Sci.* **33**, 507–513 (1968)—When egg yolk is dried and rehydrated, it loses its ability to form a stable foam. If sufficient carbohydrates are added to the egg yolk before dehydration, much of the foaming ability is retained by the rehydrated yolk. Removal of water from plain dried yolk irreversibly changes the structure of the low-density lipoproteins and foam-inhibiting free lipid is released from these lipoproteins. When yolk is co-dried with added carbohydrates, the carbohydrates partially protect the lipoproteins from this irreversible structural change.

HEAT DENATURATION OF THE OVOMUCIN-LYSOZYME ELECTROSTATIC COMPLEX—A SOURCE OF DAMAGE TO THE WHIPPING PROPERTIES OF PASTEURIZED EGG WHITE. J. A. GARIBALDI, J. W. DONOVAN, J. G. DAVIS & S. L. CIMINO. *J. Food Sci.* **33**, 514–524 (1968)—Heat denaturation of the ovomucin-lysozyme electrostatic complex results in damage to the whipping properties of pasteurized egg white. The rate of change in the whipping properties is first order with respect to both ovomucin and lysozyme concentrations, and the rate increases with increasing pH and temperature. The activation energy is 140 kcal at pH 7.5. Increase in ionic strength decreases the reaction rate. Removal of the product of the reaction, an irreversibly denatured ovomucin-lysozyme aggregate, restores whipping properties. The denaturation reaction decreases the mechanical stability of the foam. A longer whipping time is then required to obtain a satisfactory meringue. Whipping aids compensate for, but do not reverse, the denaturation.

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ALCOHOL:NAD OXIDOREDUCTASE (E.C. 1.1.1.1.) FROM PEAS. C. E. ERIKSSON. *J. Food Sci.* **33**, 525–532 (1968)—The enzyme catalyzes the oxidation of the primary aliphatic alcohols, especially 2-alken-1-ols, e.g., trans-2-hexen-1-ol, under the conditions used. It also catalyzes the reduction of aliphatic aldehydes, especially ethanal, hexanal, and unsaturated nonanals. The enzyme was used as a catalyst in experiments for determining equilibrium constants and the calculation of the free energy change of some alcohol-aldehyde systems in the presence of oxidized and reduced coenzyme. On the basis of the equilibrium constants obtained, the composition of various alcohol-aldehyde mixtures were calculated for different NAD^+:NADH ratios and different pH values.

GAMMA IRRADIATION AND ENRICHED CO_2 ATMOSPHERE STORAGE EFFECTS ON THE LIGHT-INDUCED GREENING OF POTATOES. R. ZIEGLER, S. H. SCHANDERL & P. MARKAKIS. *J. Food Sci.* **33**, 533–535 (1968)—Upon illumination with 3,000 lux for periods up to 20 days while maintaining the atmosphere, all tubers developed some greening. The tubers exposed to 200 and 400 Krad were inhibited from greening to the highest degree but suffered general quality loss. Fifty and 100 Krad caused a temporary inhibition of greening. The chlorophyll content of the lots treated with these two levels approached that of the controls after about 12 days of illumination. The levels of 10 to 20 Krad, which are suitable for the sprout inhibition of potatoes, did not cause significant inhibition of greening under any of the conditions of this experiment.

TOLERANCE OF BACTERIA FOR QUATERNARY AMMONIUM COMPOUNDS. P. R. SOPREY & R. B. MAXCY. *J. Food Sci.* **33**, 536–540 (1968)—When grown in gradient concentrations of quaternary ammonium compound, bacteria gained increasing tolerance, which resulted in more frequent occurrence of individual cells at the plateau of maximum tolerance. A similar, but reverse, pattern appeared with the loss of tolerance. *Pseudomonas fluorescens* gained tolerance more rapidly and to a higher level than *Escherichia coli.* The adapted cells were more resistant to quaternary ammonium compounds at low concentrations in germicidal effectiveness tests, but at levels of standard sanitizing recommendations there was no difference between the normal and the adapted cultures.

GAMMA IRRADIATION INFLUENCE ON THE STORAGE AND NUTRITIONAL QUALITY OF MUSHROOMS. J. D. CAMPBELL, S. STOTHERS, M. VAISEY & B. BERCK. *J. Food Sci.* **33**, 540–542 (1968)—Gamma irradiation of 100 krad markedly inhibits growth of freshly harvested mushrooms, as measured by the small number of broken veils. This could significantly increase storage life of mushrooms even under suboptimal conditions. Hedonic scores indicated that treated mushrooms would be acceptable, although untreated mushrooms were preferred. The respiration of the irradiated mushrooms accelerated up to three days after treatment, and then decelerated markedly. Diets that included 20% of irradiated mushrooms were fed to mice during late pregnancy and lactation, with no signicant effect on weight of the offspring.

INTESTINAL FLORA AND CHICKEN FLAVOR. N. D. HARRIS, D. H. STRONG & M. L. SUNDE. *J. Food Sci.* **33**, 543–547 (1968)—Chickens, including those reared under germfree, gnotobiotic (in contact only with *Clostridium perfringens, Escherichia coli,* and *Streptococcus faecalis*) or conventionel conditions were compared for flavor, utilizing the triangle taste testing technique. The results of these tests indicated a highly significant difference between the flavor of cooked meat from chickens reared under germfree conditions and those reared in the conventional manner. Less pronounced flavor differences existed between gnotobiotic birds and conventionally reared birds. No significant difference was evident between birds grown under gnotobiotic conditions and those grown under germfree conditions.

BIOLOGICAL DEGRADATION OF CHLOROPHYLL IN A SYSTEM USING BELL PEPPERS *(Capsicum frutescens).* R. F. McFEETERS & S. H. SCHANDERL. *J. Food Sci.* **33**, 547–553 (1968)—A degradation system was developed based on the incorporation of radioactive chlorophyll into Bell peppers *(Capsicum frutescens).* ^{14}C-labeled chlorophyll *a* was prepared and injected into ripening carpels for different periods of time. The distribution of radioactivity into three fractions prepared by extraction was established. Preliminary chromatography of two fractions did not permit isolation of radioactive products. The system developed provides a tool for further studies of the biological degradation of chlorophyll. Labeling facilitates isolation, identification, and establishment of origin of small amounts of breakdown products.

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Formation of N-Substituted Pyrrole-2-Aldehydes in the Browning Reaction Between D-Xylose and Amino Compounds

SUMMARY—The formation of *N*-substituted pyrrole-2-aldehydes in the browning reaction between D-xylose and selected amino acids was investigated.

In order to facilitate the separation of the reaction products, the carbonyl compounds formed in the browning solution were converted into 2,4-dinitrophenylhydrazones (2,4-DNPs) and then esterified with diazomethane. Isolation of the esterified 2,4-DNP was accomplished by column chromatography with alumina. Chemical structures of the isolated 2,4-DNPs were confirmed by elementary analyses, ultraviolet and visible absorption spectra, and nuclear magnetic resonance spectra.

The following *N*-substituted pyrrole-2-aldehydes were isolated as methyl ester of 2,4-DNP, respectively: (2-formylpyrrol-1-yl)acetic acid from the reaction mixture of D-xylose and glycine, 3-(2-formylpyrrol-1-yl)propionic acid from β-alanine, and 2-(2-formylpyrrol-1-yl)-4-methyl-valeric acid from L-leucine.

The extent of pyrrolealdehyde formation from D-xylose and selected alkylamine or amino acid was in the following order which corresponded to the extent of melanoidin formation: *n*-butylamine > methylamine, β-alanine > glycine > DL-alanine. *N*-substituted pyrrole-2-aldehyde was rather stable and was not considered to be an important intermediate of melanoidins. However, some correlationships between the formation of pyrrolealdehyde and that of melanoidins were demonstrated and discussed.

INTRODUCTION

3-Deoxyosuloses (3-deoxyosones) have proved to be an important intermediate in non-enzymic browning reaction between aldoses and amino compounds (Kato, 1960; Anet, 1960; McWeeny *et al.*, 1963).

Under acidic condition, 3-deoxyhexosuloses easily decompose to 3,4-dideoxyhexosulos-3-enes and 5-hydroxymethyl-2-furaldehyde (Anet, 1961, 1962). But, in neutral solution, the formation of furaldehyde from 3-deoxyosulose would be limited to a small amount, if any, and, in the presence of amino compounds, 3-deoxyosulose forms melanoidins (Kato, 1960).

Some investigators showed that 5-hydroxymethyl-2-furaldehyde or 2-furaldehyde was not the major intermediate in melanoidin formation (Kato, 1959, 1963a; McWeeny *et al.*, 1963; Burton *et al.*, 1963; Song *et al.*, 1966a; Song *et al.*, 1966b).

Kato (1967) isolated a new product, N-alkyl-pyrrole-2-aldehyde as its 2,4-dinitrophenylhydrazone (2,4-DNP) from the reaction mixture of D-xylose or 3-deoxy-D-pentosulose (3-DP) with alkylamine such as methylamine or *n*-butylamine heated at 55 to 100°C in aqueous or methanol solution neutralized with acetic acid. In addition, Kato (1967) also reported that, in the reaction mixture of L-rhamnose with *n*-butylamine was produced the corresponding pyrrolealdehyde, 1-*n*-butyl-5-methyl-pyrrole-2-aldehyde; D-glucose, on the contrary, under the same reacting condition, did not form any detectable amount of the corresponding pyrrole-aldehyde. However, effect of *N*-substituted pyrrole-2-aldehyde described above on melanoidin formation is not yet clear.

In the present investigation the reactions between D-xylose and several amino acids instead of alkylamines were examined and the formation of the corresponding pyrrole-aldehyde was established. *N*-Substituted pyrrole-2-aldehyde was not so unstable, indicating that it is not the major intermediate of melanoidins, but some correlationships between the formation of pyrrolealdehyde and that of melanoidins were demonstrated and discussed.

EXPERIMENTAL

Melting points were uncorrected. Ultraviolet and visible absorption spectra were recorded with a Hitachi recording spectrophotometer Model EPS-3T. Nuclear magnetic resonance (NMR) spectra were recorded with a JNM-4H-100 spectrometer in deuteriochloroform or nitrobenzene with tetramethylsilane as an internal standard.

Isolation of (2-formylpyrrol-1-yl)acetic acid

D-Xylose (15 g), glycine (7.5 g) and sodium bicarbonate (0.84 g) were dissolved in water (50 ml) and the solution (initial pH 6.5) was heated in boiling water for 1 hr. The carbonyl compounds formed in the resulted brown solution were reacted with reagent of 2,4-dinitrophenylhydrazine (2.0 g) (Shriner *et al.*, 1956) at room temperature for 2 hr.

The precipitate of 2,4-DNP (2.0 g) was filtered with suction, washed with water, then dried. This was suspended in tetrahydrofuran (30 ml), and to the suspension was added ether solution (30 ml) of diazomethane (*ca.* 0.5 g) (DeBoer *et al.*, 1956) and the mixture was allowed to stand at room temperature (*ca.* 20°C) for 2 days.

Under the condition indicated, 3-deoxypentosulose bis-2,4-DNP (Kato, 1960, 1962) decomposed in a significant rate; *N*-*n*-butylpyrrole-2-aldehyde 2,4-DNP, on the contrary, was fairly stable. Consequently, 2,4-DNPs of *N*-substituted pyrrole-2-aldehydes would be generally considered to be stable under the condition. Then, the reacted mixture given above was evaporated under reduced pressure and dried. The residue was extracted with hot benzene, filtered, and the filtrate was passed through a column of acid-treated alumina (Merck & Co., 40 g) (Kato, 1963b) to adsorb 2,4-DNP, followed by development with benzene—ethyl acetate (19:1 v/v).

The second eluted brown band was collected, evaporated, and rechromatographed by the same procedure to give crystalline residue. Recrystallization from benzene—petroleum ether yielded 35 mg of dark-red needles, methyl ester

of (2-formylpyrrol-1-yl)acetic acid 2,4-DNP, m.p. 224~225°C; λ_{max}^{EtOH} 309 (ϵ, 8,520), 406 mμ (ϵ, 26,900). *Anal.* Calcd. for $C_{14}H_{13}N_5O_6$: C, 48.42; H, 3.77; N, 20.17. Found: C, 48.42; H, 3.91; N, 19.85%.

Isolation of 3-(2-formylpyrrol-1-yl)propionic acid

D-Xylose (15 g), β-alanine (8.9 g), and sodium bicarbonate (0.84 g) were dissolved in water (50 ml) and the solution was heated in boiling water for 1 hr. After esterification of the precipitate of 2,4-DNP (3.6 g) with diazomethane as described above, chromatography was carried out on a column of alumina (Merck & Co., 50 g). At least four bands were separated by development with benzene—ethyl acetate (19:1 v/v) and the main band was collected and rechromatographed.

The crystals thus obtained were washed with benzene—petroleum ether and dried to give 88 mg of purple needles, methyl ester of 3-(2-formylpyrrol-1-yl)propionic acid 2,4-DNP, m.p. 184~186°C; λ_{max}^{EtOH} 309 (ϵ, 8,540), 406 mμ (ϵ, 28,500). *Anal.* Calcd. for $C_{15}H_{15}N_5O_6$: C, 49.86; H, 4.18; N, 19.39. Found: C, 49.56; H, 3.92; N, 19.30%.

Isolation of 2-(2-formylpyrrol-1-yl)-4-methyl-valeric acid

D-Xylose (15 g), L-leucine (13.1 g), and sodium bicarbonate (0.84 g) were suspended in water (230 ml) and heated in boiling water for 5 hr. During heating, brown precipitate was formed. The procedures for formation of 2,4-DNP (yield, 4.5 g), esterification and chromatography were similar to the case of the reaction of D-xylose and glycine. Crude *N*-substituted pyrrolealdehyde 2,4-DNP fraction (54 mg), crude isovaleraldehyde 2,4-DNP fraction (934 mg), and crude 2-furaldehyde 2,4-DNP fraction (55 mg) were separated. The crude pyrrolealdehyde 2,4-DNP fraction was purified by rechromatography and by recrystallization from benzene—petroleum ether. Pure 2,4-DNP of 2-(2-formylpyrrol-1-yl)-4-methyl-valeric acid methyl ester was isolated in the yield of 34 mg, orange needles, m.p. 158°C (shrunk at 155°C); λ_{max}^{EtOH} 310 (ϵ, 7,760), 406 mμ (ϵ, 28,700). *Anal.* Calcd. for $C_{18}H_{21}N_5O_6$: C, 53.59; H, 5.25; N, 17.36. Found: C, 53.81; H, 5.05; N, 17.14%.

Densitometry of thin layer chromatogram

Solution of D-xylose (2 or 0.5 *M*) and methylamine (2 or 0.5 *M*) neutralized with acetic acid was heated at 100°C, and to the resulted brown solution was added 2,4-dinitrophenylhydrazine reagent (Shriner *et al.*, 1956). After 2 hr at room temperature, the precipitate of 2, 4-DNP was filtered on a filter paper, washed with water and 50% ethanol, then dried. The 2,4-DNPs thus obtained were dissolved in ethyl acetate or/and tetrahydrofuran through the above filter paper, and filled up to 50 ml. 2,4-DNPs of both 3-DP and *N*-methylpyrrole-2-aldehyde in this solution were determined by the following procedure. The solution of 2,4-DNPs given above was spotted on a Toyo Chromatosheet (3 × 10 cm, polyethylene film coated with silica gel, Toyo Kagaku-sangyo Co., Ltd., Tokyo) by the aid of micropipet (0.01 ml), and developed with benzene—ethyl acetate (2:1 v/v). The developed thin layer chromatogram was recorded with Densicord Model 542 (Photovolt Corporation) with 420 mμ filter. The part of the corresponding absorption peak of the recorded paper was cut, weighed, and calculated from the standard curve of the authentic sample submitted to the similar procedure.

RESULTS AND DISCUSSION

Isolation of 2,4-DNPs of *N*-substituted pyrrole-2-aldehydes

Three amino acids, glycine which has the simplest structure, β-alanine which has a ω-amino group, and L-leucine, were selected and reacted with D-xylose in aqueous solution adjusted to initial pH 6.5 with sodium bicarbonate at 100°C, respectively. After heating, carbonyl compounds formed in the resulted brown mixture were converted into 2,4-DNPs. In case of amino acid, the *N*-substituted pyrrolealdehyde, which is expected to be formed, should have a carboxyl group in the *N*-substituted group.

In order to facilitate separation of the 2,4-DNP by column chromatography, the mixture of 2,4-DNPs obtained was treated with ether solution of diazomethane to convert carboxylic acid into its methyl ester. Thin layer chromatograms of the mixture of 2,4-DNPs before and after treatment with diazomethane were shown in Fig. 1. After esterification, a characteristic brown spot appeared respectively. Isolation of the esterified 2,4-DNP was accomplished by adsorption chromatography on a column of alumina or acid-treated alumina (Kato, 1963b). By this procedure, the following 2,4-DNPs were isolated: $C_{14}H_{13}N_5O_6$, 2,4-DNP of III in Fig. 2, dark-red needles, m.p. 224~225°C, from the reaction mixture of D-xylose and glycine; $C_{15}H_{15}N_5O_6$, 2,4-DNP of IV in Fig. 2, purple needles, m.p. 184~186°C, from β-alanine; and

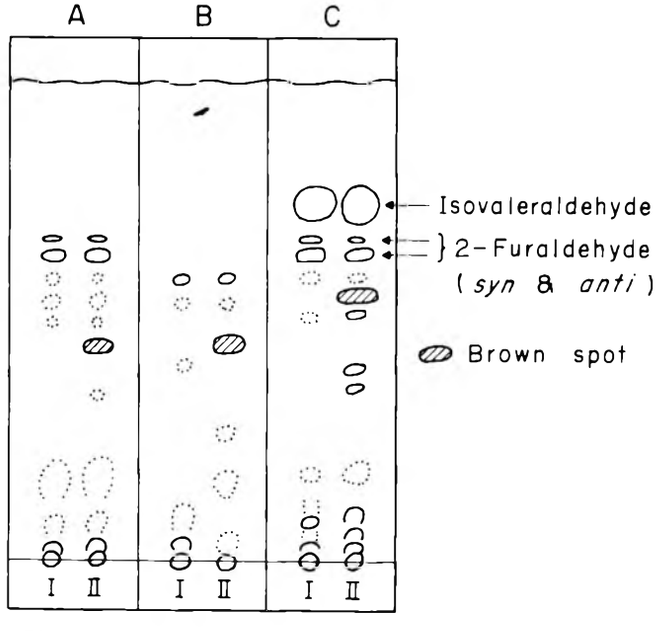


Fig. 1. Thin layer chromatograms of 2,4-DNPs of carbonyl compounds formed by the reaction between D-xylose and amino acid in aqueous solution (initial pH 6.5) before and after methylation with diazomethane. Thin layer plates coated with Silica Gel G according to Stahl were developed with benzene—ethyl acetate (19:1 v/v).
A: D-xylose and glycine, at 100°C for 1 hr.
B: D-xylose and β-alanine, at 100°C for 1 hr.
C: D-xylose and L-leucine, at 100°C for 5 hr.
I: Before treatment with diazomethane.
II: After treatment with diazomethane.

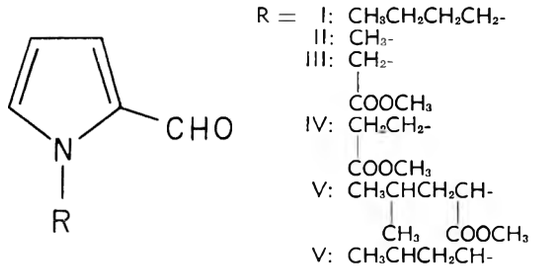


Fig. 2. Structure of N-substituted pyrrole-2-aldehydes isolated.

$C_{18}H_{21}N_5O_6$, 2,4-DNP of V in Fig. 2, orange needles, m.p. 158°C, from L-leucine.

Confirmation of the structures of pyrrolealdehydes isolated

Ultraviolet and visible absorption spectra of 2,4-DNPs of III, IV and V were given in Fig. 3. All these derivatives showed absorption peaks at 309~310 and 406 mμ.

As described in the previous paper (Kato, 1967), 2,4-DNP of *N-n*-butyl- or *N*-methyl-pyrrole-2-aldehyde (I or II in Fig. 2) also showed absorption peaks at 308~309 and 407 mμ. Accordingly, ultraviolet and visible absorption spectra of these derivatives (2,4-DNPs of I to V) are very close, indicating that these are compounds of analogous series.

With a view to confirming the structures, NMR spectra of 2,4- DNPs of III and V were recorded (Fig. 4). From the results of examination of the spectra, it was considered that their structures corresponded to *N*-substituted pyrrole-2-aldehyde formed from the parent amino acid respectively.

Summarizing the facts described above, it was concluded that III was methyl ester of (2-formylpyrrol-1-yl)acetic acid, IV was methyl ester of 3-(2-formylpyrrol-1-yl)propionic acid, and V was methyl ester of 2-(2-formyl-pyrrol-1-yl)-4-methyl-valeric acid (Fig. 2).

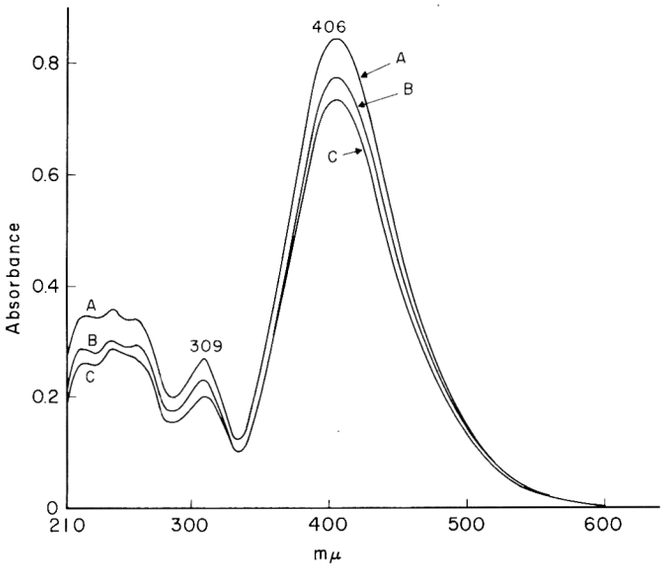


Fig. 3. Ultraviolet and visible absorption spectra of N-substituted pyrrole-2-aldehyde 2,4-DNPs in ethanol.
A: 2,4-DNP of III, 1.055 mg/100 ml.
B: 2,4-DNP of IV, 0.985 mg/100 ml.
C: 2,4-DNP of V, 1.049 mg/100 ml.

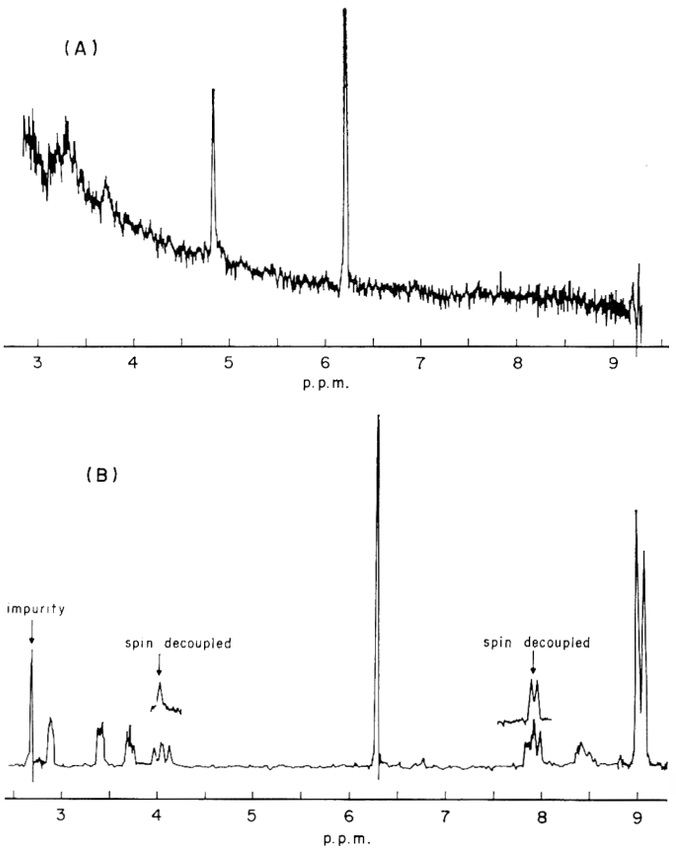
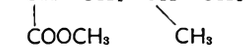


Fig. 4. NMR spectra (100 Mc) of N-substituted pyrrole-2-aldehyde 2,4-DNPs.
A: 2,4-DNP of III in nitrobenzene. N-substituted group: $-CH_2COOCH_3$.
B: 2,4-DNP of V in $CDCl_3$. N-substituted group:



Comparison of browning rate and yield of pyrrolealdehyde

As amino compounds, *n*-butylamine, methylamine, glycine, β-alanine, and DL-alanine were used, and color intensity and yield of pyrrolealdehyde in the reaction with D-xylose were compared. Amino compound (0.1 mole) was dissolved in water (50 ml) and adjusted to pH 6.5 with acetic acid or sodium bicarbonate, and then D-xylose (0.1 mole) was dissolved into the solution. After heating at 100°C for 1 hr, the corresponding *N*-substituted pyrrole-2-aldehyde 2,4-DNP was isolated. In the case of *n*-butylamine or methylamine, the isolation procedure described in the previous paper (Kato, 1967) was carried out. In the case of DL-alanine, corresponding crude 2,4-DNP was separated but further purification was not accomplished.

The results were shown in Table 1. The yield of pyrrolealdehyde was in the following order: *n*-butylamine > methylamine, β-alanine > glycine > DL-alanine. Color intensity (optical density at 470 mμ) was also in the same order. These facts indicate that ω-amino group attached to longer carbon chain has stronger reactivity, and that carboxyl group inhibits the reaction especially in the case of α-amino acid.

Table 1. Comparison of browning (color intensity) and yield of *N*-substituted pyrrole-2-aldehyde.

Amino compound reacted[1]	O.D. at 470 mμ × dilution	Structure of pyrrolealdehyde isolated	Yield	
			2,4-DNP (mg)	free aldehyde (mmole)
n-Butylamine[2]	562	I	277	0.83
Methylamine[2]	400	II	83	0.29
β-Alanine	420	IV	88[3]	0.24
Glycine	264	III	35[3]	0.10
DL-Alanine	113	/	25[3,4]	/

[1] D-Xylose (0.1 mole), amino compound (0.1 mole), and sodium bicarbonate (0.84 g) were dissolved in water (50 ml) (initial pH 6.5) and heated in boiling water for 1 hr.
[2] Neutralized with acetic acid (0.1 mole).
[3] Isolated as methyl ester.
[4] Crude powder not submitted to further purification.

Change of pyrrolealdehyde during heating

D-Xylose (2 *M*) and methylamine (2 *M*) in aqueous solution neutralized with acetic acid (initial pH 6.5) were heated at 100°C. Changes of optical density (470 mμ), 3-DP, and *N*-methylpyrrole-2-aldehyde (II) during heating were investigated (Fig. 5). Determinations of 3-DP and II were carried out by densitometry of thin layer chromatograms of their 2,4-DNPs. 3-DP, an intermediate of melanoidin formation, decreased after only 15 min at 100°C. As already reported (Kato, 1960), in the browning reaction of *N*-n-butyl-D-xylosylamine and acetic acid in methanol, 3-DP reached the maximum amount after 7 min at 55°C. On the contrary, *N*-methylpyrrole-2-aldehyde (II) was rather stable, although it decreased after longer heating time (2 hr), indicating that II is not the main intermediate of melanoidin formation.

PH dependency of the formation of pyrrolealdehyde

Solution of D-xylose, methylamine, and acetic acid (each 0.5 *M*) was adjusted to each pH (range 2 to 8) with HCl or NaOH and heated at 100°C for 1 hr. Optical density (470 mμ), 3-DP, and *N*-methylpyrrole-2-aldehyde (II) were determined (Fig. 6). Formation peak of II was around at pH 3.5 to 4.0. In the case of 3-DP, it was around at pH 4.0 to 4.5, but at higher pH, 3-DP would become more unstable to form melanoidins. At pH 2, neither II nor melanoidin was formed.

From the results shown in Fig. 6, some correlationships among melanoidin, 3-DP, and pyrrolealdehyde were expected.

Correlationship between formation of melanoidin and pyrrolealdehyde

From the result in Fig. 5, pyrrolealdehyde was not considered to be an important intermediate of melanoidin formation. However, from the results in Table 1 and Fig. 6, formation reaction of melanoidins was considered to be closely connected with the formation of pyrrolealdehyde. As shown in Fig. 1, 2-furaldehyde was formed in the cases of α-amino acids, glycine and L-leucine, from

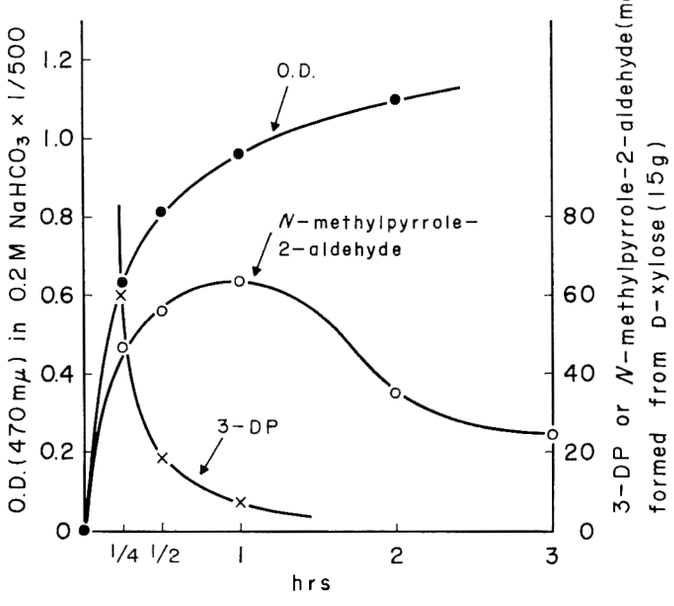


Fig. 5. Changes of optical density (470 mμ), 3-deoxypentosulose (3-DP), and N-methylpyrrole-2-aldehyde during heating (100°C) of D-xylose (2 M) and methylamine (2 M) in aqueous solution adjusted to pH 6.5 with acetic acid.

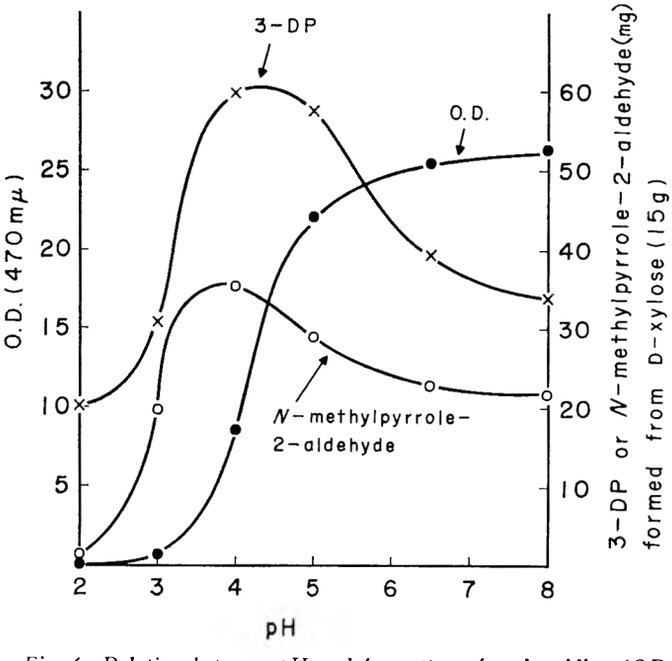


Fig. 6. Relation between pH and formation of melanoidins (OD at 470 mμ), 3-DP, and N-methylpyrrole-2-aldehyde. Aqueous solution of D-xylose, methylamine, and acetic acid (each 0.5 M) was adjusted to each pH with HCl or NaOH and heated at 100°C for 1 hr.

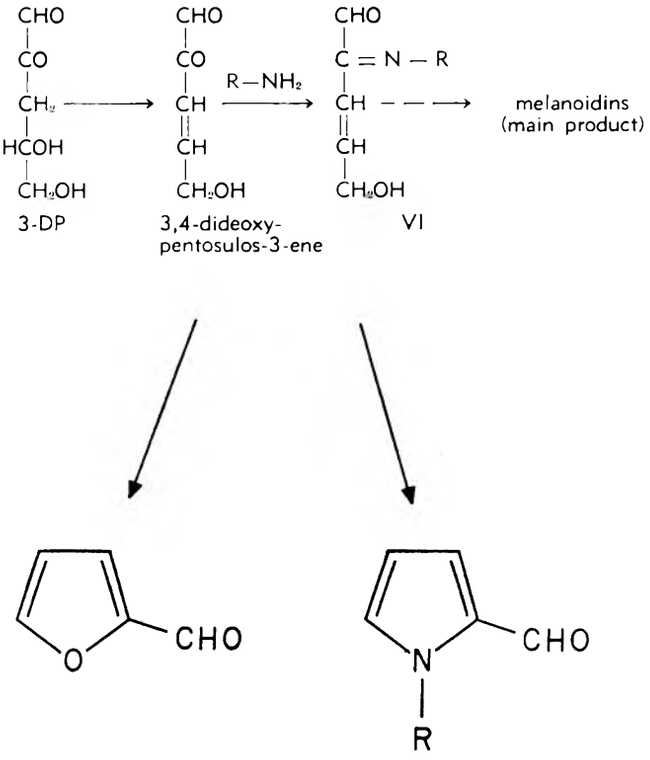


Fig. 7. Formation of 2-furaldehyde, N*-substituted pyrrole-2-aldehyde, and melanoidins from 3-DP and amino compound.*

which both melanoidins and pyrrolealdehyde were formed in less amount; on the other hand, 2-furaldehyde was not detected in the case of β-alanine, from which much melanoidins and pyrrolealdehyde were formed (Table 1).

These facts indicate that 2-furaldehyde is a by-product in melanoidin formation. As discussed in the previous paper (Kato, 1967), the key reaction for pyrrolealdehyde formation is considered to be condensation of amino group to C-2 keto group of 3,4-dideoxypentosulos-3-ene. It is reasonable that ω-amino group is more active for the condensation reaction than the amino group of α-amino acid. If the condensation reaction should not occur, a part of 3,4-dideoxypentosulos-3-ene would form 2-furaldehyde, as observed in the case of glycine or L-leucine (Fig. 1). Therefore, the condensation product (VI in Fig. 7) is postulated to form both pyrrolealdehyde, a by-product, and melanoidins, the main product. These relationships were illustrated in Fig. 7. Song *et al.* (1966b) postulated that such condensation product (VI) would form melanoidins through polymerization.

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For abbreviation, "pyrrole-2-aldehyde" was used instead of "pyrrole-2-carboxaldehyde."

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Relationships Among Shear Values, Sarcomere Lengths and Cooling Procedures in Turkeys

SUMMARY—Studies were conducted to determine the effect of different chilling procedures after slaughter on the tenderness of the breast and thigh muscles of turkeys as measured by shear press values. Measuring sarcomere lengths determined the effect of the chilling procedures on length of muscle fibrils and their correlation with shear press values. Three chilling treatments were used: (1) 16°C for three hr; (2) 16°C for 45 min, 8°C for 45 min, and 0°C for 90 min; and (3) 0°C for 3 hr. The 0°C treatment for 3 hr resulted in a significant increase in shear press values for thigh muscle in both studies. Shear values also increased for breast muscle in the same 0°C treatment group, but not significantly.

Shear values for the left thigh muscle were significantly higher than for the right in Experiment I, while in Experiment II hens had significantly higher thigh shear values than toms. In Experiment I with younger birds, shear values were significantly higher in the breast muscle of toms than in hens. The surface slice of 3 slices of breast muscle had higher shear values in both experiments. Chilling treatments resulted in a progressive shortening of sarcomere lengths in breast and thigh muscles with decreasing temperature, and the sarcomere lengths were shorter for breast muscle than for thigh. No significant correlations were found between shear values of breast and thigh muscles, or between shear values and sarcomere length.

INTRODUCTION

THERE IS REASON to believe that temperature changes of the chilling water in the poultry processing plant may affect the tenderness of the end product. In present processing procedures, the freshly killed, plucked and eviscerated birds are immersed directly into a chill tank of crushed ice and water (about 0°C to 4°C) which would probably result in maximum cold shortening. Locker *et al.* (1963) described the phenomenon of cold shortening in isolated beef muscle with maximum shortening occurring at 2°C. In his work on isolated breast muscle of chickens and turkeys, Smith (1966) observed a cold shortening effect, with the shortening at about 0°C greater than at 14–16°C. Reportedly, Dr. Cook of the University of Sidney (Briskey *et al.*, 1966) has also noticed cold shortening in chicken breast muscle.

Shortening of muscle fibers has been related to toughness. Comments by B. B. Marsh and R. C. Cassens (Briskey *et al.*, 1966) on this effect indicate that the extent of cold shortening has a very pronounced effect on muscle tenderness, and that various methods which cause muscles to shorten result in a substantial increase in toughness. Marsh *et al.* (1966) working with beef and lamb found there was excess toughening from intermediate amounts of shortening, while relatively tender meat resulted from low or very high shortening values.

Thus, there is reason to suspect that chilling procedure may influence the tenderness of poultry. Dodge *et al.* (1960) suggested that the temperature of the aging medium effects rigor mortis and the level of tenderness of the breast muscle of poultry at a given time post-mortem. However, de Fremery *et al.* (1960) found no significant difference in shear values on intact breast muscle of poultry aged at various temperatures between 0°C to 40°C. May *et al.* (1962) found that the breast muscle of 10-week-old chickens was significantly more tender at 0°C and 19°C over all periods of aging than at 37°C. There was no significant difference between aging temperatures for 72-week-old hens. Cooking was done in an electronic oven. It should be pointed out that most of the work on cold shortening has been done on excised muscle.

These studies were to determine the effect of different chilling procedures after slaughter on tenderness of the cooked turkey meat as measured by shear press values. A second experiment allowed for a longer period between killing and chilling, because it was felt this would more closely follow commercial procedures. Sarcomere lengths were measured in the second study to determine the effect of the chilling procedures on length of muscle fibrils, and their correlation with shear press values.

PROCEDURE

Experiment I

Seventy-two turkeys (equal number of young toms and hens), varying in age from 18–21 wk, were killed, eviscerated and placed in a chilling treatment tank within 10 min after slaughter. The 3 chilling treatments used throughout were: (1) 16°C for 3 hr; (2) 16°C for 45 min, 8°C for 45 min and 0°C for 90 min; and (3) 0°C for 3 hr. Equal numbers of birds were placed in the different chilling treatments on a random basis. The turkeys were killed over a 4-week period (18/wk, 6/day) with toms and hens on alternate weeks. All temperatures were maintained within ±1°C. Immediately after chilling, the birds were wrapped in foil and placed in a preheated, thermostatically controlled electric oven at a temperature of 163°C until a deep, internal breast temperature of 83°C was reached. The cooked birds were removed and chilled overnight for 12 hr in a 2°C chamber.

Shear measurements were then made with an Allo-Kramer shear press on samples from both right and left breast and thigh. Three slices were taken from the pectoralis major muscle on each side of the breast, and the biceps femoris muscle was used from each thigh for shear value measurements. A 2500-lb ring was used on the shear press with a range setting of 10 for breast and 50 for thigh, and a downstroke time of 45 sec.

Table 1. Mean shear values for thigh muscle for treatment and side for experiment I.[1]

	Treatment temperatures			
	16°C	16-8-0°C	0°C	Average
Left	10.61	11.31	13.08	11.67
Right	10.50	10.85	12.50	11.28
Average	10.55	11.08	12.79	

[1] Shear values given in kilograms per gram of muscle.

Experiment II

In this experiment 60 turkeys, equal numbers of toms and hens, varying in age from 25–28 wk were used. The birds were held for 30 min after slaughter, then placed in the chilling water at the prescribed treatment temperatures. When removed from the chilling water after 3 hr, the birds were sawed into equal right and left halves. One half was cooked, chilled and used for shear measurements as in the first experiment. Samples from the breast and thigh from the other half were used for sarcomere length measurements. Approximately 2–5 g of tissue and 50 ml of 0.08 *M* KCl were blended in a homogenizer for 1 min. The sarcomeres of 10 myofibrils were measured in each sample using a phase-contrast microscope with an eyepiece micrometer. All other conditions were the same as in Experiment I.

ANALYSIS

A COMPUTER ANALYSIS of variance for factorial design programmed by Health Sciences Computing Facility, UCLA, was conducted on all data. A correlation with transgeneration programmed by the same facilities was used to compare shear values for breast and thigh in Experiment I, and sarcomere and shear press values in Experiment II.

RESULTS AND DISCUSSION

THE CHILLING TREATMENTS did exert a significant effect on the shear values of thigh muscle in both Experiments I ($P < .001$) and II ($P < .025$). The 0°C chilling treatment for 3 hr caused a significant increase in the shear measurements (Tables 1 and 2). The shear press values for the breast muscle were not significantly affected by the chilling treatment in these two experiments, although an increase in shear values was noted for the 0°C treatment (Table 3).

For thigh muscle in Experiment I there was also a side effect which was significant ($P < .01$), with the left side having higher shear values (Table 1). There was no significant difference in shear values for sides in breast muscle, but the right side had consistently higher measurements than the left. In Experiment II the sex of the birds had a significant effect ($P < .05$) on the shear values of the thigh muscle with the values for the hens being higher (Table 2).

Table 2. Mean shear values for thigh muscle for treatment and sex for experiment II.[1]

	Treatment temperatures			
	16°C	16-8-0°C	0°C	Average
Toms	8.83	8.84	9.58	9.08
Hens	9.54	9.91	12.15	10.53
Average	9.19	9.37	10.86	9.81

[1] Shear values given in kilograms per gram of muscle.

Table 3. Mean shear values for breast muscle for treatments.[1]

	Treatment temperatures		
	16°C	16-8-0°C	0°C
Experiment I	7.50	7.47	7.81
Experiment II	10.03	9.38	11.10

[1] Shear values given in kilograms per gram of muscle.

This was contrasted with the significant sex effect ($P < .005$) on the breast muscle in Experiment I where the toms had the higher shear measurements. The mean shear values were 8.39 kg/gram of breast muscle for the toms and 6.80 kg/gram for the hens. There was a significant difference between the shear values of the 3 slices of breast muscle in both Experiments I ($P < .001$) and II ($P < .01$) (Table 4). The first (exterior) slice had the highest shear values, but the difference between slices was much greater in the younger birds of Experiment I.

In the case of the sarcomere measurements on the breast muscle in Experiment II only the weeks exerted a significant effect ($P < .025$) (Table 5). This effect is probably due to the variance caused by the unexpected drop in sarcomere length for the 3rd wk for the 16-8-0°C treatment. While the treatment effect was not significant, the breast muscle does show a decrease in sarcomere length with decreasing temperature (Table 5). A similar effect occurs with the thigh muscle with the measurements being 2.38, 2.36 and 2.21 microns for the 3 treatments in order of decreasing temperature. The sarcomere lengths for the thigh are generally longer than for the breast muscle.

No significant correlation was found between shear values for breast and thigh, or between shear values and sarcomere measurements.

These results suggest that turkey processors could possibly increase tenderness of their products by gradually chilling the birds to 0°C rather than placing them directly

Table 4. Mean shear values for breast muscle for slices.[1]

	Slice 1	Slice 2	Slice 3
Experiment I	8.23	7.17	7.38
Experiment II	10.39	10.20	9.91

[1] Shear values given in kilograms per gram.

Table 5. Sarcomere length of breast muscle for weeks and treatment.[1]

	Treatment temperatures			
Weeks	16°C	16-8-0°C	0°C	Average
1	2.05	2.21	1.41	1.89
2	2.00	1.86	2.02	1.96
3	2.08	0.92	1.51	1.51
4	2.06	2.12	1.52	1.90
5	1.74	2.09	2.08	1.97
Average	1.99	1.84	1.71	1.84

[1] Sarcomere lengths in microns.

in an ice bath of about 2°C. There was little difference between the treatments of 16°C and the 16-8-0°C in shear press values. Further studies would need to be made to determine whether these differences in chilling procedures would hold after several days' aging or after freezing.

The increase in shear values with decrease in temperature would be first expected due to the cold shortening phenomenon. However, in chilling the intact muscle, the sarcomere measurements showed only a slight amount of shortening with decreasing temperature, and this shortening was less for the thigh muscle which had the significant increase in shear values. One would suspect the increase in shear values might be due to the temperature effect on some muscle component such as connective tissue, which is higher in the thigh muscle than in the breast, rather than on muscle shortening.

Lack of a significant difference between chilling treatments in shear press values for breast muscle agrees with the work of de Fremery *et al.* (1960) and May *et al.* (1962). The authors do not know of any other studies with which to compare these results on the effects of chill temperatures on intact thigh muscle as measured by shear press.

Information on the rate of temperature decline in the breast and thigh muscles for the various treatments might be useful to explain some of the effects found or some of the bird-to-bird variability. However, this information was not obtained in these studies. The difference between the shear values for the outer and inner slices of breast muscle can possibly be explained by higher heat and denaturation effects received by the outer layers of tissues during cooking. This agrees with Experiment II where there is less difference in shear values between outer and inner slices due to the greater protective effect of the thicker skins on the older, heavier birds. Note the little difference in shear values between breast and thigh muscles in Experiment II, as compared with the wide difference in the younger birds of Experiment I. This change was due primarily to the increase in shear values for the breast muscle, although there was also a decrease in the thigh shear values from Experiment I. Age does not seem to have the same effect on thigh muscle as it does on breast muscle, as measured by shear press values.

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Essential Amino Acid Composition of Chicken Meat and Drip After 30 and 90 Days of Frozen Storage

SUMMARY—Light and dark meat from 24 heavy hens was frozen and stored for 30 and 90 days. After storage, drip was collected and moisture and fat removed. The essential amino acids in acid and alkaline hydrolyzates of dark and light chicken meat and drip were determined by microbiological assay. Acid hydrolyzates from drip were also analyzed on a Beckman/Spinco Amino Acid Analyzer. The concentration of amino acids in both dark and light meat decreased and the concentration in drip increased with increasing storage time. In addition, larger quantities of essential amino acids were detected in drip from frozen light meat than from frozen dark meat after both periods of storage. The quantitative results obtained from the analyzer were in general agreement with those obtained by microbiological assay but were somewhat higher. The concentration of each amino acid, as a percentage of total amino acids, was similar in meat and drip.

INTRODUCTION

SIMILARITIES IN THE AMINO ACID composition of various edible muscles from chicken, turkey, beef, pork, veal, lamb, shrimp, salmon, codfish and frog legs have been found by Beach *et al.* (1943), Millares *et al.* (1948, 1949), Scott (1954), and Lyman *et al.* (1949). The amino acid composition of voluntary muscle tissue, when expressed as a percentage of the muscle proteins, is similar in *Aves, Mammalia, Pisces,* and *Crustacea* (Beach *et al.,* 1943).

Freezing and frozen storage affects the amount and proximate composition of drip from muscle tissues obtained during the thawing process, Godeaux (1957), Pearson *et al.* (1959), Taylor (1931), Howard *et al.* (1960), Love (1956). Although poultry muscle protein undergoes some denaturation and proteolysis during frozen storage (Khan *et al.,* 1963; Swanson *et al.,* 1953), the amino acid composition of dark and light meat of chicken and drip from these muscles has not been reported.

This study was conducted to evaluate the effects of specific periods of frozen storage on the amino acid composition of dark and light chicken meat and drip by means of microbiological assays and an amino acid analyzer.

PROCEDURE

TWENTY-FOUR HEAVY HENS were bled, scalded and machine-picked in an automatic rubber-fingered picker. After evisceration, the birds were placed in slush ice for 18 hr. One-half of the carcasses were packaged in Cryovac bags (partial vacuum), frozen and stored at −18°C until analyzed.

The light and dark meat was separated from the carcasses of the remaining birds and packaged separately. These samples were later used for drip analyses. One-half of the birds were thawed and prepared for analysis after 30 days storage, the remaining birds after 90 days storage.

Drip was collected from appropriate meat samples which were thawed at 16°C for 18 hr as described by Wladyka *et al.* (1967).

In preparing meat samples for assay, the whole birds were thawed in the Cryovac bags at approximately 16°C. After thawing, the light meat (Pectoralis major and minor) was removed from each carcass and the thighs and drumsticks (dark meat) were deboned. Individual meat samples were ground twice through a ⅛-inch plate, spread on aluminum foil trays and frozen at −29°C. Moisture was removed from the ground meat samples by freeze drying. Fat was removed from the freezer-dried samples by Soxhlet extraction with petroleum ether for 18 hr.

The moisture-free, fat-free samples were pulverized with a mortar and pestle; 300-mg samples were analyzed for total nitrogen by the micro-Kjeldahl method. Protein values were calculated by multiplying micro-Kjeldahl values by 6.25.

Acid hydrolyzates from meat were prepared by refluxing one g of meat in 25 ml of 6 *N* hydrochloric acid for 16 hr, and from drip by refluxing 5 ml of drip in 5 ml of 12 *N* hydrochloric acid for 16 hr. The acid hydrolyzates were frozen at −29°C and held at −18°C for analyses.

Alkaline hydrolysis of the moisture-free, fat-free meat samples for the determination of tryptophane was carried out with sodium hydroxide in the presence of L-cysteine in order to prevent oxidative destruction, as recommended by Kuiken *et al.* (1947). Meat samples (0.2 g) were hydrolyzed with 15 ml of 5 *N* sodium hydroxide at 15 lb pressure for 16 hr. The hydrolyzates were cooled, made up to approximately 100 ml and neutralized with standard acid solution; the precipitates were washed and recentrifuged; the supernatants were combined, diluted to convenient volumes, frozen at −29°C, and held for analyses at −18°C.

Alkaline hydrolyzates from drip were prepard by autoclaving 4 ml of drip in 10 ml of 5 *N* sodium hydroxide at 15 lb pressure for 16 hr.

Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine and valine were determined microbiologically, using *Leuconostoc mesenteroides* P-60 ATCC #8042 as the test organism (Steele *et al.,* 1949; Anon, 1953). *Streptococcus faecalis* (ATCC #8043) was used for the determination of threonine as described by Steele *et al.* (1949) and Anon (1953). The presence of tryptophane was measured by the acidimetric growth response

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to *Lactobacillus arabinosus* 17–5 (Anon, 1953; Greene *et al.*, 1944).

The methods used specified the use of a DL-standard for isoleucine. This necessitated the correction of values obtained in the assay since the test organism responds only to the L-form (Schweigert *et al.*, 1949). The L-tryptophane standards were also corrected because of racemization of the DL-isomers during hydrolysis with sodium hydroxide and heat. L-amino acid standards were used to determine the eight remaining amino acids assayed. However, the lysine standard was made using the monohydrochloride form, and values were corrected to the pure L-isomer.

Concentrations of the amino acids in the meat hydrolyzates were calculated by reference to standard curves; grams of amino acid per 100 g of protein were subsequently calculated.

A Beckman/Spinco Model 120 Amino Acid Analyzer was used for the chromatographic determination of amino acids in chicken drip hydrolyzates. Samples were prepared according to the methods of Spackman (1960). The frozen drip hydrolyzates (dark and light meat, 30 and 90 days storage) were thawed at 3°C. Aliquots varying in size from 25 to 35 ml were evaporated to dryness, washed with distilled water, and dried again. The final solutions contained 3.5 ± 0.5 mg of protein per ml. These samples were frozen at −29°C and held at −18°C until analyzed.

For analysis, the samples were thawed and brought to room temperature. One ml of the protein solution was diluted to 5 ml with buffer (pH 2.2). Two 2-ml aliquots were used for analysis on the analyzer, one for determination of neutral and acidic amino acids and one for the basic amino acids. Total nitrogen was determined by the micro-Kjeldahl method.

RESULTS AND DISCUSSION

THE CONCENTRATION OF AMINO ACIDS determined by microbiological methods, from dark and light chicken meat after 30 and 90 days frozen storage, and after the removal of drip, is shown in Table 1. Phenylalanine is not given since considerable loss occurred during preparation of hydrolyzates. A small difference was noted in the relative concentration of each amino acid between dark and light meat. These values agree with those of Millares *et al.* (1948) and Beach *et al.* (1943).

Table 1. Concentrations[1] of specific amino acids in dark and light chicken meat after 30 and 90 days of frozen storage.

Amino acid	Dark meat		Light meat	
	30 day	90 day	30 day	90 day
	g amino acid per 100 g protein			
Arginine	6.08	5.32	5.68	4.31
Histidine	2.24	1.66	3.13	2.58
Isoleucine	5.30	4.50	5.49	4.76
Leucine	8.58	7.06	8.83	7.42
Lysine	8.88	7.97	8.99	7.91
Methionine	2.47	1.93	2.41	1.90
Threonine	4.13	2.61	4.06	3.12
Tryptophan	1.18	1.07	1.34	1.28
Valine	5.04	4.71	5.89	4.91

[1] Average concentration of two samples each containing two replicates as determined by microbiological assay.

Tables 2, 3, 4 and 5 show that the amino acid content of drip from both dark and light meat increased with storage time. The amount of amino acids exuded from light meat was greater than that from dark meat. This indicates that proteolysis probably occurred during frozen

Table 2. Essential amino acids in dark chicken meat and drip after 30 days of frozen storage and the percentage of the total contained in the drip.

Amino acid	g AA per 100 g protein in meat	g AA per 100 g protein in drip		% of original AA in meat found in drip	
Arginine	6.08	2.70[1]	3.72[2]	2.1[1]	2.8[2]
Histidine	2.24	1.36	2.94	2.9	6.1
Isoleucine	5.30	2.77	3.06	2.5	2.7
Leucine	8.58	5.60	6.32	3.1	3.5
Lysine	8.88	5.11	7.50	2.7	4.0
Methionine	2.47	1.23	1.56	2.3	3.0
Threonine	4.13	2.33	2.97	2.7	3.4
Tryptophane	1.18	1.45		3.8	
Valine	5.04	4.97	5.20	4.6	4.9
Average				3.0	3.8

[1] Average of two samples each containing two replicates as determined by microbiological assay.
[2] Average of two samples as determined by AA analyzer.

Table 3. Essential amino acids in dark chicken meat and drip after 90 days of frozen storage and the percentage of the total contained in the drip.

Amino acid	g AA per 100 g protein in meat	g AA per 100 g protein in drip		% of original AA in meat found in drip	
Arginine	5.32	2.94[1]	3.17[2]	4.4[1]	4.7[2]
Histidine	1.66	1.99	2.70	9.4	12.8
Isoleucine	4.50	3.56	3.31	6.2	5.8
Leucine	7.06	6.06	6.51	6.7	7.2
Lysine	7.97	6.14	7.86	6.1	7.8
Methionine	1.93	1.41	1.55	5.7	6.4
Threonine	2.61	2.90	3.22	8.9	9.9
Tryptophane	1.07	1.43		10.8	
Valine	4.71	5.64	5.63	9.5	9.4
Average				7.6	8.0

[1] Average of two samples each containing two replicates as determined by microbiological assay.
[2] Average of two samples as determined by AA analyzer.

Table 4. Essential amino acids in light chicken meat and drip after 30 days of frozen storage and the percentage of the total contained in the drip.

Amino acid	g AA per 100 g protein in meat	g AA per 100 g protein in drip		% of original AA in meat found in drip	
Arginine	5.68	3.30[1]	3.80[2]	3.1[1]	3.6[2]
Histidine	3.13	3.23	4.24	5.5	7.3
Isoleucine	5.49	4.26	4.17	4.2	4.1
Leucine	8.83	6.27	6.87	3.8	4.2
Lysine	8.99	6.25	7.72	3.7	4.6
Methionine	2.41	1.49	1.84	3.3	4.1
Threonine	4.06	3.18	3.95	4.2	5.2
Tryptophane	1.34	1.02		4.1	
Valine	5.89	5.56	6.06	5.0	5.5
Average				4.1	4.8

[1] Average of two samples each containing two replicates as determined by microbiological assay.
[2] Average of two samples as determined by AA analyzer.

Table 5. Essential amino acids in light chicken meat and drip after 90 days frozen storage and the percentage of the total contained in the drip.

Amino acid	g AA per 100 g protein in meat	g AA in 100 g protein in drip		% of original AA in meat found in drip	
Arginine	4.31	3.49 [1]	4.36 [2]	7.7 [1]	9.6 [2]
Histidine	2.58	3.54	4.45	13.1	16.5
Isoleucine	4.76	4.84	4.12	9.7	8.3
Leucine	7.42	6.36	6.58	8.2	8.5
Lysine	7.91	7.10	7.94	8.6	9.6
Methionine	1.90	1.73	1.73	8.7	8.7
Threonine	3.12	3.22	3.86	9.9	11.8
Tryptophane	1.28	1.27		9.5	
Valine	4.91	6.47	5.75	12.6	11.2
Average				9.8	10.5

[1] Average of two samples each containing two replicates as determined by microbiological assay.
[2] Average of two samples as determined by AA analyzer.

storage, resulting in release of amino acids in approximately the same proportions as they existed in the meat. Results from the amino acid analyzer were slightly higher than those from microbiological assay for all amino acids and for percentage of total amino acids recovered in the drip.

The amounts of specific non-essential amino acids (and ammonia) found in drip from dark and light chicken meat after frozen storage periods of 30 and 90 days are presented in Table 6. The results were expressed in terms of mg of amino acid per ml of drip collected.

The quantities of ammonia in the drip from dark and light meat after 90 days of frozen storage were slightly larger than that in the drip from dark and light meat frozen for 30 days. An increase in ammonia was also reported by Monzini (1953) from beef which had been frozen.

Results from both methods of assay were in general agreement as to the quantities of amino acids obtained from the dark and light meat samples and the increase in amounts of amino acids with increased periods of frozen storage (30 to 90 days). However, the results obtained with the analyzer were somewhat higher than those obtained by microbiological assays.

Some of the variation can be attributed to the different methods of analysis used. This loss of protein in the procedure for decolorizing the hydrolyzates should also be considered since a considerable loss of phenylalanine was indicated in this study. A similar loss of phenylalanine and an abundant loss of tyrosine was reported by Featherstone *et al.* (1964). Some protein loss may have also occurred in preparing the drip hydrolyzates for analysis on the analyzer.

A number of workers have suggested that proteolysis and/or denaturation occurs in frozen stored meat as a result of intrinsic enzymes which remained active at below-freezing temperature, (Reay, 1933; Pearson *et al.*, 1959; Khan *et al.*, 1963). Also during frozen storage some of the structural proteins appear to be modified, probably as a result of proteolysis, and exuded in drip.

Table 6. Quantity [1] of specific amino acids contained in the drip from dark and light chicken meat after frozen storage periods of 30 and 90 days.

Amino acid	30 days storage		90 days storage	
	dark (mg/ml)	light (mg/ml)	dark (mg/ml)	light (mg/ml)
Alanine	2.2	3.0	3.8	4.9
Ammonia	0.5	0.3	0.7	0.6
Aspartic acid	3.1	4.5	6.0	7.4
Glutamic acid	4.3	6.4	7.6	7.9
Glycine	1.9	2.6	3.3	4.0
Proline	0.9	1.7	2.4	2.9
Serine	0.9	1.8	1.9	2.9
ml drip per 100 g meat	4.7	5.3	7.9	9.6

[1] Average of two samples determined with the amino acid analyzer.

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Comparison of Sarcomere Length to Other Predictors of Beef Tenderness

SUMMARY—Sarcomere length in the longissimus dorsi muscles of 20 bovine animals was compared to other variables in predicting tenderness (Allo-Kramer shear) in two muscle positions (medial and lateral) and at four cooking temperatures (60, 64, 68 and 72°C). The medial muscle position was less tender, had shorter sarcomeres and had higher correlations between sarcomere length and tenderness than the lateral muscle position. In neither position did sarcomere length account for tenderness variation that was unaccounted for by combinations of other commonly-used carcass variables indicating that a single measure of sarcomere length at a given position is of little value in predicting tenderness if certain carcass parameters are known.

Comparison of sarcomere length with resistance to shear across the cross section of 5 additional longissimus dorsi muscles showed that very small differences in contraction state were associated with marked differences in tenderness in restricted muscle areas. The study emphasizes the importance of post-rigor contracture to tenderness but indicates that several estimates are necessary to characterize a single muscle.

INTRODUCTION

THE STATE of bovine muscle contraction, measured as sarcomere length, was first suggested by Locker (1960) to be associated with tenderness. Herring *et al.* (1965a,b) reported that muscles with long sarcomeres (resulting from tension during chilling) tended to have low resistance to shear. Although the measurement of sarcomere length of a post-rigor muscle may be only a gross indication of molecular arrangements within the myofibril, it represents a simple, objective method of estimating the degree of contraction. Its usefulness as a predictor of tenderness is therefore of practical interest.

The purpose of this experiment was to evaluate the importance of the contraction state of muscle, estimated as sarcomere length, as a predictor of beef tenderness and to compare it with other predictors of tenderness in muscle samples heated to different temperatures.

EXPERIMENTAL

Phase I

The carcasses of 20 Angus steers (of known age and similar nutritional and genetic backgrounds) with a live weight of approximately 1,000 lbs were used. They were chilled at 1 to 2°C. Parameters of the carcasses and their musculature, commonly used in marketing, were observed objectively and subjectively.

Forty-eight hr post-mortem. Several traits of the carcasses were rated subjectively by three persons. These traits were marbling, muscle color, skeletal maturity, conformation, and final grade. Numerical scores were assigned as shown in Table 1. Measurements were taken of the cross-sectional area of the longissimus dorsi muscle at the 12th and 13th rib space. Subcutaneous fat thickness was measured at the same cross section and opposite a point that was three-fourths of longissimus dorsi cross-sectional length beginning at the medial side.

Table 1. Symbols and means for variables used to predict tenderness.

SL—Sarcomere length, 48 hr post-mortem[1,2] (μ)—M=1.83; L=2.00
pH 48—pH, 48 hr post-mortem[1,2]—M=5.62; L=5.60
pH 6—pH, 6 days post-mortem[1,2]—M=5.60; L=5.56
Refl—Reflectance at 540 mμ, 48 hr post-mortem[1,2] (%)—M=6.57; L=6.49
Marb—Marbling score[3] (1=traces, 10=extremely abundant)—5.13
FT—Fat thickness over longissimus dorsi, ¾ of cross-sectional length[3] (in.)—0.69
Color—Color score[3] (1=light, 4=dark)—2.9
Mat—Maturity score of skeleton (1=youthful, 4=mature)—2.6
Conf—Conformation score (15=high prime, 12=high choice, etc.)—11.9
Grade—Final carcass grade (15=high prime, 12=high choice, etc.)—11.7
%M—Moisture[1,4] (%)—M=69.7; L=69.4
%EE—Ether extract[1,4] (%)—M=7.95; L=8.54
CW—Carcass weight, 48 hr post-mortem (lb)—618.8
LD—Longissimus dorsi area[3] (sq in.)—10.24
JL—Juice loss as a percent of total moisture after heating and centrifugation[1,4] (%)
60°C—M=32.3; L=31.3
64°C—M=34.7; L=34.2
68°C—M=38.1; L=37.5
72°C—M=42.6; L=41.7
Age—Age of animal (days)—578

[1] Medial (M) or lateral (L) position in longissimus dorsi.
[2] Opposite 12th thoracic vertebra.
[3] Carcass cross section between 12–13th ribs.
[4] Opposite 4–5th lumbar vertebrae.

