November-December 1968
Volume 33 : Number 6

JOURNAL of FOOD SCIENCE

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AN OFFICIAL PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS

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of FOOD SCIENCE

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BROILING, SEX AND INTERRELATIONSHIPS WITH CARCASS AND GROWTH CHARACTERISTICS AND THEIR EFFECT ON THE NEUTRAL AND PHOSPHOLIPID FATTY ACIDS OF THE BOVINE LONGISSIMUS DORSI. R. N. TERRELL, G. G. SUESS, R. G. CASSENS & R. W. BRAY. *J. Food Sci.* **33**, 562–565 (1968)—Phospholipid fraction fatty acids were affected more by broiling than were neutral fraction fatty acids. Steers had a larger percentage of C16 and C18 and a smaller percentage of C18:1 than heifers. Neutral fraction acids (C18, C18:1 and C18:2) were significantly correlated with lipid prosphorus, cholesterol, % fat trim (retail), estimated % carcass fat and estimated % carcaass lean. Low, nonsignificant correlations were found among individual fatty acids from the neutral and phospholipid fractions of bovine longissimus dorsi muscles with tenderness and juiciness scores.

MUSCLE PROTEIN COMPOSITION AND EATING QUALITY OF FRESH AND FROZEN TURKEYS. I. M. HOKE, B. K. McGEARY & F. LAKSHMANAN. *J. Food Sci.* **33**, 566–571 (1968)—Fifty-four Beltsville small white turkeys were studied fresh-unfrozen and after 5 and 10 months storage at −5°F. Quantitative changes in proteins separated from extracts made with KCl-borate buffer or with de-ionized water indicated a decrease in actomyosin nitrogen of the pectoralis major muscle and some proteolytic changes after 10 months frozen storage. Frozen-thawed turkeys required longer cooking times to reach internal temperature end-points than fresh-unfrozen turkeys. Higher moisture losses and more tender, mealy thigh meat from frozen turkeys appeared to be due to these longer cooking times rather than to quantitative changes in muscle proteins.

THE FORMATION OF CARBONYL COMPOUNDS IN CUCUMBERS. H. P. FLEMING, W. Y. COBB, J. L. ETCHELLS & T. A. BELL. *J. Food Sci.* **33**, 572–576 (1968)—A large increase in the formation of carbonyl compounds occurred when fresh cucumbers were blended with water in the presence of oxygen. The most significant increase occurred in the formation of 2,6-nonadienal, the aldehyde largely responsible for the flavor of fresh cucumbers. The evidence indicated that the characteristic flavor components of fresh cucumbers are generated enzymatically as a consequence of cutting or mechanically rupturing the fruit.

EFFECT OF LOW TEMPERATURE ON STRUCTURE AND FIRMNESS OF APPLE TISSUE. C. STERLING. *J. Food Sci.* **33**, 577–580 (1968)—Apple tissue was frozen at different temperatures and in different solutions, thawed, examined microscopically, and tested for resistance to compression (firmness). In slow freezing, ice crystals separate the cells, crush them and rupture cell walls. The firmness of frozen tissue is much lower than that of raw tissue, largely because of loss of turgor. Added solutes generally minimize ice-caused damage. The more rapid the rate of freezing (to lower temperatures) the firmer is the tissue and the less the amount of tissue disruption.

PHOTOXIDATION OF CHOLESTEROL IN SPRAY-DRIED EGG YOLK UPON IRRADIATION. E. CHICOYE, W. D. POWRIE & O. FENNEMA. *J. Food Sci.* **33**, 581–587 (1968)—Irradiation of yolk solids by radiant energy from either a 40-watt fluorescent lamp (about 280 hr) or summer sunlight (5 hr) caused the formation of at least five photoxidation derivatives of cholesterol as shown by TLC and GLC. The major reaction products were identified as 7-ketocholesterol, 7α- and 7β-hydroxycholesterols, cholesterol-5β,6β-oxide and cholestane-3β,5α,6β-triol. Insignificant amounts of these compounds were present in fresh yolk and unirradiated spray-dried yolk.

CELLULASE ACTIVITY DURING THE MATURATION AND RIPENING OF TOMATO FRUIT. G. E. HOBSON. *J. Food Sci.* **33**, 588–592 (1968)—An enzyme that attacks carboxymethyl cellulose may be extracted efficiently from tomato fruit by salt solutions. From a high initial value in small green fruit, activity fell gradually during fruit swelling. With incipient ripeness, the activity increased again and continued to rise to the full red condition. The green areas of "blotchy" ripened fruit showed 40% less activity than the adjacent red tissue. Fruit of tomato species in the sub-genus *Eriopersicon* contained considerably more activity than examples from the sub-genus *Eulycopersicon*. Firmness measurements on fruit from both sub-genera were not significantly correlated with the cellulase activities.

ANTHOCYANIN PIGMENTS OF RHUBARB, CANADA RED. R. E. WROLSTAD & D. A. HEATHERBELL. *J. Food Sci.* **33**, 592–594 (1968)—The anthocyanin pigments of rhubarb (*Rheum rhaponticum* 'Canada Red') were extracted with 0.02% methanolic HCl and partially purified by use of cation-exchange resin. The pigments were separated into two bands with paper and cellulose thin-layer chromatography. The purified pigments were characterized by their R_f values, partial acid hydrolysis, identification of the aglycones and sugars after complete hydrolysis, and their spectral properties. The main pigment (87% of the total anthocyanins) was identified as cyanidin-3-glucoside and the second (13%) is proposed to be cyanidin-3-rutinoside.

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SEPARATING AND ISOLATING AROMA AND FLAVOR CONSTITUENTS OF ROASTED PEANUTS. B. A. BROWN, K. S. KONIGSBACHER, F. E. ELLISON & G. E. MANN. *J. Food Sci.* **33**, 595–598 (1968)—A technique was developed for the extraction of quantities of peanuts and for the separation of those extracts into fractions of different chemical categories, each of which had a distinct aroma. Twelve acids were identified; the presence of hexanal, 2,4-decadienal and β-sitosterol was confirmed; and evidence was gathered that indicates the presence of aliphatic lactones. In addition, 2-oxooctanal and a dihydroxynaphthaleneacetic acid were also found to be present. Gas chromatographic examination of the acid fractions from three varieties of peanuts showed some qualitative and quantitative differences.

CHARACTERIZATION OF WATER-SOLUBLE WHEAT FLOUR PENTOSANS. F. M. LIN & Y. POMERANZ. *J. Food Sci.* **33**, 599–606 (1968)—The yield and protein contents of water-soluble pentosan preparations ranged from 0.67 to 0.84% and 15.4 to 24.2%, respectively. After soluble-starch was removed, total yield of water-soluble gums was 0.38 to 0.58%, and their protein contents ranged from 16.9 to 22.6%. Pentosans from six flours were fractionated on diethyl aminoethyl-(DEAE) cellulose columns into five fractions. Based on carbohydrate contents, fraction II, eluted with 0.0025 *M* borate was the largest; a substantial amount was eluted with 0.5 *N* NaOH (fraction V). Fraction I (eluted with water) was generally the smallest and contained no protein. Fraction III (eluted with 0.025 *M* borate) was the second smallest and was richest in protein.

CYSTEINE INHIBITION OF ENZYMATIC BLACKENING WITH POLYPHENOL OXIDASE FROM POTATOES. P. MUNETA & J. WALRADT. *J. Food Sci.* **33**, 606–608 (1968)—The initial site of cysteine inhibition of enzymatic blackening caused by tyrosine oxidation occurred at the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (dopa). Concentrations of cysteine (9.5×10^{-4}M) which inhibited tyrosine oxidation for 100 min did not inhibit dopa oxidation significantly. Higher concentrations (1.9×10^{-2}M) inhibited dopa oxidation. Cysteine did not inhibit chlorogenic acid oxidation under the conditions of our study. Cysteine did not inhibit tyrosine oxidation in the presence of chlorogenic acid.

POST-MORTEM CHANGES IN EXTRACTABILITY OF MYOFIBRILLAR PROTEIN FROM CHICKEN PECTORALIS. R. N. SAYRE. *J. Food Sci.* **33**, 609–612 (1968)—Myosin, actomyosin and other nitrogenous fractions of chicken pectoralis were extracted from muscle aged in ice for various periods from 30 min to 24 hr post-mortem. Sarcoplasmic protein, non-protein nitrogen and stromal protein were extracted at constant levels for all aging periods. Myosin extractability decreased rapidly during the first 3–4 hr post-mortem while actomyosin was extracted at a low, constant level. After 4–6 hr, actomyosin extractability began to increase as myosin extractability continued to decline. The initial loss of myofibrillar protein solubility and subsequent release of actomyosin corresponds with the time course of toughening and post-rigor tenderization in chicken muscle.

TENDERNESS AND MATURITY IN RELATION TO CERTAIN MUSCLE COMPONENTS OF WHITE LEGHORN FOWL. R. M. WANGEN & J. H. SKALA. *J. Food Sci.* **33**, 613–616 (1968)—Shear values of roasted biceps femoris increased significantly with maturity particularly between 12 and 18 months of age. Shear values of stewed thigh muscle did not change appreciably with age and were significantly lower at 12 and 18 months of age. Tenderness of pectoralis major did not vary appreciably among ages or between cooking methods. Total and residual hydroxyproline content of roasted biceps femoris were significantly correlated with shear values ($r = 0.64$ and 0.79, $P < 0.01$). The residual hydroxyproline values, which differed significantly at 18 months of age (roasted muscle higher, $P < 0.01$) while total did not, seemed to explain more satisfactorily the divergence in shear values.

PARTIAL PURIFICATION OF SALMON MUSCLE CATHEPSINS. CHAO-YUN TING, M. W. MONTGOMERY & A. F. ANGLEMIER. *J. Food Sci.* **33**, 617–621 (1968)—Salmon muscle cathepsins, which showed optimal activity at pH 3.7 with hemoglobin as substrate, were purified. Catheptic activity was extracted with 0.2 *N* KCl. After precipitation of inactive proteins at pH 5.5, further purification was obtained by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. Salmon muscle cathepsins were fractionated into two components.

ADSORPTION OF VOLATILE ORGANIC COMPOUNDS IN DEHYDRATED FOOD SYSTEMS. 1. Measurement of Sorption Isotherms at Low Water Activities. P. ISSENBERG, G. GREENSTEIN & M. BOŠKOVIĆ. *J. Food Sci.* **33**, 621–625 (1968)—Removal of water vapor during food dehydration results in losses of volatile compounds that contribute to flavor. A frontal analysis gas chromatographic apparatus has been developed to measure vapor-solid adsorption at low adsorbate concentrations. Volatile compound is sorbed from the gas stream by an adsorbent in a system designed to minimize diffusion and kinetic effects. The system includes saturator chambers, which maintain constant concentration of adsorbate in the carrier gas stream, and a flame ionization detector.

POLYCYCLIC HYDROCARBON COMPOSITION OF WOOD SMOKE. KI SOON RHEE & L. J. BRATZLER. *J. Food Sci.* **33**, 626–632 (1968)—Eleven polycyclic hydrocarbons were isolated and separated stepwise by liquid-liquid extraction, chromatography on silicic acid, thin-layer chromatography with acetylated cellulose powder and chromatography on aluminum oxide. They were characterized by ultraviolet and fluorescence studies on the fractions obtained from the aluminum oxide column. Polycyclic hydrocarbons found in the hardwood sawdust smoke include naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, 1,2-benzanthracene, chrysene, 3,4-benzopyrene and 12-benzopyrene.

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THE VOLATILE ALCOHOLS OF RIPE BANANAS. K. E. MURRAY, J. K. PALMER, F. B. WHITFIELD, B. H. KENNETT & G. STANLEY. *J. Food Sci.* **33**, 632–634 (1968)—The following 13 alcohols were identified by gas chromatography-mass spectrometry supported in some cases by IR spectral data: ethanol, propan-1-ol, 2-methylpropan-1-ol, butan-1-ol, pentan-2-ol, 3-methylbutan-1-ol, hexan-1-ol, heptan-2-ol, *cis* and *trans* hex-3-en-1-ol, *cis* and *trans* hex-4-en-1-ol and *cis* pent-2-en-1-ol (tentative). 2-Methylbutan-1-ol was shown to be associated with 3-methylbutan-1-ol in a ratio of 1:200.

PETALS OF AZTEC MARIGOLD, *TAGETES ERECTA*, AS A SOURCE OF PIGMENT FOR AVIAN SPECIES. A. U. ALAM, C. R. CREGER & J. R. COUCH. *J. Food Sci.* **33**, 635–636 (1968)—The deposition of the pigments of Aztec marigold petal was determined in the egg and tissue of laying hens fed 33.66 and 99 mg of the pigments per kg of low pigment diet. Thirty-three mg pigment per kg of feed produced yolk color which is considered acceptable to the consumer. With an increase in the amount of pigment in the feed, color deposition in the organs increased, but efficiency of utilization was lowered.

ROLE OF FLAVIN ADENINE DINUCLEOTIDE IN STABILIZATION OF CYTOSKELETON OF CHICKEN MUSCLE CELL. D. W. STANLEY, F. M. SAWYER & H. O. HULTIN. *J. Food Sci.* **33**, 637–641 (1968)—It is suggested that aging of muscle is accompanied by a breakdown of the cytoskeleton. This paper reports a detailed study of the stabilization of the cytoskeleton of chicken breast muscle by flavin adenine dinucleotide (FAD), a compound previously suggested for this role. No significant differences were found in FAD decomposition or extraction between samples handled in such a manner to produce very large differences in the extent of emptying, the measure of cytoskeletal breakdown. Similarly, adding FAD to suspensions of muscle cell segments could not inhibit emptying under conditions where the supernatant fraction of a muscle homogenate could. It was concluded that FAD plays no role in the stabilization of the cytoskeleton of chicken breast muscle.

LOW TEMPERATURE GROWTH OF SALMONELLA. J. R. MATCHES & J. LISTON. *J. Food Sci.* **33**, 641–645 (1968)—Salmonellae, which are normally incubated at mesophilic temperatures, were shown to increase in numbers at temperatures below 10°C. When grown on agar in a temperature-gradient incubator, the minimum growth temperatures obtained for serotypes ranged from 5.5 to 6.8°C. When grown in broth in a polythermostat over a temperature range of 1.1 to 12.3°C, the minimum growth temperatures obtained for *S. heidelberg*, *S. typhimurium*, and *S. derby* were 5.3, 6.2, and 6.9°C respectively. The results of growth at several temperatures indicate a growth temperature shift during extended incubation of *Salmonella* at low temperature.

DYNAMIC VISCOELASTIC CHARACTERIZATION OF SOLID FOOD MATERIALS. C. T. MORROW & N. N. MOHSENIN. *J. Food Sci.* **33**, 646–651 (1968)—Engineering parameters for the characterization of mechanical response of food materials used in processing or direct consumption are reviewed. Dynamic methods currently available—direct stress-strain measurements, transducer methods, resonance methods and wave propagation—are discussed. A relation between the structural mechanics of food material and the observed mechanical behavior appears to be desirable both as an aid in quality control and as a guide to the development of synthetic products.

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Carbonyls in Oxidizing Fat. 11. The Effect of the Pro-oxidant Activity of Sodium Chloride on Pork Tissue

SUMMARY—Oxidation of freezer-stored, sodium chloride-cured pork was characterized by a rapid rate and moderately high monocarbonyl/peroxide ratios. Increase in the concentration of NaCl accelerated autoxidation, but did not affect hydroperoxide decomposition to monocarbonyl compounds. High proportions of lean increased autoxidation and the monocarbonyl-peroxide ratios. Sodium nitrite (0.03%) catalyzed autoxidation by reaction with the meat pigment in an apparently independent effect to that exerted by NaCl. Composition of the free monocarbonyl compounds indicated linoleate specificity in peroxide decomposition, although hematin catalysis is nonspecific in its attack on unsaturated fatty acids. Possible direct effects of NaCl did not appear to involve a reactive chlorice ion.

INTRODUCTION

THE PRO-OXIDANT EFFECT of NaCl on the triglycerides in meat is one of the most puzzling influences in food science. Observations reported sometimes seem contradictory, and it is possible that a number of factors can operate jointly. Banks (1937) and Lea (1939) suggested that NaCl was not itself a pro-oxidant, but promoted the activity of lipoxidase in meats. However, Banks (1944) and Tappel (1952, 1953) showed that there is no lipoxidase in meat, and it was indicated that the activity promoting autoxidation was due to heme pigments. Separately, the pigments of meat are powerful catalysts of fatty acid and triglyceride autoxidation. However, freezer-stored fresh meat is relatively stable despite the presence of such catalysts.

There is no evidence of a direct specific effect by NaCl on oxidation catalyzed by myoglobin or hemoglobin. Chang *et al.* (1950) found NaCl did not accelerate oxidation being promoted by clear meat extracts or hemoglobin solutions. However, Lea (1939) observed a pro-oxidant effect of NaCl on oxidation caused by expressed meat juice. In this instance, salt may have acted on the tissue cells or fibers in the press fluid.

NaCl has a powerful influence on meat proteins and pigments (Coleman, 1951; Grant, 1956). Gibbons *et al.* (1951) reported that rancidity of bacon sides developed more rapidly as freezer storage temperatures were lowered. Chang *et al.* (1950) observed a direct pro-oxidant effect by 15% or higher NaCl solutions or dry salts where a large surface of contact with lard existed. It was concluded that the freezing process might be similar to a dehydration process. As an example of the effect of physical conditions, Mabrouk *et al.* (1960) found that autoxidation of aqueous emulsions of methyl linoleate were inhibited with increase in dissolved NaCl.

This work was undertaken to determine the monocarbonyl compounds formed by NaCl promoted autoxidation of pork triglycerides. It was also expected that information might be obtained concerning the mechanisms involved in this rancidity problem.

EXPERIMENTAL

PAIRED BACON SIDES were each divided into six segments as described by Gaddis (1952) to give mated samples. Mixtures of ground pork lean and back fat furnished homogenous material for selected studies and experimental setups. After dry curing with analytical grade salts, the bacon segments were sliced (commercial thickness) and stored at 0°F.

Fat for chemical examination was obtained by warming ground experimental samples to 40–50°F and immediately pressing and filtering through cheesecloth. The resulting clear fat or lard was used for monocarbonyl and peroxide determinations. The purpose of this study was to examine the intermuscular fat or triglycerides. If oxidation of polar lipids has occurred, volatile breakdown products might be expected to have diffused into the fat. This procedure of fat separation was used because solvent extraction necessitates the use of methods of carbonyl isolation which break down hydroperoxides (Gaddis *et al.*, 1965, 1966b). The very mild rendering procedure used causes little change in hydroperoxides and free monocarbonyls.

Free volatile carbonyl compounds were isolated from 10 g samples by the vacuum distillation method of Lea *et al.* (1962) as modified by Gaddis *et al.* (1960) and Gaddis *et al.* (1965). The volatile carbonyls were converted to 2,4-dinitrophenylhydrazone derivatives, and the monocarbonyl hydrazone fraction was separated by chromatography on hydrated alumina as described by Gaddis *et al.* (1959a). Monocarbonyl hydrazones were separated into alkanal-alk-2-enal, and alk-2,4-dienal classes by the method of Gaddis *et al.* (1959b). From this resolution, data enabled the determination of proportions of the classes of monocarbonyl compounds. Each class was separated into individual compounds and the compounds were estimated as described by Ellis *et al.* (1959).

Peroxides were determined by the method of Kenaston *et al.* (1955) and expressed as meq/100 g.

RESULTS AND DISCUSSION

Some characteristics of stored, cured pork

Preliminary experiments indicated that NaCl-cured, freezer-stored pork fatty tissue had much higher monocarbonyl/peroxide ratios than observed in previous oxidation studies with lard. Uncured, freezer-stored pork developed peroxides very slowly. Extensive hydroperoxide breakdown to carbonyl compounds with propagation of autoxidation is a characteristic of hematin catalysis (Tappel, 1955). Zipser *et al.* (1964) have commented on the effect of strong peroxide decomposers on TBA number/peroxide ratios. The degree of spontaneous hydroperoxide breakdown to monocarbonyls would appear of possible value in detecting changes in and judging the type of prooxidant activity involved.

Fat tissue well separated from lean in stored bacon slices (NaCl-cured) showed accelerated oxidation, but fat immediately adjacent to the lean streaks had somewhat greater peroxide values and much higher monocarbonyl values and percentage of alkanal compounds. The data suggested that the NaCl-cured lean had a special effect on tissue fat oxidation.

Paired 2% and 6% NaCl-cured bacon segments from one hog were compared after 0°F storage at intervals up to 139 days. Autoxidation was rapid in the cured samples. Uncured samples showed hardly perceptible oxidation during the 139 days of storage. Although significant differences were evident, oxidation progression was not smooth due to the wide variation in composition of the cuts.

A mixture of ground back fat and lean in 1 : 1 proportions treated with 2, 4 and 6% NaCl had fairly smooth progression in oxidation when stored at 0°F for 20, 40, 57, 90 and 110 days. Degree of oxidation was directly related to the NaCl content. Effect of NaCl concentration on measurements representative of autoxidative characteristics is shown in Table 1. No consistent differences in monocarbonyl/peroxide and alk-2-enal/alk-2,4-dienal ratio were found. The former ratio has already been discussed as an indication of degree of peroxide breakdown and type of catalytic activity. This result could be construed to indicate that the acceleration of oxidation due to increase in NaCl was not due to augmented catalytic action. Possibly this might be a clue to the direct effect of NaCl that has been suggested.

Table 1. Effect of NaCl concentration on autoxidation characteristics.

1:1 Back fat-lean-mixture 0°	Monocarbonyl μm/10 g	Ratios: Monocarbonyl to PV	Ratios: Enal to dienal
90 Days			
2% NaCl	2.0	0.1	1.0
4% NaCl	2.3	0.2	1.0
6% NaCl	2.9	0.1	0.9
110 Days			
2% NaCl	2.7	0.2	1.1
4% NaCl	2.7	0.1	0.9
6% NaCl	3.4	0.1	1.1

In this experiment, individual alkanals C_2, C_3, C_6 and C_9; alk-2-enals C_7, C_8, C_9, C_{10} and C_{11}; and alk-2,4-dienals C_7, C_9 and C_{10} were detected and measured. No important differences due to NaCl concentration were noted. C_8 Alkanal, characteristic of oleate, was not detected. There was an increase in proportion of alkanals with NaCl concentration and degree of autoxidation. Such an increase as oxidation progresses has been commonly noted (Ellis *et al.*, 1959).

Effect of proportion of lean

The influence of amount of lean at constant (4%) NaCl content was examined next. As shown in Table 2, the rate of autoxidation increased sharply with the amount of lean in the mixtures. The monocarbonyl/peroxide ratio tended to increase with the lean. A change in this ratio could mean differences in type of catalytic activity. This catalysis might be due to increase in heme pigments or some other active component in the salt-treated lean. There was a trend toward a decrease in the enal-dienal ratio. This might mean a change in the linoleate hydroperoxide isomers, more breakdown of dienal precursors, or simply a greater rate of oxidation of linoleate (Gaddis *et al.*, 1966b).

Table 2. Effect of proportions of lean on oxidation characteristics and alkanal composition of back fat-lean mixtures stored 90 days at 0°F.

	% Lean			
	10	20	50	70
Monocarbonyls μM 10 g	1.6	1.9	2.3	5.1
Ratio—monocarbonyls to PV's	0.11	0.14	0.16	0.16
Ratio—enals to dienals	1.4	1.1	1.0	1.2
% Alkanals				
C_2	6	3	5	3
C_3	8	5	4	tr
C_6	58	67	72	77
C_9	5	4	3	tr
Total	77	79	84	80

Table 2 shows that increase in salted lean concentration changed the quantitative composition of the alkanals. C_6 Alkanal increased nearly 20%, and there were definite decreases in C_3 and C_9 alkanals. However, increases in total alkanals were small. The effect of increased cured lean appeared to consist mostly in a selective action toward greater autoxidation of linoleate or breakdown of linoleate hydroperoxides. This seems significant because oleate is by far the major unsaturated fatty acid in pork fat; linoleate amounts to only 5–10% of the total. Tappel (1955) has emphasized that hematin catalysis is non-specific, and that rates are a function of the unsaturation of the lipid as in simple autoxidation.

Mixtures of the above lean-back fat proportions without NaCl had peroxide values in the region of about 17 after 365 days at 0°F. There was only a very small effect of amount of lean on degree of oxidation. Monocarbonyl/peroxide ratios were much higher than those of the salt-treated samples. There was scarcely an appreciable effect

of concentration of lean on the ratios, which were on about the same level as values found for uncured bacon fat (Table 3). The proportion of alkanals was similar to that usually observed at that level of autoxidation. These data indicate significant differences in oxidation characteristics, presumably due to type of catalysis, between cured and uncured pork tissue triglycerides.

Simple and catalyzed autoxidation

The oxidation of ground back fat containing 6% NaCl was compared with 1:1 back fat-lean portions containing 2% and 6% NaCl when stored at 0°F for 26, 42, 58, 93 and 112 days. Autoxidation progressed smoothly in all three groups of samples. Peroxide formation was rapid in the cured back fat samples. In Table 3, oxidation characteristics are compared with similar data for uncured bacon fat and rendered lard (Gaddis *et al.*, 1966b). Significant differences in degree and type of catalysis seem to be indicated by the wide range in the ratios of the four kinds of samples. Lard, presumably nearly free of catalysts, had a very low proportion of alk-2,4-dienals and a low yield of monocarbonyls.

Uncured bacon tissue triglycerides which contained potential catalysts had a very high conversion of hydroperoxides to monocarbonyls and also a low proportion of alk-2,4-dienals. The previously mentioned unsalted samples containing different levels of lean showed little effect of composition on the ratios.

NaCl had a powerful influence on the amount and rate of autoxidation in the lean-containing samples. The effect of salt on ratios resulted in a lower conversion of hydroperoxides to monocarbonyls and increase in proportion of alk-2,4-dienals. This suggests activation by NaCl of a component in the lean that changed the oxidation characteristics of pork adipose tissue. Production of more dienals may be related to changes in hydroperoxide isomer equilibrium. However, it is most likely due to increased rate of oxidation of linoleate or breakdown of its hydroperoxides or alternatively autoxidation of polyunsaturated fatty acids of the polar lipids. Dienal formation has been considered in earlier publications (Gaddis *et al.*, 1966a), and it is otherwise usually increased by heat effect on the hydroperoxides (Gaddis *et al.*, 1959a).

Differences in individual alkanals were examined (Table 4). The lard, an example of uncatalyzed autoxidation (Gaddis *et al.*, 1966b), had much lower proportions of C_6 alkanal (linoleate) and higher proportions of C_3 alkanal (linolenate) and C_8 and C_9 alkanals (oleate). C_8 Alkanal was not detected in the cured samples, and C_3, C_8 and C_9 alkanals were not present in the uncured bacon. The cured back fat was intermediate in proportion of C_6 alkanal. The wide differences in C_6 alkanal between lard, cured back fat-lean, and uncured bacon do not appear to agree with Tappel's non-specific effect of heme catalysis (Tappel, 1955).

These results indicate a selective action in the oxidation of linoleic acid, if only triglycerides are involved. However, this could be due partially to a specific linoleate hydroperoxide decomposition to C_6 alkanal by hematin. Hematin catalysis progresses by hydroperoxide decomposition, but its exact effect on the nature of carbonyl products is not known. Analysis of the free monocarbonyl compounds isolated from uncured bacon showed the C_6 alkanal present in 80% proportions. This is considerably higher than that found for cured back fat-lean samples (Table 4).

Significant differences, due to the kind of catalytic activity, are indicated between cured and uncured pork tissue fat. Data showed high ratios for oxidized glycerides of uncured pork (Table 3). This indicated a high degree of linoleate specificity, either in the fatty acid oxidative attack or hydroperoxide decomposition. Salt evidently had the effect of decreasing both ratios. In autoxidized lard there was little specificity in fatty acid attack, and the degree of hydroperoxide conversion to monocarbonyls was low.

The pro-oxidant effect of various salts

In addition to NaCl, other salts have been tested for their pro-oxidant activity. The consensus in the literature appears to indicate wide differences existed, with potassium chloride and sodium sulfate having almost no effect (Banks, 1937; Chang *et al.*, 1949, 1950; Watts *et al.*, 1947). However, one theory for direct oxidative action of NaCl is based on reactivity of the chloride ion (Hills *et al.*, 1946). If this were the case, all chlorides and halogens should behave similarly, and other salts show no effect. It is well known that a number of salts vary in the degree that they inhibit some of the respiratory enzymes of meat (Grant, 1956). Such an effect seems to agree closely with the reported pro-oxidative action of such salts.

Table 3. Effect of lean and sodium chloride on oxidation constants.

			Ratios	
	% NaCl	Monocarbonyl μm/10 g	Carbonyl to PV	Enals to dienals
112 Days at 0°F				
Back fat (PV 20)	6	1.4	0.07	1.4
1:1 Back fat-lean (PV 17)	2	2.0	0.12	0.9
1:1 Back fat-lean (PV 27)	6	3.7	0.14	0.8
300 Days at 0°F				
Uncured bacon (PV 33)	0	8.3	0.25	2.0
20°C (Light)				
Lard (PV 29)	0	0.5	0.02	1.7
Lard (PV 58)	0	0.9	0.02	2.3
Lard (PV 119)	0	2.1	0.02	2.3
Lard (PV 256)	0	5.6	0.02	3.2

Table 4. Effect of lean and sodium chloride on alkanal proportions.

	% Total monocarbonyls					
	C_2	C_3	C_6	C_8	C_9	Total alkanals
112 Days at 0°F						
Back fat 6% NaCl (PV 20)	7	10	60	....	3	80
1:1 Back fat-lean 2% (PV 17)	2	4	70	....	2	78
1:1 Back fat-lean 6%	2	4	77	....	2	85
Uncured bacon 300 days days at 0°F (PV 33)	2	tr	80	....	....	82
20°C (Light)						
Lard (PV 29)	1	17	24	9	12	67
Lard (PV 119)	tr	10	44	8	9	72

In an experiment designed to compare directly the pro-oxidative effect of salts, paired bacon segments were dry-cured with 2% NaCl and equivalent quantities of lithium chloride, sodium nitrate, magnesium chloride, calcium chloride, potassium chloride, sodium sulfate and potassium sulfate. By using paired segments, a direct comparison between each salt and NaCl was obtained. When all of the NaCl was absorbed, some of the other salts were not completely assimilated. Results may therefore be modified in the case of $CaCl_2$, K_2SO_4 and $MgCl_2$ by their degree of absorption and distribution. All of the salts, even the sulfates, showed some pro-oxidative action on storage at 0°F for 20, 41 and 65 days.

Table 5 shows the comparative oxidative activity of several of the salts. The influence of KCl was higher than expected from reports in the literature. In the case of LiCl and $CaCl_2$, monocarbonyl and peroxide comparisons did not correlate. LiCl had higher monocarbonyls and lower peroxide values than NaCl. $CaCl_2$ was the opposite in these relationships. The explanation would seem to be due to a difference in the degree of peroxide breakdown to monocarbonyls. The lack of similarity in pro-oxidant activity of the chloride salts shows that the Hills *et al.* (1946) theory of chloride ion catalysis as applied to meat is probably incorrect.

The amounts of free individual aldehydes formed by the pro-oxidant activities of NaCl and KCl are shown in Table 6. There was close similarity in the composition of these monocarbonyl compounds; also NaCl- and KCl-cured samples had similar monocarbonyl/peroxide ratios.

Pro-oxidant effect of nitrite

Zipser *et al.* (1964) have reported that nitrite when heated with meat converts the pigments to catalytically inactive ferrous nitric oxide hemochromogen. However, when NaCl was present, acceleration of oxidation occurred when the cooked meat was freezer-stored.

The following experiment was set up to measure the effect of unstabilized nitrite-myoglobin combinations on fat autoxidation. Bacon segments were dry cured separately in a paired setup with 0.03% $NaNO_2$, 2% NaCl and 0.03% $NaNO_2$ with 2% NaCl. Storage of the slices was at 0°F for 20, 41 and 65 days. Data on oxidation of these samples are shown in Table 7.

The rate of oxidation promoted by 0.03% $NaNO_2$ tended to be somewhat higher than that of 2% NaCl. When the curing was done with both 0.03% $NaNO_2$ and 2% NaCl, the oxidative effect was much higher than with 2% NaCl alone. The two influences appeared additive and were conceivably independent. It may be possible that the two compounds activate different catalytic systems. It seems certain that the NO_2-accelerated oxidation is due to the pigments, in particular myoglobin and hemoglobin (Chang *et al.*, 1949).

The strong pro-oxidant effect of practically trace amounts of NO_2 may appear surprising; yet it is not when the facts are considered. NO_2 used was more than sufficient to combine with all the myoglobin. It is known that nitrite-myoglobin combinations are extremely reactive unless stabilized to the hemochromogen by heat (Walsh *et al.*, 1956; Reith *et al.*, 1967). Zipser *et al.* (1963) have ob-

Table 5. Effect of various salts on oxidation of bacon segment in comparison to NaCl.

		% of NaCl values				
	Storage at 0°F	LiCl	$NaNO_3$	$MgCl_2$	$CaCl_2$	KCl
20 Days	Monocarb	107	85	85	67	59
	PV	0	103	0	85	41
41 Days	Monocarb	104	70	56	66	42
	PV	79	72	46	100	40
65 Days	Monocarb	121	70	61	66	75
	PV	81	78	73	119	72

served little effect of nitrite on freezer-stored, cooked meat, but strong pro-oxidant action by NaCl. This appears to further support an independent oxidative mechanism of NaCl.

The effect of NaCl and $NaNO_2$ on peroxide decomposition and monocarbonyl class relationships is shown in Table 7. There was seemingly little difference in peroxide decomposition. However, large differences were present in the effect of the two additives on the ratio of enal to dienal. The proportion of enal to dienal of the NO_2 samples was high and about the same level as found in autoxidation of lard and uncured bacon (Table 3). The significance of this is not clear, but it must be related to the hydroperoxide isomers present and mechanism of peroxide decomposition.

Individual aldehydes found for the NaCl and $NaNO_2$ samples are shown in Table 8. Proportions in the alkanal and enal classes were similar. However, the proportion of C_{10} alk-2,4-dienal was much larger for the NaCl samples, and this may account for the differences in enal-dienal ratios. Results were similar in a comparison of NaCl and $NaNO_2$ combined cure with NaCl, although the differences in monocarbonyl/peroxide and enal/dienal ratios were much smaller.

This investigation has demonstrated certain oxidation characteristics of NaCl-accelerated oxidation of freezer-stored pork tissue. The exact mechanism of the pro-oxidant influence of NaCl remains to be determined. Separately, heme compounds are very powerful catalysts of fat autoxidation (Tappel, 1952, 1953, 1955). However, as meat pigments they were relatively inactive or slowly effective in freezer-stored fresh pork.

Oxidation of unsaturated fatty acids by heme compounds has been reported (Tappel *et al.*, 1961) to be non-specific

Table 6. Comparison of free aldehydes in bacon segments oxidized by equivalent amounts of salts.

65 Days at 0°F		Alkanals			
		C_2	C_3	C_6	C_9
2% NaCl	μM/10 g	0.06	0.10	1.55	0.11
	% total	2.5%	4.5%	69%	5.0%
2.55% KCl	μM/10 g	0.04	0.08	1.18	0.77
	% total	2.2%	4.8%	69.4%	4.6%
		Alk-2-enals			
		C_7	C_8	C_9	C_{10}	C_{11}
2% NaCl	μM/10 g	0.08	0.06	0.04	0.04	0.04
	% total	3.4%	2.5%	1.6%	1.7%	1.6%
2.55% KCl	μM/10 g	0.07	0.05	0.03	0.02	0.02
	% total	4.3%	3.1%	1.8%	1.2%	0.9%
		Alk-2,4-dienals			
		C_7		C_9	C_{10}
2% NaCl	μM/10 g	0.04		0.04	0.11
	% total	1.8%		0.8%	5.0%
2.55% KCl	μM/10 g	0.04		0.04	0.06
	% total	2.2%		2.1%	3.4%

Table 7. Effect of $NaNO_2$ and NaCl on autoxidation characteristics of stored bacon.

	Animal No. 1		Animal No. 2	
Storage at 0°F	0.03% $NaNO_2$	2% NaCl	0.03% $NaNO_2$-2% NaCl	2% NaCl
20 Days				
PV	16	17	37	0
Monocarbonyls [1]	0.4	0.5	0.6	0.2
41 Days				
PV	22	29	52	28
Monocarbonyls [1]	0.7	0.6	1.1	0.7
Ratios:				
Monocarbonyls/PV [2]	0.14	0.10	0.09	0.10
Alk-2-enal/alk-2,4-dienal [2]	2.6	1.3	2.1	1.1
65 Days				
PV	61	50	68	34
Monocarbonyls [1]	1.3	0.9	1.4	0.6
Ratios:				
Monocarbonyls/PV [2]	0.10	0.08	0.09	0.07
Alk-2-enal/alk-2,4-dienal [2]	2.8	1.4	2.7	1.5

[1] Absorbance in 100 ml CCl_4 of monocarbonyl 2,4-dinitrophenylhydrazones from 10 g fat.
[2] μM of monocarbonyl per g fat.

Table 8. Comparison of free aldehydes produced by NaCl and $NaNO_2$.

			Alkanals			
			C_2	C_3	C_6	C_9
41 Days	2% NaCl	μM/10 g	0.06	0.21	2.07	1.10
		%	2.2	7.2	71.2	3.3
	0.03% $NaNO_2$	μM/10 g	0.10	0.18	2.16	0.14
		%	2.1	6.7	70.2	4.7
65 Days	2% NaCl	μM/10 g	0.08	0.17	3.14	tr
		%	2.0	4.3	79.2	tr
	0.03% $NaNO_2$	μM/10 g	0.08	0.52	4.48	tr
		%	1.4	8.9	76.3	tr

			Alk-2-enals				
			C_7	C_8	C_9	C_{10}	C_{11}
41 Days	2% NaCl	μM/10 g	0.08	0.06	0.05	0.04	0.03
		%	2.6	2.1	1.9	1.3	1.2
	0.03% $NaNO_2$	μM/10 g	0.10	0.11	0.07	0.04	0.03
		%	3.1	3.5	2.4	1.3	1.0
65 Days	2% NaCl	μM/10 g	0.08	0.10	0.06	0.07	0.09
		%	2.1	2.5	1.6	1.0	1.4
	0.03% $NaNO_2$	μM/10 g	0.17	0.12	0.14	0.07	0.09
		%	2.8	2.0	2.4	1.2	1.6

			Alk-2,4-dienals		
			C_7	C_9	C_{10}
41 Days	2% NaCl	μM/10 g	0.03	0.05	0.11
		%	1.2	1.8	3.9
	0.03% $NaNO_2$	μM/10 g	0.04	0.04	0.06
		%	1.2	1.4	1.9
65 Days	2% NaCl	μM/10 g	0.05	0.05	0.16
		%	1.2	1.2	4.0
	0.03% $NaNO_2$	μM/10 g	0.06	0.06	0.09
		%	1.0	1.0	1.5

and similar to uncomplicated autoxidation. However, oxidation of freezer-stored, cured and uncured pork tissue fat showed a linoleate specificity in oxidation characteristics and monocarbonyl compounds. The high C_6 alkanal content suggests a lipoxidase-type of selective action, but the existence of such an enzyme in meat has been largely ruled out (Tappel, 1952, 1953). The explanation may be that heme compounds have a specific decomposing action on linoleate hydroperoxide to C_6 alkanal.

Autoxidation in this study has been considered to be taking place in the triglycerides. The possible involvement of polar lipids has not been overlooked. The extremely high polyunsaturated fatty acid content of the polar lipids (Hornstein *et al*, 1961; Kuchmak *et al.*, 1963; Giam *et al.*, 1965) should be reflected in the composition of the free aldehydes (Gaddis *et al.*, 1961; Ellis *et al.*, 1966). The presence of unusual amounts of C_3 and C_7 alkanals and C_7 alk-2,4-dienal should enable the detection of an influence by oxidizing polar lipids. Furthermore, hydroperoxides have not been observed to accumulate in oxidized phospholipids. This incompletely understood type of autoxidation has been reported only in refrigerated, cooked meats (Younathan *et al.*, 1959; Zipser *et al.*, 1957), and has not been observed in freezer storage.

Nothing is known concerning the separate catalytic effect of heme pigments on the monocarbonyl products of oxidized fat or individual unsaturated fatty acids. A specific linoleate hydroperoxide to monocarbonyl decomposing action by the heme pigment may well exist. Similarly, knowledge of the independent oxidative influence of NaCl, trace metal ions, and lipoxidases would serve to clarify the mechanisms involved. There should also be advantage in the study under appropriate conditions of the volatile and free carbonyls of oxidized polar lipids.

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Ms. rec'd 8/16/67; revised 3/15/68; accepted 7/18/68.

Presented at the 27th meeting of the Institute of Food Technologists.

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Broiling, Sex and Interrelationships with Carcass and Growth Characteristics and their Effect on the Neutral and Phospholipid Fatty Acids of the Bovine Longissimus Dorsi

SUMMARY—Broiling had a greater effect on phospholipid fraction fatty acids than on neutral fraction fatty acids. The percentages of C18:3 (neutral fraction) and C14 and C15 (phospholipid fraction) were significantly smaller (5% level) in the broiled steaks. However, the percentage of C8 (phospholipid fraction) was larger in the broiled than in the raw steaks (5% level). Sex differences, restricted to the neutral fraction acids, were greater than the effect of broiling. Steers had a larger percentage of C16 and C18 and a smaller percentage of C18:1 than heifers (5% level). Neutral fraction acids (C18, C18:1 and C18:2) were significantly correlated with lipid prosphorus, cholesterol, % fat trim (retail), estimated % carcass fat and estimated % carcass lean. Phospholipid fraction acids (C16, C18, C18:3 and C20:4) were associated with average daily gain and days of animal age (5% level). Low, nonsignificant correlations were found among individual fatty acids from the neutral and phospholipid fractions of bovine longissimus dorsi muscle with tenderness and juiciness scores.

INTRODUCTION

COMMON METHODS of cookery are known to accelerate the oxidation of meat lipids (Watts, 1954). Small and generally nonsignificant differences in fatty acid distribution among raw and cooked meat samples and drip losses, have been reported (Chang *et al.*, 1952a; Siedler *et al.*, 1964; Campbell *et al.*, 1967). Isolation and characterization of muscle lipids in meat flavor research has illustrated the importance of studying the phospholipid fraction fatty acids (Hornstein *et al.*, 1960 and Hornstein *et al.*, 1961).

In many of these studies, animal history was unknown or sample numbers were too small for adequate statistical analysis. In experiments, designed to study cooking effects, it may be difficult to partition all sources of variation associated with muscle lipids. However, known history of the muscle samples would be of value in establishing those fatty acid differences resulting from effects other than those due to the method of cooking. Specifically, changes induced by cooking may be confounded with sex or other factors when history of the samples is not known. Further, consideration of the interrelationships of muscle fatty acids with carcass and growth characteristics would provide information on additional factors which may contribute to the fatty acid composition of muscle.

This project was designed to: 1) evaluate the effects of broiling on neutral and phospholipid fraction fatty acids from bovine longissimus dorsi muscle and 2) to study the interrelationship of these fatty acids with other lipid components, carcass and growth characteristics, and taste panel scores.

EXPERIMENTAL

Sample description

The data reported herein were obtained during a concurrent study on the growth and composition of bovine muscles. Ten Angus steers and 10 Angus heifers were fed, *ad libitum*, a concentrate ration from weaning to an end point weight of either 386 kg, 420 kg, or 450 kg. Age of these animals ranged from 402 to 624 days. Steers averaged 503 while heifers averaged 530 days of age. Methods for obtaining carcass data and growth characteristics have been outlined by Suess *et al.* (1966a,b). Six days postmortem, steaks from the longissimus dorsi (LD) were removed at the first and second lumbar vertebrae. All external fat, covering these steaks, was removed and the muscle samples from the first lumbar vertebra (control), were placed in polyethylene bags, flushed with nitrogen, sealed, placed in glass jars and stored at −17°C. Steaks from the second lumbar position (treated) were individually wrapped and stored overnight in a 1–3°C cooler. These steaks were then broiled to an internal temperature of 65°C (approximately 25 min) whereupon they were evaluated for tenderness, juiciness and flavor, by an experienced 18 member taste panel (nine-point Hedonic scale).

Chemical determinations

Extractable lipid (Ostrander *et al.*, 1961) was determined on 25 gm samples of the raw and broiled steaks. A CO_2 atmosphere was maintained in the blender during this extraction. The lipid extract was then placed in 2 dram glass vials, flushed with nitrogen, sealed and stored at −17°C. Fractionation of this lipid extract into neutral and phospholipid moieties was determined according to Choudhury *et al.* (1960), with the following modifications: 1) 5 gm activated silicic acid and 600 mg lipid extract were shaken with 50 ml chloroform for 15 min; 2) the lipid-chloroform-silicic acid mixture was then transferred to a sintered-glass filter and washed with 150 ml of chloroform (elute neutral lipids); 3) before washing the sintered-glass filter with methanol, care was taken in rinsing the sides and bottom of the filter to remove traces of neutral lipid; and 4) the silicic acid was then washed with 120 ml of methanol to elute the phospholipid fraction.

Approximately 60 mg of neutral lipid and 40 mg of phospholipid were esterified by a modified precedure (2% sulfuric acid-methanol) (Terrell *et al.*, 1967). Lipid phos-

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phorus (Morrison, 1964) and total cholesterol (Mann, 1961) was also determined on the raw and broiled lipid extract.

Chromatographic conditions

Gas-liquid chromatography of the methyl esters was performed on a dual column Varian Aerograph Chromatograph, Model 204, using dual flame ionization detectors. A coiled pyrex column, 2.5 m × 3.17 mm, was packed, at 18.6 kg/sq cm, with 20% DEGS (diethyleneglycol-succinate on Chromasorb W, 80–100 mesh). Operating conditions were as follows: 1) sample size = 0.15 μl, neutral fraction and 0.25 μl, phospholipid fraction; 2) column temperature = 183°C; 3) injector temperature = 225°C; and 4) detector temperature = 240°C. Quantification of peaks was determined according to Baumgardt (1964). Individual esters (Hormel Institute) were used in making a mixed reference standard, based upon literature values reported for individual fatty acids. All esters were expressed as relative percent and the precision of GLC determinations was within the limits of error described by Horning *et al.* (1964).

Statistical procedures

Statistical analyses were based on a weight, sex and treatment (raw versus broiled) model. Least-squares analysis (Harvey, 1960) was used, and means with significant F ratios were tested by Duncan's new multiple range test, as outlined by Duncan (1955) with modifications for unequal replications (Kramer, 1956). Simple correlations were calculated among all variables.

RESULTS AND DISCUSSION

Neutral and phospholipid fatty acids

Treatment means and standard deviations for the neutral and phospholipid fraction fatty acids are presented in Table 1. In the neutral fraction, broiling affected only C18:3 (1.22% raw versus 0.81% broiled). No significant differences were observed among the other neutral fraction acids. Because of the lower melting point of neutral glycerides with C18:3, it may have been lost to the fat drippings. However, Chang *et al.* (1952a,b) found no significant changes in the spectrophotometric pattern of polyunsaturated fatty acids from beef, pork and poultry, due to common methods of cookery. Fatty acids, C22 and C20:4 were not detected in the neutral fraction.

With respect to the phospholipid fraction acids (Table 1), the percentage of C8 was larger (5% level) in the broiled than in the raw LD, while the percentages of C14 and C15 were smaller (5% level) in the broiled steaks. Although broiling may be expected to have a greater effect on long-chain polyunsaturates, this apparently was not the case in the current experiment. Oxidative studies on cooked broiler tissues (Mickelberry *et al.*, 1964) have shown no alteration in iodine values and similar results, for the long-chain polyunsaturates of roasted peanuts, have been reported by Iverson *et al.* (1963).

It is interesting to note (Table 1) that broiling affected the saturated acids (C8, C14 and C15) of the phospholipid fraction and only the unsaturated acid, C18:3 of the neutral fraction. These effects may be explained on the basis of a partitioning of the phospholipids between the broiled LD and the drip (Campbell *et al.*, 1967). The broiled LD may retain a larger portion of phospholipids that are high in C8 while phospholipids containing C14 and C15 may be lost to the drip; hence the lower values for broiled samples. The fact that phospholipids are known to serve an inherent function in cellular membranes, suggest that they are tightly bound and are not easily released during the broiling process. This may explain why there were no significant

Table 1. Effect of broiling on neutral and phospholipid fraction fatty acids from the bovine longissimus dorsi.[1,2]

Fatty acid[3]	Neutral		Std. dev.	Phospholipid		Std. dev.
	Raw	Broiled		Raw	Broiled	
C8	.44	.53	.15	.63[x]	1.20[y]	.70
C10	.12	.10	.04	.12	.18	12
C12	.08	.07	.03	.13	.16	.10
C14	3.19	3.14	.37	1.61[x]	.78[y]	.58
C14:1	1.02	.90	.23	.10	.09	.15
C15	.43	.37	.17	.54[x]	.19[y]	.21
C16	33.50	33.76	1.55	29.73	30.95	3.81
C16:1	3.65	3.37	.53	2.04	2.11	.78
C17	1.17	1.04	.36	.69	.78	.54
C18	11.92	11.54	2.02	8.89	9.58	1.50
C18:1	41.43	42.32	2.28	25.86	27.55	3.66
C18:2	1.81	2.04	.71	15.45	14.27	2.63
C18:3	1.22[x]	.81[y]	.36	3.24	2.89	.78
C22	.....	.....	.....	2.20	1.82	.81
C20:4	.....	.....	.....	8.77	7.44	3.57
Sat.	50.85	50.55		44.54	45.64	
Unsat.	49.13	49.44		55.46	54.34	
Unsat./sat.	.97	.98		1.25	1.19	

[1] Means with different superscripts are different (5% level).
Means with no superscript are not different (5% level).
[2] Means expressed as relative percent of total acids measured.
[3] Carbon number and unsaturation indicated.

differences in the long-chain polyunsaturates of the raw and broiled phospholipid fractions.

Alterations in lipids during cooking appear to be, in part, a function of time and temperature. Under the conditions of broiling, neither appear to be great enough to produce marked effects. However, more work should be directed towards the transfer of heat in a multiphasic system, such as meat, and its relationship to the phospholipid fatty acids.

Effect of sex

Significant sex differences (5% level) in the percentages of C16, C18 and C18:1 from the neutral fraction, are shown in Table 2. Steers had a larger percentage of C16

Table 2. Effect of sex on neutral fraction fatty acids from the bovine longissimus dorsi.[1]

Fatty acid [2]	Steers	Heifers
C16 *	34.22	33.04
C18 *	12.51	10.95
C18:1 *	40.89	42.86

* Sex means for C16, C18 and C18:1 are different (5% level).
[1] Means expressed as relative percent of total acids measured.
[2] Carbon number and unsaturation indicated.

(34.22% versus 33.04%) and C18 (12.51% vs 10.95%) than heifers. The reverse was true for C18:1 (heifers = 42.86% and steers = 40.89%). Subcutaneous fat depots of steers were found to have larger percentages of C10, C14, C16 and C18 but smaller percentages of C18:1 than heifers (Terrell *et al.*, 1966).

Sex differences for the neutral fraction fatty acids of the porcine LD have been reported by Allen *et al.* (1967a). These workers suggested that sex differences were related to the fact that boars had a larger number of muscle fibers with the enzymatic capacity to mobilize and oxidize lipid than did gilts and barrows. In agreement with the work of Allen *et al.* (1967b), sex effects (Table 2), were totally associated with the neutral fraction fatty acids rather than the phospholipid fraction fatty acids. These data suggest that sex of the animal influences LD neutral fraction fatty acids more than broiling.

Sex differences in animal lipids are known to be associated with hormonal changes and their possible influence on enzymatic systems. However, cellular mechanisms for this relationship are not fully understood. There were no live animal weight effects observed in this study.

Correlations

Due to the concurrent nature of this experiment, the authors thought it pertinent to evaluate the interrelationship of certain neutral and phospholipid fraction fatty acids with lipid components, carcass and growth characteristics, and taste panel scores. These data are presented in Table 3. The neutral fraction fatty acids, C18 and C18:1 were related (5% level) to lipid phosphorus (r = 0.67 and −.61) and to cholesterol (r = 0.66 and −.56), respectively. These correlations indicate that approximately 31 to 47% of the variation in lipid phosphorus and cholesterol was associated with the C18 and C18:1 content of the bovine LD neutral fraction.

The percentage of C18 was negatively associated with % fat trim (retail) and estimated % carcass fat, while C18:1 and C18:2 were positively related to these carcass traits. This would indicate that as the amount of fat increases in a carcass the neutral lipid of the LD becomes more unsaturated. Waldman *et al.* (1965) reported nonsignificant correlations of LD fatty acids (diethyl ether extract, as opposed to chloroform-methanol extract) with percent carcass fat. They also found no significant relationship of LD fatty acids with days of age, which is in agreement with the neutral fraction data presented in Table 3.

Although Waldman *et al.* (1965) reported a significant correlation among taste panel juiciness scores and C14 and C16, the present study does not show this. The low and nonsignificant correlations of neutral fraction fatty acids with tenderness and juiciness scores, in the present sample of cattle, indicate that these acids are of little value in predicting organoleptic scores.

The phospholipid fraction fatty acids, C16 and C18, were negatively associated (5% level) with days of age (r =

Table 3. Simple correlations of certain neutral and phospholipid fraction fatty acids from the raw longissimus dorsi, with lipid components, carcass and growth characteristics and taste panel scores.[1]

	Neutral			Phospholipid			
Variable	C18	C18:1	C18:2	C16	C18	C18:3	C20:4
Lipid phosphorus [2]	0.67**	−.61**	−.20	−.22	−.11	0.18	0.27
Total cholesterol [2]	0.66**	−.56*	−.17	−.24	−.19	0.23	0.27
% Fat trim (retail)	−.73**	0.68**	0.45*	−.08	−.21	0.16	−.09
Est. % carc. fat	−.72**	0.53*	0.55*	−.01	−.20	0.17	−.10
Est. % carc. lean	0.67**	−.50*	−.58*	0.07	0.22	−.22	0.02
Days of age	0.11	−.11	0.23	−.48*	−.50*	0.43	0.42
Av. daily gain	−.21	−.18	−.16	0.44*	0.51*	−.51*	−.47*
Tenderness [3]	0.05	0.10	−.31	0.11	0.04	−.20	−.13
Juiciness [3]	0.33	−.19	0.06	−.13	−.13	0.14	0.22

** 1% level.
* 5% level.
[1] Twenty observations per correlation.
[2] mg/100 g, wet tissue.
[3] Taste panel, 18 members, hedonic scale 9 to 1.

−.48 and −.50), respectively, whereas these two acids were positively related to average daily gain. These data suggest that as animal age increases, the major saturated acids of the phospholipid fraction decrease. A faster rate of gain (Table 3) is associated with a decrease in C18:3 and C20:4 and an increase in C16 and C18. In a weight end point study, the faster gaining animals would necessarily be younger at the designated slaughter weight than the slower gaining animals.

One conceivable explanation for these results may be a preferential mechanism whereby muscle tissues of fast gaining animals would preferentially form phospholipids containing the saturated acids C16 and C18, at the expense of the unsaturated acids C18:3 and C20:4. The opposite may be true in regard to days of age. That is, as animal age increases, the phospholipids in the LD change from a saturated (C16 and C18) to a predominantly unsaturated (C18:3 and C20:4) lipid. Correlations of phospholipid fraction fatty acids, with tenderness and juiciness scores, were low and nonsignificant.

In comparing the neutral and phospholipid fraction acids presented in Table 3, it appears that the neutral fraction acids are significantly related to lipid components and indices of carcass fat; whereas, the phospholipid fraction acids are more closely associated with growth characteristics. These correlations suggest that there may be some metabolic alterations in phospholipid fraction acids as influenced by animal age and rate of gain. Enzymatic changes may provide a partial answer, but specific studies regarding possible enzymatic changes in the bovine LD should be conducted.

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Ms. rec'd 11/2/67; revised 4/22/68; accepted 8/1/68.

Meat and Animal Science Department. Manuscript No. 475 of the Wisconsin Agricultural Experiment Station.

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Muscle Protein Composition and Eating Quality of Fresh and Frozen Turkeys

SUMMARY—Fifty-four Beltsville small white male turkeys were studied either fresh-unfrozen or frozen-stored for 5 and 10 months. Samples of raw muscle were removed for protein analysis; the turkeys were then roasted at 325°F to end-points of 165°, 175°, and 185°F in the thigh muscles and eating quality was evaluated.

Quantitative changes in muscle proteins as separated from extracts made with KCl-borate buffer or with de-ionized water were not marked. There was a decrease in actomyosin nitrogen of pectoralis major and some indication of proteolytic Changes. The magnitude of these changes in turkey muscles stored up to 10 months was not expected to alter eating quality as much as the longer cooking times required to reach the end-point temperatures when cooking frozen-stored turkeys.

Moisture losses were higher, and cooked thigh muscles were more tender and mealy from frozen-stored than fresh turkeys. Cooked pectoralis major muscles required more force to shear after 5 months storage than at either 0 (fresh-unfrozen) or 10 months storage. There was some indication that an undesirable flavor developed in thigh meat of turkeys stored 10 months.

INTRODUCTION

IDENTIFYING FACTORS which contribute to decreased consumer acceptance of frozen turkeys would greatly aid development of procedures to improve eating quality and promote use of frozen-stored turkeys. Recently, the U. S. Department of Agriculture found consumer resistance to frozen turkeys was lessening but preference for fresh over frozen turkeys was three to one (Knott, 1966).

Goertz *et al.* (1960) found that fresh-unfrozen turkeys roasted to 95°C (203°F) in the thigh had shorter cooking times and generally had eating quality characteristics of less well done meat than turkeys stored 1 and 3 months at 0°F.

Khan *et al.* (1963) reported decreased protein extractability with KCl-borate buffer in both breast and leg muscles of chickens during frozen storage because of loss of solubility of the actomyosin fraction. The sarcoplasmic fraction decreased only after long storage. The non-protein nitrogen fraction increased.

van den Berg (1964) has reported pH changes in poultry meat frozen at −10°C for 6 months. He suggested that these changes are caused mainly by increased concentration of such components as proteins in the unfrozen phase, by precipitation of salts, by interaction of proteins with ionic substances and by enzymatic activity.

Other studies relating characteristics of eating quality of muscle such as tenderness, texture and juiciness to sarcoplasmic, myofibrillar or stroma proteins of muscle were recently reviewed by Donnelly *et al.* (1966).

In this research, differences in quantity of protein fractions from raw muscles, and in heating characteristics of fresh-unfrozen and frozen-stored turkeys were determined. The extent of changes in these factors were evaluated in terms of differences in eating quality between fresh-unfrozen turkeys and those stored up to 10 months.

EXPERIMENTAL

FIFTY-FOUR BELTSVILLE small white male turkeys were obtained from the Animal Husbandry Research Division, ARS, USDA. They were hatched from eggs of one flock collected in three lots, each 1 wk apart, then raised in confinement and fed corn-soybean diets. The turkeys were slaughtered at 16 wk by cutting throats and bleeding after which they were agitated for 30 sec in water at 138°F. Feathers were picked by hand. Carcasses were placed in ice water overnight, eviscerated and vacuum-packaged in heat-shrunk polyethylene bags. Fresh-unfrozen turkeys were evaluated on the next 2 days, and remaining turkeys were placed immediately in freezer storage at −5°F.

Design for evaluation

A completely randomized split-plot design was used. At each period of study (0, fresh-unfrozen and 5 and 10 months, frozen storage) 18 turkeys were evaluated over 3 wk so that six from each hatch lot were evaluated during 2 days each week. Each day, three turkeys were sampled for studies of raw muscle. They were then roasted at 325°F to internal thigh temperature of 165°, 175° or 185°F, and evaluated for eating quality.

Raw muscle evaluation

Wrapped, fresh turkeys were held in a refrigerator. Wrapped, frozen-stored turkeys were thawed by immersion in cold tap water for 4 hr at room temperature (75° ± 3°F) and then held about 36 hr in a refrigerator. Turkeys were removed from the refrigerator ½ hr before roasting.

For raw muscle samples, about 15 g were removed and minced. The anterior tip of one pectoralis major muscle was used as a light meat sample, and the upper portion of the gluteus primus muscle of one thigh was a dark meat sample for each turkey.

Two g of mince were used for extraction of proteins with KCl-borate buffer, (ionic strength = 1.0, pH = 7.4) at 0 and 10 months storage. The extract was fractionated at about 4°C into myofibrillar proteins, soluble at ionic strength 0.5 and insoluble at 0.08 and sarcoplasmic proteins, soluble at 0.08. The difference in the amount of soluble nitrogen at 0.5 and 0.25 was reported as actomyosin nitrogen and that at 0.25 and 0.08, as myosin nitrogen (Khan, 1962). Nitrogen content of various fractions was determined by Technicon Nitrogen Analyzer.

Ten g of mince were used at 0-, 5-, and 10-month

periods of storage to study water-soluble proteins. The mince was mixed with 5 ml chilled, de-ionized, glass-distilled water, stirred intermittently for 3 hr, then centrifuged for 25 min at 2,000 rpm (Payne, 1963). The supernatant was refrigerated overnight, then acrylamide gel disc electrophoresis was carried out in a Canalco Disc electrophoresis apparatus at room temperature. Gel tubes of standard 5 mm ID were used. The standard gel was 7½% acrylamide. Positive moving proteins were separated with standard buffer at pH 8.3 (Tris-glycine buffer). The current was 0.5 ma for each gel. Total time for electrophoresis was 30–40 min. Amidoschwarz stain was used to detect protein in the gels. Gels were examined densitometrically to determine the percentage of the individual fractions.

About 1 g of muscle mince was dried under vacuum at 55°C for 48 hr to determine moisture content of raw muscle samples. Nitrogen determinations of dried muscle mince were made by the micro-Kjeldahl method.

After raw samples were removed, skin was drawn over excised muscle areas and stitched together. Ready-to-cook weights of turkeys ranged from 5.0 to 7.8 lb with a mean weight of 6.8 lb.

Roasting

Turkeys were roasted breast side up on V-shaped racks in shallow aluminum pans in ovens preheated to 325°F. Heat penetration into turkeys was measured by thermocouples placed in each thigh. Insertion was at the distal end of the femur, extended parallel to and ½ the length of the femur so that the tip of the thermocouple was in the center of muscles posterior to the femur. Thermocouples were also placed in each pectoralis major muscle of the breasts, 1-in. deep in the thick anterior portion. The thigh used to determine the end-point of cooking at 165°, 175° or 185°F was alternated daily. Aluminum foil was placed lightly over the breast area when the thigh muscles reached 150°F (Hoke *et al.*, 1966). Turkeys and drippings were weighed following roasting. Cooking losses were calculated as percentages of ready-to-cook turkey weights.

Evaluation of cooked meat

Samples to evaluate cooked meat were taken 15 to 30 min after termination of roasting from the side of the turkey not used for raw samples, except those for determination of moisture and fat contents.

For evaluation by a taste panel of six to eight judges, samples about ⅜-in. thick were sliced across the muscle fibers at the center of the pectoralis major muscles and the composite of muscles in the posterior half of the thigh; also, a ½-in. square the thickness of the gluteus primus muscle. The meat was evaluated on a nine-point scale. Nine represented the highest intensity of odor, flavor, tenderness, juiciness and mealiness; one, the lowest. Doneness of cooked meat was scored five when optimum. Increasing overdoneness was indicated by increasing values from six to nine and underdoneness by decreasing values from four to one.

Shear force determinations were made with a Kramer shear apparatus. Duplicate samples were taken adjacent to the areas of muscles used for evaluation by taste panel. Samples of pectoralis major had a mean weight of 12 g; of thigh muscles, 8.5 g; and gluteus primus, 3.5 g. Shear values were reported as lb/g to shear across the meat fibers.

Press fluid determinations on 0.5 g samples were made with a Carver Press fluid apparatus (Hoke *et al.*, 1966). Samples of pectoralis major muscle were taken from the anterior end next to the sample for shear determination. Samples of thigh muscles were cross-sections of the gluteus primus and of the femorotibialis internus, the muscle on the medial surface of the femur.

Moisture and fat determinations were made on muscle samples from the same areas used for taste panel evaluation, but from the opposite side of the turkey. Drying was at 55°C under vacuum for 48 hr. Fat determinations were by Soxhlet extraction with petroleum ether.

Analysis of data

Data were subjected to least squares analysis of variance to determine how cooking times, cooking losses and the various measurements of raw and cooked muscles were affected by the storage periods or by the internal temperature to which the meat was cooked. Significance of differences among means was based on the standard of the error of the difference between two means.

RESULTS AND DISCUSSION

Changes in quantities of raw muscle proteins

Nitrogen values for raw pectoralis major and gluteus primus muscles and for various protein fractions at 0 and 10 months frozen storage are given in Table 1. Total

Table 1. Mean values for nitrogen content (g/100 g muscle, wet weight) of protein fractions of pectoralis major and gluteus primus muscle of turkeys, fresh-unfrozen and frozen-stored 10 months.

Component	Pectoralis major		Gluteus primus	
	fresh-unfrozen	frozen-stored	fresh-unfrozen	frozen-stored
Total nitrogen	3.66 ± .17	3.57 ± .15	2.85 ± .22	3.10 ± .11
Extractable nitrogen	1.88 ± .18	1.74 ± .34	1.45 ± .18	1.49 ± .13
Non-protein nitrogen	0.26 ± .06	0.33 ± .05	0.22 ± .05	0.33 ± .16
Actomyosin nitrogen	0.90 ± .12	0.45 ± .09	0.42 ± .07	0.49 ± .05
Myosin nitrogen	0.24 ± .08	0.15 ± .08	0.16 ± .04	0.14 ± .05
Sarcoplasmic nitrogen	0.71 ± .07	0.68 ± .09	0.58 ± .06	0.64 ± .07

nitrogen values for pectoralis major muscles were higher than for gluteus primus muscles, as previously observed in light and dark poultry meat by Khan (1962) for chickens and by Scharpf *et al.* (1946) for turkeys.

The amount of extractable nitrogen using KCl-borate buffer for 16-wk-old turkeys at both storage periods (0 and 10 months) was about 50% of the total nitrogen content of the muscles. Scharpf *et al.* (1964) reported about 48 and 56% extraction from light meat and about 37 and 41% extraction from dark meat of 11- and 26-wk-old turkeys, respectively.

Protein fractionation of the extract made with KCl-borate buffer showed that the major difference in nitrogen values of muscles studied at 0 and 10 months storage periods was in the actomyosin nitrogen for pectoralis major muscles (Table 1). Actomyosin nitrogen was 48% of total extractable nitrogen at 0 storage but only 26% at 10 months storage of the turkeys. Actomyosin nitrogen percentage in pectoralis major muscles after 10 months storage was more like that obtained for the gluteus primus muscles at either storage period (29% at 0 and 33% at 10 months storage). Myosin and sarcoplasmic nitrogen values at 0 and 10 months storage were 13 and 9%, 37 and 39%, respectively, of the total extractable nitrogen in pectoralis major muscles. These values for gluteus primus muscles were 11 and 9%, 40 and 43%.

As shown in Table 1, values for non-protein nitrogen tended to increase after turkeys were stored 10 months. Increases in this nitrogen fraction are generally considered to indicate that some proteolysis has occurred (Khan *et al.*, 1963).

Nitrogen contents for the various protein fractions obtained in this study varied from those reported by Scharpf *et al.* (1964) for turkeys. Undoubtedly, the interpretation of the method reported by Khan (1962) as to extraction times as well as differences in samples, times and temperatures for holding turkeys post-mortem contribute to these variations.

The amount of nitrogen extracted with water from raw muscles remained at 12.9% of the total nitrogen for pectoralis major and 9.5% for gluteus primus muscles throughout the study. The electrophoretic pattern obtained from water-soluble extracts of pectoralis major and gluteus primus muscles was labeled alphabetically (A through G) by areas starting with the fastest moving component, A.

The pattern for fresh pectoralis major muscles of two light bands (A and B) followed by three heavier bands (D, E, and F) was similar to that published by Maier *et al.* (1966) for water-soluble proteins of chicken breast muscles at 24 hr post-mortem. The pattern for gluteus primus muscles showed two to three fine bands in the F area, compared to only one visible in the pattern for the pectoralis major muscles. A faint band was usually evident in the center of the C area for extracts from gluteus primus, but not for pectoralis major muscles. Both muscles usually had a faint stained area just before the distinct D band. The G area in both muscles seemed to be material which did not move much beyond the immediate vicinity of origin and which was a large diffuse area extending from the distinct bands in the F area to the spacer gel. Table 2 shows the percentage of the total stained area of the gels forming bands in each area. Bands in the C area were not distinct enough to be quantitated by densitometry. The individual fractions in frozen turkey muscles stored for 5 months were not significantly different from those stored for 10 months. Values were, therefore, combined for the two storage periods. Bands in the A and B areas tended to increase slightly in intensity after frozen storage of 5 or 10 months, compared to those observed at 0 storage. Differences in amounts, however, were not statistically significant. Maier *et al.* (1966) in their study of acrylamide gel disc electrophoretic patterns of water-soluble proteins of chicken breast muscles post-mortem noted an increase in relative intensity of bands in this same area. They suggested this may represent peptide fragments of larger molecules, either sarcoplasmic or myofibrillar, which have undergone enzymatic hydrolysis during post-mortem aging. A similar change may occur in frozen-stored muscles.

Bands in the F area showed the greatest variability frequently appearing quite faint, particularly in samples from turkeys held in frozen storage.