Longissimus dorsi samples from the 12th rib region of the left carcass side were secured. pH determinations, using a Beckman combination electrode, were made on the medial and lateral portions of a freshly cut surface of the muscle. Fifteen minutes after removal of muscle samples from the carcass, the light reflectance at the two positions was measured with a Spectronic 20 color reflectance meter at 540 mμ. Suspensions of myofibrils in 0.08 *M* KCl (Locker, 1960) were prepared from samples taken near the medial and lateral muscle borders. The average sarcomere length of 25 myofibrils, measured with a phase-contrast microscope equipped with an eye-piece micrometer, was determined for each sample.

Six days port-mortem. pH was determined at the medial and lateral positions of a freshly cut muscle surface adjacent to the 48-hr pH sample. At this time the muscle was excised from the left carcass side in the 13th thoracic to 3rd lumbar region and 4 slices were prepared (5.1 cm

thick) by cutting parallel to the fibers. Each slice was then divided into approximately equal-sized medial and lateral halves and placed in cryovac bags. A thermocouple was placed in the center of each sample, the air was removed, the bag was sealed, and the sample was placed in a waterbath (60, 64, 68, or 72°C). After the sample reached an internal temperature of 1°C less than the water-bath temperature, 12 min were allowed to elapse before removal to a 4°C cooler for 24 hr. Resistance to shear across the muscle fibers was measured with an Allo-Kramer shearing device on 8 to 12 slices (4 cm × 2.5 cm × 3 mm). Results were expressed in pounds per gram of cooked weight minus weight of ether extract in equivalent weight of uncooked tissue.

Samples from the 4th and 5th lumbar region were divided into medial and lateral portions, the epimysial connective tissue was removed, and they were ground twice through a ⅛-in. plate. Samples weighing 25 g were heated for 30 min at the previously described cooking temperatures in the centrifuge tubes designed by Wierbicki *et al.* (1957). The tubes were cooled at 25°C and centrifuged for 10 min at 230 g. Juice loss as a percent of total moisture was calculated for each muscle position and cooking temperature. The values were corrected for moisture in the juice (60°C = 0.910; 64°C = 0.931; 68°C = 0.940; 72°C = 0.941). Percent moisture and percent ether extract were determined by oven drying and Soxhlet extraction.

Data analysis. The data were analyzed by analysis of variance, correlation analysis, and multiple regression analysis (Steel *et al.,* 1960). A step-wise regression analysis was used to compare the contributions made by the individual variables, when combined with other variables, to the prediction of tenderness.

Symbols for the variables used to predict tenderness are shown in Table 1.

Phase II

The second phase of the experiment was conducted to evaluate more clearly the sarcomere length-tenderness relationship as influenced by location in the muscle. The left longissimus dorsi muscles of five additional Angus carcasses were utilized for this phase. At 48-hr post-mortem, sarcomere length was determined in six locations equally spaced across the cross-section of the muscles in the 12th rib region. At 6 days, post-mortem samples were secured from the 13th rib to 3rd lumbar region and were heated to 60, 64, 68, or 72°C. Resistance to shear across the fibers was evaluated on 26 slices of the same dimensions described in Phase I. The positions of these slices are depicted in Fig. 2.

RESULTS AND DISCUSSION

Phase I

Means for the variables used to predict tenderness are shown in Table 1.

Analysis of variance performed on the Allo-Kramer shear values show significant differences due to cooking temperature ($P < .01$) and muscle position ($P < .01$). The means for these data are shown in Fig. 1.

The effects of cooking temperature on resistance to shear are similar to those of other published studies on beef. Cover *et al.* (1957) found that muscles containing little connective tissue, such as the longissimus dorsi, are most tender when cooked to a rare degree (61°C internal). Machlik *et al.* (1963) showed that heating semitendinosus muscles for 30 to 60 min at 60 to 64°C resulted in maximum tenderness.

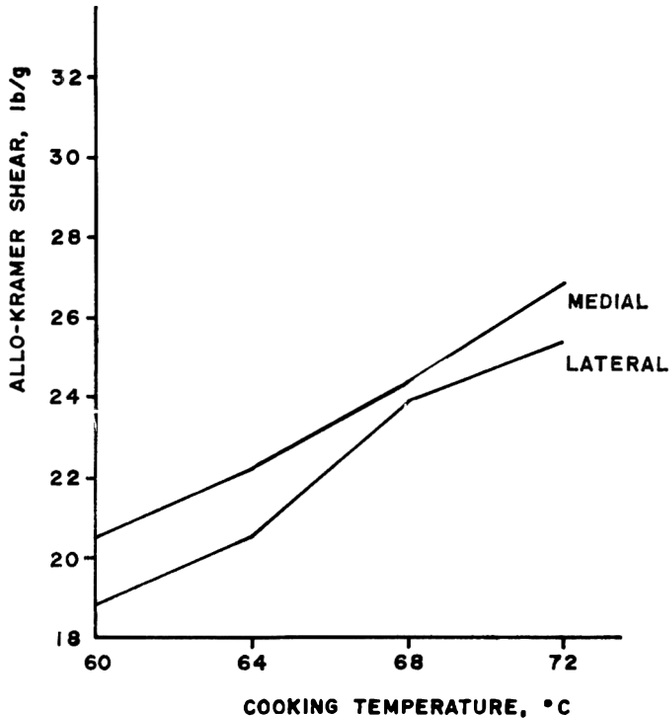

Fig. 1. Allo-Kramer shear values at four cooking temperatures in relation to position in longissimus dorsi muscle.

The data indicate that the lateral half of the longissimus dorsi muscle was more tender than the medial half. These results are in agreement with those of Carpenter *et al.* (1965) on lamb and Alsmeyer *et al.* (1965a) on pork. However, Tuma *et al.* (1962) and Alsmeyer *et al.* (1965b) found beef longissimus dorsi to be more tender in the dorsal (medial) position. Tuma *et al.* (1962) also reported that the middle of the muscle cross section was more tender than either lateral or dorsal (medial) samples. The latter finding may partially explain these conflicting results since the present study utilized the entire muscle cross section for Allo-Kramer shearing whereas the cited studies utilized cores for the Warner-Bratzler shearing device.

The analysis of variance for sarcomere length determinations showed significant ($P < .01$) differences due to position. The length of the sarcomeres on the medial and lateral borders of the muscle averaged 1.83 ± 0.09 and 2.00 ± 0.06 μ respectively. Eisenhut *et al.* (1965), using samples taken between points that were 5.0 and 7.5 cm from the spinous process, observed sarcomeres that were equal in average length to those of the lateral position of muscles in the present study. The differences in contraction state at different muscle positions could have resulted from tension differences exerted by skeletal and connective tissue attachments (Herring *et al.,* 1965a,b)

Table 2. Relation of Allo-Kramer shear values [1] to carcass variables by muscle position and cooking temperature.[2]

	Cooking temperature (°C)							
	60		64		68		72	
Variable [3]	Medial	Lateral	Medial	Lateral	Medial	Lateral	Medial	Lateral
SL	−.56**	.09	−.54*	.18	−.55*	.06	−.54*	.19
pH 48	−.15	−.05	−.28	.02	−.23	.18	.02	.36
pH 6	−.43	.13	−.43	.22	−.48*	.09	−.44*	.17
Refl	.19	.12	.27	.22	.18	.21	.06	.24
Marb	−.14	−.14	−.03	−.05	.00	−.12	−.01	.02
FT	−.45*	−.39	−.48*	−.38	−.44*	−.46*	−.43	−.48*
Color	.31	.26	.17	.18	.32	.14	.43	.11
Mat	−.58**	−.47*	−.50*	−.47*	−.59**	−.43	−.65**	−.34
Conf	.38	.28	.22	.35	.22	.16	.28	.33
Grade	−.03	−.04	.03	.07	.07	−.06	.06	.12
%M	−.20	.04	−.17	.10	−.26	.00	−.23	−.06
%EE	.20	−.10	.22	−.14	.26	−.02	.25	.06
CW	.21	−.24	−.23	−.12	−.19	−.25	−.03	−.29
LD	.33	.50*	.14	.34	.12	.22	.01	.05
JL	.51*	.11	.40	.21	.32	−.14	.07	.06
Age	−.30	−.25	−.22	−.28	−.16	−.13	−.13	−.10

[1] Lb shear/g longissimus dorsi cooked weight minus weight of ether extract in equivalent weight of uncooked tissue.
[2] Correlation coefficients.
[3] See Table 1 for description of variables.
**P < .01, *P < .05 that the variables are independent.

or by different rates of heat removal during chilling of various muscle areas (Locker *et al.*, 1963).

Correlation coefficients between resistance of the cooked muscle samples to shear and the carcass variables showed several significant relationships (Table 2). Sarcomere length was associated with tenderness, as reported by Herring *et al.* (1965a,b), but only in the medial portion of the muscle. Tests of significance, after Z transformations of the correlation coefficients, showed that medial and lateral position correlations differed (P < .05) at each cooking temperature.

Negative correlations were found between maturity score and shear values in both muscle positions, indicating that tenderness increased with advancing carcass maturity but the tenderness-age correlations were not significant (Table 2). Other studies, utilizing cattle of widely varying ages, have shown reductions of tenderness with advancing age or maturity (Palmer, 1963). However it is notable that Alsmeyer *et al.* (1959) found a positive association between tenderness and chronological age.

The only other variable that appeared to be consistently correlated with shear values was the measure of subcutaneous fat thickness. It is possible that this association of fatness with tenderness was the result of the maturity-tenderness correlation discussed above since Haecker's (1920) work showed that advancing maturity is accompanied by increased fatness.

Correlation coefficients were calculated between sarcomere length and the other carcass variables to identify relationships that might have physiological bases (Table 3). Low but significant correlations were found for some of these variables. Of interest is the relationship between lateral sarcomere length and the pH of the aged muscle (.54; P < .05). Since the sarcomeres were measured at 2 days post-mortem, and their length was unrelated to pH at that time, the relationship implies that the initial post-rigor contraction state may influence pH changes during aging. This relationship needs further study in light of the findings of Gothard *et al.* (1966) that sarcomere length also changes during aging.

In the medial muscle position, the correlation between sarcomere length and percent ether extract (−.46; P < .05) (and consequently the sarcomere length—% mois-

Table 3. Relation of longissimus dorsi sarcomere length to carcass variables by muscle position.[1]

	Position	
Variable [2]	Medial	Lateral
pH 48	.15	.04
pH 6	.40	.54*
Refl	−.29	.18
Marb	−.17	−.17
FT	.40	−.26
Color	−.30	.04
Mat	.31	.04
Conf	−.31	.16
Grade	−.19	−.18
%M	.49*	.27
%EE	−.46*	−.32
CW	.31	−.25
LD	−.30	−.22
Age	.20	−.23
JL		
60°C	−.47*	.53*
64°C	−.27	.35
68°C	−.12	.25
72°C	−.20	.39

[1] Correlation coefficients.
[2] See Table 1 for description of variables.
*P < .05 that the variables are independent.

ture correlation) is not readily explainable (Table 3). However, such a relationship might result if intramuscular lipid influenced tension development during rigor mortis. Differences in tension would subsequently influence sarcomere length (Herring *et al.,* 1965a).

The correlations between sarcomere length and juice loss after heating and centrifugation suggest that contraction state of bovine muscle influences its water-holding properties (Table 3). Why the correlations are opposite in sign for the two muscle positions is not apparent.

The usefulness of sarcomere length as a predictor of tenderness was evaluated on the basis of the variation it accounted for that was independent of other predictors. The step-wise multiple correlation and regression analysis used to rank the variables in the order of their value as predictors are shown in Tables 4 and 5 for the medial and lateral muscle positions respectively. In the medial position, sarcomere length was ranked first or second at each cooking temperature, but cooking temperature apparently did not influence its ranking among the variables. However it was deleted (i.e. did not contribute significantly to the prediction equation) when certain other variables were added. In the lateral position, sarcomere length was ranked below many of the other variables.

These results and the simple correlation coefficients (Table 2) suggest that the tenderness of the area of the muscle having relatively short sarcomeres (medial) was more closely related to contraction state than the area having relatively long sarcomeres (lateral). Yet, in no instance did the sarcomere length measurement provide knowledge of tenderness that was not provided by combinations of certain commonly-observed carcass parameters.

Phase II

The sarcomere length and shear values for the five additional longissimus dorsi muscles were superimposed on an outline of the muscle cross section (Fig. 2). Significant ($P < .05$) effects due to muscle position were noted by analysis of variance for sarcomere length and for shear values at each cooking temperature. When the 60°C cooking temperature was used, the average shear values were plotted as a smooth curve that was approximately the reverse of the sarcomere length plot. At the 64°C cooking temperature the same approximate relationship was evident. According to Machlik *et al.* (1963), collagen shrinkage occurs, but protein hardening does

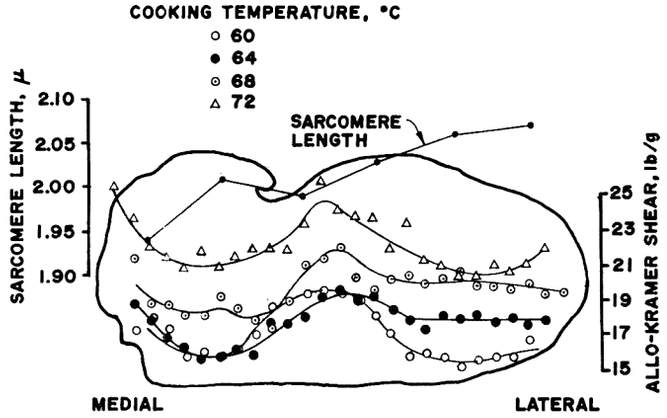

Fig. 2. Allo-Kramer shear values in relation to cooking temperature and sarcomere length in longissimus dorsi muscle cross section.

Table 4. Cumulative multiple correlations in step-wise regression analysis [1] for Allo-Kramer shear values [2] in medial longissimus dorsi.[3]

Cooking temperature (°C)											
60			64			68			72		
Inclusion	Deletion	R	Inclusion	Deletion	R	Inclusion	Deletion	R	Inclusion	Deletion	R
Mat		.58	SL		.54	Mat		.59	Mat		.65
SL		.71	Mat		.64	SL		.71	SL		.74
LD		.75	FT		.69	JL		.76	FT		.77
FT		.79	pH 6		.75	CW		.79	pH 6		.80
pH 6		.81	Refl		.78	pH 6		.83	Conf		.83
Conf		.83		SL	.78	FT		.86		SL	.83
	SL	.83	Conf		.80		SL	.86	Grade		.84
Marb		.85	pH 48		.82	Color		.87	Age		.85
CW		.89	CW		.83	Refl		.88	Refl		.87
%EE		.90	LD		.85	Age		.90	pH 48		.88
%M		.91	Grade		.86	LD		.90		FT	.88
	FT	.91	%EE		.87	SL		.91	Marb		.89
Grade		.92	%M		.88	%M		.92	JL		.90
FT		.93		FT	.88		CW	.92	Color		.90
	Marb	.93	SL		.89	Conf		.92	LD		.91
SL		.94	Marb		.89	Marb		.92	%EE		.91
Age		.94	Age		.90		LD	.92	SL		.92
Color		.94	Color		.90	pH 48		.93		pH 48	.92
Refl		.95				CW		.93	CW		.92
JL		.95				Grade		.93	FT		.93
							Marb	.93		Marb	.93

[1] The variable making the greatest reduction in the error sum of squares was added at each step. Variables deleted when mean square due to regression divided by error mean square <0.1.
[2] LB shear/g longissimus dorsi cooked weight minus weight of ether extract in equivalent weight of uncooked tissue.
[3] See Table 1 for description of variables.

Table 5. Cumulative multiple correlations in step-wise regression analysis[1] for Allo-Kramer shear values[2] in lateral longissimus dorsi.[3]

Cooking temperature (°C)											
60			64			68			72		
Inclusion	Deletion	R	Inclusion	Deletion	R	Inclusion	Deletion	R	Inclusion	Deletion	R
LD		.49	Mat		.47	FT		.46	FT		.48
Mat		.71	LD		.61	Mat		.58	pH 48		.55
Conf		.76	Conf		.70	LD		.62	LD		.59
Refl		.79	Refl		.77	pH 48		.65	Mat		.63
Color		.80	SL		.79	Refl		.69	Refl		.66
%M		.82	Age		.80	%M		.70	%M		.70
Grade		.86	Color		.80	Color		.72	SL		.73
Age		.88	%M		.81	pH 6		.74	Conf		.75
%EE		.89	CW		.83	Marb		.75	Color		.76
FT		.90	Grade		.84	Age		.81	%EE		.78
JL		.90	pH 6		.85		pH 48	.81		Conf	.78
pH 48		.91	pH 48		.86		FT	.81	pH 6		.80
Marb		.91		SL	.86	SL		.82		FT	.80
	Grade	.91		LD	.86		LD	.82	Marb		.86
pH 6		.92	Marb		.87	JL		.84		pH 48	.86
SL		.92		Grade	.87	FT		.85		SL	.86
	JL	.92				%EE		.86	JL		.87
	LD	.92					%M	.86	Age		.88
	FT	.92								LD	.88
CW		.92							CW		.89
									Conf		.89

[1] The variable making the greatest reduction in the error sum of squares was added at each step. Variables deleted when mean square due to regression divided by error mean square <0.1.
[2] LB shear/g longissimus dorsi cooked weight minus weight of ether extract in equivalent weight of uncooked tissue.
[3] See Table 1 for description of variables.

not occur in bovine semitendinosus muscle heated to the 60 to 64°C range.

At the 68 and 72°C cooking temperatures the shear values were more variable than those of samples heated to lower temperatures (Fig. 2). Hardening of the fibrillar proteins presumably occurred in these samples, yet the curves are still inverse to the sarcomere length line. The difference in average sarcomere length across the muscle cross section was only 0.13 μ but there was a relatively wide range in resistance to shear. This observation appears to be consistent with those of Vorober *et al.* (1962) who reported that moderate tension on frog sartorius muscle increases its resistance to thermal denaturation. It is possible that the contraction state of muscle tissue is highly associated with its tenderness, but the observation of sarcomere length at a single point (as performed in Phase I of this study) does not adequately reflect the overall contraction state of the muscle.

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Arbutin and a Related Glucoside in Immature Pear Fruit

SUMMARY—Arbutin (p-hydroxyphenyl-β-D-glucoside) was separated from other phenols in an extract of immature fruit of Pyrus c.v. Kieffer by preparative paper chromatography and isolated as the penta-acetyl derivative. A monoacetylarbutin (p-hydroxyphenyl-6-0-acetyl-β-D-glucoside) was identified in the same pear extract. In addition, arbutin was found in both mature and immature pears, but at much higher levels in the immature fruit.

INTRODUCTION

DURING A STUDY of core browning in pears and apples in this laboratory, some naturally occurring phenols were tested with polyphenoloxidase preparations to see if brown pigments were formed. Arbutin, the glucoside occurring in pear leaves, produced reddish-brown color in this enzyme's presence. This was demonstrated in buffer solutions of the glucoside incubated with pear enzyme and by spraying chromatograms of arbutin with a suspension of the enzyme prepared according to Siegelman (1955). Hattori *et al.* (1963) found that isolated chloroplasts of arbutin-containing plants oxidized arbutin to a dihydroxy glucoside which was identified on paper chromatograms as 3,4 dihydroxyphenyl-β-D-glucoside. These findings indicate that arbutin is a polyphenoloxidase substrate.

Arbutin was thought to be a constituent only of pear leaves, bark and possibly seeds (Williams, 1957). If it occurs in the fruit, it may contribute to pear core browning, not only because it is a substrate for polyphenoloxidase but because hydrolysis of this glucoside yields quinol. Quinol is easily oxidized, enzymatically or otherwise, to benzoquinone and subsequently to brown pigments (Hattori *et al.*, 1963).

The present work was done to see if arbutin or related compounds were present in the fruit tissues.

EXPERIMENTAL

Materials

Four commercial varieties of pears were used: Kieffer, Phileson, Beurre d'Anjou and Bartlett. The latter 2 were obtained on the local market. Phileson was obtained from a tree, made available through the courtesy of the Ottawa Research Station, Canada Department of Agriculture, and Kieffer samples were obtained from Dr. R.E.C. Layne, Research Station, Canada Department of Agriculture, Harrow, Ontario.

Penta-acetylarbutin was prepared by acetylation of arbutin (Nutritional Biochemicals Corp.) using acetic anhydride in the presence of dry pyridine (Shriner *et al.*, 1948). After recrystallization from 95% ethanol the penta-acetate melted at 144–145°C, agreeing with the reported value (Cornforth, 1938).

Monoacetylarbutin (6-0-acetylarbutin) was synthesized from arbutin by a mild acetylation procedure outlined by Entlicher *et al.* (1967). The reaction mixture was applied to chromatograms and the products identified by their R_f values in butanol-acetic acid-water (4:1:1 v/v). The products were 6-0-acetylarbutin, R_f 0.65 and some 2-0-acetylarbutin, R_f 0.78, agreeing with those of Entlicher *et al.* (1967). This mixture was used as a chromatographic standard.

Chromatographic identification of arbutin and monoacetylarbutin

Cold ethyl acetate extracts of core, cortex, peel and seeds obtained from the 4 different commercial pear varieties were prepared according to a method already described (Durkee *et al.*, 1965). Streaks and spots of these extracts were chromatographed on Whatman 1 and 3 MM paper and developed with the following solvent systems: (a) butanol-acetic acid-water (4:1:1, v/v); (b) isopropanol-ammonia-water (8:1:1, v/v); (c) 2% glacial acetic acid; (d) benzene-propionic acid-water (organic phase) (2:2:1, v/v) and (e) isopropanol-water (8:2, v/v). After drying, papers were sprayed with diazotized sulfanilic acid reagent (Block *et al.*, 1958) and red spots or bands, with R_f values identical with authentic arbutin, were located in all the above extracts.

One of these bands, presumed to be arbutin, was eluted with 95% ethanol, taken to dryness and treated with 0.2 *N* hydrochloric acid for 1 hr on a boiling water bath. The aqueous solution was dried on the rotary evaporator and the residue taken up in 70% ethanol for re-chromatography in butanol-acetic acid-water (4:1:1, v/v). Two chromatograms were prepared. Quinol was detected as the aglycon, by spraying one paper with diazotized sulfanilic acid. The second paper was sprayed with ammoniacal silver nitrate reagent (Block *et al.*, 1958), where well-separated spots of glucose (R_f 0.20) and quinol (R_f 0.83) were identified by comparison with standard markers. Quinol reacted immediately to the reagent, whereas the color due to glucose did not appear until the chromatogram was heated for 1 min at 100°C.

Another compound found in extracts of immature Kieffer and Phileson pears was not rigorously characterized, but was identified tentatively as a native monoacetylarbutin by its color reaction with diazotized sulfanilic acid and R_f values in 3 solvent systems. Comparison with chromatograms of the products obtained by mild acetylation of arbutin (see materials) is shown in Table 1.

Estimation of arbutin

Arbutin concentrations in the 4 pear varieties were estimated spectrophotometrically at 286 mμ, after elution of the bands obtained from chromatograms of extracts (50 g samples) and comparison of the absorbance readings with a standard curve set up for varying arbutin concentrations. According to this method, immature Kieffer

pears contained about 400 ppm, while the mature Anjou contained only 30 ppm arbutin (fwb). The 4 pear varieties studied contained arbutin in the following decreasing concentration: Kieffer (immature) > Phileson (immature) > Beurre d'Anjou (mature) > Bartlett (mature).

Isolation of arbutin from immature Kieffer pears

One kg (fresh weight) of sliced immature pears, with the stems removed, was extracted in 3 batches in a gallon-capacity Waring blendor using ethyl acetate (pre-cooled to −20°C). All operations prior to evaporation of the extracts were carried out in a cold room at 0°C. The details of this procedure have already been described (Durkee *et al.*, 1965).

The extracts were combined (6.5 L) and then divided into smaller portions which were evaporated to dryness at 40°C, using a rotary film evaporator, and the residues taken up in small volumes of 95% ethanol. These were streaked on 18 Whatman 3 MM papers, which were developed overnight in isopropanol-ammonia-water (8:1:1, v/v). Thin strips cut out of the center of the papers were sprayed with diazotized sulfanilic acid, followed by 20% sodium carbonate. Wide bands consisting of arbutin and related glucosides were located, cut into small pieces, and eluted with boiling 95% ethanol (1 hr). The eluates were filtered and evaporated to small volumes for rechromatography.

The combined eluates from the 18 papers were subdivided again (4 portions), streaked on 3 MM paper and developed overnight in butanol-acetic-acid-water (4:1:1, v/v). In this solvent system, the related compounds were well separated from the arbutin. The arbutin bonds were eluted with 95% ethanol as previously described, and the combined alcoholic solutions were dried at 40°C (rotary evaporator).

This residue was treated with 3 ml dry pyridine (dried over KOH and re-distilled) and 1.5 ml acetic anhydride. The mixture was boiled for 4 min under reflux and, after cooling, poured into 5 ml of ice-water and placed in the refrigerator. After 1 hr, the crude acetyl derivative had precipitated. It was then collected on a Buchner funnel, and washed with dilute HCl and cold water. After drying in the air, the crystals melted at 139–143°C. Recrystallization from 95% ethanol yielded a small amount of pure penta-acetylarbutin m.p. 144–145°C, agreeing with that of the synthetic derivative. The yield as acetylated glucoside before recrystallization was 150 mg/kg pear fruitlets (fresh weight).

The immature Kieffer pears were not de-seeded for the isolation procedure, but analytical results on 50 g samples, with and without seeds, as well as on 10 g seeds removed from a 1000 g of Kieffer pears, indicated that approximately 10% of the isolated derivative would be due to the seed content.

Comparison of the UV spectra, on the Bausch and Lomb 502 Spectrophotometer, of the synthetic acetate and the acetate obtained from the pear substance showed that both substances had a major absorption peak at 274 mμ. Eluates from chromatograms of pear extract and authentic arbutin absorbed at a maximum wave length of 286 mμ (95% ethanol as reference). Acetylation of the sugar hydroxyls apparently caused a hypsochromic shift of 12 mμ. The pear extract eluates (before acetylation) and authentic arbutin gave bathochromic shifts of 20 mμ, after treatment with a few drops of sodium ethoxide (Saxby, 1964) (Table 1).

RESULTS AND DISCUSSION

ARBUTIN (p-hydroxyphenyl-β-D-glucoside) was identified in pear fruit extracts by paper chromatography using 5 solvent systems. The R_f values, color tests, hydrolysis products and UV spectra agreed with those of authentic arbutin. The glucoside was found in extracts of seeds, peel, stems, core and cortex of the pear fruit, and was isolated as the crystalline penta-acetate from the whole immature fruit. These experiments firmly establish arbutin's presence in the fruit tissue.

At the same time, a related substance was identified in the immature fruit tissues. The chromatographic behavior of this substance in 3 solvent systems (Table 1) suggests that it is p-hydroxyphenyl-6-0-acetyl-β-D-glucoside. Entlicher *et al.* (1967) recently isolated p-hydroxyphenyl-2-0-acetyl-β-D-glucoside from young pear leaves and showed that the 2-0-acetyl derivative was easily transformed to the 6-0-acetyl derivative in the presence of weak alkali. Because in our isolation of arbutin it was necessary to use isopropanol-ammonia-water as the first chromato-

Table 1. Chromatographic data and properties of arbutin and related substances from pear.

Substances	M.P. (Acetate)	R_f solvents[1]					Color Diaz S[2]	Acid hydrolysis products	UV spectra (mμ)		
		BAW	IAW	HAc	BPW	IW			MAX	after NaOET[3]	after Acetylation
Unknown I	144–45°C	0.46	0.62	0.81	0.01	0.64	Red	Quinol, glucose	286	306	274
Arbutin	144–45°C	0.45	0.62	0.80	0.01	0.61	Red	Quinol, glucose	286	306	274
Unknown II	.....	0.69	0.75	0.80	.....	.....	Red	.....	.....	.....	.....
Acetylated arbutin mix											
2-0-Acetylarbutin		0.78	0.76	0.80	.....	.....	Red	.....	.....	.....	.....
6-0-Acetylarbutin		0.69	0.76	0.80	.....	.....	Red	.....	.....	.....	.....

[1]-Solvent systems—BAW butanol-acetic acid-water (4:1:1), IAW isopropanol-ammonia water (8:1:1), HAc 2% glacial acetic acid, BPW benzene-propionic acid-water (2:2:1) (organic phase), IW isopropanol-water (8:2).
[2] Diaz S—diazotized sulfanilic acid.
[3] NaOET—Sodium ethoxide.

graphic developing solvent, the end product was the more stable 6-O-acetylarbutin.

Methylarbutin (the monomethyl ether of arbutin) is thought to occur along with arbutin in some plant tissues and is not easily separable from it (Cornforth, 1938; and Hattori *et al.*, 1963). Entlicher *et al.* (1967) and the present authors did not identify it in either pear leaf or fruit—the monoacetylarbutin being the only other related compound identified on chromatograms. Since no authentic sample of the methyl derivative was available for comparison purposes, it is not possible at this time to state that the methyl ether was absent from the tissue.

Methylarbutin may lead to yellow discolorations, due to indirect oxidation (Hattori *et al.*, 1963), but it cannot be classed as a polyphenoloxidase substrate, due to the presence of the methoxyl group. The fact, however, that arbutin is present throughout the pear fruit in relatively high concentrations and can be enzymatically transformed to easily oxidized products either by the action of a β-glucosidase or by a phenol oxidase, does suggest that pear fruit browning is due to oxidative breakdown products derived mainly from arbutin and is analogous to the blackening of senescent pear leaves.

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Microscopic Characteristics of Cooked Muscles Subjected to Stretch-Tension during Rigor

SUMMARY—Frozen sections, representing the entire cross-sectional area at the site of shear force determination, were prepared from cooked muscle samples previously subjected to two degrees of stretch-tension during rigor. Histological observations revealed very few consistent relationships between structure at the shearing site and shear force values. Sections prepared from control samples exhibited significantly greater total amounts of perimysial connective tissue than sections prepared from stretched samples. Stretched sections tended to show greater amounts of perimysial tissue denaturation as indicated by the degree of tissue granulation. These results suggest that a portion of the increased tenderness previously reported for stretched muscle samples may be accounted for by a mechanical thinning of connective tissues due to stretching which results in a decrease in force required for shearing.

INTRODUCTION

GREATER AMOUNTS of connective tissue, as determined histologically and chemically, have been related to increased shear values and decreased tenderness by many workers (Moran *et al.*, 1929; Mackintosh *et al.*, 1936; Ramsbottom *et al.*, 1945; Strandine *et al.*, 1949; Hiner *et al.*, 1950 and Parrish *et al.*, 1961). It is well established that cooking results in a hydrolysis of collagenous tissues which is partly responsible for increased tenderness. Several workers have reported that collagen denatured by heat reacts abnormally to differentiating histological stains, not staining at all in some cases (Harrison *et al.*, 1949; Paul *et al.*, 1944 and Paul, 1963).

The decreased shear values associated with stretched muscle samples reported previously (Buck *et al.*, 1967) may have been influenced by physical changes in connective tissues resulting from the stretching or cooking. This portion of the investigation was designed to determine the relationship between histological structure and resistance to shear.

Samples of muscle at the shear point were evaluated histologically. Both the amount of connective tissue and the denaturation of the connective tissue in the cooked samples, as determined by the staining reaction, were assessed.

EXPERIMENTAL

Origin and preparation of samples

The tissues employed in this investigation were from the same samples used in a previous study (Buck *et al.*, 1967). Muscle strips, prepared from seven pairs of longissimus dorsi muscles obtained from cull dairy animals varying in age from 3 to 9 years, were subjected to two degrees of stretch-tension during rigor. Weighted muscle strips were not disturbed for 72 hours to insure that the rigor process was complete. At the end of this period, portions of the muscle strips for future objective tenderness measurements and histological observation were wrapped in plastic film and frozen at −30°C.

Frozen samples were thawed overnight at 5°C, removed from the refrigerator and held until internal temperatures reached 15°C. They were then placed in a bath of corn oil maintained at 135°C and cooked to an internal temperature of 65.5°C. Maximal internal temperature reached after removal from the oil bath was 73.9°C. All temperatures were recorded by means of thermocouples and a Leeds and Northrup Recording Potentiometer.

Samples were prepared for shear force determinations and immediately following shearing on an Allo-Kramer Shear Press, one-half of each sheared strip, with a few drops of saline solution, was frozen to the object disc of a Lipshaw Cryotome (Model No. 1500). The cold chamber containing the microtome was operated at −20°C. After several sections were removed to square the sheared end of the tissue strip, three consecutive 10 μ cross sections were removed and affixed to glass slides with Zwemer's Chrome Glycerine Jelly (Gatenby *et al.*, 1950). Sections were air-dried for 10 min before staining.

Structural components of interest were stained with a modified Mallory's Triple Stain. Alteration of Mallory's method involved a thorough rinsing of sections in a 1% solution of phosphomolybdic acid after staining with acid fuchsin and before staining with the aniline blue-orange G mixture. Sections were taken from Mallory's Solution II and placed directly in 70% alcohol for 1 min, 95% alcohol for 1 min and absolute alcohol for 1 min. They were then cleared in xylene and mounted. This method was very rapid and a mounted section could be obtained in approximately 10 min.

Histological analysis of sections

Stained sections were examined and scored for amounts and denaturation of perimysial and endomysial connective tissues, presence and size of arteries and fat islands, and any other structural differences which might be related to the shear force value associated with the particular section being examined.

Connective tissue and denaturation were each scored on an arbitrary 1 to 3 scale with 1 indicating small amounts and 3 indicating large amounts of connective tissue or denaturation. Arteries and fat islands were recorded as to number and size (small, medium or large). All scoring

was relative, and to avoid bias, slides were coded and scored several times with the mean value recorded.

A second procedure for histological examination was also used. A section of control muscle and a section from the corresponding area in the paired stretched muscle were examined side by side on the stage of a dissecting microscope at a magnification of 50X. Two experienced operators determined which slide of the pair had the greater amount of perimysial connective tissue and heat denaturation of the connective tissue.

RESULTS AND DISCUSSION

INITIAL STAINING TRIALS in which albumin was used as an adhesive presented some difficulties in that tissue sections became detached from the slides during staining. Under the conditions of this experiment, Zwemer's Chrome Glycerine Jelly (Gatenby *et al.*, 1950) proved to be an excellent adhesive for frozen sections prepared from cooked muscle tissue.

Shear force determinations were made at three to five locations within each sample. Three stained sections were prepared from the sheared surfaces at each shearing location. Figs. 1, 2 and 3 are representative of the approximately 160 slides prepared and examined.

The muscle cells of sections prepared from cooked tissues stained a very dark red. Perimysial connective tissues showed wavy bands of dark blue collagenic fibers surrounded, in most cases, by a pinkish-red granular material which was interpreted to be hydrolyzed collagenous tissue (Fig. 1). The endomysial connective tissues were almost always completely denatured appearing granular and pinkish-red in color. No elastic fibers were observed in the endomysial spaces and only a few pink elastic fibers appeared in perimysial spaces.

Figs. 1 and 2 represent sections prepared from similar shearing sites in paired control and stretched muscles, respectively. The greater amounts of perimysial connective tissue in the control section (Fig. 1) resulted in its receiving a score of 2 as compared to its paired stretched section (Fig. 2) which received a score of 1. Several dark wavy bands of stained collagenic fibers may be seen in Fig. 1 while Fig. 2 is practically devoid of them, implying a larger degree of tissue granulation in the section in Fig. 2. In this particular example the greater shear force is associated with the muscle sample from which the control section (Fig. 1) was prepared.

Approximately 75% of the sections showed one or more small to large fat islands. Arteries were observed in the perimysial spaces of approximately 10% of the sections and they were always surrounded by dense areas of dark blue collagenic and pinkish-red elastic fibers. One would think that fat islands and arteries, as seen in Fig. 3, would influence shear force values, yet this was not the case under the conditions of this study. Fat islands and/or

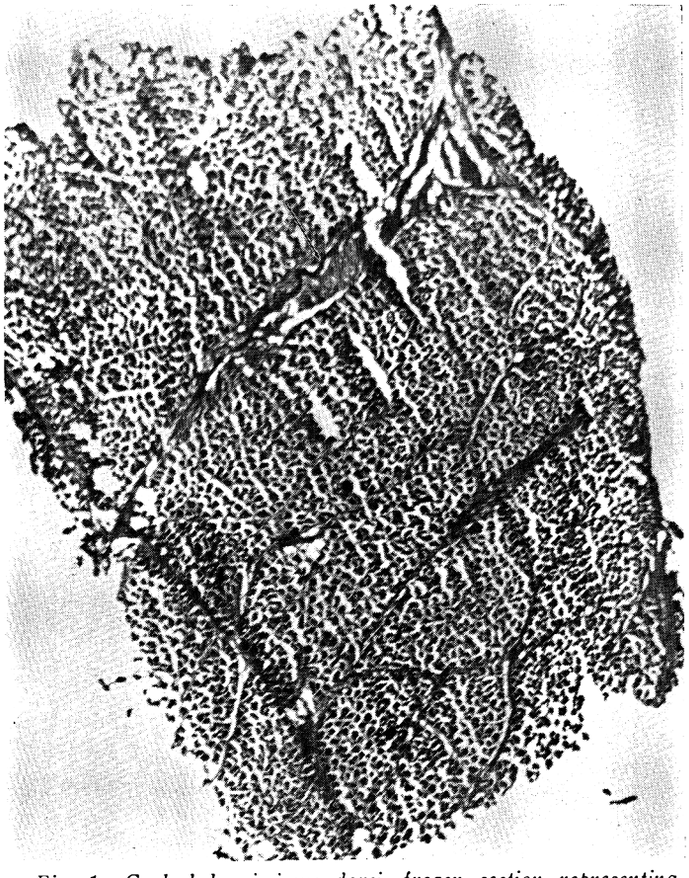

Fig. 1. Cooked longissimus dorsi, frozen section representing entire cross-sectional area of control sample at site of shear force determination. Note dark wavy band of stained collagenic fibers (arrow) surrounded by granular tissue. Shear force, 8.70 lbs. Magnification × 16.3.

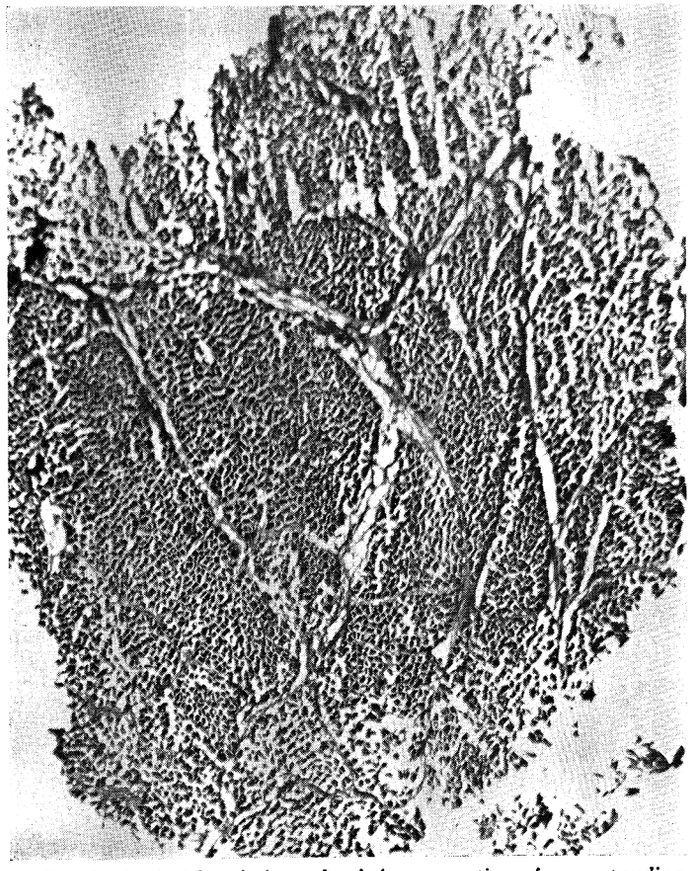

Fig. 2. Cooked longissimus dorsi, frozen section of corresponding area (Fig. 1) in paired stretched muscle. Shear force, 5.40 lbs. Magnification × 16.3.

arteries were not consistently associated with either increased or decreased shear forces.

It is interesting to note that the collagenous framework of the fat islands showed very little denaturation and stained an intense blue. Some pink elastic fibers were also visible. Considering the high heat carrying capacity of fat one would expect to find more granulation of the collagenous tissue.

The staining properties of the collagenous tissues in the present study are similar to those reported by Paul *et al.* (1944) and Paul (1963) who found that the staining properties of collagen changed as heating progressed eventually resulting in structural change from fibrous to granular. Skelton *et al.* (1963) reported that collagenous tissue appeared as long fibrous strands in sections of raw meat and as masses of granular tissue dispersed throughout the sections of cooked tissue.

Subjective scoring of sections prepared from control and stretched muscle strips on a 1–3 scale failed to reveal any relationship between degree of granulation or perimysial connective tissues, amounts of endomysial connective tissue and shear forces. There was a definite trend, however, for sections from control muscle strips to score higher for total amounts of perimysial connective tissues. This trend was brought into sharper focus by comparing a control section with a stretched section from the corresponding location in the paired muscle. Two operators made identical choices when asked to indicate which slide of the pair showed greater amounts of total perimysial connective tissue, and also which section showed greater denaturation or granulation of the perimysial connective tissues. Results proved to be 100% reproducible with coded sections. The results of the paired comparison are shown in Table 1.

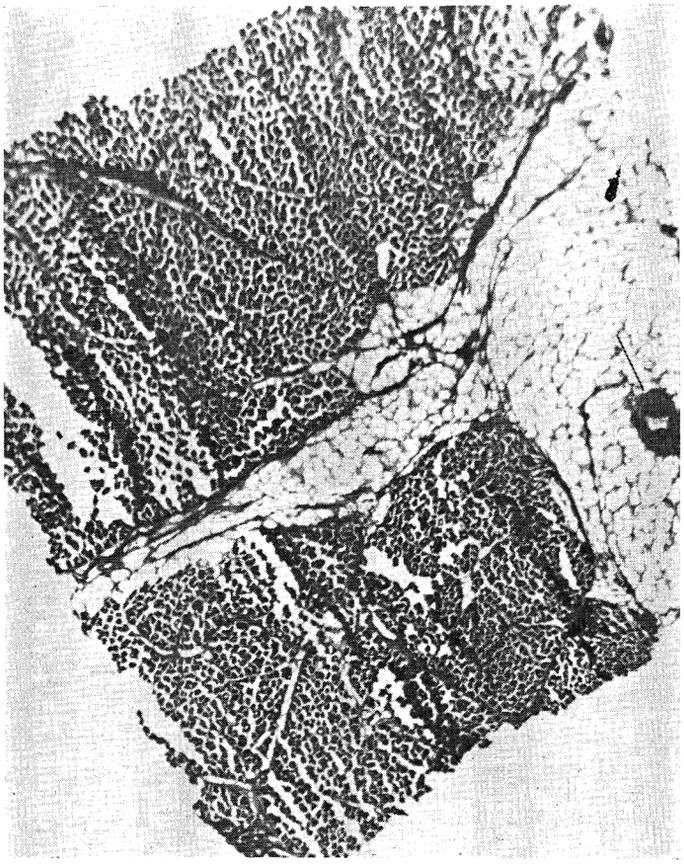

Fig. 3. Cooked longissimus dorsi, frozen section representing entire cross-sectional area of a control sample at site of shear force determination. Note artery (arrow) surrounded by a large fat island. Shear force, 7.30 lbs. Magnification × 16.3.

With the exception of Trial 5, more control sections than stretched sections exhibited greater total amounts of perimysial connective tissue. The chi-square test was employed and these differences proved to be significant at the 1% level. These data tend to offer support to those workers who have demonstrated a relationship between decreased tenderness and increased amounts of connective tissue since the greater shear force values are associated with the control slides in Trials 3 through 7. As was reported previously (Buck *et al.*, 1967), stretched muscle samples from Trials 1 and 2 were inadvertently cooked to a higher internal temperature and it was suggested that this higher temperature had a toughening effect on the myofibrillar proteins which was able to mask any connective tissue effects present.

The data in Table 1 show a slight trend for the greater amount of perimysial tissue denaturation to be associated with sections from stretched muscle samples and therefore, lower shear values. Assuming the granular areas represent hydrolyzed collagenous tissue, this would be expected; however, the data are not clear on this point and differences did not prove to be statistically significant.

The histological observations reported in this paper suggest that increased tenderness resulting from pre-rigor stretching of muscle is due, in part, to changes in connective tissue. The condition of myofibrillar proteins undoubtedly also contributes to tenderness, or a lack of it; however, the proportional contribution of each remains to be determined.

The decreased amounts of connective tissues associated with the stretched muscle samples in the present study might have been due to an elastic effect or a mechanical thinning of perimysial connective tissues which resulted in decreased resistance to shear.

The results reported here should be interpreted cautiously since evidence is accumulating which suggests that amounts of connective tissue may not play as significant a role in muscle toughness as previously thought, at least insofar as muscles containing smaller amounts of connective tissue are concerned. Weidemann *et al.* (1967) concluded that tenderness in beef muscle was produced by a disruption of the actin filaments and by a breaking down of the linkages between the actin and myosin filaments in the sarcomeres, provided the effects of gross connective tissue were small.

Herring *et al.* (1967b) found collagen content did not differ among longissimus dorsi muscles from bovine animals in A, B and E maturity groups and that it was not significantly related to tenderness. These workers also reported that neither collagen content nor collagen solubility was affected by post-mortem contraction state; however, tenderness was markedly decreased by increased contraction.

Herring *et al.* (1967a) found that post-mortem contrac-

Table 1. Histological comparison of the sheared surfaces of control and stretched muscle strips.

Trial No.	No. sections showing greater amounts of perimysial con. tissue[1]		No. sections showing greater granulation of perimysial con. tissue[1]		Average Shear force (lbs.)	
	Control	Stretched	Control	Stretched	Control	Stretched
1	2	1	2	1	5.47	6.63
2	3	0	0	2	3.47	4.70
3	4	1	1	3	5.81	4.50
4	4	0	1	3	6.71	5.45
5	1	3	3	1	7.20	6.23
6	3	0	2	1	7.04	5.96
7	4	0	0	4	7.70	5.28

[1] When compared to the corresponding section from the opposite group.

tion of muscles was very effective in causing decreased tenderness and that as muscles were shortened they had a larger percent area of fibers and, in contrast to the results of the present study, a smaller percent area of both endomysial and perimysial material.

It seems apparent at the present time that both connective tissue and intra-fiber molecular changes are involved in the tenderness pattern of a particular muscle; however, the proportional contribution of each and the conditions under which one may mask the other, remain to be resolved.

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Photomicrographic Studies of Dynamic Changes in Muscle Fiber Fragments. 1. Effect of Various Heat Treatments on Length, Width and Birefringence

SUMMARY—Photomicrographs were made of muscle fiber fragments as the stage of the microscope was heated from room temperature to 80°C, or as fibers were held at 37, 45, 53, 61, 69, or 77°C on the heated stage for an hour. The possible relationship of changes in width, length and in birefringence brought about by heating to loss of moisture, water-holding properties, loss of acidic groups, and changes in tenderness are discussed. Changes in width appeared to be related to changes in water-holding capacity. Changes in length and loss of birefringence were related to loss of acidic groups, to coagulation of proteins, to volume change in cooked meat, and to changes in tenderness.

INTRODUCTION

A REVIEW of literature reveals a continued interest in the effect of heat on muscle proteins. The rate of temperature rise in ground beef as affected by fat content was studied by Irmiter *et al.* (1967), and in longissimus dorsi steaks as affected by ante-mortem stress by Lewis *et al.* (1967). Weidemann *et al.* (1967), Paul (1965), and Lowe *et al.* (1961), noted changes in the histology of muscle tissues after cooking. Many studies (Bramblett *et al.*, 1964; Gilpin *et al.*, 1965; Hamm *et al.*, 1960; Pengilly *et al.*, 1966; Ritchey *et al.*, 1965; Rogers *et al.*, 1967; and Tuomy *et al.*, 1964) have been centered around changes in physical and chemical properties of muscles as they were heated by various methods to different temperatures for varying lengths of time. Hamm (1966), summarized the work on the heating of muscle systems.

This paper presents methods for the direct observation of changes in muscle fiber fragments when they are heated. In the first method, fibers were heated gradually by increasing temperature with time. In the second method, fibers were held at a constant temperature for a period of time. The changes in fibers observed by each method of heating are described.

EXPERIMENTAL

Materials and techniques

One-inch-thick slices of longissimus dorsi muscle from beef carcasses aged 7 days at 2°C were frozen and stored at −23°C until needed. Fragments of muscle fibers were obtained by placing a small piece of muscle in a Waring blender with enough saline solution (0.9% NaCl) to cover the blades of the blender. After running the blender for 15 to 30 sec, the separated fibers were poured into a Petri dish. Fiber fragments were picked up with a medicine dropper and placed on a glass slide.

A square approximately 18 × 18 mm was outlined on a glass slide, using vacuum grease. Muscle fiber fragments were then placed in the center of the square and covered with a 22 × 22 mm cover glass which was pressed into the vacuum grease to make a seal.

The prepared slide was then placed on a microscope stage (Leitz) equipped with both an electric heating element and a coil for circulating hot or cold liquids.

Increasing temperature with time. After suitable fiber fragments in one field were found, a photomicrograph was taken of that field at room temperature using either Kodak Panatomic X black and white film, or Kodachrome II color film. The heating element in the stage was then turned on and photomicrographs of this same field were taken after each 3-degree rise in temperature from 29 to 50°C, and every 2 degrees from 50 to 80°C. Approximately 45 min were required to heat the stage from room temperature to 80°C.

Constant temperatures with time. After a photomicrograph was taken of fibers at room temperature, heated water from a constant temperature bath was circulated through the coil in the stage. Photomicrographs were taken every minute for the first 10 min, every 2 min for the next 10 min, and every 5 min thereafter, for a total of 60 min. The temperature of the stage was recorded each time a photograph was taken. Approximately 10 min were needed to bring the stage to each of the following temperatures: 37, 45, 53, 61, 69 and 77°C. At 5 min the temperatures were within 4 degrees of 77°C and within 2 degrees of 37°C.

Measurements of dimensional changes of fibers were made from prints of the black and white negatives or from projected images of color slides. It was usually possible to have four or five fiber fragments in a field that could be measured through an entire series of photographs. The width and length of the fragments were measured. All values are given as percent change from the original width and length of the fragments at room temperature.

Birefringence of muscle fiber. The heating stage was transferred to a polarizing microscope equipped with a half-wave filter for red. The muscle fibers appeared yellow, red or blue-green against a red background, depending on their orientation with respect to the crossed Nicol prisms of the polarizing microscope. Color transparencies using Kodachrome II film were taken during the course of heating. The degree of change in birefringence of the fibers from one temperature to the next was determined by measuring the percent transmission through the color transparency of the fiber by means of a Photovolt densitometer (Model 542). A blue-green filter ($\lambda = 485$ mμ) was used and the densitometer adjusted so that the area

of a fiber allowing the most light to reach the photoelectric cell gave a reading approximately 80% full scale, while no light gave a zero reading. Although the values in Fig. 4 cannot be interpreted quantitatively, they indicate clearly the point where prominent changes in birefringence occurred.

RESULTS

FIG. 1 REPRESENTS mean values of the shrinkage characteristics of 32 individual fibers, and clearly shows that fiber fragments decreased in both width and length as heat was applied according to the first method (increasing heat with time). Gradual, but small, decreases in width began soon after heating was begun and continued to 45°C. Between 45 and 62°C, a more rapid decrease in width occurred. At 62°C, the process appeared complete, since very little further decrease in width occurred.

Shortening of the muscle fibers proceeded very slowly or not at all as the temperature was raised to 55°C. Between 55 and 65 °C, most of the fiber fragments shortened rather suddenly to about 80 percent of their original length. Increasing the temperature from 65 to 80°C resulted in an additional decrease of 10% of the original fiber length.

The second study (Fig. 2) in which desired temperatures were held constant, showed only a small (2.5%) decrease in width during the first 8 min at 37°C. Another 2 to 3% change took place in the next 8 min, but after this, little change took place. At 45°C, a 10% decrease in fiber width took place during the first 7 min, and during the next 18 min there was another 5% decrease. Little change in width occurred thereafter. At higher temperatures (53, 61, 69, and 77°C) most of the decrease in fiber width (23 to 27%) took place during the first 5 min of heating, with little change thereafter.

The length of the fiber fragments decreased very little (Fig. 3) as fibers were heated for 1 hr at 37 and 45°C. Some decrease in length took place during the time required for them to reach 53°C, but little after this. At 61°C, 10% shortening took place during the first 9 min and continued very slowly for the next 20 to 25 min, with very little change during the last 25 min of the heating period. At temperatures above 61°C, 25% shortening took place and was essentially complete in a little over 5 min.

When viewed under polarized light, muscle fibers appeared brilliantly birefringent. Upon heating, the birefringence gradually decreased (Fig. 4) for all muscle fiber fragments observed. Some fragments became isotropic by

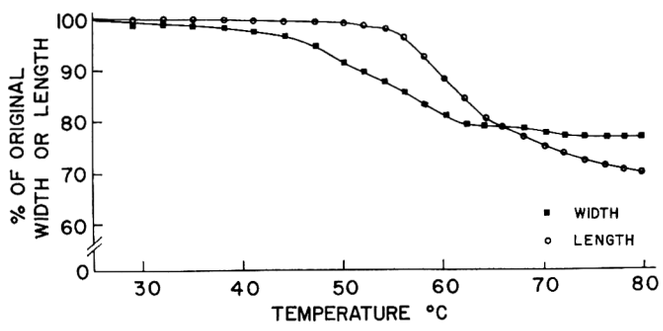

Fig. 1. Change in width and length of fiber fragments with increasing temperature.

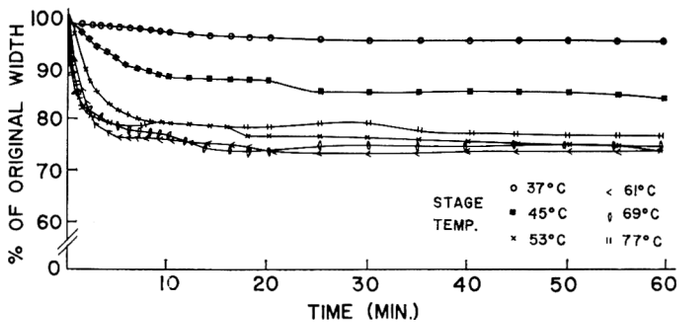

Fig. 2. Change in width of fiber fragments with time at different temperatures.

the time they reached 58 to 60°C, while others retained some birefringence at 66 to 68°C.

DISCUSSION

THE CHANGES in muscle fibers observed in these experiments seem to be related most closely to the water-holding properties of muscle proteins as described by Hamm *et al.* (1960) and Hamm *et al.* (1962). As proteins coagulate after being denatured by heat, their water-holding capacity is lost. Hamm states that the juice lost as drip during this process is that water held immobile by the myofilaments, but is not water of hydration. When the proteins of the myofilaments denature, the immobile water is freed, escapes from the intermyofibrillar space, and carries with it some soluble sarcoplasmic proteins. This material, visible in our observations as amorphous particles, appeared around the entire fiber, not at the cut ends only. The fiber simultaneously became narrower, this process being essentially complete at 53°C.

Results of our time-temperature studies on isolated fibers (Fig. 2) also showed that the diameter decreased in a manner parallel to the loss of water-holding capacity as demonstrated by Hamm *et al.* (1962). Thus the decrease in diameter may be explained as the early stages of heat denaturation where unfolding of peptide chains has occurred, causing a loss of water-holding capacity but little disturbance in the parallel array of myofilaments.

The changes in length of fibers do not conform to this behavior pattern, but instead resemble more closely the behavior described by the curves Hamm *et al.* (1960) have shown for the decrease or loss of the acidic groups. Fiber shortening does not begin until the temperature reaches 50° or more, when birefringence (Fig. 4) is noticeably

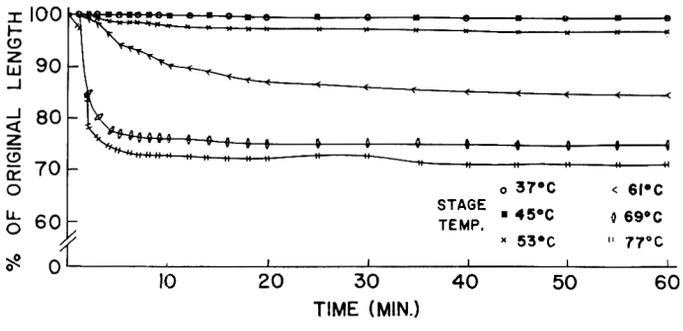

Fig. 3. Change in length of fiber fragments with time at different temperatures.

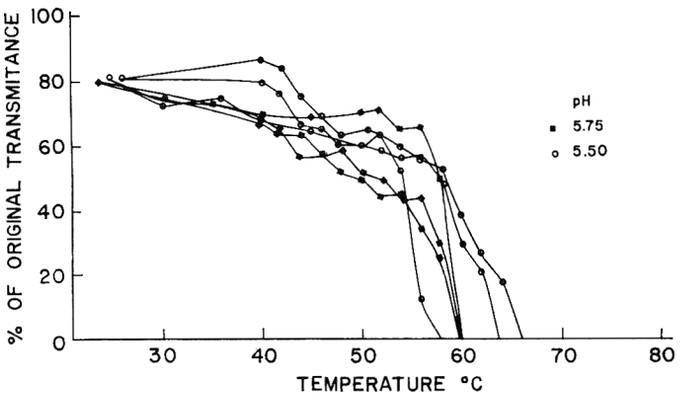

Fig. 4. Change in birefringence of fiber fragments with increasing time, measured as change in transmittance of light (λ = 485 mμ) passing through color transparency of fiber fragment.

diminishing (54 to 56°C). At these temperatures myosin is supposedly coagulated (Locker, 1956), myofibrillar proteins are rearranging and forming stable cross-linkages (Hamm, 1966), and sarcoplasmic proteins are rapidly coagulating (Lee *et al.*, 1966). These changes coincide with the observed changes in birefringence and shortening, and suggest that the fiber shortening is associated with the actual coagulation processes of the various proteins in the muscle. The loss of birefringence further indicates that the A-Band structure is being disturbed when the fibrillar proteins coagulate and are thrown out of alignment.

It can be assumed that changes in the length and width of muscle fibers also take place in meat being cooked since volume changes after cooking have been noted by several workers (Ritchey *et al.*, 1965; Lowe *et al.*, 1961). Ritchey *et al.* (1965) in work on the comparison of steaks oven-broiled to 61, 68, 74 and 80°C, found that a greater weight loss per minute of cooking time took place between 61 and 68°C than between the other temperature intervals. This is soon after the muscle fiber fragments observed in the present study began to shorten, and corresponds to the temperatures at which the greatest shortening of the muscle fiber fragments took place.

Machlik *et al.* (1963) measured changes in shear force of one-half inch cores of semitendinosus muscle with time at one-degree intervals between 50 and 90°C. At temperatures from 50 to 54 °C, there was little change in shear with time. It was only in the range 55–59°C that the decrease in shear seemed to be a function of time as well as temperature. This is again in the range where shortening began in isolated LD muscle fibers and it is also within the range where the shortening of the muscle fiber fragments seemed to be a function of time as well as temperature.

Machlik *et al.* (1963) further showed that at higher temperatures the decrease in shear was primarily a function of temperature. In our work, shortening of the muscle fiber fragments also became a function of temperature above 61°C. The question of whether the results of the two studies can really be compared can not be answered with certainty until one or both of the tests are run with samples from the other muscle.

Ritchey *et al.* (1964) found that the shear values of cores from LD were higher after being heated to 80°C than they were at 61°C. There is little evidence of this in the work of Machlik *et al.* (1963) on semitendinosus. Thus it remains to be seen whether shortening of muscle fibers due to heating can be associated with toughening in one muscle and no change in another. One factor which may enter into this question may be the possible difference in reaction to heat between free floating isolated fibers and those whose movement may be more or less restricted, as would be the case in a muscle. Perhaps the manner of relative position or restriction would influence the tenderness.

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Quantitative Methods for Anthocyanins. 4. Determination of Individual Anthocyanins in Cranberry and Cranberry Products

SUMMARY—A new method was developed to determine the quantities of the four major anthocyanins in cranberry and cranberry products. The pigments were streaked on Whatman No. 1 paper and separated by multiple ascending chromatography with 1-butanol-benzene-formic acid-water (100:19:10:25). The individual bands were measured by transmission densitometry at 525 nm. The ratio of individual pigments was calculated from the densitometric peak areas. The linearity of densitometric response for anthocyanins was established. The amount of each individual anthocyanin present was calculated from the total anthocyanin content and the ratio of individual anthocyanins. The reproducibility of the methods was 6% for the two arabinosides and 4 to 5% for the two galactosides expressed as the coefficient of variability.

INTRODUCTION

DUE TO VARIATIONS in the nature and position of the aglycon, sugar, and acyl moieties, probably several hundred different anthocyanins (Acys) exist in nature. A recent listing of all known Acys (Harborne, 1967) gave the source for 119 Acys. Most fruits, examined so far, contain more than one anthocyanin (Acy) and the number of these pigments within one fruit can be as high as 16 (Francis *et al.*, 1966a).

It has been demonstrated on solutions of pure individual anthocyanins (I Acys) that variation in the structure of Acys has a definite effect on the behavior and chemical stability of these pigments (Lamort, 1958; Horubala, 1964; Robinson *et al.*, 1966). Thus it is important to identify the Acy pigments in fruits and to determine the stability of each pigment during processing and storage. Information on the influence of varietal differences and growing conditions on the I Acy content would help in breeding varieties, and developing cultivation and processing practices which would result in a high degree of color stability in red fruit products. The quantitative Acy pattern can also serve as an indicator of adulteration for fruit products.

The lack of adequate methods for the quantitative determination of I Acys in a pigment mixture has greatly hampered research. Such a method should fulfill the following requirements:

1. It should be simple and applied readily to large numbers of samples.
2. Each Acy present should be determined quantitatively. The loss during manipulation, if unavoidable, should be proportionally the same for each Acy present in the particular product.

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The four major cranberry anthocyanins (Cy Acys) were identified by Sakamura *et al.* (1961) and Zapsalis *et al.* (1965) as cyanidin 3-monogalactoside (Cy 3-Ga), cyanidin 3-monoarabinoside (Cy 3-Ar), peonidin 3-monogalactoside (Pn 3-Ga) and peonidin 3-monoarabinoside (Pn 3-Ar). Because these Acys are closely related chemically, it is difficult to determine the quantity of individual pigments. The aim of this research was to establish a method, which fulfills the above requirements, for the quantitative estimation of the major Acys in cranberry (Cr) and its products. This method is expected to be used in establishing degradation rates for the I Acys in Cr products and to determine their quantity in different strains and varieties of Cr and related species. If one or two of the pigments should prove to be more stable than the others, then it might be possible to produce new varieties with improved color stability through selection and breeding. This work will also help to establish methods for the quantitative estimation of I Acys for other fruits, berries and their products.