Cooking times

Cooking times in min/lb of ready-to-cook turkey weights were significantly longer for frozen-stored than for fresh turkeys. Difference in cooking times for turkeys frozen 5 or 10 months was not significant (Table 3). Goertz *et al.* (1960) have reported shorter cooking times and lower volatile cooking losses for fresh-unfrozen than for turkeys frozen up to 3 months.

Increases in cooking times as the end-point temperatures for cooking increased were highly significant between 165° and 175°F with no further significant increases by cooking to an end-point of 185°F (Table 3).

The temperature of all turkeys at the start of roasting was 48° ± 4°F. During the first hour of roasting, the

Table 2. Percentage of total stained area of acrylamide gel disc electrophoretic patterns of water soluble proteins from fresh and frozen-stored turkeys.

Muscle	Percentage of total stained areas [1]						
	A	B	D	E	F		G
Pectoralis major—fresh-unfrozen	1.2	1.1	10.3	18.8	6.2		62.2
frozen-stored	3.4	2.6	10.5	20.5	5.2		57.8
					F_1	F_2	
Gluteus primus—fresh-unfrozen	0.2	3.2	9.8	27.7	8.4	7.1	43.6
frozen-stored	1.2	4.3	9.6	29.2	7.2	5.8	43.1

[1] C bands were too indistinct to be quantitated.

Table 3. Mean cooking times for fresh-unfrozen and frozen-stored turkeys.[1]

Cooking end-point	Storage months at −5°F		
	0	5	10
°F	min/lb	min/lb	min/lb
165	17.8	21.8	28.3
175	22.1	28.4	34.0
185	25.2	32.7	34.6

[1] Based on ready-to-cook weight of 6 turkeys with mean weight of 6.8 lb.

Table 4. Final temperatures in pectoralis major muscles of turkeys cooked to end-point temperatures in thigh muscles.

Cooking end-point	Storage months at −5°F		
	0	5	10
°F	°F	°F	°F
165	187	184	183
175	194	187	187
185	196	194	195

heat penetration rate into thigh muscles of fresh turkeys was 1.5°/min compared to 1.2°/min for frozen-stored turkeys. During the next hour, the rate of heat penetration was about 0.5°/min and for the remainder of the cooking time about 0.25°/min for all turkeys.

In the first hour the rate of temperature rise in pectoralis major muscles was 1.6°/min for fresh turkeys and 1.4°/min for those which were stored. In the next hour the rate was 0.8°/min and for the remainder of the cooking time, 0.2°/min for all turkeys. Final temperatures at each end-point of cooking for the three storage periods are summarized in Table 4.

Temperatures of right and left sides of a turkey during roasting were quite variable. Twenty-one of the 54 turkeys studied had differences of 10° or more; 18 of the turkeys had differences of 5° or less between right and left thigh muscles at the end-point of cooking. During the latter part of cooking, when the heat rise in thigh muscles was about 0.25°/min, a 10° difference could mean as much as 40 min cooking time to reach a selected end-point temperature. Such differences in the rate of heat penetration with heat from a conventional household-type oven and with variations in insertion of thermocouples emphasize the difficulty in establishing precise roasting time for turkeys when thigh muscle temperature is used to indicate the cooking end-point.

Forty of the 54 turkeys studied had differences of 5° or less and only four had differences to 10° or more between right and left pectoralis major muscles at the end-point of cooking.

Moisture changes

Measurements of moisture in cooked turkeys indicated decreased moisture levels as cooking times were increased with frozen storage or with increases in the end-point temperature to which they were cooked (Table 5).

Total cooking losses were less for fresh than for frozen-stored turkeys. There was no significant change in cooking losses between turkeys held for 5 and 10 months. Drippings remained constant with a mean value of 2.3%. Evaporation losses paralleled the changes observed in total cooking losses.

Mean total moisture content of raw meat samples from pectoralis major was 74.7% and gluteus primus muscles, 77.0% throughout the study. As indicated in Table 5, some change in total moisture content of cooked pectoralis major and gluteus primus muscles was attributable to frozen storage of the turkeys. For both muscles, the change occurred between fresh-unfrozen and frozen-stored turkeys, and not between turkeys held 5 and 10 months. The significant changes in total moisture content of pectoralis major muscles as a result of end-point temperature occurred between 165° and 175°F with no further change by cooking to 185°F. The change in total moisture content of a composite of thigh muscles, as internal end-point

Table 5. Mean measurements for moisture contents of fresh-unfrozen and frozen-stored turkeys roasted at 325°F.

Storage—months at 5°F	0			5			10			Significance of F values [1]	
Cooking end-points —°F	165	175	185	165	175	185	165	175	185	Storage	Cooking
Total cooking losses, % [2]	18.2	22.8	26.0	22.5	27.9	31.1	24.6	29.7	31.0	**	**
Moisture content, %											
pectoralis major	67.1	65.2	64.8	65.4	64.7	64.7	65.2	64.8	62.9	*	**
thigh muscles	68.9	68.2	67.1	67.5	66.5	66.1	67.8	66.5	65.2	ns	*
gluteus primus	70.2	69.3	67.8	67.6	67.3	66.4	71.8	68.3	66.7	**	**
Press fluid yield, %											
pectoralis major	48.7	50.2	46.9	44.1	46.9	39.2	46.5	45.9	45.4	*	**
thigh muscles	52.4	50.6	50.0	50.2	49.2	44.1	54.6	48.9	45.0	*	**
gluteus primus	48.9	46.2	44.9	47.9	46.3	46.1	48.8	46.9	47.7	ns	*
Juiciness-score [3]											
thigh muscles	6.7	6.2	6.1	6.5	6.3	5.8	6.7	5.9	5.6	ns	*
gluteus primus	6.0	6.0	6.4	6.7	6.1	6.1	6.8	5.8	5.5	ns	*

[1] * denotes significant differences at 5% level of probability; **, significant at 1% level ns, non-significant.
[2] Percentages of ready-to-cook turkey weights.
[3] 9 (very juicy) to 1 (very dry).

temperatures increased, was very slight; values at 165° and 175°F were similar and at 175° and 185°F, similar. The decrease in total moisture content of gluteus primus muscles was significant with each increase in end-point temperature.

Press fluid yields of pectoralis major and of the composite of thigh muscles were different at each storage period. In both cases, press fluid yields for turkeys at 5 months frozen storage were lower than values for turkeys stored 10 months, and both values were less than those for fresh turkeys. The changes in press fluid yields obtained by cooking turkeys to different end-point temperatures followed the pattern described for total moisture contents of the muscles (Table 5).

The scores for juiciness of cooked turkey meat by the taste panel did not change significantly over the storage periods for the turkeys. As might be expected from the slight changes in moisture values observed by objective measurements, the taste panel could not detect these changes on samples judged at 5-month intervals. Differences in juiciness observed in turkeys cooked to the different end-point temperatures were significant only for thigh muscle samples. For both the composite of thigh muscles and for the gluteus primus muscles, the change was significant between 165° and 175°F, with no further decrease in juiciness when the end-point temperature was increased to 185°F.

Moisture content of cooked skin decreased with each increase in the end-point temperature. These values, as end-point temperatures increased, were 50.2, 47.2 and 43.9% and did not change significantly after holding turkeys in frozen storage.

Fat contents of cooked samples of pectoralis major, a composite of thigh muscles, gluteus primus, and of skin were 0.6, 6.2, 5.9 and 27.9%, respectively, based on wet sample weights. There was no significant variation during storage.

Doneness, odor, flavor, and texture changes

Significant changes in measurements of eating quality of cooked turkey meat other than those of moisture are summarized in Table 6.

Scores for doneness of cooked turkey meat from the pectoralis major and a composite of thigh muscles increased for turkeys held in frozen storage, as compared to those not stored. Scores for doneness of the gluteus primus muscles, which lie on the outer surface of the thighs, were near optimum (mean score 5.3) throughout the study.

Scores for doneness of cooked pectoralis major muscles indicated the meat was at optimum doneness or overdone (183° to 187°F) even when turkeys were cooked to only 165°F in the thigh muscles. There was a significant increase in doneness when 175°F was the end-point for cooking, but no further change when the end-point for cooking was 185°F.

Increases in doneness of cooked thigh muscles were significant with each increase in end-point for cooking. Thigh muscles were underdone at all end-points of cooking for fresh turkeys. For frozen-stored turkeys, cooked thigh muscles were near optimum doneness at 175°F and very slightly over optimum at 185°F. These differences in levels of doneness for fresh and frozen-stored turkeys cooked to the same end-point temperature suggest that cooking rate has an effect. That is, fresh turkeys which show a fast rate of heat penetration require a higher end-point temperature for comparable doneness to frozen-stored turkeys which take a longer time to reach a selected end-point temperature.

It may be possible to remove many of the differences in eating quality of cooked meat from fresh and frozen-stored

Table 6. Mean measurements for doneness, odor, flavor and texture of fresh and frozen-stored turkeys roasted at 325°F.

Storage—months at −5°F	0			5			10			Significance of F values [1]	
Cooking end-points −°F	165	175	185	165	175	185	165	175	185	Storage	Cooking
Score											
Doneness [2]											
pectoralis major	5.7	6.4	6.7	6.7	7.0	7.4	6.4	7.2	7.6	*	**
thigh muscles	3.6	4.1	4.4	4.5	4.9	5.3	4.1	4.7	5.2	*	**
Odor [3]											
gluteus primus	4.3	4.4	4.8	6.1	6.0	6.0	5.5	5.9	6.0	**	ns
Flavor [3]											
gluteus primus	4.9	4.8	5.5	6.0	6.3	6.4	5.8	5.7	6.3	*	ns
Tenderness [3]											
thigh muscles	5.8	6.0	5.7	5.6	6.1	6.9	6.3	6.3	6.7	*	ns
Mealiness [3]											
pectoralis major	5.7	6.4	6.7	6.7	6.5	7.8	6.0	7.3	7.4	ns	*
thigh muscle	1.9	1.8	2.1	2.3	2.6	3.0	2.4	2.8	3.1	*	**
gluteus primus	2.7	2.8	2.8	3.4	3.9	3.8	3.5	3.8	4.3	*	ns
Shear force—lb/g											
pectoralis major	7.5	6.6	8.2	9.2	8.5	9.7	7.3	6.9	7.9	*	*

[1] * denotes significant differences at 5% level of probability; **, significant at 1% level; ns, non-significant.
[2] 5 denotes optimum doneness; 6 to 9, increasing overdoneness; 4 to 1, increasing underdoneness.
[3] 9 denotes high intensity; 1, low intensity.

turkeys by cooking turkeys to the same level of doneness. This laboratory has reported that when the rate of heating frozen, thawed, boneless turkey roasts was varied by oven temperature from 250° to 400°F, the internal end-point required for optimum doneness of the cooked roast was not changed (Hoke *et al.*, 1967). However, it may be that the rate of coagulation by heating is sufficiently different between fresh and frozen muscles of whole turkeys to require different end-points for the same degree of doneness.

Mean scores for odor and flavor of cooked pectoralis major muscles were 6.7 and 6.2, respectively, and for the composite of thigh muscles, 6.0 and 5.9. These scores described slightly less than full characteristic odor and flavor which did not vary significantly throughout the storage study. Odor and flavor of gluteus primus muscles were higher for stored than for fresh samples (Table 6). Goertz *et al.* (1960) observed that flavor scores for light meat from turkeys were highest when meat was scored most done, but flavor of dark meat was highest for fresh turkeys which had the shortest cooking times compared to turkeys cooked longer but held in frozen storage for 1 and 3 months.

In this study, the scores for flavor of cooked pectoralis major muscles tended to increase with the longer cooking times associated with frozen storage of turkeys and higher end-points for cooking. Flavor scores of a composite of thigh muscles and for gluteus primus muscles were higher for turkeys cooked to higher end-point temperatures and for turkeys cooked longer at 5 months as compared to the shorter cooking fresh turkeys. However, flavor of cooked dark meat was lower for turkeys stored 10 months than for those stored only 5 months. Although this trend in dark meat flavor was not significant, it may indicate that an undesirable flavor change in dark meat takes place in turkeys stored for 10 months and is not compensated by increased cooking times that tend to increase odor and flavor to desirable intensities (Hoke *et al.*, 1967).

Texture changes in cooked turkey meat, significantly detected by the taste panel, were increased tenderness for thigh muscles, increased mealiness of cooked thigh and gluteus primus muscles from frozen-stored as compared to fresh turkeys, and significant increases in mealiness of both cooked pectoralis major and thigh muscles with each increase in end-point temperature for cooking. Mean tenderness score for pectoralis major muscles was 7.6 and for gluteus primus muscle, 7.0.

There was a significant increase in the force to shear cooked pectoralis major muscles from turkeys stored 5 months compared to that from fresh turkeys or from those stored 10 months. Significantly lower shear values were obtained for cooked pectoralis major muscles from turkeys cooked to 175°F rather than to 165° or 185°F (Table 6). Shear values for the composite of thigh muscles had a mean value of 9.3 lb/g and of gluteus primus muscles, 12.0 lb/g.

Overall, results of this study suggest that optimum cooking of either fresh or frozen-stored turkeys is of prime importance for high consumer acceptance. There does not seem to be a significant change in the muscle proteins studied from which marked changes in characteristics of eating quality between fresh and frozen turkeys could occur.

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Ms. rec'd 12/21/67; revised 3/8/68; accepted 6/11/68.

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The Formation of Carbonyl Compounds in Cucumbers

SUMMARY—The carbonyl contents of benzene extracts of aqueous cucumber homogenates were estimated spectrophotometrically as the 2,4-dinitrophenylhydrazones. A large increase in the formation of carbonyl compounds occurred when cucumbers were blended with water in the presence of oxygen. This formation of carbonyl compounds was prevented by three methods: blending the cucumbers at pH 1.0; blending in an oxygen-free atmosphere; and heating whole cucumbers to an internal temperature of 77°C before blending.

Chromatographic assays indicated that negligible amounts of the 2-enals, 2,6-nonadienal, 2-nonenal, and 2-hexenal are present in intact cucumbers; but a rapid synthesis of this class of carbonyl compounds occurred when fresh cucumbers were blended in the presence of oxygen. The most significant increase occurred in the formation of 2,6-nonadienal, the aldehyde largely responsible for the flavor of fresh cucumbers. There were indications that ethanal and propanal were present in appreciable levels in intact cucumbers.

These observations suggest that the characteristic flavor components of fresh cucumbers are generated enzymatically as a consequence of cutting or mechanically rupturing the fruit.

INTRODUCTION

IT IS WELL KNOWN that the metabolic activity of plants is altered when the tissue is cut or crushed, sometimes resulting in the formation of new products (Virtanen, 1962). This phenomenon, which may be considered an artifact by the plant physiologist, is of practical as well as theoretical interest to those concerned with the flavor and other organoleptic properties of fruits and vegetables.

Evidence indicates that certain compounds important to the flavor of metabolically active fruits and vegetables may be formed when the tissue is physically damaged (Weurman, 1963). A classic example of this phenomenon is the formation of garlic and onion flavor compounds when the bulbs are crushed (Stoll *et al.*, 1951; Schwimmer *et al.*, 1961).

More closely associated with the work reported here is the evidence that 2-hexenal is not present in several plant materials including certain leaves (Nye *et al.*, 1943), strawberries (Winter *et al.*, 1964), bananas (Hultin *et al.*, 1961), and apples (Drawert *et al.*, 1965); but this aldehyde is formed when the above products are crushed.

The flavor of fresh cucumbers has been attributed largely to aldehydes and to a lesser extent certain corresponding alcohols (Forss *et al.*, 1962). The pleasant element was attributed to 2,6-nonadienal, while two other unsaturated aldehydes, 2-hexenal and 2-nonenal, and three saturated aldehydes, ethanal, propanal, and hexanal were considered to contribute secondarily to the overall flavor. The objectives of the present study were to learn if the above carbonyl compounds are present in intact cucumbers and to study the effect of grinding cucumbers in a blender on the formation of these compounds.

MATERIALS AND METHODS

FRESH MODEL variety pickling-type cucumbers, approximately 1¼ in. in diameter, were used in this study.

The carbonyl contents of cucumbers were quantitatively estimated by extracting aqueous homogenates of cucumbers with benzene, conversion of the carbonyls in the extracts to 2,4-dinitrophenylhydrazones and subsequent spectrophotometric assay. Cucumbers were peeled (1 to 2 mm thickness of the skin, stainless steel knife) and ground with an equal weight of distilled water in either a Waring Blendor or a Sorvall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The slurry was then saturated with sodium chloride and shaken in a separatory funnel with 1/5 volume of benzene.

After centrifugation to break the emulsion, a portion of the benzene layer was removed and assayed for carbonyl content by the Henick *et al.* (1954) procedure. Reagent grade benzene (Fisher Scientific Co., ACS Certified) was found to be sufficiently carbonyl free for these extractions without further purification. One to five ml of benzene extract and sufficient benzene to make a total of 5 ml were added to 50 ml volumetric flasks containing 3 ml of 4.3% trichloroacetic acid in benzene and 5 ml of benzene saturated with 2,4-dinitrophenylhydrazine (Matheson Coleman and Bell). The flasks were glass-stoppered and heated for 30 min at 60°C and then cooled. Ten ml of potassium hydroxide in ethanol (4%, w/v) was added slowly while mixing and absorbances at 430 and 460 mμ were determined 10 min later.

Calculations for concentrations of saturated and unsaturated carbonyl compounds in these flasks were based on the extinction coefficients and equations reported by Henick *et al.* (1954) for the analysis of hexaldehyde and crotonaldehyde mixtures. From these calculations and correction for dilution, the total contents of carbonyls in the extracts were expressed as μ moles per 100 g fresh cucumber. The proportion of unsaturated carbonyls of this total was expressed as mole %.

Spectrophotometry was performed with a Bausch and Lomb 505 spectrophotometer.

Gas chromatographic analyses were performed with a Barber-Colman Model 10 gas chromatograph equipped with a hydrogen flame detector. A stream splitter permitted 50% of the column effluent to exist through the collector port for odor monitoring. Separations were ac-

complished with Apiezon L, 5%, on Chromosorb G (60 to 80 mesh, AW-DMCS, Applied Science Laboratories, Inc.) contained in a 6-ft, ¼-in. internal diameter glass column. Conditions for the analyses were: column, 120°C; cell, 200°C; nitrogen carrier gas, 17 psi inlet, 10 ml/min through collector; hydrogen, 15 psi; oxygen 25 psi.

Column chromatographic separations of 2,4-dinitrophenylhydrazones were carried out by adsorption on magnesia as a modification of the Schwartz *et al.* (1963) procedure. Fifteen g each of Seasorb 43 and Celite 545 (Fisher Scientific Co.) were slurried in 150 ml of chloroform and poured through a funnel into a glass column, 2.5 × 51 cm, containing a small wad of glass wool at the base. The column was packed under 2 to 3 psi nitrogen, allowing the solvent to reach a level of about 5 mm above the packed portion. Benzene extracts of cucumbers were diluted to the same carbonyl concentration, 2.5×10^{-4}M. These solutions were saturated with 2,4-dinitrophenylhydrazine and combined with an amount of 4.3% trichloroacetic acid proportionate to that defined in the Henick *et al.* (1954) assay procedure. The solutions were heated for 30 min at 60°C, cooled, and then washed four times with two volumes of distilled water to remove the acid.

After drying the solutions over sodium sulfate, the benzene was removed by evaporation *in vacuo* at 40°C. A 2-ml sample containing 10 micromoles of the 2,4-DNPH derivatives contained in chloroform was pipetted onto the column, allowed to be adsorbed, and then eluted with a sequence of solvents consisting of 200 ml chloroform, 100 ml each of 1, 3, 6, 15 and 50% methanol in chloroform, respectively, and finally 200 ml methanol. Five-ml fractions were collected with an LKB type 3402B automatic fraction collector and absorbances determined at 360 mμ.

Thin-layer chromatography (TLC) of 2,4-dinitrophenylhydrazones was performed with kieselguhr G plates (20 × 20 mm) coated with 2-phenoxyethanol as described by Urbach (1963). The plates were spotted 3 cm from the origin and developed in heptane which had been saturated with 2-phenoxyethanol. Four ascents of the solvent to about 3 cm from the top of the plate were allowed, the plate being removed and dried a few minutes before return to the developing tank for a subsequent ascent. This procedure was used by Urbach for the separation of hydrazones of an homologous series and was a valuable aid in identifying the components in eluates from class separations by column chromatography.

RESULTS AND DISCUSSION

Total carbonyl content of cucumber extracts

Over 80% of the unsaturated carbonyl compounds of fresh cucumbers was extracted into benzene in the first extraction when one part benzene to 5 parts of a sodium chloride-saturated cucumber slurry was shaken in a separatory funnel and the emulsion centrifuged. The saturated carbonyl compounds were less completely extracted into benzene and were present in the aqueous phase in appreciable quantities after three successive extractions. It was assumed that the more hydrophilic carbonyl compounds, e.g. ethanal and acetone, were responsible for the less complete extraction of the saturated carbonyls into benzene. Since the longer chain unsaturated carbonyls were of primary interest, a single benzene extraction was adopted for use in quantitative estimations. Benzene extracts of peeled cucumbers absorbed negligibly when appropriately diluted and read at the wavelengths specified in the assay. With unpeeled cucumbers the assay was influenced slightly due to the presence of pigments in or near the skin, hence the reason for removing the skin prior to extraction.

Earlier studies in this laboratory indicated that the carbonyl content of intact fresh cucumbers is comparatively low. This conclusion was made after a comparison of the carbonyl content of fresh cucumbers blended in distilled water with that of cucumbers blended in acidified water (final pH about 1.0). It was assumed, therefore, that the major portion of carbonyl compounds are formed enzymatically as a consequence of crushing the cucumbers. Several prior reports have shown that 2-hexenal is formed enzymatically upon crushing plant leaves and certain fruits. Nye *et al.* (1943) found that 2-hexenal is formed by leaves of *Ailanthus glandulosa* upon grinding in the presence of air, but is not present in the intact leaves. A similar observation was reported by Major *et al.* (1963) for leaves of the tree, *Ginkgo biloba*. In both instances, oxygen was essential for the formation of 2-hexenal and an enzymatic mechanism was assumed to be involved.

An experiment was designed to learn if oxygen is essential for the formation of carbonyl compounds in ground cucumbers. Cucumbers were blended under controlled aeration with a modified Sorvall Omni-Mixer. The stainless steel cover of the apparatus was fitted with two 1-in. ports (3 mm I. D.) for inlet and outlet of gases. A 100-g sample of peeled and coarsely diced (about 1-in. cubes) cucumbers and 100 ml of distilled water were placed in a pint Mason jar and then the jar was coupled to the cover and blade assembly by means of an adapter ring. One sample was purged with nitrogen for 5 min and then blended. The slurry was acidified to approximately pH 1.0 by slowly adding 1 *N* HCl with a syringe through the outlet port; nitrogen gassing continued. The sample was then removed from the jar, saturated with sodium chloride and immediately extracted with benzene. The data in Table 1 show that the total carbonyl content of this extract was only about 20% of that when the cucumbers were blended in the presence of oxygen before acidifying. The cucumber slurry was still capable of forming carbonyls after being blended under a nitrogen atmosphere.

After gassing with nitrogen and blending as described above, a sample was then gassed with oxygen for 5 min before acidifying. It was concluded from these results that the activity of an enzymatic, oxygen-requiring, carbonyl-forming system in cucumbers is greatly increased when the tissue is mechanically ruptured; this system may be incapable of operating in the intact tissue. The system operates rapidly at room temperature as no appreciable increase in carbonyl content occurred beyond 5 min after blending the cucumbers.

The enzymatic formation of carbonyl compounds in cucumbers was prevented by another means, heating prior to blending. Whole cucumbers were packed into ½-gal jars, 1 kg per jar. The jars were then filled with distilled

Table 1. Control of carbonyl formation in blended cucumbers.

Treatments[1]	Total carbonyl content (μmoles/100 g)[2]	Unsaturated carbonyl content (mole %)
Aeration control during blending		
Blended under N_2, then acidified	8.5	31.2
Blended under N_2, gassed with oxygen, then acidified	45.9	39.7
Blended under oxygen, then acidified	42.2	39.5
Heat inactivation of enzymatic activity		
Heated whole cucumbers, then blended	10.3	19.4
Blended to allow carbonyl formation, then heated	39.5	13.1
Blended, not heated	44.1	42.7

[1] See text for detailed description of procedure.
[2] Expressed on a fresh weight basis of the cucumbers.

water, capped ("Twist-Off" cap, White Cap Co., Chicago, Ill.) and immersed and heated in an 82°C water bath to an internal temperature of 77°C. A control jar containing a thermometer placed in the center of a cucumber served to indicate the internal temperature. The jars were cooled to room temperature and the cucumbers were removed, peeled and then ground in a Waring Blendor and extracted as described earlier. The total carbonyl content of the heated whole cucumbers was less than 25% that of the fresh, unheated cucumbers (Table 1). Also, the proportion of unsaturated carbonyls was lower in the heated cucumbers.

A third sample served to assess the effects of heat on destruction of carbonyls. Peeled cucumbers were blended with an equal weight of distilled water to allow carbonyl formation. This homogenate was transferred to ½-gal. jars, and then the jars were capped and heated to an internal temperature of 77°C as were the whole cucumbers. It was found that the total carbonyl content of this sample was slightly reduced when compared to the fresh, unheated cucumbers. The proportion of unsaturated carbonyl compounds was appreciably lower, indicating partial destruction of this class of carbonyl compounds by heat (Table 1).

Gas liquid chromatography of cucumber benzene extracts

Portions of benzene extracts from the heat inactivity study reported in Table 1 were assayed by gas chromatography (GLC). The benzene from 50 ml of extract, representing the carbonyl content of 125 g of cucumbers, was removed by evaporating slowly at 40°C *in vacuo* in a flash evaporator. The concentrate from the "heated whole" cucumbers had a hay-like, cooked vegetable odor; no odor of fresh cucumbers was evident. The concentrate from the fresh unheated cucumbers had a pleasant and strong odor of fresh cucumbers. The concentrate from cucumbers blended prior to heating had a hay-like, cooked vegetable odor but in addition an odor of fresh cucumbers.

The oily residues were taken up in 0.2 ml of re-distilled carbon disulfide. Five microliters of these samples were gas chromatographed on Apiezon L (isothermally, 120°C). There is a striking contrast in the GLC profiles of Fig. 1.

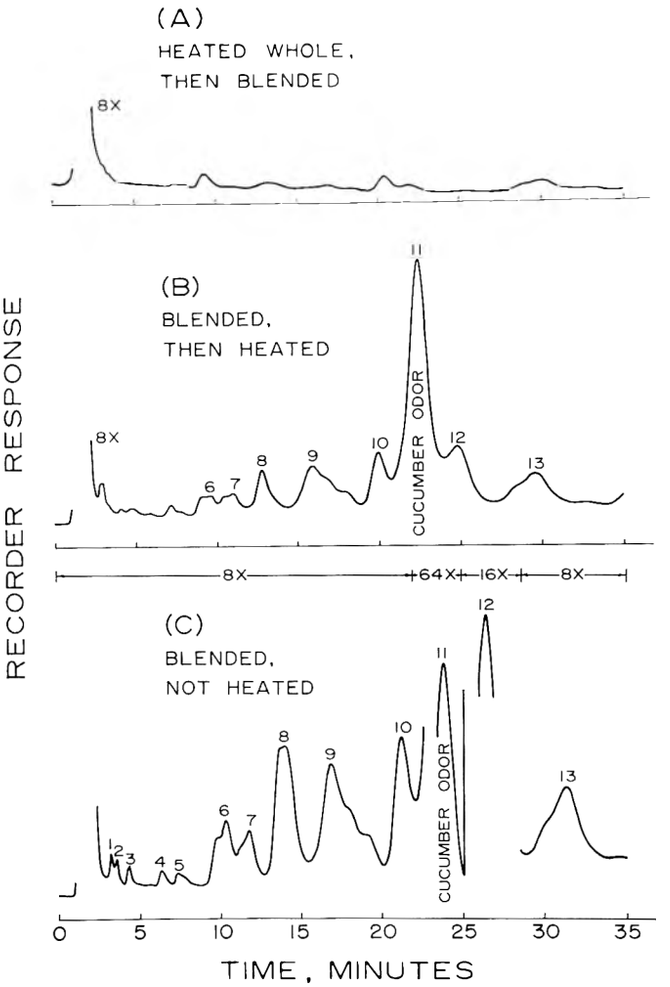

Fig. 1. Gas chromatograms of benzene extracts of cucumbers. The extracts were prepared from the samples included in the heat inactivation study of Table 1. All three samples represented extracts from equal quantities of cucumbers.

The profile of the "heated whole" cucumbers is devoid of most of the peaks showing in the fresh cucumber concentrate, and most of those appearing are greatly reduced. The concentrate from cucumbers heated after blending indicates the presence of most of the compounds of the fresh sample, but at lower levels. Most of the lower boiling compounds were undoubtedly lost during concentrating; however, some of the higher boiling carbonyls, such as the 9-carbon aldehydes, should have been retained.

The odors of the components emerging from the stream splitter of the gas chromatograph were monitored. Peak no. 11 of the fresh cucumber concentrate had a strong, definite fresh cucumber odor. Peak nos. 1, 2, 9, 10 and 12 were described as either aldehyde or cucumber-like. These peaks, although smaller, also were present in the sample from cucumbers heated after blending. Synthetic 2,6-nonadienal (Haarmann and Reimer Corp., Union, New Jersey) had the same retention time as did peak no. 11. The "heated whole" cucumber sample gave no peak or odor in the region where cucumber and aldehyde-like odors emerged with the other two samples.

Chromatography of 2,4-DNPH derivatives

It was desired to learn the relative amounts of carbonyl classes, as well as individual carbonyl compounds, in the fresh as compared to the heated cucumber samples (heat inactivation study, Table 1). Ten micromoles of 2,4-dinitrophenylhydrazones, prepared from the benzene extracts of these three samples, were pipetted onto Seasorb 43-Celite 545 columns and eluted with chloroform containing increasing concentrations of methanol (Materials and Methods). The carbonyl compounds of interest were eluted by the time the 15% methanol in chloroform solution had passed through the column. Only a relatively small quantity of carbonyls was eluted with higher concentrations of methanol and the components of these eluates were not studied.

Elution profiles for the three samples are shown in Fig. 2. Peak tubes were evaporated to dryness and the hydrazones dissolved in chloroform. Absorption spectra of these fractions were determined and the wavelength maximum for each of the peaks is indicated in Table 2. The assignment of peak fractions to carbonyl classes was made on the basis of the absorption maximum and verification by TLC analysis of the components. The slight deviation in the absorption maxima reported in Table 2 from the values reported for the pure 2,4-DNPH classes (Schwartz *et al.*, 1962) was expected since the chromatographic procedure did not render complete resolution. A blank containing the appropriate reagents and solvents was carried through the same procedure as the samples. From this blank it was ascertained that peak no. 1 of Fig. 2 was a reagent or solvent impurity; otherwise, only negligible amounts of carbonyl impurities were present.

After determining the absorption maxima, the peak tube samples were again evaporated to dryness, redissolved in 0.1 ml of chloroform and assayed by thin layer chromatography. Identification of the DNPH's (Table 2) was made by a comparison of R_f values with pure derivatives. There was a strikingly low level of 2-enal compounds in the "heated whole" cucumbers as revealed by column fractionation (Fig. 2) and the TLC data confirmed this observation. The "heated whole" cucumbers contained only a trace of 2,6-nonadienal and no detectable level of 2-nonenal and 2-hexenal whereas unheated cucumbers contained a significant amount of the former two aldehydes and a trace of 2-hexenal.

Heating caused a reduction in the level of 2-enals as evidenced by the cucumbers that were blended to allow carbonyl formation prior to heating (Fig. 2 and Table 2); but, TLC revealed a significant quantity of 2,6-nonadienal and 2-nonenal in the "blended, then heated" cucumbers. Appreciable quantities of ethanal and propanal, as well as a detectable amount of hexanal, were present in the "heated whole" cucumbers. These and foregoing observations (Table 1) indicate that the 2-enals, if present in intact cucumbers, are there in comparatively low amounts; a large increase in the formation of these aldehydes occurs when fresh cucumbers are blended in the presence of oxygen. The levels of alkanals also increased when fresh cucumbers were blended but they were also present in detectable levels in the intact fruit. Acetone was present

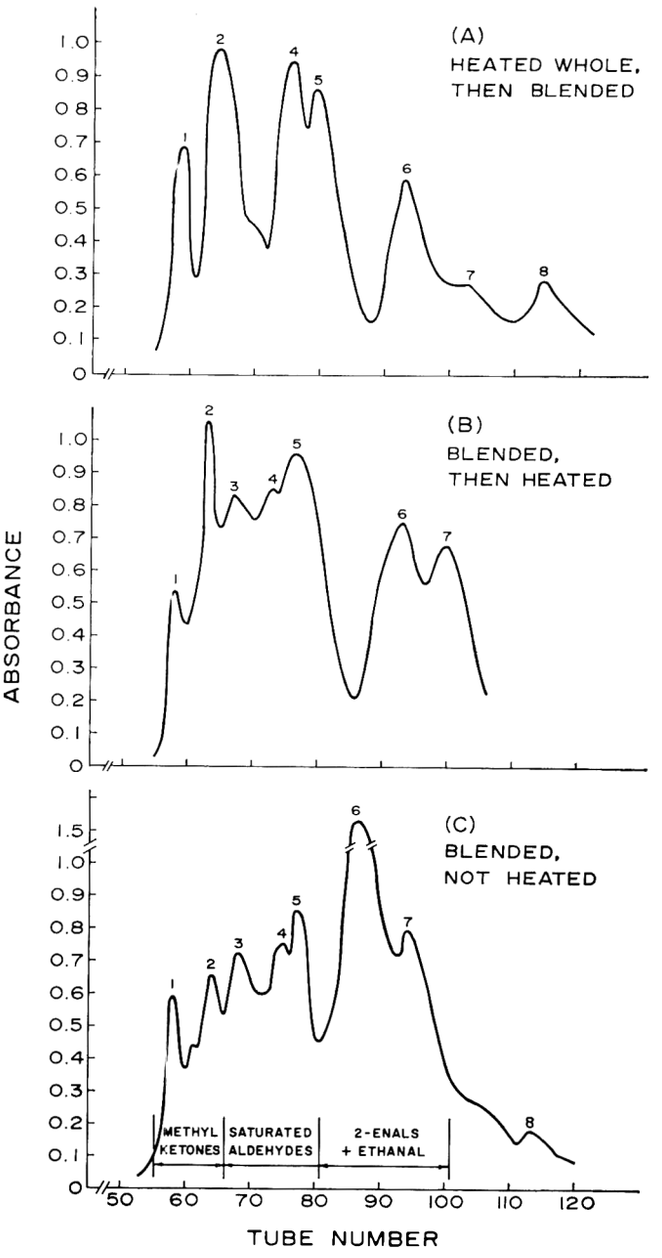

Fig. 2. Column chromatography of 2,4-dinitrophenylhydrazones from benzene extracts of cucumbers. The extracts were prepared from the samples included in the heat inactivation study of Table 1 and correspond to the samples that were analyzed gas chromatographically (Fig 1). The profiles represent relative proportions of three carbonyl classes for each sample, when 10 μ moles of hydrazone were chromatographed. A quantitative comparison among samples may be made by considering the total carbonyl contents (Table 1).

in a relatively large amount in the "heated whole" as compared to the unheated cucumbers. Apparently acetone is present in intact cucumbers; but heating may have caused an additional amount of this compound. Several compounds were detected by TLC but were not identified. Three such compounds of possible significance were present in peak No. 3 of the "unheated" and the "blended, then heated" cucumbers. These compounds were not present in the "heated whole" cucumbers, and may be metabolic intermediates related to the formation of 2-enals.

Table 2. Identification of 2,4-dinitrophenylhydrazones in fractions from column chromatography.[1]

Peak no.	λ max	Carbonyl class	Tentative compounds	Detection of fractionated cucumber DNPH's by TCL[2]		
				Blended not heated	Blended then heated	Heated whole then blended
1	366	methyl ketone	unknown	+	+	+
2	362	methyl ketone	acetone	++	++	++
3	358	unknown	unknown	+	+	−
4	358	alkanal	hexanal	+	+	+
			nonanal	+	+	−
5	358	alkanal	propanal	++	++	++
6	372	2-enal	ethanal	+	++	++
		(+ ethanal)	2,6-nonadienal	++	−	−
			2-nonenal	+	−	−
			2-hexenal	trace	−	−
7	375	2-enal	2,6-nonadienal	++	++	trace
			2-nonenal	+	+	−
8	372	unknown	unknown	+		+

[1] These data were obtained from eluates of the column fractionations depicted in Fig. 2. Peak No. refers to the fraction so numbered in Fig. 2 and λ max values refer to the wavelength of maximum absorption of the fractions from the unheated cucumber sample (Fig. 2 C).

[2] The presence of a compound in the fraction, as indicated by TLC, is designated by a "+," compounds of highest concentration in the fraction (visual estimation) by a "++," and the absence by a "−."

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Ms. rec'd 1/19/68; revised 6/12/68; accepted 6/24/68.

Paper number 2515 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

The authors wish to thank P. J. Hauser and R. M. Swindell for their valuable technical assistance in this study. Gratitude is expressed to the Mount Olive Pickle Company, Mount Olive, N. C., and Perfect Packed Products, Inc., Henderson, N. C., for supplying cucumbers throughout this study.

Mention of firms or products does not imply endorsement.

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Effect of Low Temperature on Structure and Firmness of Apple Tissue

SUMMARY—Apple tissue was frozen at different temperatures and in different solutions, thawed, examined microscopically and tested for resistance to compression (firmness). In slow freezing, ice crystals separated the cells, crushed them, and ruptured cell walls to produce radial splits in the tissue. The mechanical freezing damage occurs during freezing rather than during thawing. The firmness of frozen tissue was much lower than that of raw tissue. The effect of most added solutes was to minimize ice-caused damage and to increase firmness with increasing concentration. The lower the temperature of freezing, the firmer was the tissue and the less was the amount of tissue disruption. The maximum firmness of frozen tissue was about half that of raw tissue, with the major decrease in strength due to loss of turgor. Firmness was increased to about twice that of raw tissue by immersion of the raw tissue in salt solutions at −2°C or at lower temperatures if the solutions remained unfrozen.

INTRODUCTION

Meryman (1956, 1966) has reviewed the broad outlines of ice formation in living tissues at different rates of freezing. During slow freezing, intercellular ice is formed, and there is virtually no rupture of membranes—only the separation of cells. Often animal tissues will survive slow freezing if the temperature does not go so low as to permit intracellular ice to crystallize. With rapid freezing, however, intracellular ice is formed, and most cells are permanently and irreparably damaged, but again there is said to he usually no microscopic tearing of membranes.

Although there has been a great interest in the mechanism of ice formation in plant tissues, few studies give a clear picture of the change in cellular relations as a result of freezing. Woodroof's (1938) pioneering investigation on this topic is illustrated principally by drawings and low-power photomicrographs. The microscopic investigations of Lee *et al.* (1949) and Maurer et al. (1951) were made by reflected light with free-hand sections, and the results are not as clear-cut as are desirable.

The sections shown in more recent studies (Brown, 1967; Hulle *et al.*, 1965) again have low magnification. Moreover, there are conflicting reports that there is either little or no cell wall breakage (Levitt, 1941; Meryman, 1966) in extracellular freezing or that there is indeed cell wall rupture (Woodroof, 1938; Cox *et al.*, 1942; Brown, 1967). It seemed warranted, therefore, to conduct a careful histological study, supplemented by measurements of firmness, on the changes in plant tissue at different low-temperature exposures. Because of the uniformity of their tissues and their structural stability in storage, apples were chosen as the test material.

MATERIALS AND METHODS

THE APPLE VARIETIES, Newtown Pippin and Red Delicious, were obtained from Watsonville, California and stored at +2° and 0°C respectively, while being used. (These apples were kindly donated by Mr. R. Travers of Watsonville, Calif.) During a 6-month period of storage, changes in firmness (as measured by resistance to compression on a General Foods Texturometer, see below), were almost negligible.

At least 50 radial cylinders of tissue, 1 cm in diameter, were cut from previously cored fruit and trimmed to 1 cm length. The specimens were immersed in brass cups of test solutions, either directly or after first being placed in cage-like, screened cells. The immersion media were, besides water, aqueous solutions of sodium chloride, ethanol, glycerol, ethylene glycol, polyethylene glycol 400, dimethyl sulfoxide (DMSO), sorbitol, corn syrup solids, polyvinyl pyrollidone (PVP) and an inert, fluorocarbonic fluid (3M Co. L-1644). Many of these solutes will protect animal cells against freezing injury, and their possible mode of action has been discussed by Greaves *et al.* (1965).

The cups with immersed apple cylinders were held for 2 days at temperatures from +2° to −78.5°, the latter being provided by dry ice in acetone or ethanol. Rates of freezing in tissue in the center of the cup (in rapidly-stirred refrigerant) are shown in Fig. 1. The curves represent the temperature/time relationship at the center of a cylinder of apple tissue, 1 cm in diameter, immersed in a brass cup of inert, fluorocarbonic fluid that does not freeze at these temperatures. The immediate sub-zero plateau of each curve occurs at −1.5°C. Note that in curve D the tissue reaches this plateau in 3 min and remains there 1 min before dropping rapidly. In curve A, the plateau is reached after 10 min, and the tissue remains at −1.5° for 20 min before dropping gradually lower. Curves B and A are offset 10 and 20 min, respectively, from curves C and D (each dot occurs at a 30 sec interval).

Temperatures down to −45° were produced by mechanical refrigeration. Several lots (untreated) were frozen by immersion in liquid nitrogen (−196°C). The tissues were all thawed in distilled water at room temperature, and measurements of firmness were made.

Each cylinder of tissue was compressed along its longitudinal axis between two test plates of a General Foods Texturometer (Friedman *et al.*, 1963). The force necessary to compress a cylinder from 1 cm to 0.5 cm was recorded for each specimen. That force, in arbitrary "G. F. units," is an index of a physical quality that will be called

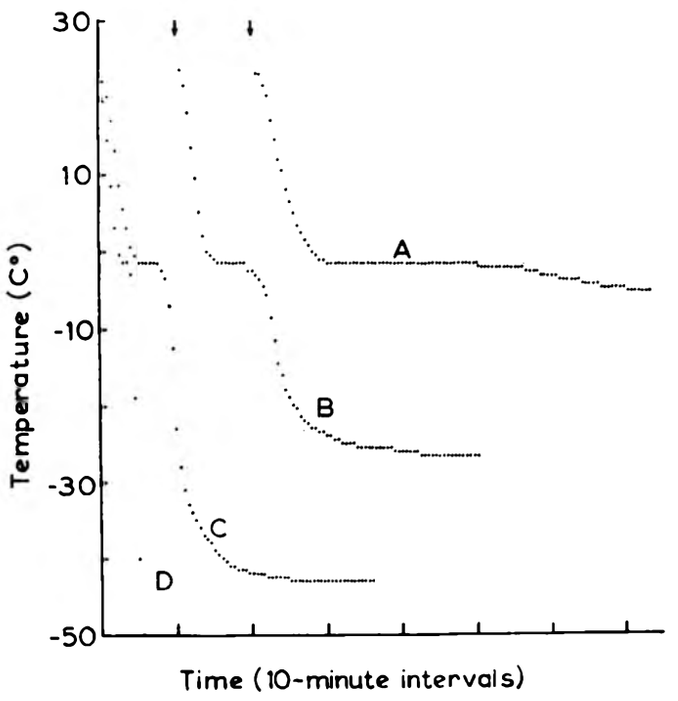

Fig. 1. Course of freezing in rapidly stirred refrigerant at several representative, ultimate temperatures: A, *−5°C;* B, *−25°C,* C, *−45°C;* D, *−78°C.*

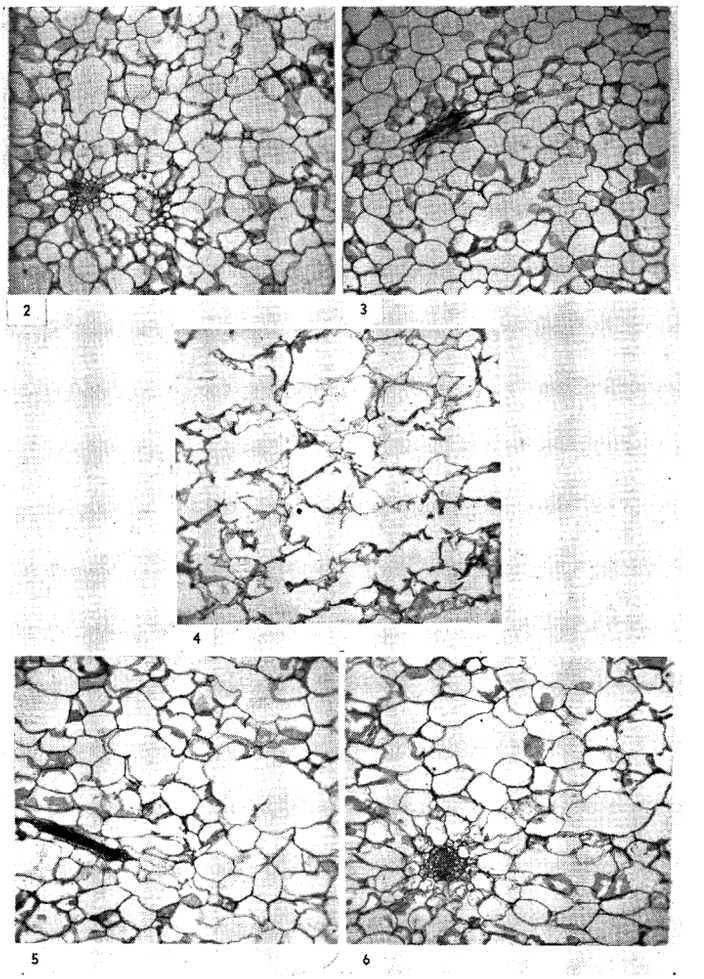

Fig. 2. Tangential section of raw Newtown Pippin apple. × 51. Fig. 3. Radial section of raw Newtown Pippin apple. Note elongated radial intercellular spaces extending from left to right. × 51. Fig. 4. Radial section of Red Delicious apple frozen in air at −17°C. Orientation of this and all other radial sections is the same as in Fig. 2. Many cells are compressed or distorted and many cell walls are broken. × 51. Fig. 5. Radial section of Red Delicious apple frozen at −78°C. × 51. Fig. 6. Radial section of Red Delicious apple frozen at −196°C. × 51.

"firmness," *i.e.,* the larger the number of units needed to cause the defined compression, the firmer the tissue.

When the results of the mechanical measurements appeared interesting, a second set of samples was frozen under the same conditions for histological study. The microtechnique involved the standard paraffin method, with sections cut at 15 μ and stained in methylene blue.

RESULTS

THE STRUCTURE of the raw apple tissue is shown in Figs. 2 and 3, which represent, respectively, tangential and radial sections through the parenchymatous flesh. As shown by these figures, the cells of the flesh are more or less isodiametric, loosely arranged, and surrounded by intercellular spaces that, according to Reeve (1953), amount to about 21% of the fruit volume. These spaces are usually elongated in a radial direction and may best be seen in radial section. Vascular bundles are usually small and relatively isolated.

When the untreated tissues are frozen slowly in air or water, extensive cell separation, compression and rupture occur (Fig. 4). The displaced cells are separated by radial spaces—indeed, the compressed cell remnants tend to lie in radial rows. Cells are compressed tangentially, and rupturing of the walls occurs in a tangential direction. These events prolong the radial dimension of the space. Note that the radially elongated spaces in the frozen fruit are much larger than those in the raw fruit. In slow freezing, ice is formed in the tissues at −1.5°C (Fig. 4). The longer the tissue remains at this temperature, the larger the ice crystals can grow (Meryman, 1956, 1966). The more rapid the rate of freezing, the less is the amount of cell distortion and separation. At −78°C (Fig. 5) and −196°C (Fig. 6) there is virtually no microscopic evidence of damage to the cell wall, and cell size is preserved.

Compression tests on tissues frozen in rapidly-stirred refrigerant (Table 1) indicated that the firmness of the tissue dropped from the raw value (100 G.F. units) as the tissue was frozen, with some samples reaching a minimum firmness at a final freezing temperature of −25°C (the rates of freezing are those of Fig. 1). Below that temperature, the compressive strength in all cases increased progressively, the lower the temperature. A maximum firmness is attained that is about half the raw value (the standard deviation of the values in Table 1 is about 10 G.F. units). Tissues frozen in liquid nitrogen experienced extensive tissue rupture, involving large cracks that fragmented the tissue. These tissues had very low values of firmness (*cf.* Wolford *et al.,* 1965).

Table 1. Firmness of thawed Red Delicious apple (G. F. Texturometer units).

Freezing environment	Freezing temperature (C°)					
	−5	−15	−25	−35	−45	−78[1]
Air						44
10% ethanol	27	41	58	64	54	40
20% ethanol	41	40	26	38	52	46
20% glycerol	43	21	22	.....	29	43
L-1644	24	27	33	31	37	42

[1] Data at −78°C from Newtown Pippin apples, converted to same scale as Red Delicious apples. Freezing rates corresponding to these temperatures are shown in Fig. 1. Firmness of raw apple: 100.

Table 2. Firmness of thawed Red Delicious apples (frozen at −17°C)[1] (G. F. Texturometer units).

Freezing environment	Concentration (%)			
	5	10	15	20
Glycerol	27	35	45	53
Polyethylene glycol 400	21	28	31	41
Ethylene glycol	15	23	38	38
Sorbitol	10	15	20	21
Corn syrup solids	10	13	13	15
Sucrose	13	15	18	18
DMSO	13	21	23	31
Ethanol	21	26	31	33
PVP	8	5	5	3

[1] Freezing rate described in text. Firmness of raw apple: 100.

Table 3. Firmness of thawed Newtown Pippin apples (G. F. Texturometer units).

Freezing environment	Freezing temperature (C°)[1]					
	+2	−2	−4	−6	−8	−10
Air		103	12	17	13	9
Water		42	11	17	14	16
L-1644		65	73	57	9	7
20% Sucrose		114	108	41	19	13
1 M $MgSO_4$		138	61	35	15	10
1 M NaCl		178	69	51	34	18
2 M NaCl	94	203	190	195	186	115
2 M NaCl + 10% Sucrose		216	197	210	100	21

[1] Freezing rate at −5° shown in Fig. 1. Firmness of raw apple: 100.

The effects of different percentages of added solutes were examined principally by slow freezing for 2 days in a cold room held at −17°C. The temperature in the tissue reached −1.5°C in 20 min and remained at this level for 15 min more. These results are given in Table 2, in which the values may be compared with the firmness of apple tissue frozen in air or water: 9 G.F. units. Fig. 7 and 8 show apple tissue immersed in 5% and 20% glycerol, respectively, as an example. Fig. 9 shows tissue from 10% ethanol. Most solutes do exercise a protective effect: the tissues are firmer, and the amount of permanent cellular damage is smaller than in unprotected tissue, shown in Fig. 4. Only in PVP was there a weakening of the tissue, which was accompanied by an intensive browning reaction. At −78°C (Table 1), none of the immersive solutions exercised any noteworthy effect different from that of freezing in air.

Solutes were also applied at temperatures in the vicinity of 0°C in rapidly stirred refrigerant (Table 3). Although this table concerns a different variety of apple from that in Table 2, both varieties are referable to a firmness of 100 G.F. units in the raw tissue. The dramatic drop in firmness can be correlated with the visible evidence of ice formation: in 20% sucrose there was no ice at −4°C, but ice was present below this temperature. A unique secondary phenomenon was found in salt solutions, namely that a special firming effect occurs at about −2°C. This effect

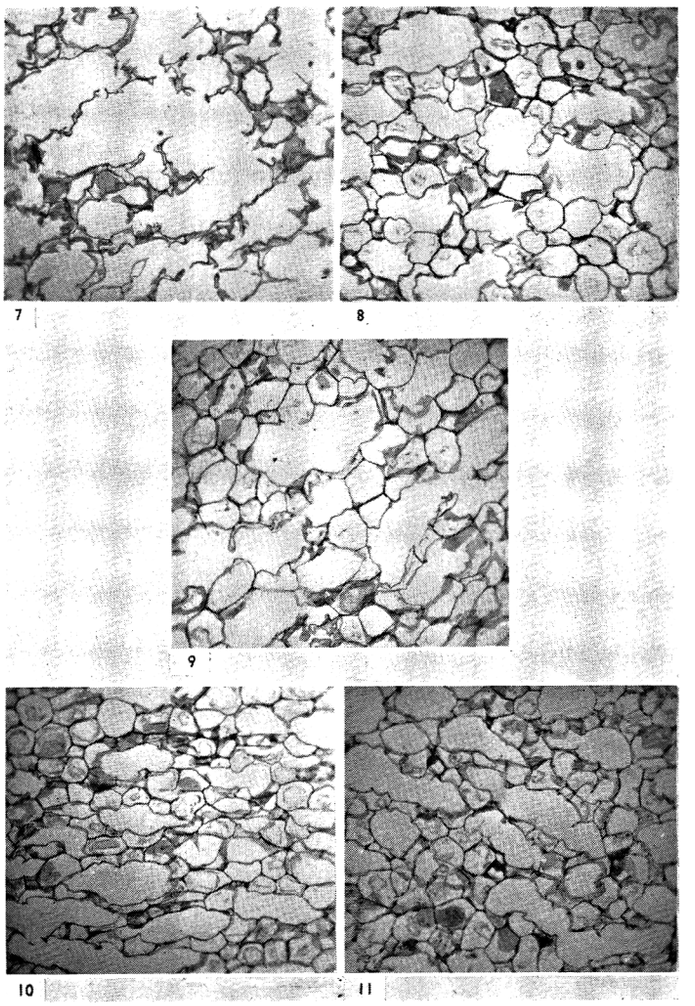

Fig. 7. Radial section of Red Delicious apple frozen in 5% glycerol at −17°C. × 51. Fig. 8. Radial section of Red Delicious apple frozen in 20% glycerol at −17°C. × 51. Fig. 9. Radial section of Red Delicious apple frozen in 10% ethanol at −17°C. × 51. Fig. 10. Radial section of Newtown Pippin apple in 2 M NaCl at −2°C. × 51. Fig. 11. Radial section of Newtown Pippin apple in 2 M NaCl at −10°C. Ice crystals had formed at this temperature. × 51.

occurs in 1M solutions of both NaCl and $MgSO_4$, and it appears to increase with increasing concentration: in 2M NaCl the effect extends to −8°C. However, when sucrose is added, the salt effect appears to be somewhat diminished at lower temperatures. Sections of the salt-treated tissues seemed to take up the methylene blue stain more avidly than those not immersed in salt solution. Fig. 10 and 11 show the tissues at −2° and −10°C.

DISCUSSION

THE MICROSCOPIC SECTIONS show structural alterations that are attendant on freezing. It is apparent that, in part, the loss of firmness is due to disruption of the tissues, particularly in slow freezing. Ice in the intercellular spaces forces the cells apart, compressing cells, frequently rupturing the middle lamella in large part, but also tearing across cell walls. The tissue weakens as its cohesiveness is destroyed by the growth of the crystalline, intercellular ice masses. A similar event seems to be shown in carrot (Hulle *et al.*, 1965), green bean (Brown, 1967; Wolford *et al.*, 1965) and onion (Shimazu *et al.*, 1965). Thus, unlike many animal cells (Meryman, 1966), plant cells can be torn by growth of ice crystals during slow freezing (*cf.* Woodroof, 1938). It is not known whether the tearing is due to penetration by a growing ice crystal or whether it is secondarily induced by tensile forces caused by expansion of ice crystals in a nearby intercellular space. The fact that all frozen tissues were thawed in the same way shows that the cellular damage occurs during freezing rather than during thawing.

The remainder of the loss of firmness still must be explained. It is apparent that with the most favorable freezing conditions, the firmness of the frozen tissue is at best about half that of the raw tissue. The most reasonable supposition is that turgor pressure in the raw tissue is responsible for its greater firmness. Indeed, when the raw tissue is killed by immersion in cold 20% ethanol without being frozen, the firmness of the now flaccid tissue is 50 G.F. units. Hence the loss of turgor, as the cells are killed in freezing, is mainly responsible for that portion of the loss of firmness.

Microscopic evidence for the protective role of solutes in freezing is that the size of the ice-formed intercellular cavities is reduced and that the tissue-rupturing activity of the ice crystals is mitigated. Ethanol appears to produce further tissue-firming effects in high concentration (firmness of 75–80 G.F. units at concentrations of 50 and 60%). This additional firming is probably attributable to dehydration and consequent hardening of the cell wall substances (it appears that PVP does not protect but rather weakens apple tissue, possibly by accelerating the oxidation of the cell wall materials).

The effect of salt in enhancing the firmness was marked in the Newtown Pippin apple but smaller in the Red Delicious apple. It did not occur in the freestone peach or the strawberry. Various possibilities, either separately or in conjunction, may be involved: (1) stimulation of pectic esterase activity in the cell wall, thereby permitting the formation of a salt-linked ionic pectate gel. This agrees with the observation on increased uptake of methylene blue and has also been shown for brined cherries (McCready *et al.*, 1954; Buren, 1967); (2) stimulation of salt accumulating activity in the still living cell, to increase the turgor over that of the untreated cell when immersed in water before testing; (3) dehydration of the cell wall.

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Ms. rec'd 2/19/68; revised 8/2/68; accepted 8/30/68.

Thanks are due to Miss Shiu-mei Huang for preparing the microscopic sections and to General Foods Corporation for financial support.

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Photoxidation of Cholesterol in Spray-dried Egg Yolk Upon Irradiation

SUMMARY—Photoxidation products of cholesterol in spray-dried yolk exposed to nonionizing radiation were examined by thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and infrared spectroscopy. Irradiation of yolk solids by radiant energy from either a 40-watt fluorescent lamp (about 280 hr) or summer sunlight (5 hr) brought about the formation of at least 5 photoxidation derivatives of cholesterol as demonstrated by TLC and GLC. The major oxidation products were identified as 7-ketocholesterol, 7α- and 7β-hydroxycholesterols, cholesterol-5β,6β-oxide and cholestane-3β,5α,6β-triol. Neither fresh yolk or unirradiated spray-dried yolk (held at 25°C for 1 year) contained significant amounts of typical autoxidation products of cholesterol.

INTRODUCTION

IONIZING AND NONIONIZING radiant energy enhance the autoxidation of cholesterol to structurally-related steroids (Bergström *et al.*, 1961). Schulze *et al.* (1904) were the first researchers to note that crystalline cholesterol, irradiated with sunlight in the presence of air, underwent decomposition. The reaction products were not identified. According to Dauben *et al.* (1956), crystalline cholesterol, stored at room temperature in air but in the absence of radiant energy, was stable for up to a year. But when crystalline cholesterol was held at a temperature of 82°C in the dark, Fioriti *et al.* (1967) detected some autoxidation products after several weeks. Pure cholesterol in aqueous suspension without irradiation was not oxidized by atmospheric oxygen even when the temperature was raised to the boiling point (Blix *et al.*, 1928). However, alkaline soap brought about extensive cholesterol autoxidation when the aerated dispersion was held at 90°C for 1 hr.

Several investigators have demonstrated that ionizing radiations, such as X-ray and γ-ray, cause autoxidation of cholesterol in the crystalline and aqueous dispersed states. Upon irradiation of aqueous dispersions of cholesterol by X-rays, Keller *et al.* (1950) isolated cholestane-3β, 5α,6β-triol and 7-ketocholesterol. The acetates of these decomposition compounds were formed when suspended cholesteryl acetate was irradiated by X-rays. In both cases, the triol was the predominant degradation product. When Horvath (1966) irradiated crystalline cholesterol at 50°C with γ-rays from a Co^{60} irradiator, up to 85% of the total cholesterol was decomposed. The degradation compounds were tentatively identified as cholestane-3β,5α,6β-triol, 7-hydroxycholesterols, 7-ketocholesterol and 3,5-cholestadien-7-one. Radiation-induced oxidation of cholesterol occurred when C^{14}-labeled cholesterol was stored in the presence of air (Dauben *et al.*, 1956). The 7-hydroxy, 7-keto and 5α,6β-dihydroxy derivatives of cholesterol were produced. The degree of decomposition was dependent on the duration of crystal storage.

Nonionizing radiation (ultraviolet, visible, sunlight) can bring about photoxidation of cholesterol. During the studies on the ultraviolet irradiation of cholesterol for the preparation of antirachitic factor, incomplete recovery of ultraviolet irradiated crystalline cholesterol by digitonin precipitation was noted by Hess *et al.* (1926), Rosenheim *et al.* (1926) and Sheer *et al.* (1927). Using the digitonin-precipitable compounds of ultraviolet irradiated cholesterol, Windaus *et al.* (1941) identified 7α-hydroxycholesterol and 4-cholestene-3,6-diol as reaction products.

Schenck *et al.* (1957) reported that 5α-hydroperoxy-6-cholestene-3β-ol was formed to the extent of 49% yield during the ultraviolet irradiation of a pyridine solution of cholesterol exposed to oxygen and in the presence of hematoporphyrin as a sensitizer. Undoubtedly, the absence of water in the system accounted for the restricted decomposition of the hydroperoxide to commonly encountered autoxidation products. Upon ultraviolet irradiation of crystalline cholesterol, Beckwith (1958) indicated that 25-hydroxycholesterol was formed. Fieser *et al.* (1957) detected this 25-hydroxy compound in a 4-yr-old sample of crystalline cholesterol. However, the environmental conditions were not presented. According to Fioriti *et al.* (1967), the irradiation of crystalline cholesterol at room temperature with ultraviolet light caused the formation of 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, 1,4-cholestadiene-3-one and 5-cholestene-3-one.

When crystalline cholesterol was exposed to radiant energy in the visible range (from a fluorescent light), 7α- and 7β-hydroxycholesterols, as well as 25-hydroxycholesterol, were identified by Claude *et al.* (1966). Hais *et al.* (1965) found that during the exposure of aqueous dispersions of radioactive cholesterol (26-C^{14}) to daylight, compounds more polar than cholesterol were formed. Photoxidation was greatest when the dispersions were strongly acidic or alkaline. Moreover, the radioactive cholesterol in the dry state was oxidized rapidly upon exposure to daylight. According to Acker *et al.* (1963), the loss of cholesterol in egg-containing foods was very high when fine powders were exposed to sunlight. The degradation products were cholesterol hydroperoxides and 7-hydroxycholesterol.

Since very little information is available on the autoxidation of cholesterol in food products, this study was initiated to isolate and identify photoxidation products of

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spray-dried egg yolk. Egg yolk was selected for experimentation because the total cholesterol content of yolk solids is high. According to Okey (1945), dried egg yolk with a moisture content of 3.7% had a cholesterol content of 3.9%.

MATERIALS AND METHODS

Materials

A commercial sample of spray-dried egg yolk with a moisture content of 3% was used throughout this study. Fresh yolk was obtained from an egg not more than 24-hr old. The 7-ketocholesterol was prepared by t-butyl chromate oxidation of cholesterol acetate (Ruiz, 1958; Chicoye *et al.*, 1968b). The epimeric 7-hydroxycholesterols were synthesized by the reduction of purified 7-ketocholesterol with $LiAlH_4$ (Fieser *et al.*, 1949; Ringold *et al.*, 1952). The epimers were separated by preparative TCL (Chicoye *et al.*, 1968b). A method similar to that of Davis *et al.* (1949) was used for the preparation of cholesterol-β-oxide. Cholesterol-α-oxide and cholestane-3β,5α,6β-triol were purchased from Steraloids, Inc., Pawling, N. Y. All steroids were purified by preparative TLC.

Irradiation of dehydrated egg yolk

Spray-dried egg yolk was exposed to either radiant energy from a fluorescent lamp or summer sunlight. A thin layer (about 3 mm) of yolk powder was placed 6 in. below a 40-watt cool white fluorescent tube in a diffusing fixture and irradiated at about 25°C for approximately 280 hr. Using direct July sunlight as the radiant energy source, egg powder (about 5 mm layer thickness) was irradiated for 5 hr.

Preparation of nonsaponifiable matter from irradiated egg yolk powder

About 24 g of irradiated egg yolk solids were mixed with a solution consisting of 34 g KOH, 48 ml of water and 480 ml of ethanol. The mixture was held at either 25°C for 16 hr or refluxed for 2 hr. After dilution of the mixture with 1000 ml of water, the nonsaponifiable matter was extracted with 3 portions of 1000 ml ethyl ether. The combined ether extracts were washed first with water and then 4 times with 0.5 N KOH to remove fatty acids. After washing to neutrality with water, the ether extract was dried over anhydrous Na_2SO_4 and the solvent was distilled under reduced pressure. The residue was dissolved in chloroform for further studies.

Thin layer chromatography (TLC)

Silica gel without binder (Adsorbosil-2, Applied Science Laboratories, Inc.) was used for analytical and preparative thin-layer chromatography. One part of silica gel powder was slurried with 1.5 parts of water for spreading on 200 × 200 mm glass plates with a Desaga/Brinkmann adjustable applicator. An adsorbent thickness of 0.25 mm was used for analytical determinations while 1 mm thickness was employed for isolation and purification of the autoxidation products. Each plate was activated at about 110°C for approximately 1 hr.

For analytical TLC, the plates were spotted with a steroid-containing extract by a micropipette. Ethyl ether was used to develop the analytical plates. The separated steroids on analytical TLC plates were visualized by UV fluorescence of the spots after spraying with 0.2% dichlorofluorescein (in ethanol) and by color development of spots after spraying the plates with 50% H_2SO_4. The plates were heated for 2 hr at 110°C to enhance coloration of ketone and oxide spots (yellow-brown hue). For complete charring of the spots, TLC plates were held at 110°C for about 12 hr. To tentatively identify the spots, R_c values were computed. R_c is defined as distance of the sample spot from the origin/distance of the cholesterol spot from the origin.

The oxidation products of cholesterol were separated by preparative TLC-double development procedure as described below. Application of a large amount of nonsaponifiable matter to preparative TLC plates was facilitated by a capillary tube applicator consisting of a series of 40 permanently spaced capillary melting-point tubes. The nonsaponifiable matter from 24 g of irradiated yolk solids was applied to a total of about 30 TLC plates. The plates were developed first with a solvent system of ethyl ether and cyclohexane (90:10, v/v). After solvent evaporation at 25°C, the plates were developed again with ethyl ether.

The steroid bands on the preparative TLC plates, sprayed with 0.2% dichlorofluorescein, were located with the aid of UV light. Each desired steroid band was scraped to form a powder which was swept into a sintered-glass funnel. The steroid of each band was extracted from the adsorbent with methanol-chloroform mixture (50:50, v/v). The dichlorofluorescein was also extracted with this solvent system. Purified steroid fractions were prepared by applying each concentrated steroid extract to preparative TLC plates and developing with ethyl ether. The dichlorofluorescein remained at the origin of each chromatogram. Band positions were detected by applying 50% H_2SO_4 to a confined center channel. Again, purified steroid was extracted from the adsorbent band with methanol-chloroform (50:50, v/v). After solvent evaporation, the steroids were crystallized from methanol.

Gas liquid chromatography

Gas liquid chromatography was carried out with Barber-Colman Model 10 gas chromatograph with a hydrogen flame ionization detector. Pyrex glass columns (U-shaped, 6 ft long, 4 mm I. D.) were packed with 1% SE-30 or 1% QF-1 on Gas Chrom Q (silane-treated support), 80/100-mesh. The operating conditions were as follows: nitrogen carrier gas flow rate, 95 ml/min for SE-30 and 120 ml/min for QF-1; column temperature, 212°C; flash evaporator temperature, 280°C. A 10 μl Hamilton syringe was used to inject a suitable volume supply (usually 2 μl) of steroid solution. Retention times were calculated relative to cholestane.

Infrared spectroscopy

Infrared spectra of steroids were obtained with Beckman IR-4 Infrared Spectrophotometer. The steroids were dissolved in either chloroform or carbon tetrachloride to prepare approximately 1% solutions.

RESULTS

TLC and GLC analyses

Analytical thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) were used to determine the presence of oxygenated cholesterol compounds in fresh yolk, unirradiated spray-dried yolk and irradiated spray-dried yolk. The yolk lipids were saponified at 25°C rather than refluxing temperature (81°C) to prevent steroid decomposition. With ether as the development solvent, cholesterol and related steroids in the nonsaponifiable matter of the above-mentioned yolk samples separated as distinct spots on the analytical TLC plates (Fig. 1). Ether, as the TLC solvent, was selected on the basis that synthetic oxygenated cholesterol compounds, commonly found in autoxidized cholesterol systems, were resolved effectively below the uppermost cholesterol spot.

As shown on the chromatograms in Fig. 1, the major steroid in all yolk samples was cholesterol. With regard to chromatogram A (Fig. 1) for fresh yolk, four minor spots were detected. Spots 2, 3, 4 and 5 with R_c values of 0.77, 0.73, 0.46 and 0.36, respectively, were not identified as typical autoxidation products of cholesterol. Although spot 5 had an R_c value similar to that of 7α-hydroxycholesterol, a typical blue coloration of diol spots with 50% H_2SO_4 spray treatment was not detected.

According to Pennock *et al.* (1962), 3,5-cholestadiene-7-one was identified as a minor steroid in the nonsaponifiable matter of fresh yolk without detailed substantiating evidence. With our TLC procedure using ether as the development solvent, a 3,5-cholestadiene-7-one standard migrated ahead of the cholesterol spot. Thus, this steroid was not one of the four minor spots below cholesterol in chromatogram A. The long streak (spot 6) below the minor spots in chromatogram A was colored yellow on the unsprayed plate and presumably consisted of carotenoid pigments. No steroids were detected in spot 6 by GLC.