LITERATURE REVIEW

IDEALLY THE DETERMINATION of I Acys should be done directly on the natural pigment mixture by measuring the optical density (OD) at a selected wavelength where the interference from the other Acys present would be minimal. However, the absorption spectra of most of the Acys are so similar (Harborne, 1967) that spectral measurements are of little use for such purpose. The methods described in the literature for the quantitative determination of I Acys were all done after the Acys were separated.

The methods applied for the quantitative determination of I Acys may be classified according to the method used for the measurement of the separated pigments.

Spectrophotometric methods

The concentrations of the separated Acys are determined by measuring the OD at the wavelength of maximum absorption. Such methods can be applied only on solutions, which makes their application slow and troublesome.

Effluent methods. Sondheimer *et al.* (1956) separated the strawberry Acys on a silicic acid column and determined the Acy concentration in each of the collected fractions.

Eluate method. Francis *et al.* (1966b) eluted the huckleberry Acys separated by paper chromatography and measured the Acy concentration in the eluate. The ratio of the four Acys was calculated as wt % by assuming the same E value for each pigment.

In situ methods

The quantities of I Acys are determined directly on the chromatogram. The rapidity of the *in situ* methods makes them particularly suitable for analysis of large numbers of samples.

Visual estimation. The color intensity of the spots can be roughly evaluated by the visual comparison. Such method was employed by Ribéreau-Gayon *et al.* (1954) to estimate the relative quantities of grape Acys separated by two-dimensional paper chromatography. This method was further refined by taking into consideration the spot size as well as the intensity (Ribéreau-Gayon, 1955).

Densitometric methods. Two different techniques have been employed for photometry of Acys *in situ*: photometry in transmitted light and in reflected light. Both techniques involve either the evaluation of the whole or the center of an individual spot, or the scanning of the chromatogram. In the latter case, the concentration of a certain substance is determined by integrating the area below the densitometric curve.

Reflectance densitometry. Gombkötö (1964) scanned the chromatograms of grape Acys with an instrument measuring the intenisty of the reflected light after passing through a green filter.

Transmittance densitometry. Ribéreau-Gayon *et al.* (1957) used such a method first for Acys. They used an automatic scanning transmission photometer employing monochromatic light to measure the Acy bands on one dimensional chromatograms of partially separated grape pigments. Since the pigments were not completely separated, identified, or their extinction coefficients determined, the results were expressed for each separate band as percent of the total peak area. A similar method was employed by Albach *et al.* (1959) but they corrected the densitometric readings for the difference in absorbancy between the different Acys by using "average standard peak areas," a corrected relative quantitative term.

The presence of a large amount of added sugar, such as that found in most preserved products, makes the separation of Acys rather difficult. Daravingas *et al.* (1965) employed an effluent method for the determination of Acy content of canned red and black raspberries. The Acys were extracted with methanol and separated on cellulose powder columns. The development was started in an ascending direction but after the solvent front reached one-third of the column's length, it was continued in a descending manner. The Acy content of each eluted band was determined using the Sondheimer *et al.* (1948) pH differential method. Fitelson (1967) and Mattick *et al.* (1967) used transmittance densitometric methods for Concord grape juice pigments. Fitelson (1967) purified the pigments by precipitating the Acys with neutral lead acetate and ethyl ether, while Mattick *et al.* (1967) used the untreated juice. Both authors used paper chromatography to separate the Acys into groups.

MATERIALS AND METHODS

THE SOURCES for the Cr and cranberry juice cocktail (CrJ) were reported previously (Fuleki *et al.*, 1968a,b) as well as the preparation of the pure individual Cr Acys (Fuleki *et al.*, 1967a).

Solvents

The following solvents were used:

BBFW—1-butanol-benzene-formic acid-water (100:19:10:25), aged 3 days, upper phase (Fuleki *et al.*, 1967b).

BAW—1-butanol-glacial acetic acid-water (4:1:5), upper phase.

EtOH-1.5 *N* HCl (85:15)—95% ethanol-1.5 *N* HCl (85:15). The acidity of the solvent was adjusted with conc. HCl to pH 1.0.

Instruments

A Hitachi Perkin-Elmer, Model 139 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) was used to determine the Acy content of the I Acy stock solutions. A Photovolt Densicord, Model 542, transmission densitometer (Photovolt Corp., New York, N. Y.) was used to measure the separated Acys on the chromatograms.

Chromatography room

The chromatography was carried out in a windowless, well-ventilated room kept at constant temperature (73 ± 1°F). Kurz-Miramon all-glass ascending assemblies in 12 × 12 × 24 in. tanks (Kensington Scientific Corp., Berkeley, Calif.) were used for quantitative chromatography.

Quantitative separation of cranberry anthocyanins

Crude Cr Acy was prepared by purifying and concentrating the Acys from CrJ or an ion exchange resin column (Fuleki *et al.*, 1968c). The crude Cr, Acy or the Cr extract (Fuleki *et al.*, 1968a) was applied on Whatman No. 1 paper as 4 cm long streaks in such a way that the direction of development was perpendicular to the machine direction of the paper. The chromatograms were equilibrated over the aqueous phase of BBFW for about 12 hr, transferred to another jar and developed ascendingly with the organic phase of BBFW. The atmosphere in the jar was saturated with the solvent prior to the development by lining the side of the jar with filter paper and soaking it with the developing solvent. The development was terminated as soon as the solvent front reached the area within 1 to 2 cm from the top of the paper. The chromatograms were air-dried for 20 min and the development was repeated.

Preparation of the chromatograms for densitometry

The upper frame of the sample holder was used as an aid in selecting and cutting out the strips from the chromatograms for scanning. The area passing over the slit was selected to give a uniform lateral distribution, always excluding the outer 2 to 3 mm of the streaks. The attenuancy of the Acys was intensified by fuming the chromatograms with hydrochloric acid. The fuming was carried out by suspending the strips from a glass frame placed in a chromatography jar, over approximately 6 *N* HCl for 5 min.

Establishment of operating parameters for the densitometer

The interference filter was selected by scanning a chro-

matogram of the Cr Acys with each of the wide-band filters supplied with the instrument. The wavelengths of maximum transmission for the filters were at 420, 445, 465, 485, 505, 525, 545, 570, 595, and 610 nm. The filter which gave the greatest response (525 nm) was selected for further work with the instrument.

The slit aperture was selected by scanning a chromatogram of the separated Cr Acys with the commercially supplied apertures: 0.1 × 15; 0.1 × 6, 1 × 6 and 1 × 3 mm. The narrower slits gave the best resolution. Although the sensitivity was slightly better with the 0.1 × 6 mm slit, the one with 0.1 × 15 mm dimensions was chosen because of the statistically better distribution of pigments over a larger area. The standard scanning speed (2 in. per min) was adequate with the selected slit.

The instrument was equipped with a variable response recording feature with switch positions from 0 to 12. The proper setting was selected by scanning a series of triplicate chromatograms of known amounts of Pn 3-Ga.

The No. 5 setting, where the quantity of Acy applied on the chromatogram vs. the densitometric peak area curve showed the best linearity, was selected as the "Response" switch setting for the instrument. This is a logarithmic setting (Levy, 1963).

Evaluation of the densitograms

Those densitograms in which the separation between the two lowest peaks reached beyond the half height of the smallest peak or where the height of the peaks was not between the 5 and 90 division marks were discarded. Taking into consideration the slightly tailing shape of the peaks the outline of each peak area was traced in the region where they overlapped. The area of each peak was measured twice with a planimeter and the readings were averaged.

Standard curves for the cranberry anthocyanins

Accurately measured volumes of freshly purified I Acys were applied in triplicate on Whatman No. 1 paper as carefully defined 4 cm streaks. The chromatograms for the standard curve were developed twice with BBFW as described previously. The concentration of Acy in the applied solution was measured in the EtOH-1.5 *N* HCl medium using the appropraite E values (Fuleki *et al.*, 1968a). The chromatograms were evaluated densitometrically and the average peak areas in planimeter units were plotted against the amounts of Acy applied on the streaks.

RESULTS AND DISCUSSION

Quantitative separation of cranberry anthocyanins

The quantitative determination of I Acys present in Cr is not feasible without prior separation of the pigments. Zapsalis (1963) suggested silicic acid column chromatography for the quantitative separation of Cr Acys. Column chromatography, particularly with silicic acid, is difficult to use with large numbers of samples. Paper or thin-layer chromatography is well suited for the handling of large numbers of samples and they allow the *in situ* measurement of the separated pigments. Since paper chromatography is the best method available for the separation of Acys (Harborne, 1967), major effort was directed toward the development of a method based on this technique.

The requirements of densitometry governed primarily the selection of operating conditions for the chromatographic separation. In order to have optimum results from densitometry, the chromatograms had to fulfill the following requirements:

1. The bands should be completely separated and should not be diffused.
2. The separated bands should be straight without any tailing or zigzag patterns.
3. The concentration of Acy in the bands should not exceed at any one point the concentration range where the densitometric response is linear.
4. The applied streak should be longer than the slit.
5. The background should be optically uniform and the thickness of the support should be such that the sensitivity of the detection is not overly reduced.
6. The measurement should be carried out at a pH level where the attenuancy of Acys are at their maximum and small variations do not appreciably effect the attenuancy.

Solvent. More than 50 solvent systems were tested but none of them were able to separate the four major Cr Acys. Finally, a new solvent system was developed (BBFW) which gave excellent separations (Fuleki *et al.*, 1967b).

Paper. The conditions which were found best with BAW, (Fuleki, 1967) namely Whatman No. 1 paper, developed across the machine direction were adapted for the quantitative work. However, No. 20 paper could be used with equal success. The separation as well as the uniformity of the background was good with both papers.

Equilibration. During routine use of the new solvent system to separate the Cr Acys, poor separation occurred during one period. The pigment bands were diffused and moved slower, while the solvent front traveled faster than usual. The charts showing the influence of benzene content (Fuleki *et al.*, 1967b) indicated that the anomalous behavior of the solvent system was due to a decrease in the water content. The cause of this was eventually found in the chromatographic paper used. The paper was stored under very dry conditions which decreased the water content of the paper considerably. Since the paper absorbed a larger than usual amount of water during development, the water content of the solvent was reduced with the consequent distortion of the usual pattern of development. This situation was remedied by equilibrating the chromatogram over the aqueous phase prior to the development. A similar phenomenon may have occurred with BAW in a few cases, in which the four Cr Acys were completely separated by this solvent (Fuleki, 1967).

Size of the streak. It was found that 4-cm streaks gave the best separation and at the same time allowed flexibility in choosing the area for densitometric scanning.

Mode of development. Descending development had the advantage of speed and complete separation. However, more uniform bands could be obtained on ascending development. The faster descending development frequently gave zigzag or tailing V-shaped bands which were unsuitable for densitometric measurement.

To improve the separation on ascending development, a multiple development technique was tried. After the first development was completed the chromatograms were air dried and developed again in the same solvent. The separation was greatly improved with this technique and it was adapted for use in the quantitative method. The additional manipulations (drying, longer development time, etc.) had no deleterious effect on the Acys (Fuleki, 1967).

Densitometric measurement of the separated anthocyanins. A densitometric method was favored over elution and spectrophotometric measurement of individual pigment concentration because it was simple and fast. Also, densitometry avoids the loss due to irreversible adsorption of Acys on cellulose (Roux *et al.*, 1950) and the overlapping part of the bands could be easily assigned to the appropriate pigments.

A careful selection of the chromatograms and the area which will be scanned was very important. The errors which could occur at this stage were as follows:

1. Poorly separated or excessively diffused chromatograms were retained for densitometric measurements.
2. The scanning area covered pigment bands with uneven lateral distribution. In this case the areas of two adjacent bands or the band and the pigment free area overlapped in certain slit positions due to slanted, zigzag or a tailing V-shape of the band.

Uneven lateral distribution of Acys results in an error because the Densicord as well as the other commercially available densitometers does not integrate attenuances across the width of the strip (Bush, 1963). In some cases, although the pigment bands were completely separated on the chromatogram, two of them passed over the slit at the same time because of the slanted or tailing V-pattern of the bands. This resulted in a poor separation on the densitogram. To prevent uneven lateral distribution, the Acys were applied on the paper as streaks instead of spots. Furthermore, those chromatograms in which a scanning area giving uniform distribution could not be selected were rejected.

Since the absorbance of the Acys varies greatly with pH, (Fuleki *et al.*, 1968b) it was necessary to assure a uniform pH of the paper at the time of measurement. Fuming of the paper with hydrochloric acid was quite satisfactory. It assured a uniform acidity in the pH region below 1.0, where small changes in acidity did not influence the absorption noticeably. Furthermore, the low pH resulted in maximum absorbancy for the Acys.

In order to demonstrate the tracing of the peak area and to show the separation achieved, a densitogram obtained with the ion exchange purified Cr, is shown on Fig. 1. Since the background absorption due to tannins (Albach *et al.*, 1959) was low and the instrument was carefully zeroed before the area with the pigments was scanned, the zero line was used as baseline. Planimetry was found to be the best means to measure the peak areas on the densitogram.

Densitometric standard curves. The establishment of the standard curves (Fig. 2) served three purposes. First, it was necessary to determine whether the densitometric response of Acys on paper followed Beer's law. It was also necessary to establish the limits of the response range for the instruments in which a linear response can be expected. The standard curves also made it possible to account for differences, if any, in the densitometric response between the four Cr Acys.

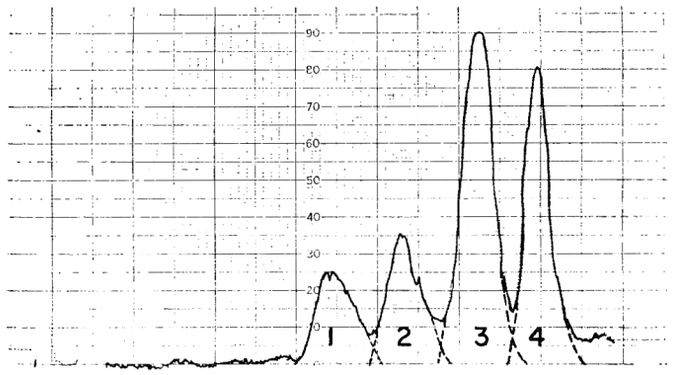

Fig. 1. A densitogram of the separated major cranberry anthocyanins. The numbering was started with the fastest moving pigment, therefore the numbers 1, 2, 3 and 4 stand for Pn 3-Ar, Cy 3-Ar, Pn 3-Ga and Cy 3-Ga respectively.

The standard curves for the four Cr Acys (Fig. 2) showed that the densitometric response followed Beer's law. The peak area plotted on the graph could not be used directly for setting limits of acceptability for the densitogram peaks because the deviation from linearity was a direct function of peak height and not that of peak area. Since the extent of diffusion and overlapping varied, peaks having equal areas did not necessarily have the

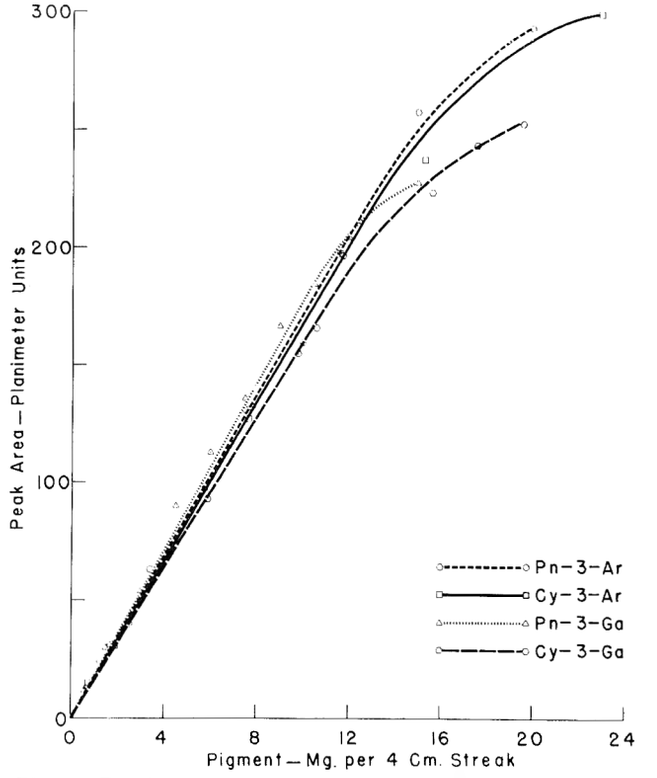

Fig. 2. Densitometric standard curves for the major cranberry anthocyanins (Densicord: Response = 5; Filter = 525 nm; Slit = 0.1 × 15 mm).

Table 1. Densitometric peak heights at the last determined point on the linear and the first determined point on the non-linear portions of the standard curves for the major cranberry anthocyanins.

Pigment	Last linear	First non-linear
	Peak height	
Pn 3-Ar	81	91
Cy 3-Ar	70	93
Pn 3-Ga	91	92
Cy 3-Ga	88	90

same height. This is reflected in the standard curves where deviation from linearity appeared at a lower concentration for the galactosides than for the arabinosides. On the basis of their molecular weights, the opposite would be expected. However, the galactosides moved slower, therefore, they produced less diffuse bands. As a consequence of this, the densitometric peak height per unit pigment was greater for the galactosides, hence the standard curves leveled off at a lower pigment quantity. Consequently, the limit for acceptability was set on the basis of peak height. This was accomplished by measuring the peak heights on the densitograms produced for the standard curves (Fig. 2) at the last determined point where the response was still linear and also at the first point on the non-linear portion of the curves. (Table 1).

The above data in Table 1 show the peak heights between which the deviation from linearity occurred. No data were available for the arabinosides at peak heights near 90 divisions. However, the limit could be set using the data available for the galactosides because their densitometric behavior appeared to be quite similar. The data indicated that the response was linear up to 90 divisions of peak height. Beyond that point, linearity was not expected because the instrument has a "break-off" point at the 90 division mark.

Although the lower ends of the standard curves were linear, it was necessary to set a lower limit of acceptability in order to reduce the error due to variation in the background. Considering the relative quantity of the pigments and the upper limit, the lowest acceptable peak height was set at the 5-division mark.

The results in Fig. 2 show very little difference in the attenuances for the four Cr Acys. The densitometric responses in planimeter units for 10 μg Acy applied as a 4-cm streak, under the conditions described for the operation of the Densicord, are shown in Table 2. The difference in attenuancy was within the variation between individual determinations; therefore, no correction factor was established to adjust the densitometer readings before the calculation of the percent contribution of individual pigments.

Table 2. Densitometric response for the major cranberry anthocyanins.[1]

Pigment	Response peak area
Pn 3-Ar	172
Cy 3-Ar	166
Pn 3-Ga	178
Cy 3-Ga	160

[1] The densitometric responses (Densicord: Response = 5; Filter = 525 nm; Slit = 0.1 × 15 mm) are given for 10 μg anthocyanin applied as 4 cm streak on Whatman No. 1 paper.

With other fruits where the pigments may exhibit greater differences in attenuance, such a correction factor could be established by dividing the densitometric response for the Acy with the highest attenuance by the densitometer response for Acy "X." The densitometer readings for Acy "X" should be multiplied with the correction factor before the calculation of the percent pigment composition. With mixtures containing monoglycoside and diglycosides of the same aglycone, the correction may be avoided by establishing the standard curves and calculating with molar instead of absolute quantities.

The values from the standard curves could be used for the direct determination of the quantity of I Acys. Such a method would eliminate the need for a separate Total Anthocyanin (T Acy) determination and the actual quantities, instead of the percentage, for the individual Acys would be obtained directly from the densitograms. However, such a direct method would require the utmost care to reduce losses on manipulation and great accuracy in applying the pigments for chromatography. The exacting nature of such a method outweighs its advantage of eliminating the separate T Acy determination, therefore, this approach was not used.

Calculation of individual anthocyanin content

The T Acy content in mgs, the Total Optical Density (TOD) or Optical Density Difference (ΔOD) were available from the analyses described previously for Cr and CrJ (Fuleki *et al.*, 1968a,b). The ratio of I Acys were obtained from the densitometric readings as follows:

$$\text{No. 1 Acy, \%} = \frac{\text{densitometric peak area for No. 1 Acy} \times 100}{\text{densitometric peak area for all four pigments}} \quad [1]$$

Since the calculations were essentially the same for all four pigments, the equations and the example are given only for the No. 1 Acy.

Simplified calculation. The quantity of I Acys was calculated by distributing the mgs of T Acy between the four Cr Acys according to their percentages.

$$\text{No. 1 Acy mg/100 g (ml)} = \text{No. 1 Acy, \%} \times \frac{\text{T Acy, mg}}{100} \quad [2]$$

Successive approximation. This calculation may be accomplished by determining the TOD or ΔOD for a hypothetical sample containing 100 mg T Acy and having the same type and percent I Acy composition as that of an actual sample. The OD or ΔOD values for the amounts of each pigment present in the hypothetical sample were calculated by using the appropriate E or ΔE values (Fuleki *et al.*, 1968a,b). The percentages of each pigment were taken as mg Acy in 1 ml solution and the OD or ΔOD value calculated for each of them (Equation 3). Since the E and ΔE values were given for a 1% solution (10 mg/ml), while the results were desired in mg/ml, only one-tenth of the E or ΔE values were used in the calculation.

$$\text{OD for No. 1 Acy (hypothetical)} = \text{No. 1 Acy, \%} \times E_{\text{No. 1 Acy}}/10. \quad [3]$$

$$\text{TOD (hypothetical)} = OD_{\text{No. 1 Acy}} + OD_{\text{No. 2 Acy}} + OD_{\text{No. 3 Acy}} + OD_{\text{No. 4 Acy}} \quad [4]$$

The summed OD or ΔOD value was related to the TOD or ΔOD value of the actual sample in order to obtain the Calculation Factor (CF).

$$CF = TOD\ (sample)/TOD\ (hypothetical) \quad [5]$$

The absolute quantities of I Acys were calculated by multiplying the quantities of each Acy in the hypothetical sample (which is equal to the percentage of the pigment) with the CF.

$$\text{No. 1 Acy, mg/100 g (ml)} = \text{No. 1 Acy, \%} \times CF \quad [6]$$

Example

An example is given in the following. The actual data were rounded off for ease of calculation. The T Acy content of a Cr sample was calculated in a previous example (Fuleki *et al.,* 1968a) as 101.8 mg per 100 g (TOD = 10,000). The densitometric areas for the four pigments (starting with the No. 1 pigment) were 40, 80, 160, and 120 planimeter units.

$$\text{No. 1 Acy, \%} = \frac{40 \times 100}{400} = 10\% \quad [1]$$

Simplified calculation

$$\text{No. 1 Acy} = 10 \times \frac{101.8}{100} = 10.18 \text{ mg per 100g} \quad [2]$$

The same equation was applied to the other pigments and the results were 20.36, 40.72, 30.54 mg per 100 g Cr respectively.

Successive approximation

$$\text{OD for No. 1 Acy (hypothetical)} = 10 \times \frac{981}{10} = 981 \quad [3]$$

$$\text{OD for No. 2 Acy (hypothetical)} = 20 \times \frac{1002}{10} = 2004$$

$$\text{OD for No. 3 Acy (hypothetical)} = 40 \times \frac{985}{10} = 3840$$

$$\text{OD for No. 4 Acy (hypothetical)} = 30 \times \frac{958}{10} = 2874$$

$$TOD\ (hypothetical) = 9699 \quad [4]$$

$$CF = \frac{10{,}000}{9699} = 1.031 \quad [5]$$

$$\text{No. 1 Acy} = 10 \times 1.031 = 10.31 \text{ mg per 100 g.} \quad [6]$$

The same equation was applied to the other three pigments and the results were 20.62, 41.24, and 30.93 mg per 100 g Cr respectively. The quantities for the I Acys were added up to obtain an accurate value of the T Acy content (103.1 mg per 100 g Cr) as indicated in the first two articles of this series (Fuleki *et al.,* 1968a,b). The calculations are similar for Cr products but ΔOD and ΔE values are used instead of the OD and E values.

The calculation process for Cr and CrJ was selected by calculating the I Acy content for Cr and CrJ samples with both methods. The results showed a negligible difference between the two methods, therefore the use of the more involved successive approximation would not be justified. The reason for the close agreement was that the differences in E values between the four Cr Acys were small. In cases with greater differences between the E or ΔE values of the Acys present in a particular mixture, or where the highest accuracy was required, the successive approximation should be used. The simplified calculation procedure is recommended for use in the method for the determination of I Acy content in Cr and CrJ.

Evaluation of the developed methods

The method recommended for the determination of individual anthocyanin content in cranberry cocktail. As shown in the schematic diagram (Fig. 3) the method consists of two separate procedures:

Determination of T Acy content
Determination of the ratio of I Acys

The results of the two determinations are used for the calculation of the I Acy contents in absolute quantities.

Determination of the total anthocyanin. The developed pH differential method (Fuleki *et al.,* 1968b) was used for the determination of the T Acy content. Aliquots of CrJ were diluted with sufficient amounts of pH 1.0 and 4.5 buffers to bring the pH to the required levels and to permit the OD measurements in the optimum range. The OD measurements were carried out at 510 nm after an equilibration period of 2 hr. From the OD readings the T Acy content was calculated.

Determination of the relative individual Acy content. The CrJ was purified on an Amberlite CG-50 ion exchange column (Fuleki *et al.,* 1968c). The column was washed with water and the Acy was eluted with 0.25% HCl in methanol. The Acys were separated by paper chromatography and the relative amount of each pigment was measured densitometrically as described in previous chapters of this paper. The quantities of the I Acys were calculated with the simplified procedure.

The method recommended for the determination of the individual anthocyanin content in cranberry. As the schematic diagram (Fig. 3) shows the procedure was essentially the same for Cr, except that the extraction of the Acy was the first step, the purification step was omitted and the single pH T Acy determination method was used (Fuleki *et al.,* 1968a).

The reproducibility of the methods was determined by carrying out the analyses outlined above for CrJ on 10 samples drawn from the same bottle. Only minor variations occurred in the first part (T Acy determination) of the analyses (Fuleki *et al.,* 1968b). The determination

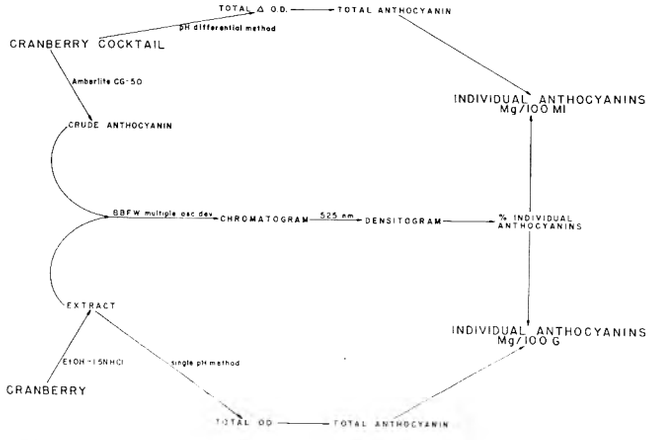

Fig. 3. Schematic diagram of the methods developed for the determination of individual anthocyanin content in cranberry and cranberry juice cocktail.

Table 3. Results of the individual anthocyanin determinations by the developed method on ten identical cranberry cocktail samples.

Sample No.	Individual Acy			
	Pn 3-Ar	Cy-3-Ar	Pn 3-Ga	Cy 3-Ga
	mg/100 ml			
1	0.76	1.94	3.60	3.60
2	0.85	1.88	3.74	3.32
3	0.97	1.93	3.33	3.64
4	0.89	1.85	3.63	3.55
5	0.91	2.04	3.41	3.41
6	0.90	1.61	3.86	3.55
7	0.90	1.95	3.39	3.79
8	0.86	1.82	3.43	3.74
9	0.85	1.85	3.56	3.62
10	0.86	1.81	3.58	3.65
Mean	0.87	1.87	3.55	3.59
Coeff. var.	6.28	6.15	4.70	3.91

of the percent I Acy content was the major source of variation for the whole method (Table 3) due primarily to the densitometric step. The coefficient of variability showed that the reproducibility of the developed method was at an acceptable level. The reproducibility may be improved by carrying out the densitometric analyses with as many replicates as desired for the required accuracy.

The two requirements listed in the Introduction for a quantitative I Acy determination method were fulfilled to a different extent by the developed methods. The developed methods are relatively simple and can be easily applied to a large number of samples. The method for CrJ has already been used to evaluate a storage study involving several hundreds of samples (Starr *et al.*, 1968). The method developed for Cr is currently in use to investigate the effect of variety and cultural conditions on the pigment ratios of Cr.

The second requirement was fulfilled only to a limited extent. The presence of several Acys occurring in trace quantities in Cr was revealed in the course of this investigation. Two of these pigments were identified as Cy 3-Gl and Pn 3-Gl (Fuleki *et al.*, 1967a). These glucosides were not separated from the corresponding galactosides in the quantitative method; therefore their quantities were included in the figure determined for the corresponding galactosides. The other minor Acys moved slower than the slowest moving major pigment (Cy 3-Ga). A small peak was frequently registered on the densitograms due to these unidentified pigments. However, this group of slow-moving minor pigments was disregarded because their quantity could not be measured reliably and their content was much less than 1% of the total. Descending development for an extended period of time would separate the pigments occurring in trace quantities but the limitation imposed by their low concentrations would still be present. Experiments with known quantities of pigments on the chromatograms showed that losses occurred to the same extent for all four pigments.

The method developed for Cr and CrJ could be applied with slight modifications to other fruits, fruit products and any Acy containing plant material as well. The basic design of the methods was that the total quantity and the ratio of I Acys were determined separately (Fig. 3). This approach is recommended for other Acy containing materials as well, because it frees the analyst from the burden of conducting a series of quantitative determinations requiring the highest accuracy.

The CrJ could be successfully purified and concentrated on Amberlite CG-50 ion exchange resin column. Extracts with high sugar content can be purified and concentrated also with the method employing basic lead acetate (Fuleki *et al.*, 1968c). In either case, before such a method can be used routinely, it is necessary to determine whether any alteration in the ratio of the particular combination of I Acys occurs as a side-effect of the treatment.

The separation of I Acys was the crucial part of the method. At present, paper chromatography is the best for quantitative separation of Acys. The separation on paper cannot always be accomplished with the conventional developing solvents. The system developed for Cr (BBFW) should be useful to accomplish many difficult separations but in some cases it may be necessary to develop new solvents. Other approaches such as those investigated to some extent in the course of this work (multiple development with 1-butanol-2*N* HCl (1:1) followed by BAW; quantitation of the aluminum chloride reaction may offer a solution for some other difficult Acy combinations (Fuleki, 1967).

In spite of its limitations, densitometric evaluation of the chromatograms is superior to the elution method when dealing with large numbers of samples. Careful selection of the operating conditions such as the filter, slit, paper, scanning area, etc, are very important in optimizing the performance of the densitometric method. It is also important to determine whether the pigments occurring in a particular mixture have similar attenuances. If they differ appreciably, it would be necessary to establish a correction factor. The possibility of carrying out the calculations on a molar basis should be also examined to eliminate the need for a correction factor.

The calculation procedure should follow one of those given for Cr. The selection will depend on the accuracy required and on the pecularities of the particular Acy mixture. Pigments, for which E values are not available, present a special problem. If the absorption coefficients cannot be established, then the best way to handle this problem is to calculate the T Acy content using an E value for a known pigment.

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Food Volatiles: Gas Chromatographic Determination of Partition Coefficients in Water-Lipid Systems

SUMMARY—The distribution of volatile solutes between two immiscible solvents can be determined by a study of the vapor pressures in the corresponding single solvent systems, since the partition coefficient is related to the Henry's constants: $K_p = k_I/k_{II}$. Application of this principle makes possible the gas chromatographic determination of partition coefficients involving nonvolatile solvents. The method was applied to a solvent pair consisting of paraffin oil and aqueous sodium sulfate. An approximate semilogarithmic relationship exists between the partition coefficient and number of carbon atoms within each homologous series of aldehydes, ketones, alcohols and esters. Saturation with sodium sulfate increased the partial pressure of volatiles from 12 to 20 times. The partial heat of vaporization of volatiles in paraffin oil decreased with increasing temperature and was appreciably smaller than that of the pure compound at its boiling point. These observations may be utilized in quantifying food volatiles in operations involving extraction and gas stripping prior to analysis.

INTRODUCTION

NAWAR (1966) has drawn attention to the various factors that affect vapor pressure of volatiles in contact with food media or with extracting solvents. Both from theoretical considerations of physical chemistry and from available experimental evidence (Lea *et al.*, 1958; Jellinek, 1959; Patton, 1964) it is clear that temperature, nature and complexity of suspending medium and of solute, concentration of solute, interaction of solutes, etc. play major roles in determining vapor pressures. These factors not only affect the interpretation of gas chromatographic headspace analyses, but also have to be considered in quantitative analyses based on procedures involving the use of extraction, stripping, concentration or other manipulations of the original sample.

We have reported elsewhere (Nelson *et al.*, 1966) on a method for the quantitative determination of food volatiles utilizing a nonaqueous, "non-volatile" solvent (paraffin oil) as the extractant. When a mixture of volatiles is partitioned between an aqueous and a non-aqueous phase, the quantity of volatiles in each phase at equilibrium will be determined by the partition coefficients of the individual components. We are here describing a gas chromatographic procedure for obtaining partition coefficients in such systems. Certain observations of factors that affect the magnitude of partition coefficients as well as of vapor pressure in pure solvents are also reported.

METHODS, MATERIALS AND THEORETICAL

PREDOMINANTLY AQUEOUS foods, such as most fruits, vegetables, milk and meat, contain volatile components in extremely dilute solution (ppm to ppb). The behavior of these solutions is therefore close to ideal and the vapor pressure of the volatile solutes is governed by Henry's law. If a lipid material is added (e.g., paraffin oil) or is already present (fats), volatile solutes will distribute themselves between the two phases as governed by the common vapor pressure. Under these circumstances and at equilibrium:

$$p = k_o \cdot c_o = k_w \cdot c_w \qquad [1]$$

where p, k and c are partial pressure, Henry's constant, and concentration in the liquid phase respectively, and the subscripts $_o$ and $_w$ refer to the two phases—oil and water. Since the partition coefficient is defined as $K_p = c_o/c_w$, it follows that $K_p = k_w/k_o$. Determination of partition coefficients is thereby reduced from a problem of determining concentrations in a binary solvent system to a problem of determining Henry's constants in a single solvent system. These were determined gas chromatographically as follows:

A known quantity of a pure volatile substance was allowed to vaporize completely in a 545-ml vapor flask (Hoff *et al.*, 1964). A quantity of 10 μl was used for the majority of the materials tested. A headspace vapor aliquot of 10 ml was removed and injected into another vapor flask held at constant temperature.

After agitation, aided by glass beads in the flask, a 2-ml aliquot of the second flask was injected into the gas chromatograph. Next, 10 ml of either paraffin oil or of a saturated sodium sulfate solution were injected into the flask. After adequate mixing to establish equilibrium, a 2-ml headspace aliquot was again injected into the gas chromatograph. Under our conditions, quantities of vapor injected were faithfully represented by peak heights. The decrease in peak height between the first and the second injection then represented the quantity of the volatile in the solvent.

The quantities of volatile solutes used in this study were selected to give concentrations in the liquid phases roughly corresponding to those found in nature. The maximal concentrations in the liquid phases, assuming that all the solute was absorbed, would be 10 ppm. The quantities injected into the gas chromatograph (Research Specialties, Model 600), were maximally 0.8×10^{-6} g (with no liquid phase present) and were detected by means of a flame ionization detector. A 6 ft × ¼ in. Ucon Non-Polar, LB 1715 column was used.

Henry's constant was calculated as follows:

$$k = p/c = PH' \cdot V_l/(PH - PH') \cdot V_g \qquad [2]$$

where PH and PH′ refer to the peak heights before and after addition of the solvent, and V_l and V_g refer to the

volume of the liquid and of the vapor space respectively. Henry's constants were expressed, not in absolute units, but as the ratio between the concentration in the vapor phase and the concentration in the liquid phase, g per ml vapor/g per ml liquid. This procedure allowed, for practical purposes, Henry's constant to be considered as formally nondimensional.

The aqueous phase was saturated with sodium sulfate (6 g per 10 ml water) to simulate the conditions of the extraction procedure. The vapor flasks containing vapor and the liquid phases being tested were maintained at 28°C unless otherwise stated. At this temperature the solubility of sodium sulfate is close to maximal, and the salting-out effect is reproducible. Lack of reproducibility was encountered when the temperature was allowed to transgress the decomposition point of the decahydrate (32.4°C).

Preliminary studies revealed that volatiles were dissolved in the oil. Steam distillation at reduced pressure was used to remove the entrapped components. The purified oil was stored in a brown reagent bottle under nitrogen.

The effect of temperature on the vapor pressure of volatiles dissolved in oil was investigated separately. A two-necked, 2 l round bottom flask equipped with a mercury-sealed mechanical stirrer and a serum cap to allow sampling of the vapor space with syringe and needle was maintained at the desired temperature in a controlled temperature bath. One hundred ml oil and 10 μl of a known compound were added to the flask. Vigorous agitation with the mechanical stirrer produced a thin oil film on the large internal surface of the flask. This aided in obtaining rapid heat and mass transfer. After mixing for a time sufficient to obtain equilibrium (usually 5 min) 2 ml of headspace were injected into the gas chromatograph and peak heights recorded. The temperature was adjusted from 30°C to 90°C with samples taken at 10°C intervals. Pressure was maintained constant by allowing headspace gases to escape after each 10° rise in temperature. Corresponding corrections were made in the calculations of partial pressures utilizing the ideal gas equation.

RESULTS AND DISCUSSION

Effect of sodium sulfate

The salting-out effect exerted by sodium sulfate is well known. The efficiency of this particular compound is probably associated with the binding of water molecules in the decahydrate with a corresponding reduction in solvent-solute interaction. The effect on Henry's constants of four compounds is shown in Table 1. The vapor pressure of these compounds in dilute solution is increased 12 to 20 fold when the aqueous phase is saturated with sodium sulfate. These effects are somewhat larger than found by Nawar (1966), but the experimental conditions were different. We worked at substantially lower concentrations and probably at higher temperatures than this investigator. The importance of maintaining defined conditions is illustrated by the effect of concentration of the salt on Henry's constants. Since the solubility of sodium sulfate is sharply affected by temperature up to the decomposition point of the decahydrate, strict control of temperature is imperative in order to obtain reproducible results.

Table 1. Effect of sodium sulfate concentration on Henry's constants at 28°C. $\left(\frac{\text{g/ml vapor}}{\text{g/ml liquid}}\right)$

	Water only	Water:salt 1:0.3	Water:salt 1:0.6	k_w Na_2SO_4 / k_w H_2O
	k_w	k_w	k_w	
Acetone—	.0013	.0052	.014	15
Ethyl acetate—	.0071	.024	.117	17
Isovaleraldehyde—	.020	.070	.241	12
2-pentanone—	.0044	.020	.082	20

Partition coefficients

Henry's constants and partition coefficients for selected compounds in aqueous solution and in paraffin oil at 28°C are given in Tables 2 through 5. The average of

Table 2. Partition coefficients and Henry's constants of selected alcohols at 28°C.

	k_w[1]	k_o[2]	K_p[3]
Methanol	.0014	.1229	.0114
Ethanol	.0030	.0916	.0332
Propanol	.0069	.0304	.2088
Butanol	.0133	.0057	2.386
Pentanol	.0454	.0014	34.5
Isopropanol	.0070	.0571	.1242
Isobutanol	.0163	.0113	1.442
Isopentanol	.0237	.0031	7.64
2-butanol	.0056	.0210	.2667

[1] k_w = Henry's constant for saturated Na_2SO_4 soln.
[2] k_o = Henry's constant for mineral oil.
[3] K_p = Partition coefficient.

Table 3. Partition coefficients and Henry's constants of selected esters at 28°C.

	k_w[1]	k_o[2]	K_p[3]
Methyl acetate	.0951	.0377	2.58
Ethyl acetate	.1169	.0098	11.84
Butyl acetate	.1750	.0010	167.2
Amyl acetate	.1958	.0008	248.
Propyl propionate	.1081	.0014	77.21

[1] k_w = Henry's constant for saturated Na_2SO_4 soln.
[2] k_o = Henry's constant for mineral oil.
[3] K_p = Partition coefficient.

Table 4. Partition coefficient and Henry's constants of selected aldehydes at 28°C.

	k_w[1]	k_o[2]	K_p[3]
Acetaldehyde	.0240	.1039	.2377
Propanal	.0367	.0175	2.103
Butanal	.1513	.0167	9.03
Pentanal	.1818	.0028	65.08
Hexanal	.4257	.0019	223.9
Isovaleraldehyde	.2415	.0057	42.36
Furfural	.0027	.0009	3.0

[1] k_w = Henry's constant for saturated Na_2SO_4 soln.
[2] k_o = Henry's constant for mineral oil.
[3] K_p = Partition coefficient.

Table 5. Partition coefficients and Henry's constants of selected ketones at 28°C.

	k_w[1]	k_o[2]	K_p[3]
Acetone	.0144	.0416	.345
2-butanone	.0410	.0130	3.21
2-pentanone	.0820	.0039	20.7

[1] k_w = Henry's constant for saturated Na_2SO_4 soln.
[2] k_o = Henry's constant for mineral oil.
[3] K_p = Partition coefficient.

duplicate samples of two trials are shown. A semilogarithmic plot of the partition coefficients versus carbon number gave essentially straight lines for each homologous series tested (Fig. 1). It is observed that partition coefficients are not only influenced by polarity, but also by angular orientation of intramolecular groups. Thus, branched and secondary alcohols have appreciably lower partition coefficients than primary, straight chain alcohols. Similarly, ketones, although being generally considered as less polar than aldehydes, exhibit a greater preference for the aqueous phase than aldehydes. All the tested compounds, except isoamyl alcohol, with five or more carbon atoms are extracted by the paraffin oil to an extent of 95% or greater. Considering other sources of error in quantitation of volatile compounds, the extraction may for these compounds be taken as complete, and the partition coefficient disregarded.

Effect of temperature on Henry's constant

Food volatiles that are partitioned into paraffin oil from an aqueous system are in our extraction procedure removed and isolated by means of stripping with an inert gas (helium). The efficiency of this process is determined by the partial pressures of the volatiles and affected to a major extent by temperature. If the increase in partial pressure relative to that of a reference temperature is considered and the latent heat of vaporization is assumed to be constant, one may write the Clausius-Clapeyron equation in the following form:

$$\log(P_T/P_{303}) = -\Delta H_v/4.576 \cdot T + C, \qquad [3]$$

where the P_T and P_{303} refer to the partial pressures at T and 303°K respectively. This procedure facilitates plotting and interpretation of the data (Fig. 2). When the temperature is increased from 30°C to 90°C, the vapor pressure of acetone increases by a factor of 2.3, while that of hexanol increases by a factor of 12.2. Raising the temperature of stripping will therefore primarily benefit the efficiency of removal of relatively high-boiling compounds.

The nonlinearity of the plots in Fig. 2 shows that the assumption made in integrating the Clausius-Clapeyron equation does not hold. ΔH_v decreases with increasing temperature. This deviation is more pronounced for low-boiling compounds than for high-boiling compounds. If ΔH_v is calculated based on the slope between 30°C and 40°C, it is seen (Table 6) that the heat of vaporization

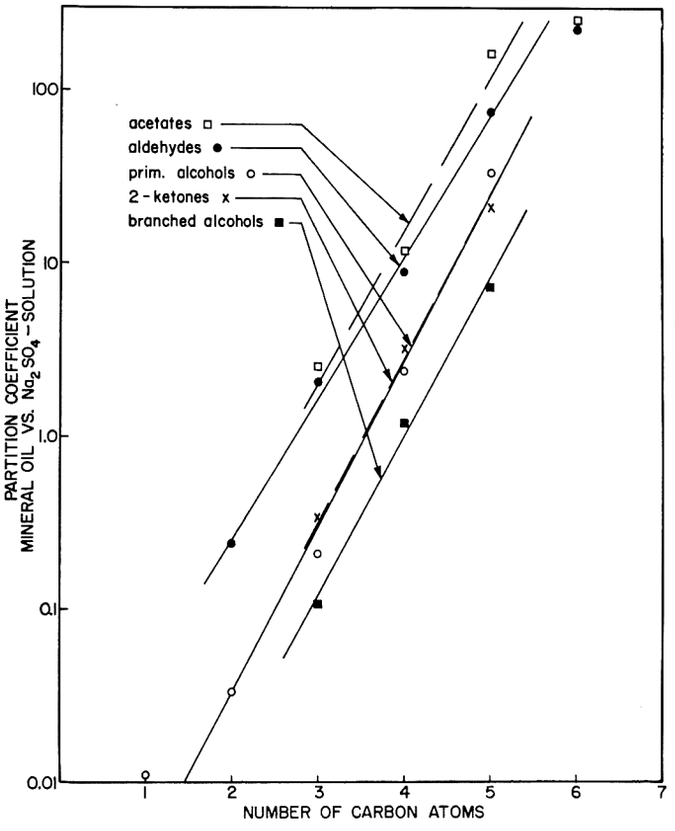

Fig. 1. Relation between partition coefficients and carbon number of selected components.

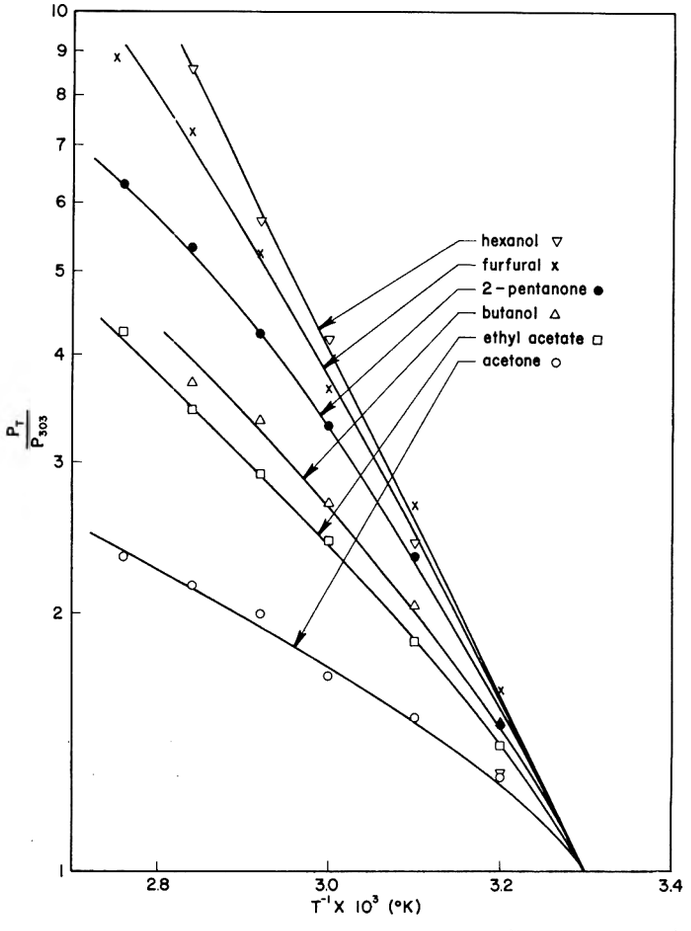

Fig. 2. Relation between vapor pressure and temperature of selected components in paraffin oil.

Table 6. Latent heats of vaporization of selected compounds in dilute solution in paraffin oil.

Compound	$-\Delta H_v$ (cal/g) in oil (30-40°C)	$-\Delta H_v$ (cal/g) Pure compound at 1 atms	Polarity Index
	(1)	(2)	(2):(1)
Hexanol	89.5	105.5 [2]	1.18
Furfural	85.7	107.5 [1]	1.25
2-Pentanone	82.1	92.8 [2]	1.13
Butanol	92.0	141.3 [1]	1.53
Ethyl acetate	69.0	102.0 [1]	1.48
Acetone	98.2	123.5 [1]	1.25

[1] (Perry, 1950).
[2] (Union Carbide, 1961, 1964).

in dilute solution in paraffin oil is in all cases appreciably smaller than the heat of vaporization of the pure compounds at their boiling points. This is reasonable since molecular interaction is largely absent in the dilute paraffin oil solutions. The difference or the ratio between these values expresses the magnitude of the molecular interaction. This ratio as shown in Table 6 could therefore be used as a "polarity index."

Data such as those given in Fig. 2 may be utilized for the determination of the time necessary for removing a volatile solute from a nonvolatile solvent by stripping with an inert gas. If it is assumed that equilibrium is established between the gas phase and the liquid phase when a gas is passed through a solution, then:

$$-\frac{dC}{dt} = K \cdot p \cdot F \qquad [4]$$

where C,t,p and F refer to concentration of solute, time, partial pressure of solute and gas flow rate respectively, and where K is a constant dependent on the system but independent of the specific nature of the solute. Substituting for p according to Henry's law, and integrating between the limits of C_0 (the initial concentration at $t = 0$) and C, one obtains,

$$t = \ln\frac{C_0}{C} \cdot \frac{1}{K \cdot k \cdot F} \qquad [5]$$

where k is Henry's constant at the operating temperature. The constant K may be obtained by determining the recovery of a compound whose Henry's constant is known after stripping for any convenient length of time. The recovery of any other compound of known Henry's constant can then be computed.

Stripping may be considered to be complete when $\frac{C_0}{C} > 100$; i.e. when less than 1% of the solute remains in the solvent. Under these conditions,

$$t_{completion} = 4.6 \cdot \frac{1}{K \cdot k \cdot F} \qquad [6]$$

It is seen that completion time is inversely proportional to Henry's constant.

Under our conditions, operating at 40°C, the completion time for acetone was 38 min, while that of hexanal was 500 min.

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Intensity - Concentration Relationships for Sugar and Salt Solutions

SUMMARY—Intensity of sweetness or saltiness was examined as a function of concentration and logarithm of concentration in solutions of sugar and salt in water and orange or tomato juice. Solutions were judged in linear or logarithmic concentration increments. No clear-cut evidence was found to show that the logarithmic function described the relationship better than the linear function. In the juice experiments, the failure to demonstrate a logarithmic function apparently resulted from the panel's insufficient discriminating power relative to deviations from regression arising from fitting a linear function to logarithmic data. In the water experiments, where such discriminating power was present, some evidence that the judging was on a linear continuum was found.

INTRODUCTION

IN A STUDY of a response surface approach to the evaluation of interrelationships between sugar and acid concentrations and the sweetness of orange juice, it was found that the relationship could be described by a first-order polynomial, and the higher order terms that would be required if the relationship were non-linear did not contribute a significant component to the variation associated with levels of sugar and acid (Gordon, 1965). In any curve-fitting procedure, establishing empirical relationships does not disprove the existence of more complex theoretical relationships; it merely establishes that, within the rules of the chosen statistical strategy, the empirical relationship is an adequate description of the data. Such may have been the case in this study since the usually accepted view is that intensity of response is proportional to the logarithm of the stimuli.

Several explanations for the relative simplicity of the empirical relationship between intensity and concentration could be suggested, and this paper reports a series of experiments designed to investigate this relationship further.

One explanation might be found in the performance of judges in their use of the rating device. If they view the assignment of scores as one step beyond ranking, and, after having established a rank-order for the series, they divide the scoring continuum in an essentially linear fashion, there would be a linear relationship between scores and whatever function of concentration had been presented to them. Alternately, the same result could be obtained if the physiological differentiation between various functions of concentration required a sensory acuity beyond the judges' capabilities.

In the studies reported here, the samples were presented to the judges in two series, one with equal intervals on a linear concentration scale, and one with equal intervals on a logarithmic concentration scale. The relationship between intensity and concentration, and intensity and logarithm of concentration was examined in the expectation that the intensity judgments in the linear series would be best described by a linear function of concentration and as a linear function of log C in the logarithmic series, if these factors were operative. Supplemental paired comparison and ranking tests were made to determine the extent to which judges could recognize differences between levels within a given series and between series.

Another explanation for the simplicity of the empirical relationships may lie in the nature of the theoretical function and the statistical test used to establish significance of regression and deviations from it for any given function of concentration. With a function such as y=f (log C), a considerable portion of the variation associated with regression appears as a linear component if the data are fitted to the function, y=f (C), and a relatively small component remains for testing the significance of deviations from regression. Given the variability associated with the judging situation, inherent in the variation between judges and successive judgments by the same judge, it is conceivable that the "deviations from regression" component would not be statistically significant even though it were of the order of magnitude generated by the theoretical function. This aspect of the problem was considered in the examining the structure of the test of significance of deviations from regression when the intensity scores were fitted to the two functions, y=f (C) and y=f (log C).

EXPERIMENTAL

Sugar-water

Sugar concentrations ranged from 1 to 5.06 g per 100 ml. This range was chosen to bring the lowest levels above the threshold reported by Feeney *et al.* (1966) for young adult females and the total range within that reported by Pangborn (1961). Since $1.5^4 = 5.0626$, a logarithmic series with 5 levels could be established by using the levels 1, 1.5, 1.5^2, 1.5^3, 1.5^4. The linear series was constructed by dividing the range, 1 to 5.06, into four equally spaced intervals.

Sugar-orange juice

The range of sugar additions was the same as that in the 2-factor experiments previously reported (Gordon, 1965), 0 to 0.2 *M* or 0 to 8.55 g per 100 ml. Concentrations were expressed as total concentrations based on estimated initial concentrations of 12.40 g per 100 ml in Exp. 1 and 10.30 g per 100 ml in Exp. 2 and Exp. 3. Initial concentrations were measured, after inversion, as reducing sugar by ferricyanide-thiosulfate titration or spectrophotometrically by the method of Ting (1956). Frozen orange juice reconstituted with tap water in 1:3 ratio was used throughout the experiment. The total concentration

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range was divided into equally spaced intervals for the linear series and the range of log C (1.09342 to 1.32015 in Exp. 1, and 1.01284 to 1.2753 for Exp. 2 and Exp 3), into equal intervals for the logarithmic series.

Salt-water

The range of salt concentrations was the same on a molar basis as that for the sugar-water experiments since the molar thresholds for sodium chloride and sucrose, as summarized by Amerine *et al.* (1965) are quite similar. The range was 0.029 *M* to 0.148 *M* or 1.71 g per 1000 ml to 8.65 g per 1000 ml. This range was divided, as in the sugar experiments, into equally spaced increments for the linear series and the range of log C (0.23300 to 0.93702) was divided into equal intervals for the logarithmic series.

Salt-tomato juice

The same range of salt additions was used in the tomato juice experiments. The increments were calculated on the basis of an initial salt concentration of 7.32 g per 1000 ml in tomato juice used in the linear series and 7.36 in that for the logarithmic series. Initial salt concentration was estimated from the sodium concentration determined with a flame photometer (Instrumentation Laboratories, Boston, Massachusetts, Model 143) with lithium as the standard.

Judging procedure

A series of ranking and paired comparison tests was completed before beginning each group of intensity experiments. Ranking in incomplete blocks (Bradley *et al.*, 1952) was used to evaluate differences between levels within each water series and ranking in complete blocks for levels within each juice series. Results for the pairs generated by the Bradley-Terry procedure were evaluated for significant differentiation based on tabulated values for significant differentiation in paired comparison tests (Amerine *et al.*, 1965). Ranks assigned in complete blocks were evaluated by procedures based on Kendall's Coefficient of Concordance (Amerine *et al.*, 1965). Simple paired comparison tests were used to evaluate differences between levels across linear and logarithmic series.

Intensity was scored on a 9-point scale with guide words of none, slight, moderate, strong and extreme. In the sugar-water, sugar-orange juice, and salt-water experiments, a linear series and a logarithmic series of samples were judged at each sitting, which was considered a replication. The presentation order of the two series and the order of samples within each series was randomized for each judge. In the tomato juice experiments, all of the replications of the linear series were completed first, followed by the logarithmic series.

Two complete experiments were done for the sugar-water series: four replications by eight judges in the first experiment and four replications by nine judges in the second. Three complete experiments were performed for the orange juice series. The first and third experiments consisted of four replications by nine judges and the second of five replications by nine judges. Single experiments of five replications by eight judges and five replications by nine judges were completed for salt-water and salt-tomato juice series.

The judges were undergraduate women, most of them foods and nutrition majors. The initial group had participated in a number of experiments with orange juice, and replacements were added as these experiments progressed. Since evaluation of saltiness was a new experience for the entire panel, the hypothesis that the individual judges supplied a homogeneous estimate of error for the total experiment was tested before further analysis of the data. Bartlett's test (Steel *et al.*, 1960) was applied to the level × replication interaction associated with individual judges. The calculated values of χ^2 were 5.08, 7 D.F., and 7.11, 7 D.F. for the linear and logarithmic series of salt-water. These values were less than χ^2 of 14.1 for 7 D.F. and 5% level of significance necessary to reject the hypothesis of homogeneity of variance.

Analysis of variance was applied to intensity scores. Regression of intensity scores as a function of concentration and the logarithm of concentration was calculated for both linear and logarithmic series. Significance of mean squares attributed to regression and deviations from regression was tested against the mean squares for judges × replication interaction.

For each experiment (sugar-water, sugar-orange juice, salt-water and salt-tomato juice), values of $\hat{y}$ for concentrations used in each experiment were estimated from the regression equation, $\hat{y} = a + b \log C$, where $\hat{y}$ is the estimated intensity score, a and b are fitted constants, and C is the concentration. Mean squares associated with regression as a function of concentration (rather than log C), and mean squares for deviations from this regression were then calculated. The latter mean square was designated "hypothetical deviations from linear regression." The maximum mean square for error possible if significance were to be established for deviations from linear regression was calculated by solving the equation:

$$F = \frac{\text{Hypothetical deviations from linear regression}}{\text{Maximum mean square for error}}$$

where F was the tabulated F value at 5% level of significance for the appropriate degrees of freedom.

RESULTS

Results of the ranking tests for samples within each series showed that the judges were able to differentiate between the concentrations occurring within each series. In the sugar-water series, eight judges made no errors in selecting the sweeter sample in each of the 10 pairs generated by the Bradley-Terry ranking procedure in which each sample is paired with every other sample. Considering the test as a simple paired comparison test, seven correct identifications would be required for differentiation at the 5% level of significance (Amerine, *et al.*, 1965).

In the salt-water series, the judges made no more than two errors in 17 judgments in the linear series, and no errors in 18 judgments in the logarithmic series, results well within the 13 correct judgments needed for differentiation at the 5% level in 17 and 18 trials. In the sugar-orange juice and salt-tomato juice experiments in which ranking in complete blocks was used, the large value of F associated with the test of significance of W, Kendall's Coefficient of Concordance, indicated agreement among the judges on the order of the samples.

Results of the paired comparison tests between levels across linear and logarithmic series are shown in Table 2.

The judges differentiated between levels in the sugar-water and salt-water experiments but were less successful in differentiating between levels across the linear and logarithmic series in sugar-orange juice or salt-tomato juice experiments. Thus, in the juice experiments, it was likely that the linear and logarithmic series would appear

Table 1. Rank sums for sugar-orange juice and salt-tomato juice experiments.

	Rank sum [1]							
	Sugar-orange juice				Salt-tomato juice			
	Linear		Log		Linear		Log	
Sample	1	2	1	2	1	2	1	2
A	54	54	53	52	50	54	49	47
B	45	44	42	47	38	43	45	49
C	35	37	35	35	39	26	39	33
D	27	25	32	26	23	27	25	28
E	19	19	17	19	22	26	18	21
F	9	10	10	10	17	13	13	11
F ratio [2] (5,38 D.F.)	300	161	62	107	11	23	28	27

[1] Number of judges, 9. Rank of 1 assigned to the sweetest (or saltiest) sample. A refers to the lowest concentration.

[2] $F = \frac{(k-1)W}{1-W}$, where k = number of judges, W = Kendall's Coefficient of Concordance defined as $\frac{\text{product s.s.} - (1/k)}{\text{total s.s.} + (2/k)}$

D.F. $= \frac{(n-1)-(2/k)}{(k-1\,[(n-1)-(2/k)]}$, where n = number of samples.

Table 2. Paired comparisons between levels of linear and logarithmic series.

	Number of correct judgments				
Pair Linear-log	Sugar-water	Sugar-orange juice		Salt-water	Salt-tomato juice
B—B	23	26	23		10
C—C	23	28	23	17	12
D—D	22	29	27	17	12
E—E		18	22	16	6
A—B	21				
B—C	20			16	
C—D	21			16	
D—E				16	
No. judgments	24	34	36	17	18
No. correct judgments for differentiation at 5% level	17	23	24	13	13

Table 3. Mean intensity scores for sugar-water experiments.

	Linear series			Log series		
	C g/100 ml	Mean intensity score		C g/100 ml	Mean intensity score	
Sample		Exp. 1[1]	Exp. 2[2]		Exp. 1[1]	Exp. 2[2]
A	1.00	1.5	1.8	1.00	1.5	1.6
B	2.02	3.0	3.0	1.50	2.1	2.2
C	3.03	4.6	4.6	2.25	3.5	3.6
D	4.05	6.1	6.0	3.38	5.3	5.1
E	5.06	7.3	7.2	6.06	7.2	7.1

[1] Mean of 4 replications by 8 judges.
[2] Mean of 4 replications by 9 judges.

Table 4. Mean intensity scores for sugar-orange juice experiments.

	Linear series					Log series				
	Exp. 1[1]	Exp. 2 & 3				Exp. 1[1]	Exp. 2 & 3			
Sample	C g/100 ml	Mean intensity score	C g/100 ml	Mean intensity score		C g/100 ml	Mean intensity score	C g/100 ml	Mean intensity score	
A	12.40	1.8	10.30	2.6	2.1	12.40	2.0	10.30	2.3	2.0
B	14.11	3.3	12.01	3.6	2.9	13.76	3.3	11.62	3.4	3.1
C	15.82	4.7	13.72	4.8	4.4	15.28	4.5	13.13	4.3	4.2
D	17.53	5.9	15.43	5.8	5.3	16.96	5.6	14.80	5.5	5.2
E	19.24	6.8	17.14	6.9	6.4	18.83	6.9	16.70	6.5	6.2
F	20.90	7.5	18.85	7.4	7.1	20.90	7.7	18.85	7.3	7.2

[1] Means of 4 replications by 9 judges, Exp. 1; 5 replications by 9 judges, Exp. 2; 4 replications by 9 judges, Exp. 3.

Table 5. Mean intensity scores for salt-water.

	Linear series		Log series	
Sample	C g/1000 ml	Mean intensity score[1]	C g/1000 ml	Mean intensity score[1]
A	0	1.0	0	1.1
B	1.71	2.0	1.71	1.9
C	3.45	3.5	2.56	2.6
D	5.18	4.9	3.85	4.0
E	6.93	6.2	5.77	5.7
F	8.65	7.2	8.65	7.3

[1] Mean of 5 replications by 8 judges.

Table 6. Mean intensity scores for salt-tomato juice.