Chromatogram B in Fig. 1 for the nonsaponifiable matter of unirradiated yolk powder (stored at 25°C for 1 year) has four very minor spots, three of which were identified tentatively as cholesterol-5,6-oxide (spot 2, R_c = 0.71), 7β-hydroxycholesterol (spot 3, R_c = 0.51) and 7α-hydroxycholesterol (spot 5, R_c = 0.36). Upon spraying the analytical TLC plates with 50% H_2SO_4, spots 3 and 5 instantly became blue. This color change is typical for the hydroxycholesterols.

The hydroxycholesterols, but not cholesterol oxide, have been found as supposedly naturally-occurring steroids in some unirradiated biological systems (Bergström *et al.*, 1961). Without irradiation, cholesterol in spray-dried yolk was not decomposed significantly to oxygenated cholesterol compounds over a 1 year period. Cholesterol stability in dried yolk may be attributed, in part at least, to the presence of naturally-occurring antioxidants. Pennock *et al.* (1962) reported that a yolk, weighing 21.9 g, contained 1 mg of total tocopherol.

According to the scheme proposed by Bergström *et al.* (1961) for the autoxidation of cholesterol, a hydroperoxide would form at C_7. Subsequently, the unstable hydroperoxide would decompose to numerous compounds including 7-ketocholesterol, and 7α- and 7β-hydroxycholesterols. Moreover, the epoxidation at C_5–C_6 of cholesterol was hypothesized as an intermediate towards the formation of cholestane-3β,5α,6β-triol. It was suspected that cholesterol in spray-died egg yolk, upon exposure to nonionizing radiant energy, would undergo photoxidation to at least some of the above-mentioned reaction products. Thus, experimentation was directed towards identifying some of the degradation compounds of cholesterol in dehydrated yolk irradiated for about 280 hr at approximately 25°C with low-energy light from a 40-watt fluorescent lamp or for 5 hr in the direct summer sunlight. As shown in Fig. 2, most of the radiant energy of the fluorescent lamp had wave lengths between 400 and 700 mμ.

A very small fraction of the total radiant energy was in the ultraviolet range between 325 and 400 mμ. In contrast to the few, small spots below cholesterol on the chromatogram B (Fig. 1) for unirradiated spray-dried yolk, 10 distinct spots were distributed below cholesterol in the chromatogram C of Fig. 1 for dehydrated yolk irradiated with fluorescent light. When the sunlight was used as the radiant energy (5 hr exposure), a TLC pattern similar

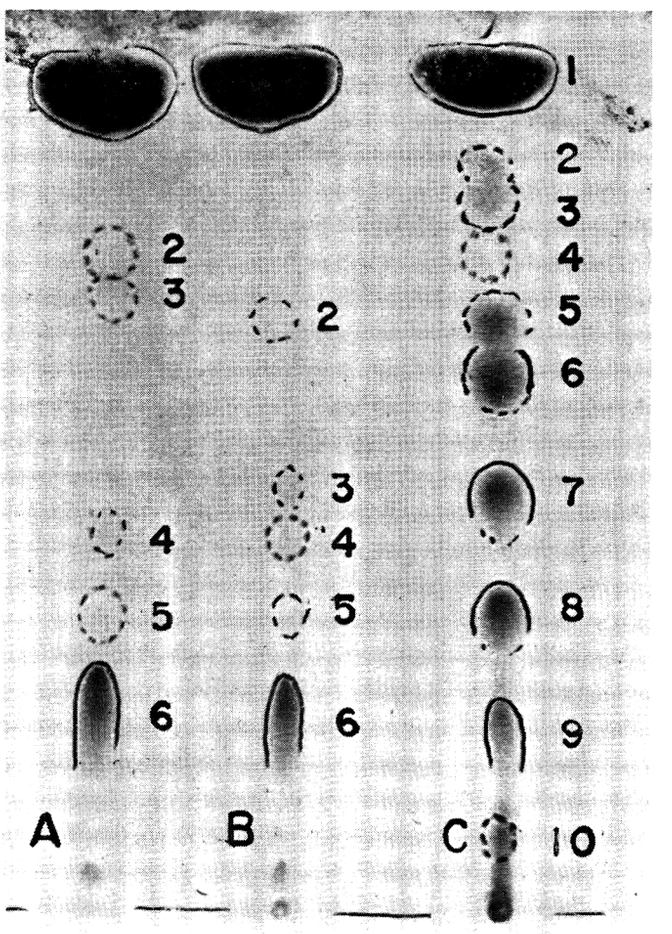

Fig. 1. Thin-layer chromatograms of steroids in the nonsaponifiable matter of egg yolk. A: fresh egg yolk, B: unirradiated one-year-old spray-dried yolk, and C: spray-dried yolk irradiated with fluorescent light. Identified compounds in chromatogram C are spot 1, cholesterol; spot 5, cholesterol-5,6-oxide; spot 6, 7-ketocholesterol; spot 7, 7β-hydroxycholesterol; spot 8, 7α-hydroxycholesterol; spot 9, carotenoids; spot 10, cholestane-3β,5α-6β-triol. Ethyl ether was used as the developing solvent.

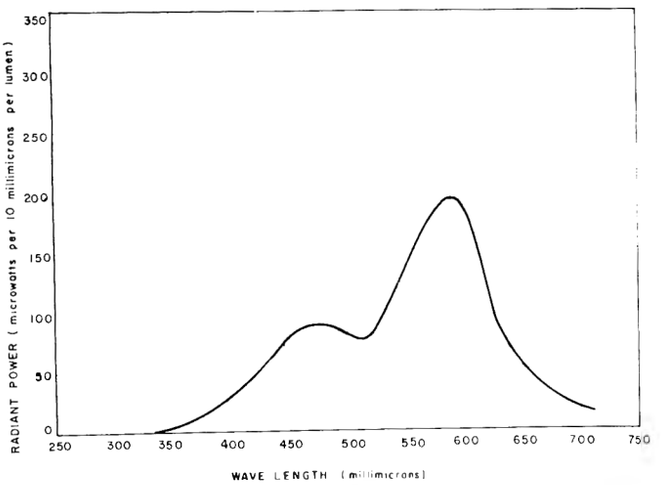

Fig. 2. Spectral energy distribution of a 40-watt cool white fluorescent lamp (data from Bulletin TP-11, General Electric Company).

to the chromatogram C pattern was obtained. Apparently, nonionizing radiant energy from either a fluorescent lamp or sunlight can accelerate autoxidation of yolk cholesterol through the same reaction pathways. For studies described hereafter, spray-dried yolk irradiated with a fluorescent lamp was used. It is of interest to note that the photoxidation products of dried yolk (chromatogram C, Fig. 1) were the same as autoxidation products in an aerated dispersion of cholesterol in water (Fig. 3).

Some of the steroid compounds in the nonsaponifiable fraction of irradiated yolk solids were identified tentatively by the position (R_c) and coloration (50% H_2SO_4 spray) of the TLC spots: spot 5, cholesterol-5,6-oxide (R_c = 0.71); spot 6, 7-ketocholesterol (R_c = 0.64); spot 7, 7β-hydroxycholesterol (R_c = 0.50); spot 8, 7α-hydroxycholesterol (R_c = 0.35); spot 10, cholestane-3β,5α,6β-triol (R_c = 0.11). Evaluation of the darkness and size of charred spots on the heated H_2SO_4-sprayed plate (chromatogram C, Fig. 1) indicated that 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol and cholesterol oxide were the major photoxidation products, whereas cholestane-3β,5α,6β-triol and unidentified steroid spots were somewhat minor compounds. The approximate amount of each diol isomer in irradiated yolk was calculated by the spot area method to be around 1.7 mg/g of yolk solids. According to Acker *et al.* (1963), only cholesterol hydroperoxides and 7-hydroxycholesterol were found as photoxidation products of dried egg-containing foods irradiated by sunlight.

The gas chromatogram of the nonsaponifiable matter of irradiated yolk solids is presented in Fig. 4. Using 1% SE-30 as the liquid phase, six major symmetrical peaks were resolved with no undesirable overlapping within a reasonable elution time. The small peaks, immediately following the solvent peak, are undoubtedly yolk hydrocarbons. When the nonsaponifiable matter of unirradiated yolk was subjected to gas chromatography under the same conditions, only one major peak along with a few minor peaks were traced. The major peaks in the gas chromatogram were identified tentatively by comparison of the relative retention times (RRT) and steroid number with those of authentic steroid standards. The sequence of identifiable peaks beyond the cholesterol peak was 7-ketocholesterol, cholesterol-5,6-oxide, 7-hydroxycholesterol and cholestane-3β,5α,6β-triol. One major peak could not be identified but probably represented one or more C_{27} steroids.

The α- and β-isomers of cholesterol-5,6-oxide and 7-hydroxycholesterol could not be resolved on either SE-30 or QF-1. Indeed, no TLC method has been found to separate the cholesterol α-oxide from the β-oxide. QF-1, as a liquid phase, was considered to be inadequate for effective GLC separation of the autoxidation products of cholesterol since 7-hydroxycholesterols decomposed to such an extent that numerous peaks were obtained. According to Fioriti *et al.* (1967), cholesterol-5,6-oxide was degraded on QF-1. As

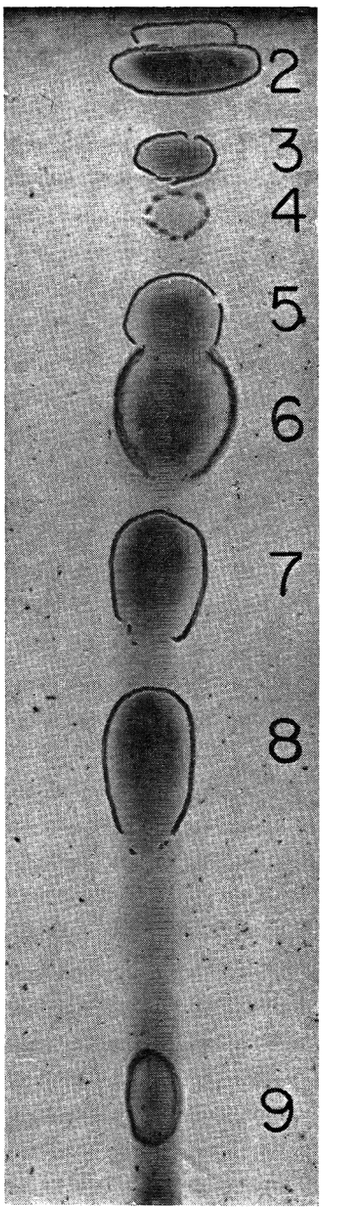

Fig. 3. Thin-layer chromatogram of autoxidation products of aerated cholesterol sol. Identified compounds are spot 2, cholesterol; spot 5, cholesterol-5,6-oxide; spot 6, 7-ketocholesterol; spot 7, 7β-hydroxycholesterol; spot 8, 7α-hydroxycholesterol; spot 9, cholestane-3β,5α,6β-triol. Ethyl ether was used as the developing solvent.

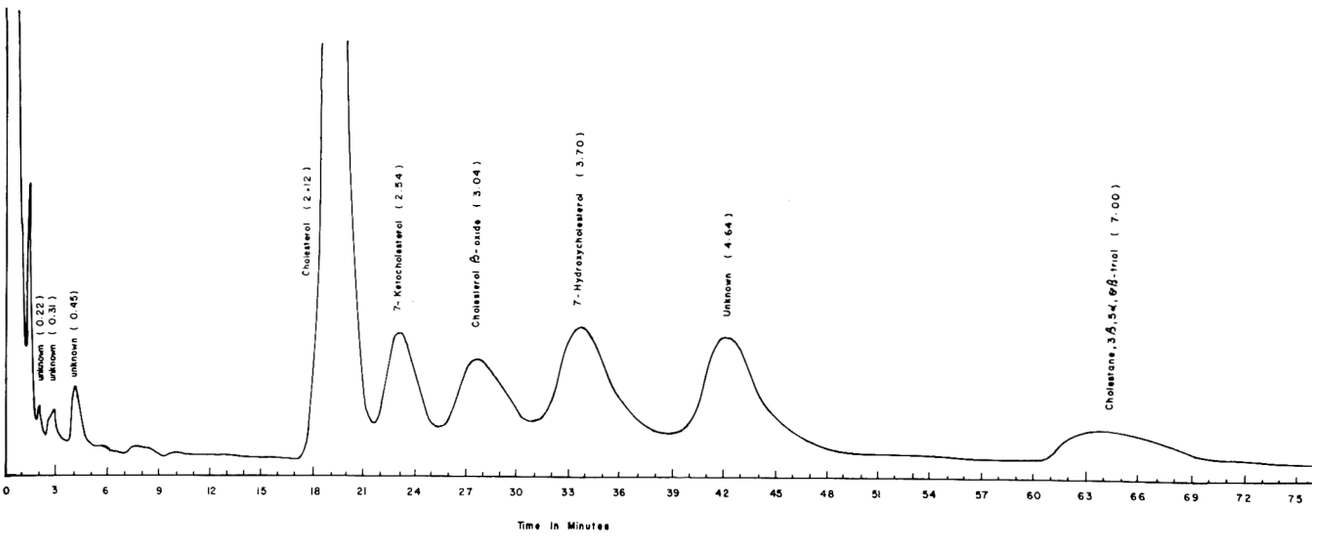

Fig. 4. Gas chromatogram of nonsaponifiable matter from spray-dried yolk, irradiated with fluorescent light. Conditions: 1% SE-30 on Gas Chrom Q, 80/100 mesh; 6 ft × 4 mm I.D. glass column; 212°C column temperature. Instrument: Barber-Colman Model 10 with hydrogen flame ionization detector.

a consequence, they prepared trimethylsilyl ether derivatives of cholesterol autoxidation products, prior to GLC, on a QF-1 column. Although this silylation treatment prevented steroid degradation, the resolution of the major autoxidation products was inadequate, particularly for quantitative studies.

Isolation and infrared spectroscopy of photoxidation products

To positively identify some of the major oxygenated cholesterol compounds in irradiated egg yolk, the infrared spectrum of a purified steroid fraction, procured by the preparative TLC technique, was compared to that of the proposed authentic standard. The lipids in the irradiated yolk were saponified with KOH at either 25°C or 81°C. With a saponification temperature of 25°C, decomposition of oxygenated cholesterol compounds was insignificant. Saponification at 25°C was carried out with irradiated yolk solids prior to the isolation of the 7-hydroxycholesterol isomers. When irradiated yolk solids were treated with hot KOH solution (81°C), the 7-ketocholesterol decomposed, whereas the cholesterol-5,6-oxide and other oxygenated compounds were not structurally rearranged.

Numerous degradation products, including 3,5-cholestadiene-7-one as the major compound, were produced when purified 7-ketocholesterol was heated (81°C) with a KOH solution. Bergström *et al.* (1941) mentioned the instability of 7-ketocholesterol in hot alkaline medium and suggested 3,5-cholestadiene-7-one as the reaction product. However, supporting data for this reaction was not provided. With either a single development (ethyl ether) or a double development (ethyl ether-cyclohexane, 10:10 v/v and ethyl ether) in our study, the hot saponification degradation products of 7-ketocholesterol had R_f values much higher than that of cholesterol-5,6-oxide. The ketone degradation and movement of the products above cholesterol oxide zone on TLC plates was fortunate since purified cholesterol oxide normally could not be isolated easily due to the overlapping bands of the ketone and oxide on preparative TLC plates. Thus, hot saponification was an important step in the isolation procedure for preparing purified cholesterol oxide from irradiated yolk powder.

Using the nonsaponifiable matter from irradiated yolk solids, distinct bands of tentatively identified 7α- and 7β-hydroxycholesterols were separated on preparative TLC plates by a double development technique using ethyl ether-cyclohexane (90:10, v/v) and ethyl ether. Rechromatography was used for further purification. The purity of the two isolates was determined by analytical TLC. With each diol isolate, only one spot was detected on the H_2SO_4-sprayed plate. The R_f value of each spot corresponded to that of the proposed authentic diol isomer. The infrared spectrum of each isolated diol along with the matched authentic steroic are presented in Figs. 5 and 6. The spectrum of each purified isolate (A) was identical to that of the synthetic standard (B).

In the functional group region between 5000 and 1300 cm^{-1}, the pattern of absorption bands was the same for both 7α- and 7β-hydroxycholesterols. The spectral differences between the diol isomers were in the fingerprint region of 1200–900 cm^{-1}.

Preparative TLC was used successfully for the separation of the tentatively identified cholesterol-5,6-oxide from other steroids of irradiated spray-dried yolk. Infrared analyses of the isolate and the synthetic α- and β-oxide standards were carried out. As shown in Fig. 7, the IR spectra of the oxide isolate (A) and authentic β-oxide were the same. Spectral comparison of the α- and β-oxides revealed differences in band positions within the 1200–900 cm^{-1} region. Both isomers had one major band in this frequency region with maximum absorption at 1060 cm^{-1} for the β-oxide and 1030 cm^{-1} for the α-oxide (Chicoye *et al.*, 1968a).

Since 7-ketocholesterol could not be easily isolated from TLC plates due to the overlapping of this steroid band with that of cholesterol oxide, the technique of Smith *et al.*

(1967) was used to obtain further evidence for the presence of the ketone. A developed analytical TLC plate with the separated spot of tentatively identified 7-ketocholesterol was sprayed with 1% sodium borohydride in methanol. After a spray treatment with 50% H_2SO_4, the ketone spot turned blue. This procedure provided further assurance that 7-ketocholesterol was a photoxidation product in dried yolk. The borohydride reduced the 7-ketocholesterol to 7-hydroxycholesterol which, when sprayed with H_2SO_4, turned blue.

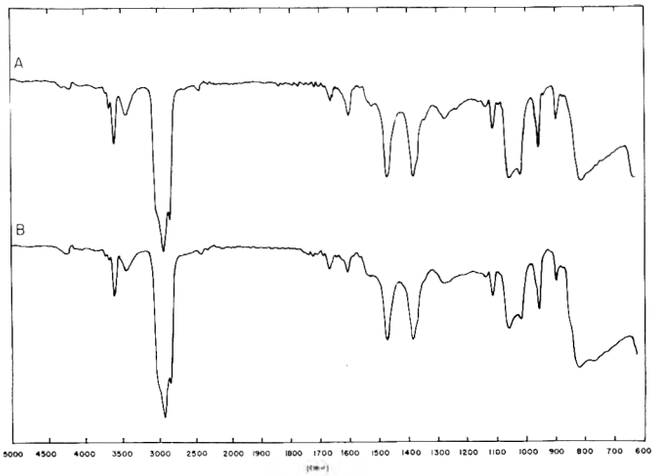

Fig. 5. Infrared spectra of 7α-hydroxycholesterol. A: isolated from irradiated yolk solids; B: synthetic sample.

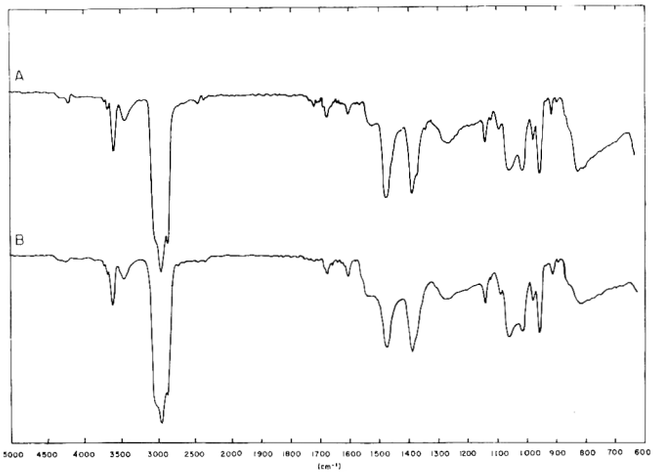

Fig. 6. Infrared spectra of 7β-hydroxycholesterol. A: isolated from irradiated yolk solids; B: synthetic sample.

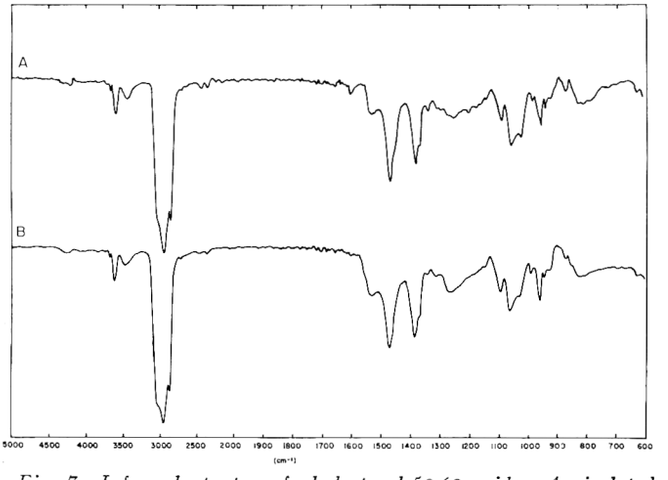

Fig. 7. Infrared spectra of cholesterol-5β,6β-oxide. A: isolated from irradiated yolk solids; B: synthetic sample.

DISCUSSION

THE DISTRIBUTION and physical state of cholesterol in spray-dried yolk undoubtedly are major factors governing the extent of cholesterol oxidation and the types of autoxidation products. Cholesterol in egg yolk is present in the low-density lipoprotein micelles (LDL) and the high-density lipoproteins (HDL) of the granules (Cook *et al.*, 1962). According to Sugano *et al.* (1961), the LDL (84% lipid) contained 4% cholesterol on a lipid basis whereas lipid in HDL (20% lipid) consisted of 5%. Cholesterol molecules in LDL presumably are linked with phospholipid molecules on the surfaces of the high molecular weight micelles (Gurd, 1960).

The organization of cholesterol in HDL has not been elucidated. When most of the water is removed from liquid yolk by spray drying, surface cholesterol of LDL should be oxidized easily in the dried yolk because of the ease of oxygen accessibility to reactive sites. However, even though atmospheric oxygen was present in the spray-dried yolk used in this study, irradiation was found to be essential for the occurrence of extensive autoxidation of yolk cholesterol. Aerated egg yolk solids stored for a period of about one year in the dark contained only trace amounts of 7-hydroxycholesterols (Fig. 1).

Presumably organic compounds other than cholesterol were decomposed during the exposure of yolk solids to irradiation. The color of irradiated yolk samples was pale yellow in contrast to the deep yellow color of the unirradiated product. Irradiation of food by light can cause carotenoid destruction (Borenstein *et al.*, 1966).

The mechanism of photoxidation of yolk cholesterol may include the creation of hydroperoxy groups at C_7 of cholesterol. Although 7-hydroperoxycholesterols have not been detected in autoxidized cholesterol systems, 5α-hydroperoxy-6-cholestene-3β-ol has been isolated from irradiated cholesterol in pyridine by Schenck *et al.* (1957). Since the 5α-hydroperoxide in chloroform rearranges to 7α-hydroperoxide (Lythgoe *et al.*, 1959), perhaps 5α-hydroperoxide is an intermediate in the conversion of cholesterol to 7-hydroperoxide in dried yolk. It is of particular interest to note that Acker *et al.* (1963) found cholesterol hydroperoxides in egg-containing foods irradiated by sunlight, but detailed hydroperoxide structures were not presented. Since 7α- and 7β-hydroxycholesterols as well as 7-ketocholesterol were identified as photoxidation products in yolk solids (Fig. 1), 7-hydroperoxides would be expected as precursors.

Cholesterol-5β,6β-oxide has never been identified as an autoxidation product of cholesterol. Recently, Fioriti and Sims (1967) identified, by gas chromatography, the α-isomer of cholesterol oxide as a compound in heated crystalline cholesterol. The preferential formation of the β-isomer of cholesterol-5,6-oxide in irradiated yolk solids cannot be explained at the present time. Cholestane-3β,5α,6β-triol in

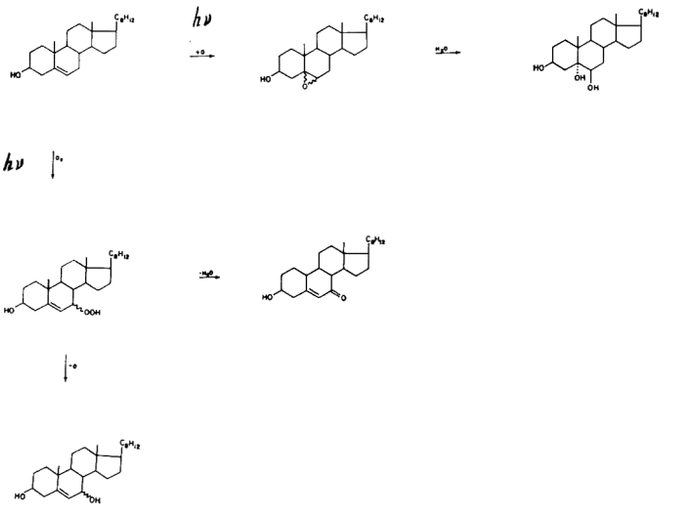

Fig. 8. Reaction scheme for the photoxidation of cholesterol in spray-dried yolk.

dehydrated yolk after irradiation probably was derived from the β-oxide by fissure of the epoxide ring in the presence of moisture (Fieser *et al.*, 1959). A simple reaction scheme is offered in Fig. 8 for explaining the formation of photoxidation products.

In general, the rate of photochemical oxidation declines as the wave length increases. It would be expected, then, that radiant energy with wave lengths in the ultraviolet region would be more effective for the photoxidation of cholesterol than visible radiation. Sunlight consists of radiation with wave lengths in the ultraviolet, visible and infrared regions. The wave lengths in the ultraviolet region extend down no lower than 295 mμ (Koller, 1965). The solar energy rises with an increase in wave length from 295 to 500 mμ and then gradually decreases. The presence of ultraviolet radiation in sunlight probably accounts for the rapid photoxidation of yolk cholesterol in our study. In subsequent studies, ultraviolet and γ irradiation of yolk will be carried out to assess the rate of photoxidation.

Irradiation may be useful for reducing the cholesterol content of food as well as for producing products of nutritional value. Recently, Kanazawa *et al.* (1966) obtained a U. S. patent on the oral use of cholestane-3β,5α,6β-triol and some esters for the prevention and treatment of atherosclerosis. When the triol diformate was administered orally, the serum cholesterol levels of healthy male adults were not altered, but the cholesterol levels of volunteers with hypercholesterolemia were reduced significantly.

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Ms. rec'd 1/5/68; revised 8/6/68; accepted 8/30/68.

This study was supported by a grant from the Wisconsin Heart Association and an institutional grant of the American Cancer Society.

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Cellulase Activity during the Maturation and Ripening of Tomato Fruit

SUMMARY—An enzyme that attacks carboxymethyl cellulose may be extracted efficiently from tomato fruit by salt solutions. From a high initial value in small green fruit, activity fell gradually during fruit swelling. With incipient ripeness, the activity increased again and continued to rise to the full red condition. The green areas of "blotchy" ripened fruit showed 40% less activity than the adjacent red tissue. Fruit of tomato species in the sub-genus *Eriopersicon* contained considerably more activity than examples from the sub-genus *Eulycopersicon*. Firmness measurements on fruit from both sub-genera were not significantly correlated with the cellulase activities, and from this and other evidence it is concluded that cellulase is not a major factor controlling the softening of tomato fruit, at least during the ripening period.

INTRODUCTION

THE SOFTENING OF FRUIT during ripening is usually explained in terms of a solubilization and partial degradation of the pectic substances in the middle lamella by the action of a complex mixture of enzymes known collectively as the protopectinases and the pectinases (Joslyn, 1962). This loss of structure by the middle lamella allows cell slip to take place, and the fruit soften. In a number of varieties of tomato fruit at the orange-red stage of ripeness, it has been shown that there is a positive relation between the firmness and the activities of the pectic enzyme polygalacturonase present (Hobson, 1965).

The degradation of cell wall components other than the pectic substances may also contribute to softening as was suggested by the work of Kertesz *et al.* (1959) on apples. Hall (1964) and Dickinson *et al.* (1964) have shown that cellulase is present in ripening tomatoes, so the possible effect of this enzyme on the loss in firmness of the fruit, complementing that of the pectic enzymes, has been investigated. To examine the part played by cellulase in various ripening disorders of the tomato, the activity of this enzyme has also been measured in fruit showing the conditions of "blotchy" ripening (Bewley *et al.*, 1926) and "blossom-end" rot (Spurr, 1959).

EXPERIMENTAL

Fruit samples

"Harbinger" was the variety used in connection with Tables 1–3 and "Potentate" with Table 4. All fruit were grown in heated glasshouses and a description of the color stages during ripening has already been given (Hobson, 1959). Two distinct methods of sampling tissue from "blotchy" tomatoes were employed: "Whole fruit" samples consisted of segments of tissue cut from the red and green parts of the same affected fruit. "Outer locular wall" samples imply that comparable areas of the walls were cut from contrasting regions of "blotchy" fruit. With specimens showing "blossom-end" rot, horizontal slices of tissue were taken from the blackened regions and compared with similar slices of red tissue cut from the more normal parts of the same fruit.

Dry matter determinations

Representative samples of fresh tissue were dried in a forced draft oven at 80°C for 48 hr.

Enzyme studies

Fruit samples were stored in sealed jars at −20°C for not more than 2 mo. These conditions did not affect the enzyme activity. After thawing, 20 g of macerated tissue were thoroughly mixed with 1 g of a 4:1 (w/w) mixture of sodium chloride and disodium ethylenediamine tetraacetic acid (EDTA). The whole was brought to pH 8.0 with 1*N* NaOH and kept at 2°C for 1 hr. The slurry was then centrifuged at 2000 × G for 3 min, the supernatant liquid filtered under reduced pressure through muslin and Whatman No. 5 paper, and the residue extracted twice more for 15 min each time with 10 ml portions of a 5% solution of a 4:1 mixture of NaCl with disodium EDTA.

After the last extraction the solid material was squeezed dry by means of the muslin. A total of about 35 ml of enzyme solution was collected. Cold acetone was slowly added with stirring to the enzyme solution until it was 70–72% with respect to the ketone (Nisizawa, 1955). After an overnight stand at −20°C the material was centrifuged for 3 min and the supernatant layer discarded. The last traces of acetone were removed by leaving the container at −20° for a minimum of 12 hr. Subsequently the enzyme was redissolved in 10% ammonium sulfate solution and the insoluble material removed by centrifugation.

The assay medium consisted of 0.8% sodium carboxymethyl cellulose (British Drug Houses): degree of substitution about 0.5 (Reese *et al.*, 1950), degree of polymerization about 400; 0.02*N* sodium acetate buffer, pH 4.80; and 1 ml of enzyme solution in a total volume of 10 ml. Immediately after enzyme addition an aliquot was removed and further aliquots were taken according to the activity expected up to 16 hr. The experiments were carried out at 30°C. The enzyme was inactivated by heating the samples in a boiling water bath for 25 min, after which the denatured protein was removed by centrifugation. The remaining procedures were as given previously (Hobson, 1962) except that glucose was used as the

standard sugar, and the tubes were diluted with water instead of ethanol.

A unit of activity is regarded as the amount of enzyme contained by 100 g of fresh fruit tissue that would degrade a solution of carboxymethyl cellulose to produce an increase in reducing power equivalent to 1 mg of glucose per hr at 30°C and pH 4.80.

Firmness measurements

A description of the firmness machine used and its method of operation was given by Shafshak *et al.* (1964).

Statistics

Statistical analyses of the data were carried out according to Snedecor (1956).

RESULTS

IN THIS STUDY the term "cellulase" is used to describe the activity observed using a soluble cellulose derivative, carboxymethyl cellulose, as substrate, and it is assumed that the effect on undegraded cellulose would be quantitatively related. It seems likely that "cellulase" activity as reported here is really the summation of the effects of several different enzymes probably possessing individual specificities and characteristics (Jermyn, 1952; Nisizawa *et al.*, 1963). The situation perhaps parallels the exo- and endopolygalacturonases in the pectic enzyme complex of ripening tomatoes (Hobson, 1964).

Efficiency of extraction of the enzyme

In two previous methods for the desorption of cellulase from tomato tissue (Dickinson *et al.*, 1964; Hall, 1964), macerated fruit was extracted once with up to 10% NaCl. It was found, however, that much more enzyme could be desorbed by carrying out a series of extractions using a mixture of NaCl and disodium EDTA. Neutralization of the tissue to pH 8.0 as recommended by Dickinson *et al.* (1964) was adopted. The percentages of the total enzyme activity obtained by five successive extractions employing this modified procedure are given in Table 1. The first three extractions brought out 96% of the total activity and the data in the rest of the tables are based on this number of extractions.

The effects of buffer identity and strength on the activity of cellulase were both investigated in detail. Various methods for deactivating the enzyme were also examined (Stone, 1961; Mandels *et al.*, 1963); heating in boiling water for 25 min was both convenient and effective (Hallinwell, 1961). The pH optimum of tomato cellulase in this particular system was 4.80.

Table 1. Relative activity brought out by successive extractions of cellulase from red tomato fruit (figures are the means of three experiments).

Extraction no.	Extractant per 20 g tissue	Activity (% of total in five extractions)
1	1 g of NaCl-EDTA disodium salt) (4:1 mixture)	64.5
2	20 ml of a 5% (w/v) solution of the above mixture	23.3
3	20 ml of the same solution	7.8
4	20 ml of the same solution	3.3
5	20 ml of the same solution	1.1

Cellulase during maturation and ripening of tomatoes

Cellulase activity at various stages during growth is shown in Table 2. The activity fell progressively as the fruit increased in size. Differences in the activities between the size categories were all statistically significant except for the two smallest and the two largest sizes analyzed. Mature green fruit, on the other hand, showed a resurgence of activity, and further increases occurred up to the red stage. Differences were significant except between the green and the orange-green stages. From limited experimental work cellulase did not appear to continue to increase in activity into the overripe condition as was found with the enzyme polygalacturonase (Hobson, 1964).

About one-third of the cellulase was free in the juices that could be expressed from mature green tomatoes but the proportion fell to one-sixth at the red stage. This observation parallels the case of polygalacturonase and indicates that strong adsorption of the enzyme on the cell wall occurs as ripening proceeds.

Following results with evenly ripening tomatoes, investigations were extended to "blotchy" fruit. Using whole segments of fruit cut from the differently colored sides, the red tissue contained quite typical activity while the green (blotchy) tissue showed about 40% less (Table 3). Outer locular wall tissue taken from the red and the green areas

Table 2. Cellulase activity during the growth and ripening of tomato fruit (activities based on five determinations).

Fruit size or ripening stage	Units of activity	Logarithm of activity[1]	Percentage dry matter
½ in.	1.06	0.02	7.65
½–1 in.	0.99	−0.01	7.42
1–1½ in.	0.12	−0.93	7.22
1½–2 in.	0.06	−1.20	6.37
Significance of the F-test		0.01	0.01
L.S.D. and P = 0.05		±0.74	±0.65
Green	0.65		6.62
Orange-green	0.86		6.30
Red	1.57		6.54
Significance of the F-test	0.01		n.s.
L.S.D. at P = 0.05	±0.50		±0.28

[1] Mean values were obtained by averaging the logarithms of individual experimental values in which form statistical evaluation was carried out so that the error variances of the values at various sizes were kept about the same.

Table 3. Cellulase activity in the red and green regions of tomato fruit showing "blotchy" ripening (five pairs of samples analyzed).