	Linear series		Log series	
Sample	C g/1000 ml	Mean intensity score[1]	C g/1000 ml	Mean intensity score[1]
A	7.32	2.9	7.36	2.8
B	9.03	3.9	8.60	3.7
C	10.77	4.6	10.04	3.9
D	12.50	5.6	11.73	5.5
E	14.25	6.4	13.70	6.3
F	15.97	7.2	16.01	7.3

[1] Mean of 5 replications by 9 judges.

Table 7. Significance of deviations from regression of intensity as a function of C and log C for linear and logarithm series.

Variation due to:	D.F.	Linear series C M.S.	F	Linear series Log C M.S.	F	Log series C M.S.	F	Log series Log C M.S.	F
				Sugar-water Exp. 1					
Regression	1	703.64		675.89		703.59		688.07	
Deviations	3	0.44	<1.00	9.69	5.01**	2.05	1.50	7.22	5.27**
J × R	21	1.93				1.37			
				Sugar-water Exp.2					
Regression	1	685.44		646.98		709.10		693.64	
Deviations	3	0.54	<1.00	13.36	6.85**	1.71	1.43	6.86	5.72**
J × R	24	1.95				1.20			
				Salt-water					
Regression	1	683.95		665.73		783.72		779.86	
Deviations	3	1.01	<1.00	7.08	2.92	4.40	3.21*	5.69	4.15*
J × R	28	2.42				1.37			
				Sugar-orange juice Exp. 1					
Regression	1	832.89		845.52		828.07		839.78	
Deviations	4	4.01	1.06	0.85	<1.00	3.72	1.54	0.80	<1.00
J × R	24	3.77				2.42			
				Sugar-orange juice Exp. 2					
Regression	1	790.75		793.80		802.51		812.84	
Deviations	4	1.64	<1.00	0.88	<1.00	3.08	1.50	0.50	<1.00
J × R	32	1.66				2.05			
				Sugar-orange juice Exp. 3					
Regression	1	672.96		674.02		659.20		665.81	
Deviations	4	1.46	1.33	1.19	1.08	1.76	<1.00	0.10	<1.00
J × R	24	1.10				2.00			
				Salt-tomato juice					
Regression	1	562.14		557.32		665.81		660.80	
Deviations	4	0.21	<1.00	1.42	<1.00	3.10	1.10	4.35	1.54
J × R	32	3.46				2.83			

* F significant at 5% level. ** F significant at 1% level.

to the judges as merely replications when intensity scores were being assigned.

Mean intensity scores are given in Tables 3 to 6, and tests of significance of regression and deviations from regression are given in Table 7. The constants fitted to $y = f(C)$ and $y = f(\log C)$ are summarized in Table 8.

Table 8. Comparison of regression equations. Linear and logarithmic experiments.

Experiment		$y = a + b\,C^1$ a	$y = a + b$	$y = a + b \log C^1$ a	b
Sugar-water	1 Linear	0.07	1.46		
	Log	0.07	1.44		
	2 Linear	0.41	1.36		
	Log	0.30	1.37		
Salt-water	Linear	0.88	0.75	0	7.34
Sugar-orange juice	1 Linear	−6.26	0.68	−26.08	25.58
	Log	−6.01	0.67	−25.70	25.45
	2 Linear	−3.36	0.59	−16.94	19.16
	Log	−3.51	0.59	−17.27	19.36
	3 Linear	−4.10	0.60	−18.08	19.74
	Log	−3.86	0.60	−17.76	19.59
Salt-tomato juice	Linear	−0.58	0.49	− 7.97	12.44
	Log	−1.04	0.53	− 9.13	13.57

[1] C expressed as g/100 ml in sugar experiments, and g/1000 ml salt experiments.

In the sugar-water experiments, deviations from regression were not significant for $y=f(C)$ for either the linear or logarithmic series, but deviations were significant for $y=f(\log C)$. Furthermore, the fitted constants for each set of linear and logarithmic series were very similar; for example, a = 0.07 and b = 1.46 and 1.44 for the linear and logarithmic series, respectively, in the first experiment. These results suggest, then, that the judges placed all the samples, whether judged in linear or logarithmic concentration increments, on the same linear scoring continuum. In the salt-water experiment, the deviations from regression were not significant for both functions in the linear series, and significant for both functions in the logarithmic series.

In the orange juice and tomato juice experiments, deviations from regression fitted to either function were not significant. Thus, in the juice experiments, intensity data could be fitted to either function, $y=f(C)$ or $y=f(\log C)$, within the chosen level of significance.

The question may then be raised whether sufficient discriminatory power was present to differentiate between linear and logarithmic functions. The data summarized in Table 9 are an approach to answering this question. Mean

Table 9. Hypothetical mean squares for discrimination between functions.

Experiment		Mean square for: Hypothetical deviations from regression [1]	Maximum error for significance [2]	Observed error
Sugar-water	Linear Series	11.8	3.8	1.9
	Log Series	11.8	3.8	1.4
Salt-water	Linear Series	11.8	4.0	2.4
	Log Series	11.6	3.9	1.4
Sugar-orange juice	Linear Series	1.3	0.5	3.8
	Log Series	1.3	0.5	2.4
Salt-tomato juice	Linear Series	1.4	0.5	3.5
	Log Series	1.8	0.7	2.8

[1] Regression equations: Sugar-water $y = 0.95 + 8.3 \log C$; Salt-water $y = 0 + 7.3 \log C$; Sugar-orange juice $y = -26 + 26 \log C$; Salt-tomato juice $y = -8 + 12.4 \log C$

[2] Maximum error M.S. $=$ $\frac{\text{M.S. Hypothetical Deviations from Regression}}{F}$

$F = 3.07$, 2.95, 2.78, and 2.67 for sugar-water, salt-water, sugar-orange juice, and salt-tomato juice respectively for 5% level of significance.

squares for deviations from linear regression fitted to data generated from logarithmic functions are summarized in the table for each experiment. The table gives maximum values for error mean squares above which F values for the ratio of mean square for deviations from regression to error mean square would not be significant. The observed mean squares estimated from the judge × replication interaction are also given in the table.

The estimates of error supplied by the interaction term in the juice experiments were very much larger than the maximum value possible for discrimination. Thus, differentiation between the two functions, $y=f(C)$ and $y=f \log (C)$ appeared to require a discriminating power beyond that of the panel.

The hypothetical deviations were larger and the experimental error was smaller in the water experiments than in the juice experiments. Consequently, enough discriminating power was present to differentiate between the function in the water experiments. In none of these experiments did the logarithmic function give a better description of the data than the linear function. The manner of presenting samples caused some differences in the pattern of significance, but there was no evidence of a better fit to log C in the logarithmic presentations than in the linear presentations, as would have been expected if the judges were simply dividing the intensity continuum in a rank-order fashion. In addition, each set of constants from comparable linear and logarithmic series (Table 8) was quite similar.

These experiments, then, have not resolved the question of the theoretical relationship between intensity and concentration, but, as a consequence of the examination of the empirical relationship, have suggested some factors to be considered in designing experiments to evaluate the relationship.

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Limonin Monolactone, the Nonbitter Precursor Responsible for Delayed Bitterness in Certain Citrus Juices

SUMMARY—Tissues of early-season navel oranges and grapefruit were found by paper electrophoretic procedures to contain a nonbitter precursor of limonin, but no significant amounts of limonin. Limonin is the intensely bitter triterpenoid dilactone responsible for the bitterness which develops in certain navel orange juices on standing. The nonbitter precursor was identified as limonin monolactone by comparison with the authentic compound prepared by partial hydrolysis of limonin and by acid-catalyzed conversion into limonin. Limonin monolactone is stable in the tissues of the intact fruit (which are not bitter) because it is apparently not in direct contact with the acidic juice. It is slowly converted into limonin (and the juice becomes bitter) when the fruit tissues come in contact with the juice, after the juice is expressed from the fruit. Limonin monolactone was not detected in late-season navel oranges or grapefruit. This agrees with the fact that juice made from these fruits does not contain limonin. Work is in progress to determine whether the naturally occurring limonin monolactone is the A-ring monolactone, the D-ring monolactone, or a mixture of the two.

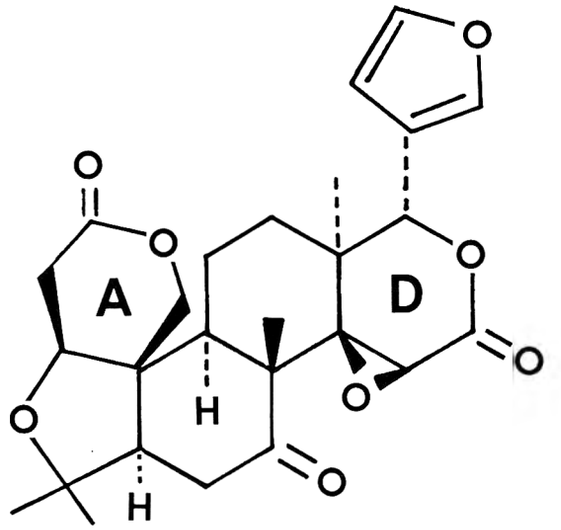

Fig. 1. The structure of limonin showing the A-ring lactone and the D-ring lactone.

INTRODUCTION

A SERIOUS PROBLEM in the production of juice from certain varieties of orange (early- to mid-season Washington Navel, Shamouti and Australian Valencia oranges) is the gradual development of bitterness in the juice after extraction from the fruit. When eaten fresh, these fruits are normally nonbitter. This phenomenon is generally referred to as delayed bitterness. For reviews, see Kefford (1959) and Joslyn *et al.* (1961).

The compound responsible for the bitter taste of the juice from fruits exhibiting delayed bitterness is the triterpenoid limonin (Fig. 1). Limonin was first isolated from Washington Navel orange juice by Higby (1938); it was shown to be the sole bitter limonoid in navel orange juice by Emerson (1949); and its complete structure was reported by Arigoni *et al.* (1960). In addition, Maier *et al.* (1965) isolated small amounts from grapefruit juice.

Although numerous attempts have been made to determine the cause of delayed bitterness, the problem has defied conclusive experimental proof. However, indirect evidence gathered over the years has led to the formulation of two theories. For convenience these can be designated the precursor theory and the diffusion theory.

The precursor theory was first put forth by Higby (1938). He proposed that the fruit tissues contain a nonbitter, water-soluble substance which, after disruption of the fruit tissues in juice manufacture, is extracted into the juice where it is slowly converted into limonin. Emerson (1949) studied the dihydroxy diacid formed on base hydrolysis of limonin and concluded that this compound lactonized too slowly to be the precursor. He suggested that the precursor might be one of the monolactone acids or perhaps a glycoside and that enzymes might be involved in conversion of the precursor into limonin. However, Emerson was unable to isolate or identify the precursor substance.

Chandler (1958) also attempted to isolate the precursor without success. This caused doubts to arise about the precursor theory and led Kefford (1959) to put forth the diffusion theory. He argued that limonin itself is present in the fruit tissues but because of its low solubility it takes an appreciable time to diffuse from the tissue fragments of the juice into solution and to reach a concentration sufficient to impart a bitter taste.

Samisch *et al.* (1950) felt that bitterness developed from a nonbitter precursor because filtration of juice immediately after reaming prevented development of bitterness. However, Kefford (1959) argued that this evidence could also be cited in support of the diffusion theory.

Because of the importance of the delayed bitterness problem to navel orange juice manufacture, we have reexamined the delayed bitterness phenomenon. Since much of the earlier evidence favored the precursor theory, it was felt that the best approach to the problem would be to search for and identify the precursor, if one existed. On the basis of the chemistry of limonin it appeared that the

simplest possible precursor would be one of the hydrolyzed forms of the compound.

Barton *et al.* (1961) had shown that limonin (Fig. 1) is a modified tetracyclic triterpenoid with a furan-ring side chain and that the A- and D-rings are δ-lactones (cyclic esters) which can be opened reversibly with excess alkali. [The dihydroxy diacid form of limonin will be referred to here as limonin disalt or the disalt. The A-ring monolactone hydroxy acid (or salt) and the D-ring monolactone hydroxy acid (or salt) forms of limonin will be referred to here collectively as limonin monolactone or the monolactone.]

Higby (1938) showed that limonin disalt undergoes lactonization to limonin under mildly acidic conditions when heated. Emerson (1949) reported that the disalt of limonin is water-soluble, but unstable in the acid form.

Apparently no direct studies of the monolactone forms of limonin have been made. In order to test for the natural occurrence of limonin disalt and monolactone it was necessary, therefore, to prepare the authentic compounds and to develop an extraction and detection procedure which would not, of itself, cause the compounds to lactonize. This has now been done and we report here direct evidence of the existence and identity of the nonbitter precursor of limonin in navel oranges and grapefruit.

EXPERIMENTAL AND RESULTS

Analytical procedures

The different forms of limonin were separated from each other by high voltage paper electrophoresis (PE) on Whatman No. 1 filter paper using 0.05 *M* citrate buffer, pH 5.5 and 113 volts/cm for 40 min. Limonin remained stationary and limonin monolactone and limonin disalt migrated toward the positive pole 6.5 and 14.5 cm, respectively. Each of the compounds gave the same distinctive orange-brown color when sprayed with Ehrlich's reagent (paper sprayed with a 1% solution of *p*-N-dimethylaminobenzaldehyde in methanol, then fumed with HCl), a selective spray for limonoids (Dreyer, 1965a,b). Limonin was distinguished from other limonoids by thin-layer chromatography (TLC) on silica gel-G, using the upper phase of benzene-ethanol-water-acetic acid, 200:47:15:1 (Maier *et al.*, 1965) and ethyl ether-acetic acid-water, 15:3:1. See Table 1.

Table 1. R_f values of limonoids on silica gel-G thin-layer chromatography.

Limonoid [1]	Benzene-ethanol-water-acetic acid (200:47:15:1, upper)	Ethyl ether-acetic acid-water (15:3:1)
Deacetylnomilin	0.27	0.73
Deoxylimonic acid	0.25	0.75
Deoxylimonin	0.37	0.63
Ichangin	0.26	0.67
Isoobacunoic acid	0.20	0.83
Limonexic acid [2]	0.27	0.61
Limonilic acid	0.30	0.92
Limonin	0.48	0.73
Limonin diosphenol	0.57	0.87
Nomilin	0.41	0.64
Obacunoic acid	0.20	0.84
Obacunone	0.58	0.86

[1] Limonin is the only limonoid reported to occur in navel orange and grapefruit juices (see: Introduction). Deacetylnomilin, deoxylimonin, ichangin, limonin, nomilin, and obacunone are the only limonoids reported to occur in *Citrus* seeds (Dreyer, 1965b, 1966). See Dreyer, 1965a for structures of these limonoids.

[2] Uncertainty exists as to whether limonexic acid is a natural constituent of *Citrus* seeds or an artifact (Dreyer, 1965a). Limonexic acid is negative with Ehrlich's reagent, but positive with periodate-permanganate.

Preparation of limonin monolactone and limonin disalt

Limonin was refluxed with a slight excess of 0.1 *N* NaOH for 1.5 hr to achieve partial hydrolysis and for 2 hr to achieve complete hydrolysis. Assay by paper electrophoresis (PE) showed that the partial hydrolyzate contained approximately equal amounts of monolactone (migration distance 6.5 cm) and disalt (migration distance 14.5 cm) and the complete hydrolyzate contained only the disalt. Proof that the hydrolyzates contained no decomposition products was obtained by reconverting the monolactone and disalt into limonin. A portion of each hydrolyzate was adjusted to pH 3.5 with dilute HCl and refluxed for 30 min. PE and thin-layer chromatography on silica gel-G (see analytical procedures) showed only the presence of limonin. In addition, each solution was intensely bitter. Other portions of the partial hydrolyzate were adjusted to pH 7.0 and 3.2 and diluted with water to a concentration of 100 ppm monolactone plus 100 ppm disalt. When tasted by individuals who could detect limonin bitterness at 2 to 7 ppm, the hydrolyzate was judged nonbitter. In like manner the complete hydrolyzate diluted to 100 ppm disalt was nonbitter.

The stability of limonin disalt to the conditions of the PE procedure was tested. A solution containing 96 ppm limonin disalt was adjusted to pH 5.0 (a pH slightly lower than that of the citrus tissue extracts) and 0.25 ml was applied as an 8-cm long streak to a PE paper (with a Rodder Streaker) under a continuous stream of air to aid evaporation. PE was run in the standard manner and the dried paper was sprayed with Ehrlich's reagent. Only one limonoid band was present. It corresponded to limonin disalt in migration distance. Also, 100 ppm each of limonin monolactone and disalt in 0.05 *M* citrate buffer at pH 5.0 formed no detectable limonin when held for 48 hr at 29°C, although limonin was formed on longer standing. Limonin did not hydrolyze (to monolactone or disalt) in 0.05 *M* citrate buffer at pH 5.0 or 5.6 even when boiled several hours.

Early-season Navel oranges

A group of Washington Navel oranges (*Citrus sinensis* (Linn.) Osbeck) were harvested November 13 for use in the following experiments. Their average weight was 211 g, and their average equatorial diameter was 6.7 cm. Several fruit were peeled and the endocarp portion was carefully separated into a carpellary membrane fraction (no seeds, albedo, or juice vesicles) and a juice vesicle fraction. The carpellary membranes were rinsed with water to remove any acid from broken juice vesicles. The albedo was collected from the peel by shaving off the pigmented flavedo. Juice was extracted from several other fruit from the same lot with a kitchen handreamer.

Carpellary membranes. A 28-g portion of the rinsed

membranes was blended with 100 ml water at high speed for 3 min in an Omni Mix at 5°C. The homogenate was rapidly filtered through celite (1 min) into a receiver immersed in an ice bath and gave a clear, almost colorless solution, pH 5.1 which was just perceptibly bitter. It was held at 5°C and divided into three portions for the following tests:

(1) Immediately after preparation, 500 μl of extract were applied as a 9-cm streak to an electropherogram paper, along with authentic limonin, monolactone, and disalt. After PE and treatment with Ehrlich's reagent, the extract showed a limonoid band that was identical to authentic monolactone in color and migration distance. No disalt or limonin was detected.

(2) A 30-ml portion of the extract was heated on a steam bath for 20 min and cooled. It was very bitter. PE (500 μl as a 9-cm streak) showed the presence of monolactone and limonin in the approximate ratio 3:2. (On longer heating the monolactone eventually disappeared and only limonin was found.)

(3) A 65-ml portion of the extract was adjusted to pH 3.0 with 1 *N* HCl, heated on a steam bath for 20 min and cooled. It was appreciably more bitter than the unacidified sample after 20 min of heating. PE (500 μl as a 9-cm streak) showed the presence of limonin and the absence of monolactone. Twenty-five ml of the acidified, heated extract was extracted with $CHCl_3$ (5 × 10 ml), the $CHCl_3$ was dried with Na_2SO_4 and concentrated to 2 ml. TLC and PE (40 μl as a 9-cm streak) showed the presence of a substantial amount of limonin and the absence of monolactone and disalt.

A test was run to confirm that limonin monolactone was not being formed enzymically or chemically (by acid catalysis) from limonin disalt during the extraction and PE procedures. Membrane tissues were blended to a slurry in 0.05 *M* phosphate buffer, pH 8 at 5°C. The ice-cold slurry was filtered rapidly and a portion of the filtrate was brought to a boil almost instantaneously. After boiling 3 min, it was cooled rapidly. PE of both the heated and unheated filtrates showed the presence of limonin monolactone and the absence of limonin disalt and limonin. Identical treatment of authentic limonin disalt caused no change in this compound.

Albedo. Ten grams of albedo were blended and extracted with 50 ml of water in the same manner as was the carpellary membrane fraction. After filtration the clear extract had a pH of 5.6 and was only very slightly bitter. It was held at 5°C and used in the following tests:

(1) Immediately after preparation, 150 μl was applied as a spot to an electropherogram paper. PE analysis showed limonin monolactone to be present and limonin and its disalt to be absent.

(2) 25 ml of aqueous extract were extracted with $CHCl_3$ (5 × 10 ml), the $CHCl_3$ was dried over Na_2SO_4 and concentrated under vacuum to 5 ml. PE and TLC (60 μl/spot) showed the presence of only a trace of limonin and no monolactone or disalt. The $CHCl_3$ extracted aqueous fraction was adjusted to pH 3 with 1 *N* HCl and refluxed 30 min. It was again extracted with $CHCl_3$ as above. PE and TLC (30 μl/spot) of the concentrated $CHCl_3$ extract showed the presence of a substantial amount of limonin and the absence of the monolactone.

Juice vesicles. This fraction was blended to break up the vesicles and release the juice. Immediately after preparation, the slurry (pH 3.2) was not bitter. After heating to 100°C over 14 min and cooling for 30 min it was extremely bitter. The slurry was filtered through celite giving a clear filtrate which was only slightly bitter.

Juice. This fraction (pH 3.3) was not bitter immediately after it was prepared. It was heated to boiling in 3 min and allowed to cool for 27 min, at which time it was only slightly bitter. After standing 2.5 hr at 23°C it was very bitter, as well as after 4.5 hr. A portion of the juice was filtered through celite giving a clear, light yellow solution. When compared with the very bitter unfiltered juice, the filtrate was rated as slightly bitter.

Another portion of the bitter juice was mixed with seven volumes of acetone, brought to a boil and filtered. The residue was treated with acetone once again, and the combined acetone extracts were concentrated under vacuum to an aqueous residue. The aqueous residue was extracted with $CHCl_3$, the $CHCl_3$ was dried over Na_2SO_4 and evaporated to dryness. The semisolid residue was mixed several times with hexane, centrifuged, and the remaining residue was treated with CH_2Cl_2, filtered, and concentrated. TLC of the CH_2Cl_2 concentrate gave a spot with Ehrlich's reagent in both developing solvents that was identical in R_f and color to authentic limonin.

Seeds. To test whether the extraction procedure would solubilize limonin if it were present in tissues, Valencia orange seeds which are known to contain limonin (orange seeds when chewed are extremely bitter) were examined. Five g of seeds were blended and extracted with 25 ml water in a manner identical to that used for the fruit tissues. The slurry and clear filtrate (pH 6.4) were both extremely bitter immediately after preparation and PE and TLC (15 μl/spot) of the clear extract showed substantial amounts of limonin and moderate amounts of monolactone to be present. No disalt was seen.

Early-season Marsh grapefruit

Albedo. A 10-g portion of albedo from an immature Marsh grapefruit (*Citrus paradisi* Macf.) (6.6-cm equatorial diameter, harvested July 4) was blended and extracted with 150 ml water in the same manner as was the orange carpellary membrane fraction. After filtration, the clear extract had a pH of 5.6. It was held at 0°C and used for the following tests:

(1) Immediately after preparation, 80 μl of extract were applied as a spot to an electropherogram paper, along with authentic compounds. After PE and treatment with Ehrlich's reagent the extract showed a limonoid spot that was identical to authentic monolactone in color and migration distance. No disalt or limonin was detected.

(2) After standing overnight at 0°C, 25 ml of extract were extracted with $CHCl_3$ (4 × 25 ml), the $CHCl_3$ was dried and concentrated to 2 ml. PE and TLC (19 μl/spot) of the $CHCl_3$ fraction showed the presence of only a trace of limonin. PE (80 μl/spot) of the aqueous fraction (after $CHCl_3$ extraction) showed the presence of the monolactone in an amount essentially unchanged from (1) above.

(3) The $CHCl_3$-extracted (limonin free) aqueous fraction was adjusted to pH 2.1 with 1 *N* HCl and boiled 30 min. It was extracted with $CHCl_3$ (4 × 25 ml), and the $CHCl_3$ was dried and concentrated to 2 ml. PE and TLC (6.4 μl/spot) gave a strong positive test for limonin and a negative test for monolactone and disalt. The aqueous fraction following $CHCl_3$ extraction gave a negative test (150 μl/spot) for limonin, monolactone and disalt by PE.

Carpellary membranes. A 7-g portion of membranes from a Marsh grapefruit (7.7-cm equatorial dia., harvested Oct. 13) was blended and extracted with 50 ml water by the method already given. About 500 μl of this clear extract (pH 5.0) was immediately applied as an 11-cm long streak to an electropherogram paper along with authentic compounds. PE showed a strong band which was identical to authentic monolactone in color and migration distance. No bands corresponding to limonin or limonin disalt were visible. A portion of the clear aqueous extract was acidified, heated and extracted with $CHCl_3$ as in (3) above. TLC and PE (18 μl as a 5-cm streak) of the concentrated $CHCl_3$ extract showed the presence of limonin and the absence of the monolactone and disalt.

Later-season Navel oranges and Marsh grapefruit

Navel orange. A 28-g portion of carpellary membranes from fruit harvested in April, extracted and filtered as before, gave a clear, almost colorless solution, pH 5.4. The solution was nonbitter and when tested by PE (using identical procedure and amounts as used with the early-season fruit) neither limonin monolactone, limonin disalt nor limonin bands were found. The solution was adjusted to pH 3.0 with HCl and heated 20 min on a steam bath. The cooled solution was nonbitter. Twenty-five ml of the heated extract was extracted with $CHCl_3$ (5 × 10 ml) and the $CHCl_3$ was dried and concentrated to 2 ml. TLC showed the presence of only a trace of limonin. The heated whole juice from portions of these fruit was also nonbitter.

Marsh grapefruit. A 7-g portion of carpellary membranes from fruit harvested in February was blended and extracted with 50 ml water by the method already given. It was tested by PE in exactly the same manner as the early-season grapefruit membrane extract and no limonin monolactone, limonin disalt or limonin was detected.

DISCUSSION

Paper electrophoresis (PE) proved to be an effective method of separating limonin disalt, limonin monolactone, and limonin because of charge differences exhibited by these compounds at pH 5.5. On PE the doubly charged authentic disalt migrated roughly twice as far as the singly charged authentic monolactone, while authentic limonin with zero charge was unmoved. All forms of limonin were stable during PE and were readily detected with Ehrlich's reagent, a selective spray for limonoids (Dreyer, 1965a,b). Limonin monolactone and disalt were differentiated from other mono- and di-carboxylic acid limonoids by acid-catalyzed conversion into limonin. Limonin was differentiated from other limonoids such as obacunone, nomilin, deacetylnomilin, deoxylimonin, ichangin, limonin diosphenol, obacunoic acid, isoobacunoic acid, limonexic acid, deoxylimonic acid and limonilic acid by thin-layer chromatography (TLC) on silica gel-G, Table 1. Taste tests showed that authentic limonin disalt and monolactone were nonbitter in neutral or acidic aqueous solutions at 100 ppm, while limonin was detectably bitter at 2 to 7 ppm. Taste tests also permitted limonin to be differentiated from obacunone, deacetylnomilin, deoxylimonin, and limonexic acid, all of which are tasteless (Dreyer, 1965a, 1965b, 1966).

The carpellary membranes of the endocarp and the albedo of the peel proved to be the best portions of the fruit for analysis. Aqueous extracts of these tissues were only mildly acidic (pH 5.0 to 5.7) and were low in interfering substances. In addition, previous workers had shown that bitterness is associated with the tissue fragment portion of navel orange juice. Analysis of early-season Washington Navel orange and Marsh grapefruit albedo and carpellary membrane extracts showed that limonin monolactone was the primary limonoid present.

Tests showed that limonin monolactone was not formed from limonin disalt by acid or enzyme catalysis nor from limonin by hydrolysis during extraction and analysis. Traces of limonin were detected in the chloroform extracts of the aqueous tissue extracts, but the limonin was probably formed from the monolactone during the liquid:liquid extraction step. Limonin monolactone was identified by electrophoretic comparison with authentic monolactone, by its color with Ehrlich's reagent, by its nonbitter taste, and by acid-catalyzed conversion into limonin. The identity of the limonin so formed (no other limonoid was detected) was confirmed by taste, PE, TLC, and color with Ehrlich's reagent.

If limonin itself had been present in the tissues in significant amounts, the clear aqueous extracts of the finely ground tissues should have been quite bitter (as was the seed extract) rather than essentially tasteless, and limonin rather than limonin monolactone should have been detected by PE. Treatment of the nonbitter, clear extracts with heat or with acid and heat caused the monolactone to disappear, limonin to appear, and the extracts to become bitter. These experiments show that a nonbitter precursor of limonin exists and that the precursor present in extracts of albedo and carpellary membranes of Washington Navel oranges and Marsh grapefruit is limonin monolactone. It would be reasonable to expect limonin monolactone also to be the nonbitter precursor of limonin in the tissues of other citrus fruits that exhibit delayed bitterness.

Whole juice prepared by reaming early-season navel oranges or juice made by blending the juice vesicles from similar fruit was not bitter immediately after preparation. After boiling and cooling, however, both juices became bitter. The presence of limonin in these bitter juices was confirmed by solvent extraction and TLC. No other limonoids were detected. When the boiled juices were filtered, the filtrates were less bitter than the unfiltered juices, indicating that a portion of the limonin was associated in some way with the tissue fraction of the juices. In addition, these results agree with the fact that before contact with acid, carpellary membranes are good sources of limonin monolactone. This experiment indicates that

limonin monolactone is also present in the walls of the juice vesicles.

Mid- to late-season navel oranges frequently produce juice that does not become bitter. Tests of later-season navel orange carpellary membrane tissue extracts showed that limonin monolactone was not present. When the extract was acidified and heated, no bitterness developed and only traces of limonin could be found in concentrated chloroform extracts of the aqueous solution. In addition, whole juice from these fruit after heating and standing for 5 hr was not bitter. Therefore, as navel oranges mature, limonin monolactone disappears from the tissues, and the juice made from these fruit does not become bitter after standing or heating. Tests of later-season grapefruit carpellary membrane tissue extracts showed that limonin monolactone was not present at this stage of maturity. Thus, grapefruit are similar to navel oranges in that limonin monolactone disappears as the fruit matures.

On the basis of this work it can be concluded that limonin monolactone is the primary naturally occurring limonoid present in the albedo and endocarp tissues of Washington Navel oranges and Marsh grapefruit at about the time they first reach commercial maturity. Minor amounts of limonin disalt may also occur, but only at levels beyond the sensitivity of the methods used here or at earlier stages of fruit development. Free limonin does not appear to occur to any significant extent as a natural constituent of the albedo or endocarp tissues of healthy intact navel oranges or grapefruit, although it is a natural constituent of citrus seeds. Free limonin might be found as a constituent of intact fruit tissues in instances where the fruit has been damaged and the released acids or enzymes have caused conversion of the monolactone to limonin. This may account for the bitterness of oranges damaged by frost. Since limonin is a dilactone, it is clear that two limonin monolactones are possible, the A-ring monolactone, and the D-ring monolactone. Work is now in progress to determine which of these forms occurs naturally.

The following explanation of the delayed bitterness phenomenon emerges from the work reported here. Limonin monolactone is present in the albedo and endocarp tissues of Washington Navel oranges and Marsh grapefruit. Since it is a nonbitter compound, the navel oranges when eaten fresh are nonbitter. However, when the juice is expressed from the fruit, in juice manufacture, cells and tissues are disrupted, and the tissue fragments (which contain limonin monolactone) come in direct contact with the acidic juice. Over a period of time, which is shortened if the juice is heated, the monolactone undergoes lactonization to the bitter dilactone, limonin, and the juice becomes bitter. The monolactone is stable in the intact fruit probably because it is located in a region of relatively neutral pH.

Higby (1941) reported that bitterness in navel orange juice could be minimized by avoiding tissue maceration and by immediately separating the coarser tissue fraction from the extracted juice. The chemical basis of this process is now clear. Avoiding maceration retards extraction of limonin monolactone into the liquid phase and at the same time facilitates removal of this potential source of bitterness before limonin is formed. It is hoped that this new knowledge will both aid and stimulate the commercial application of this process.

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Calcium and Oxalate Ions Effect on the Texture of Canned Apricots

SUMMARY—Softening of canned apricots was accompanied by increase in soluble pectin and syrup viscosity. Calcium ions have the ability to decrease, to some extent, the movement of pectic material from the fruit to the syrup. This might be explained by the ability of calcium ions to make a bridge between polygalacturonic acid. units, thus producing larger molecules which bind the cells together at the middle lamella. Added oxalate ions removed calcium from pectin in the cell wall, causing an increase in water-soluble pectin in the syrup and softening of texture. The mineral contents of canned apricots from three growing areas were determined by flame spectrophotometry. Low potassium in the fruit seems to be related to low pH in the cell sap, causing hydrolysis of pectic materials through hydronium ion catalysis and softening during heat processing and storage.

INTRODUCTION

THE CANNING INDUSTRY is occassionally faced with the problem of soft texture in canned apricots. Softening takes place in the can after processing or during storage. In severe cases the apricot halves nearly completely disintegrate to a mushy appearance. Luh *et al.* (1966) reported that harvest maturity and processing time are related to softening in canned apricots. Kertesz (1951) suggested that soft texture in canned tomatoes can be remedied, to some extent, by adding calcium ions. Collins *et al.* (1963) studied the influence of added calcium ions on the texture of thermally processed apple slices. Hoos *et al.* (1956) reported on the effect of 2,4,5-trichlorophenoxyacetic acid spray on the organic acids, pectin, and quality of canned apricots. The chemical composition of apricots has been reported by Strachan *et al.* (1951).

This study covers the effect of calcium and oxalate ions on the texture and pectin of canned apricots.

EXPERIMENTAL MATERIALS

Apricots

Blenheim apricots grown on the Wolfskill Ranch, near Winters (Yolo County), California, were harvested at canning ripeness. They were used to study the effect of added calcium and oxalate ions on the texture and pectin in canned apricots. Blenheim apricots grown at Suisun (Solano County) and Cupertino (Santa Clara County) were also used to determine whether a change in some chemical or physical property of fruit as brought about by environment might be associated with the softening of canned apricots. Calcium chloride was added to the syrups at 0, 50, 100, and 200 ppm calcium during the canning process. In a separate experiment, ammonium oxalate was added at 0, 0.25, and 0.50% levels to the syrup during canning.

Canning procedure

Canning was done in the pilot plant of the Department of Food Science and Technology, University of California, Davis. Green and overripe fruits were removed before canning. The apricots were washed with cold tap water and then halved and pitted in a Felice and Perrelli pitting machine. The halves were processed in No. 2½ cans made with differential electrolytic tin plate. The fill weight was 18 ± 0.5 oz. Enough 40° Brix syrup made with sucrose and distilled water was added to maintain a headspace of 5/16 to 7/16 in. The cans were sealed in a double seamer under a vacuum of 16 in. Hg, and heat processed in an Anderson-Barngrover continuous rotatory cooker at 210°F for 19 min. The cans were cooled in a rotatory water cooler, and stored at 70°F. Each sample consisted of 40 No. 2½ cans.

ANALYTICAL METHODS

Texture determinations

Twelve readings were made on four cans of each sample with a L.E.E.-Kramer shear press. Then 150 g of fruit was placed in the regular test cell. A 3,000-lb ring was used for testing the fresh fruit, and a 500-lb ring for testing the canned product. The dial settings for the fresh fruit ranged from 300 to 1,000 lb, depending on the ripeness of the product. For the canned apricots, a 200-lb dial setting was employed. The readings were converted to the 100-lb basis. The time interval from the beginning of the downward motion of the ring until it passed through the product in the test cell was 1 min ± 5 sec. The average area under the curve, in square inches, represents the firmness of the product. (Luh *et al.*, 1966).

Soluble solids

A Zeiss-Opton refractometer was used to determine the soluble solids in the samples. Results are expressed as °Brix at 20°C.

pH

A Leeds and Northrup glass electrode pH meter, model 7664-A1, was used to measure the pH of the blended samples.

Titratable acidity

A Beckman automatic titrator, model K, was used to determine titratable acidity. The fruit and syrup were blended for 1 min. Ten grams of the slurry were weighed into a 250-ml beaker, diluted with 150 ml of distilled water, and then titrated with 0.1 *N* NaOH to pH 8.0. Results are expressed as percent citric acid.

Extraction and determination of pectic substances

The syrups from four cans were mixed. To 100 ml of sample were added 5 g of Super-Cel. The resulting mixture was stirred and then filtered under suction through Whatman No. 1 filter paper. To 10 ml of clear filtrate were added 5 drops of 0.5 *N* HCl and 30 ml of 95% ethanol. The precipitated pectin was stirred thoroughly, separated from the suspending medium by centrifugation, and then washed successively with 95% and 70% ethanol. The precipitate was suspended in 25 ml of 0.05 *N* NaOH for 1 hr, by which time the pectin had completely dissolved. The resulting solution was diluted with distilled water to 1 L. The pectin content in the extract was determined by the carbazole colorimetric method described by McComb *et al.* (1952) and McCready *et al.* (1952).

Syrup viscosity

The fruit from four cans was drained on an 8-mesh screen for 2 min, and the syrup thus obtained was centrifuged for 30 min at 10,000 rpm (12,000 × g). The viscosity of 5 ml of the centrifugate was determined at 30°C in a No. 300 Ostwald-Finske capillary viscometer. A pycnometer was used to measure the specific gravity of the syrup. A precision electrical timer with an accuracy of 0.1 sec was used to measure the flow time in seconds. The viscosities are reported in centipoises at 30°C, with distilled water used as a reference.

Mineral content

The concentration of calcium, magnesium, and potassium was determined according to the method described by Luh *et al.* (1959). The canned apricots were drained to remove the syrup and then disintegrated in a blender. Ten grams of pulp were weighed into a silica dish, partially dried on a steam bath, and then dried in a vacuum oven at 70°C. The dried material was ashed to a constant weight in an electric muffle furnace at 550°C. The ash was weighed and dissolved in 0.15 *N* HCl. The resulting solution was made to 100 ml with the same solvent, and then filtered through Whatman No. 1 filter paper.

A Beckman DU spectrophotometer was adopted for flame photometry with the following wavelengths: calcium 422.2 mμ low sensitivity, magnesium 285.2 mμ high sensitivity, and potassium 767.0 mμ high sensitivity with a blue filter. The oxygen pressure was 10 psi. A phosphorous sulfur-nitrogen reagent was used for better resolution of the peaks of the emission curve. This reagent contained 50 ppm of $P(H_3PO_4)$, 20 ppm of $S(H_2SO_4)$, and 10 ppm of $N(HNO_3)$. Twenty ml of the sample solution were pipetted into a test tube, and 1 ml of the phosphorus-sulfur-nitrogen reagent was added. The mixture was thoroughly shaken before being introduced into the flame photometer. The results are expressed as mg per hundred g of initial sample.

In the case of syrup, 20 ml of clear syrup were pipetted into a silica dish, weighed, and treated as above.

RESULTS AND DISCUSSION

Texture of canned halves

The L.E.E.-Kramer shear press was used to measure texture changes in the canned apricot halves. The work needed to break the tissues in the shear press was recorded as the area under the force-time curve. Fig. 1 shows the changes in texture associated with the addition of calcium ions to canned Blenheim apricot halves stored at 70°F. The apricots were grown at Winters and were canned with 40° Brix sucrose syrup containing 0, 50, 100, and 200 ppm calcium ions and 0.25 and 0.50% ammonium oxalate. All samples softened gradually during storage. Samples canned with syrups containing added calcium ions were slightly firmer than the control sample. Fig. 2 shows that the samples containing oxalate were softer than untreated controls.

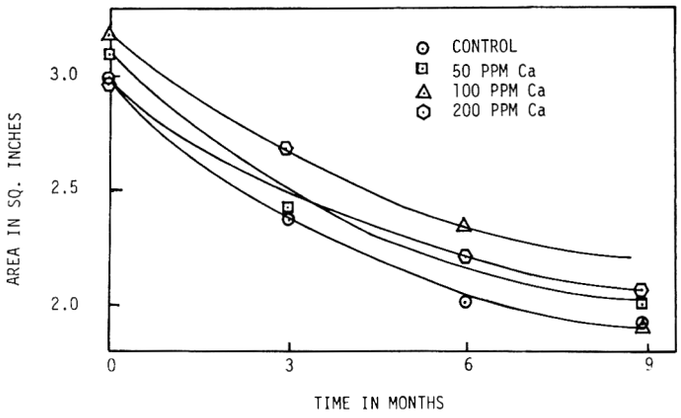

Fig. 1. Effect of storage at 70°F on texture of canned apricots from Winters. The syrups were treated with 0, 50, 100, and 200 ppm calcium during canning.

Trends were similar in apricots from the Suisun and Cupertino areas. The apricots from the Winters area had initial L.E.E.-Kramer readings of 3.0 to 3.2 sq. in., which decreased gradually to 1.8 to 2.1 sq. in. after 9 months at 70°F (Fig. 1). The sample from the Suisun area had an initial reading of 1.9 to 2.1 sq. in. (Fig. 3). It appears that the beneficial effect of calcium ions on texture was more pronounced in the fruits from the Suisun and Cupertino areas, especially after 9 months at 70°F. Maximum firming effect was observed when 100 ppm calcium was added to the syrup during canning. No further improve-

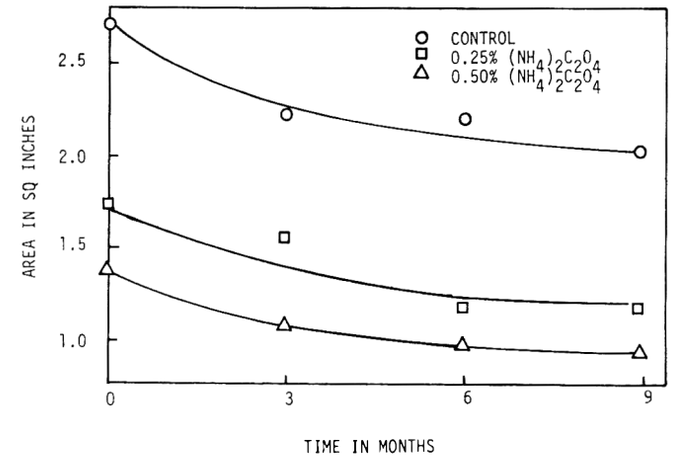

Fig. 2. Effect of storage at 70°F on texture of canned apricots from Winters. The syrups were treated with 0, 0.25, and 0.50% $(NH_4)_2C_2O_4$.

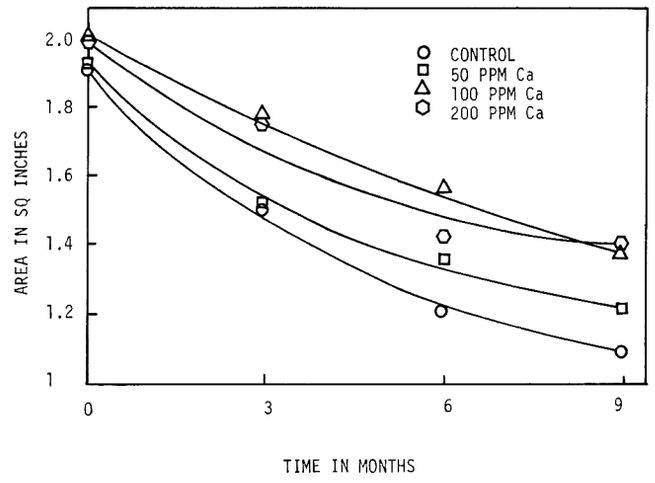

Fig. 3. Effect of storage at 70°F on the texture of canned apricots from Suisun. The syrups were treated with 0, 50, 100, and 200 ppm calcium during canning.

ment was observed when more calcium chloride was added. The phenomenon may be explained by the fact that calcium chloride is a salt of a weak base and a strong acid. It tends to be hydrolyzed to yield HCl which may counteract the effect of calcium ions.

The firming effect of the calcium ions on the texture of canned apricots may be explained by the linking of the carboxyl groups in the polygalacturonic acid units with calcium ions. This caused a strengthening of the binding force between cells, forming a firmer texture. Hanglein (1947) postulated that protopectin is formed by the association of polygalacturonic acid chains among each other, and perhaps even with cellulose, exclusively through calcium linkage. This was further evidenced by work of Postlmayr *et al.* (1956), who reported that firmness in clingstone peaches is related to the retention of protopectin. The relationship between calcium, pectin quality, and texture appears to be applicable to canned peaches and apricots.

That calcium ions play an important role in the texture of canned apricots was further shown by adding ammonium oxalate to the syrup. Fig. 2 shows the softening caused in canned apricots from Winters by adding 0.25 and 0.50% ammonium oxalate to the syrup during canning. The softening effect may be explained by the removal of calcium ions from the pectin in the cell walls to form calcium oxalate. This weakens the binding force between cells because of the reduction in binding between calcium and polygalacturonic acids. The samples with oxalate ions were extremely soft and judged unacceptable.

Effect of pH and acidity

Table 1 shows the firmness, pH, and titratable acidity of the fresh apricots used. Table 2 shows the effect on pH and titratable acidity of adding calcium chloride to the canning syrup. The results show that the samples from Suisun and Cupertino, which were characterized by considerable softening after canning, were higher in acidity and lower in pH than the Winters samples. It is possible that the hydrogen ions in the cell sap, activated by the thermal processing, caused softening due to hydrolysis of large pectin molecules into smaller units and solubilization of the cementing materials between cell walls. A possible approach to the problem would be determination of the pH and total acidity of the fresh fruit before canning. Fruit lots that prove unusually high in acidity and low in pH values may be diverted to the fresh-fruit market or used for apricot nectar and concentrates.

The relationship between the characteristics of the fresh apricots and the texture of canned products may be represented by the equation:

$$Y = -7.2021 + 0.0585\ X_1 + 2.7103\ X_2 - 0.1135\ X_3$$

where Y = Shear press readings (sq. in.) of the canned apricot halves after storage at 70°F for 9 months.

X_1 = Shear press readings (sq. in.) of the fresh apricots.

X_2 = pH of the fresh fruit.

X_3 = Soluble solids (°Brix at 20°C) of the fresh fruit.

When the X_1, X_2, and X_3 values of the fresh apricots are known, one may predict the shear press values of the canned product from the above equation. The pH value (X_2) appears to be an important factor influencing the texture of canned product.

Table 2. Effect of calcium chloride on pH and acidity of canned apricot halves. Stored at 20°C for 90 days.

Code	Growing area	Calcium ion added to syrup (ppm)	pH	Titratable acidity as citric acid (5)	Brix at 20°C
A1	Winters	0	3.90	0.608	25.0
A2	Winters	50	3.95	.612	25.0
A3	Winters	100	3.90	.640	24.6
A4	Winters	200	3.90	.674	24.5
A5	Suisun	0	3.52	.809	26.1
A6	Suisun	50	3.52	.829	26.0
A7	Suisun	100	3.48	.864	25.5
A8	Suisun	200	3.52	.867	25.5
A9	Cupertino	0	3.53	.856	24.6
A10	Cupertino	50	3.55	.859	24.6
A11	Cupertino	100	3.50	.866	24.6
A12	Cupertino	200	3.50	.860	25.2

Table 1. Chemical and physical properties of Blenheim apricots at canning ripeness.

Sample	Growing area	L.E.E.-Kramer shear press reading (sq in.)	Pressure test (lb) (7/16 in. plunger)	pH	Titratable acidity as citric acid (%)	Brix at 20°C	Av wt per fruit (oz)
A1	Winters	6.0	3.6	3.92	1.177	16.3	1.43
A5	Suisun	7.2	3.8	3.55	1.336	15.0	2.11
A9	Cupertino	6.9	3.9	3.50	1.348	14.1	1.87
L.S.D. p = .05		1.2	0.8	0.21	.062	.60	.20

Syrup viscosity

A close relationship was found between syrup viscosity and the texture of canned apricots. The effect of storage on the syrup viscosity of canned apricot halves from Winters as influenced by calcium ions is shown in Fig. 4, and that by oxalate ions in Fig. 5. The syrup viscosity of the canned product varied from 5 to 10 centipoises initially, increased rapidly during the first 3 to 6 months of storage, and increased more slowly on further storage.

Table 3 shows that the syrup viscosity was higher in samples from Suisun and Cupertino than in samples from the Winters area. Syrup viscosity gradually increased during storage at 70°F. Samples showing softening were higher in syrup viscosity. It seems likely that one of the factors in softening might be movement of high-molecular-weight pectin from the apricot tissue to the surrounding syrup. The increase in syrup viscosity appears to be accompanied by a decrease in firmness of the apricot halves. Addition of calcium ions to the syrup caused binding of the pectin molecules in the cell wall, which inhibits their movement from the fruit tissue to the surrounding syrup. This was also suggested by the experiment in which oxalate ions were added to the syrup.

The increase in syrup viscosity was faster and proceeded further in the oxalate-treated samples than in the control. This may be explained by the extraction of calcium from the protopectin in the apricot tissue, causing solubilization of pectic material, which diffuses into the syrup. It should be understood, however, that calcium ion was not the only factor affecting texture. Hydrogen bonds between hydroxyl groups at the C_2 and C_3 positions between galacturonide units in protopectin and glucoside units in cellulose also contribute to the binding force between the cell walls.

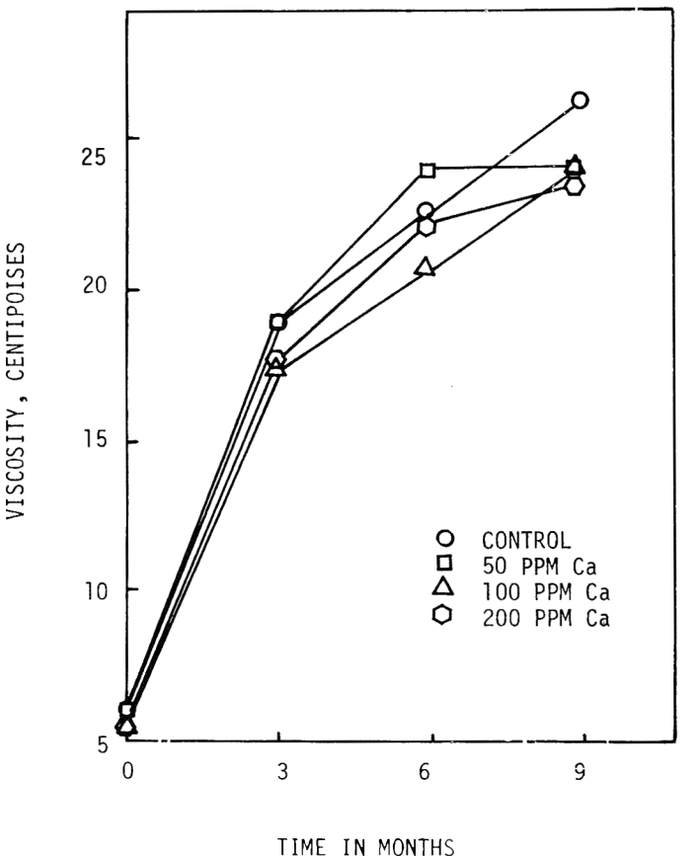

Fig. 4. Effect of storage at 70°F on syrup viscosity of canned apricots from Winters. The syrups were treated with 0, 50, 100, and 200 ppm calcium during canning.

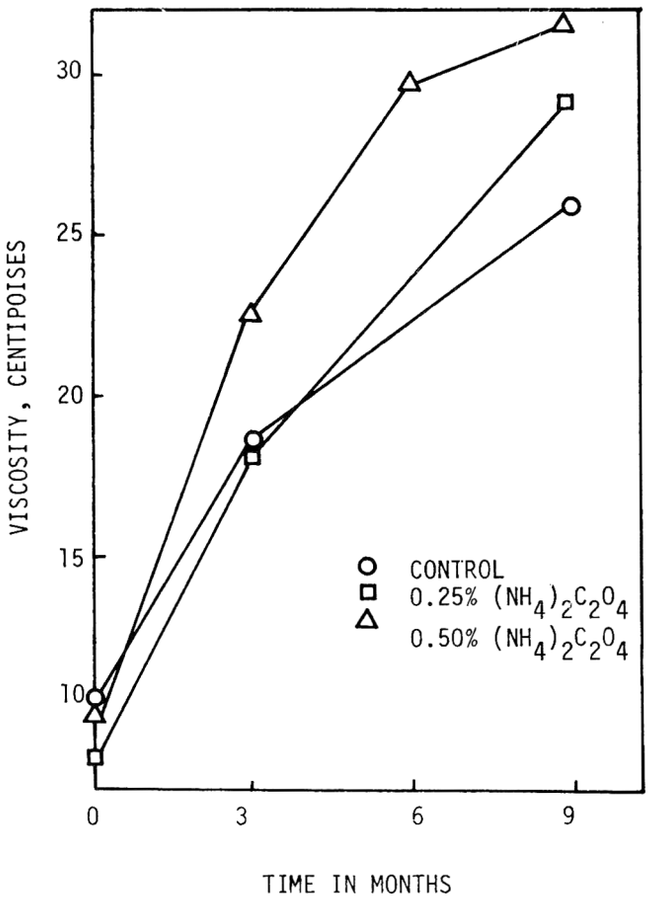

Fig. 5. Effect of storage at 70°F on syrup viscosity of canned apricots from Winters. The syrups were treated with 0, 0.25, and 0.50% $(NH_4)_2C_2O_4$ during canning.

Table 3. Effect of calcium ions on syrup viscosity of canned apricot halves.

Sample	Growing area	Calcium added to syrup (ppm)	Syrup viscosity (cps) Storage at 70°F (months) 0	3	6	9
A1	Winters	0	6.17	18.93	22.70	26.18
A2	Winters	50	5.82	18.71	24.01	24.00
A3	Winters	100	5.30	17.25	20.71	24.23
A4	Winters	200	5.35	17.85	22.39	23.66
A5	Suisun	0	13.93	32.49	62.14	63.45
A6	Suisun	50	12.26	35.02	66.89	73.84
A7	Suisun	100	11.52	44.62	54.29	70.95
A8	Suisun	200	10.19	27.39	42.59	61.95
A9	Cupertino	0	14.01	37.43	57.58	53.31
A10	Cupertino	50	21.64	37.15	41.52	58.60
A11	Cupertino	100	13.65	35.74	47.45	50.09
A12	Cupertino	200	11.75	35.64	55.18	55.00

Water-soluble pectin

Since protopectin in fruit tissues acts as a binding material between cell walls and is responsible at least in part for the firm texture of fruit tissues, the softening of apricots appears to be related to the conversion of protopectin to water-soluble pectin. This action reduces the binding force between cells and results in soft fruit tissue. The gradual softening of canned apricot halves was shown to be correlated with the increase in water-soluble pectin in the syrup.

Table 4 shows a rapid increase in water-soluble pectin in the syrup of canned apricots from Winters when the canned product was stored at 70°F. One day after canning, the concentration of pectin in the syrup was 119–134 mg/100 g. The increase was rapid during the first 3 month storage period, and then tapered off. The trends were similar to the increase in syrup viscosity, as was shown previously.

It can be postulated that there was a gradual conversion of protopectin to water-soluble pectin in the apricot tissue, which caused an increase in syrup viscosity resulting from diffusion of the water-soluble pectin from the tissue into the surrounding syrup. Addition of calcium ions appears to inhibit, to some extent, the diffusion of the pectin from the tissue into the syrup. Results were similar with the samples from Suisun and Cupertino. The increase was much slower after 6 months of storage.

Table 4. Effect of calcium ions on syrup pectin of canned Blenheim apricots.

Code	Growing area	Calcium added to syrup (ppm)	Syrup pectin mg/100 g			
			1 day	3 months	6 months	9 months
A1	Winters	0	119.5	286.8	316.5	327.6
A2	Winters	50	133.9	278.0	287.3	310.5
A3	Winters	100	120.0	233.6	284.2	275.1
A4	Winters	200	119.1	280.2	296.9	313.4
A5	Suisun	0	167.3	252.6	302.1	306.4
A6	Suisun	50	158.0	268.2	299.8	304.7
A7	Suisun	100	148.3	272.5	299.4	292.2
A8	Suisun	200	135.4	249.7	288.9	292.1
A9	Cupertino	0	166.7	287.4	319.5	319.1
A10	Cupertino	50	164.8	291.9	311.7	313.1
A11	Cupertino	100	151.7	264.4	305.7	300.7
A12	Cupertino	200	174.0	264.5	303.3	319.1

Mineral constituents

The potassium, magnesium, and calcium concentrations of canned apricots from Winters, Suisun and Cupertino are shown in Table 5.

Potassium. The sample from Winters (A1), which had a normal texture after canning, was higher in potassium content than those from Suisun (A5) and Cupertino (A9), which were unusually soft after canning and storage. Potassium is needed in many of the biological processes involving growth, metabolism, and synthesis in plants. It can combine with the organic acids formed in the biosynthesis processes, to form salts. When the potassium supply in the soil is low, the apricot may have a low pH and higher free acid content in the cell sap. The problem may be approached by checking the availability of potassium in the soil, and proper application of potassium fertilizers. Whatever the cause of higher acidity, the softening in canned apricots can be attributed, in part, to acid hydrolysis of the pectic or hemicellulose material in the fruit tissue during heat processing and storage.

Magnesium. Magnesium is one of the minerals needed in many biological processes in plants and in chlorophyll synthesis for photosynthetic activities. Table 5 shows the magnesium content of canned apricots from the three sampling areas. The sample from Winters (A1), which was normal in texture after canning, was slightly higher in magnesium content than the samples from Suisun (A5) and Cupertino (A9), which showed softening.

Calcium. Calcium is one of the important constituents related to the texture of canned fruits, since it binds pectic materials in the middle lamella. Removal of calcium from the tissue by oxalate ions caused softening in the canned product. Table 5 shows the calcium ion content of canned apricots from the three growing areas. The respective calcium contents in the fruit samples from Winters (A1), Suisun (A5), and Cupertino (A9) were 15.4, 9.7, and

Table 5. Distribution of K, Mg, and Ca in the fruit and syrup of canned Blenheim apricots stored at 32°F for 4 weeks after processing.

Sample	Growing area	Calcium added to syrup (ppm)	Minerals in mg/100 g					
			Fruit			Syrup		
			K	Mg	Ca	K	Mg	Ca
A1	Winters	0	254.7	6.8	15.4	251.1	6.0	6.0
A2	Winters	50	247.6	6.4	16.2	202.6	5.2	5.5
A3	Winters	100	240.3	5.7	19.9	234.9	6.2	10.0
A4	Winters	200	280.6	7.1	23.4	188.0	5.1	7.2
A5	Suisun	0	177.1	4.2	9.7	157.9	4.5	6.0
A6	Suisun	50	158.4	4.6	13.7	150.0	4.7	9.2
A7	Suisun	100	143.0	4.7	16.3	147.1	4.8	10.3
A8	Suisun	200	170.7	4.0	19.3	123.4	3.8	8.7
A9	Cupertino	0	182.8	4.5	19.9	145.1	4.1	4.6
A10	Cupertino	50	163.6	4.5	21.4	154.2	5.6	8.3
A11	Cupertino	100	178.3	4.0	21.9	133.3	3.4	6.7
A12	Cupertino	200	163.9	3.9	26.1	130.4	4.4	11.5

19.9 mg/100 g. The calcium in the syrup of the same samples was respectively 6.0, 6.0, and 4.6 mg/100 g.

Although the addition of calcium ion to the syrup caused some firming of the canned product, as shown in an earlier section, the differences in texture among the three lots cannot be attributed to their difference in initial calcium content. The sample from Cupertino (A9) was unusually soft in texture, yet its calcium content was 19.9 mg/100 g, considerably higher than the value of 15.4 mg/100 g in the sample from Winters (A1), which was normal in texture.

It seems likely that pectic materials in the middle lamella are not the only ones that serve as a cement to bind the cells together. McClendon and Somers (1960) reported that when calcium is extracted from fruit tissues, cell separation may not be complete, presumably because other bonds may be holding the cells together. Besides the calcium bridges in calcium pectinate gel, hydrogen bonds and other secondary valence forces may also hold the cells together. Breakdown of the pectic substances in the middle lamella by acid hydrolysis and by hydrogen-bond rupture can lead to weaknening of the intercellular cement and cause softening of the tissue. It remains to be investigated whether the biosynthesis of pectin and other carbohydrate polymers in the cell wall was changed by environmental conditions which may influence the texture of canned apricots.

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Salting and Drying Fish. 3. Diffusion of Water

SUMMARY—Drying of salted fish was studied in the falling rate period. It was found that this period consisted of two distinct phases, each of which was characterized by a Fickian diffusion coefficient. The coefficient for the first phase was, in all cases, greater than that for the second phase. The effect, on both diffusion coefficients, of the following variables was studied: degree of salting, drying temperature, and degree of hydration of the muscle (varied by addition of acid, base and sodium trypolyphosphate to the muscle). The first diffusion coefficient was found to increase, pass through a maximum, and then decrease with degree of salting. The second coefficient was found to decrease with degree of salting within the range for which it was determined.

The first diffusion coefficient was found to be directly correlated with the degree of hydration of the muscle; a plot of the coefficient versus water content of the muscle (expressed as pounds of water per pound of bone-dry solids) yielded a straight line, the regression equation of which was found to be $D = 0.16W + 0.09$, where D is the diffusion coefficient in $cm^2/sec \times 10^5$ and W is the water content. Variations in the diffusion coefficient due to degree of salting and due to addition of acid, base, and phosphate could be explained by the hydration-dependence of the coefficient. The temperature variation of both coefficients was found not to be great.

INTRODUCTION

THE FIRST TWO PAPERS in this series have been concerned with salting of fish. This paper considers some aspects of the drying of salted fish.

A number of studies on drying of fish have been concerned with fixing of "optimum conditions" of temperature, air relative humidity and speed for drying. Thus Fougere (1948) found that fresh cod could be optimally dried by an air stream of linear velocity 150 cm/sec at 55°C and 60% relative humidity or by a stream of velocity 250 cm/sec at 26.7°C and 50% relative humidity. He also found that drying times were inversely related to the thickness of the fish.

Del Campo *et al.* (1956) found that fatty herrings took longer to dry than lean ones, and that considerable fat oxidation occurred during the process. Finally, Linton *et al.* (1945), studying the drying of heavily-salted cod, reported that the optimum drying conditions for this product were: air velocity of 100 to 125 cm/sec, air temperature of 26°C and relative humidity of 45 to 55%. They noted that high drying potentials inhibited drying due to the formation of an impervious salt-protein crust on the surface of the fish. Other studies exist which report essentially the same or similar results (MacPherson, 1933; Hampton, 1937).

The salt-protein crust which is formed during drying of heavily-salted fish has also been noted by Jason (1958). According to Wood (1964), the drying rate retardation effect caused by the presence of this crust may be circumvented by a process which consists of alternating periods of drying with periods of press-piling to break the film.

Jason (1958) conducted a number of theoretical studies on the drying of fish, including heavily-salted fish. He found that drying occurred in two stages, a constant-rate period and a falling-rate period. Drying in the constant-rate period was found to be controlled solely by the conditions of the ambient atmosphere, being equal to that from a saturated water surface of the same shape; drying in the falling-rate period was found to occur in two distinct phases, each of which was characterized by a Fickian water diffusion coefficient.

Concerning these two diffusion coefficients, both were found to be isotropic and to depend upon the fat content of the fish; both also followed an Arrhenius-type variation with temperature. The coefficient for the first falling-rate period was found to be greater than that for the second falling-rate period.

Jason also studied drying of heavily-salted cod in the falling-rate period. He found that diffusion coefficients for the first falling-rate period were approximately equal for both heavily salted and unsalted fish, while coefficients for the second falling-rate period were considerably lower for salted than for unsalted fish. He also found that salted fish entered the second falling-rate period sooner than unsalted fish.

Jason (1965) also investigated the effects of fat content on the diffusion coefficients. He found that both were sensitively dependent upon the amount of fat present in the muscle. The equation which correlated the variation of either coefficient with fat content for various species was found to be $D^{-1} = a + bF$, where D is the diffusion coefficient, D^{-1} is the "diffusion resistivity," F is fat content, expressed as percent of net weight, and a and b are constants.

The drying studies reported in this work were concerned with (1) determination of the effect of degree of salting on the diffusion coefficients for both falling-rate periods in drying of salted fish muscle; (2) determination of the effect, on the diffusion coefficients, of the addition of acid, base, and phosphate to the salted muscle; and (3) determination of the effect of temperature on both diffusion coefficients.

The studies described in point (2) were carried out because it is known that acid, base, and phosphate have an appreciable effect upon the degree of hydration of muscle and because it was desired to determine the effect of the latter parameter on the diffusion coefficients.

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PROCEDURE

TRANSPORT OF WATER during drying occurs in two successive steps: diffusion from the interior of the material to be dried to the surface, and evaporation at the surface. The rate of the second step depends upon air velocity, air relative humidity and temperature, while that of the first step depends upon temperature and magnitude of the diffusion coefficients.

Drying occurs in the constant-rate period if surface evaporation controls; it occurs in the falling-rate period if diffusion controls. Drying can be made to occur in either period by making the corresponding step the rate-limiting one. Thus, low air velocities lower surface evaporation rates with respect to diffusion rates and cause drying to occur in the constant-rate period. High air velocities, on the other hand, increase surface evaporation rates with respect to diffusion rates and cause drying to occur in the falling-rate period. In this work, the constant-rate period was suppressed and drying was begun in the falling-rate period by using high air velocities.

Swordfish *(Xiphias gladius)* was used in all cases. Samples of muscle were selected so as to assure that all were of approximately the same fat content (2 to 3%).

Swordfish slices of uniform shape (4 cm diameter × 0.5 cm thickness) were salted in brines of different salt concentrations (0.5 molal, 2.5 molal and saturated) by a method described in a previous paper (Del Valle *et al.*, 1967). Slices were also salted in brines of the same above concentrations which contained, in addition, hydrochloric acid (0.05 molar), sodium hydroxide (0.05 molar) and sodium tripolyphosphate (5%). After desalting, the slices were removed from the brines and were blotted dry. Dimensions of the slices were determined after blotting. Concentration of salt in the slices was also determined by a method described in another paper (Del Valle *et al.*, 1967).

The procedure followed in all drying runs was as follows. The slices to be dried were placed on a tray inside a drying tunnel and their wet weight was read in a Mettler balance to which the tray had been attached (Fig. 1). The balance had been previously tared to zero weight with the tray alone. Following this, drying was commenced by turning on the fan, and was allowed to proceed for 3 hr, with readings of the weight of the slices being taken every 10 min. At the end of the drying run, the slices were removed from the tray and were put in a closed aluminum moisture cup for final moisture determination. Drying air temperatures were measured by inserting a thermometer in the tunnel as shown in Fig. 1. Runs were conducted at 40 and 55°C.

Data taken during the drying runs were processed as follows. Total weights of the slices obtained as functions of time in the different runs were first converted to water contents (weight water per weight dry solids) in the following manner:

$$w - w_S = w_{H_2O} \quad [1]$$

$$W = w_{H_2O}/w_S \quad [2]$$

where w is total weight of the slices, w_S is their dry weight (obtained by drying the slices to constant weight in a vacuum oven at 65°C), w_{H_2O} is the net weight of water in the slices, and W is the water content.

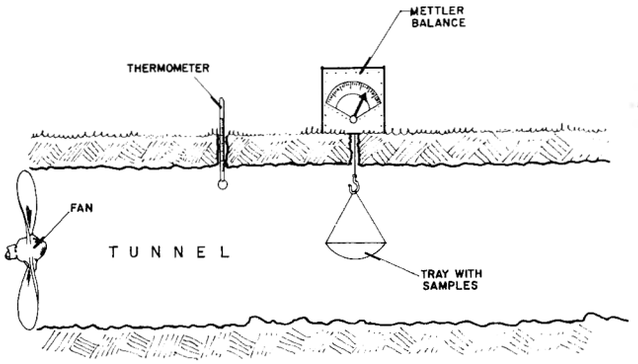

Fig. 1. Sketch of experimental drying tunnel.

Diffusion coefficients for the first falling-rate period were calculated from the W versus time data by making use of the Sherwood equation (Sherwood, 1929), graphically represented in Fig. 2, which expresses the functional relationship between the parameters $X = (W - W_e)/(W_0 - W_e)$ and $\ominus = Dt/L^2$, where W_0 is the initial water content, W_e is the equilibrium water content, D is the water diffusion coefficient, t is time and L is the half-thickness of the slice. A preliminary determination of the equilibrium water contents W_e had shown that these were negligible in comparison with the water contents W, so that the parameter X became W/W_0. Each W was therefore converted to X by dividing the corresponding initial water

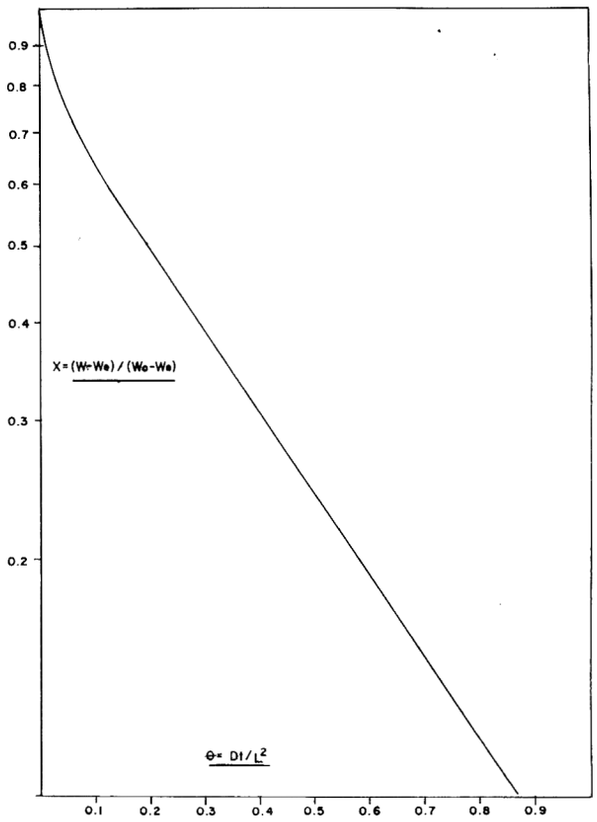

Fig. 2. Graphical representation of Sherwood equation used in calculation of diffusion coefficients.

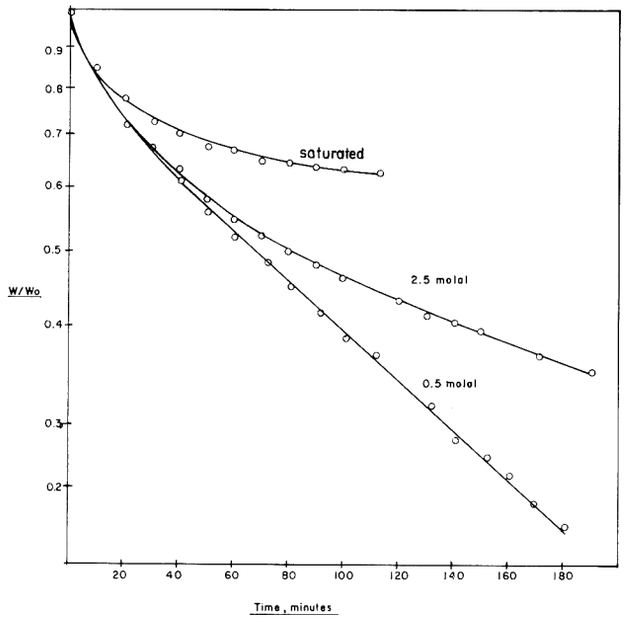

Fig. 3. Typical drying curves at 55°C for fish slices salted in brines of different concentrations.

content. The corresponding $\ominus$ value was next determined for each X by use of Fig. 2. Finally, since t values for each W—and hence for each X—were known, the diffusion coefficients were calculated from

$$D = \ominus L^2 t. \qquad [3]$$

Diffusion coefficients for the second falling-rate period were determined from the linear part, at high times, of plots of log (W/W_0) versus time (Fig. 3). The Sherwood equation shows that such plots, at high times become straight lines with slopes equal to $(D/L^2)(\pi^2/4)$, where D is the diffusion coefficient and L is the half-thickness of the slice. Therefore,

$$D = (\text{Slope})(L^2)(4/\pi^2). \qquad [4]$$

It should be noted that linearity of these plots at high times did not necessarily assure that drying was already in the second falling-rate period. It happened in some cases that the first falling-rate period was much prolonged and covered the entire 3-hr length of the drying run. In such cases, diffusion coefficients determined from Eq. 4 were identical with those determined from Eq. 3.

RESULTS AND DISCUSSION

TABLE 1 GIVES A SUMMARY of all results obtained in the drying experiments at 55°C and also includes data on initial moisture and water contents of the slices which were dried. Table 2 compares data obtained at 40°C with corresponding data obtained at 55°C. The following points are of interest:

(1) The falling-rate period in drying of salted fish was found to be characterized by two water diffusion coefficients, with the first being greater than the second.

(2) Due to the long drying times required, the determination of the diffusion coefficient for the second falling-rate period was subject to rather large errors. Values obtained, therefore, serve mainly to indicate trends in the behavior of this variable. Tables 1 and 2 and Fig. 4 show that the second diffusion coefficient behaved in essentially the same manner as the first.

Table 2. Effect of temperature on diffusion coefficient of water in drying of salted fish muscle in falling-rate period.

Conc. of brine in which muscle was salted, molal	Temperature, °C	Diffusion coefficient cm²/sec × 10⁶	
		First period	Second period
Fresh fish	40	0.30	Note (1)
	55	0.39	Note (1)
0.5 m	40	0.36	Note (1)
	55	0.62	Note (1)
2.5 m	40	0.54	0.22
	55	0.87	0.24
Saturated	40	0.26	0.10
	55	0.33	0.09

Note (1): Drying did not enter second falling-rate period in duration of experiment.

Table 1. Summary of data obtained in drying experiments at 55°C.

Conc. of brine in which muscle was salted, molal	Conc. of additional substance in brine	Percent water in muscle	Weight water / Weight solids	Diffusion coefficient cm²/sec × 10⁶	
				First period	Second period
Fresh fish		72.8	2.69	0.39	Note (1)
0.5 m	Control	78.4	3.72	0.62	Note (1)
	0.05M HCl	75.1	3.03	0.60	Note (1)
	0.05M NaOH	80.9	4.23	0.69	Note (1)
	5% Phosphate*	82.0	4.80	0.78	Note (1)
2.5 m	Control	74.5	2.92	0.87	0.24
	0.05M HCl	70.4	2.38	0.61	0.24
	0.05M NaOH	75.7	3.11	0.60	0.20
	5% Phosphate*	75.7	3.11	0.67	0.26
Saturated	Control	62.2	1.65	0.33	0.09
	0.05M HCl	61.0	1.56	0.32	0.11
	0.05M NaOH	65.3	1.89	0.30	0.09
	5% Phosphate*	64.8	1.84	0.26	0.11

Note (1): Drying did not enter second falling-rate period in duration of experiment.
Note (*): "Phosphate" refers to sodium tripolyphosphate.

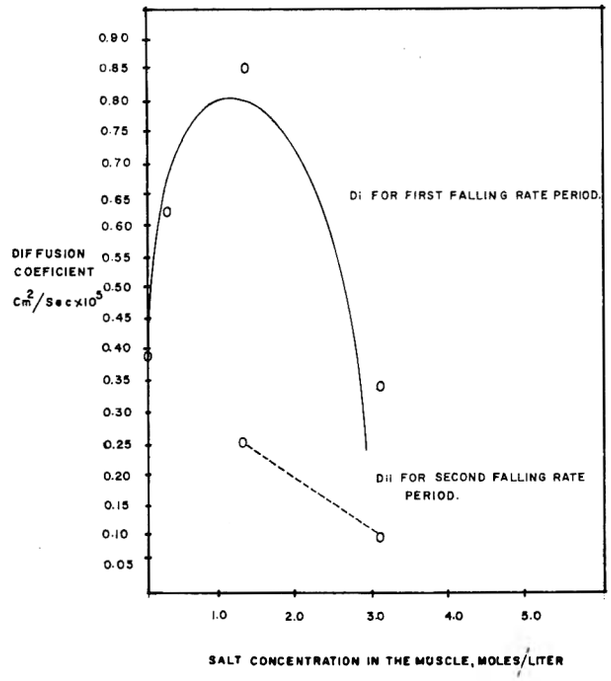

Fig. 4. Variation of water diffusion coefficients in drying with salt content of salted fish muscle at 55°C.

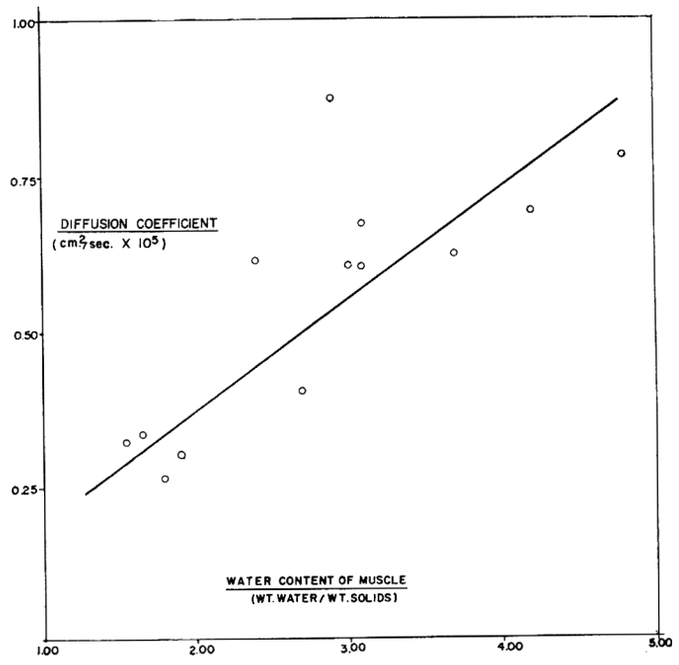

Fig. 5. Regression of diffusion coefficient for first falling-rate period in drying of salted fish on initial water content of muscle.

(3) Fig. 4 shows that the diffusion coefficient for the first falling-rate period was a function of the degree of salting of the muscle, increasing initially, passing through a maximum, and subsequently decreasing with degree of salting.

(4) Depending upon the degree of salting, addition of acid, base, and/or phosphate affected the diffusion coefficient for the first falling-rate period differently. At a low degree of salting, addition of acid decreased, and addition of base and phosphate increased the diffusion coefficient; at a very high degree of salting, no effect was noted from the addition of either of these substances. At an intermediate degree of salting, addition of the three substances apparently resulted in a decrease of the coefficient.

(5) The diffusion coefficient for the first falling-rate period was found to increase with temperature for all degrees of salting studied.

Observations (1) and (5) agree with and extend the results of Jason (1958).

It was felt that observations (3) and (4) might both point to a possible correlation between the diffusion coefficient and the water content of the muscle, as reference to Table 1 will show. To verify this possibility, the correlation coefficient was calculated and Fig. 5 was plotted with the data from Table 1. A correlation coefficient of 0.82 (significant at the 99% level) and Fig. 5 both reveal that a good linear relationship indeed existed between both variables. The regression straight line, calculated by the method of least squares, was found to be:

$$D = 0.16W + 0.09 \qquad [5]$$

where D is the diffusion coefficient in $cm^2/sec \times 10^5$ and W is the water content in pounds water per pound dry solids.

A possible explanation of the above observations might be the following. During drying of salted fish muscle, water molecules migrate from the interior to the outside of the muscle. This migration is, however, hindered by a number of retardatory forces, including protein-water and salt-water interactions. It is known that the effect of some of these forces—notably the last ones—is strongly concentration-dependent; i.e., as water molecules are brought closer to protein molecules and salt ions (as a result of decreasing the water concentration), the respective interaction forces increase, thus retarding further the migration of the water molecules. Since the diffusion coefficient is a measure of the ease of this migration, it should also decrease with decreasing water concentration and vice-versa.

An objection to the above explanation might be raised at this point in the sense that, if the diffusion coefficient is water concentration-dependent, why was it found to remain essentially constant during the drying runs, when the water concentration was continually decreasing? The explanation of this phenomenon, which has been observed in many drying experiments, remains obscure. Jason (1958) points out that it could be possibly due to a fortuitous cancellation of concentration-dependence effects by shrinkage effects.

Some further observations may be made on the results obtained in the drying experiments:

(1) Jason's observation that the diffusion coefficient for drying of unsalted fish in the first falling-rate period was approximately equal to that for drying of fully salted fish in the same period was verified by the results of these experiments, as reference to Table 1 will show.

(2) The effect of temperature on the diffusion coefficients was generally not very great. A plot of the logarithm of the diffusion coefficient versus the inverse of the absolute temperature to determine activation energies

for water transport was not attempted because it was felt that two temperature levels alone were insufficient for this calculation.

(3) Drying of fully salted fish was observed to be accompanied, at both temperatures, by syneresis of a highly concentrated salt solution from the slices. This solution eventually covered the entire surface of the slices and probably interfered with drying because drying rates were observed to drop appreciably after the surfaces were covered. If the solution was scraped from the surface of the slices, drying rates immediately increased, although they rather quickly dropped again to their former levels. The syneresis solution is probably a manifestation of the "salt-protein crust" already referred to, and reported by a number of investigators (Linton *et al.*, 1945; Wood, 1964; Jason, 1958). Syneresis was possibly caused by shrinkage of the slices during drying as well as by loss of water from the muscle as a result of coagulation of the protein by high salt concentrations and by temperature.

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Penetration Gradients of Sodium Nitrite and Sodium Tripolyphosphate in Haddock Fillets

SUMMARY—Studies were carried out to determine the penetration gradients of sodium nitrite, containing sodium 24, and sodium tripolyphosphate, containing mostly phosphorus 32 in addition to sodium 24. In a parallel experiment, the penetration of sodium nitrite was also determined colorimetrically. Results on this technique compare closely with those obtained from the radiological method.

INTRODUCTION

INVESTIGATIONS (Schmidt, 1964 and 1965; Schmidt *et al.*, 1964) have shown that when *Clostridium botulinum* organisms are inoculated into comminuted fish flesh, their development is inhibited or delayed by various salts; e.g., nitrites and polyphosphates.

Little information is available, however, on the absorption characteristics or distribution gradients of salts in whole fish fillets that have been dipped into a salt solution. Neither is there much information on precise laboratory procedures for measuring these gradients.

Such information is needed to permit the establishment of dipping procedures that will insure compliance with limits prescribed by Federal and State regulatory agencies.

This paper discusses a radioactive tracer technique developed to measure the absorption and distribution of certain additives in fish flesh. The accuracy of this method is compared, in the case of sodium nitrite, with a modified colorimetric procedure based upon the procedure for nitrite anion present in meats (A.O.A.C., 1955).

EXPERIMENTAL

Radioactive tracer method

Irradiation with neutrons yielded radioactive salts. This was done in the Massachusetts Institute of Technology (MIT) reactor having a flux of 2×10^{12} neutrons per cm^2 per sec. With analytical samples of solid sodium nitrite, 130 mg, the only radioactivity obtained after 1 min, about 100 μc, was due to sodium 24 which has a half-life of 14.8 hr.

Irradiation of sodium tripolyphosphate ($Na_5P_3O_{10}$) 300 mg, for 30 min also gave phosphorous 32, estimated to have a radioactivity of 67μc and half-life of 14.3 days. In addition, an estimated 6,400 μc of radioactivity was contributed by sodium 24. This material was allowed to decay about 5 days before use, whereby, about 51μc of phosphorous 32 remained with only about 25 μc of sodium 24.

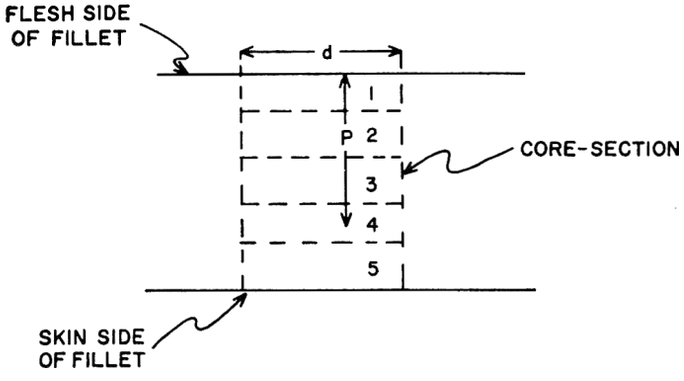
Dip solutions of a desired concentration and radioactivity were prepared from mixtures of the radioactive salt and the non-radioactive salt.

At the time of analyses, determining the radioactivity of the dip solution expressed by the ratio, C.P.M. per gram, enables one to calculate readily the salt quantity in a given sample of flesh from its radioactivity, i.e., its C.P.M. divided by the ratio.

The weight percentage of salt introduced into a core section by dipping was considered to be the average concentration of the salt at the center of the section. All data were corrected for background radiation, about 18 C.P.M.

The volume of the core section in cm^3 is the weight of the section in grams. The distance from the center of the section to the surface of the fillet was equal approximately to ½ the weight of the core-section plus the weights of the aggregate core-sections above the particular section divided by the cross-sectional area of the sample core, i.e., by that of the cork borer, 2.85 cm^2 (Fig. 1).

In a typical experiment, 200 ml of radioactive salt solution were prepared, and a 10 ml portion was withdrawn for later assay. Portions of haddock fillets were dipped into the solution both for 10 sec, and for 10 min, drained and stored at 33°F in a moist desiccator for 24 hr. The fillets were lightly frozen on a piece of dry ice to facilitate cutting. Core samples were taken with a cork borer and sectioned with a razor blade. The sections were weighed on planchets, and their radioactivity was deter-



$$\text{SECTION \#4, P cm} = \frac{wt_1 + wt_2 + wt_3 + wt_4/2}{\pi d^2/4}$$

Fig. 1. Average penetration defined.

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mined directly by counting. At the same time, a 50 μl aliquot of the dip solution was placed on a planchet and its radioactivity determined.

Colorimetric method

A standard sodium nitrite solution was prepared by diluting a 1 ml aliquot of a sodium nitrite solution, 0.1093 g of analytical grade sodium nitrite per 100 ml, to 1 L. The concentration of sodium nitrite obtained was, therefore, 1.093×10^{-6} g per ml.

Aliquots of this standard solution, 1–5 ml, were diluted to 5 ml in biuret tubes and 3 ml of Griess reagent added. Color development was complete after 5 min.

Griess reagent was prepared as follows: α-naphthylamine, 0.4 g, was dissolved in 2 ml of glacial acetic acid and diluted with 50 ml of distilled water. The solution was decolorized by warming with 1 g of norite charcoal, cooling and filtering. It was then combined with a solution of 0.5 g sulfanilic acid dissolved in 100 ml of distilled water to which was added 20 ml of glacial acetic acid. Prepared in the above manner, Griess reagent is almost colorless and will remain so if stored in an amber bottle.

In a typical experiment, sample core-sections were combined after their radioactivity was determined. They were then placed in a 250 ml Erlenmeyer flask with some distilled water and broken up with a spatula. Following a few minutes' boiling, 5 ml of a saturated solution of mercuric chloride, a 10% solution, were added. After standing overnight, the solution was suction-filtered, and the filtrate was made up to a standard volume.

The weight of sodium nitrite in a given core-section was determined by calculating the optical density for this standard volume from the determined optical density of the sample volume and multiplying by 1.02×10^{-5}.

The accuracy of the colorimetric procedure was determined through some blank experiments using 100 ml of standard sodium nitrite solution with 5 g samples of haddock. About 90–95% of the nitrite was accounted for in the resulting analysis.

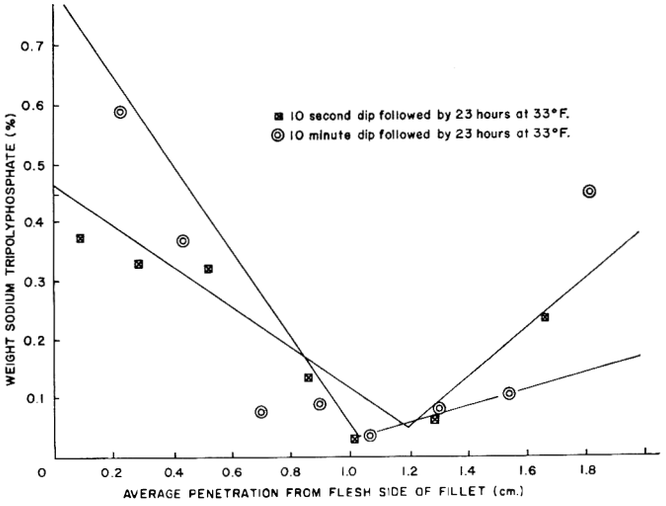

Fig. 2. Penetration of sodium tripolyphosphate into haddock fillets (skin on) after dipping in 12.1% sodium tripolyphosphate solution.

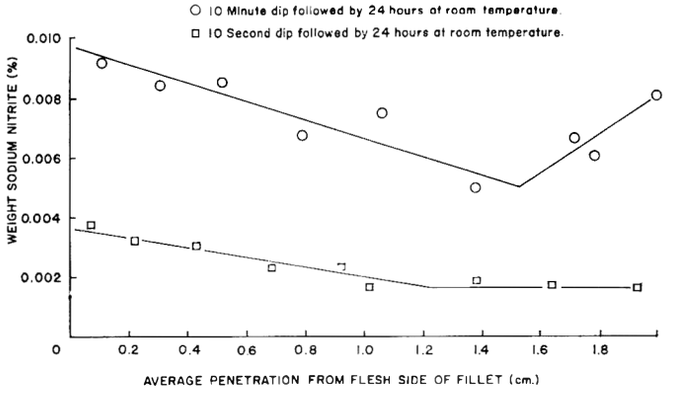

Fig. 3. Penetration of sodium nitrite into haddock fillets (skin on) after dipping into 0.135% solution of sodium nitrite.

RESULTS AND DISCUSSION

THE DATA for the penetration of sodium tripolyphosphate into haddock fillets from 12.1% sodium tripolyphosphate solution are expressed graphically in Fig. 2. The data show that the penetration of sodium tripolyphosphate to the center of the fillet is slight compared with that of the other salt evaluated. As expected, the concentration is highest at the surface and decreases toward the center. It is interesting to note that although the concentration near the surface increased with an increase in dip time, the concentration at the center remained about the same.

Data for the penetration of sodium nitrite into haddock fillets are shown graphically in Figs. 3 and 4, corresponding to dip concentrations of 0.135 and 0.262 weight percentages, respectively.

When the weight percentages of sodium nitrite in the core sections were multiplied by the ratio of the above dip concentrations, 0.262/0.135, and the projected data plotted with the data at the higher dip concentration, the data appear identical within experimental error as a function of the depth. Thus, the concentration of the salt which is some function of the depth of penetration appears directly proportional to the concentration of salt in the dip solution.

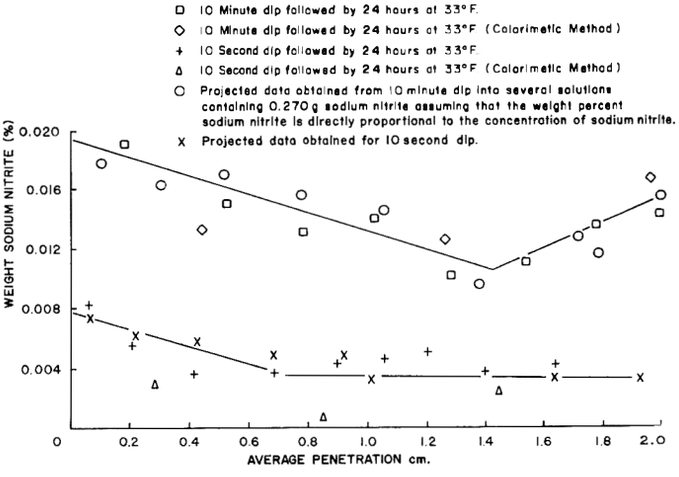

Fig. 4. Penetration of sodium nitrite into haddock fillets after dipping into 200 ml of solution containing 0.525 g of sodium nitrite.

Table 1. Concentrations of sodium nitrite and sodium tripolyphosphate in dipped haddock fillets.

Salt	Concentration of salt in bath (wt. %)	Total concentration of salts in fillets dipped for 10 seconds (wt. %)	Total concentration of salts in fillets dipped for 10 min (wt. %)
Sodium nitrite	0.135	0.0021	0.0080
	0.262	0.0045	0.014
Sodium tripolyphosphate	12.1	0.21	0.28

The overall absorption of salts calculated from the data is given in Table 1.

All of the radioactive tracer data show a difference in penetration of salt with respect to the skin side and flesh sides of the fillet. The point of minimum penetration is evidently shifted toward the skin side, indicating that penetration through the skin is reduced.

Figs. 3 and 4 show that penetration after 10 min is only about twice that obtained after 10 sec although the time span is 60 times greater. The initial rate of penetration is, probably, very rapid. The reproducibility of separate experiments particularly for 10-sec dips might, therefore, be poor. For this reason, among others, the comparison of the radioactive tracer and colorimetric methods with sodium nitrite was made directly in parallel experiments with identical material.

Losses in the colorimetric method might result from the retention of nitrite ion in the pieces of filtered and discarded fish and from a possible side-reaction between the nitrous acid liberated in the colorimetric reaction and free-amino-functional groups in the water-soluble components of the flesh, such as amino acids. These water-soluble components probably are not precipitated by the addition of the mercuric chloride.

A plot of ml standard solution vs optical density at 520 mμ was linear corresponding to a slope of 1.02×10^{-5} g sodium nitrite per unit of optical density.

Fig. 4 shows the colorimetric method as a parallel experiment with the radioactive tracer method. The colorimetric data plotted with the data obtained from the radioactive tracer method show general agreement.

CONCLUSIONS

The radioactive tracer method for the determination of distribution gradients appears to be a general method of analysis whenever the salt to be investigated can be made radioactive by irradiation with neutrons. Isotopes, however, must be obtained that have sufficiently long half-lives for experimental purposes.

The distribution gradients can be determined by simple calculations if the standard solution is counted at the same time as the sample. The more complex calculations based on the laws of radioactive decay are, therefore, unnecessary.

The colorimetric method used is specific to determine nitrite ion and might provide in this case an accurate alternative method.

General agreement between the tracer and colorimetric methods for sodium nitrite shows that they are analytically comparable.

The penetration of the nitrite anion, therefore, must be the same as that of the observed radioactive sodium 24 upon which the calculation of nitrite anion concentration is based. The tripolyphosphate anion, however, can be determined directly, since radioactive sodium tripolyphosphate can be prepared that contains essentially only radioactive phosphorous 32.

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Co-dried Carbohydrates Effect on the Performance of Egg Yolk Solids

SUMMARY—When egg yolk is dried and subsequently rehydrated, it loses its ability to form a stable foam. If sufficient carbohydrates are added to the egg yolk before dehydration, much of the foaming ability is retained by the rehydrated yolk. Evidence has been presented that, in plain-dried yolk, the removal of water irreversibly changes the structure of the low-density lipoproteins and that foam-inhibiting free lipid is released from these lipoproteins. When yolk is co-dried with added carbohydrates, the carbohydrates partially protect the lipoproteins from this irreversible structural change, probably by replacing the water of hydration at its binding sites during the drying process.

INTRODUCTION

VARIOUS SUGARS and related polyhydroxy compounds have been added to egg yolk and whole egg before spray-drying since early in World War II. At that time, Brooks *et al.* (1943) reported that these carbohydrates minimized the loss of foaming power which otherwise occurred in egg yolk or whole egg immediately after spray drying. It was suggested by Brooks *et al.* (1944) that the added sugars stabilized an unidentified complex between lipids and proteins. Since that time, sugar-dried egg products have gained a prominent industrial role. To date, however, there has been no satisfactory explanation of the chemical or physical-chemical role that the added co-dried carbohydrates play in improving the aerating ability of the dried products.

Brooks *et al.* (1943) reported that, if 10% sucrose was added to egg yolk or whole egg and the liquid mixture spray-dried, the resulting product would foam voluminously, while the plain spray-dried egg would not form a foam. They also noted that a small percentage of the total lipids present in the sugar-dried products was extractable with fat solvents, while nearly all the lipid present in the plain-dried egg was extractable. The easily extractable lipid was removed from plain spray-dried egg yolk with acetone by Joslin *et al.* (1954), who found that the resulting product would then foam when rehydrated and beaten. They also found that, if a very small amount of the lipid that had been extracted by the acetone was added to a foam produced from fresh yolk or the defatted dried yolk, the foam would collapse. If the lipid were added to fresh yolk before it was whipped, no foam would form.

An extensive study of the properties of yolk-containing solids with added carbohydrates was reported by Kline *et al.* (1964). They found that the addition of increasingly greater amounts of sugars before spray-drying gradually increased the specific volume of the foam prepared from the dried material. They also reported that, as the level of carbohydrates added to the liquid egg before spray-drying is gradually increased, an abrupt transition occurs in the spray-dried products wherein the lipids go from a state of being easily extractable with a nonpolar fat solvent to a virtually unextractable state.

The level of added carbohydrates, when this transition occurred, varied from 7 to 15%, depending on the molecular species of carbohydrate added. This transition state in lipid extractability corresponded to a similar transition in the flavor stability of the dried substances. Consequently, a level of added carbohydrates that gives a dried product with the best foaming power cannot be used because of ensuing flavor instability.

Martin *et al.* (1964) reported that more than 90% of the total lipid present in egg yolk is associated with the low-density fraction lipoproteins. They suggested a spherical structure for these lipoproteins, with an internal "lipid-core" and a hydrophilic surface composed of protein and phospholipid. A water of hydration content of between 10 and 30 percent of the total weight of the lipoprotein micelle was suggested. Saari *et al.* (1964) also isolated and described these low-density yolk lipoproteins. Bellairs (1961), in a detailed electron-microscopic study of the structure of egg yolk, described spheres about 250Å in diameter, which she believed to be protein. Since Martin *et al.* (1964) reported the low-density lipoproteins averaged about 250Å in diameter, as calculated from ultracentrifugal flotation analyses, it is probable that they are the protein structures reported by Bellairs (1961).

The objective of this project was to elucidate the physiochemical role that added carbohydrates play in preventing the loss of foaming power in sugar-dried eggs. With additional knowledge of the basic chemistry of the associations between added carbohydrates and the yolk lipoproteins, it might be possible to design new products that would combine good functional performance with satisfactory flavor stability.

EXPERIMENTAL

Egg yolk

The egg yolk used in these experiments was of two basic types—commercially and laboratory dried. Egg yolk was prepared in the laboratory from eggs obtained fresh from the Iowa State University Poultry Farm. The eggs were stored at 3 to 4°C until broken, always within one week of being laid. The yolks were rolled on paper towels, the chalazea removed with tweezers, the membranes punctured, and the contents drained into a beaker.

A commercial spray-dried yolk source was also used in some experiments. The liquid yolk to be spray dried was commercially prepared from machine-broken eggs. The

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yolk was dried in a commercial, modified Rogers type spray drier. The liquid yolk contained 44% solids. Sugar-dried commercial yolk was prepared from the same batch of liquid yolk as the plain-dried yolk. The final sugar-dried yolk contained 74.5% egg solids, 20.5% 30-dextrose equivalent corn syrup solids, and 4.2% moisture. The plain-dried yolk contained 4.7% moisture.

Samples of plain and sugar-dried yolk with different viscosities were prepared by varying the feed rate of the dryer. Three different viscosities of plain yolk and two different viscosities of sugared yolk were prepared. Differences in viscosity were confirmed by rehydrating 60 g of dried yolk with 80 ml of water and measuring the viscosity with a Brookfield viscometer.

Samples of laboratory-dried eggs were prepared with a lyophilizer and by rotary evaporation. Preliminary experiments with rotary evaporation showed no difference between samples dried at 55 mm Hg absolute pressure and samples in which the vacuum was gradually increased during drying. If the pressure was reduced below 55 mm Hg in the early stages of drying, the yolk foamed out of the rotary evaporator flask which was submerged in a water bath maintained at 35°C.

Low-density lipoprotein isolation

Low-density lipiproteins were isolated from fresh or dried yolk by the procedure illustrated in Fig. 1. The procedure used is a slight modification of that described by Martin *et al.* (1964).

Foam volume tests

Foam volume tests were run by adding 18 g of granulated sucrose to egg products equivalent to 26.0 g of egg solids and enough water to make a total of 74.0 ml of water in the whole mixture. The temperature of the ingredients was adjusted to 25°C in all cases. The ingredients were then mixed with a Hobart Kitchen-Aid Model K5-A mixer for 3 min at speed 8. The weight of 250 ml of foam was recorded. The specific volume of the foam is reported in milliliters per gram. In the case of sugar-dried yolk, the amount of sucrose was reduced an equivalent amount. Sixty g of fresh yolk was diluted with 50 ml of water in the tests to measure the foam-inhibiting properties of added lipid.

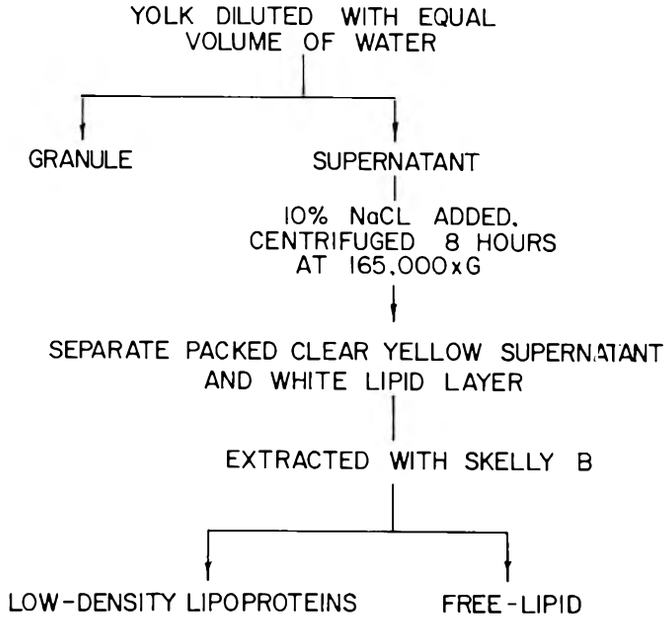

Fig. 1. Flow diagram for the isolation of the low density lipoprotein and free lipid from egg yolk.

Photomicrographs

Photomicrographs were made with a Zeiss photomicroscope equipped with an automatic exposure device. The drops of yolk to be photographed were placed on a microscope slide, which was on the microscope stage, and dried with low radiant heat. The drops were photographed at intervals as they dried.

Free lipid extraction

Free lipid was extracted by a rapid-extraction procedure with redistilled petroleum ether (Skelly B, B.P. = 66 to 68°C). Skelly B was combined with the sample to be extracted in a 450-ml teflon-lined Lourdes homogenizer container. The contents were then mixed on a Lourdes model VM volu-mix homogenizer and mixed at a speed of approximately 100,000 rpm for one minute. The homogenized mixture was then transferred to a funnel and filtered through Whatman Number 1 filter paper into a tared 250-ml beaker. The homogenizer container was rinsed with Skelly B, and the filter paper rinsed three more times with portions of Skelly B. After filtration, the solvent was evaporated on a steam bath and the remaining lipids were further dried in a forced-air oven for 30 min at 105°C. The beaker containing the dried fat was reweighed, and the percentage of theoretical fat extracted from the sample was calculated.

Heat treatments

Fresh egg yolk and low-density fraction lipoproteins isolated from fresh yolk were heat-treated to determine the amount of lipid released due to heat denaturation of the proteins. The samples to be heat-treated were weighed into tared 250-ml beakers, a teflon covered magnetic stirring bar was added to the beaker, and the beaker was covered with Parafilm. The beaker was then about two-thirds submerged in a bath of glycerol maintained at the proper temperature. The yolk or lipoprotein was stirred in the glycerol bath for 10 min, the sample removed and cooled, and the yolk or lipoprotein extracted with petroleum ether.

Analytical ultracentrifugation

Analytical ultracentrifugations were made with a Beckman model E analytical ultracentrifuge. Epon-aluminum, 2½°, 12 mm, double sector centerpieces were used in an AN-D rotor at a speed of 29,500 rpm. Schlieren optics were used, and the rotor was maintained at a temperature of 26°C. A lipoprotein concentration of 1.5% (W/W) was dissolved in a 1,745 molal sodium chloride solvent (density = 1.063 g/ml at 26°C).

RESULTS AND DISCUSSION

Effect of free lipid on foaming

Fresh egg yolk, diluted with water, foams quite voluminously (Fig. 2). However, when small amounts of

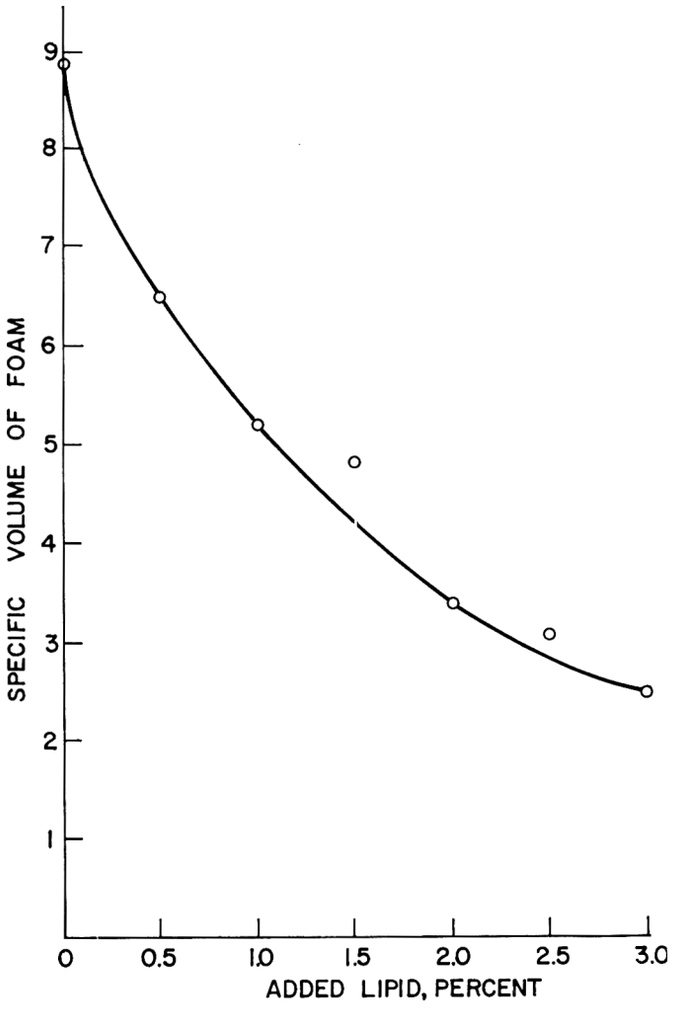

Fig. 2. Effect of added lipids on foaming ability of fresh egg yolk.

lipid, isolated from plain spray-dried egg yolk with Skelly B, were added to the egg yolk, the specific volume of the foam was rapidly reduced. The Skelly B was evaporated, and the lipids were cooled to room temperature before they were added to the fresh yolk. Thus, isolated egg lipids, when re-added to fresh yolk, possess considerable antifoaming properties.

Effect of moisture level on amount of free lipid

If fresh egg yolk is extracted with Skelly B under the conditions described earlier, essentially no free lipid is extracted from the yolk. Egg yolk was dried to different moisture levels by interrupting rotary evaporation after various times. Thus, egg yolk was obtained containing from about 5% to nearly 50% moisture (i.e., the moisture content of the fresh yolk). This egg yolk was extracted with Skelly B as described earlier. The egg yolk containing from 50% down to about 33% moisture had essentially no fat extracted from it (Fig. 3). When the moisture content was reduced below 33%, however, the amount of lipid extracted rapidly increased until, at a moisture content of about 5%, more than 55% to the lipid theoretically present in the yolk was extracted.

Low-density lipoproteins isolated from fresh yolk by the method outlined in Fig. 1 were dissolved in water so that the final moisture content was 50% to permit distribution on the walls of the rotary evaporation flask. The lipoproteins were then dried in a manner identical to the fresh yolk and extracted with Skelly B. The results are also shown in Fig. 3. The pattern of lipid release versus moisture content for the low-density lipoproteins is similar to the one for fresh egg yolk.

The lipoproteins, however, do not begin releasing free lipid until their moisture content drops below 30%. The lipoproteins dried to 5% moisture released more free lipid than did egg yolk dried to 5% moisture by rotary evaporation. In general, the curve of free lipid release as a function of moisture content is similar, and the slight differences are easily understandable in light of the different nature of the two materials.

Thus, it appears that rather mild drying conditions (those that would ordinarily not be expected to cause protein denaturation) cause lipid to be released from the lipoprotein micelles. That is, as the moisture content is reduced, the lipid goes from a state of being unextractable, under the conditions used here for extraction, to a state where it is easily extractable.

Effect of sugar on retarding free lipid release

The coalescence of lipid released during dehydration can actually be observed through a microscope if a drop of yolk is dried on a microscope slide. Fig. 4-A is a photomicrograph of fresh yolk immediately after being placed

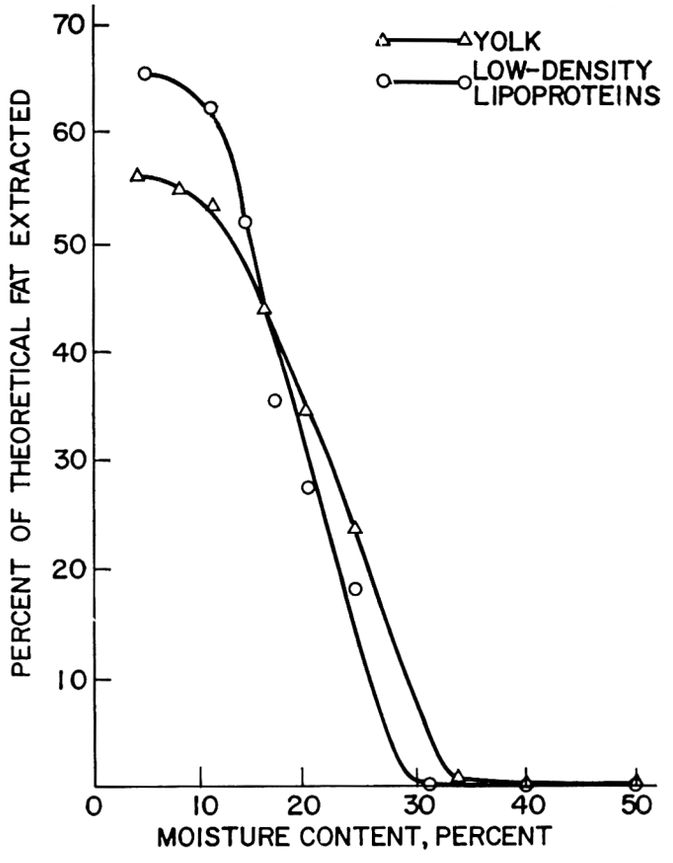

Fig. 3. Effect of moisture level on lipid extractability of egg yolk and low density lipoproteins.

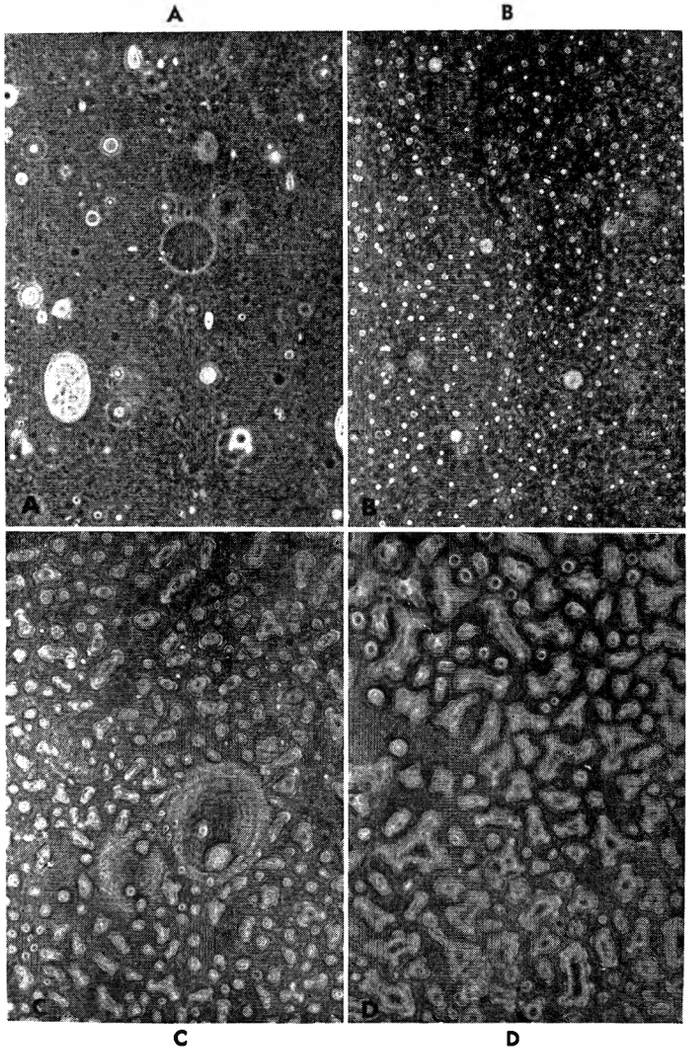

Fig. 4. Photomicrographs (x187) of drying egg yolk. (A) Fresh liquid egg yolk; (B) fresh yolk after drying has begun, showing small fat droplets beginning to form; (C) drying yolk showing fat droplets beginning to coalesce; (D) dried yolk showing coalesced fat nearly covering surface of the yolk.

on the slide. The yolk granules and globules described by Romanoff *et al.* (1949) are readily visible. In Fig. 4-B, small droplets of fat have begun appearing on the surface of the drying yolk. It was demonstrated that the small round droplets seen in the picture are fat by the fact that they were readily stained by osmium tetroxide vapors. The small droplets of fat have begun to coalesce in Fig. 4-C. In Fig. 4-D, the yolk is quite dry, and the fat has coalesced on the surface of the yolk until the surface is nearly covered.

Fig. 5-A shows yolk containing 10% maltose dried on a microscope slide. Even though it is so dry that a film has formed over the surface of the drop of yolk and the yolk granules and globules can no longer be seen, there is no lipid coalesced on the surface of the droplet. Figs. 5-B and 5-C show egg yolk dried with 4% and 2% added maltose, respectively. Increasingly larger amounts of free lipid coalesce as the amount of sugar is decreased. Fig. 5-D is of low-density lipoprotein dried in a manner similar to than described for the egg yolk. In this photograph the yolk is at an intermediate stage of drying, and free lipid can be seen coalescing on the surface of the lipoproteins. The lipoproteins behave and look much like fresh egg yolk, except initially the granules and globules are not present in the isolated lipoproteins, as they are in fresh yolk.

It appears that, first, when plain yolk dries (even under mild dehydration conditions), free lipid is released and coalesces. Second, evidence has been developed to support the theory that the low-density lipoproteins are the source of this free lipid. Third, sugars added to liquid yolk prior to dehydration tend to greatly reduce the amount of free lipid released by the yolk during dehydration.

The effect of sugar in reducing the amount of easily extractable lipid present in dried yolk can be seen in Fig. 6. Yolk with varying amounts of added sucrose was lyophilized, and the dried material extracted with Skelly B. The dried yolk contained approximately 3% moisture. The level of added sucrose has a definite effect on the amount

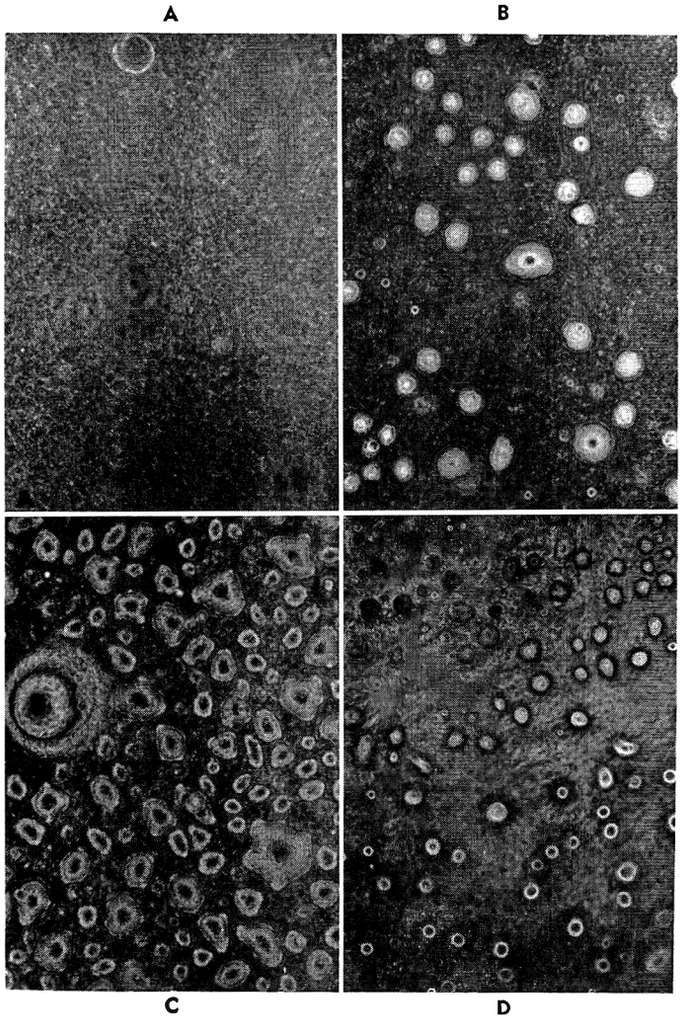

Fig. 5. Photomicrographs (x187) of dried egg yolk and lipoproteins. (A) Dried yolk with 10% maltose added prior to drying, illustrating that no coalesced fat is present; (B) dried yolk with 4% maltose added prior to drying, illustrating only a small amount of coalesced fat; (C) dried yolk with 4% maltose added prior to drying, illustrating an intermediate amount of coalesced fat on the surface of the yolk; (D) dried low density lipoproteins, illustrating coalescing fat.

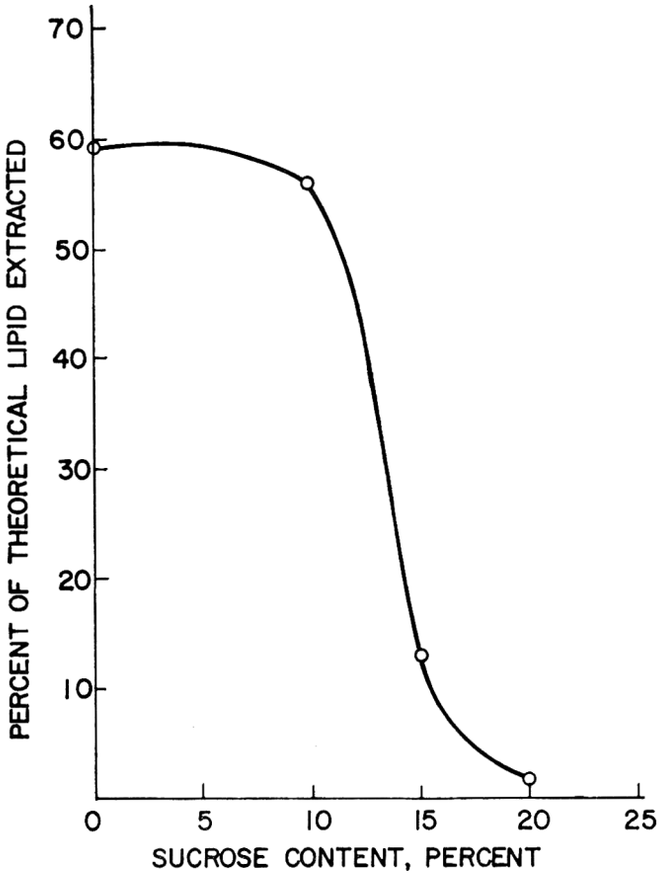

Fig. 6. Effect of co-dried sucrose on lipid extractability of dried egg yolk.

of easily extractable lipid in the yolk after drying (Fig. 6).

From this evidence, the hypothesis was developed that, during drying, the added carbohydrates, through their hydroxyl groups, replace the water of hydration in the outer protein layer of the lipoprotein micelle. Since this water is probably bound to the protein overwrap through hydrogen bonding, the hydroxyl groups on the carbohydrates, through induced charges, could occupy the charged sites on the lipoprotein micelle and thus stabilize it against a disruption which otherwise occurs upon the dehydration of the lipoprotein micelle. When the dried yolk is rehydrated, the water again replaces the sugar and rehydrates the lipoprotein micelle surfaces. Upon dehydration, if no sugar is added, the lipoprotein micelle releases lipid formerly in the center of the micelle. Then, upon rehydration, the lipoproteins in the plain-dried yolk do not resume their native structure as well as do the lipoproteins in the sugar-dried yolk.

Effect of heat on free lipid

An experiment was designed to determine whether heat denaturation of the protein coat on the micelle surfaces would cause the release of free lipid. As previously indicated, Skelly B extracts no lipid from fresh egg yolk. In these experiments, samples of fresh yolk and fresh yolk plus 10% sucrose, yolk plus 10% lactose, yolk plus 10% glucose, and yolk plus 10% maltose were heated at 60°, 70°, 80°, 90°, 100° and 110°C for 10 min in a glycerol bath. In addition, a sample of lipoprotein also was heated at these temperatures. As fresh egg yolk is heated to increasingly higher temperatures, larger amounts of free lipid are released (Fig. 7). The low-density lipoprotein behaves similarly, except that it appears even more labile to heat than is fresh yolk.

The yolk with added sugars does not release free lipid as rapidly on heating as does fresh yolk. If the release of lipid is due to heat denaturation of proteins associated with these lipids, then it would appear that the added sugars tend to partially protect these proteins from heat denaturation. It is known that carbohydrates can protect proteins against denaturation. Brosteaux *et al.* (1935) reported that changes in the molecular structure of proteins caused by drying were retarded in the presence of sugars. It appears that the mol ratio of carbohydrates to proteins is important since the monosaccharide, glucose when added at a level of 10% by weight, protected the proteins against denaturation to a greater extent than did an equal amount of the disaccharides. The three disaccharides, all added at the same level by weight, gave identical results.

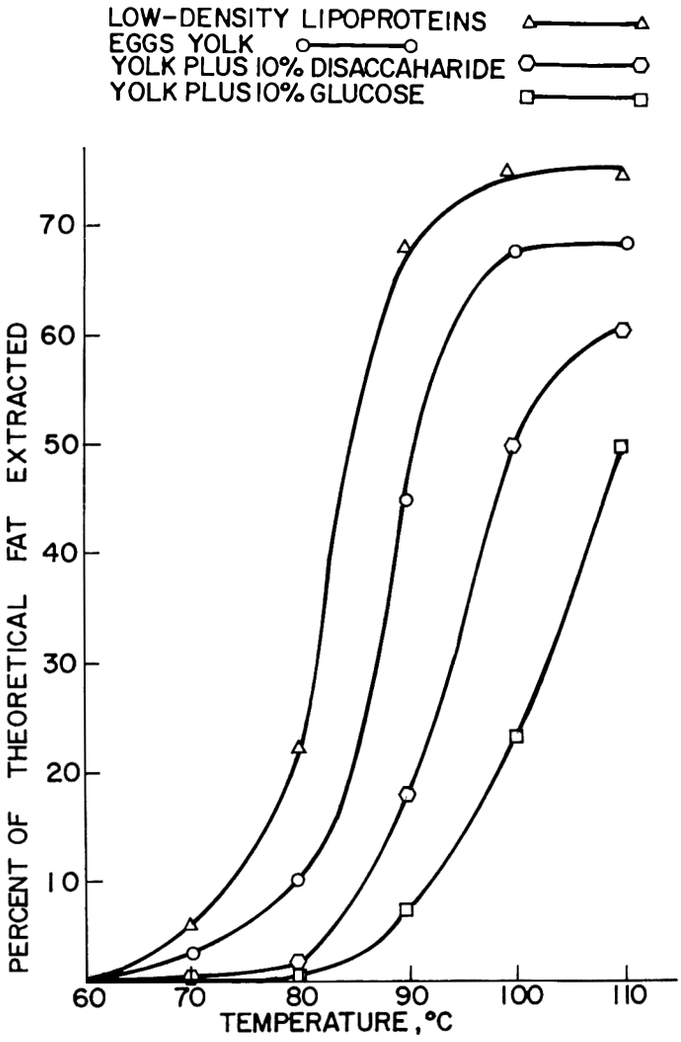

Fig. 7. Effect of heat treatment and added sugars on the lipid extractability of egg yolk and low density lipoproteins.

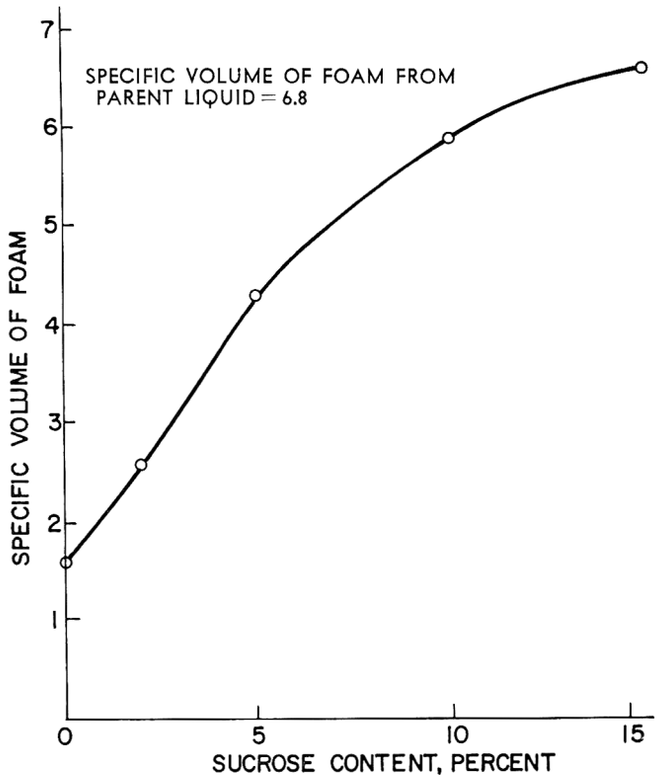

Fig. 8. Effect of co-dried sucrose on the foaming ability of dried egg yolk (adapted from Kline et al., *1964; Fig. 1).*

Effect of added sugar and time on foaming ability

When sugar is added to yolk before it is dried, the specific volume of the foam that can be formed with the rehydrated material is gradually increased as the level of sugar increases. As shown in Fig. 8, adapted from Fig. 1 of Kline *et al.* (1964), egg yolk dried with 15% sucrose foams nearly as well as fresh egg yolk. Thus, in contrast to the sharp break-point that occurs in the pattern of easily extractable lipid from dried yolk, a gradual increase in foaming occurs. Fig. 8 is nearly a mirror image of Fig. 2, which could indicate that as the level of sugar increases, the amount of lipid remaining free in the rehydrated yolk is gradually reduced.