Type of sample	Color of tissue		Significance of the *t*-test[1]	L.S.D. at $P = 0.05$
	Red	Green		
Whole fruit	1.55	0.88	0.05	±0.54
% Dry matter	6.14	5.93	n.s.	±0.52
Outer locular walls	1.19	0.68	0.01	±0.22
% dry matter	6.04	5.79	n.s.	±0.44

[1] Using Student's method for paired samples.

produced a similar contrast in activities, although the level of activity in each case was somewhat lower than for whole-fruit samples. Thus the parts of the fruit interior to the wall tissue must have a higher activity per unit weight than the outer tissue. Hall (1964) has investigated the distribution of cellulase in tomatoes in detail.

Symptoms of "blossom-end" rot include the progressive breakdown and blackening of the whole of the fruit starting from the distal end. In extreme cases the whole fruit may be affected leading to complete tissue disintegration. In milder instances the more proximal parts of the fruit are apparently unaffected except that this tissue ripens more quickly than usual. Contrary to expectation, cellulase activities in both red and blackened regions of affected fruit were of quite normal proportions for variety Potentate (Tables 4 and 5) and the progress of the disorder would appear to be unconnected with cellulose breakdown.

As cellulase may have some bearing on the extent to which tomato fruit soften, a particularly firm variety, Potentate, containing low polygalacturonase activity was compared with variety Harbinger, which lacks firmness and has high polygalacturonase activity. Firmness measurements and cellulase assays were carried out on samples of these two varieties and the results are given in the first part of Table 5. The firmer variety, Potentate, contained more than twice as much activity as the softer, Harbinger, and this would suggest that the enzyme is not quantitatively important in the softening process.

To confirm this conclusion, further investigations were carried out with fruit from two sub-genera of *Lycopersicon*. *Eriopersicon* contains species whose fruit do not turn red when ripe but slowly assume a yellowish-green color. Fruit of species in the sub-genus *Eulycopersicon* change color and ripen normally; all commercial varieties of tomato belong to this group. Results given in Table 5 indicate that at comparable stages of ripeness, fruit of neither sub-genus could be said to be consistently softer than the other. Cellulase activity, however, was very much higher in the sub-genus *Eriopersicon*, and on this evidence with fruit of these species the contribution of the enzyme to the loss of firmness with ripening is likely to be of minor importance.

Table 4. Cellulase activity in tomato fruit showing "blossom-end" rot (ten pairs of samples analyzed).

Type of tissue	Units of activity	Percentage dry matter
"Blossom-end" rot	3.17	7.72
Control	3.17	7.98
Significance of the t-test [1]	n.s.	n.s.
L.S.D. at P = 0.05	±1.32	±0.43

[1] Using Student's method for paired samples.

DISCUSSION

Of the methods available for the estimation of cellulase activity (Halliwell, 1963), it is generally accepted that viscometry and reducing sugar assays give comparable results (Levinson *et al.*, 1950; Hash *et al.*, 1958). Since sugar assays are easier to carry out on a routine basis, they were used exclusively in this study.

Previous workers have assumed that cellulase activity as measured with a soluble substrate represents the action of the enzyme on native cellulose. The difficulties in relating activities on one substrate that is soluble with another that is insoluble are fully recognized (Levinson *et al.*, 1950), and it is presumed that the figures in the tables have some bearing on the actual rate of dissolution of cellulose in tomato fruit. Cellulase from fungi may be resolved into a number of separate components (Whitaker, 1963; Hashimoto *et al.*, 1963) and it would be surprising if the enzyme from tomato fruit did not also consist of a mixture of multiple forms.

Investigations on cellulases from various organisms have revealed a number of additional factors that might affect

Table 5. Cellulase activity in relation to the firmness of fruit of tomatoes of various species (activity figures in the first part of the table are based on five determinations and in the second part on six).

	Units of activity	Compression (mm)	Percentage compression	Percentage dry matter
Lycopersicon esculentum				
var. Potentate	3.13	1.49	3.24	6.02
var. Harbinger	1.44	2.47	6.32	7.30
Significance of the t-test [1]	0.05	0.001	0.001	0.05
L.S.D. at P = 0.05	±1.24	±0.24	±0.60	±0.81
Sub-genus *Eriopersicon*				
L. hirsutum	9.99	2.32	11.67	12.43
L. hirsutum var. *glabratum*	41.52	2.79	16.25	10.89
L. peruvianum	10.36	2.11	10.89	13.15
Sub-genus *Eulycopersicon*				
L. pimpinellifolium (B 6009)	2.88	2.50	18.03	10.17
L. pimpinellifolium (B 34)	3.71	2.19	15.78	12.50
L. esculentum var. *cerasiforme*	4.11	1.77	10.59	9.44
Significance of the F-test	0.001	0.001	0.001	0.001
L.S.D. at P = 0.05	±3.62	±0.19	±1.22	±1.16

[1] Using Student's method for paired samples.

the activity of tomato cellulase. Myers *et al.* (1959), for instance, showed that protein in the form of albumin stabilized the enzyme during long incubation periods. Similar additions to the substrate medium in the present investigation did not improve the activity; neither did the addition of cysteine during extraction, nor sodium fluoride during assay, as recommended by Horton *et al.* (1967). Hall *et al.* (1965) made the valuable observation that the chemical sterilization of tomato tissue did not alter the cellulase activity, indicating that the enzyme does not stem from bacteria in the fruit.

A concentration of 0.8% sodium carboxymethyl cellulose was sufficient to saturate tomato cellulase, and, provided that the enzyme concentration was adjusted so that less than the equivalent of 5 mg of glucose was produced per 10 ml assay solution, the inhibiting effect of this sugar on the activity (Myers *et al.*, 1959) was negligible.

A wide range of substances have been found to inhibit cellulase of fungal origin (Jermyn, 1952; Mandels *et al.*, 1963). Of four of the most important phenolic acids shown to be present in tomato fruit (Walker, 1962), only chlorogenic acid showed any activity against tomato cellulase when added to the assay medium at a final concentration of 0.02%. "Blotchy" tissue does not contain abnormally high concentrations of phenolic acids (Hobson, 1967), so it is unlikely that the low cellulase activity in "blotchy" areas is due to inhibition by chlorogenic acid.

Two previous accounts of cellulase activity in ripening tomato fruit (Dickinson, 1962; Hall, 1964) indicated that in general the activity rose quite quickly with coloration. The figures in Table 2 confirm these findings and show that red fruit have more than double the activity of mature green samples. Cellulase activity of the green areas of tomatoes showing "blotch" was little more than half the value for ripe fruit. This fact, although probably of less importance than the severe attenuation of the pectic enzymes in these areas, must contribute to some extent to the pronounced lack of softening in "blotchy" tissue.

Various species of *Lycopersicon*, previously used for compositional studies by Davies (1966), were used to provide ripe fruit tissue, which, on analysis, showed a wide range of cellulase activities. There was, however, no significant relation between these activities and values for either the compression or percentage compression of the species of fruit. It must therefore be concluded that, whereas the activity of cellulase rises with normal ripening, the action of this enzyme does not appear to be a primary cause for the loss in firmness during this period and there is therefore no reason at the moment to suggest that this property is other than directly controlled by the pectic enzyme complex.

This conclusion is entirely in accord with studies on pears (Jermyn *et al.*, 1956) and peaches (Sterling, 1961), where it was found that the cellulose micelles undergo only a very limited degradation during the ripening period. The cellulose content of apples also changes little with storage (Kertesz *et al.*, 1959), and during the postharvest period pectic transformations were thought to account for the softening. Whereas cellulase activity is highest near the beginning and towards the end of the growth cycle of tomato fruit, on present evidence its effect on tissue firmness might be of more importance during fruit swelling, since with incipient ripeness this property then appears to be under the control of the pectic enzyme complex.

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Ms. rec'd 2/8/68; revised 7/8/68; accepted 7/29/68.

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Anthocyanin Pigments of Rhubarb, Canada Red

SUMMARY—The anthocyanin pigments of rhubarb (*Rheum rhaponticum* 'Canada Red') were extracted with 0.02% methanolic HCl and partially purified by use of cation-exchange resin. The pigments were separated into two bands with paper and cellulose thin-layer chromatography. The purified pigments were characterized by their R_f values, partial acid hydrolysis, identification of the aglycones and sugars after complete hydrolysis, and their spectral properties. The main pigment (87% of the total anthocyanins) was identified as cyanidin-3-glucoside and the second (13%) is proposed to be cyanidin-3-rutinoside.

INTRODUCTION

RHUBARB VARIETIES EXHIBIT a wide range of colors from light green to pink to beetroot-red, with the development of the red pigmentation being most noticeable in the stalks towards maturity. The presence of leucoanthocyanins in rhubarb has been reported by Racz *et al.* (1959) and by Fujita *et al.* (1958). In 1931 Robinson *et al.* reported the pigment of rhubarb petiole skin to be a cyanidin-3-bioside. Recently, Gallop (1965) in an extensive study on the chemical composition of several varieties of rhubarb reported that the two red pigments are cyanidin-3-glucoside and cyanidin-3-rutinoside.

This investigation covers the extraction, purification, and identification of the anthocyanin pigments of rhubarb (*Rheum rhaponticum* 'Canada Red'). Moreover, previous work is extended in that relative amounts of the two pigments are reported.

EXPERIMENTAL

Rhubarb

Fully mature rhubarb stalks were harvested in the fall of 1966 from the Oregon State University horticultural farm, Corvallis. The stalks were thoroughly washed and stored under nitrogen in polyethylene bags at −26°C.

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Paper chromatography

Whatman No. 3 paper was used for preparative chromatography, and No. 1 for determining R_f data. Descending chromatography was used in all cases except sugar determinations. All runs were performed in the dark at 21 ± 1°C.

Thin-layer chromatography

Cellulose plates of 0.25 mm thickness were prepared by spreading a homogenate of 20 g of cellulose powder (MN-300, without binder) and 110 ml of distilled water on 20 × 20 cm plates. The coated plates after setting at room temperature for 1–4 hr were dried at 100°C for ½ hr and stored in a desiccator. Development was carried out in an ascending manner under the same conditions as specified for paper chromatography.

Borate-impregnated silica gel G plates were prepared for sugar separations as described by Jacin *et al.* (1964).

Chromatographic solvent systems

1. BAW 415: less-dense phase of *n*-butanol, glacial acetic acid, water (4:1:5). Equilibrated 3 days for R_f data.
2. AWHCl: glacial acetic acid, water, conc. hydrochloric acid (15:82:3).
3. 1% HCl: conc. hydrochloric acid, water (1:99).
4. Formic: 90% aqueous formic acid, conc. hydrochloric acid, water (5:2:3).
5. BPW: *n*-butanol, pyridine, water (6:3:1).
6. BAW 541: *n*-butanol, glacial acetic acid, water (5:4:1).

Chromogenic spray reagents

Molybdate spray reagent for pigments with vicinal phenolic groups was prepared as described by Albach *et al.* (1965).

Sugars separated on borate-impregnated silica gel G plates were detected with 2-napthol spray reagent made up as outlined by Jacin *et al.* (1965).

Partridge's reagent used for visualization of sugars on paper chromatograms was formulated as described by Albach *et al.* (1965).

Knowns

Cyanidin-3-glucoside and cyanidin-3-rutinoside were isolated from Bing cherries (*Prunus avium* L. var. Bing) harvested from the Oregon State University horticultural farm. This source was originally reported by Lynn *et al.* (1964). Cyanidin chloride was purchased from Calbiochem, Los Angeles, California.

Isolation of pigments

Essentially, the procedure of Smith *et al.* (1965) was used. Six hundred g of the red pigmented outer skin was extracted with 0.02% methanolic HCl, adsorbed onto 500 g of Dowex 50W × 4 cation-exchange resin in the hydrogen form, and thoroughly washed with pure methanol and distilled water. The anthocyanins were eluted from the resin by successive extractions with 0.05, 1, and 3% methanolic HCl. The combined extract was concentrated nearly to dryness on a Buchi rotary evaporator using a water bath temperature of 40°C. The concentrate was taken up in 0.01% methanolic HCl, flushed with nitrogen gas, and stored at −20°C.

Chromatographic purification

Chromatograms of the pigment concentrate that had been developed in AWHCl were dried at room temperature in the dark. Bands were cut into approximately 1 cm square pieces and extracted with 0.01% methanolic HCl. The extract was filtered through Whatman No. 1 filter paper and flash evaporated.

Pigments were also purified using thin-layer cellulose chromatography. After development in AWHCl and drying at room temperature, the bands containing the pigment were scraped off the plate and extracted with 0.01% HCl, filtered, and concentrated.

Partial acid hydrolysis

Less than 1 mg samples of the two purified pigments were hydrolyzed in 2 ml of 10% aqueous HCl at a temperature of 98°C. The procedure followed was that described by Albach *et al.* (1963). Aliquots withdrawn at 5 min intervals were spotted on cellulose TLC plates and developed in AWHCl.

Complete acid hydrolysis

The aglycones and sugars were obtained by heating less than 1 mg of pigment with 10% aqueous HCl for 1 hr at a temperature of 98–102°C. After cooling, the aglycones were extracted with 1–2 ml of isoamyl alcohol. The acid was removed from the remaining aqueous phase by extracting with 2 ml portions of 10% di-*n*-octylmethylamine (obtained from K & K Laboratories, Inc., Plainview, N.J.) in chloroform. Fifty μl samples of the solution were spotted along with samples of authentic monosaccharides on borate-impregnated silica gel thin-layer plates and on Whatman No. 1 paper. The thin-layer plates were developed with BAW 541, the paper in BPW.

Determination of spectra

Spectra of purified bands were determined on a Beckman DK-1 spectrophotometer using 0.01% HCl in spectro grade methanol as a solvent. After determining the spectra one drop of 5% $AlCl_3$ in ethanol was added to the 3 ml cuvette, mixed, and the spectrum immediately recorded in the visible region.

The relative amounts of the two pigments were estimated from a thin-layer chromatogram of the total anthocyanin extract. The well-resolved bands were extracted with 0.01% methanolic HCl and made up to a volume of 10 ml. The optical density of the solutions was recorded at the wavelength of maximum absorbance.

RESULTS AND DISCUSSION

THE RESULTS OF THE CHROMATOGRAPHIC investigation of the pigments are shown in Table 1. AWHCl separated the pigment concentrate into 3 distinct spots, all of which gave the color change from red to blue characteristic of pigments with vicinal phenolic groups. The two main bands (designated rhubarb 2 and 3) were separated by preparative paper chromatography using AWHCl. Each

Table 1. Chromatographic investigation of pigments.

Solvent	Pigment	Rf Paper	Rf TLC	Molybdate test
BAW 415				
	Rhubarb 2	.38	.43	+
	Rhubarb 2 aglycone		.78	+
	Rhubarb 3	.28	.41	+
	Rhubarb 3 aglycone		.76	+
	Cyanidin chloride		.80	+
	Cyanidin-3-glucoside	.36	.49	+
	Cyanidin-3-rutinoside	.35	.42	+
AWHCl	Rhubarb 1		.09	
	Rhubarb 2	.30	.33	+
	Rhubarb 2 aglycone		.07	+
	Rhubarb 3	.46	.51	+
	Rhubarb 3 aglycone		.06	+
	Cyanidin chloride	.10	.07	+
	Cyanidin-3-glucoside	.29	.32	+
	Cyanidin-3-rutinoside	.46	.48	+
1% HCl				
	Rhubarb 1		.03	+
	Rhubarb 2	.11	.13	+
	Rhubarb 3	.22	.27	+
	Cyanidin chloride	.02	.03	+
	Cyanidin-3-glucoside	.11	.12	+
	Cyanidin-3-rutinoside	.19	.20	+
Formic				
	Rhubarb 1		.26	
	Rhubarb 2 aglycone		.25	
	Rhubarb 3 aglycone		.25	
	Cyanidin chloride		.25	

Table 2. Partial acid hydrolysis of rhubarb anthocyanins.

Pigment	No. of simpler products	Components	Rf AWHCl (TLC)
Rhubarb 2	1	Original pigment	.30
		Aglycone	.09
Rhubarb 3	2	Original pigment	.53
		Intermediate	.31
		Aglycone	.10

Table 3. Chromatographic investigation of sugar moiety.

Sugar	R_f Paper BPW	Color Partridges reagent	R_f TLC BAW 541	Color 2-Naphthol reagent
Rhubarb 2 sugar	.20	Olive	.26	Blue
Rhubarb 3 sugars	.19; .42	Olive; Orange	.28; .41	Blue; Orange
Hydrolysis product of cyanidin-3-glucoside	.20	Olive		
Hydrolysis products of cyanidin-3-rutinoside	.19; .41	Olive; Orange		
Glucose	.20	Olive	.27	Blue
Galactose	.17	Olive	.23	Blue
Rhamnose	.43	Orange	.42	Orange
Arabinose	.26	Red	.29	Blue
Xylose	.30	Red	.40	Blue

chromatographed as one spot in BAW 415, AWHCl, and 1% HCl. Hence, they were not further purified for hydrolysis studies.

The results of partial acid hydrolysis of rhubarb 2 and 3 (Table 2) indicate that rhubarb 2 is a monoglycoside and 3 a diglycoside. The R_f data, while only obtained from one solvent system, suggest that the intermediate formed from rhubarb 3 may be the same pigment as rhubarb 2.

The R_f data of the aglycones obtained from complete hydrolysis of rhubarb pigments are included in Table 1 and the chromatographic data for the sugars obtained are in Table 3. The R_f data and color reactions show glucose to be the rhubarb 2 sugar. Rhubarb 3 contains 2 sugars, glucose and rhamnose. The chromatogram of the sugars from rhubarb 3 was indistinguishable from the hydrolysis products of cyanidin-3-rutinoside in terms of R_f value, color reaction, and the relative intensities of the two spots.

The R_f values of the minor pigment, rhubarb 1, indicated that it was cyanidin chloride. It was present in small quantities (less than 1%) and its concentration increased slightly with storage time. It is assumed to be to an artifact formed from the hydrolysis of rhubarb 2 and 3.

The spectral properties of the pigments are shown in Table 4. The E_{440}/E_{max} values of rhubarbs 2 and 3 indicate that the 5 position is unsubstituted (Jurd, 1962). The λ max of the aglycones of rhubarb 2 and 3 is in agreement with that of cyanidin chloride. The presence of one absorption peak in the ultraviolet spectrum of rhubarb 2 and 3 indicates that the pigments are not acylated with hydroxy-cinnamic acids (Jurd, 1962). Other spectral similarities between rhubarb 2 and cyanidin-3-glucoside and rhubarb 3 and cyanidin-3-rutinoside are evident from Table 4.

Table 4. Spectral characteristics of pigments.

Pigment	nm Max	E_{1m}/E Max vis	AlCl₃ shift (nm)
Rhubarb 2	527, 281	.23	+44
Rhubarb 2 aglycone	537		+37
Rhubarb 3	529, 275	.31	+42
Rhubarb 3 aglycone	536		+39
Cyanidin chloride	537		+35
Cyanidin-3-glucoside	526, 279	.24	+41
Cyanidin-3-rutinoside	531		+48

R_f data, identification of the sugar moiety and the aglycone, partial hydrolysis studies, and spectral data confirm Gallop's identification of cyanidin-3-glucoside. This pigment (rhubarb 2) was found to be 87% of the total anthocyanin pigments.

The pigment, which accounts for the remaining 13% of the anthocyanins is a cyanidin-3-diglycoside, the two sugars being glucose and rhamnose. It is chromatographically and spectrally similar to cyanidin-3-rutinoside isolated from Bing cherries. Therefore, the identification of cyanidin-3-glucoside and cyanidin-3-rutinoside is confirmed by the use of different procedures and their relative amounts established.

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Ms. accepted 3/14/68.

Technical paper No. 2377, Oregon Agricultural Experiment Station.

This work was supported in part by a Grant-in-Aid from the Nutrition Foundation, Inc. The technical assistance of Miss Kathy Johnston is also acknowledged.

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Separating and Isolating Aroma and Flavor Constituents of Roasted Peanuts

SUMMARY—A technique was developed for the extraction of quantities of peanuts and for the separation of those extracts into fractions of different chemical categories, each of which had a distinct aroma. Twelve acids were identified; the presence of hexanal, 2,4-decadienal and β-sitosterol was confirmed; and evidence was gathered that indicates the presence of aliphatic lactones. In addition, 2-oxooctanal and a dihydroxynaphthaleneacetic acid were also found to be present. Gas chromatographic examination of the acid fractions from three varieties of peanuts showed some qualitative and quantitative differences.

INTRODUCTION

A NUMBER of studies of the components of peanuts have been made; however, few of these identify or characterize the flavor and aroma constituents. An extensive study of the browning reactions during roasting was made by Pickett *et al.* (1952). Lee *et al.* (1963) and Mason *et al.* (1964) examined flavor precursors. Tocopherols in peanuts have been reported by Oliver *et al.* (1944), Lambertsen *et al.* (1962), and Jasperson *et al.* (1947), who also found a high molecular weight alcohol. Lefort (1953 and 1956) identified two aldehydes: hexanal and 2,4-decadienal. Using gas chromatography, Pattee *et al.* (1965) identified 11 components of high-temperature-cured off-flavor peanuts. Mason *et al.* (1966) used nuclear magnetic resonance, ultraviolet spectrophotometry, and mass spectrophotometry to identify several cyclic amines in peanuts.

The methods described by these workers generally result in the isolation of very small quantities of material for identification. In order to improve this situation a scheme was developed (Fig. 1) for the examination of large quantities of peanuts. Throughout this paper, Roman numerals in parentheses refer to those in Fig. 1.

EXPERIMENTAL

FIVE-HUNDRED POUNDS of roasted peanuts were ground with dry ice in a comminuter to a coarse size. The peanuts were air-roasted by the Planters Peanuts Division of Standard Brands, Inc., employing commercial procedures under conditions designed to yield a product of maximum organoleptic acceptability. The ground nuts were then extracted with 70 gal of boiling methanol four times. The methanol extract was cooled in an acetone-dry ice bath. After the peanut oil had solidified, it was separated from the methanol phase.

The residue (VII) from this distillation was repeatedly extracted with hot chloroform until the extracts were almost colorless in order to separate the chloroform-insoluble sucrose from the extract (IX). The chloroform was removed by distillation under vacuum, leaving a residue (XI), which was dissolved in ether. The ether solution (XII) was finally extracted with 5% solutions of $NaHCO_3$, NaOH, and HCl. During these extractions, the formation of intractable emulsions required the addition of NaCl; these emulsions were finally broken by a freezing and thawing cycle. For each extract, the pH of the aqueous solution was reversed (i.e., acidic to basic; basic to acidic), and the solution was reextracted with ether. The

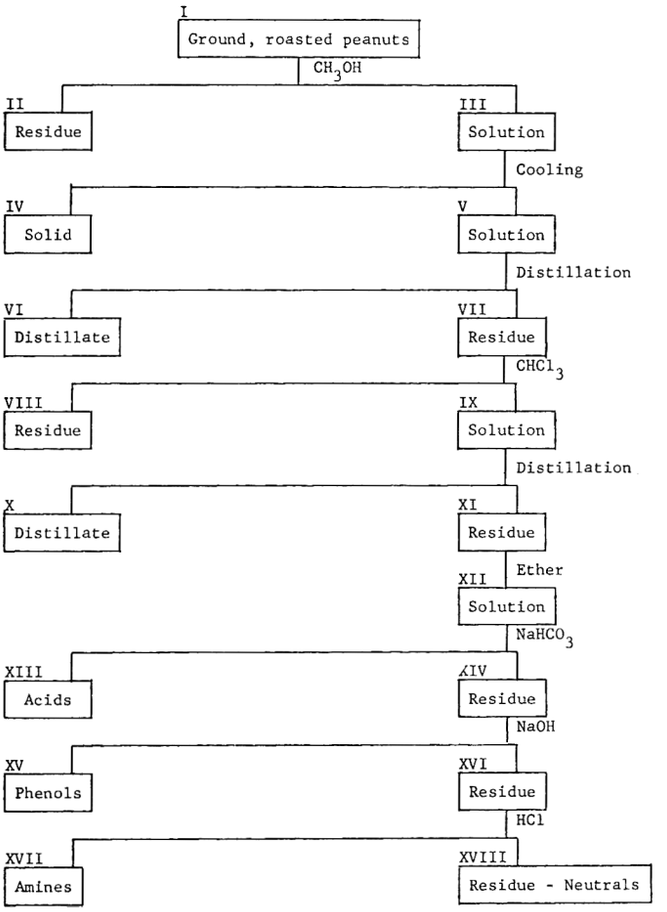

Fig. 1. Scheme for extraction and fractionation of peanuts.

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removal of the ether left four fractions: acid (XIII), phenol (XV), amine (XVII), and neutral (XVIII).

The acid fractions of several batches of peanut extracts were examined by the gas chromatographic method (polyester plus phosphoric acid columns) employed by Shelly *et al.* (1963).

Analysis of the acid fractions from Virginia, Runner and Starr peanuts for C_2-C_7 acids

Apparatus. Flame ionization gas chromatograph (F & M Model 609); Recorder: 1 millivolt (Minneapolis Honeywell), chart speed 3 min/in.

Column. 8 ft × ¼ in. stainless steel filled with 10% LAC-296 plus 5% phosphoric acid on potassium hydroxide washed Chromasorb W.

Conditions. Column temperature, 150°C; injection port rheostat setting, 40; detector block heater rheostat setting, 50. Flow meter settings: N_2, 2.1; H_2, 5.0; air, 5.1. Range: 100; attenuation, 16X; sample size 5 μl.

Analysis of the acid fraction from Runner peanuts for C_8-C_{16} acids

Apparatus. As above.

Column. As above.

Conditions. Column temperature, 200°C; injection port rheostat setting, 40; block heater rheostat setting, 50. Flow meter settings: N_2, 7.3; H_2, 11.2; air, 9.2. Range: 100; attenuation, 16X; sample size, 10 μl.

Analysis of the acid fraction from Starr peanuts for C_8-C_{18} acids

Apparatus. Wilkens Aerograph A-350, equipped with a Wilkens A-500-B hydrogen flame ionization detector kit. Recorder: 6.25 millivolt (Leeds and Northrup) Speedomax G, chart speed, 4 min/in.

Column. 2 ft × ⅛ in. stainless steel filled with 20% ethylene glycol succinate plus 3% phosphoric acid on alkali washed Chromosorb W.

Conditions. Column temperatures, (1) 165°C (first 4 min); (2) temperature increased at 1°/min to 200°C; (3) held at 200°C for the remainder of the chromatogram. Injection port temperature, 225°C. Flow rates: H_2, 25 ml/min; He, 40 ml/min; air, 250 ml/min. Input impedance, 10^9; output sensitivity, 1X; attenuation, 8X; sample size, 1 μl.

The mixture of acids was also examined by paper chromatography, employing the method of Reid *et al.* (1951). Using an adaptation of the column chromatography of Kirchner *et al.* (1946), the p-phenylphenacyl esters of the acids were separated by thin layer chromatography. The layer consisted of 0.25 mm of Brinkmann silica gel G and the developing solvent was a mixture of benzene, petroleum ether (60 to 90°), and ethyl acetate (10:5:1). The spots were visible with and without ultraviolet illumination. Derivatives of known acids were used for comparison.

Analytical data were gathered for an acid which precipitated from the mixture (XIII) on standing and which has tentatively been identified as a dihydroxynaphthaleneacetic acid:

Dihydroxynaphthaleneacetic acid

Melting point 217–218°C after recrystallization from water.

Negative tests for halogen, nitrogen, and sulfur.

Analysis. Calcd, C, 66.05; H, 4.62. Found: C, 66.81; H, 4.65.

Infrared spectrum (KBr pellet). max. 3340, 1667, 1625, 1602, 1510, 1446, 1309, 1240, 1208, 1166, 979, 825, 689 cm^{-1}.

Ultraviolet spectrum. $\lambda_{Max.}^{MeOH}$ 289 (ε 44,000) with shoulders at 299 (43,000) and 304 (41,000).

The methyl ester of this acid was formed with diazomethane:

Melting point 178°C after sublimation.

Analysis. Calcd, C, 67.00/H, 5.54. Found: C, 66.99; H, 5.24.

Infrared spectrum (KBr pellet) showed a shift in the carbonyl peak from 1667 cm^{-1} to 1690 cm^{-1} but retention of the hydroxyl peak at 3350 cm^{-1}.

The mixture of acids (XIII) was converted to methyl esters with diazomethane. Some fractionation of the methyl esters was obtained by vacuum distillation. The distillates were treated with ammonia-saturated methanol in sealed flasks to obtain the corresponding amides. Removal of solvents from one such flask left a residue, which, when recrystallized from CCl_4 had a melting point of 159 to 160°C. A mixed melting point with phenylacetamide was not depressed and the infrared spectra of the two substances were superposable.

A portion of the phenolic fraction (XV) was developed on a silica gel G thin layer plate with a mixture of benzene and methanol (95:5). On visualization with *p*-nitrobenzenediazonium fluoborate, six spots were observed, none of which was identified.

A second portion of this fraction was converted to a mixture of the aryloxy derivative, using a standard procedure (Shriner *et al.*, 1956). Chromatography of this mixture on Whatman No. 1 paper, using 1-butanol saturated with 1.5 *N* NH_4OH, revealed five compounds. The visualization agent was 0.1% bromcresol purple in 50% ethanol adjusted to pH 10 with NH_4OH.

The fraction was also separated on a column of neutral alumina using benzene for elution. Zones were visible with ultraviolet light. The lowest three zones, when extracted with methanol, proved to be unresponsive to the usual test for phenol, and had no absorption bands in the hydroxyl region of their infrared spectra. In addition, the spectra had strong peaks at 1728, 1725, and 1735 cm^{-1}, respectively.

A portion of the neutral fraction (XVIII) was vacuum steam-distilled. The distillate was saturated with sodium chloride and extracted with ether. When treated with 2,4-dinitrophenylhydrazine, the residue from the ether extract formed a precipitate with a wide melting range, which was resolved chromatographically on an alumina column into the dinitrophenylhydrazones of hexanal and 2,4-decadienal.

The neutral fraction (XVIII) contained about 90% peanut oil, most of which was removed by using an alumina column (Crossley *et al.*, 1962, 1964). The oil was eluted from the column with hexane; other components were eluted with the upper layer of a 9:1 mixture of hexane and methanol, and then with methanol alone. β-Sitosterol precipitated from the hexane-methanol eluates.

The combined residues of the methanol eluates were treated with 2,4-dinitrophenylhydrazine on columns according to the method of Mookherjee *et al.* (1963). One fraction obtained from this was crystallized from chloroform, melting point 174 to 176°C; analysis found: C, 47.41; H, 4.76.

The compound isolated from 2,4-dinitrophenylhydrazine columns was tentatively identified as 2-oxooctanal by comparison with data reported by Rinzema *et al.* (1959).

RESULTS AND DISCUSSION

Separation of the extracts of peanuts into acidic, phenolic, basic, and neutral fractions resulted in fractions of unique aromas that did not vary, regardless of how often this scheme was repeated. The characteristic aromas are listed in Table 1.

Although no attempt was made to isolate the fractions with sufficient accuracy to permit a quantitative breakdown by functional groups, in some cases it was possible to indicate differences in relative concentrations of components within individual fractions.

Gas chromatography of the acid fractions from three varieties of peanuts (Virginia, Runner, and Spanish Starr) identified the following acids: acetic, propionic, isobutyric, isovaleric, valeric, hexanoic, heptanoic, decanoic, lauric, and myristic. Comparison of the chromatograms of these varieties showed qualitative and quantitative differences. The most striking differences were observed among the acids that are as yet unidentified.

Table 2 summarizes the data for the C_2-C_7 acids. Within this group of acids, the major differences were indicated in the chromatograms thus: the isovaleric acid peak was much larger than the acetic acid peak on the chromatogram of fractions XIII from Runner peanuts, whereas both acids showed relatively large peaks on the gas chromatograms of the corresponding fractions XIII from both Virginia and Starr peanuts. No evidence of acids C_8 through C_{10} was found on the gas chromatograms of the acid fractions from Virginia and Runner peanuts.

At a later time, when these fractions were examined for the C_{12} through C_{18} acids, not enough of fraction XIII from Virginia peanuts was left for chromatography. Tables 3 and 4 summarize the results of gas chromatography for fraction XIII from Runner and Starr peanuts, respectively. Study of the two gas chromatograms indicates the following:

(1) Lauric acid was found in fraction XIII from Runner, but not in the same fraction from Starr peanuts.

(2) A comparison of the relative retention times of peak C on the gas chromatogram of fraction XIII from Runner and peak W on the gas chromatogram of fraction XIII from Starr suggests that these peaks may have been produced by the same compound.

(3) On the gas chromatogram of fraction XIII from Runner, there was no indication of the uncharacterized peak (designated Y; largest peak on the chromatogram) that was found on the gas chromatogram of fraction XIII from Starr.

(4) The gas chromatographic conditions used for the examination of fraction XIII from Runner peanuts were not capable of detecting either stearic or oleic acid. It is

Table 1. Aromas of peanut fractions.

Fraction	Aroma
Residue XII	Rich, chocolate-like, developed a peanut aroma on warming with H_2O
Acid XIII	Foul, sharp; aromas of the C_3-C_6 acids seemed to predominate
Phenol XV	Fruity, sweet
Amine XVII	Amine-like; especially similar to heterocyclic amines
Neutral XVIII	Vaguely resembled roasted peanuts

Table 2. Analysis of fraction XIII (Acids) from Virginia, Runner, and Starr peanuts for C_2-C_7 acids.

Acid	Retention time (min)	Virginia	Runner	Starr
Acetic	4.5	+[1] (major component)	+	+ (major component)
Propionic	5.4	+	−	+ (trace)
Isobutyric	5.9	+	+	+ (trace)
Butyric	7.1	+ (trace)	+ (trace)	−
Isovaleric	8.1	+ (major component)	+ (major component)	+ (major component)
Unknown	10.5	+ (trace)	−	−
Hexanoic	14.4	+ (trace)	+	−
Heptanoic	20.7	−	+ (trace)	−

[1] + = detected; − = undetected.

Table 3. Analysis of fraction XIII (acids) from Runner peanuts for C_8-C_{18} acids.

Known acids	Retention times	Runner
Octanoic	2.3	
Nonanoic	2.9	
Decanoic	3.7	
Lauric	6.5	6.2 (A)
		7.7 (B)[1]
		9.2 (C)[1]
Myristic	11.5	
Palmitic	19.0	

[1] Unidentified peaks.

Table 4. Analysis of fraction XIII (acids) from Starr peanuts for C_8-C_{18} acids.

Known acids	Retention times	Starr
Octanoic	3.6	
Nonanoic	4.4	
Decanoic	6.2	5.6 (U)
		8.6 (V)[1]
Lauric	11.4	
		15.0 (W)[1]
Myristic	18.2	19.0 (X)
		23.6 (Y)[1]
Palmitic	27.6	
Stearic	38.0	
Oleic	41.2	40.6 (Z)

[1] Unidentified peaks.

probable that either stearic or oleic acid is present in fraction XIII from Starr peanuts; it is not known if either of those acids is present in fraction XIII from Runner peanuts.