Kline *et al.* (1964) reported that dried plain egg yolk would readily foam if rehydrated immediately after spray drying. Results shown in Table 1 confirm this observation. The sugared yolk contained 10% added corn syrup solids on a liquid basis. Both plain and sugar-dried yolk foam quite well when rehydrated and whipped immediately after being spray dried. Within 24 hr of being dried, however, all the foaming power of the plain yolk is lost, and yolk containing the amount of sugar added here loses much of its foaming power.

Table 1. Lipid extractability and foaming power of freshly-dried yolk.

	Plain		Sugared	
Time after drying	Foam specific volume	% Free lipid	Foam specific volume	% Free lipid
10 Min	5.37	0.4	4.99	0.2
1 Hr	3.56	0.7	4.98	0.1
3 Hr	3.46	0.5	5.04	0.3
24 Hr	1.13	4.1	2.58	0.3
7 Day	1.10	4.0	2.71	0.3

Thus, it was reasoned that, if the lipoproteins were rehydrated soon after losing their water of hydration, they had not undergone irreversible changes. The free lipid in Table 1 was isolated by ultracentrifugation as shown in Fig. 1. Again, there is a relationship between the amount of free lipid remaining in the rehydrated yolk and the foaming power of the rehydrated yolk.

Effect of drying under various commercial conditions

Low-density lipoproteins were isolated from commercially dried egg yolk of different viscosities. The amount of lipoprotein isolated was determined, and the results reported are lipoproteins recovered as a percentage of theoretical lipoproteins present in an equal amount of fresh yolk solids.

Nearly 100% of the theoretical lipoprotein present was recovered from the sugar-dried yolk, while only about 80% of the theoretical lipoprotein present was recovered from the plain spray-dried yolk (Fig. 9). There was very little free lipid isolated from the sugar-dried yolk, while from 5 to 7% of the lipid was recovered as free or easily extractable lipid in rehydrated plain-dried yolk. The lipoprotein in the sugar-dried yolk thus survives the spray

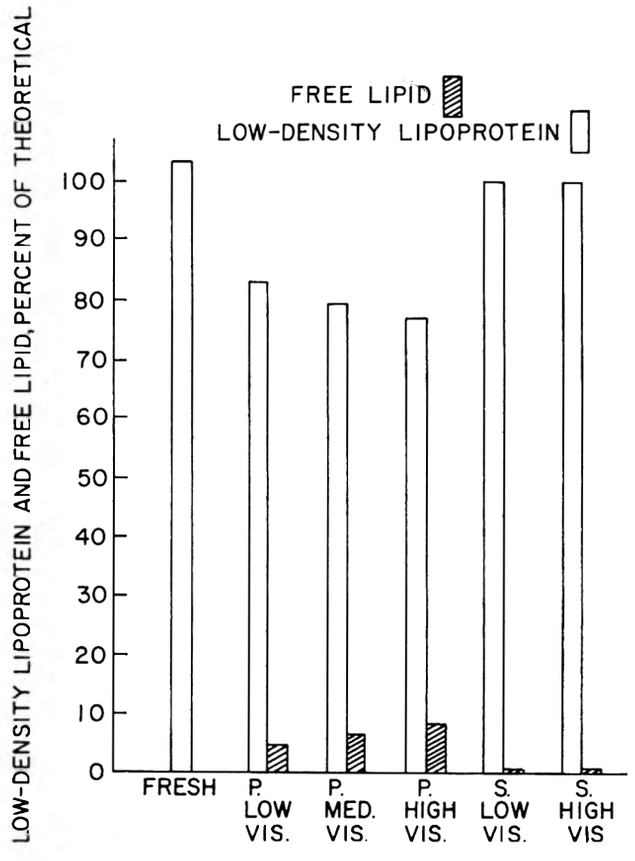

Fig. 9. Effect of drying treatment on the lipoprotein and free lipid content of rehydrated egg yolk. P = plain dried egg yolk; S = sugar dried egg yolk (20.5 corn syrup solids, dry basis); Vis. = viscosity of rehydrated powder, varied by controlling liquid feed rate to dryer.

drying and rehydrating steps much better than do the lipoproteins in the yolk without added sugar.

Additional evidence that the lipoproteins from sugar-dried yolk are not changed as much as the lipoproteins from plain-dried yolk can be seen in Fig. 10. The ultracentrifugal-flotation pattern of the low-density lipoproteins isolated from the sugar-dried yolk spreads less and is more symmetrical than that of the lipoprotein isolated from the plain-dried yolk. The flotation pattern of the lipoprotein isolated from the sugar-dried yolk is quite similar to the flotation pattern of lipoprotein isolated from fresh egg yolk. The greater spreading in the pattern of the lipoprotein isolated from the plain-dried yolk indicates a considerable amount of heterogeneity in these lipoproteins.

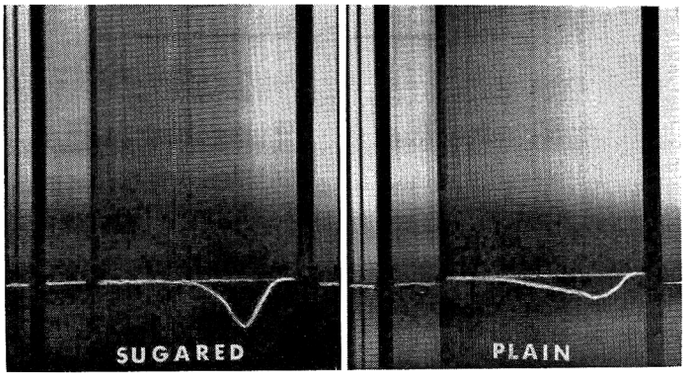

Fig. 10. Ultracentrifugal flotation pattern of low density lipoprotein isolated from spray dried egg yolk.

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Heat Denaturation of the Ovomucin-Lysozyme Electrostatic Complex—A Source of Damage to the Whipping Properties of Pasteurized Egg White

SUMMARY—A longer whip time is usually required to obtain a meringue of the same specific gravity from pasteurized egg white as from unpasteurized egg white. We have determined the rate at which this change in whipping properties occurs as a function of heating time and pH. The rate of damage is minimal at neutral pH. The activation energy for whipping property damage at pH 7.5 is 140 kcal. Experiments in which either ovomucin or lysozyme concentration of egg white was increased and decreased showed that the reaction producing damage to the whipping properties is first order with respect to both ovomucin and lysozyme concentration. Since an increase of 0.33 in the ionic strength of egg white produces a ten-fold decrease in the rate of whipping property damage, the reactants are probably present as the ovomucin-lysozyme electrostatic complex. The product appears to be an irreversibly denatured ovomucin-lysozyme aggregate or network. Removal of the product restores the whipping properties of the egg white. The whipping property damage is a decrease in the mechanical stability of the foam. For this reason a longer time is needed to whip pasteurized egg white to a satisfactory meringue. Whipping aids such as triethyl citrate or triethyl phosphate compensate for the damage to the whipping properties, but do not appear to reverse the reaction producing damage to the whipping properties of the egg white.

INTRODUCTION

HEAT TREATMENT of stabilized egg white for only a few minutes at temperatures near 60°, such as is recommended for the pasteurization of egg white (Lineweaver *et al.*, 1965) is known to produce a significant increase in the time required to whip egg white to a meringue of definite specific gravity (Atkin, 1966). The purpose of this study was to determine the cause of this change in the whipping properties of egg white which occurs upon pasteurization.

The experiments described below were carried out on egg white rather than with any of the recognized fractions of egg white, such as the albumins or globulins. It has been shown by MacDonnell *et al.* (1955) that the foaming properties of egg white reside primarily in the globulins, while the stability of an egg white foam depends primarily on the ovomucin. However, the physical properties of egg white also depend upon interactions among the different components, both those of high molecular weight (proteins), and those of low molecular weight (salts, sugars, etc.). These interactions have not been studied in relation to the foaming or whipping properties, although such an interaction between lysozyme and ovomucin has been studied in relation to the viscosity and rigidity of egg white (Cotterill *et al.*, 1955; Brooks *et al.*, 1959).

When it became evident from preliminary experiments that the lysozyme-ovomucin interaction was the principal cause of the changes produced in the whipping properties of egg white by heat, attempts were made to study the separated lysozyme-ovomucin complex. This complex, in the absence of other egg white components, was insoluble, as reported by Hawthorne (1950), and formed a sticky mass when heated above 50°. The failure to duplicate the results of heat denaturation in the absence of the other components of egg white is a good example of the importance of these intermolecular interactions. Thus, the properties of egg white may not merely be the sum of the properties of its individual components when these are measured in isolation.

MATERIALS

EGG WHITE was obtained from a local egg product manufacturer. Shell eggs for breaking and separating were procured by the manufacturer directly from producers and had been held at refrigeration temperatures for at most 1 to 2 weeks from day of lay. At the time of breaking, the pH of the egg white was observed to be between pH 9.0 and 9.3.

Lysozyme chloride was prepared from fresh egg white by the method of Alderton *et al.* (1946), and checked for purity by starch gel electrophoresis. The substrate, *M. lysodeikticus,* and the buffers for the lysozyme assays were obtained from Difco Laboratories.

N-acetyl neuraminic acid used as standard for the sialic acid assay was Calbiochem Lot #30165.

METHODS

Stabilization of egg white

The egg white was stabilized using aluminum sulfate and lactic acid, as described by Cunningham *et al.* (1965). The final pH was 7.3 ± 0.2, except where noted.

Pasteurization of egg white

The stabilized egg white was pasteurized by heating it to 60°C (140°F) in a commercial plate pasteurizer and holding it at this temperature for 3.5 min (Cunningham *et al.*, 1965).

Sample selection and storage of egg white samples

Where direct comparisons are made, the treated egg white sample was taken from the same batch of egg white as the control egg white, and thus no variation from one batch of egg white to another affected the results. Usually, egg white was frozen and held at −18° until used, although some experiments were performed with unfrozen egg white. No differences were observed between frozen and unfrozen

egg white samples given equivalent heat treatments. The frozen egg white, when thawed, had the same appearance and turbidity as the unfrozen egg white. No insoluble material was present.

Heat treatment of egg white

Sixty g portions of egg white, in capped bottles with only a few ml of head space to avoid CO_2 loss with resulting pH change, were heated at the stated temperature ($\pm 0.03°$) in a water bath of 20-l capacity. The samples were preheated 15 to 20 min at temperatures 10° to 12° below experimental temperatures to keep "come-up time" to a minimum (about 3 min). Zero time (control) samples were also preheated. Hot water was added to the bath to compensate for temperature drops of more than a few tenths of a degree when large numbers of samples were immersed in the bath. After treatment, the egg white was cooled by agitating the bottles in a cold water bath. The whipping properties were usually determined within 4 hr after heat treatment. The pH change on heating was less than 0.05 pH units.

Separation of high and low molecular weight components

Stabilized egg white, both pasteurized and unpasteurized, was filtered under one atmosphere pressure at 4° through Visking size 36 dialysis tubing supported by a polyester fabric mesh in an apparatus described by Davis *et al.*, 1967. After 3 days, 60% of the volume of egg white had passed through the membrane. Since the filtration rate had become negligible, the filtration was halted. The filtrates and retentates from both egg whites were collected. The retentates were shown by gel electrophoresis to contain essentially all the high molecular weight components of egg white. The filtrates were essentially free of such compounds.

Ovomucin analyses

Method of Hoover (1940). The ovomucin content of egg white was determined by a modification of the procedure described by Hoover (1940). The sample of egg white was brought to pH 7 with acetic acid, and three volumes of water were added. The precipitate was dispersed, sedimented by centrifugation for 10 min at 2000 × G, and washed successively with 0.01 *M* NaCl containing acetic acid, 0.01 *M* NaCl, and distilled water. The precipitate was weighed after drying overnight in air at 105°.

Acid-precipitable sialic acid. The ovomucin content of lysozyme-depleted egg white was calculated from results of sialic acid analyses of acid-precipitable material. Two to four ml aliquots of egg white were diluted with three volumes of water. The pH of the diluted white was adjusted to 4.5 with 5 *M* acetic acid. The suspension was centrifuged and the supernatant liquid discarded. The unwashed precipitate was resuspended in 4 ml of 0.12 *N* sulfuric acid and heated at 80° for one hour. Control experiments indicated that this treatment released all the sialic acid of ovomucin. After centrifugation, aliquots of the supernatant liquid were analyzed for sialic acid using p-dimethylaminobenzaldehyde (Werner *et al.*, 1952).

The absorption at 533 mμ was used to calculate the concentration of sialic acid, since standards read at this wavelength showed no deviation from Beer's law. Since lysozyme-free ovomucin prepared in this laboratory contained 4.0% by weight of *N*-acetyl neuraminic acid, ovomucin content of egg white was calculated on this basis.

Lysozyme-depleted egg white

Egg white depleted in lysozyme was used to determine the contribution of lysozyme to the change in whipping characteristics of egg white on heat treatment. This lysozyme-depleted egg white was prepared by dispersing 35 g of the acid form of carboxymethyl cellulose (CMC) in 800 ml of egg white. The suspension was adjusted to pH 9.0 with 5 *M* NaOH and stirred slowly at 4° overnight. Successive centrifugations at 650 × G and 4,500 × G yielded a lysoxyme-depleted egg white free of CMC.

"Ovomucin-free" egg white

Both pasteurized and unpasteurized egg white were centrifuged in a Beckman Model L-2 preparative ultracentrifuge. Aliquots of 250 ml were centrifuged at 19,000 RPM (equivalent to 53,000 × G at R_{max}) in a 19 rotor at 4° for 16 hr. Supernatants were easily separated from the firmly packed pellet. The two fractions from each sample of egg white were weighed and stored at 4° until used.

Starch gel electrophoresis

Horizontal starch gel electrophoresis was carried out using the discontinuous buffer system of Poulik (1957). The gels, pH 8.5, were 0.075 *M* in tris(hydroxymethyl) aminomethane, 0.005 *M* in citric acid, 2 *M* in urea and contained 10% starch. Buffer for the electrode vessels was 0.03 *M* in sodium borate, 0.18 *M* in boric acid, pH 8.1. Normally, aliquots of 0.05 ml of egg white were placed in the slots at the origin in the gel. The samples were subjected to electrophoresis at room temperature for 15 to 17 hr at a potential gradient of 8 volts per cm. The gel was then stained with 1% amido black in 5% acetic acid and destained electrophoretically.

Whip test

To obtain true comparisons of the whipping properties of egg white samples, overwhipping must be avoided. In these experiments several portions of the control egg white (which always has the shortest whipping time) were whipped for different time periods to determine the minimum time necessary to obtain a meringue with specific gravity of 0.15 g/ml, called the "soft peak stage."

The time period so determined for the control egg white was chosen as the whipping time for all samples. Since the control material has the shortest whipping time, no sample is overwhipped. All egg white samples from each experiment were whipped for identical times, and under identical whipping speed conditions. The specific gravities of the resulting meringues were used as the basis of comparison of the whipping properties.

The whip test was conducted as follows: 60 g of egg white at 25° was whipped at speed 10 in a Hobart model K4B mixer for the time determined for the control sample as described above. The mixer was stopped, the speed changed to 6, and 46 g sucrose were added in three equal portions with a 4-sec mix after each addition. The specific gravity of the meringue was calculated from the weight

of the meringue in a container of known volume. Potassium acid tartrate was added before whipping in predetermined amounts to give meringues with a pH of approximately 6.

Lysozyme assays

Egg white was analyzed for lysozyme turbidimetrically, by a modification of the method of Smolelis *et al.* (1949). Difco bacto-lysozyme buffer (phosphate, pH 6.2) was made up in 0.1 *M* KCl. Lysozyme standards and egg white were diluted 3:10,000 with this buffer. Approximately 30 mg of Difco bacto-lysozyme substrate (UV killed *M. lysodeikticus*) were shaken thoroughly with 25 ml of buffer, and allowed to settle for 30 min. The supernatant suspension was decanted and used for the assay. All solutions were equilibrated at assay temperature before use. Turbidimetric assays were carried out at 22° in the thermostated cell compartment of a Cary Model 15 spectrophotometer at 540 mμ, using the synchronous mode. Two ml of substrate were mixed with 1 ml of diluted sample in a 1-cm cell. The optical density was recorded for 10 min, and the slope of the resulting line determined. Approximately 2% reproducibility was attained. The slope was linearly proportional to lysozyme concentration of the undiluted samples to at least 7 mg of lysozyme per ml (twice the concentration of lysozyme in egg white).

Viscosity measurements

Viscosity measurements were made using a size 100 Cannon-Fenske viscometer, in a Cannon M-1 constant temperature bath at 25°.

Surface tension measurements

Surface tension measurements of unpasteurized and pasteurized egg white were made with a DuNouy Tensiometer.

Meringue stability measurements

The meringue from 60 g of egg white was placed on cheesecloth in a large funnel at room temperature, and the volume of the liquid ("drip") collected in a 50-ml graduate was noted at 10-min intervals for an hour.

Foam stability comparisons

Five ml each of egg white samples were placed in 100-ml graduated cylinders. A fixed volume of air was bubbled at approximately the same rate into each sample, producing 70 ml of foam in each graduated cylinder. The stability of the foam against mechanical damage was qualitatively tested in two ways: (1) by insertion of a glass rod the length of the graduate, followed by movement of the rod across the diameter of the cylinder; (2) by striking the open top of the graduate with the palm of the hand, causing a sharp rise of pressure within the graduate.

RESULTS

Size of molecule responsible for change in whipping properties

Filtrates and retentates obtained by the fractionation of pasteurized and unpasteurized egg white through Visking dialysis tubing were recombined and cross-combined in their proper volume ratio and whipped for 40 sec (Table 1). Only the recombinations containing the retentate from the pasteurized egg white or its equivalent gave meringues of higher specific gravity, i.e., damaged whipping properties. Recombination of filtrates and retentates from each starting material yielded an egg white with whipping properties identical to the original. Therefore no change in whipping properties occurred during the filtration process.

Table 1. Whipping properties of recombined ultrafiltered materials.

Sample	Meringue SP. GR. (40 secs)
Original stabilized egg white (S)	0.156
Pasteurized-stabilized egg white (PS)	0.219
Filtrate *Retentate*	
S Filtrate + S Retentate	0.158
PS Filtrate + PS Retentate	0.211
PS Filtrate + S Retentate	0.158
S Filtrate + PS Retentate	0.202

Rate of change in whipping properties

The whipping properties of egg white heated for various periods of time at 45°, 54°, 56°, and 58° were determined. Since the specific gravity appeared to be a nearly linear function of heating time in the early stages of the heating period (Fig. 1), all kinetic experiments were reduced to an initial velocity basis, by measuring the change in meringue specific gravity produced in samples heated 30 to 60 min, or from the initial slope of curves such as those in Fig. 1.

The activation energy for the reaction which produces whipping property damage, calculated from the slope of the line drawn in Fig. 2, is 140 kcal/mole. The point at 60° in Fig. 2 was obtained from an egg white sample pasteurized with commercial plant equipment (60° for 3.5 min).

Changes in starch gel electrophoresis patterns

The change in the whipping property of egg white heated 4 hr at 54° (Fig. 1) is approximately equivalent

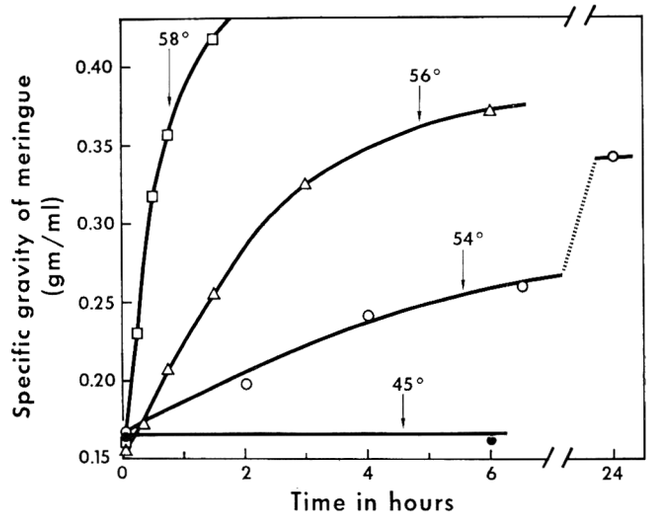

Fig. 1. Change in the whipping properties of egg white with time at several temperatures. The specific gravity of the meringue is plotted against time of heating (preheating not included). The pH of the egg white was 7.3, whipping time 40 sec.

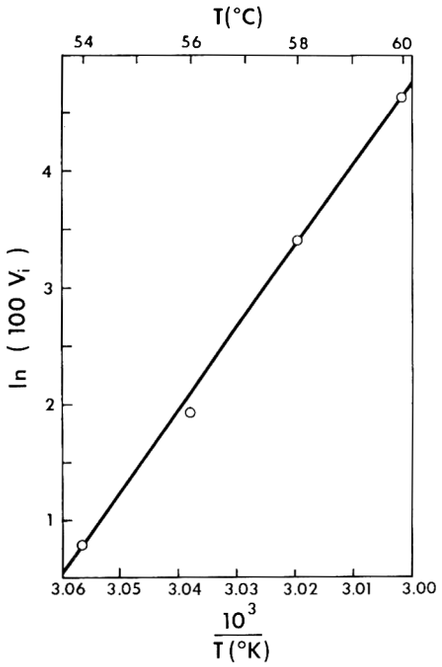

Fig. 2. Temperature dependence of the rate of change in whipping properties of egg white. The natural logarithm of the initial velocity (V_i) of the reaction, in units of specific gravity change per hour, is plotted against reciprocal absolute temperature (°K). The point at 60°C was determined for the egg white pasteurized in a commercial pasteurizer (60° for 3.5 min). The calculated activation energy is 140 kcal.

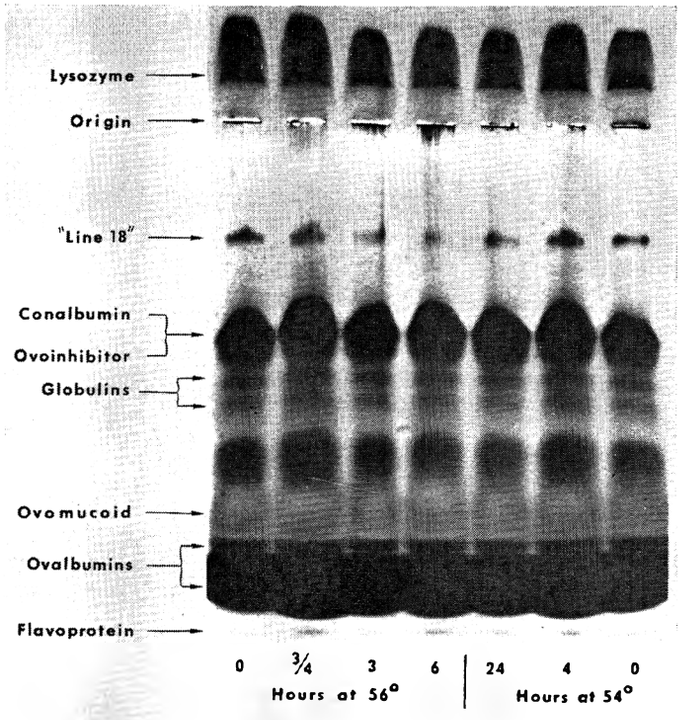

Fig. 3. Starch gel electrophoretic patterns of stabilized egg white samples heated at 56° and at 54°.

to that of egg white heated 3.5 min at 60° (Table 1). Electrophoretic patterns of unheated egg white and of egg white heated at 54° and 56° are shown in Fig. 3. Three changes in these patterns are apparent. The first and most obvious is the decrease in intensity of the band designated "line 18" by Lush (1961); the second is the slight decrease in lysozyme intensity (see also Table 2); and the third is the increase in material near the slots at the origin (probably heat-denatured protein). Since the amounts of material placed in the slot origins may differ by as much at 25%, changes in amounts of the egg white components must be evaluated by comparison with a non-changing reference material, such as conalbumin or ovalbumin, in the same pattern.

pH dependence of the rate of damage to the whipping properties

Stabilized egg white was adjusted to the desired pH with either lactic acid or ammonia, and heated for various times at 56° or 58°. The ratio of the initial velocity at each pH to that at pH 6.5 was calculated and is shown as a function of pH in Fig. 4.

Centrifugation of egg white

Egg white, upon centrifugation at 53,000 × G for 16 hr, separated into two fractions consisting of a clear supernatant and a firm pellet, 6.2% by weight of the egg white. Starch gel electrophoretic patterns (Fig. 5) showed that the egg white and its supernatant had essentially the same concentrations of protein stainable with amido black. Assays for lysozyme activity (Table 2) showed that the supernatant had a lower lysozyme concentration. Ovomucin is not detected in starch gel patterns. Therefore both unsedimented egg white and supernatant therefrom were analyzed for ovomucin, using the method described by Hoover (1940). Less than 5% of the ovomucin of egg white is found in the supernatant fraction of centrifuged egg white (Table 2). The calculated composition of the pellet is also given in Table 2.

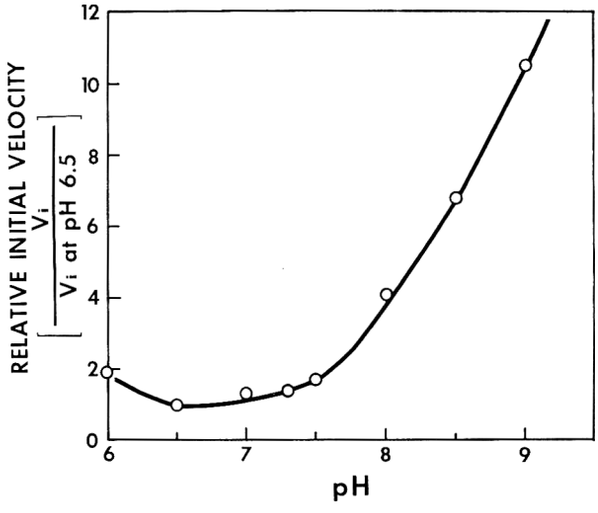

Fig. 4. The pH dependence of the rate of heat damage to the whipping properties of egg white. The initial velocity (V_i) of the reaction at each pH was determined from the change in specific gravity of the meringue with heating time. Initial velocities are expressed relative to the initial velocity at pH 6.5.

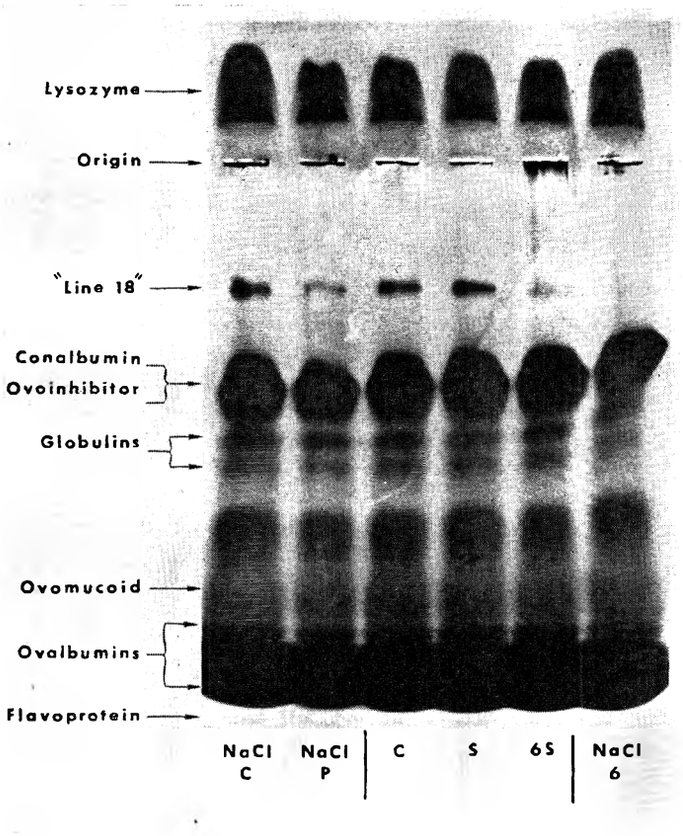

Fig. 5. Starch gel electrophoretic patterns of stabilized egg white samples. From left to right: Egg white containing ½% added salt, NaCl C, unheated control; NaCl P, pasteurized. Unheated egg white control, C; 53,000 × G supernatant from this egg white, S; Supernatant heated at 56° for 6 hr, 6S. Egg white made 0.33 M in NaCl, then heated at 56° for 6 hr, NaCl 6. The increasing distortion of the patterns from left to right was caused by a sample in a seventh slot (not shown) containing large amounts of added salt.

Recombination of fractions from centrifuged egg white

The supernatants and sediments from both stabilized and stabilized-pasteurized egg whites were recombined and cross-combined in the proper ratio. Results of whipping property evaluation of these combinations and of the supernatants are given in Table 3. Removal of sedimentable material from pasteurized egg white gave a supernatant with whipping properties approaching those of unpasteurized egg white. Therefore, some factor must be formed during pasteurization that is detrimental to the whipping properties of pasteurized egg white. This factor must be of sufficient size to be readily sedimentable at 53,000 × G.

These recombinations of sediment from unheated egg white with supernatant from either heated or unheated egg white gave a reconstituted egg white with good whipping properties. However, recombination of sediment from pasteurized egg white with either supernatant gave inconsistent results. This has been our experience with recombinations of sediment from pasteurized egg white or of sedimentable material heat treated separately after centrifugation.

Dependence of the heat damage on ovomucin content

Since the heat damage to the whipping properties of egg white depleted in ovomucin (53,000 × G supernatant) was markedly reduced (Fig. 6), the effect of ovomucin concentration on the heat damage to egg white was investigated. The ovomucin concentration of the supernatant from centrifuged egg white is essentially zero (Table 2). By mixing egg white and supernatant in different ratios, samples of egg white with intermediate ovomucin concentrations were prepared. The ovomucin content of egg white has been estimated to be from 1.6% of the total protein (Lanni *et al.*, 1949) to 4.7% (Fernández-Paláez,

Table 2. Analyses of egg white fractions obtained by sedimentation at 53,000 × G.

A. Ovomucin analysis		
Control egg white [1] (11.3% solids)	0.332 g/100 g egg white	
53,000 × G supernatant	0.014 g/94 g supernatant	
53,000 × G pellet (by difference)	0.318 g/6.2 g pellet	
B. Lysozyme content		
	Relative activity	g Lysozyme/100 g
Control egg white [1]	100	0.35
53,000 × G supernatant from control	84	0.29
Pasteurized egg white [2]	85	0.30
53,000 × G supernatant from past.	65	0.23
C. Composition of 53,000 × G pellet from control egg white [3]		
1. Total solids in pellet: 1.10 g [4]		
2. Calculated composition of solids content:		
Lysozyme [5]	0.08 g	
Ovomucin [6]	0.32	
Occluded egg white proteins [7]	0.66	
	1.06 g	

[1] Stabilized unpasteurized egg white.
[2] Pasteurized 3.5 min at 60°.
[3] 6.2 g pellet from 100 g egg white. The same weight pellet was obtained from pasteurized egg white. Composition was presumed to be nearly the same as that of the pellet from the control egg white.
[4] 6.2 g × 17.7% solids.
[5] From loss in lysozyme activity of supernatant (see B).
[6] By difference between control egg white and supernatant (see A).
[7] Weight of lysozyme plus ovomucin in pellet is 0.4 g. Total weight of pellet minus 0.4 g leaves 5.8 g occluded egg white. 5.8 g × 11.3% solids gives 0.66 g occluded egg white proteins.

Table 3. Whipping properties of egg white samples.

		Meringue specific gravity (45 sec)	
A. Original samples			
Stabilized, unpasteurized		0.153	
Stabilized, pasteurized [1]		0.316	
B. Supernatants obtained at 53,000 × G			
Stabilized, unpasteurized		0.131	
Stabilized, pasteurized		0.153	
C. Recombinations [2]			
Supernatant	Pellet		
Unpasteurized	Unpasteurized	0.153	
Pasteurized	Pasteurized	0.197	
Unpasteurized	Pasteurized	0.165,	0.374 [3]
Pasteurized	Unpasteurized	0.162,	0.162 [3]

[1] Pasteurized 3.5 min at 60°.
[2] All samples stabilized.
[3] Duplicate experiments.

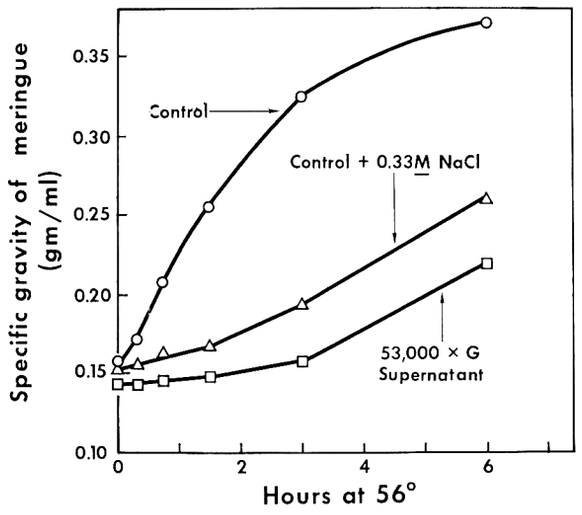

Fig. 6. Effect of salt and high speed centrifugation on the heat damage to the whipping properties of egg white. Solid sodium chloride was added to stabilized egg white to attain the indicated concentration before heat treatment. The supernatant (lowest curve) was obtained from stabilized egg white by centrifugation for 16 hr at 53,000 × G. The control experiment was performed on stabilized, but otherwise untreated, egg white. All samples in this figure were aliquots from the same batch of egg white. Whipping time 40 sec.

1960). Since there appears to be no general agreement on the amount present, ovomucin concentration is here represented in terms of a fraction of the amount present in egg white.) The whipping properties of these samples were determined before and after heat treatment at 56° (Fig. 7). The heat-damage reaction is evidently first order with respect to the ovomucin concentration.

Effect of lysozyme on the whipping property damage

The rate of loss of whipping properties was determined for control egg white, egg white reduced in lysozyme content, and both egg white and 53,000 × G supernatant with added lysozyme. Starch gel electrophoretic patterns (Fig. 8) showed that removal of lysozyme from egg white by carboxy-methyl cellulose treatment did not alter the concentrations of the other egg white proteins. Analyses (see below) indicated that the ovomucin content was unchanged. The stabilized samples were heated at 56°, cooled, and lysozyme added to some of them. Heat damage is reduced when lysozyme concentration is less than that normally found in egg white (Fig. 9).

The amount of heat damage as a function of lysozyme concentration is shown in Fig. 10. The slope of the line drawn through the points is unity. The reaction which produces heat damage is thus also first order with respect to lysozyme concentration.

Lysozyme must be present during heating for the damage to occur. Addition of lysozyme after heat treatment had no effect on the damage produced. Similarly, addition of lysozyme to unheated egg white produced only a slight increase in the specific gravity of the meringue formed. Ovomucin must be present so that this effect of lysozyme concentration can be observed. When the lysozyme concentration of 53,000 × G supernatant (in which the ovomucin concentration is very low) is doubled, no effect of heating upon the whipping properties of the supernatant is observed.

Effect of ionic strength on heat damage

Addition of salt markedly reduces heat damage in the earlier stages of heating (Fig. 6). After 3 hr of heating at 56°, there is an increase in rate. It has not been deter-

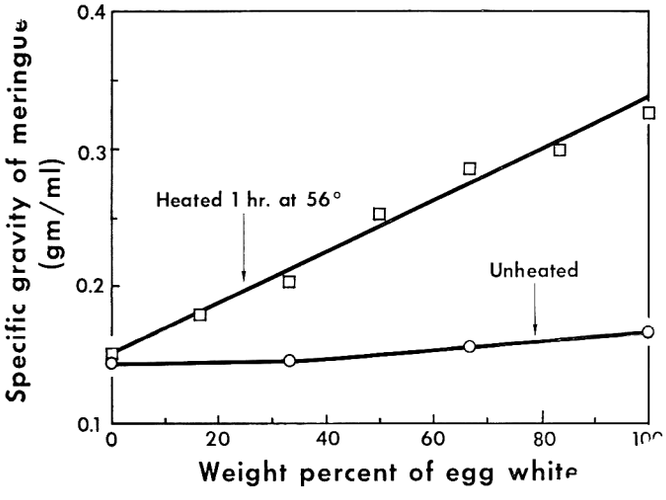

Fig. 7. The effect of ovomucin concentration on the heat damage to the whipping properties of egg white. Samples of different ovomucin concentrations were prepared by mixing egg white and supernatant from 53,000 × G centrifuged egg white. Ordinate: specific gravity of meringue obtained by whipping these mixtures for 50 sec, both before and after one hour of heating at 56°. Abscissa: the weight percent of egg white, which is directly proportional to the ovomucin concentration of the mixture.

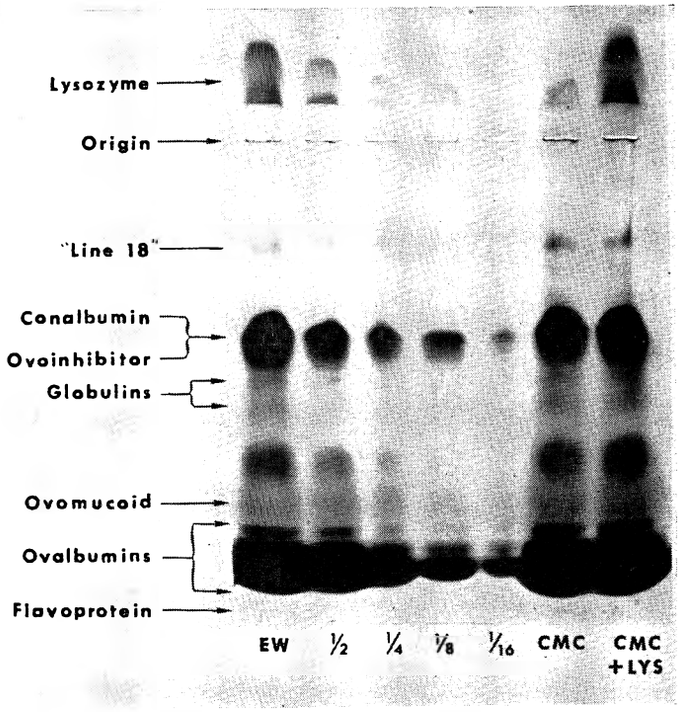

Fig. 8. Starch gel electrophoretic patterns. On the left are patterns of undiluted and of successive two-fold dilutions of unstabilized (unheated) egg white. The sixth pattern is of egg white treated with carboxymethyl cellulose to remove lysozyme. The pattern on the right is that of this carboxymethyl cellulose-treated egg white after lysozyme was added back to the original concentration.

mined whether this later damage is similar to that which occurs in the control egg white sample.

Effect of pasteurization on the lysozyme activity of egg white

The decrease in lysozyme activity in pasteurized egg white and in the 53,000 × G supernatants (Table 2) is in agreement with starch gel electrophoresis patterns (Figs. 3 and 5) which suggest smaller amounts of lysozyme are present in pasteurized egg white, in egg white heated at 54° and 56°, and in the supernatant from unpasteurized egg white. The electrophoretic patterns presented by Seideman *et al.* (1963) show a decrease in staining for lysozyme in egg white heated at 62° for 3 min at pH 7.0 to 9.5, when compared to the electrophoretic patterns for unheated egg white.

Ovomucin content of untreated egg white

When egg white was analyzed for ovomucin using the procedure described by Hoover (1940), in which the ovomucin is precipitated at pH 7, then washed at pH 4.5 (see above), 0.33 g was obtained per 100 g of egg white (Table 2). This result is similar to that obtained by Hoover (1940) and Balls *et al.* (1940). However, samples of egg white depleted to one-third the normal lysozyme content, when analyzed by the Hoover procedure, appeared to have only one-third the ovomucin of the untreated egg white. Hawthorne (1950) and Cotterill *et al.* (1955) have shown that the precipitation of ovomucin at pH 7 is facilitated by an increase in lysozyme concentration. The solubility of ovomucin in buffer solutions at pH 7 (Lanni *et al.*, 1949) suggests that in the absence of lysozyme, all the ovomucin would not be precipitated from egg white at pH 7.

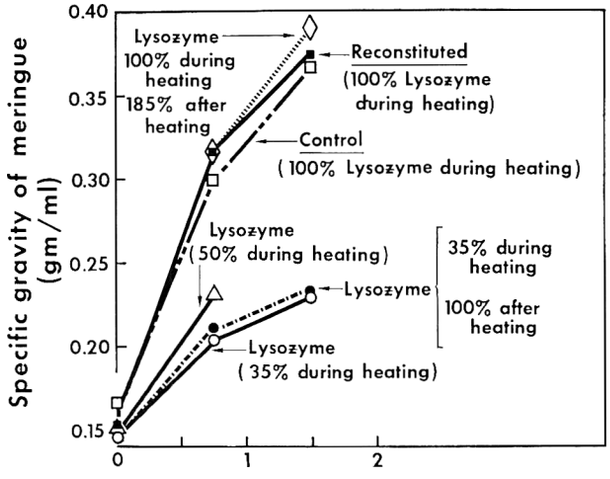

Fig. 9. Effect of lysozyme on the heat damage to the whipping properties of egg white. The samples were stabilized, adjusted to pH 7.3, heated at 56°, and whipped for 65 sec without tartrate. The sample designation is as follows:

- ⊡ *control egg white.*
- ⊙ *egg white depleted to 35% of its original concentration of lysozyme.*
- ■ *depleted egg white with lysozyme added back to the original concentration* prior *to heat treatment.*
- ● *depleted egg white with lysozyme adjusted to the original concentration* after *heat treatment and cooling.*
- △ *depleted egg white with lysozyme adjusted to 50% of the original concentration* prior *to heat treatment.*
- ◇ *control egg white with lysozyme adjusted to 185% of the original concentration* after *heat treatment and cooling.*

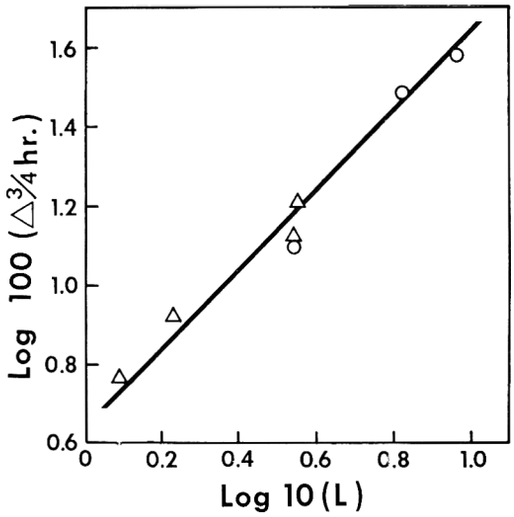

Fig. 10. The effect of lysozyme concentration on the heat damage to the whipping properties of egg white. The logarithm of the initial velocity, expressed as the specific gravity change for 45 min of heating, is plotted against the logarithm of the lysozyme concentration, in g per hundred ml of egg white. The triangles are data from control egg white and egg white reduced in lysozyme concentration; the circles are for control egg white and egg white enriched in lysozyme. The line drawn through the points has a slope of unity. Heating carried out at 56°; sample pH was 7.3 to 7.5.

Accordingly, an alternative method of analysis was devised in which ovomucin was precipitated at pH 4.5 and the ovomucin content of the precipitate calculated from analysis of its sialic acid content (see Methods). Although other proteins may be precipitated from egg white at this pH, no attempt was made to remove these, since ovomucin not only contains most of the protein-bound sialic acid of egg white, but also appears to be the only acid-precipitable protein containing significant amounts of sialic acid (Feeney, 1964).

Duplicate determinations of lysozyme-depleted egg white gave 188 and 186 μg of acid-precipitable sialic acid (as *N*-acetyl- neuraminic acid) per ml of egg white. Untreated control egg white contained 185 μg of acid-precipitable sialic acid per ml. This amount of sialic acid corresponds to 0.46 g of ovomucin per 100 ml of egg white. A preparation carried out in this laboratory yielded 0.42 g of ovomucin per 100 ml of egg white. These results suggest that not only is lysozyme required for precipitation of ovomucin from egg white at pH 7, but that precipitation of ovomucin is incomplete even in the presence of lysozyme.

Viscosity and turbidity of heated egg white

The turbidity of the egg white samples increased with heating and paralleled qualitatively the whipping property damage. Also, the viscosity of the solutions increased with both time of heating and with increasing pH. Samples heated for the longer times without stirring became gel-like. Increases in viscosity upon pasteurization were reported by Cunningham *et al.* (1965), and Kline *et al.* (1965, 1966). Samples of egg white, initially somewhat turbid, increased in turbidity during heating. The turbidity could be sedimented at 2000 × G. The volume of the

sediment, measured in calibrated centrifuged tubes, is plotted against heating time at 58° in Fig. 11. The specific kinematic viscosity of the supernatant liquid above this sediment together with the specific gravity of the meringue from egg white samples heated at 58° is also plotted in Fig. 11.

Samples of egg white which had been centrifuged at 5000 × G prior to heat treatment, although suffering half the usual loss of whipping properties, did not yield a sediment when centrifuged at 2000 × G after heat treatment. These solutions showed the usual increased turbidity but only a slight increase in viscosity upon heating.

Effect of pasteurization on stability of meringues and foams

Measurements of stability of meringues made from unpasteurized egg white, pasteurized egg white and supernatant from pasteurized egg white showed that the amount of liquid draining from the meringue during the first hour was approximately a linear function of time, and did not show a consistent dependence upon the specific gravity of the meringue (Table 4).

Qualitative measurement of the mechanical stability of foams revealed that foams from pasteurized egg white were much more susceptible to mechanical damage than foams from unpasteurized egg white. Foams from pasteurized egg white had a "dry" appearance, the lamellae appearing very thin in comparison to the lamellae in the foam from an unpasteurized egg white. Surface tension measurements of unpasteurized and pasteurized egg white showed no difference (61 ± 1 dynes/cm). Addition of

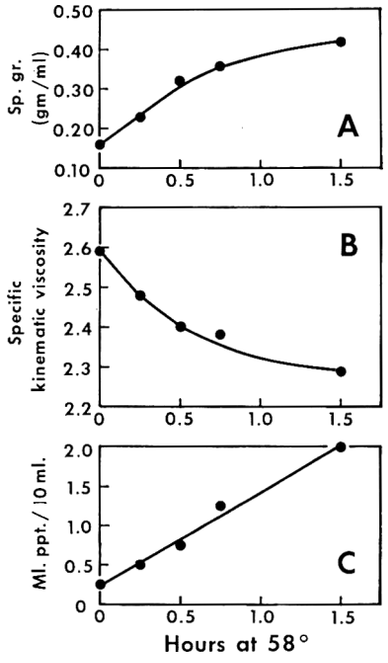

Fig. 11. Aggregation of the product of the reaction formed on heating egg white at pH 7.3. A, the specific gravity of the meringue as a function of heating time at 58°. B, the specific kinematic viscosity, determined at 25°, with respect to water, of the egg white remaining above the material sedimented at 2000 × G after heating. C, the volume of the sediment obtained at 2000 × G after heating, as a function of heating time.

Table 4. Stability of meringues[1] from pasteurized and unpasteurized egg white.

Sample	Whipping time (sec)	Meringue specific gravity (g/ml)	Draining rate (ml/hr)
Unpasteurized	40	0.156	7
Pasteurized	70	0.202	17
Pasteurized	100	0.170	9
53,000 × G			
Supernatant from pasteurized	40	0.146	29

[1] 60-g sample plus 0.7 g tartrate plus 46 g sucrose.

triethyl citrate, a whipping aid, to the pasteurized egg white before foam formation greatly increased the stability of the foam and abolished the "dry" appearance of the lamellae. Lysozyme assays both before and after addition of triethylcitrate to the pasteurized egg white showed no difference in activity.

DISCUSSION

The membrane filtration experiments presented in Table 1 suggest that the component in egg white responsible for heat damage has a molecular weight greater than 10,000, and is thus one or more of the proteins of the egg white. The change in specific gravity of the meringue with time of heating was used to evaluate the temperature dependence of the rate of heating damage. The activation energy of the reaction producing the damage to the whipping properties was 140 kcal, calculated using the van't Hoff equation: $d\ln k/d(1/T) = -E_a/R$. In the temperature range of 6° shown in Fig. 2 (54° to 60°), the rate of the heat-damage reaction increases approximately 45-fold. This high activation energy is typical of protein denaturation.

Centrifugation at high centrifugal forces separates ovomucin from the other proteins of egg white (Lanni *et al.*, 1949; Forsythe *et al.*, 1950). The pellet obtained after centrifugation at 53,000 × G contained nearly all the ovomucin in the egg white (Table 2). In addition, starch gel electrophoresis showed that the pellet contained all the other proteins in the egg white in their normal ratios, except for lysozyme, in which the pellet was enriched 4-fold compared to egg white (Table 2).

Whipping property tests of the supernatant and recombinations of supernatant and pellet from undamaged and heat-damaged egg white (Table 3) showed that whipping damage could be eliminated by removal of the material sedimentable after heat treatment. Removal of sedimentable material prior to heat treatment also greatly reduced the damage produced by heat (Fig. 6). These results strongly implicated ovomucin as the protein damaged by heat. However, ovomucin has been reported to be stable to heating, even to 100° for 2 hr above pH 9 (MacDonnell *et al.*, 1953; Cotterill, 1954; Cunningham *et al.*, 1965).

The rate of heat damage to the whipping properties of 53,000 × G supernatant from undamaged egg white is one-twentieth that of the original material (Fig. 6). The ovomucin content of the supernatant, as measured by Hoover's procedure, is approximately one-twentieth that of the original material. Starch gel electrophoresis pat-

terns of samples of the supernatant and original egg white are nearly indistinguishable (Fig. 5), but assays (Table 2) show a reduction in lysozyme concentration in the supernatant. Thus, ovomucin, which does not stain on starch gel with amido black, and lysozyme, which stains readily, are the only proteins present in egg white in significant quantity which are fractionated by sedimentation. Damage to ovomucin and/or lysozyme must be the cause of damage to the whipping properties of egg white.

This conclusion is supported by the results of experiments (Figs. 7 and 9) which showed that the reaction which produced whipping property damage was first order in ovomucin concentration and also first order in lysozyme concentration. The addition of salt, which would be expected to diminish the interaction between these oppositely charged proteins, reduced the heat damage to the whipping properties.

The protein designated "line 18" by Lush (1961) and isolated and characterized by Miller *et al.* (1966) disappears from the starch gel electrophoretic patterns of heated egg white (Fig. 3) at a rate roughly proportional to the extent of heating (compare Fig. 1). Thus, line 18 would appear to be implicated in the process of heat damage. However, the 53,000 × G supernatant has essentially the same line 18 content as unsedimented egg white (Fig. 5), although there is a 20-fold difference in their susceptibility to heat damage (Fig. 6). This observation eliminates both denaturation of line 18 and denaturation of a complex between lysozyme and line 18 as the source of damage.

The following scheme is proposed to account for the heat damage to the whipping properties of egg white:

1. $M + nL \overset{K_B}{\rightleftharpoons} ML_n$
2. $ML_n\ (\text{native}) \overset{K_D}{\rightleftarrows} ML_n\ (\text{denatured})$
3. $ML_n\ (\text{denatured}) \xrightarrow{k'} \text{Aggregate}$

Here, M represents ovomucin and L, lysozyme.

In Step 1, n lysozyme molecules bind to one ovomucin molecule in a reversible equilibrium characterized by the binding constant K_B. In view of the opposite net charges on lysozyme and ovomucin at neutral pH, it seems reasonable to suppose that this binding is primarily electrostatic and takes place at many sites on the large ovomucin molecule (see below).

In Step 2, the lysozyme-ovomucin electrostatic complex undergoes heat denaturation, which is here arbitrarily represented as being reversible.

Step 3, which is assumed to be rapid and to obey pseudo-first order kinetics, is the aggregation of the denatured complexes, possibly at the sites of denaturation.

The kinetics of the reaction scheme described above would be as follows:

$$\frac{d\,(\text{Aggregate})}{dt} = k' \quad [ML_n\ (\text{denatured})].$$

Using the relations

$$K_D = \frac{[ML_n\ (\text{denatured})]}{[ML_n\ (\text{native})]}$$

and

$$K_B = \frac{[ML_n\ (\text{native})]}{[M]\ [L]_n}$$

there is obtained:

$$\frac{d\,(\text{Aggregate})}{dt} = k'\,K_D\,K_B\,[M]\,[L]^n.$$

This scheme leads to a reaction rate which is first order in ovomucin concentration, as observed experimentally. The order with respect to lysozyme will be n if n sites at which lysozyme is bound are denatured simultaneously. Since the reaction is observed to be first order with respect to lysozyme, a single site on ovomucin at which a lysozyme molecule is bound becomes heat denatured in Step 2.

If, as assumed here, Step 3 is not rate-limiting, then the temperature dependence of the overall process will be determined by Steps 1 and/or 2. Since Step 1 is electrostatically favorable and probably diffusion controlled, its activation energy can be expected to be at most a few kcal/mole. The observed activation energy of 140 kcal/mole is undoubtedly that of a large conformational change characteristic of protein denaturation, and must be the activation energy for Step 2. The activation energy reported by Hamaguchi *et al.*, (1965) for lysozyme denaturation near pH 5 is approximately 130 kcal. The agreement between this value and that observed in the present experiments is suggestive of heat denaturation of a lysozyme molecule bound to ovomucin.

The effect of added salt appears to be in accord with the above scheme, since reduction of the strength of the electrostatic interaction in Step 1 will lead to a reduction in rate of the overall process. One of the main features of the scheme above is the introduction of the ovomucin-lysozyme electrostatic complex as a reactant. Both lysozyme and ovomucin have been reported not to undergo denaturation at the temperatures employed here.

The observations of MacDonnell *et al.* (1953) and Cunningham *et al.* (1965) indicate that in the absence of other proteins, ovomucin will withstand heating at 100° for several minutes. Lysozyme does not show evidence of thermal unfolding below 60° at pH 7 in either 0.1 *M* phosphate (Steim, 1965) or in 2 *M* lithium bromide (Hamaguchi *et al.*, 1965). Reported transition temperatures (T_m) are 69° and 67°, respectively. Thus, the formation of the ovomucin-lysozyme electrostatic complex renders one or both of these proteins susceptible to heat denaturation at reduced temperatures. The mere formation of an electrostatic complex may be sufficient to produce this change in stability. However, it may be necessary for some chemical reaction, not indicated in the above scheme, to produce a covalent attachment of lysozyme to ovomucin before denaturation will occur.

The pH dependence of the heat damage to the whipping properties (Fig. 4) appears to correlate closely with the pH dependence of the viscosity increase reported by Cunningham *et al.* (1965). It is not apparent how this pH dependence arises from the reaction mechanism proposed above. Step 1 is presumably strongly dependent upon differences in the net charge of the two reactants. The equilibrium constant for association, K_B, can be expected to be a maximum near a pH midway between the isoelec-

tric points of the two reactants, i.e., approximately at pH 8. The observed pH dependence of the reaction rate is probably determined by Step 2.

The approximate composition of the lysozyme-ovomucin electrostatic complex may be calculated from the composition of the pellet (Table 2). From 100 g of egg white, approximately 0.32 g of ovomucin is found in the pellet. The amount of lysozyme in the pellet, in excess of the amount usually found in this weight (6.2 g) of egg white, is 0.06 g. Thus, the weight ratio of lysozyme to ovomucin in the pellet is 1:5. Using the molecular weights of 14×10^3 for lysozyme (Canfield, 1963) and 8×10^6 for ovomucin (Lanni *et al.*, 1949), the molar ratio of lysozyme bound to ovomucin in the pellet is 100:1.

Pasteurized egg white shows a decrease in lysozyme activity of approximately 15% (Table 2). Upon centrifugation at 53,000 × G, an additional loss of 20% of the lysozyme activity from the supernatant is observed. Thus, even in pasteurized egg white, a large quantity of active lysozyme is sedimented by centrifugation and is presumably bound to the ovomucin of the pellet.

A large loss of lysozyme from the supernatant of heat-treated egg white is easily accounted for by the reaction scheme proposed above. When a "lysozyme-ovomucin interaction site" is heat-denatured, the lysozyme may then no longer be free to dissociate, and thus the site may become fully saturated. As more interaction sites are denatured, the molar ratio of lysozyme to ovomucin in the complex increases.

The association of lysozyme and ovomucin in egg white has been termed "cross-linking of ovomucin by lysozyme" by Brooks *et al.*, (1959). Sharp *et al.*, (1950) have termed the coherent structure in the thick portion of the egg white, which has a high ovomucin content, a "fibrous mesh." Some of this coherent structure still remains in egg white after it has been milled to lower its viscosity (Forsythe *et al.*, 1951). Since the viscosity of egg white increases both with temperature and with time of heating (Seideman *et al.*, 1963; Kline *et al.*, 1965; Cunningham *et al.*, 1965), the product of the reaction of lysozyme and ovomucin may be the source of the increase in viscosity.

In one experiment (Fig. 11), a qualitative increase in viscosity of the samples upon heating was readily apparent when the samples were poured from the reaction bottles for whipping tests. However, after low speed centrifugation at 2000 × G, a sediment was obtained in every case, even for the unheated sample. The volume of the sediment (not well compacted) was a linear function of the heating time (Fig. 11C). The viscosity of the supernatant above this sediment *decreased* with increased amount of sediment formed (Fig. 11B), suggesting that the sedimentable material produced the increase in viscosity of the heated sample.

A duplicate control egg white sample, when subjected to centrifugation at 5000 × G *before* heat treatment, did not yield a sediment when subjected to centrifugation at 2000 × G *after* heat treatment. Since this sample suffered essentially the same loss in whipping properties, it appears that the heat-damaged lysozyme-ovomucin complex either becomes attached to the original sedimentable "fibrous mesh," or that this mesh serves as the point of development of a network of damaged ovomucin-lysozyme complex. The development of this damaged ovomucin-lysozyme network, giving gel-like, or semi-solid, properties to the egg white may be the first step in the coagulation of egg white upon heating.

The results presented in Table 4 indicate that heat damage to the whipping properties of egg white is not a damage to the stability of the meringue formed from egg white so pasteurized. When pasteurized egg white was whipped for a length of time sufficient to produce a "soft peak" meringue, the stability of the meringue, as measured by rate of drain of liquid from the foam, was found to be nearly the same as that of unpasteurized egg white. The drain rate of the meringue made from the 53,000 × G supernatant from unpasteurized egg white is very high, although the specific gravity of this meringue was nearly identical with that of the meringue from the unpasteurized egg white. This observation is in accord with previous suggestions that ovomucin stabilizes a foam, but contributes little to its formation (MacDonnell *et al.*, 1955; Nakamura *et al.*, 1964).

The results of qualitative estimates of the stability to mechanical damage of foams formed from unpasteurized and pasteurized egg whites indicate the physical mechanism of the heat damage to the whipping properties of egg white. The formation of a damaged network of the ovomucin-lysozyme complex produces a "dry" foam, easily damaged by mechanical action. Thus, the increased time required for the whipping of a pasteurized egg white is needed because the foam is being broken down by mechanical action of the beaters during the same beating process in which the foam is being produced. The rate of production of foam is obviously greater than the rate of destruction, since an acceptable foam is eventually obtained. However, the rate of destruction of foam is significantly greater for pasteurized egg white than for unpasteurized, since in some cases the whipping time must be increased to three times normal.

The results presented above suggest the mode of action of whipping aids such as triethylcitrate. Triethylcitrate, added to heat-damaged egg white, does not appear to reverse the heat damage, but appears to alter some physical property of the egg white, perhaps its surficial viscosity, to produce a foam which is less susceptible to mechanical damage.

In addition to interacting with ovomucin, lysozyme must associate with most of the other proteins in egg white, since these are almost all acidic, and lysozyme very basic. Lysozyme associates with conalbumin (Ehrenpreis *et al.*, 1956), ovalbumin (Nichol *et al.*, 1964) and ovomucoid. The association constants of these complexes, as expected, are all markedly decreased with increase in ionic strength. Much of the lysozyme in egg white may be complexed with these other proteins, and little of it left free. These interactions may have significant influence on both the chemical and physical properties of egg white.

The actual conformation of an isolated protein is probably the most thermodynamically stable conformation (White, 1961). The most stable conformation of an association of two or more proteins is probably some other conformation than that of the separated proteins. Thus,

it is not surprising that the ovomucin-lysozyme complex is heat-denatured at a lower temperature than that observed for either protein separately. However, for the chicken, the sacrifice in heat stability in the formation of the ovomucin-lysozyme complex is presumably compensated by other factors, such as the increase in rigidity of the egg white produced by this association.

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Alcohol: NAD Oxidoreductase (E. C. 1.1.1.1.) from Peas

SUMMARY—The substrate specificity of the enzyme alcohol: NAD oxidoreductase from seeds and pods of the pea plant *(Pisum sativum)* was investigated. The enzyme catalyzes the oxidation of primary aliphatic alcohols especially 2-alken-1-ols *e.g.*, trans-2-hexen-1-ol, under the conditions used. It also catalyzes the reduction of aliphatic aldehydes especially ethanal, hexanal and unsaturated nonanals. The reaction product was routinely identified by mass spectrometry.

The enzyme activity was found to be inhibited competitively by fatty acids, methanol, imidazol and L-histidine.

The enzyme was used as a catalyst in experiments for determining equilibrium constants and the calculation of the free energy change of some alcohol-aldehyde systems in the presence of oxidized and reduced coenzyme. On the basis of the equilibrium constants determined, the composition of various alcohol-aldehyde mixtures were calculated for different NAD^+:NADH ratios and different pH values.

The enzyme preparation could not be separated into fractions with altered substrate specificity by ammonium sulfate precipitation or by ion exchange chromatography.

INTRODUCTION

SEVERAL OF THE VOLATILE compounds found in fruits, berries and vegetables have been identified as alcohols, aldehydes and ketones (Ralls *et al.*, 1965; Gould, 1966; Anjou *et al.*, 1967a,b; Murray *et al.*, 1968). Most of this work has been done during the last 10 years in the field of flavor research. The alcohols and their corresponding aldehydes and ketones often occur simultaneously in the material. The chemical reactions that lead to an equilibrium between an alcohol and its corresponding aldehyde or ketone are biocatalyzed by alcoholdehydrogenase (ADH)[a] together with one or both of the nicotinamide-adenine-dinucleotide coenzymes according to the formula:

$$\begin{matrix}\text{Primary alcohol}\\ \text{Secondary alcohol}\end{matrix} + NAD^+(NADP^+) \overset{ADP}{\rightleftharpoons} \begin{matrix}\text{Aldehyde}\\ \text{Ketone}\end{matrix} + NADH(NADPH) + H^+ \quad [1]$$

Knowledge of these reactions and the equilibria obtained with different alcohols, aldehydes and ketones is important for the understanding of the biosynthesis of such volatile compounds.

ADH occurs in tissues of many animals, higher plants and in microorganisms. Our knowledge about this enzyme has been reviewed (Sund *et al.*, 1963). LADH and YADH are the most intensively investigated, and it is noteworthy that the substrate specificity is very low for both. Attempts have been made to separate alcoholdehydrogenase preparations into fractions with different substrate specificity. These attempts have failed but in one case. Steroid activity was found to be associated with a minor subfraction of LADH. This fraction—$LADH_S$—also showed ethanol activity whereas the main subfraction —$LADH_E$—was found to be free from steroid activity (Theorell *et al.*, 1966). These findings led to the conclusion that LADHs contains two different binding sites for the two substrates in the same enzyme molecule.

PADH has also been studied earlier. The coenzyme has been found to be NAD^+ whereas $NADP^+$ was ineffective (Adler *et al.*, 1937). The enzyme activity rises in pea seeds during germination (Virtanen *et al.*, 1944; Goksöyr *et al.*, 1953) and the activity has been followed in various parts and organs of a pea plant during its life cycle (Davison, 1949). The enzyme activity is inhibited by hydroxylamine (Kaplan *et al.*, 1953) and by thiol reagents, metal-binding agents and metals (Suzuki, 1966). There are, however, no observations for PADH on the very interesting inhibitory effect of fatty acids on LADH (Winer *et al.*, 1959, 1960). In a preliminary report a low substrate specificity was shown to be characteristic also for PADH, which catalyzed the oxidation of especially trans-2-hexen-1-ol, at a high rate, compared with hexan-1-ol and ethanol (Eriksson, 1967).

Equilibrium constants have been determined for ethanol-acetaldehyde (Racker, 1950; Bäcklin, 1958), isopropanol-acetone (Burton *et al.*, 1953) and cyclohexanol-cyclohexanone (Merritt *et al.*, 1959).

The solubilities of aliphatic primary alcohols and aldehydes with up to 10 and 7 carbon atoms, respectively, have been determined previously (Table 1). The correlation between the logarithm of the molar solubility and the number of carbon atoms in a homologous series of alcohols or aldehydes is linear. The solubility of longer chain molecules was estimated by extrapolation. The experimental and extrapolated values are given in Table 1, from which it is clear that the solubility of alcohols and aldehydes used in the present investigation were never exceeded.

The purposes of the present investigation were to study the substrate specificity of ADH from pea seeds with special reference to alcohols and aldehydes possibly contributing to the flavor of vegetables, to measure the enzyme activty in the fresh green seeds and pods, to assess the inhibitory effect of some naturally occurring compounds and to determine the equlibrium constants for some alcohol-aldehyde pairs in the presence of oxidized and reduced coenzyme.

Data are also given about the free energy change (ΔF°) and oxidation-reduction potential (E'_o). The ability of

[a] Abbreviations: ADH for alcohol: NAD oxidoreductase; PADH, LADH and YADH for the enzymes from peas, horse liver and yeast, respectively; THAM-HCl for tris-(hydroxymethyl) aminomethane-hydrochloric acid.

Table 1. Solubility (moles/l of water) of alcohols and aldehydes (E = extrapolated value).

	(von Erichsen, 1952) 30°C	(Kinoshita *et al.* 1958) 25°C	(Saracco *et al.* 1958) 20°C
Butan-1-ol	1.79	0.97	1.28
Pentan-1-ol	0.44	0.25	0.31
Hexan-1-ol	0.10	0.059	0.058
Heptan-1-ol	0.025	0.0146	0.010
Octan-1-ol	E 0.0062	0.0038	E 0.002
Nonan-1-ol	E 0.0015	0.00097	E 0.0004
Decan-1-ol	E 0.0004	0.00023	E 0.00008
Butanal	.....	.....	0.60
Pentanal	.....	.....	0.15
Hexanal	.....	.....	0.04
Heptanal	.....	.....	0.01
Octanal	.....	.....	E 0.003
Nonanal	.....	.....	E 0.0007
Decanal	.....	.....	E 0.0002

LADH and YADH to catalyze the oxidation of unsaturated alcohols is compared with that of PADH. In connection herewith, the relative reaction rates for different alcohols are discussed on the bases of some simple experiments on such rates obtained at different substrate concentrations, pH values and temperatures.

An attempt was also made to separate the enzyme preparation into fractions by ammonium sulfate precipitation and ion exchange chromatography in order to find out whether it contained several enzymes with different substrate specificity.

EXPERIMENTAL

THE ENZYME was extracted from fresh green peas and pods as well as from ripe dry peas. The latter was the main enzyme source. The green peas in their pods were collected by hand in the field at the normal harvesting time for commercial deep freezing. The material was chilled to about 5°C and stored for about 10 hr. Just before analysis, the fresh green peas were shelled by hand. Forty g each of green peas and pods were immediately frozen in solid CO_2 in a Waring blender and ground to powder. While still cold, the powders were washed on a Büchner funnel with cold acetone and diethyl ether and then dried in a desiccator under vacuum.

The enzyme was extracted with 50 ml of 0.1 *M* sodium-potassium phosphate buffer, pH 7.5, at 0°C. The buffer contained 10^{-3}M reduced glutathione and 2% polyvinyl-pyrrolidone (PVP, MW = 10,000). After centrifugation at 14,500 × G for 30 min, the supernatant was assayed for ADH activity. In a parallel experiment an attempt was made to extract the enzyme directly into buffer without acetone-ether treatment, followed by ammonium sulphate precipitation. This procedure, however, yielded preparations with much lower activity or with no activity at all.

One hundred g of dry peas were treated in the way described for fresh green peas. The defatted and decolorized powder was placed overnight in a desiccator under vacuum. The dry powder was then sieved to remove large fragments. This procedure yielded 88 g of pea powder, which was used as starting material for several enzyme preparations. One such preparation was made by extracting 12 g of the powder for 1 hr with 100 ml of the above mentioned glutathione-PVP buffer at 0°C. The slurry obtained was filtered through Pyrex wool and then centrifuged at 200 × G for 5 min at 0°C. The supernatant was dialyzed four times at 3°C against 500 ml of 0.1*M* sodium-potassium phosphate buffer, pH 7.5, containing 10^{-4}M reduced glutathione.

The bag content was centrifuged at 14,000 × G for 20 min at 0°C. The supernatant solution was fractionated by precipitation with solid ammonium sulphate at 35, 45, 55 and 60% saturation at 0°C. The precipitation was allowed to continue for 30 min at each step in an ice bath. After centrifugation at 14,000 × G for 15 min at 0°C the precipitates were dissolved in 0.1 *M* sodium-potassium phosphate buffer, pH 7.2, containing 10^{-3} *M* reduced glutathione. The solutions were assayed for ADH activity and then divided into aliquots, each containing 0.5 ml, which were frozen separately. The enzyme maintained 90% of its original activity for at least four months when stored at −20°C. A 35% saturation precipitate was used for column chromatography, but in most experiments the precipitate obtained by 60% saturation was used.

Commercial YADH and LADH were purchased from C. F. Boehringer & Soehne GmbH, Mannheim, Germany, as crystal suspensions (analytical reagent grade), YADH 30 mg in 1 ml of 2.4 *M* ammonium sulphate solution, and LADH 10 mg in 1 ml of 0.02 *M* phosphate buffer, pH 7, containing 10% ethanol. Before use, 150 μl of the liver enzyme was dialyzed against 0.02 *M* phosphate buffer, pH 7.0, to remove the ethanol.

Alcohols and aldehydes were purchased from several suppliers and the purity of each batch was checked by gas chromatography on the analytical columns listed in Table 2. When necessary, they were purified by preparative gas chromatography and again analyzed by gas chromatography, by mass spectrometry and, in the cases of cis-trans-isomerism, also by infrared spectrophotometry. The conditions under which analytical and preparative gas chromatography were performed are also given in Table 2.

The enzyme assay method used was the normal spectrophotometric procedure based upon the increase in absorbancy at 340 nm due to the formation of NADH (Eq. 1). As a rule the temperature was kept constant at 25.0 ± 0.1°C. The reaction mixture contained 2.8 ml of 0.01 *M* sodium pyrophosphate buffer, pH 8.5, 0.1 ml of 0.015 *M* NAD^+, and 0.010 ml enzyme solution. Before addition of 0.1 ml water solution of alcohol, the reaction rate was checked at zero. After this addition the initial rate of the reduction of NAD^+ was taken as a measure of the enzyme activity.

In control runs alcohol, enzyme or NAD^+ were replaced by a corresponding volume of buffer. The assays for control of the enzyme activity in new preparations, precipitates and chromatography fractions were performed with 0.1 ml of 3 *M* ethanol. Deviations from the assay scheme are noted in the tables and legends. When inhibitors were used, 0.1 ml of the buffer was replaced by the same volume of inhibitor solution. The assay with aldehydes instead of alcohols was made in 2.6 ml of 0.1 *M* phosphate buffer

Table 2. Conditions used in analytical and preparative gas chromatography for purification and analysis of alcohols and aldehydes used for reaction rate and equilibrium measurements.

Type	Length, feet	Diameter, in.	Stationary phase	Supporting material	Carrier gas	Detector	Temperature °C
Analytical	50	0.02	Carbowax 20 M	SCOT-Column	N_2	Flame	110
Analytical	9	⅛	PG 1000 18% Tween 80 0.5%	Chromosorb WAW DMCS 80–100	N_2	Flame	80
Analytical	9	⅛	FFAP 5%	Chromosorb WAW DMCS 80–100	He	Ion current. Masspectrometer LKB 9000 70 eV.	90–150/ 4°/min
Preparative	10	⅜	Carbowax 20 M 25%	Chromosorb WAW 60–80	He	Thermal conductor	isothermal 100–120

pH 6.9, 0.1 ml of 0.003 *M* NADH, 0.005 or 0.025 ml of enzyme solution. After checking that no degradation of NADH had occurred, 0.3 ml of 0.001 *M* aldehyde solution was added. When necessary, alcohols, aldehydes and inhibitors of low solubility were dissolved in, and added with, the buffer, and the reactions were started by addition of the enzyme. All measurements were made at least three times.

The reaction rates of alcohols and aldehydes were calculated relative to ethanol and n-hexanal, respectively. The reaction rates of these two compounds, when used in the same concentrations as the other alcohols and aldehydes, were considered as unity. The equilibrium constants were determined in a medium of low ionic strength following procedures outlined by Burton *et al.* (1953).

To check that the correct reactions had occurred, the reaction product—aldehyde or alcohol—was extracted with redistilled diethyl ether and identified with the aid of a combined gas chromatograph–mass spectrometer (LKB 9000). The conditions under which this analysis was performed are given in Table 2.

Column chromatography was done on a DEAE-cellulose weak anion exchanger in a standard laboratory column 2.5 cm in diameter and 45 cm long equipped with a cooling jacket (Sephadex Column K 25, Pharmacia Fine Chemicals, Uppsala, Sweden). The ion exchanger was packed to a height of 35 cm and was pretreated with NaOH and a strong chloride solution (Semenza, 1960). In this case, 0.1 *M* pyridinium chloride solution, pH 4.4, containing 20% NaCl was used. After such treatment the ion exchanger was equilibrated with 0.01 *M* THAM-HCl, pH 7.2, containing 10^{-4} M glutathione.

An enzyme solution obtained from the 35% ammonium sulfate saturation step in the way described above was used after dialysis against the above THAM-glutathione buffer and centrifugation. After assay, 13 ml of this solution was placed on the top of the column. The elution was performed with a linear gradient to 0.6 *M* KCl in the above THAM-HCl buffer. The flow rate, 1 ml per min, was kept constant by means of a peristaltic pump. The content of UV-absorbing substance in the eluate was recorded continuously in a UVICORD (LKB-Produkter AB, Sweden) at 254 nm.

Fractions, collected every 5 min, were assayed for ADH activity, and the absorption at 280 nm was measured. Both the column and the fractions were kept at 0°C during the run.

RESULTS AND DISCUSSION

Substrate specificity

Table 3 shows a number of primary alcohols used in the specificity test of PADH-NAD^+. Methanol was not oxidized but acted as a weak competitive inhibitor in ethanol

Table 3. Relative reaction rates obtained with alcohol:NAD oxidoreductase from pea seeds and alcohols at two different concentrations [NAD^+] = 5.10^{-4} M, pH = 8.5. Specific activity of enzyme (ethanol oxidation) = 30 μmoles min^{-1} g dry weight. [Ethanol] = 0.001 *M*.

Alcohol	Alcohol concentration 0.01 *M*	0.001 *M*	Saturated[1]
Methanol	0.00	0.00	
Ethanol	1.00	1.00	
Propan-1-ol	0.18		
2-propen-1-ol (allyl alcohol)	1.41		
2-propyn-1-ol (propargyl alcohol)	0.00		
Butna-1-ol	0.27	0.22	
Trans-2-buten-1-ol	0.55	0.49	
Cis-2-buten-1-ol	0.20		
2-methyl-2-propen-1-ol	0.02		
Pentan-1-ol	0.06		
3-methyl-butan-1-ol	0.01		
3-methyl-2-buten-1-ol	0.46		
Hexan-1-ol		0.12	
4-methyl-pentan-1-ol		0.00	
Trans-2-hexen-1-ol		2.93	
Trans-3-hexen-1-ol		low	
Cis-3-hexen-1-ol		0.15	
5-hexen-1-ol		low	
Trans, trans-2,4-hexadien-1-ol		2.43	
Heptan-1-ol			0.33
Octan-1-ol			0.21
Nonan-1-ol			0.18
3-phenyl-2-propen-1-ol (cinnamic alcohol)		0.22	

[1] Compared with the rate of ethanol at 0.001 *M*.

and trans-2-hexen-1-ol oxidation (Fig. 1a). 2-alkenols, such as 2-propen-1-ol (allyl alcohol), trans-2-hexen-1-ol and trans-2-buten-1-ol, were oxidized more rapidly—the former more so than the latter—than their saturated analogues propan-1-ol, hexan-1-ol and butan-1-ol. However, 2-propyn-1-ol (propargyl alcohol) with a triple bond in the same position did not react. Double bonds further from the functional group, as shown in the C-6 series (Table 3), again lowered the reaction rate. (See e.g., trans- and cis-3-hexen-1-ol, 5-hexen-1-ol and trans,trans-2,4-hexadien-1-ol where, in the last mentioned case, the C-4 double bond slightly counteracted the effect of the C-2 double bond.)

Methyl branches substantially decreased the reaction rate,which can be seen from the vaues for 3-methyl-butan-1-ol, 4-methyl-pentan-1-ol and 2-methyl-1-propen-1-ol. In the last case, the rate increasing effect of the double bond was completely neutralized by the methyl group between the hydroxyl end and the double bond. It was interesting to observe that, by contrast, in the case of 3-methyl-butan-1-ol and 3-methyl-2-buten-1-ol the double bond effect neutralized the effect of the methyl branch. The configuration around C-2 is evidently important for the formation of ADH-NAD^+-alcohol complex.

With the exception of 2-propen-1-ol the oxidation of unsaturated alcohols has not been investigated before. However, the effect of the double bond and the methyl branches in the alcohols reported here might be compared with the fact that fatty acid amides competitively inhibit aldehyde reduction by LADH by the formation of ternary enzyme-coenzyme-inhibitor complexes (Winer *et al.*, 1959; 1960). The inhibition by propionamide (K_i = 0.439 *m*M) was lowered by the double bond in acrylamide (K_i = 5.32 mM) (Woronick, 1961). Woronick also showed that methyl substitution on C-2 with formation of 2-methyl-acrylamide almost neutralized the effect of the double bond (K_i = 0.814 mM).

Other types of alcohols, such as secondary alcohols, diols, terpene alcohols, cyclic and aromatic alcohols, with the exception of cinnamic alcohol, were oxidized very slowly or not at all with this pea enzyme preparation. The side chain of cinnamic alcohol is allyl alcohol, where again, the double bond increased the reaction rate (Table 3).

Though not quite comparable, the results obtained in the forward reaction were not mirrored in the reverse (Eq. 1). This is most striking when comparing the rates of hexanal and trans-2-hexenal in Table 4. On the whole, the picture obtained with aldehydes was more complex. The interesting thing for the flavor chemist is, however, in the first line: Aldehydes of these kinds were attacked by the enzyme with consequently rather high reaction rates.

The identification of the reaction product, the aldehyde or the alcohol, proved that the correct reactions were followed spectrophotometrically. For identification, most of the spectra obtained were compared with existing reference spectra, but, in a few cases, previously unpublished spectra were also obtained. They are trans-3-hexenal, trans-2-hepten-1-ol, trans-2-nonen-1-ol, trans-6-nonen-1-ol, trans-2-decen-1-ol, trans,trans-2,4-heptadien-1-ol, trans,trans-2,4-decadien-1-ol and trans,cis-2,4-decadien-1-ol. (Complete spectra can be obtained from the author.)

Table 4. Relative rates obtained with alcohol:NAD oxidoreductase from pea seeds and aldehydes at two different enzyme concentrations [Aldehyde] = 10^{-4} *M*, pH = 6.9. Specific activity as in Table 3.

Aldehyde	Enzyme addition 0.005 ml	0.025 ml
Ethanal	6.52	
Propanal	0.20	0.15
Butanal	0.52	0.24
Hexanal	1.00	1.00
Trans-2-hexenal		0.16
Heptanal		0.30
Trans-2-heptenal		0.19
Trans,trans-2,4-heptadienal		0.18
Octanal		0.41
Trans-2-octenal		0.19
Nonanal		0.18
Trans-2-nonenal		0.27
Trans-6-nonenal		1.01
Decanal		low
Trans-2-decenal		0.32
Trans,trans-2-4-decadienal		0.25
Trans,cis-2,4-decadienal		0.49

ADH activity in unripe green peas and pods

The substrate specificity of pea ADH was studied with preparations from ripe dry peas because of the higher yield and better stability of the enzyme obtained. The maximum specific activity of the dry peas is in the order of 25 μmoles of ethanol oxidized per min and g of dry peas. This activity will, as mentioned in the introduction, rise to a maximum after a few days of germination. Both the pea seed and the pod will synthesize ADH during ripening (Davison, 1949), and the specific activity of the unripe material might be a function of the degree of ripeness. Several enzyme preparations from the unripe peas and pods were used to obtain the data in Table 5. They all differed in specific activities. On average, the activities of ripe peas, unripe peas and unripe pods had the ratio 100:10:2 in these measurements.

The interesting thing however, was the possibility of confirming the ADH-activity previously found in unripe peas and pods and to find out whether the substrate specificity was similar to that of the enzyme from ripe seeds. Such experiments were made with selected saturated and unsaturated alcohols, and the activity of enzymes obtained from the fresh material was compared with the activity of ADH from ripe dry peas, yeast and liver. The two last mentioned enzymes were included to ascertain whether the effect of ADH on unsaturated alcohols was something specific for the pea enzyme.

Roughly speaking, the pea enzymes behaved similarly, i.e., they catalyzed the oxidation of unsaturated alcohols, which were oxidized at higher rates than the saturated analogues (Table 5). There were deviations, e.g., the enzyme from unripe pods seemed to be less active than the others against trans-2-buten-1-ol and trans-2-hexen-1-ol compared with butan-1-ol and hexan-1-ol. The effect

Table 5. The activity of ADH in different pea preparations, and of YADH and LADH on various saturated and unsaturated alcohols. [Alcohol] = 10^{-3} M. Specific activity (ripe seeds) as in Table 3.

Alcohol	Ripe seeds	PADH unripe seeds	Unripe pods	YADH	LADH
Ethanol	1.00	1.00	1.00	1.00	1.00
Propan-1-ol	0.18	—	—	0.14	1.25
2-propen-1-ol	1.41	2.29	1.41	1.81	1.64
Butan-1-ol	0.22	0.36	0.54	0.03	1.08
Trans-2-buten-1-ol	0.49	0.64	0.62	0.86	1.71
Hexan-1-ol	0.12	0.22	0.06	0.03	0.79
Trans-2-hexen-1-ol	2.93	3.49	1.78	1.02	1.31
Trans,trans-2,4-hexadien-1-ol	2.43	2.31	1.24	—	—

of YADH and LADH on the unsaturated alcohols was similar to though less strong than that of pea enzymes.

The values obtained for saturated alcohols with the liver and yeast enzymes deviated somewhat from earlier results (Sund *et al.*, 1963), probably because the alcohol concentrations and pH used in the present investigation were not identical with those used previously since they were selected for comparison with the pea enzyme. Both in this and previous papers the reaction rates of an alcohol or an aldehyde relative to those of ethanol or hexanal seem to be reproducible only under identical experimental conditions. In other words, the reactions of various alcohols or aldehydes may not be equally influenced by changes in pH, temperature, substrate concentration and substrate inhibition. Ethanol is known to inhibit at concentrations about 10mM and above. If the other alcohols inhibit less or not at all the relative reaction rates for these alcohols will increase with the substrate concentrations. The results presented in Tables 6 and 7 support these assumptions as to why relative reaction rates must not be looked at uncritically.

Inhibition of pea ADH

Imidazol and fatty acids are known to be competitive inhibitors in connection with LADH imidazol (Theorell *et al.*, 1961) only when its concentration is high compared with those of alcohol and NAD^+. In low relative concentrations it produced the opposite effect, i.e., it increased the reaction rate. It was thought worthwhile to see whether compounds of these types, some of which occur in peas, also compete with the binding of alcohols to PADH. Methanol was included in this investigation because, though it is the most common alcohol in peas except for ethanol, it was not oxidized by PADH.

Table 6. Relative reaction rates with alcohol : NAD oxidoreductase from yeast and three alcohols at different concentrations and two pH values. Standard assay. Specific activity (ethanol oxidation) = 180 μmcl min^{-1} mg $protein^{-1}$.

pH	8.2			9.2		
Alcohol concentration, *M*	0.001	0.01	0.1	0.001	0.01	0.1
Ethanol	1.00	1.00	1.00	1.00	1.00	1.00
Propan-1-ol	0.13	0.20	0.32	0.14	0.26	0.46
Butan-1-ol	0.04	0.05	0.14	0.04	0.06	0.20

Table 7. Relative reaction rates obtained with alcohol : NAD oxidoreductase from horse liver and three alcohols at different temperatures. Standard assay. Specific activity (ethanol oxidation) = 2.7 μmol min^{-1} mg $protein^{-1}$.

Temperature °C	20	25	25	30	35
Alcohol concentration, *M*	0.01	0.01	0.001	0.01	0.01
Ethanol	1.00	1.00	1.00	1.00	1.00
Propan-1-ol	0.79	0.77	1.05	0.72	0.97
Butan-1-ol	0.44	0.72	1.11	0.50	0.54

In Fig. 1, double reciprocal plots are presented for the competitive inhibition of ethanol oxidation by methanol (1a) L-histidine and imidazol (1b), octanoic acid and decanoic acid (1c), and for the inhibition of trans-2-hexen-1-ol by methanol and decanoic acid (1d). The Michaelis (K_m) and inhibitor (K_i) constants were calculated and are shown in the same figure.

Methanol and especially L-histidine were found to be very weak inhibitors. The inhibition with octanoic and decanoic acid—the latter a stronger inhibitor than the former—indicated that the regulatory effect of carboxylic acids and acid amides proposed for LADH (Theorell, 1959) might be valid also for PADH. It also confirmed a lipophilic character of the binding site of PADH, something that is supported by the fact that decanoic acid competes less with the more lipophilic trans-2-hexen-1-ol than with the less lipophilic ethanol (Figs. 1c, d).

Equilibrium constants

The equilibrium constant K was calculated as

$$K = \frac{[\text{Aldehyde}] \cdot [\text{NADH}] \cdot [H^+]}{[\text{Alcohol}] \cdot [NAD^+]} \qquad [2]$$

The equilibria were approached from both sides in each determination when both the aldehydes and the alcohols were available sufficiently pure, in the case of cis-3-hexen-1-ol, equilibrium was approached from only one side because it was impossible to purify the corresponding aldehyde enough. The constants obtained for some alcohol and aldehyde pairs are listed in Table 8 together with the calculated change in free energy (ΔF°) and the oxidation-reduction potential (E'_o). The equilibrium constant increased with the chain length of the alcohols and aldehydes involved. The value especially noticed is, however, that of the trans-2-hexen-1-ol :trans-2-hexenal system for which

a drastic rise in the equilibrium constant was observed. This result could be predicted from the forward and reverse reaction rates obtained with this alcohol and aldehyde, compared with those of hexan-1-ol and hexanal (Tables 3 and 4).

By rearrangement of Equation 2, the ratio between an alcohol and its corresponding aldehyde at pH 7.0 could be estimated as a function of the NAD^+:NADH ratio, when K had been determined. This was done in Fig. 2 where the calculated alcohol:aldehyde ratio is plotted against the logarithm of assumed NAD^+:NADH ratios. A lowering of pH one unit will move all curves one log unit to the right.

The actual ratio between the NAD^+ and NADH concentrations in plant tissues depends on several factors, including the physiological state. Developing tissues should have a high ratio because the energy-requiring anabolic processes

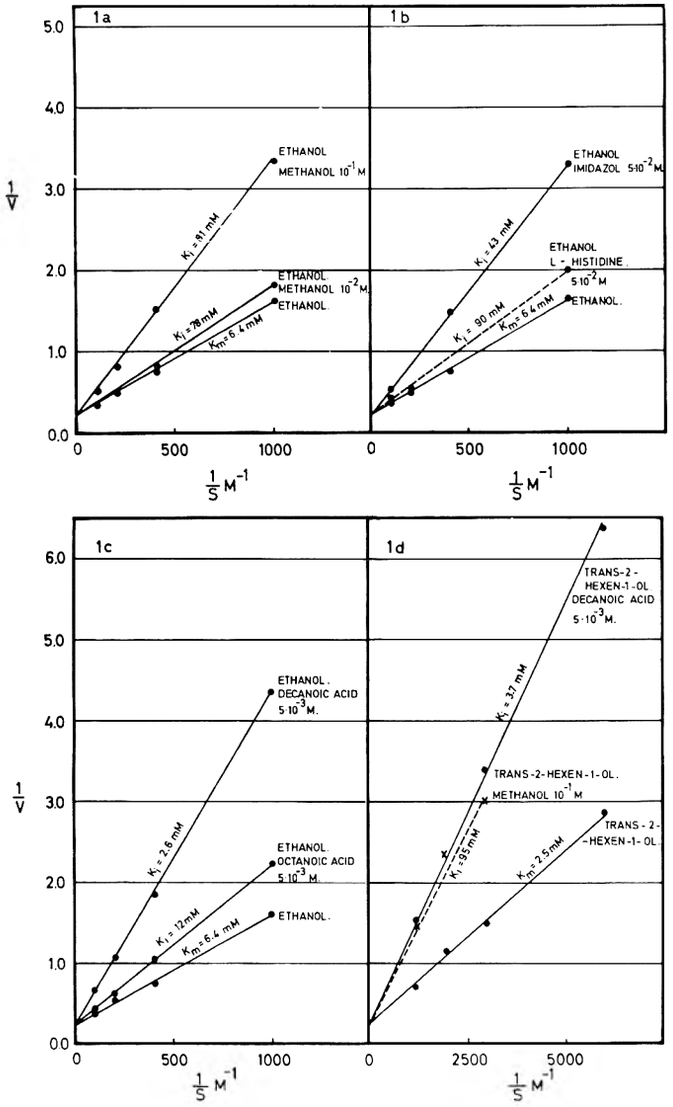
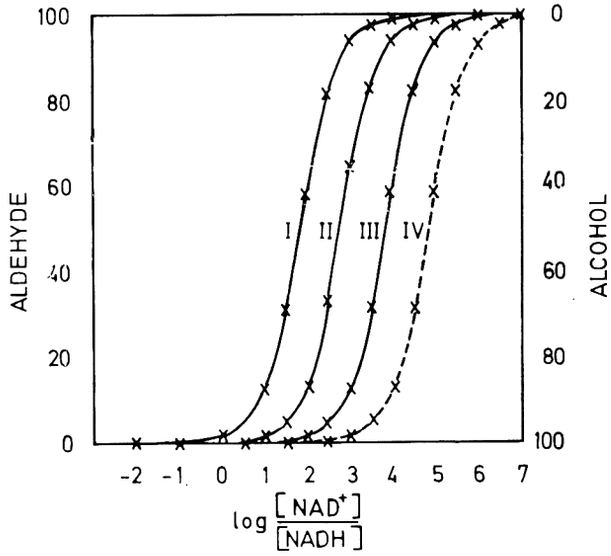


Fig. 1. Double reciprocal plots showing the effect on the reaction rate of the concentration of ethanol (a-c) and trans-2-hexen-1-ol (d) alone and in the presence of different inhibitors. Temperature 25°C. Specific activity (ethanol oxidation) = 34 μmoles min⁻¹ g dry weight⁻¹.



I TRANS-2-HEXEN-1-OL: TRANS-2-HEXENAL p'H 7.0

II NONAN-1-OL: NONANAL pH 7.0

III HEXAN-1-OL: HEXANAL pH 7.0

IV HEXAN-1-OL: HEXANAL pH 6.0

Fig. 2. Diagram showing the influence of the NAD⁺:NADH ratio on the ratio of different alcohols and aldehydes in equilibrium mixtures at pH 7, temperature 25°C. Lowering of the pH results in a shift of the curves to the right one log unit per pH unit; a rise in pH results in a corresponding shift to the left.

Table 8. Equilibrium constants (K) and change in free energy ($\Delta F°$) obtained with different alcohol-aldehyde:NAD^+-NADH systems at pH 8.2–8.4 in the presence of alcohol:NAD oxidoreductase from pea seeds. K is defined as in equation 2. Oxidation-reduction potentials (E'_o) were calculated for the reaction: alcohol $\rightleftharpoons$ aldehyde + H_2. T = 25.0 ± 0.1°C $\Delta F°$ for $NAD^+ + H_2 \rightleftharpoons$ NADH + H^+ determined to 5.49 kcal mole⁻¹ (Bäcklin, 1958; 5.47 kcal mole⁻¹).

Alcohol-aldehyde	Number of determinations	K. 10^{12}	Standard deviation 10^{12}	$\Delta F°$ kJ. mole⁻¹	$\Delta F°$ Kcal. mole⁻¹	E'_o volts
Butan-1-ol-butanal	10	9.1	0.6	63.01	15.06	−0.208
Hexan-1-ol-hexanal	13	14.4	1.5	61.88	14.79	−0.202
Trans-2-hexen-1-ol-trans-2-hexanal	9	1420	53	50.50	12.07	−0.143
Cis-3-hexen-1-ol-cis-3-hexenal	11	3.6	0.22	65.31	15.61	−0.220
Octan-1-ol-octanal	6	53	3.5	58.64	14.02	−0.185
Nonan-1-ol-nonanal	13	153	30	56.01	13.39	−0.171

will keep the concentration of NADH low by reoxidizing it to NAD^+ in the respiratory chain. If there is any interplay between the alcohol-aldehyde systems and the anabolic reactions, the aldehyde content might be higher in growing or very freshly harvested green peas.

On the other hand, when the peas are stored after harvest and are subjected to various treatments, the net reaction pattern of the tissues will change to catabolism and fermentation. Under these conditions the NAD^+:NADH ratio should decrease and also pH. Both changes will favor alcohol formation, at the same time as precursors of alcohol:aldehyde systems e.g., unsaturated fatty acids are released, with the result that the alcohol:aldehyde concentration level will rise, a well known observation.

Both alcohols and aldehydes will, however, certainly take part in other equilibrium reactions whose characteristics must be known before any definite statements can be made about the alcohol:aldehyde situation in green peas.

Fractionation

Fractionation of the pea seed preparation by precipitation with ammonium sulphate did not indicate the presence of different kinds of PADH. For the precipitates and supernatant solutions the relative rates obtained with ethanol, trans-2-buten-1-ol and trans-2-hexen-1-ol were constant. The recovery of enzyme activity was 80, 99, 99.8 and 100% in the precipitates obtained at 35, 45, 55 and 60 percent saturation, respectively. The remaining 20 and 1% were measured in the supernatant solutions of the first two steps.

The further fractionation of the 35% saturation precipitate on a cellulose ion exchanger, as shown in Fig. 3, gave the same result when all tubes containing PADH were assayed with the alcohols mentioned. The figure represents one of four almost identical chromatograms. The recoveries ranged between 90 and 75% of the total activity put on the column. The activity dropped rapidly to about 50% in 24 hr, while the activity of the alcohols mentioned had to be monitored during the run. The specificity was unchanged even after a heavy drop in activity.

These results do not exclude the presence of several enzymes of the ADH type with the substrates used, but they do indicate that, in the event of such enzymes, they are not easily separated. Conclusions about the general effect on alcohols can therefore often be drawn from measurements of only one or a few.

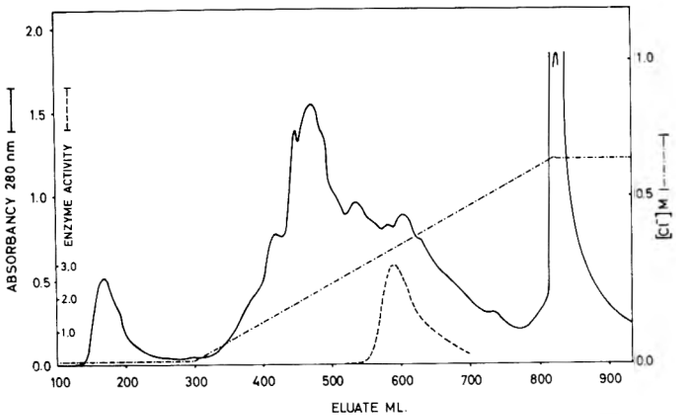


Fig. 3. Ion exchange chromatography of a pea extract containing alcohol: NAD oxidoreductase. Temperature 0°C. Recovery about 90%.

CONCLUSIONS

1. Alcohol:NAD oxidoreductase from peas has a low substrate specificity. The ratio between the reaction rates of different substrates are valid only for one set of experimental conditions; especially the substrate concentration is important. Under the conditions used in this investigation 2-alken-1-ols, especially trans-2-hexen-1-ol, were oxidized at the highest rates. Of the aliphatic aldehydes, ethanal, hexanal and unsaturated nonahals were reduced at the highest rates.

2. The same unspecific enzyme occurs in fresh green peas harvested for deep-freezing, in the pods of such peas and in ripe dry peas.

3. Commercial ADH from yeast and horse liver also give higher reaction rates with 2-alken-1-ols than for the saturated analogues but less pronounced than the pea enzyme.

4. The action of ADH from peas on ethanol is competitively inhibited by octanoic and still more by decanoic acid. In this respect the pea enzyme resembles the liver enzyme. The inhibitory action of the fatty acids also occurs when the substrate is trans-2-hexen-1-ol. A weak inhibitory action is exerted by L-histidine, the naturally occurring imidazol compound, and by methanol.

5. The equilibrium constants obtained at pH 7 are low and in the order of 10^{-9} to 10^{-11}. This means that at neutral pH and when the ratio between NAD^+ and NADH is below 10, in most aliphatic alcohol-aldehyde systems the alcohol partner is predominant. The systems rapidly respond to changes in the NAD^+:NADH ratio as well as in pH. Such changes can occur during the working up of plant material for flavor analysis as well as during heat treatment and storage of peas, if ADH is still active. In most cases the changes will favor alcohol formation, a fact which should be taken into consideration in flavor analysis.

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Gamma Irradiation and Enriched CO_2 Atmosphere Storage Effects on the Light-induced Greening of Potatoes

SUMMARY—Michigan-grown Kennebec and Sebago potato tubers were exposed to gamma irradiation in the dose range of 0 to 400 Krad and subsequently stored in atmospheres containing 0.03% to 15% CO_2. Upon illumination with 3,000 lux for periods up to 20 days while maintaining the atmosphere, all tubers developed some greening. The tubers exposed to 200 and 400 Krad were inhibited from greening to the highest degree but suffered general quality loss. The levels of 10 to 20 Krad, which are suitable for the sprout inhibition of potatoes, did not cause significant inhibition of greening under any of the conditions of this experiment. Storage in enriched CO_2 atmospheres inhibited the greening to the extent of 50% at 15% CO_2 only upon prolonged illumination (12 days), while irradiation (40 Krad) was only effective after a short period (4 days) of illumination. Irradiation in the 0–40 Krad range did not increase the inhibition caused by CO_2. The inhibition of potato greening by irradiation was effective through a period of 5 weeks storage in the dark prior to illumination.

INTRODUCTION

POTATO TUBERS exposed to light over a period of several days turn green due to the formation of chlorophyll (Larsen, 1949). Susceptibility to greening appears to be a varietal characteristic, but no commercial variety is immune to it (Akeley *et al.*, 1962). Kroener *et al.* (1942) conclude that greening significantly reduces the marketability of potatoes, especially because the green color of potatoes is associated with bitter taste and the presence of a toxic alkaloid, solanine. Potato chips made from green potatoes also show discoloration.

Gull *et al.* (1958) studied the greening of several varieties of potatoes exposed to fluorescent light in retail stores and found that light intensities of 50 to 100 foot-candles exert the same strong greening effect, but 25 foot-candles result in significantly less greening.

Hardenberg (1954) finds that packing potatoes in transparent plastic bags or nets to satisfy the consumer's desire to see the product has aggravated the greening problem. A dark-colored polyethylene bag has been recommended for preventing potato greening by Newman (1966).

Schwimmer *et al.* (1958) found that gamma irradiation only slightly reduced the light-induced greening of Russet-Burbank potatoes grown on the west coast.

Forsyth *et al.* (1968) reported that exposure of potatoes to light in atmospheres containing 15% CO_2 or more resulted in very little or no greening.

The objective of the present work was to study the effect of gamma irradiation and storage in CO_2-enriched atmospheres, separately and in combination, on the light-induced greening of Michigan-grown potatoes. It would be desirable if through a combination of effects, doses effective in preventing the sprouting of potatoes, as reported by Desrosier *et al.* (1960), also inhibited the greening.

MATERIALS AND METHODS

Potatoes

Michigan-grown potatoes of the varieties Kennebec and Sebago were used. The tubers were selected for uniformity in size and freedom from defects. Most of the results were obtained with potatoes which had been stored in the dark at 5–7°C for 5 or more months.

Irradiation

The 50,000 curie ^{60}Co facility of the Food Science Department was used for gamma irradiation. The potatoes were arranged in isodose circles around the radiation source, and when half of the dose had been received, they were turned 180° for uniform dose distribution over the entire tuber. Doses of 0 to 400 Krad were applied. The dosimetry was performed by the Fricke method, reported by Am. Soc. for Testing Materials (1959).

Illumination

After irradiation with the ^{60}Co source, potatoes were exposed to continuous light of 3,000 lux for 4 to 20 days in a temperature-controlled chamber at 23 ± 1°C. The light source was a combined bank of 12 fluorescent tubes (Sylvania F48T12-CW-VHO) and 3 incandescent lamps.

CO_2 Treatment

For controlled atmosphere storage the potatoes were placed in stainless steel trays (45 × 30 × 6 cm), covered with a plastic film nearly impermeable to CO_2, and a slow continuous stream of gas mixture was maintained flowing through the trays. Normal air (0.03%), 0.5%, 5% and 15% CO_2 in air (v/v) (Mathieson gases) were applied. The gases were saturated with moisture, by passing them through wash bottles, to avoid a drying effect of the gas stream.

Chlorophyll extraction and determination

Ten discs of 9.2 mm diameter and 3 mm thickness each were cut at random from the light exposed surface of each tuber with a corkborer, avoiding the areas of the eyes. These discs were ground together in a small mortar with quartz sand and 10 mg of $MgCO_3$. After complete disinte-

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gration, one g anhydrous Na_2SO_4 was added, and grinding continued until the sample was a dry powder. The powder was mixed with cold acetone and the suspension filtered through a fritted funnel under reduced pressure into a 10 ml volumetric flask.

The extraction was repeated with a small amount of acetone. To the combined extracts, 2 ml of distilled water were added and made to volume with acetone. Chlorophyll was determined according to Ziegler *et al.* (1965) in a Beckman DU spectrophotometer. In some cases, centrifugation of the extract was necessary for clarification. The whole procedure was performed at room temperature and dim light. The results were expressed as averages of three determinations performed on three potatoes; the differences among triplicates did not exceed 10%.

RESULTS AND DISCUSSION

THE EFFECT of gamma radiation on the inhibition of chlorophyll formation in Kennebec and Sebago potatoes stored for 5 months after harvest and exposed to 3,000 lux of continuous illumination, post-irradiation, for 12 days is illustrated in Fig. 1. The data indicate: (a) Increasing doses of irradiation in the range of 0 to 400 Krad resulted in increasing inhibition of greening. (b) While the nonirradiated potatoes reached their maximum chlorophyll content in approximately one week of continuous illumination, the irradiated potatoes generally continued forming chlorophyll throughout the 12-day period of illumination. At 20 days (not shown in the graph) there was no significant difference in chlorophyll content between the tubers irradiated with 50 and 100 Krad and the nonirradiated control. The samples which had received 200 and 400 Krad did not completely recover their chlorophyll synthesizing system during the entire observation period; these levels, however, destroy the general quality of the potatoes. (c) Chlorophyll formation appeared inhibited by irradiation slightly more in the Kennebec than in the Sebago variety.

In an attempt to find conditions of irradiation under which considerable inhibition of greening might be accomplished with doses not much higher than those applied for the prevention of sprouting of potatoes, (10–15 Krad), a combination treatment of irradiation and enriched CO_2 atmosphere storage was explored. Sebago tubers 11 months old were exposed to 0, 10, 20 and 40 Krad of gamma irradiation and subsequently stored in normal air (0.03% CO_2), and air containing 0.5%, 5% and 15% CO_2 under 3,000 lux of illumination. In Fig. 2, the increase of chlorophyll is shown for the four most widely differing treat-

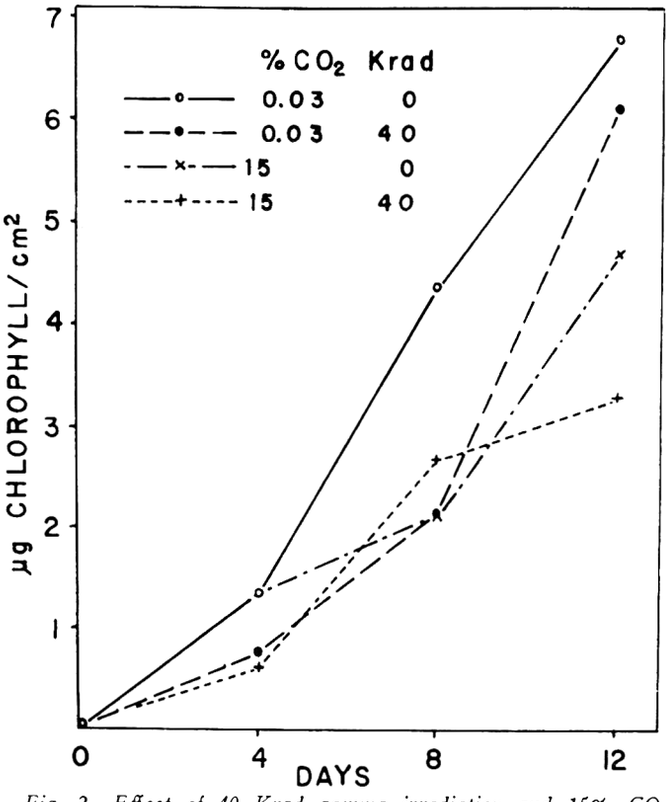

Fig. 2. Effect of 40 Krad gamma irradiation and 15% CO_2 atmosphere post-irradiation storage on the chlorophyll formation of Sebago potatoes during 12 days of illumination with 3,000 lux.

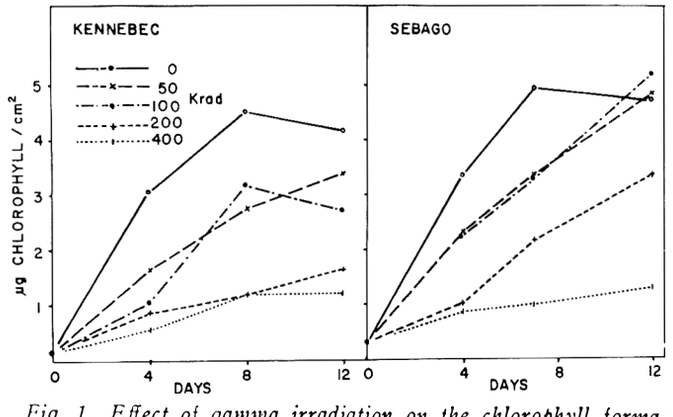

Fig. 1. Effect of gamma irradiation on the chlorophyll formation in Kennebec and Sebago potatoes during 12 days of illumination with 3,000 lux.

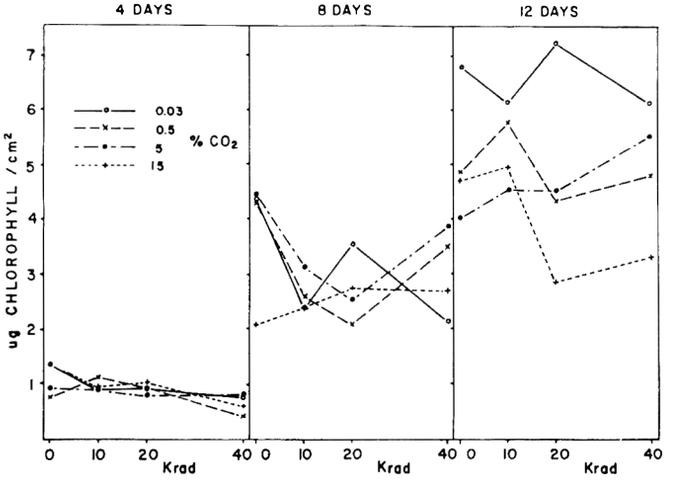

Fig. 3. Effect of four doses of gamma irradiation (0, 10,20 and 40 Krad) and four levels of CO_2 concentration (0.03, 0.5, 5 and 15%) in the air of post-irradiation storage on the chlorophyll formation of Kennebec potatoes exposed to 3,000 lux of illumination for 4, 8 and 12 days.

ments. At the end of 4 days of illumination there was an inhibiting effect on greening found in the irradiated samples; whereas at 12 days the CO_2 enriched atmosphere was more effective.

In Fig. 3, the results of all treatments are shown. Two trends are apparent: one is the decrease of greening with irradiation, irrespective of CO_2 treatment, at 4 days of illumination; and the other is the decrease of greening with increasing CO_2 in the atmosphere, irrespective of irradiation, at 12 days of illumination. Statistically different at the level $P = 0.05$ were only the average chlorophyll content of unirradiated tubers stored at all four different atmospheres and the same value (avg. of the values at all four different atmospheres) irradiated at 40 Krad. On the other hand, the average chlorophyll content of all levels of irradiation in normal air and the same for 15% CO_2 atmosphere were significantly different only after 12 days.

In the last experiment the tubers were exposed to 50 Krad of gamma irradiation and subsequently stored in darkness for 1, 2, 3 and 5 weeks at 7°C. At the end of these periods they were exposed to 3,000 lux continuous illumination for four days, and the chlorophyll formed was determined. Fig. 4 shows that for the first 2 weeks of dark storage the radiation-induced greening inhibition did not change appreciably. The chlorophyll formed during this period was 45% of the unirradiated control. At longer storage the inhibition decreased with time, indicating the possibility of repair in the chlorophyll biosynthetic mechanism. After 5 weeks the chlorophyll formed was 60% of the control.

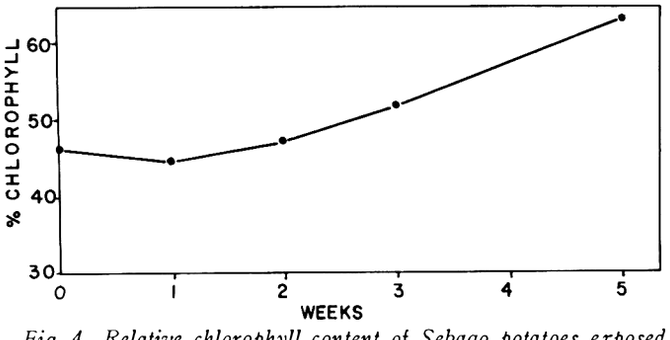

Fig. 4. Relative chlorophyll content of Sebago potatoes exposed to 50 Krad gamma irradiation, subsequently stored in darkness for 1, 2, 3 and 5 weeks at 7°C and then illuminated with 3,000 lux for 4 days. One hundred percent is the chlorophyll content of similarly treated but not irradiated potatoes determined with every sampling. The average chlorophyll content of these controls was 5.2 μg chlorophyll/cm² at the beginning and 4.0 μg/cm² at the end of the 5 week period.

Schwimmer *et al.* (1958) reached similar conclusions concerning the effect of irradiation alone on the inhibition of potato greening, although they reported much higher (up to 10 times) concentrations of chlorophyll in the green potatoes. The differences in chlorophyll concentration may be due to methodology or potato variety.

Regarding the CO_2 effect alone, Forsyth *et al.* (1960) reported that 15% CO_2 in the storage atmosphere resulted in very slight greening. Using the same potato variety, Sebago, and 15% CO_2, but higher intensity of illumination, (3,000 lux vs. 2,260) a chlorophyll content not less than 50% of the control was found.

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Tolerance of Bacteria for Quaternary Ammonium Compounds

SUMMARY—*Escherichia coli* and *Pseudomonas fluorescens* followed similar patterns of adaptation to tolerate quaternary ammonium compounds. *E. coli* approached a tolerance at 28 μg/ml in nutrient broth after 12 to 14 daily transfers. *P. fluorescens* adapted more rapidly in a similar medium, reaching a level of 120 μg/ml in 12 days. During the adaptation process, there was a gradual buildup of numbers of individual cells that tolerated the essential plateau of maximum tolerance. A reverse process was true during loss of tolerance from growth in the absence of quaternary ammonium compounds.

The cells of adapted cultures were more resistant when low exposures of quaternary ammonium compounds were used to determine germicidal effectiveness. There was no apparent difference, however, between the non-adapted and the adapted cultures, when concentrations and exposures approaching standard recommendations were used. The results indicate adaptation is an unlikely contributor to an objectionable microflora on properly cleaned food handling equipment.

INTRODUCTION

THE USE OF quaternary ammonium compounds in formulated cleaning and sanitation products is common in all segments of the food industry. Quaternary ammonium compounds are considered effective bactericides when used at an appropriate concentration. They have some major advantages (Foster *et al.*, 1958; Klimek *et al.*, 1956) such as sanitizing effectiveness where trace amounts of organic material are present. Another favorable feature of quaternary ammonium compounds is that they are considered noncorrosive to equipment.

Cousins (1963) has shown that residual quaternary ammonium compound remains on equipment and the quantity depends on processing and cleaning operations. The quantity of organic material present depends upon the food processing operation as well as the cleaning and rinsing process. The interaction of varying quantities of residual soil and quaternary ammonium compound contributes to the varying microenvironment, which in turn influences microbial growth (Maxcy, 1966b).

Quaternary ammonium compounds are more effective against the gram-positive than against the gram-negative bacteria (Foster *et al.*, 1958; Newton, 1958). The latter group includes the genera *Escherichia* and *Aerobacter* as well as most of the psychrophilic bacteria. In addition, certain gram-negative bacteria have been shown to acquire a tolerance to quaternary ammonium compounds during continued exposure (Chaplin, 1951, 1952; Crocker, 1951; McGregor *et al.*, 1958). The effect of residual quaternary ammonium compound on food handling equipment seemed to be of significance in developing a selected microflora (Thomas *et al.*, 1964). They found a more frequent occurrence of gram-negative bacteria associated with milk from farms where quaternary ammonium compounds were used as the sanitizer.

The general phenomenon of selective effect gave rise to fear of a generally resistant microflora. There was a consequent reluctance on the part of regulatory agencies to approve the use of quaternary ammonium compounds. Further caution was warranted by the lack of understanding of the mechanisms by which the bacteria acquired tolerance.

This study was conducted to obtain a better understanding of the process of gain and loss of tolerance by bacteria for quaternary ammonium compounds. Consideration was also given the effect of acquired tolerance on the results of the accepted standard method for evaluating germicidal effectiveness (Chambers, 1956).

MATERIALS AND METHODS

Media and cultures

The primary source of quaternary ammonium compound was a commercial preparation containing n-alkyl dimethyl benzyl ammonium chloride and n-alkyl dimethyl ethyl benzyl ammonium chloride (Klenzade Products, Inc.) as 10% active ingredient. Other quaternary ammonium compounds were used to show that all had a similar behavior. In reporting the results, the concentrations were expressed as active ingredient. Nutrient broth (8.0 g dehydrated medium by Difco per liter) served as the basal medium to which various concentrations of quaternary were added.

Pure cultures of *Escherichia coli* and *Pseudomonas fluorescens* maintained in the Department of Food Science and Technology were used as examples of bacteria having been reported to acquire resistance to quaternary ammonium compounds.

Prior to use, the cultures were carried through three daily transfers in nutrient broth at 30°C. The various concentrations of quaternary were inoculated with the actively growing cultures for judging growth, as vigorous growth was the phenomenon being studied. Commonly growth was determined by examining for visual turbidity after 24 hr. Where visual turbidity did not serve as an adequate criterion for growth, direct microscopic observations and enumeration by plate culture methods were used.

Assessment of tolerance

In assessing the tolerance of the bacteria a series of tubes with varying quantities of quaternary ammonium compounds were inoculated. The tube with the highest concentration of quaternary ammonium compound in which there was growth after 24 hr was considered as showing the maximum tolerance and was used for subsequent inoculations. This system of transfer into another series of still higher concentration was continued until the culture used

could tolerate essentially its upper limit. The cultures growing in this concentration were referred to as tolerant or adapted cells.

The nature of tolerance was also assessed by measuring the number of cells that were capable of tolerating the essential upper limit for the culture. The number of tolerant cells was determined by the dilution technique given in Standard Methods (American Public Health Association, 1960). A series of three 10-fold dilutions within an appropriate range was made from which 1.0-ml portions were transferred to the tubes containing 9.0 ml of nutrient broth and the appropriate concentration of quaternary ammonium compound, e.g., 28 μg/ml for *E. coli*. Each dilution was made into five replicate tubes at three concentrations. Those that gave visible turbidity after 48 hr were considered positive. The most probable numbers per ml of inoculum were determined by the use of appropriate tables (American Public Health Association, 1960). The number thus obtained indicated the tolerant cells present. The ratio of tolerant cells to the total count was determined by comparing the tube dilution technique to the plate count.

The tolerant cultures were caused to lose tolerance by growing them at 30°C in nutrient broth free of quaternary ammonium compound. After 24 hr of incubation at 30°C the tolerance was determined and transfers were made into fresh broth. Under specific conditions, to be given with the experiments later, the time required to lose tolerance, and the ratio of tolerant cells to the total count were determined for each culture.

Comparison of germicidal action

The comparative germicidal effectiveness of the quaternary ammonium compound against the tolerant and non-tolerant cells was determined by the Chambers Modification of the Weber and Black Method (Chambers, 1956).

RESULTS

Developing tolerance to quaternary ammonium compounds

To determine the pattern of adaptation of *E. coli* to tolerate quaternary ammonium compounds, daily sub-cultures in increasing quantities were made. The initial tolerance was 4 to 5 μg/ml. After a period of 12 to 14 days the culture had acquired a capacity to tolerate 28 μg/ml, which represented an essential plateau of the maximum tolerance. Slight additional tolerance could be attained only after 1 to 2 months of daily sub-cultures. The results of three separate trials are given in Table 1, which shows the pattern of developing tolerance and the variation to be expected between trials.

P fluorescens showed a similar pattern of developing tolerance. The results of three trials are given in Table 2. The initial tolerance was 15 μg/ml, but after 12 days the tolerance had reached 120 μg/ml, which appeared to be a plateau of maximum tolerance.

P. fluorescens acquired tolerance at a more rapid rate and to a higher level than *E. coli*.

The occurrence of fully tolerant cells

The manner of developing tolerance and the frequency of occurrence of fully tolerant cells could be of practical significance in the development of the microflora on food-handling equipment where residues exist. The process of gaining tolerance was examined by repeating the normal development of tolerance while comparing the numbers of essentially fully tolerant cells at various stages to the total numbers of cells.

Table 1. The acquired tolerance of *E. coli* to a quaternary ammonium compound through daily subculture in progressive concentrations.

Days subcultured	Maximum tolerance in μg/ml for growth in 24 hr			
	Trial 1	Trial 2	Trial 3	Average
1	5	4	4	4.3
2	5	6	4	5.0
3	7	6	7	6.6
4	9	8	10	9.0
5	12	8	12	10.6
6	19	10	12	13.6
7	19	12	14	15.0
8	23	18	14	18.3
9	25	18	18	20.3
10	25	18	25	22.6
11	27	20	27	24.6
12	28	22	27	25.6
13	28	24	28	26.0
14	28	28	28	28.0

The relationship between the cells capable of tolerating 28 μg/ml and the total numbers of cells is shown in Table 3, which represents an average of 3 trials. Even when the culture was in the early stage of developing tolerance, some cells were able to tolerate 28 μg/ml. In the early transfers, the number of tolerant cells was very small as compared to the total count. As the process of adaptation continued the number of tolerant cells approached the total count. Thus, the process of acquiring tolerance was within the individual cells rather than with the culture as a whole.

The process of developing an increased tolerance in *P. fluorescens* is shown in Table 4 which represents an average of three trials. *P. fluorescens* developed tolerance much more rapidly than *E. coli*.

The loss of acquired tolerance

The stability of the tolerant cells is an important factor in evaluating the significance of tolerance in a sanitation program. To study the stability of acquired tolerance, adapted cultures of *E. coli* capable of tolerating 28 μg/ml

Table 2. The acquired tolerance of *P. fluorescens* to a quaternary ammonium compound through daily subculture in progressive concentrations.

Days subcultured	Maximum tolerance in μg/ml for growth in 24 hr			
	Trial 1	Trial 2	Trial 3	Average
1	15	15	15	15.0
2	25	25	25	25.0
3	30	35	35	30.3
4	35	40	45	40.0
5	40	55	45	46.6
6	50	60	65	58.3
7	60	65	70	65.0
8	75	75	85	78.3
9	90	95	100	95.0
10	105	105	105	105.0
11	115	110	115	113.3
12	120	120	120	120.0

Table 3. The effect of daily subculturing in progressive quantities of a quaternary ammonium compound on the numbers of *E. coli* that tolerated 28 μg/ml.

Days subcultured	Tolerance in μg/ml	Total count per ml	Numbers tolerating 28 μg/ml	Ratio of tolerant organisms to total count
1	4	71×10^8	0	0
3	6	87×10^5	0	0
6	10	94×10^4	0	0
9	14	15×10^5	23	1.5×10^{-5}
12	22	32×10^5	49×10^2	1.5×10^{-3}
15	28	55×10^5	49×10^5	0.89

Table 4. The effect of daily subculturing in progressive quantities of a quaternary ammonium compound on the numbers of *P. fluorescens* that tolerated 60 μg/ml.

Days subcultured	Tolerance in μg/ml	Total count per ml	Numbers tolerating 60 μg/ml	Ratio of tolerant organisms to total count
1	15.0	21×10^3	.....	.....
3	30.3	14×10^5	.....	.....
6	58.3	60×10^2	.....	.....
9	95.0	20×10^1	30×10^1	7.40×10^{-4}
12	120.0	53×10^1	44×10^4	0.846

of the quaternary ammonium compound were reverted by growing in daily transfers to quaternary-free nutrient broth. After each third transfer in nutrient broth, observations were made on maximum tolerance, number of cells tolerating 28 μg/ml of quaternary ammonium compound, and the total count.

Results of reversion of tolerant *E. coli* cells are given. Table 5 shows typical results with cells having newly acquired tolerance. Table 6 shows typical results with cells that had been sub-cultured daily for 45 days in medium containing 28 μg/ml of quaternary ammonium compound. With each example there was a gradual decrease in the total tolerance, but the newly adapted cells lost tolerance more rapidly. The numbers of individual cells capable of tolerating 28 μg/ml decreased more rapidly in the newly adapted cultures. Even though the older culture of adapted cells did not show a loss in tolerance by the traditional transfer method, the number of cells capable of tolerating 28 μg/ml showed a continuous decrease.

Table 5. The loss of tolerance by *E. coli* with newly acquired resistance to the presence of a quaternary ammonium compound through subculture in media without quaternary ammonium compound.

Days subcultured	Tolerance in μg/ml	Total count per ml	Numbers tolerating 28 μg/ml	Ratio of tolerant organisms to total count
0	28	54×10^8	49×10^5	0.91
1	28	79×10^7	70×10^7	0.88
3	28	84×10^7	23×10^4	2.7×10^{-4}
6	28	37×10^1	54×10^8	1.5×10^{-4}
9	28	11×10^7	13×10^8	1.2×10^{-4}
12	26	56×10^7	70×10^2	1.2×10^{-5}
15	26	63×10^7	33×10^1	5.2×10^{-7}
18	24	84×10^7	13.0	1.6×10^{-8}
21	20	97×10^7	0	0
24	20	58×10^7	0	0

Table 6. The loss of acquired tolerance by an old adapted culture of *E. coli* (subcultured daily for 45 days in medium containing 28 μg/ml of a quaternary ammonium compound) to the presence of quaternary ammonium compound through subculturing in nutrient broth.

Days subcultured	Tolerance in μg/ml	Total count per ml	Numbers tolerating 28 μg/ml	Ratio of tolerant organisms to total count
0	28	91×10^8	84×10^5	0.92
1	28	53×10^7	46×10^7	0.87
3	28	78×10^7	17×10^7	0.22
6	28	65×10^7	13×10^7	0.20
9	28	76×10^7	13×10^7	0.13
12	28	96×10^7	79×10^8	8.2×10^{-2}
15	28	62×10^7	24×10^4	3.9×10^{-4}
18	28	77×10^7	23×10^4	3.0×10^{-4}
21	26	61×10^7	13×10^5	2.1×10^{-4}
24	26	96×10^7	17×10^4	1.8×10^{-4}

Using the same procedures, stability of tolerant *P. fluorescens* was studied. The average of 3 trials is shown in Table 7. *P. fluorescens* lost its tolerance to 60 μg/ml of quaternary ammonium compound after 21 transfers in nutrient broth. The results showed that *P. fluorescens* tolerated more quaternary ammonium compound, but the loss of tolerance was in the same general manner as was true for *E. coli.*

Comparative germicidal action against normal and tolerant cells

Since the adaptation process produces a greater tolerance for quaternary ammonium compound, the adapted cells might be more resistant to quaternary ammonium even at germicidal concentrations. Tests for comparative germicidal effectiveness were therefore made. The average results of three trials are given in Table 8.

At concentrations of 30 μg/ml and above, the results with the normal and tolerant cells were essentially the same. At the lowest treatment, however, the adapted cells were more resistant. In Table 8 it can be seen that within 15 sec about 46% of the tolerant cells were killed, whereas, within the same time 96% of normal cells were killed. With an increase in the time of action the tolerant cells were susceptible to the germicidal action.

Similar tests were performed with *P. fluorescens.* The average results of three trials are given in Table 9. Tolerant and non-tolerant cells were susceptible to the action of quaternary ammonium compound. At the concentrations

Table 7. The loss of acquired tolerance by a culture of *P. fluorescens* to the presence of a quaternary ammonium compound through subculture in nutrient broth.