The presence of acetic, propionic, butyric, valeric, or isovaleric, hexanoic, and lauric acids was confirmed by thin layer and paper chromatography, using known acids as standards.

Assumption of the presence of a dihydroxynaphthaleneacetic acid in peanuts is based entirely on the analytical, infrared, and ultraviolet spectral data listed in the Experimental. The only other similar compound known to occur naturally is 4-hydroxy-8-methoxy-2-naphthoic acid (Haber *et al.*, 1956).

The presence of phenylacetic acid in peanuts was shown by the isolation of phenylacetamide from the mixture (VIII) after conversion of the acids to their methyl esters and subsequently to the amides.

Chromatograms of the phenol fraction (XV) and of its aryloxyacetic acid derivatives showed the presence of at least six phenols; however, none of these was isolated or identified. During attempts to separate these phenols by chromatography on an alumina column, small quantities of at least three additional components, which were not phenolic, were isolated. Their infrared spectra resembled those of aliphatic esters, but the fact that they were extracted from the ether solution by sodium hydroxide eliminates that possibility.

Carbonyl peaks at 1728, 1725, and 1735 cm^{-1}, respectively, led to the conclusion that these compounds were 6-membered lactones (Bellamy, 1958; and Nakanishi, 1962). Recent studies have indicated that 6-membered lactones are contained in other foodstuffs: aliphatic lactones, for example, are an important factor in the flavor and aroma of heated butter (Stoll *et al.*, 1964), and 6-membered aliphatic lactones have been isolated from coconut oil (Allen, 1965). Recently, Parliment *et al.* (1966), in describing the synthesis of a group of δ-lactones, reported that the lactones all exhibited seven characteristic absorption bands in the infrared spectra. We did not observe all seven bands in the spectra reported here.

While the amine fraction (XVII) had an aroma suggestive of a cyclic amine, such a compound could not be identified; no further evidence was obtained from this fraction. Mason *et al.* (1966) recently identified some of these cyclic amines.

The method of isolating hexanal and 2,4-decadienal is somewhat similar to that employed by Lefort (1953, 1956), who questioned whether these are true flavor constituents of peanuts or artifacts created during the isolation. This point was not resolved by the present study.

Morris *et al.* (1961), reported isolating β-sitosterol glucoside from peanut flour by a series of solvent fractionations. Apparently our procedure led to the hydrolysis of some of the glucoside since we isolated the sterol itself.

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This is a report of work done under contract with Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, and authorized by the Research and Marketing Act.

The authors are indebted to Planters Peanuts Division of Standard Brands, Incorporated, for assistance in the roasting of the Virginia and Runner Peanuts. The support of the U.S. Department of Agriculture through the Southern Utilization Research and Development Division is gratefully acknowledged.

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Characterization of Water-Soluble Wheat Flour Pentosans

SUMMARY—The yield and protein contents of water-soluble pentosan preparations ranged from 0.67 to 0.84% and 15.4 to 24.2%, respectively. After soluble-starch was removed, total yield of water-soluble gums was 0.38 to 0.58%, and their protein contents ranged from 16.9 to 22.6%.

Pentosans were fractionated on diethyl aminoethyl cellulose columns. Based on carbohydrate contents, fraction II, eluted with 0.0025 *M* borate was the largest. Fraction I (eluted with water) was generally the smallest and contained no protein. Fraction III (eluted with 0.025 *M* borate) was the second smallest and was richest in protein. Effects of amylase, protease, and of pentosanases, and of column fractionation on composition of pentosans were followed by infrared spectroscopy.

INTRODUCTION

PREVIOUS INVESTIGATIONS from this laboratory (Tao *et al.*, 1967) concerned effects of water-soluble pentosans on breadmaking. Pentosans isolated from wheat flours were shown by Neukom and co-workers (Kuendig, 1962; Neukom, 1964; and Neukom *et al.*, 1962, 1967) and by Wrench (1965) to constitute a mixture of carbohydrates and glycoproteins.

The effects of enzymes on water-soluble pentosans were studied by Tracey (1964) and Cawley (1964); modifications of water-insoluble pentosans were reported by Kulp *et al.* (1963a,b), Providoli (1965), Upton *et al.* (1966), and Kulp (1967). Fractionations of water-soluble or solubilized pentosans on DEAE-cellulose columns were described by Kuendig *et al.* (1961) and Wrench (1965). This investigation was to isolate and characterize by fractionation and enzyme modification water-soluble pentosan preparations from various flours.

MATERIALS AND METHODS

Flours

Two hard red winter wheat samples ("Qv-Tm × Mql-Oro," C.I. 12995; and a "Chiefkan × Tenmarq" selection, KS 501099) and a hard red spring wheat sample ("Marquis," C.I. 3641) were composited by variety from equal portions of wheat from several locations. Single samples of soft red winter, "Seneca" (C.I. 12529); durum, "Wells" (C.I. 13333); and soft white club wheat, "Omar" (C.I. 13072) were obtained respectively from agricultural experiment stations in Wooster, Ohio; Fargo, North Dakota; and Pullman, Washington. The wheat samples were from the 1965 crop, and were milled in 1966 on a Miag "Multomat." Certain chemical and breadmaking characteristics of the flour samples are given in Table 1.

Pentosan preparations

Preparation of crude extract. Crude pentosans were isolated in duplicate from the six flours from the 1965 wheat crop according to the procedure of Cawley (1964) and Kuendig *et al.* (1961) with certain modifications. Samples were removed at five purification steps and were lyophilized. The scheme is outlined in Fig. 1.

Treatment with α-amylase. The crude pentosan extracts (Extract IV, in Fig. 1) were treated with α-amylase to remove the water-soluble starch according to the procedure of Kuendig *et al.* (1961) with slight modifications. Fifteen mg of twice-crystallized bacterial α-amylase (Nutritional Biochemicals Corp.) were dissolved in 30 ml of a 0.02 *M* sodium phosphate buffer solution, pH 7.2, containing 0.04 *N* sodium chloride and allowed to act in a dialysis bag on 600 mg of a lyophilized crude pentosan extract (Extract IV) dissolved in 30 ml distilled water. The mixture was dialyzed at room temperature for 48 hr against dilute (1:1) buffer solution. The enzyme was removed by precipitation with 5% trichloroacetic acid followed by centrifugation for 10 min at 20,000 G. The supernatant was further dialyzed against distilled water for 2 days and lyophilized (Extract V).

Fractionation on DEAE-cellulose columns. The DEAE-cellulose, a product of Carl Schleicher & Schuell Co., Keene, N. H., (exchange capacity 0.82 meq/g), was prepared according to the method of Neukom *et al.* (1960). The purified DEAE-cellulose was suspended in 0.1 *N* sodium hydroxide, and poured into a chromatography column (2 × 33 cm) containing a glass wool plug and a layer of sand about 2 cm deep. After the DEAE-cellulose had settled, the column was washed with water under slight pressure until the effluent was neutral. During chromatography, no pressure was used. The borate-form was prepared by washing the column with 8-10 column volumes of a 0.125 *M* $Na_2B_4O_7$ solution, the excess borate being washed out with distilled water.

Ten ml of α-amylase-treated, crude water-soluble pentosan solution (Extract V) containing 100 mg of dry material were placed on top of the column. The elution was accomplished stepwise with distilled water, 0.0025 *M*, 0.025 *M*, 0.125 *M* $Na_2B_4O_7$, and 0.5 *N* NaOH. The eluate was collected on a fraction collector in 20 ml quantities at about 1.0 ml per min. Elution of polysaccharide fractions was followed colorimetrically with the phenol-sulfuric

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Table 1. Chemical composition[1] and bread-making characteristics[2] of flours milled from wheat varieties grown in 1965.

Class	Variety or selection	C.I. number[4]	Moisture (%)	Ash (%)	Protein % (N × 5.7)	Baking absorption (%)	Farinograph water absorption (%)	Mixing time (min)	Bromate requirement (mg %)	Loaf volume (cc)	Crumb grain[5]
Hard red winter	Qv-Tm x Mql-Oro[3]	12995	12.5	0.42	12.3	63.2	66.4	5⅛	1	922	S
Hard red winter	Chiefkan x Tenmarq	KS 501099	12.3	0.43	12.6	65.1	72.8	1⅝	4	764	U
Hard red spring	Marquis	3641	12.3	0.52	13.0	62.1	68.0	3	2	877	S
Soft red winter	Seneca	12529	12.0	0.39	8.5	54.2	67.2	3	1	740	Q–S
Durum	Wells	13333	12.0	0.73	10.3	59.5	53.2	2⅛	2	443	U
Club	Omar	13072	12.1	0.35	8.1	47.3	65.0	2⅜	2	588	Q–U

[1] Expressed on a 14% moisture basis.
[2] Per 100 g flour.
[3] Quivira-Tenmarq x Marquillo-Oro.
[4] Cereal Investigation or Kansas Agricultural Experiment Station Number.
[5] S = satisfactory, Q = questionable, U = unsatisfactory.

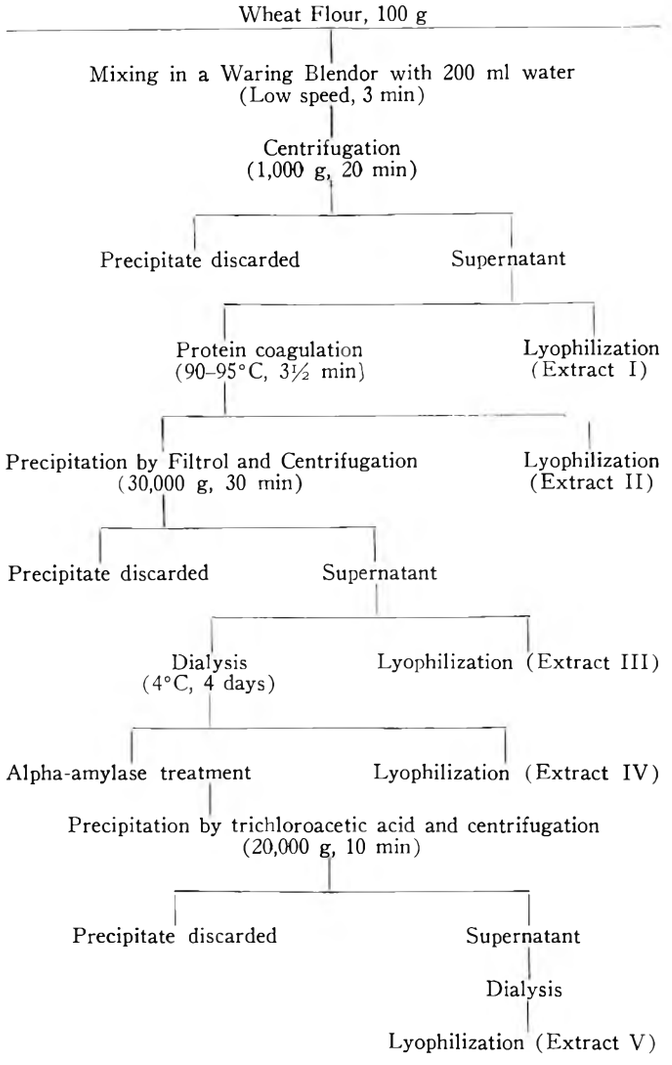

Fig. 1. Outline of preparation and stepwise purification of water-soluble wheat-flour pentosans.

acid method of Dubois *et al.* (1956). After the positions of the individual fractions were located, the appropriate eluates were combined, dialyzed against distilled water and lyophilized. The fractions eluted with 0.5 *N* NaOH were neutralized with HCl before dialysis.

Analytical methods

Protein contents of crude pentosan extracts (Extract IV) and of extracts treated with α-amylase (Extract V) of six flours, and the intermediate preparations (Extracts I, II and III) of the hard red spring wheat flour were determined by a micro-Kjeldahl method; moisture, ash, and micro-Kjeldahl protein were determined by the AACC procedures (1962). The protein contents of eluates from the DEAE-cellulose ion-exchange column were determined by the Folin-Ciocalteau reaction (Lowry's method) as described by Bailey (1962).

Determinations of carbohydrate contents of eluates from DEAE-cellulose columns were affected by the presence of borate (Lin *et al.*, 1968). To determine the distribution of carbohydrates in various fractions, the results were computed according to calibration curves of solutions containing borate.

To determine composition of carbohydrates in fractions separated on DEAE-cellulose columns, 3 mg of lyophilized material were dissolved in 3 ml 0.1 *N* H_2SO_4, the solution was then sealed in a test tube and hydrolyed at 100°C for 4 hr, and subsequently deionized with a commercial mixed-bed-ion-exchanger.

The eluate was freeze-dried. Approximately 500 γ of hydrolyzate were used for chromatographic identification of sugars. Whatman filter paper No. 4 was used with the solvent system butanol-acetic acid-H_2O (4:1:1). The paper was developed in the solvent for about 32 hr and air-dried. The sugars were located after dipping the paper in the alkaline silver nitrate solution described by Linko *et al.* (1960). Distribution of sugars was calculated by scanning the paper chromatograms on a Photovolt Densi-

tometer Model 530 (Photovolt Corporation, New York, N.Y.).

Infrared spectra were obtained on a Perkin-Elmer Infrarecord, Model 137. The reference beam contained air only. Determinations were made on pellet discs obtained by pressing a mixture of 5 mg lyophilized sample and 0.5 g optically pure potassium bromide (Harshaw Chemical Co., Cleveland, Ohio). Spectra in the ultraviolet and visible range (200–400 mμ) were obtained on a Cary 14, Recording Spectrophotometer (Applied Physics Corp., Monrovia, Calif.) by running a 0.05% aqueous solution of lyophilized sample with distilled water as reference.

Enzymatic modifications of crude pentosan preparation

Crude water-soluble pentosan extract (Extract IV) from hard red spring wheat flour was subjected to various enzyme modifications. The enzymes used were crystalline bacterial α-amylase, crystalline Pronase (proteolytic enzyme from *Streptomyces griseus*), Xylanase S173C from *Trichoderma viride,* Xylanase S175K from *Sporotrichum pruinosum* (courtesy E. T. Reese), and β-glucuronidase from limpet (Gallard-Schlesinger Chemical Mfg. Corp.). A 0.02 *M* sodium phosphate buffer solution at pH 7.2, containing 0.4 *M* NaCl, was used for α-amylase, at pH 8.0 for Pronase, and at pH 4.5 for both xylanases and for β-glucuronidase.

The enzymes were dissolved in appropriate buffer solution, and mixed with equal volumes of 2% pentosans in buffer solutions. Three treatments were made in duplicate for each enzyme. The first treatment involved inactivation in a 90°–95°C water bath for 3½ min immediately after mixing the enzymes and substrate. In the second treatment, the mixture was incubated at 37°C for 48 hr before inactivation. In the third preparation from enzyme-treated pentosans, the mixture was centrifuged at 20,000 × G for 20 min and dialyzed against distilled water for 4 days following the second treatment. To reduce microbial growth, two drops of toluene were added to each mixture prior to dialysis. The samples were lyophilized and refrigerated until used.

RESULTS AND DISCUSSION

FLOUR MILLED *from Marquis*—a standard, high quality, breadmaking wheat—was treated by the procedure described in Fig. 1. Table 2 summarizes yields and protein contents of the crude pentosans isolated. The solids of Extract I comprised 2.7% of the original flour and contained 24% protein. The closely similar figures for Extract II show that, as expected, boiling caused little change in yield or protein content.

Table 2. Yields and protein contents of crude pentosans from stepwise extraction of Marquis flour.

Extraction step	Total yield (% of original flour	Kjeldahl protein contents (5.7 × N) (%)
Original flour	.....	12.8
I—Crude extract	2.70	24.1
II—Boiled extract	2.74	24.0
III—After Filtrol precipitation	2.13	9.2
IV—After dialysis	0.80	22.0
V—After amylase digestion	0.55	22.6

After adding "Super Filtrol," to precipitate part of the protein, and after subsequent centrifugation at high speed, the yield (Extract III) was reduced 23%, and was accompanied by a substantial decrease in protein contents. About 62% of Extract III was removed during subsequent dialysis. The large increase in protein contents in Extract IV indicates that nonprotein substances were primarily removed during dialysis. About 31% of Extract IV seemed to be water-soluble starch, since that amount was removed after α-amylase treatment.

The infrared spectra of the extracts are shown in Fig. 2. The top curve represents the spectrum of Marquis flour from which the extracts were prepared. In the spectrum of Extract I, the absorption at 920 cm^{-1} (type I ring-stretching), 865 cm^{-1}, and 770 cm^{-1} (type III ring-breathing vibration) were weaker than those in the spectrum of the original flour. Those three absorption peaks are characteristic of starch (Lin *et al.,* 1965). The increase in absorption at 1625 cm^{-1} (amide I) and 1550 cm^{-1} (amide II) agrees with the higher protein contents determined by the Kjeldahl-nitrogen method. Boiling Extract I produced no significant changes in the spectrum.

Decreases in absorption at 1625 cm^{-1} (amide I) and 1550 cm^{-1} (amide II) in the spectrum of Extract III resulted from removing proteins by precipitation with Filtrol. Subsequent increase in the protein absorbance band (1625 cm^{-1}), after dialysis and removing water-soluble starch, is shown in the spectra of Extracts IV and V, respectively. A new absorption band at 897 cm^{-1} was observed in Extract IV. According to Marchessault *et al.* (1962), this absorption band is relatively sharp and intense in xylans. As a result of α-amylase treatment, starch absorption bands at 920 cm^{-1}, 865 cm^{-1}, and 770 cm^{-1} disappeared, and the pentosan absorption at 897 cm^{-1} became more distinct in the spectrum of Extract V.

In addition to infrared spectra, ultraviolet spectra of the five stepwise extracts of crude pentosan preparations were obtained. The resolution was poor and no specific bands, indicative of changes in composition, were recorded.

Yields of crude water-soluble pentosans (Extract IV) isolated from the six flours ranged from 0.67% to 0.84% (Table 3); the highest yield was from Omar (soft white, club), and the lowest from Wells (durum) and Qv-Tm x Mql-Oro (hard red winter). Protein contents ranged from 15.4% to 24.2% (Table 3); it was lowest in durum and highest in Qv-Tm x Mql-Oro. After α-amylase treatment, the yield ranged from 0.38% to 0.58%. The highest yield was from the soft red winter wheat and the lowest from durum; the protein contents ranged from 16.9% to 22.6% with durum lowest, and hard red spring highest.

Decrease in yield after α-amylase treatment in the six flours ranged from 0.20% to 0.32% (Table 3); hydrolysis of water-soluble starch and dialysis of products of amylolytic cleavage would be expected to increase the protein contents, but no increase was observed (Table 3). It is concluded, therefore that substantial amounts of protein were lost following the α-amylase treatment of crude pento-

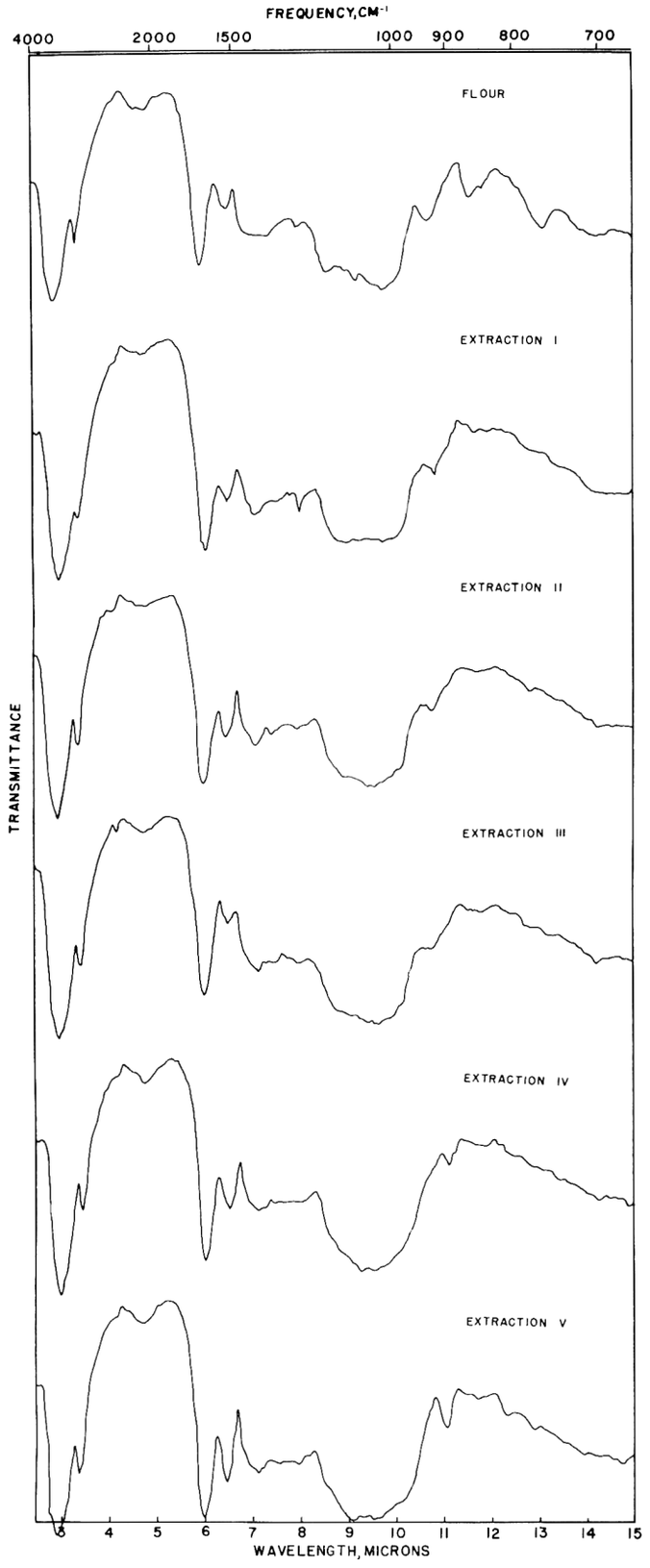

Fig. 2. Infrared spectra of water-soluble wheat-flour pentosans from Marquis flour, at various isolation and purification steps.

Table 3. Pentosan yields and protein contents in extracts of indicated flours before and after amylase treatment.

Pentosan source	Extract IV (after Filtrol precipitation and dialysis)		Extract V (after amylase treatment)	
	Yield (%)	Protein contents (%)	Yield (%)	Protein contents (%)
Qv-Tm x Mql-Oro	0.67	24.2	0.47	19.9
Chiefkan x				
Tenmarq	0.83	19.7	0.51	19.2
Marquis	0.80	22.0	0.55	22.6
Seneca	0.82	20.5	0.58	18.9
Wells	0.67	15.4	0.38	16.9
Omar	0.84	22.0	0.57	22.5

sans. Trichloroacetic acid used to precipitate α-amylase might also precipitate some flour proteins or glycoproteins. It is possible that the trichloroacetic-precipitated proteins or glycoproteins either differed from the moieties removed with Filtrol, or that treatment with α-amylase after precipitation with Filtrol made some proteinaceous flour components more available to precipitation with trichloroacetic acid.

The clear and viscous starch-free pentosan preparations (Extract V) from the six flours were fractionated on DEAE-cellulose ion-exchange columns (borate form). Fractionation by ion-exchange chromatography was carried out at room temperature. Absorbance of carbohydrates and proteins in 20 ml eluates was plotted against effluent volumes. Five fractions differing in total carbohydrate and protein contents, and in sugar composition were obtained.

The results of column fractionation of pentosans from the strong hard red winter wheat flour (Qv-Tm x Mql-Oro) are shown in Fig. 3. Distribution of total carbohy-

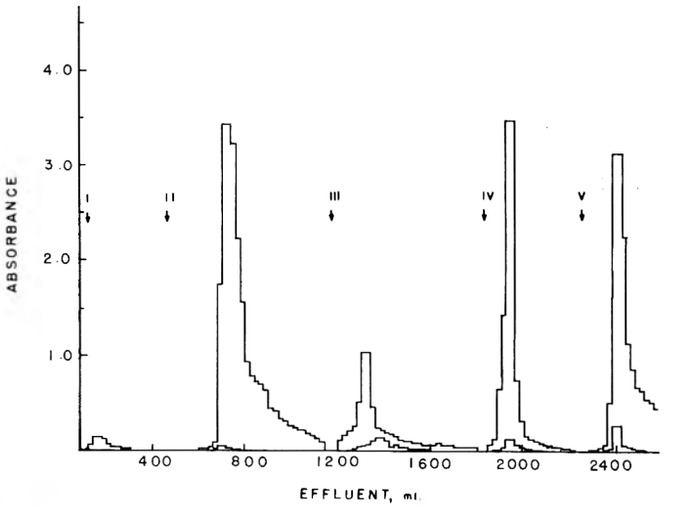

Fig. 3. Fractionation on DEAE-cellulose columns of α-amylase-treated pentosans from the hard red winter flour CI 12995 (Qv-Tm x Mql-Oro). Average of two fractionations of 100 mg each. Top curve, carbohydrates; lower curve, proteins. Elution patterns of 20 ml fractions; I, with water; II, 0.0025. III, 0.025; IV, 0.125 M sodium tetraborate; and V, 0.5 N NaOH.

Table 4. Distribution of carbohydrates and proteins[1] in fractions eluted from DEAE-cellulose-columns.

Pentosan source	Carbohydrate in fraction					Protein in fraction				
	I	II	III	IV	V	I	II	III	IV	V
Qv-Tm x Mql-Oro	2.0	37.9	10.4	18.1	33.3	0	9.0	34.6	23.7	32.7
Chiefkan x Tenmarq	7.2	40.5	4.7	9.6	38.0	0	8.7	54.9	18.2	23.4
Marquis	1.1	38.2	6.7	12.5	41.5	0	8.7	36.0	25.3	30.0
Seneca	0.4	40.0	6.8	9.1	43.7	0	9.8	30.1	20.9	39.2
Wells	3.0	48.6	6.4	14.3	27.7	0	8.9	14.8	32.6	43.7
Omar	12.8	38.6	9.6	14.8	24.2	0	5.9	29.7	31.9	32.5

[1] As % of total eluted from the column.

drate and protein contents in fractions from the six flours is summarized in Table 4. The fractions were characterized by two elution curves; the higher one of carbohydrates and the lower one of proteins. Fraction I had a single carbohydrate elution curve as no proteins were detected in this fraction.

Arrows in Fig. 3 denote changes in eluant composition or concentration. The first fraction was eluted with distilled water; the second, third and fourth with 0.0025 *M*, 0.025 *M* and 0.125 *M* sodium tetraborate solutions, respectively. The fifth fraction was eluted with 0.5 *N* NaOH. After correction for the effects of borate on carbohydrate determination, recoveries of material applied to the column ranged 72–91% (average 80%). Percentage distribution in various fractions—average of at least two determinations—is summarized in Table 4. The results were computed by measuring the area under the elution curve. Comparable results were obtained by measuring absorbance in pooled solutions of effluents of various fractions.

Determinations of sugars in hydrolyzed DEAE-cellulose fractions of pentosans from Marquis flour are summarized in Table 5.

The polysaccharide, which did not complex with borate, was eluted with water as Fraction I and is believed to be a pure arabinoxylan free of protein and galactose (Kuendig *et al.*, 1961; Cole, 1967); it corresponded to the pentosan studied by Perlin (1951) and Montgomery *et al.* (1956). The work of Perlin and co-workers (Ewald *et al.*, 1959; Goldschmid *et al.*, 1963) indicated that Fraction I, the pure arabinoxylan, consists of a straight cellulose-like xylan chain, to which residues of single arabinofuranose units are attached. The arabinose residues are responsible for the water solubility, and their removal by mild acid treatment (Perlin, 1951), or by selective enzymatic action (Neukom *et al.*, 1967) insolubilizes the xylan.

The yield of Fraction I (up to 12.8%) was substantially lower than that reported by Kuendig *et al.*, (1961) and Wrench (1965) (respectively 46% and above 50%). Fraction I—a pure arabinoxylan—is likely to be insolubilized during extraction. Thus, Neukom *et al.* (1960) originally found only a small amount of Fraction I and substantially large amounts in subsequent fractionation work (Kuendig, 1962). Kuendig *et al.* (1961) reported a yield of 1.106 g (per 100 g flour) with 8.8% protein (N × 6.25) in their lyophilized flour extract; the preparations used by Tracey and co-workers (Cawley, 1964) contained about 25% protein (N × 5.7).

Fraction I from DEAE-cellulose columns isolated by Kuendig et al. (1961) contained no protein, whereas, quite significant amounts were reported in Fraction I by Wrench (1965). Similarly, the protein:carbohydrate ratio in the various fractions isolated by the two groups varied significantly. Heterogeneity of the various fractions (indicated by lack of symmetry and differences in shape and position of peaks in protein and carbohydrate elution curves) dictate that claims regarding the presence of defined glycoproteins of fixed composition, size, and structure be considered with utmost caution.

Fraction II contained about 38–49% of total carbohydrates eluted from DEAE-cellulose columns; the protein contents of Fraction II ranged from about 6 to 10% of the total recovered from the DEAE column (Table 4). No glucose was present in Fraction II (Table 5); arabinose and xylose were present in approximately equal concentrations along with a small amount of galactose.

Table 5. Sugar composition (% of total separated by paper-chromatography) of hydrolyzed carbohydrates of fractions separated on DEAE-cellulose columns.

Fraction	Reference*	Galactose	Glucose	Arabinose	Xylose
I	(b)	8	0	43	49
	(c)	0	0	45	55
	(d)	.....	1	27	66
II	(a)	8	0	43	49
	(b)	17	0	41	42
	(c)	26	0	36	38
	(d)	.....	5	32	66
III	(a)	39	0	40	21
	(b)	65	0	35	0
	(c)	54	0	46	0
	(d)	.....	trace	53	53
IV	(a)	56	0	38	6
	(b)	57	0	43	0
	(c)	49	0	43	8
	(d)	1	3	45	50
V	(a)	0	47	22	31
	(c)	2	0	17	81
	(d)	trace	13	20	35

(*) (a) Present data from Marquis flour.
(b) From Wrench (1965).
(c) From Kuendig (1961).
(d) From Cole (1967) on flour hemicellulose (NaOH-soluble).

Fraction III contained little carbohydrates (about 5–10%) but its protein contents were the highest among the five fractions. Fig. 3 shows that the effluent volume of the carbohydrate peak differed from that of the protein peak. A similar shift is found on close scrutiny of elution curves reported by Kuendig *et al.* (1961). The possibility of noncovalent bonds (i.e., hydrogen bonds or hydrophobic bonds, rather than covalent bonds, in linkages between proteins and carbohydrates) cannot be excluded. The results strongly suggest elution of a heterogenous mixture of proteins, or of proteins and glycoproteins.

High concentrations of arabinose were found in Fraction III of both water and NaOH-soluble preparations (Table 5) (reported by Cole, 1967). None of the preparations contained glucose. Whereas the water-soluble pentosans were rich in galactose, the hexose was absent from the water insoluble preparation. Fraction III of the water-insoluble pentosan was very rich in xylose. Wrench (1965) and Kuendig *et al.* (1961) reported no xylose in Fraction III; Table 5 shows about 21% of xylose in this fraction. Sugars in Fraction IV of the water-soluble pentosans from three sources were rich in galactose and arabinose, contained little xylose, and no glucose.

Fraction IV of the NaOH-soluble pentosan (Cole, 1967) differed substantially in sugar composition from the composition of Fraction IV of the water-soluble pentosans. No galactose was detected in Fraction V; the strong glucose spot on the paper chromatogram likely resulted from hydrolysis of cellulose particles from the DEAE-column.

Infrared spectra were run to follow changes in the α-amylase treated pentosan preparation (Extract V) during fractionation on the DEAE-cellulose column. Fig. 4 shows the spectra of Extract V (after amylase treatment) and Fractions II to V of DEAE-cellulose-fractionated material. The yield of Fraction I was too low to determine infrared spectra. Intensity of protein absorption peaks (1625 cm^{-1}, amide I; 1570 cm^{-1} to 1530 cm^{-1}, amide II; 1300 cm^{-1} to 1240 cm^{-1}, amide III) varied in the four fractions. The variations confirm the data given in Table 4; Fractions III and IV contained substantially more protein than did Fraction II. Compared with Fractions III and IV, Fraction II has a very weak peak at 1540 cm^{-1}, but a strong band around 1240 cm^{-1} indicating possible differences in conformational structure of the involved proteins.

Kretschmer (1957) reported that treatment of wheat protein which tended to denature it or decrease its solubility caused the 1240 cm^{-1} band to increase in intensity at the expense of the other amide III bands. Absorption at 897 cm^{-1} reflected the xylose content of the various fractions. Its intensity was in decreasing order in Fractions II, V and III. Absorption at 897 cm^{-1} is characteristic of xylans (Machessault *et al.*, 1962).

Infrared spectra were run to follow changes in enzyme-modified pentosan preparations. Pronase, α-amylase, Xylanase S173C from *Trichoderma viride* (Xylanase A), Xylanase S175K from *Sporotrichum pruinosum* (Xylanase B), and β-glucuronidase were used to modify pentosan Extract IV (dialysis after Filtrol precipitation). Spectra were determined in dialyzed and lyophilized enzyme-treated

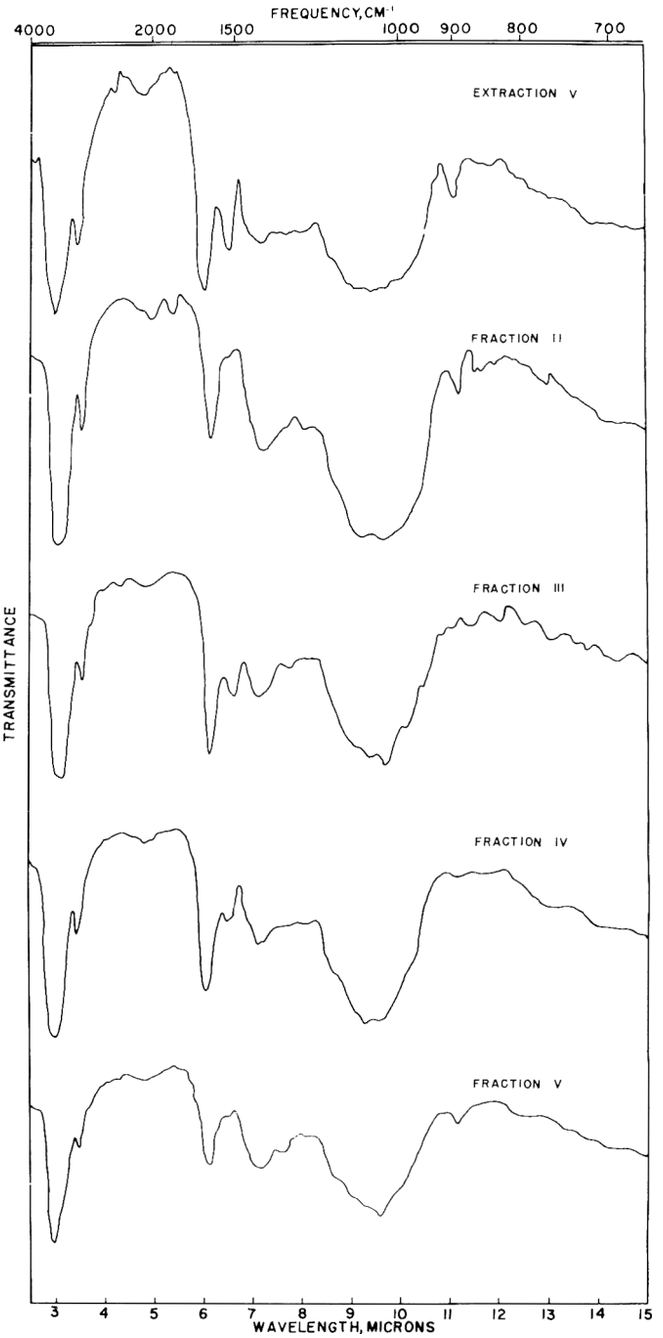

Fig. 4. Infrared spectra of DEAE-cellulose-column fractions of water-soluble pentosans from Marquis flour.

preparations. The infrared spectra of modified pentosan preparations are presented in Fig. 5. The top curve is the spectrum of Extract IV from the hard red spring flour. Curves of preparations treated with α-amylase, Xylanase A, Xylanase B, and β-glucuronidase, show that removing carbohydrate increased the protein content, and intensified the protein absorption peaks at 1625 cm^{-1}, 1540 cm^{-1}, and 1240 cm^{-1} (amides I, II and III); Pronase treatment somewhat decreased absorption at 1540 cm^{-1} (amide II). The residual absorbance around 1540 cm^{-1} indicates that proteins were only partly broken down during proteolytic digestion.