Days subcultured	Tolerance in μg/ml	Total count per ml	Numbers tolerating 60 μg/ml	Ratio of tolerant organisms to total count
0	60	36×10^7	33×10^7	0.93
1	60	48×10^7	35×10^7	0.72
3	60	57×10^7	12×10^7	0.20
6	60	54×10^7	39×10^5	7.34×10^{-3}
9	60	58×10^7	10×10^6	1.52×10^{-3}
12	60	42×10^7	54×10^8	2.10×10^{-4}
15	58	62×10^7	42×10^7	6.98×10^{-7}
18	57	68×10^7	23×10^1	3.52×10^{-7}
21	57	52×10^7	10×10^7	1.92×10^{-7}

Table 8. The sensitivity of normal and tolerant cultures of *E. coli* to a quaternary ammonium compound.

Culture	Original cell count $\times 10^6$	Quaternary ammonium compound μg/ml	Percent organisms killed in exposure periods of: 15 sec	30 sec	60 sec	120 sec
Normal	137	40	99.999	99.999	100.000	100.000
		35	99.999	99.999	100.000	100.000
		30	99.999	99.997	100.000	100.000
		15	99.984	99.998	99.999	99.999
		5	95.285	98.318	99.480	99.940
Tolerant	130	40	99.999	99.999	100.000	100.000
		35	99.996	99.999	99.999	100.000
		30	99.995	99.998	99.999	100.000
		15	99.886	99.972	99.983	99.988
		5	46.556	73.974	98.166	98.660

of 30 μg/ml and higher, tolerant and non-tolerant cells were susceptible to the same extent. At the lower concentrations tolerant cells were less susceptible. In general, the results of germicidal action against *P. fluorescens* were similar to those of *E. coli.*

DISCUSSION

E. coli, P. fluorescens, and certain other gram-negative microorganisms are able to develop a tolerance for quaternary ammonium compounds. While this phenomenon may appear, on the surface, to be of major importance in the sanitizing process, further consideration of the conditions required to develop the tolerance is warranted.

The conditions reported to allow development of tolerance are based on a progressively increasing gradient of quaternary ammonium compound at a challenging but nonlethal concentration. In addition, nutrients, temperature, and time are critical because it is the progeny that has the tolerance. With the laboratory conditions for development of tolerance the number of cells is indeed large, which is a most unlikely situation except on equipment being totally neglected. In addition, there is a reversal of the adaptation process when the cultures are not exposed to challenging concentrations of quaternary ammonium compound.

Even with relatively ideal laboratory conditions 12 days are required for *E. coli* to reach its plateau of maximum tolerance. The *P. fluorescens* required 12 days to develop a tolerance of 120 μg/ml. While individual microorganisms may differ in their exact behavior toward quaternary ammonium compounds, the general pattern is the same as exemplified by the results with *E. coli* and *P. fluorescens.*

The plateau of maximum tolerance may appear to be relatively high when considering the normal recommended concentration for sanitizing operations, but the conditions for developing tolerance and the conditions for sanitizing operations are quite different. The former provides an adequate growth environment while the latter assumes cleaned equipment free of nutrients for growth. Furthermore, in the presently accepted test for evaluating sanitizers, tolerant cultures of *E. coli* and *P. fluorescens* were no more resistant than normal cultures.

The results of this work indicate that a selectively resistant microflora from the use of quaternary ammonium compounds as sanitizers is most unlikely. When a preponderance of gram-negative bacteria is found where quaternary ammonium compounds are used, a logical approach may be to seek a previously unrecognized harborage of soil (Maxcy, 1966a).

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Table 9. The sensitivity of normal and tolerant cultures of *P. fluorescens* to a quaternary ammonium compound.

Culture	Original cell count $\times 10^6$	Quaternary ammonium compound μg/ml	Percent organisms killed in exposure periods of: 15 sec	30 sec	60 sec	120 sec
Normal	130	40	99.999	100.000	100.000	100.000
		35	99.999	100.000	100.000	100.000
		30	99.996	99.999	100.000	100.000
		15	99.995	99.997	99.998	100.000
		5	99.003	99.121	99.287	99.654
Tolerant	109	40	99.997	100.000	100.000	100.000
		35	99.996	99.999	100.000	100.000
		30	99.994	99.999	100.000	100.000
		15	99.992	99.998	99.999	99.999
		5	47.827	78.244	98.443	98.650

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Gamma Irradiation Influence on the Storage and Nutritional Quality of Mushrooms

SUMMARY—Growth of freshly harvested mushrooms subjected to 100 krad of gamma irradiation is markedly inhibited, as measured by the small number of broken veils. This could significantly increase the storage life of mushrooms even under suboptimal conditions. Untreated mushrooms were preferred to the treated, but hedonic scores indicated that the treated mushrooms would be acceptable. There was no significant change in rate of moisture loss, or in reducing sugar or dry matter content of the irradiated mushrooms. Respiration of irradiated mushrooms is accelerated up to about 3 days after treatment and then slows markedly. The inclusion of 20% irradiated mushrooms in mouse diets fed in late pregnancy and lactation had no significant effects on the average mouse weight of the offspring 40 days old or on their daily food intake and weight gain in a subsequent 6-wk feeding period.

INTRODUCTION

THE COMMERCIAL production of the most widely grown mushroom in the U. S. A. and Canada, *Agaricus campestris, (Agaricus bisporus)* (Singer, 1961) has increased almost elevenfold during the past 36 years to a total of 190,402,000 pounds in 1965 (Lambert, 1958; Dominion Bureau of Statistics, 1966; Kneebone, 1966).

Top-grade mushrooms are harvested before the veil is broken. At room temperature they continue to grow; the stem elongates, the veil breaks, exposing the dark-colored gills, and the white cap and stem become brownish in color. Such changes make mushrooms unattractive to the consumer, and reduce their economic value.

Relatively low dosages of gamma radiation prevent sprouting of vegetables such as potatoes (Sparrow *et al.*, 1954) and onions (Dallyn *et al.*, 1959). The possible use of irradiation to extend the storage life of mushrooms was investigated. The initial objective was to determine the optimum irradiation dosage that would prevent post-harvest growth of mushrooms. Freedom from possible toxic effects and maintenance of the nutritive value of treated mushrooms were also considered. Accordingly, the effects of incorporating treated mushrooms in the diet of test mice were investigated. For preliminary information on physiological effects, the CO_2 output of irradiated and untreated mushrooms was compared.

In preliminary investigations of the influence of gamma irradiation on the storage and nutritional quality of mushrooms, three experiments were undertaken.

EXPERIMENT 1

Freshly harvested mushrooms were irradiated at different levels in a mobile gamma irradiation unit with a Co^{60} (15,000 curies) energy source. The mushrooms were placed in wire baskets into lead buckets (40 × 40 × 75 cm). These were moved on conveyers to the irradiation chamber. A ferris wheel arrangement enabled controlled exposure of the mushrooms to the gamma radiation. Dos-

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age levels were 0, 6.25, 12.5, 25, 50, 75, 100, 200 and 300 kilorad (krad).

The treated mushrooms and 2 kg of untreated mushrooms were stored at 1°C and 85% R.H. (optimal) for 1 wk, followed by suboptimal storage at 21°C and 30% R. H. Based on comparative effects on external appearance, growth changes and flavor, 100 krad was selected as the optimum dosage. The appearance of treated mushrooms improved when the dosage exceeded 75 krad, but the taste ratings of mushrooms treated at dosage levels of 200 krad were lowered. The mushrooms were sliced uniformly, fried in butter in stainless steel pans and served warm in randomized order for rating on a 9-point hedonic scale, where 9 represented "like extremely" (Amerine *et al.*, 1965).

Taste ratings by a panel of 17 untrained persons were 6.1, 6.7, 5.5, 6.3, 6.0 and 6.1 for mushrooms treated at 0, 6.25, 12.5, 25, 50 and 75 krads respectively. The ratings by a 16-member panel were 5.6, 5.6, 4.7 and 5.7 for mushrooms treated at 0, 100, 200 and 300 krads respectively. The mushrooms irradiated at a dosage of 200 krads were significantly inferior in taste appeal by Tukey's W-procedure (Steel *et al.*, 1960).

EXPERIMENT 2

Mushrooms treated at the 100 krad dosage were stored for 4 days at 1°C and 85% R.H. followed by 2 days at 21°C and 30% R.H. Growth as measured by broken veils was recorded. The content of reducing sugars was measured by the arsenomolybdate method (Colowick *et al.*, 1957). Also determined were percent of weight loss and dry matter content of the mushrooms after storage.

Table 1 shows that the growth of the treated mushrooms was markedly inhibited, as indicated by the small number of broken veils. The average contents of reducing sugars of the treated and the control groups were 0.14% and 0.09% respectively, and was statistically insignificant. The comparative effects on weight loss and dry matter were similarly insignificant.

A taste panel of 21 untrained persons judged coded samples of treated and untreated fried mushrooms in a paired preference test (Amerine *et al.*, 1965). Four replicates were prepared and served at one sitting. Thus, each panelist judged 4 pairs in which the order of samples was alternated between pairs. The statement of "no preference" was discouraged.

The taste tests showed a significant preference ($P = 0.01$) for the untreated mushrooms. Fifteen of the 21 panelists were consistent in their preferences in 3 or 4 of the 4 pairs. Twelve of those 15 preferred the untreated mushrooms. Some panelists (14%) commented that the untreated mushrooms were more flavorful; 17% of the panel members detected a stronger flavor in the treated mushrooms. With regard to texture, 10% of the panelists judged the untreated samples to be more tender than the treated mushrooms. During sample preparation, the irradiated mushrooms appeared softer both before and after cooking and were slightly darker than the untreated mushrooms after cooking. Although this experiment indicated a definite preference for the untreated mushrooms, it did not show the degree of preference. Experiment 1, admittedly without replication, showed no difference in scores based on degree of liking between the untreated samples and those treated at a dosage of 100 krads. Thus, it is felt that further research would show equal acceptability between untreated and 100-krad treated mushrooms.

EXPERIMENT 3

A gamma cell equipped with a Co^{60} irradiation unit (Gammacell Model No. 220, Atomic Energy of Canada, Ottawa, Ontario) was used. The dosages used in the initial experiment were repeated, and similar results were obtained. Freshly harvested mushrooms were treated in quadruplicate amounts at 100 krad. The treated and untreated materials were dried, ground in a Braun grinder, and incorporated into pelleted rations to feed to mice. The rations were similar to the semi-purified mouse diet of Bell (1960). To facilitate pelleting, corn starch was replaced by sucrose for a gestation-lactation test.

A preliminary experiment showed that the addition of 20% dried mushrooms (to replace sucrose) had no adverse effect on the daily food consumption or body weight of young adult mice. Accordingly, untreated and irradiated mushrooms at a 20% level in the diet were compared in the gestation-lactation test.

Twelve pregnant albino (CF no. 1) mice (2–5 days before term) were divided into two groups of six to study effects of irradiated mushrooms on the mothers and their offspring. One group received the diet containing the irradiated mushrooms; the second group received the diet with untreated mushrooms. The offspring of both groups were weaned at an average age of 24 days, fed a standard laboratory chow, and weighed individually at 40 days. While more of the offspring of mothers fed irradiated

Table 1. The influence of 100 krad of gamma irradiation on mushrooms after 4 days storage at 1°C and 85%R. H.

	Broken veils (per 100 mushrooms)		Reducing sugars (%, dry weight basis)		Weight loss (%)		Dry matter content (%)	
Replicate	Irrad.	Check	Irrad.	Check	Irrad.	Check	Irrad.	Check
1	3	83	0.23	0.06	10.96	12.00	9.68	11.18
2	11	90	0.11	0.05	10.55	10.88	9.60	11.85
3	5	86	0.18	0.12	11.44	12.22	10.55	11.75
4	2	76	0.04	0.13	11.76	11.56	12.40	11.15
Mean	5.3	83.7	0.14	0.09	11.18	11.70	10.56	11.48

mushrooms died before weaning, there was no significant difference after 40 days between the average mouse weight of the offspring from female mice fed irradiated dried mushrooms compared to untreated dried mushrooms. The coefficient of variability in this series was 16.8%.

Sixteen offspring (8 males and 8 females) from each treatment were subsequently fed their mother's diet for 6 wk during their 8- to 14-wk life stage. Two males and one female on the irradiated mushroom diet and two males on the untreated mushroom diet died during the test. No significant difference in increase in body weight was observed in the remaining mice. For both treatments, the average daily intake was 2.4 g per mouse.

Since preliminary experiments showed that mushroom growth was strongly inhibited by 100 krad of gamma irradiation, it was considered that measurement of the effect of this dosage on CO_2 output during respiration of mushroom tissue would be informative. Such measurements were made in all three experiments.

Preliminary tests indicated that fresh mushrooms should be placed directly into glass bottles, sealed and treated to avoid subsequent contamination. Replicated samples (50 g) were placed in bottles fitted with gas-tight rubber stoppers containing rubber septums through which the air within the bottles could be sampled with a gas-sampling syringe. The bottles were kept in hermetic storage at 4°C for 4 days. Measurements were terminated after 4 days because it was considered that the accumulated CO_2 content in the bottles would unduly interfere with normal respiration.

Four replicates were irradiated at a dosage of 100 krad in the Gammacell 220. The CO_2 content of 2-cc air samples obtained from the bottles was determined by gas chromatography. The method used (Berck, 1967) employed an F and M Model 500 linear temperature programmed gas chromatograph (F and M Scientific Corporation, Avondale, Pennsylvania), with a thermal conductivity detector. Samples were taken at 3 hr, 4½ hr, and 1, 2, 3 and 4 days after treatment.

Fig. 1 shows that the level of accumulated CO_2 was considerably higher for the irradiated mushrooms than for the untreated mushrooms at the 1- and 2-day sampling period. However, the difference in CO_2 level became progressively less thereafter, and at 4 days there was no statistical significance. It should be noted that the rate of rise in CO_2 of the irradiated mushrooms diminished very appreciably after 3 days, and this was not the case with the untreated mushrooms. This was also shown in the first two experiments wherein suppression of CO_2 output was obtained for the treated samples.

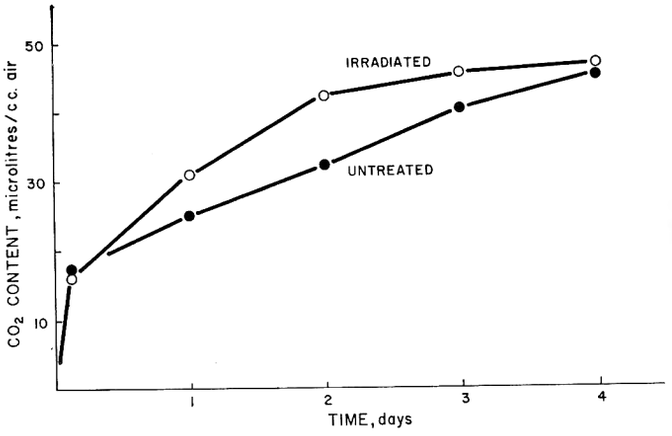

Fig. 1. CO_2 content of air of irradiated and untreated mushrooms during storage at 4°C in closed systems.

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Intestinal Flora and Chicken Flavor

SUMMARY—Chickens, including those reared under germfree, gnotobiotic (in contact only with *Clostridium perfringens*, *Escherichia coli*, and *Streptococcus faecalis*) or conventional conditions were compared for flavor utilizing the triangle taste testing technique. Dark and light meat were evaluated separately. The results of these tests indicated a highly significant difference between the flavor of germfree and conventional chicken meat, a difference of lower significance between gnotobiotic and conventional chicken meat and no significant difference between the flavor of gnotobiotic and germfree chicken meat. Meat from the conventionally-reared chickens had a stronger and more characteristic chicken flavor than that from germfree chickens. These results indicate that bacteria present in the intestinal tract do affect flavor of the meat of the chicken.

INTRODUCTION

THE FACTORS WHICH ESTABLISH the flavor of poultry have not been completely defined. In considering these factors it seemed feasible that intestinal bacteria could synthesize compounds or alter compounds present in the gut of the bird in such a way as to produce substances which might contribute to flavor.

Baker *et al.* (1956) compared the flavor of eviscerated and uneviscerated birds and found that flavor deterioration was more rapid in uneviscerated birds. Since flavor differences existed between eviscerated and uneviscerated carcasses it appeared that flavoring compounds were carried to the muscle. That compounds such as hydrogen sulfide are carried to muscles is demonstrated by the "green struck" phenomenon which occurs in uneviscerated birds following multiplication of bacteria in the gut (Nickerson *et al.*, 1939). Barnes *et al.* (1957) investigated the causes of greening in poultry and concluded that during storage hydrogen sulfide diffused through the gut wall and finally reached the muscle tissue where it reacted with the heme pigments of blood and muscle and, in the presence of air, formed the green pigment sulfmyoglobin.

Shrimpton (1966) speculated that the gamey flavor which developed in uneviscerated birds was caused by microbiological activity in the intestine and the subsequent acquisition by the skeletal muscle of the metabolites so formed. Earlier experiments by Shrimpton *et al.* (1965) indicated that flavor components are transferred from the gut to the muscle of poultry. They tentatively identified 15 flavor components of chicken breast meat all of which were also found in the ceca of cannulated birds. They further observed that the amounts of these substances increased in the breast muscle of birds held after death at 15°C with their viscera in place. When the level of the substances was high, there was a concurrent "gamey element" in the flavor. It appeared that at least some of the gamey flavor of the birds had its origin in the metabolic reactions that occurred in the cecal flora.

The studies presented here were designed to give another approach to the question of whether the bacterial flora of the intestinal tract affect the flavor of chicken muscle. The flavor of cooked muscle of germfree chickens was compared with that of chickens reared in the conventional manner. This procedure was designed to determine whether flavor compounds produced by bacteria could be carried from the intestine to the muscle tissue while the bird was alive. When the presence of microorganisms was found to affect flavor, additional experiments were carried out in which selected microorganisms were fed to germfree birds to determine their effect on chicken flavor.

MATERIALS AND METHODS

ARBOR ACRE FEMALES were mated to Peterson males to produce broiler-type chickens. Incubated fertile eggs were obtained from a local hatchery on the 18th day of incubation.

Control chickens were reared in the conventional manner under non-germfree conditions. The 18-day-old eggs were transferred from the commercial hatchery incubator to a 37°C incubator at the University. After hatching, chicks were transferred to wire cages equipped with electrically-heated batteries. A gradual reduction in temperature began and was continued for approximately 2 weeks when room temperature was reached and maintained.

In order to hatch germfree birds, the 18-day-old embryonated eggs were washed with a brush in a 2% (w/v) detergent solution maintained at 37°C distilled water. Eggs were placed in a nylon stocking and immersed for six min in a 1.5% (w/v) mercuric chloride solution maintained at 37–39°C. The eggs were then transferred with a wire hook through a tube without exposure to the non-sterile air into a previously sterilized germfree isolator. Inside the isolator the eggs were removed from the stocking and placed in a special pan for hatching. Ten eggs yielded from six to eight chicks. To prevent overcrowding only three chicks were retained in each isolator for the experiment.

This procedure was carried out in a room maintained at 37°C. Two days after hatching, a gradual reduction in temperature began and room temperature (22°C) was reached and maintained after two weeks.

The germfree isolator was modeled after that of Trexler (1959). Each isolator was equipped with a collapsible cage constructed of stainless steel ½-in. mesh wire.

All chickens were fed ration and water *ad libitum*. The diet was designed to compensate for losses due to the heat treatment in sterilization and for the fact that vitamins

Table 1. Composition of diet for all birds.

Ingredient	g
Ground corn	548.0
Soybean meal (50% protein)	300.0
Fish meal	25.0
Dried whey	50.0
Alfalfa meal	25.0
Meat scrap	25.0
Salt	5.0
Feeding oil	2.0
Calcium carbonate	7.5
Dicalcium phosphate	7.5
Manganese sulphate (monohydrate)	0.3
Vitamin premix *	5.0
D-L methionine	0.5
Ethoxyquin	1.0
* Vitamin premix	
Thiamine-HCl	10.0
Niacin	10.0
Riboflavin	1.6
Ca Pantothenate	10.0
Vitamin B_{12} (mg/gm)	2.0
Pyridoxine-HCl	0.6
Biotin	0.06
Folic acid	0.4
Inositol	10.0
Vitamin A (250,000 IU/gm)	4.0
Vitamin D_3	0.5
Menadione	1.0
Sucrose	449.84

normally synthesized by the microbial flora would not be produced in germfree birds. The composition of the diet for all birds is presented in Table 1.

The above mixture of vitamins was blended together with enough sucrose to equal 500 g. A high vacuum autoclave with an oil-sealed vacuum pump was employed for sterilizing all the feed. Feed was placed on cloth-lined mesh trays to a depth of one in. Trays were placed in a steel sterilizing cylinder which was then sealed with Mylar film. The cylinder was placed in the autoclave and the following sterilizing cycle was carried out:

1. The chamber was evacuated to 12 mm Hg residual pressure and held for 15 min.
2. The vacuum was broken by steam and the feed was held at 121°C for 25 min.
3. A vacuum cycle of 15 min was employed to remove excess moisture.

When it was necessary to add items to the isolator the transfer sleeve was attached and the items as well as the transfer sleeve were sprayed with 2% peracetic acid. Items to be added were allowed to set for a minimum of 30 min before they were admitted to the germfree isolator. When it was necessary to remove items from the isolator the transfer sleeve was attached, sprayed with peracetic acid and allowed to set for 30 min before the inside door was opened.

Tests for the sterility of the apparatus and the birds were usually scheduled to coincide with the time when food, water, and other materials had to be introduced into or removed from the isolator. Tests were made for sterility of the chicken by swabbing various parts of the bird and by obtaining samples of excreta. Swabbings were also taken from cage surfaces and samplings were made of feed and water. Two types of liquid media, brain heart infusion broth and fluid thioglycollate medium, were employed in these tests. Inoculated tubes of these media were removed from the isolator for plating in brain heart infusion broth with 1.5% agar added. Plated cultures were incubated aerobically or anaerobically at 37°C and room temperature (about 22°C). Transfers were also made from the inoculated tubes into freshly prepared fluid thioglycollate medium, and these cultures, along with the inoculated tubes which were removed from the isolator, were incubated at 37°C or room temperature for 48 hr. After incubation, plates were examined for the growth of bacterial colonies and the liquid media were examined for turbidity. Groups that became contaminated were eliminated from the experiment.

In this paper, "gnotobiotic" refers to those birds associated with only three organisms, from those which are predominant in the intestinal flora of chickens. These were *Escherichia coli (gratia), Streptococcus faecalis,* and *Clostridium perfringens 1168.* The three strains were administered simultaneously when the birds reached three weeks of age.

E. coli was propagated on sterile tryptone glucose extract agar slants for 24 hr at 37°C. Bacterial cells were then washed from two slants with 100 ml sterile 0.1% peptone water. The suspension was adjusted to an optical density reading of 0.175 at 660 μ on a spectrophotometer to produce a concentration of approximately 300×10^6 cells per ml. Twenty-five ml of this suspension were added to the drinking water of the chickens.

S. faecalis was propagated in sterile brain heart infusion broth for 24 hr at 37°C. Sterile distilled water was added to the broth to adjust the optical density of the suspension to 0.230 on a spectrophotometer at 660 μ. An optical density reading of 0.230 indicated approximately 150×10^6 cells per ml. Fifteen ml of this suspension were added to the birds' drinking water.

C. perfringens 1168 was propagated in sterile fluid thioglycollate medium (FTG) for 6 hr at 37°C. Ten ml of the FTG and cell suspension along with one ml thiamine HCl was inoculated into each of two bottles of sterile SEC broth, a sporulating medium (Angelotti *et al.,* 1962). The SEC broth was incubated for 18 hr at 37°C. The broth was then removed from the incubator and bacterial cells were concentrated by centrifuging for 10 min at 13200 × g. After decantation of the supernatant broth, cells were washed with sterile saline and then placed in smaller tubes for centrifugation at 27000 × g for 10 min. After decantation of the supernatant, enough sterile saline was added to the cells to produce an optical density reading of 0.2 on the spectrophotometer at 420 μ. This reading indicated the presence of approximately 5.9×10^7 cells per ml. Twenty-five ml of the suspension were administered to the birds in their drinking water.

During the week following inoculation, tests were carried out to ascertain whether the organisms had established themselves in the intestinal tract. Eosin methylene

blue agar and E.C. medium were both used to confirm the presence of *E. coli*. Sulfite-polymyxin-sulfadiazine agar was selected to test for the presence of *C. perfringens*. To test for the presence of *S. faecalis* two examinations were completed. In the first test, azide dextrose broth and ethyl violet azide broth were used. In the second test, yeast water mannitol with 0.04% potassium tellurite was employed as a test medium.

Gram stains made on the test suspension from gnotobiotic isolators always clearly revealed the presence of three organisms with morphological characteristics identical to those used for the inoculation.

Eight weeks after hatching, the chickens were slaughtered and dressed at the University of Wisconsin Poultry Research Laboratory. The birds were allowed to bleed for 2 min before they were scalded in an automatic rotary scalder at 54°C for 21 sec. Feathers were then plucked by hand and the birds were eviscerated. A maximum time lapse of 90 min occurred between slaughtering and the completion of the evisceration process. Carcasses were placed in refrigerated storage (5°C) about 18 hr and were then roasted whole in a preheated 191°C oven for 75 min. Aluminum foil loosely covered the birds during the first hr of cooking. After cooling, meat was removed from the bones, the white meat from the breast portion being kept separate from the dark meat of the leg muscle. White meat from all birds in a particular group was mixed together and dark meat was treated likewise. Skin and pan fat drippings were included in the sample. Skin composed approximately 4% and fat approximately two percent of the total sample. The meat was finely sliced with an electric chopper for 10 sec to produce a reasonably uniform sample. The meat was immediately placed in sterile petri plates in a freezer maintained at −18°C where it remained for 3 weeks.

A triangle taste test (Amerine *et al.*, 1965) was administered to a taste panel of 11 graduate students, who had participated in several practice sessions prior to actual tests. Dark and light meat were judged separately. The meat was at room temperature when served. Two triangle sets were presented at each tasting session. Chi-square tests were used as a basis for determining significant differences.

RESULTS

THREE FLAVOR COMPARISONS were completed: (1) chickens reared under germfree conditions and compared with those reared in the conventional manner, (2) chickens which were associated with only three organisms compared with those reared in the conventional manner, and (3) chickens which were associated with three known organisms compared with those reared under germfree conditions.

Germfree vs. Conventional

While the range in significance for the three replicates varied from .05 to .001, (Table 2), the total level of significance was very high (.001) indicating that a difference existed between the flavor of birds reared under germfree conditions and those reared conventionally. These data were consistent in indicating significant flavor differences. Panel members were asked to describe the flavor difference between odd and like samples. They generally described the meat from chickens reared in the conventional manner as having a stronger flavor or a more characteristic chicken flavor than the meat from chickens reared under germfree conditions.

Gnotobiotic vs. Conventional

Table 3 presents the results of flavor tests between these two groups. Panel members appeared more able to detect flavor differences in the first replicate than in the second and third replicates. They were able to detect flavor differences in the light meat more often than in the dark meat samples. The total levels of significance for differences were .01 for light meat and no significance for dark meat samples. It appeared from word descriptions of flavor differences that panel members could detect little difference between meat from birds reared under gnotobiotic conditions and those reared under conventional conditions.

Gnotobiotic vs. Germfree

Table 4 shows the results of three tests. It should be pointed out that seven was the lowest number of correct judgments which could be considered significant. Thus, significant differences, when they did occur, were minimal. The total results of the three replicates indicated no significant differences between the flavor of birds reared under gnotobiotic and germfree conditions when light and dark meat were judged separately. Word descriptions of flavor differences existing between odd and like samples further indicated that panel members were generally unable to distinguish flavor differences in meat from birds grown under gnotobiotic or germfree conditions.

There was little difference in the general appearance of all birds except those contained in isolators had dirtier

Table 2. Flavor difference between germfree and conventional chickens as determined by triangle tests.

Replicate	Light meat[1]			Dark meat[1]		
	No. of tasters	No. of correct judgments	Level of significance	No. of tasters	No. of correct judgments	Level of significance
1	11	8	.01	11	8	.01
2	11	9	.001	11	9	.001
3	8	7	.01	8	6	.05
Total	30	24	.001[2]	30	23	.001[2]

[1] Samples were a composite of muscle, skin, and fat from a minimum of three birds.
[2] Indicates significance of total correct judgments.

Table 3. Flavor difference between gnotobiotic[1] and conventional chickens as determined by triangle tests.

Replicate	Light meat[2]			Dark meat[2]		
	No. of tasters	No. of correct judgments	Level of significance	No. of tasters	No. of correct judgments	Level of significance
1	11	9	.001	11	7	.05
2	11	5	N.S.	11	4	N.S.
3	11	5	N.S.	11	1	N.S.
Total	33	19	.01[3]	33	12	N.S.[3]

[1] Gnotobiotic birds were in contact with only *Escherichia coli, Clostridium perfringens* and *Streptococcus faecalis.*
[2] Samples were a composite of muscle, skin and fat from a minimum of three birds.
[3] Indicates significance of total correct judgments.

Table 4. Flavor differences between gnotobiotic[1] and germfree chickens as determined by triangle tests.

Replicate	Light meat[2]			Dark meat[2]		
	No. of tasters	No. of correct judgments	Level of significance	No. of tasters	No. of correct judgments	Level of significance
1	11	2	N.S.	11	7	.05
2	11	7	.05	11	2	N.S.
3	11	4	N.S.	11	4	N.S.
Total	33	13	N.S.[3]	33	13	N.S.[3]

[1] Gnotobiotic birds were in contact with only *Escherichia coli, Clostridium perfringens* and *Streptococcus faecalis.*
[2] Samples were a composite of muscle, skin and fat from a minimum of three birds.
[3] Indicates significance of total correct judgments.

feathers than those reared under conventional conditions. It appeared that germfree birds consumed slightly more feed than others. Mean weights of the different groups are presented in Table 5. These data indicate that as the number of strains of organisms was reduced to three and then to germfree, the weights of the birds increased. All birds were fed the same diet and kept for the same length of time.

DISCUSSION

THIS STUDY INDICATED that microbial intestinal flora do have an effect on the flavor of chicken muscle since flavor judges were consistently able to detect a flavor difference betwen the cooked muscle of birds grown under germfree as compared with those grown under conventional conditions. Flavor differences between birds grown under gnotobiotic conditions and those grown in the conventional manner were less than those in the first study since the overall flavor comparisons indicated no significant difference between dark meat samples and a difference at the .01 level of significance for light meat samples.

Table 5. Mean weights of conventional, gnotobiotic[1] and germfree birds live and ready to cook.

Group	No. of birds	Mean weights (g)	
		Live	Ready to cook
Conventional	33	1190	737
Gnotobiotic	17	1304	822
Germfree	19	1418	992

[1] Gnotobiotic birds were in contact with only *Escherichia coli, Clostridium perfringens* and *Streptococcus faecalis.*

Birds grown under gnotobiotic procedures and compared for flavor with birds grown in a germfree environment showed no overall significant difference. This indicated that the flavor of meat from birds grown under gnotobiotic conditions more closely resembled that of meat from birds grown under germfree conditions than that from birds grown in the conventional manner.

It is possible that other organisms in the intestinal tract contribute more to flavor than the ones tested. The organisms included in this study may have reacted differently in the presence of a number of varieties of the microorganisms found in the intestinal tract. It is also possible that a synergist effect may occur in which case the three organisms tested may contribute more to flavor when the total intestinal flora are present than when the three alone are present. In any case these results tend to show that the combined presence of *C. perfringens, E. coli* and *S. faecalis* in the gut do not produce the same flavor as the total flora of the intestinal tract.

This study substantiates Shrimpton's (1966) theory that some flavor components in the flesh of the bird are of microbiological origin and synthesized by the intestinal flora. This study seems to further indicate that certain flavor components, metabolites of bacteria in the intestine, can be absorbed and carried to the muscle while the bird is alive.

One of the major problems confronting flavor science appears to be a knowledge of the source and the importance of the compounds identified. Once the exact role of certain intestinal microorganisms in determining flavor is established, flavor could perhaps be better controlled.

The breed of bird, its age and diet, as well as antibiotics, might be employed in such a way as to produce maximum numbers of bacteria which contribute desirable flavor compounds.

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Biological Degradation of Chlorophyll in a System Using Bell Peppers (Capsicum frutescens)

SUMMARY—A degradation system was developed based on the incorporation of radioactive chlorophyll into a ripening bell pepper carpel.

Pure chlorophyll *a* in aqueous triton X-100 injected into green bell peppers *(Capsicum frutescens)* variety 035 was degraded up to 50% by the end of ripening, versus a control in buffer pH 5.4 not exceeding 7% loss in 2 weeks. Variety and stage of ripeness affected the amount of degradation.

Labeled chlorophyll *a* with a specific activity of 7 to 8 × 10^5 dpm/mg was then prepared from young wheat plants, fed $^{14}CO_2$ and injected in amounts of 0.2 to 0.3 mg. The distribution of activity in pepper extracts after pigment degradation was evaluated. The acetone water extract remaining after transfer of lipid material to petroleum ether acquired activity withir 2 days of injection, but the amount remains fairly constant for 12 days. The activity of the extraction residue, and of an 80% ethanol extract thereof, increased throughout the experiment. The residue containing increasing amounts of protein had the largest amount of radioactivity of the three fractions at the conclusion of the experiment.

Preliminary chromatography did not yield isolated radioactive products.

Extracts of pepper show no activity when substituted for soybean extract in a system containing chlorophyll and linoleic acid.

The degradation of chlorophyll by ripening bell peppers provides a tool for further studies for degradation in a physiological system.

Labeling facilitates isolation, identification, and establishment of origin of small amounts of breakdown products.

INTRODUCTION

ONE OF THE MOST obvious changes during the senescence of fruit and other plant tissues is the disappearance of chlorophyll. Control of this reaction would be of interest, for example, since bananas sometimes do not degreen, rendering them unsaleable, while on the other hand, the shelf life of green bell peppers might be extended if degreening could be prevented.

Freezing of such products as peas and beans preserves the color for a certain period of time, but color deterioration still occurs before other quality factors such as flavor or texture become unacceptable.

While some empirical control over these reactions is possible, little is known of the biochemical mechanism of chlorophyll disappearance. Better control of color quality in food products would result from the understanding of this mechanism. This knowledge would also permit fur-

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ther investigations concerning its biological significance. For instance, it is not known whether chlorophyll breakdown products are of physiological significance (Spencer, 1965). If intermediates in this process were known, it would be possible to establish the presence or absence of chlorophyll turnover in mature tissues by following the appearance and disappearance of these products. Preliminary experiments indicated that the study of this process in whole tissues was not feasible. Therefore, an investigation was made to find a system which would be suitable for the isolation and identification of intermediates and products of the physiological degradation of chlorophyll.

Schanderl *et al.* (1966), Co *et al.* (1967b), Michel-Wolwertz *et al.* (1965) and Bacon *et al.* (1967) have all reported the existence of some chlorophyll-type compounds in plant extracts. However, the relationship of these compounds to the biological breakdown of chlorophyll has not been established. Even less is known about any steps leading beyond the destruction of the porphyrin ring.

Though some chlorophyll degradation in nature may be a result of photooxidation of the pigment, the fact that ripening fruits show loss of chlorophyll either in the light or dark indicates that the degradation *in vivo* is at least partially enzymatic. This is supported by the work of Hoyt (1966), who found that rye grass *(Lolium perenne)* lost most of its chlorophyll within 6 days after cutting. Low temperature during incubation, boiling or freezing the tissue prior to incubation, and desiccation of the grass greatly reduced chlorophyll loss.

Wagenknecht *et al.* (1952) observed degradation of chlorophyll in frozen peas and attributed this loss to the action of lipoxidase. Walker (1964) came to similar conclusions about the disappearance of chlorophyll in French beans. Holden (1965) reported a system, based upon that of Blain *et al.* (1953), which contained legume seed extracts and long-chain fatty acids and degrades chlorophyll rapidly to products without chlorophyll-type spectra.

This system should prove useful for the study of the degradation of chlorophyll in some food products. However, this type of degradative system may be limited to legumes or to tissues which are damaged by processing, such as freezing or extraction. Two observations which argue against the general operation of such a lopoxidase-related degradation are: (1) in ryegrass (Hoyt, 1966) the chlorophyll destruction was inhibited by freezing or grinding, whereas rapid degradation is observed in previously frozen legumes or in legume extracts, and (2) the lipoxidase-related degradative system bleaches carotenoids (Blain *et al.,* 1953) as well as chlorophyll, but in Bell peppers, for instance, a large increase in carotenoids is observed at the same time as chlorophyll disappears during ripening (Curl, 1964), indicating the absence of very low activity of a carotenoid degradative system. These considerations made this system appear unsuitable as a model for the study of the physiological degradation of chlorophyll.

Seybold's (1943) failure to find compounds in autumn leaves obviously derived from chlorophyll and the failure of attempts in our laboratory to find such products in ripening fruits made it necessary to develop a system which would degrade exogenous chlorophyll. This would allow degradation of labeled chlorophyll in the system so that radioactive products could be detected and isolated.

Earlier experiments in this laboratory had shown no degradation, other than pheophytin or pheophorbide formation, when chlorophyll was added to isolated chloroplasts or to acetone powders prepared from ripening bananas or peppers. In view of the lability of the chlorophyll degradative system in rye grass (Hoyt, 1966), it appears that such treatments probably destroyed the activity of degradative enzymes.

It was known that mature green peppers *(Capsicum frutescens)* lose their chlorophyll very rapidly once the ripening process begins and that no chlorophyll remains at advanced ripening stages (Schanderl *et al.,* 1966). Despite the obvious difficulties of incorporation of a large molecule such as chlorophyll into the cell and the possibility that it would be degraded in a nonphysiological way, it was thought that a rapidly ripening pepper might degrade exogenous chlorophyll or chlorophyll derivatives. The results of such experiments are described in this paper.

METHODS AND MATERIALS

Preparation of chlorophyll *a*

Chlorophyll *a* was prepared according to the procedure of Strain *et al.* (1966).

Spinach leaves were dropped in a Waring Blendor with enough acetone to give a final concentration of 80% acetone. Oxalic acid was added after grinding. The solution was allowed to stand at room temperature until the color changed from green to gray, indicating that most of the chlorophyll had been converted. The solution was filtered through a Büchner funnel and the residue washed with acetone to remove most of the remaining pigments. This was overlayered with ether, and water or concentrated sodium chloride solution was added until the pigments were transferred to the ether layer, which was then washed several times with water to remove acetone.

The ether solution was dried in a vacuum evaporator to remove any remaining water, and the pigments were taken up in petroleum ether and applied to a column packed with powdered sugar containing 3% starch. The column was developed using 3% acetone in petroleum ether. Pheophytin was a gray band which moved behind alpha and beta carotene. After the pheophytin band showed enough separation from the other components, the column was sucked dry and the pheophytin part cut out and diluted with ether.

Preparation of ^{14}C-labeled chlorophyll *a*

Two hundred wheat seeds were planted in sterile soil in an aluminum pie plate. The wheat was grown in dim light for 3 to 4 days until the primary leaves were approximately 1½ in. through the coleoptile tip. The wheat was placed into a desiccator, and a 6 lb/per sq in. vacuum was pulled. A small round-bottom flask which contained 3 millicuries (11.5 mg) of $Ba^{14}CO_3$ had previously been connected to the desiccator. Four ml of 5 *N* lactic acid was slowly added to the barium carbonate, and the system

was sealed completely until the $^{14}CO_2$ generation was completed. The flask was flushed with air until only a slight vacuum remained in the system. The desiccator was sealed and kept in a hood under constant light from two 30-watt fluorescent lamps at a distance of 3 ft until the wheat grew to 7 to 9 in. This took 3 to 4 days. The wheat was cut and the chlorophyll extracted according to the procedure described for chlorophyll *a* preparation. The only modifications made were that the wheat was ground in an omnimixer slowly during the extraction, and four or five extractions with methanol-petroleum ether 4:1 were made. The wheat tissue was more difficult to extract than spinach leaves, so these changes were necessary to get a complete extraction.

The specific activity was determined after each chromatography. If the specific activity of the chlorophyll *a* was the same after each chromatography and the chlorophyll was spectrally pure, it was used for further experiments.

One preparation of the size described gave approximately 5 mg chlorophyll *a* with a specific activity of 7 to 8×10^5 dpm/mg.

Extraction of peppers

Peppers were cut into pieces, placed in an omnimixer cup, and acetone was added to give at least an 80% acetone concentration, assuming the tissue was water. This was ground for 1 min. The ground material was filtered with suction through a fine fritted funnel. The residue was returned to the mixing cup, and 100 ml acetone was added. It was ground for 1 min and again filtered through the fritted funnel. The residue was washed with acetone until the filtrate was free of pigment, and all filtrates were combined in a separatory funnel.

After adding approximately 100 to 150 ml of petroleum ether, followed by 50 ml of water, the funnel was shaken gently until the petroleum ether and acetone-water layers were separated. The lower layer was removed, and the petroleum ether layer was washed several times with water to remove remaining acetone. The petroleum ether solution was evaporated to dryness in a vacuum evaporator with a few additions of small amounts of acetone to aid evaporation. The dried pigments were taken up and made into a known volume with either diethyl ether or acetone for pigment determinations or other analysis. Approximately 95%of the remaining pigment is recovered with this method.

For radioactive measurements the residue obtained after pigment extraction was boiled for 1 hr with 80% ethanol, The mixture was filtered hot, through a fine fritted funnel. The filtrate was evaporated and made to 10 ml with water. The residue was dried and weighed. The acetone-water layer and the washings from the petroleum ether layer were combined, evaporated to a small volume, and made to 10 ml with water.

A schematic diagram of the extraction procedure is given in Fig. 1.

Determination of chlorophyll *a* and pheophytin *a*

All quantitative absorbance measurements were made using a Beckman DU spectrophotometer with a Gilford readout attachment.

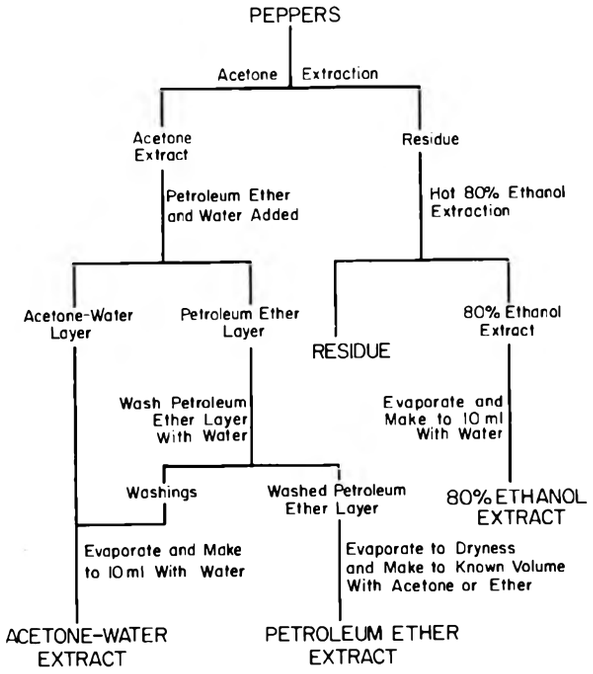

Fig. 1. Schematic diagram of the extraction procedure used.

The readings of stock solution of chlorophyll *a* were made in diethyl ether, and an absorptivity value of 100.9 $lg^{-1}cm^{-1}$ at 662 nm (Smith *et al.*, 1955) was used for calculations. The ratio of the absorbance at 429 nm to the absorbance at 662 nm was used as indication of the spectral purity of the chlorophyll *a*.

Chlorophyll *a* was completely converted to pheophytin *a* by adding 2 mg/ml oxalic acid to the solutions for 3 hr before readings were taken (Vernon, 1960). Absorbancy readings were taken at 667 nm and at 700 nm to control the light scattering of the solution. The difference of the two readings was used to calculate the pheophytin concentration using the absorptivity of 58.7 $lg^{-1}cm^{-1}$ (Wilson, *et al.*, 1962). If pheophytin *a* was injected into a pepper, the same procedure was followed without the oxalic acid addition. The chlorophyll *a* concentration was obtained by multiplying by a factor of 1.025 to correct for molecular weight difference.

When the determination was made in 80% acetone, the absorptivity for pheophytin *a* of 55.2 $lg^{-1}cm^{-1}$, uncorrected for magnesium loss, (Vernon, 1960) was used.

Thin-layer chromatography

A. *Co and Schanderl (1967a):* Plates were coated with silica gel G (E. Merck, Darmstadt, Germany), using a Desaga spreader. Thirty g of silica gel were mixed with 60 ml of water to make five 20 × 20 cm plates 0.25 mm thick. The plates were dried at room temperature. The solvent system was benzene-petroleum ether-acetone (10:2.5:2 by volume).

B. *Hager and Bertenrath (1962):* Five 20 × 20 cm 0.25 m thick plates were made by mixing 12 g kieselgur G, 3 g kieselgel, 3 g $CaCO_3$, and 0.02 g $Ca(OH)_2$ with 50 ml 8×10^{-3} *N* ascorbic acid solution and spreading with a Desaga spreader. The plates were dried for 1½ hr at 50 to 60°C. A solvent system consisting of 100 ml hep-

tane, 12 ml isopropanol and 0.25 ml water was used for development.

C. *Schneider (1965): Fifteen g* MN cellulose 300 (Macherey, Nagel & Co.) were mixed with 100 ml water in an omnimixer. Five plates (20 × 20 cm) 0.4 mm thick were made using a Desaga spreader. The development solvent was methanol-dichloromethane-water (100:18:20, by volume).

D. *Bacon (1965):* Plates were made, as with the Schneider method. The development solvent was petroleum ether-acetone-n-propanol (90:10:0.45, by volume).

Injection of peppers

Quantitative injection of pigment was possible using a 60 to 70% acetone solution. However, some tissue damage occurred as a result of the high acetone concentration. This was used in a few experiments.

Chlorophyll *a* was dissolved in 0.2 ml acetone and then suspended in 3 to 5 ml of water containing 0.2% triton X-100. The chlorophyll suspension was injected into the pepper with a sterile syringe. The test tube and syringe were washed with 0.1 ml acetone and 0.5 ml water containing triton X-100, which were also injected into the pepper. No visible damage to the pepper tissue was observed with this method.

Characteristics of the degradation

Chlorophyll *a* was injected into the peppers which were picked and placed in the dark until extracted. Yellow-green 035 peppers and very dark green variety 044 peppers were injected. The peppers were extracted and the chlorophyll degradation was measured according to the procedures given above.

Effect of pH on chlorophyll degradation

Chlorophyll *a* (0.26 mg) was suspended as before, except that 5 ml acetate buffer at pH 5.4 was used in place of water. The suspended pigment was kept in the dark at 26 to 27°C in test tubes. At six sampling times the contents of three test tubes were transferred to 25 ml volumetric flasks and made to volume with acetone to give a final acetone concentration of 80%. The chlorophyll remaining was determined as described. The average chlorophyll recovery from three determinations, expressed as percent chlorophyll recovery at zero time, was calculated.

Chlorophyll bleaching using Holden's system

A variety 035 pepper, with about one-fourth of the surface orange, was cut into small pieces after removing the stem and seeds. Forty grams of tissue were ground in a mortar with 120 ml of acetate buffer pH 5.9. This extract was centrifuged for 10 min at 5,000 g. The supernatant was used as the enzyme extract. The substrate consisted of 0.35 mg chlorophyll *a* dissolved in 0.3 ml acetone. Sufficient acetate buffer pH 5.9 containing 0.2 triton X-100 was added to suspend the chlorophyll and give a final sample volume of 25 ml. Either 1 ml of 0.5% linoleic acid in ethanol or 1 ml ethanol was added to the suspension. The reaction was initiated by adding enzyme extract and vigorously stirring the reaction mixture for 2 min.

The reaction was stopped after 15 min by adding 25 ml acetone. The mixture was transferred to a separatory funnel, and 25 ml petroleum ether was added. The funnel was agitated until the chlorophyll *a* was transferred to the petroleum ether phase. The acetone-water phase was discarded. After several washings with water, the petroleum ether was evaporated to dryness. The chlorophyll was made to 25 ml with 80% acetone, and the remaining chlorophyll was determined.

Time study of chlorophyll degradation

Twelve 035 peppers were each injected with 0.26 mg ^{14}C-chlorophyll *a* suspended in 5 ml 0.2% triton X-100. All peppers were at the same stage of ripeness with a small portion of the peppers orange-colored, indicating the beginning of ripening. The peppers were incubated in the dark at 26 to 27°C and sampled at six different times. One pepper showed irregular ripening and was discarded.

The peppers were extracted according to the procedure given above. The petroleum ether extract was dried and made to 25 ml with acetone, and the recovery of the injected chlorophyll *a* was determined. The acetone-water extract and 80% ethanol extract were made to 10 ml, and the residue after 80% ethanol extraction was dried and weighed before radioactivity determinations.

A Nuclear Chicago low background planchet counter was used for radio-activity measurements.

The average counting rate of five determinations on a known amount of sample was calculated for all samples. The residue was a powdery material; therefore, to prevent contamination of the counting chamber, the planchets were covered with a thin plastic wrap. The average loss of counts caused by the plastic wrap was determined to be 44% by counting several samples of chlorophyll *a* before and after covering the planchets. The counting rate obtained from the residue samples was adjusted to correct for this loss.

Protein determination

The protein content of the residue fractions was determined using the method of Lowry *et al.* (1951). Casein was used for the standard curve. Protein from approximately 1.5 to 2.5 mg of residue was dissolved in 0.4 ml of NaOH for 45 min before determination.

RESULTS AND DISCUSSION

THE DATA in Table 1 demonstrate the characteristics of the degradation. Variety 035 peppers when injected at an early ripening stage degraded a significant portion of the injected chlorophyll *a*. Variety 044 peppers degraded only a small portion of the injected chlorophyll. When injected at later stages of ripening, both varieties caused very small chlorophyll losses, indicating that fully ripe peppers are no longer capable of carrying on chlorophyll degradation. When a ripe pepper was injected with chlorophyll *a* and immediately extracted, only a 2% loss of chlorophyll was observed. This shows that the losses observed in the 035 peppers injected at the beginning of ripening did not occur during the extraction procedure. The fact that the amount of degradation depended upon the pepper variety and that chlorophyll *a* could be left inside a ripe pepper for 5 days

Table 1. Degradation of chlorophyll *a* by two varieties of peppers during ripening in darkness.

Pepper variety	Stage of ripening at injection and extraction[1]	Percent degradation of injected chlorophyll *a*
035	1	46
035	1	32
044	1	15
044	1	4
035	3	10
035	2	6
044	2	6
044	4	2

[1] 1. Injected at early ripening stage; extracted at completion of ripening.
2. Injected when ripe; extracted after 5 days.
3. Injected at 2/3 ripeness; extracted at completion of ripening.
4. Injected when ripe; extracted immediately after injection.

with little degradation indicated that the degradation was not a result of air oxidation of chlorophyll dried on the endocarp of the pepper.

The pH of the juice squeezed from a green 035 pepper is approximately 5.5. Therefore, it seemed a reasonable possibility that the observed breakdown was caused by some acid-catalyzed reaction and did not depend upon enzymatic activity in the pepper.

The results in Fig. 2 show that a significant chlorophyll loss is only observed after 11 days, and the 7% loss observed is not nearly as large as the degradation observed in the peppers.

Another reason for the chlorophyll degradation observed in peppers could have been a reaction caused by enzymatic lipid oxidation (Holden, 1965). If this were true, it would indicate that the degradation was a result of tissue injury. In this case, Holden's degradative system would probably be a better model system with which to study the reaction. This possibility was tested by substituting an extract of tissue from a ripening pepper for the soybean extract in her system. The details of this experiment are described above.

The chlorophyll loss, expressed as percent of the control which contained neither enzyme extract nor linoleic acid, was only 3% for a 15 minute incubation and is considered to be within experimental error. Holden observed a 69% chlorophyll degradation during 2-min incubation when soybean extract was used. If such a chlorophyll-bleaching system exists in pepper tissue, it is either much less active than the soybean enzyme, or it is active under different conditions. Blain (1953) showed that carotenoids are also bleached under these conditions. Since a large increase in carotenoids is observed during ripening of peppers (Curl, 1964), this is one argument against such a bleaching system being active in the tissue. It was concluded that the observed degradation of the injected chlorophyll probably occurs by some pathway other than that in Holden's bleaching system.

Information about the products derived from the chlorophyll degraded by the peppers could be obtained using labeled chlorophyll. Chlorophyll *a*, assumed to be randomly labeled with carbon-14 (Perkins *et al.*, 1962), was prepared and injected into twelve 035 peppers. The chlorophyll recovery, determined by spectrophotometric measurement expressed as percent recovery of injected chlorophyll, is shown in Fig. 3.

At day zero the peppers were just beginning to ripen, as evidenced by a small orange spot on the pepper. This variety of peppers had only a small amount of chlorophyll naturally present in the tissue, but enough was present to give recoveries of greater than 100% at zero and two days. By day five the peppers were orange, and nearly all endogenous chlorophyll had disappeared. At day fourteen the peppers were dark red and beginning to shrivel.

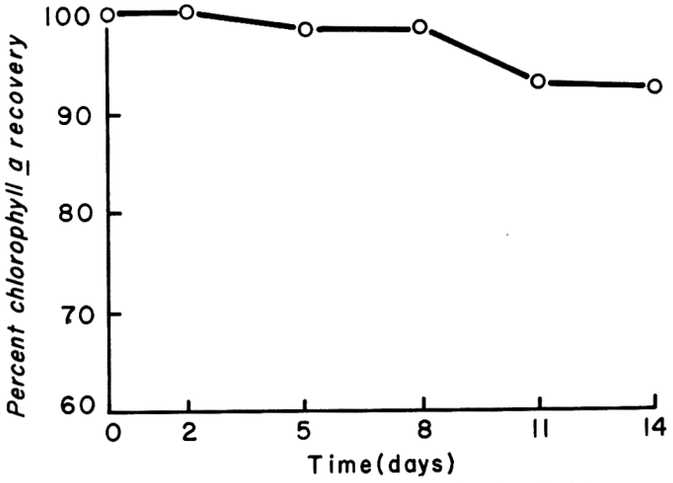

Fig. 2. Recovery of chlorophyll a suspended in pH 5.4 acetate buffer expressed as percent chlorophyll content at zero time.

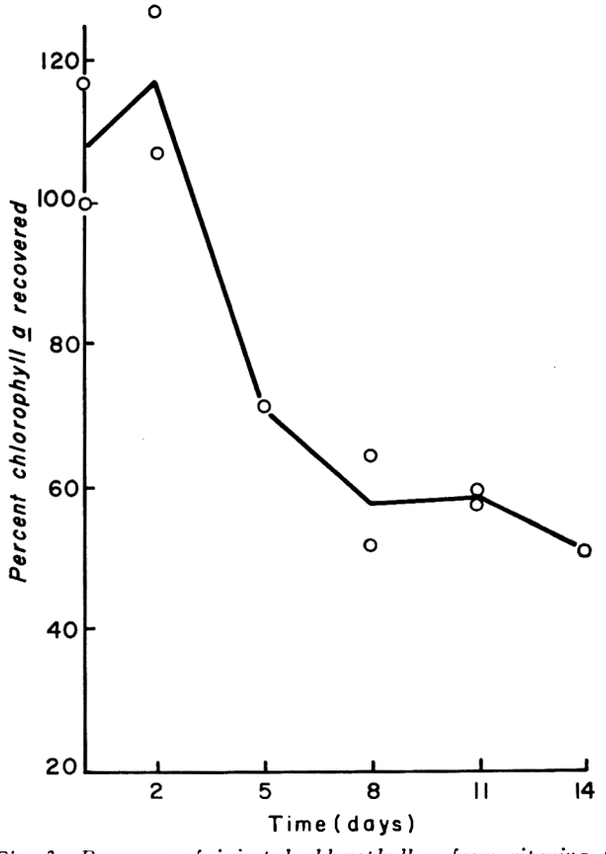

Fig. 3. Recovery of injected chlorophyll a from ripening 035 peppers as determined spectrophotometrically.

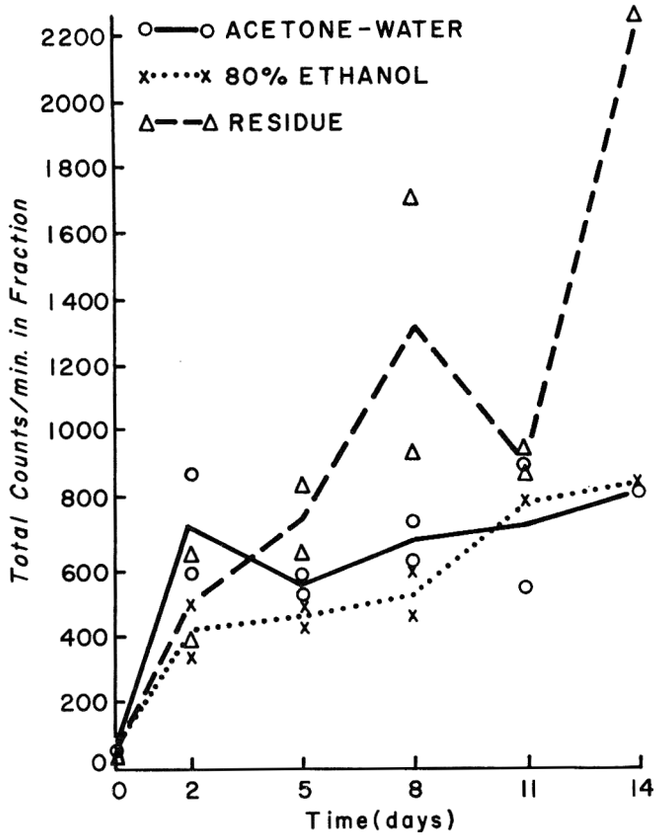

Fig. 4. The distribution of ^{14}C from injected ^{14}C-chlorophyll a in fractions prepared from ripening peppers.

Fig. 4 gives the total counts per minute of the acetone-water, 80% ethanol, and residue fractions.

The counting procedures were subject to errors which did not allow a quantitative measurement of activity. The residues were powdery, and the size of the particles varied with the ripeness of the pepper. Therefore, there was undoubtedly some loss from sample self-absorption. The same problem of self-absorption was also present for the acetone-water and 80% ethanol extracts. However, much less material was on the planchets for these extracts than for the residue, so the self-absorption was probably much less. Therefore, the curves in Fig. 4 give an estimate of the relative incorporation of activity into the fractions with time, but the absolute incorporation is likely to be somewhat greater, especially in the residue fractions.

The combined activity in the acetone-water, 80% ethanol, and residue fractions in general is considerably less than would be expected if all the products formed from the chlorophyll *a*, which disappeared in the spectrophotometric determination, had been extracted in these fractions. Therefore, it appears that either activity was lost as $^{14}CO_2$ or some lipid soluble product(s) was formed which remained in the petroleum ether extract.

The activity in the acetone-water extract increased rapidly after injection and remained relatively constant after the second day. This fraction contains components of the pepper which are soluble in 90% acetone, but are hydrophilic enough not to be transferred to the petroleum ether layer. The 80% ethanol extract contains the material from the pepper which is insoluble in 90% acetone, but soluble in boiling 80% ethanol. The activity of this fraction increases throughout the ripening period. The residue, after hot 80% ethanol extraction, contains protein and polysaccharide material. An iodine test indicated the presence of little starch, especially at the later ripening stages.

The protein content, determined by the Lowry method, for each residue is shown in Fig. 5. The period of protein increase in the residue corresponds quite closely to the period of most rapid chlorophyll degradation (see Fig. 3). The chlorophyll degradation nearly stopped after 8 days when the loss of protein becomes rapid. However, increase of radioactivity in the residue fraction continues during the period of protein loss. Enzymes for conversion of chlorophyll degradation products to substances which remain in the residue must be more stable than enzymes which are involved in the initial steps of the degradation.

The next step in the study of this system is to isolate and identify the products formed from chlorophyll *a*. Some attempts have been made to isolate products from the petroleum ether and acetone-water extracts by thin-layer chromatography with the methods of Co *et al.* (1967a), Hager (1962), Schneider (1965), and Bacon (1965). However, it has not been possible to isolate distinct radioactive products, other than pheophytin *a*, from the petroleum ether extract. Except for one instance which could not be repeated, when copper pheophorbide *a* was obtained from an acetone-water extract, no radioactive products were isolated from this fraction.

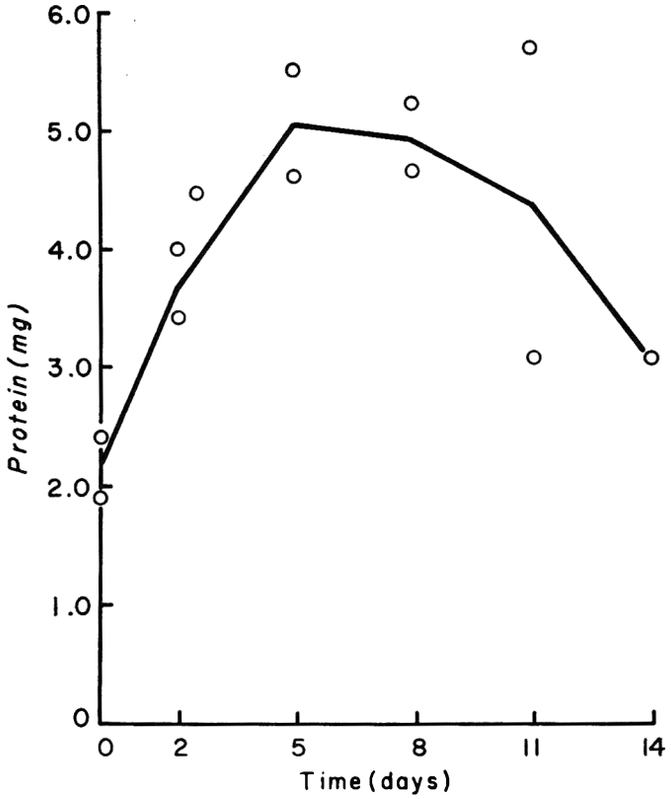

Fig. 5. Protein content of residue fractions during ripening of variety 035 peppers injected with ^{14}C-chlorophyll a. The content is expressed in mg/g fresh weight of the pepper at the time of picking.

There are two explanations for the inability to find distinct radioactive bands. Either the methods used were not appropriate for separation of the chlorophyll degradation products formed, or the plates were too overloaded to give a good separation. The amount of carotenoids in ripe pepper extracts is much greater than the amount of injected chlorophyll. In order to apply enough activity onto a chromatogram so that minor components would be detectable, it was necessary to overload the chromatograms with carotenoids. No work has been done to isolate products from the 80% ethanol extract or the residue fraction.

The data presented are consistent with the theory that the observed degradation was accomplished by a physiologically important mechanism.

This system permits a more direct study of the degradation of chlorophyll than was previously possible. The identification of small quantities of unknown compounds is still a problem, but the use of labeled chlorophyll makes isolation and proof of their origin from chlorophyll considerably easier.

Proof that this is a physiological system will have to wait until some compound, which is formed from the exogenous chlorophyll, is demonstrated *in vivo,* and its appearance is correlated with the loss of endogenous chlorophyll.

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