Incomplete breakdown of wheat flour proteins by Pronase has been observed previously (Lin *et al.*, 1964). Absorption at 897 cm^{-1} (possibly related to β-1, 4 linkage of xylan) was intensified by Pronase, α-amylase, and β-glucuronidase treatments. That was expected since removing by dialysis of non-xylan components correspondingly increased the relative xylan content. Decrease in absorbance at 897 cm^{-1} confirmed the capacity of Xylanase A and Xylanase B to degrade wheat flour pentosans. Pentosans treated with β-glucuronidase had a distinct peak around 920 cm^{-1}, indicating cleavage occurred at different sites from those in xylanase-treated samples.

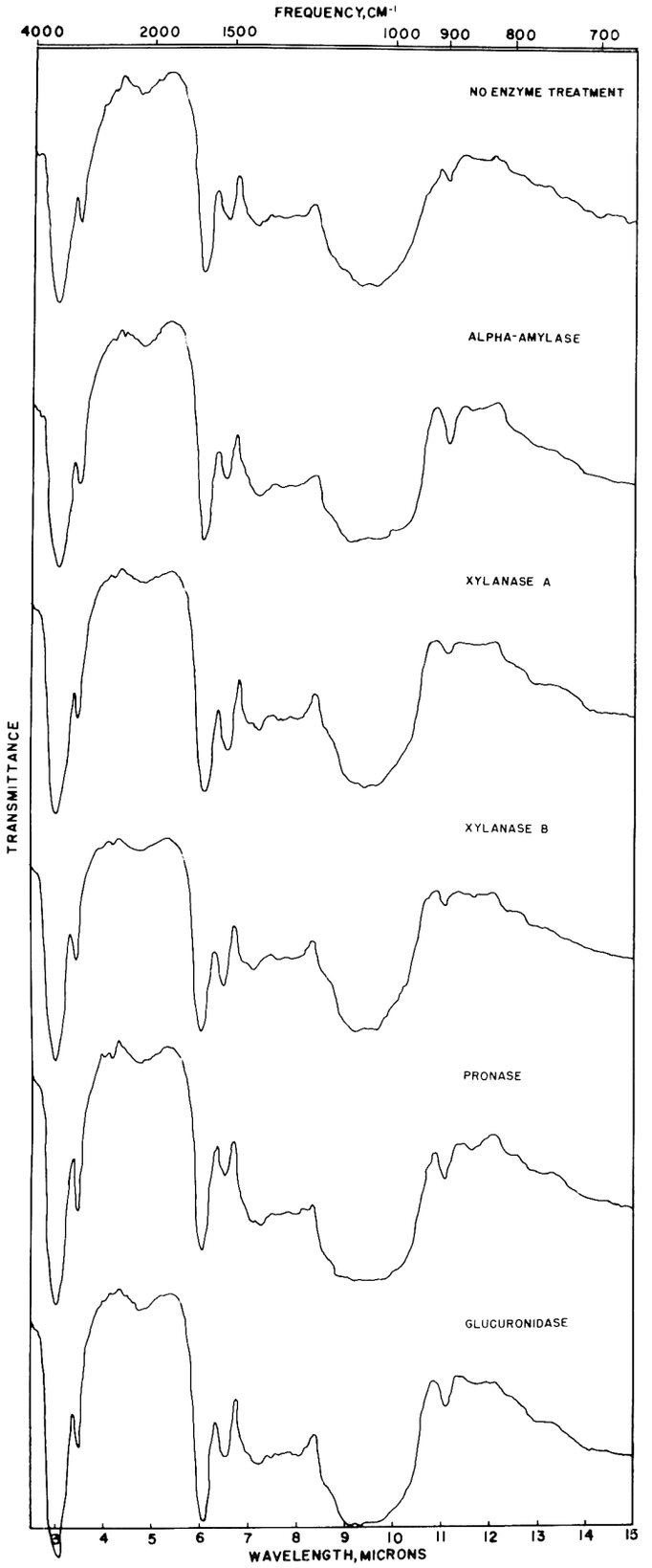

Fig. 5. Infrared spectra of enzyme-modified pentosans from Marquis flour.

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Ms. rec'd 11/22/67; revised 3/4/68; accepted 7/19/68.

Paper presented at the 28th Annual Meeting of the Institute of Food Technologists in Philadelphia.
Cooperaive investigations between Department of Grain Science and Industry, Kansas State University and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. Contribution No. 626, Kansas Agricultural Experiment Station, Manhattan.
Mention of firms or products does not imply endorsement. The authors thank E. T. Reese, U. S. Army Natick Laboratories for samples of xylanases.

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Cysteine Inhibition of Enzymatic Blackening with Polyphenol Oxidase from Potatoes

SUMMARY—The possible sites of cysteine inhibition of enzymatic blackening using polyphenol oxidase from potatoes were studied. The initial site of cysteine inhibition of enzymatic blackening caused by tyrosine oxidation occurred at the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (dopa). Following the initial induction period, the oxygen uptake paralleled those treatments with no cysteine. Concentrations of cysteine (9.5×10^{-3}M) which inhibited tyrosine oxidation for 100 min did not inhibit dopa oxidation significantly. However, higher concentrations (1.9×10^{-2}M) inhibited dopa oxidation. Cysteine did not inhibit chlorogenic acid oxidation under the conditions of our study. Oxygen uptake with chlorogenic acid plus cysteine was higher than in the absence of cysteine. Cysteine concentrations which effectively inhibited tyrosine oxidation did not inhibit oxidation in the presence of tyrosine plus chlorogenic acid.

INTRODUCTION

ENZYMATIC BLACKENING or browning is of great economic importance in the discoloration of many fruits and vegetables. A grey, tan, brown or black discoloration may appear at the site of a cut or bruise or when the plant material is physiologically injured. The enzyme involved is polyphenol oxidase or tyrosinase. In fruits and vegetables, tyrosine or one of its oxidation products are the substrates most often implicated in blackening, while the browning reaction substrates are more often o-hydroxyphenols such as chlorogenic acid. Both chlorogenic acid and tyrosine are found in potato tubers (Juul, 1949; Mulder, 1949; Craft *et al.*, 1958). Tyrosine is thought to be the substrate for enzymatic blackening of potatoes (Nelson *et al.*, 1944; Mulder, 1949).

Cysteine and other related sulfhydryl compounds can be used to inhibit enzymatic discoloration (Lerner, 1953). Henze (1956) stated that cysteine inhibited enzymatic browning caused by chlorogenic acid oxidation by combining with the quinone formed during the reaction. According to Bouchilloux *et al.* (1960) and Roston (1960), cysteine inhibits enzymatic blackening caused by the oxidation of tyrosine or 3,4-dihydroxyphenylalanine (dopa) by forming addition products with the quinones. Although the addition products of oxidized tyrosine or dopa with cysteine were isolated, it is possible that cysteine may also inhibit the oxidation of tyrosine to dopa or of dopa to dopaquinone. If dopachrome formation is delayed by reduction or formation of colorless products, then spectrophotometric experiments which determine the red pigment of dopachrome will not give useful information on tyrosine oxidation.

Therefore, oxygen uptake experiments with polyphenol oxidase from the potato, tyrosine, dopa and/or chlorogenic acid in the presence and absence of cysteine were performed to determine the mechanism of cysteine inhibition.

EXPERIMENTAL

THE POTATOES USED in this study were the Russet Burbank variety grown in southern Idaho and stored at 3–4°C until used. The polyphenol oxidase was obtained from an acetone precipitation of an aqueous extract of raw potatoes (Muneta, 1966). The final dialyzed polyphenol oxidase was prepared in a 0.1 *M* NaOH-NaH maleate buffer (pH 6.3). Various lots of potatoes exhibited different enzyme activity and only the most enzymatically active potatoes were used.

The following stock solutions were used: 1×10^{-2} *M* L-tyrosine, D,L-dopa, and chlorogenic acid, 1×10^{-1} *M* cysteine (free base), 20% KOH (0.2 ml) for CO_2 absorption. All substrate and inhibitor solutions were adjusted to

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pH 6.3 with NaOH. Tyrosine was put in the side arm unless stated otherwise. The pre-incubation time for the cysteine and the enzyme in the main compartment was approximately 1 hr, and the tyrosine was tipped in from the side arm.

Total nitrogen was determined by micro-Kjeldahl procedure with HgO as the catalyst. Oxygen uptake was determined in a Warburg apparatus set at 30°C and 120 oscillations per min.

RESULTS AND DISCUSSION

FIG. 1 SHOWS the enzymatic oxidation of tyrosine in the presence and absence of cysteine. The presence of cysteine resulted in an induction period before oxidation of the tyrosine begins. Following the induction period, the oxygen uptake essentially paralleled the curve where no cysteine was present. No inactivation of the enzyme is evident when 0.2 ml cysteine (9.5×10^{-3} *M*) was used. This contrasts with the inactivation of the enzyme by bisulfite (Muneta, 1966), the most commonly used inhibitor in commercial practice. The concentration of cysteine required for comparable inhibition periods was 5-fold higher with cysteine than for bisulfite. Fig. 1 also shows the effect of incubating the enzyme with cysteine. The induction period for oxygen uptake is decreased with pre-incubation. The presence of an enzymatic or non-enzymatic system which may change the cysteine to inactive forms is indicated.

With 0.2 ml cysteine (9.5×10^{-3} *M*) tyrosine oxidation is inhibited for over 2 hr, but this same amount did not inhibit the oxidation of dopa significantly (Fig. 2). However, with 0.4 ml cysteine (1.9×10^{-2} *M*) the oxidation of dopa is inhibited. Since cysteine will form addition products with oxidized dopa, higher concentrations of these addition products may result in enzyme inhibition.

These experiments show that the initial effect of the cysteine is to inhibit the oxidation of tyrosine to dopa. The oxidation of tyrosine to dopa is more sensitive to cysteine inhibition than the oxidation of dopa to dopaquinone.

If the initial inhibition was due to the reaction of cysteine with a quinone, then oxygen uptake should have occurred with no induction period. This would be expected because oxygen is required for both the oxidation of tyrosine and dopa before the first possible reactive quinone is formed. Addition products formed by reaction of cysteine and oxidized dopa may result in inhibition at a later stage due to a competitive mechanism.

Fig. 3 shows that chlorogenic acid is oxidized very rapidly by the polyphenoloxidase, reaching a plateau

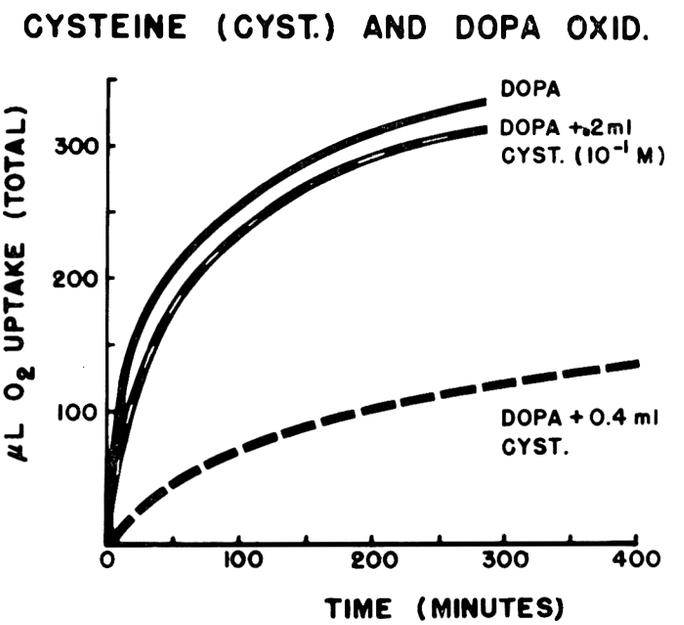

Fig. 2. Cysteine (cyst) inhibition of the enzymatic oxidation of dopa. The reaction mixture contained 0.8 ml enzyme (1.6 mg N); 0.8 ml 10^{-2}M dopa; 0.0, 0.2, or 0.4 ml 10^{-1}M cysteine; H_2O was added to a final reaction volume of 2.1 ml.

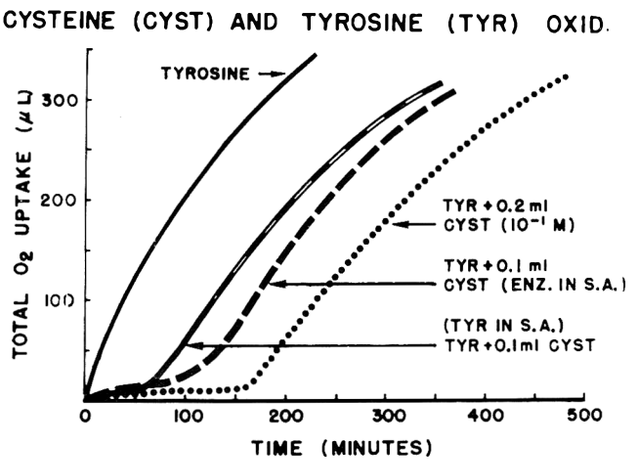

Fig. 1. Effect of preincubation of cysteine (cyst) on the enzymatic oxidation of tyrosine (Tyr). The reaction mixture contained: 0.8 ml enzyme (1.8 mg N); 0.8 ml 10^{-2}M tyrosine; 0.0, 0.1, or 0.2 ml 10^{-1}M cysteine with and without preincubation with enzyme + tyr in side arm (S.A.); H_2O to a final reaction volume of 2.1 ml

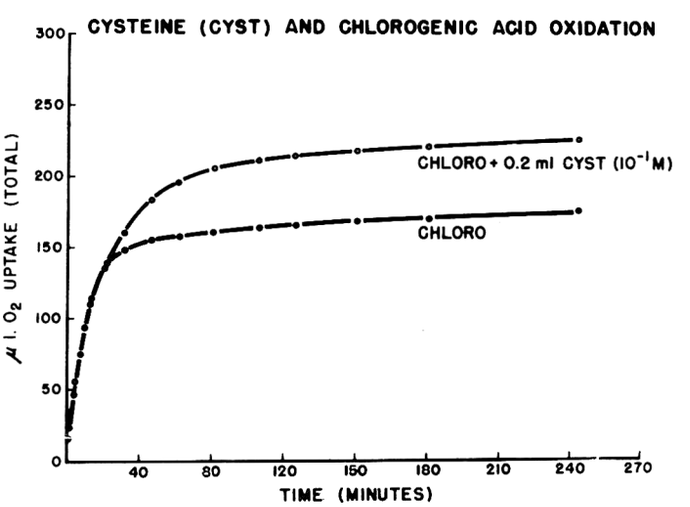

Fig. 3. Cysteine (cyst) inhibition of the enzymatic oxidation of chlorogenic acid (chloro). The reaction mixture contained: 0.8 ml enzyme (1.6 mg N); 0.8 ml chlorogenic acid, 0.0, or 0.2 ml 10^{-1}M cysteine; H_2O to a final reaction volume of 2.1 ml.

equivalent to 1 molecule of oxygen uptake per molecule of chlorogenic acid. The oxidation of chlorogenic acid is not inhibited by the 0.2 ml cysteine (9.5×10^{-3} *M*) used in these experiments. When 0.4 ml of cysteine (1.9×10^{-2} *M*) was used, essentially the same oxygen uptake occurred as with the 0.2 ml cysteine (9.5×10^{-3} *M*).

Oxygen uptake reached a higher plateau when cysteine was added to the reaction mixture. There are several possible explanations for this. The cysteine may act as a reducing agent on the quinone to give significantly higher oxygen uptake than with chlorogenic acid alone. The cysteine may also prevent or slow down the rate of reaction inactivation. Total oxygen uptake may be affected by the relative rates of quinone reduction and the formation of inactive products from the addition of cysteine to the quinone. The reaction between cysteine and oxidized chlorogenic acid does not give colorless products but forms a light yellow-tan pigment. This supports Henze's (1956) evidence that cysteine inhibits enzymatic browning by combining with the quinone. However, enzymatic oxidation can occur with little pigment formation because of the formation of sulfhydryl addition products.

These experiments give further evidence that cysteine inhibits enzymatic browning caused by the oxidation of o-hydroxyphenols by combining with the quinones which are formed, rather than by inhibiting the initial oxidation. However, cysteine can also inhibit enzymatic blackening by inhibiting the oxidation of tyrosine to dopa.

Simple o-hydroxyphenols have a catalytic effect on tyrosine oxidation. Since both chlorogenic acid and tyrosine are found in the potato tuber, interaction between the two compounds and cysteine was studied (Fig. 4). In cysteine's absence, the oxygen uptake rate is increased when tyrosine and chlorogenic acid are used as substrates. Oxygen uptake occurs immediately and proceeds rapidly in the initial oxidation stages. Mapson *et al.* (1963) showed that the rate of pigment formation from tyrosine oxidation is increased in the presence of chlorogenic acid.

The induction period when 0.2 ml cysteine (8×10^{-3} *M*) is added to the tyrosine is over 100 min. When chlorogenic acid, tyrosine and cysteine are used, oxygen uptake is not inhibited. Oxygen uptake is rapid even after the plateau level for chlorogenic acid-cysteine oxidation is reached. The cysteine's inhibitory level has been lowered to such a degree that the oxidation of tyrosine can proceed rapidly. The oxidized chlorogenic acid may have lowered the cysteine concentration by reacting with it to form either addition products (Henze, 1956) or oxidized cysteine compounds (Winkler, 1949).

This type of interaction may be very important from a practical standpoint. For although the tyrosine oxidation may be the main cause of the discoloration of cut or bruised potatoes, the chlorogenic acid concentration and its oxidation products are important in determining the rate of tyrosine oxidation and the level of inhibitor which may be necessary to prevent enzymatic blackening.

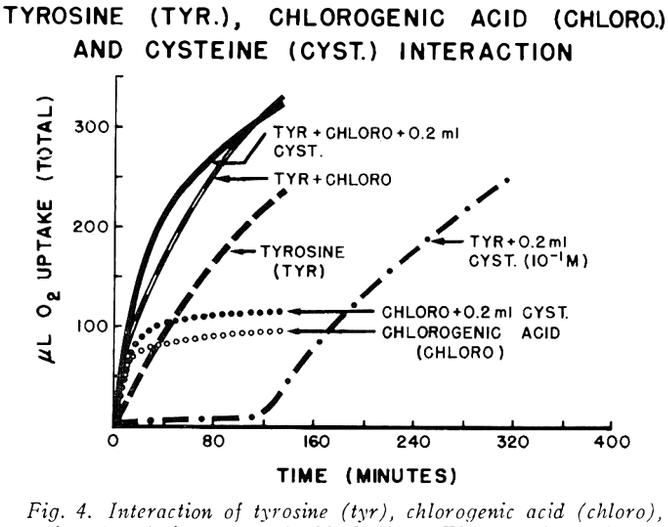

Fig. 4. Interaction of tyrosine (tyr), chlorogenic acid (chloro), cysteine (cyst) in enzymatic blackening. The reaction mixture contained 0.8 ml enzyme (1.8 mg N); 0.0, or 0.8 ml 10^{-4}M tyrosine; 0.0 or 0.6 ml 10^{-2}M chlorogenic acid; 0.0, or 0.2 ml 10^{-1}M cysteine; H_2O to a final reaction volume of 2.5 ml.

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Ms. rec'd 9/8/67; revised 2/16/68; accepted 5/27/68.

Research Paper 737 of the Idaho Experiment Station.

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Post-Mortem Changes in Extractability of Myofibrillar Protein from Chicken Pectoralis

SUMMARY—Protein extractability from chicken pectoralis was measured after the muscle had aged in ice for various periods from 30 min to 24 hr post-mortem. The extraction solution was KCl in phosphate buffer at pH 7.0 and ionic strength 1.0. Residue from the salt extraction was treated with 0.1N NaOH to remove additional myofibrillar proteins and the remainder was considered stromal protein.

Sarcoplasmic protein, non-protein nitrogen and stromal protein remained constant for all aging periods at 33%, 16% and 7% of the total nitrogen, respectively, Myosin extractability decreased rapidly during the first 3–4 hr of aging while the alkali soluble protein increased and actomyosin was extracted at a low, constant level. Following 4–6 hr of aging the alkali soluble protein became constant, and actomyosin appeared in the extract in increasing quantities as myosin continued to decline. The sum of myosin, actomyosin and alkali soluble protein was constant for all aging times at 44% of the muscle nitrogen. Actomyosin formation was accompanied by increased hydration of the myofibrillar proteins as indicated by increased swelling of the residue from salt extraction with greater aging time.

The initial accumulation of alkali soluble protein and subsequent release of actomyosin correspond to the time course of toughening and tenderization in chicken muscle. Both of these observations may reflect initial binding of myosin to the nonextractable thin filaments, followed by disintegration or detachment of these filaments from the Z membrane.

INTRODUCTION

EXTRACTABILITY of muscle proteins has been studied as an indication of changes taking place in muscle after death. In the post-mortem conversion of muscle into meat, the retention of water-binding properties and the development of a pliable muscle which will be tender when cooked are of prime importance. Both of these factors are dependent to a large extent on the condition of the myofibrillar proteins. Protein solubility and juice retaining properties in pig muscle were extensively decreased by rapid pH decline prior to reduction of the muscle temperature (Sayre *et al.*, 1963). Rapid post-mortem glycolysis has been shown to result in toughening of muscle in chicken (de Fremery *et al.*, 1960, 1963).

Khan *et al.* (1964) reported that nitrogen extractability from chicken breast muscle decreased to a minimum at 4–5 hr post-mortem, then increased to a maximum at 1–1½ days. They also reported that the changes in extractability were due mostly to changes in the myofibrillar proteins, but the nature of the extracted myofibrillar protein was not examined. Weinberg *et al.* (1960) extracted chicken pectoralis at 30 min and 24 hr post-mortem and found no difference in sarcoplasmic protein or non-protein nitrogen (NPN) extractability. They did note a decrease of myosin and a marked increase of actomyosin in their 24 hr extracts.

Post-rigor tenderization occurs more rapidly in poultry muscle than in mammalian muscle. Pool *et al.* (1959) reported that little tenderization occurred after 12 hr; van den Berg *et al.* (1964) found that the tenderness of chicken breast muscle increased rapidly during the first 24 hr of aging and did not change after 1–2 days. Recently, de Fremery (private communication) cooked chicken pectoralis after various aging periods and found that the force required to shear strips of constant cross section increased to a maximum at 3 hr post-mortem then decreased to a minimum at 12 hr. Tenderness did not change further during the remainder of the 192 hr aging period. The present study was designed to investigate the effect of aging time on the extractability of myosin, actomyosin and other nitrogenous fractions of chicken pectoralis during the first 24 hr post-mortem.

EXPERIMENTAL

Sample source and preparation

Six commercial fryers (1.8 kg live wt) were electrically stunned and the carotid arteries severed. After bleeding 3 min, the skin over the breast was removed and the pectoralis muscles excised. Excess fat and connective tissue were cut away, and both left and right muscles were cut into dorsal, medial and ventral strips parallel to the keel bone. The strips were wrapped in plastic film and placed in drained ice at 12 min post-mortem. Samples were frozen in liquid nitrogen after aging 0.5, 1.5, 3, 6, 12 and 24 hr in ice. The order of freezing muscle strips according to anatomical location was varied to obviate position effects.

In a second experiment, ten 1.4 kg commercial fryers were slaughtered as above, scalded 1 min at 54°C and hand picked. The eviscerated carcasses were placed in drained ice at 10 min post-mortem. Samples of the pectoralis in the sequence of left posterior, left anterior, right posterior and right anterior were removed from the carcass and frozen in liquid nitrogen at 1, 2, 4 and 6 hr post-mortem.

The frozen muscle was powdered in a cold blender with dry ice and passed through a 20-mesh sieve according to the method of Borchert *et al.* (1965). Samples were stored in sealed plastic bags at −195°C prior to extraction and were not thawed before addition of the cold extraction solution. Freezing of the muscle strips in liquid nitrogen and thawing of the fine muscle powder in the extraction

solution were both accomplished within a few seconds. These rapid transitions into and out of the frozen state as well as storage of the muscle powder well below the eutetic point of −50° to −60°C (Reidel, 1961) were thought to prevent any appreciable alterations in extractability of the muscle components. All extraction procedures were conducted in a cold room at 3°C using chilled equipment and solutions.

Extraction procedure

The extraction and fractionation procedure was a modification of the type reported by Mommaerts (1958) and Khan (1962). Duplicate 2 g portions of muscle powder from each sample were extracted three times for 3, 3 and 2 hr successively, with 10 volumes of the following salt solution: 0.76M KCl, 0.10M K-phosphate, 1mM EDTA, pH 7.0, ionic strength 1.0. The residue was sedimented for 10 min at 30,000 X G between extractions and the supernatants were combined. Following the final salt extraction, the residue was weighed and further extracted with 15 ml of 0.1*N* NaOH for 24 hr. The residue was again sedimented at 30,000 X G and its weight was determined. A final overnight extraction with 7.5 ml of 1.0*N* NaOH was performed and the solution was centrifuged as before. The supernatants from the two alkali extractions were saved for protein estimation. Astomyosin in the salt extract was sedimented at an ionic strength of 0.25 by diluting with 3 volumes of water and centrifuging 15 min at 40,000 X G. Myosin was fractionated from the supernatant by further dilution with 5.25 volumes of water to attain an ionic strength of 0.04 and centrifugation at 20,000 X G for 5 min. The protein remaining in the supernatant was considered to be sarcoplasmic protein. The precipitated actomyosin and myosin were dissolved in 0.1*N* NaOH for protein determination. Protein in the various fractions was measured using the biuret reaction or the procedure of Lowry *et al.* (1951) standardized against Kjeldahl nitrogen. NPN was determined in an aliquot of the salt extract after precipitation of the protein in a 5% solution of trichloroacetic acid. Total muscle nitrogen and NPN were measured by the Kjeldahl method. Analysis of variance was calculated and differences determined by Duncan's new multiple range test (Steel *et al.*, 1960).

RESULTS

Nitrogen values of the different fractions from birds in the first experiment are shown in Table 1. Sarcoplasmic protein extractability remained essentially constant at 33% of the total nitrogen through the 24 hr aging period. Although the data indicate a slight trend for decreased solubility, differences were not significant at the 5% level of probability. NPN averaged 16% of the total muscle nitrogen and did not change during 24 hr aging. The nitrogen extracted by 1.0*N* NaOH from residues previously extracted with 0.1*N* NaOH also remained constant at a low level. These results indicated that any proteolysis was limited and/or specific, and that 0.1% NaOH extracted practically all of the myofibrillar protein which was not extracted by the salt solution.

When nitrogen values for actomyosin, myosin and 0.1*N* NaOH extract were combined for each bird within a time interval, the total was constant at 44% of the muscle nitrogen for all aging times. The proteins in the 0.1*N* NaOH extract were considered to be primarily salt-insoluble myofibrillar proteins. Myosin concentration in the salt extract (Fig. 1) dropped sharply during the first 3 hr and continued to decline throughout 24 hr of aging. During the first 3–4 hr post-mortem, the decrease in myosin extractability was countered by a rapid increase in nitrogen extracted from the salt residue by 0.1*N* NaOH. However, after 3–4 hr the amount of salt-insoluble myofibrillar proteins stopped increasing and was constant for the remainder of the 24 hr aging period. Despite fine division of the muscle, high salt concentration, and long extraction, actomyosin did not appear in the extract prior to 6 hr of aging except at low levels. After 6 hr, actomyosin concentration in the salt extract began to increase at a rate inversely proportional to myosin extractability.

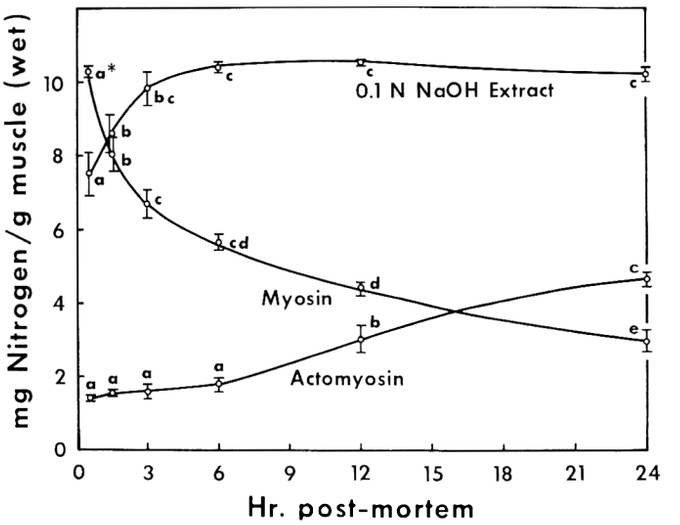

*Fig. 1. Myofibrillar protein fractions. *Points on a line marked by the same letter are not different at the 1% level of probability.*

Table 1. Nitrogen containing fractions of chicken pectoralis (mg N/g wet muscle).[1]

	Hr post-mortem						$S_{\bar{x}}$
	0.5	1.5	3	6	12	24	
Sarcoplasmic protein	14.0	13.6	13.5	13.5	13.4	13.3	0.1
Non-protein nitrogen	6.5	6.4	6.4	6.4	6.4	6.4	0.1
1.0*N* NaOH extract	1.2	1.3	1.3	1.4	1.4	1.3	0.1
Total myofibrillar fractions	18.7	18.2	18.2	17.9	18.0	17.9	0.2

[1] Average values for 6 birds.

Data for the myofibrillar protein fractions from birds in the second experiment are presented in Table 2. Although only aging times between 1 and 6 hr were used, the rates

Table 2. Myofibrillar protein fractions of chicken pectoralis (mg N/g wet muscle).[1]

	Hr post-mortem							
	1		2		4		6	
	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$	$\bar{x}$	$S_{\bar{x}}$
	*							
Actomyosin	1.2^{a}	± 0.1	1.3^{a}	± 0.1	1.6^{ab}	± 0.2	1.9^{b}	± 0.2
Myosin	7.0^{a}	± 0.3	5.0^{b}	± 0.3	4.3^{bc}	± 0.2	3.9^{c}	± 0.1
0.1*N* NaOH extract	8.1^{a}	± 0.5	10.4^{b}	± 0.5	10.6^{b}	± 0.3	10.7^{b}	± 0.2

[1] Average values for 10 birds.
* Values in a row marked by the same letter are not different at the 1% level of probability.

of change of the various fractions were similar to those shown in Fig. 1. Actomyosin extractability did not change significantly prior to 4 hr of aging, but the quantity extracted at 6 hr was significantly greater ($P < 0.01$) than that extracted at 2 hr. The salt-insoluble myofibrillar protein, after an initial rapid increase during the first 2 hr of aging, did not change significantly. Myosin content in the salt extract decreased at a diminishing rate during the entire period of observation.

Sedimented residue from the salt extraction increased markedly in volume with longer aging of the muscle prior to extraction. Fig. 2. presents the weight of the sedimented

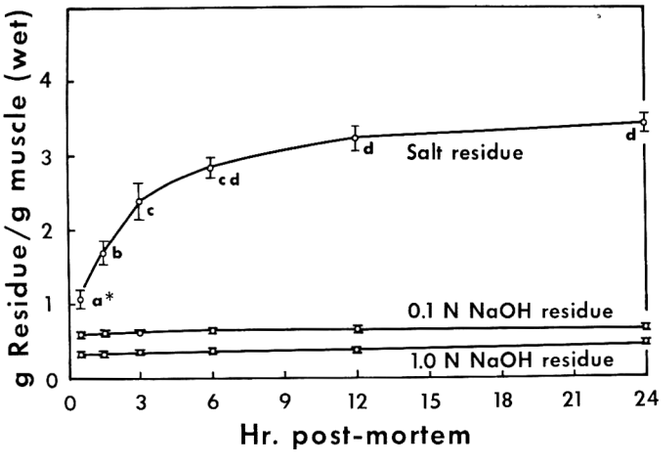

*Fig. 2. Extraction residue. * Points on a line marked by the same letters are not different at the 1% level of probability.*

residues following the three successive types of extraction. The residue weights from the two alkali extractions were constant for all aging periods, and connective tissue was considered to be the major protein component. The nitrogen in these residues amounted to 7% of the total muscle nitrogen. The salt residue weight increased rapidly during the first 3 hr of aging, but changes were not significant at the 1% level of probability after 6 hr.

The difference between the residue weight after salt extraction and that following 0.1*N* NaOH extraction was attributed to myofibrillar proteins and the water bound to them. When this myofibrillar residue weight was expressed per unit of nitrogen extracted from the salt residue by 0.1*N* NaOH (Table 3), the residue per mg *N* increased from

Table 3. Salt insoluble myofibrillar protein hydration.

	Hr post-mortem					
	0.5	1.5	3	6	12	24
	*					
mg residue/mg N[1]	69^{a}	122^{b}	174^{bc}	210^{cd}	245^{de}	269^{e}
Standard error	4	12	18	12	19	10

* Values marked by the same letter are not different at the 1% level of probability.
[1] Mg myofibrillar residue (salt residue wt–0.1*N* NaOH residue wt) per mg N in 0.1*N* NaOH extract.

69 mg at 30 min to 269 mg at 24 hr. The most rapid swelling of the salt-insoluble myofibrillar proteins occurred during the first 3 hr post-mortem, but hydration continued at a slower rate through the entire 24 hr aging period.

DISCUSSION

Both excised muscle (Experiment I) and muscle restrained on the carcass (Experiment II) were used in this study. Extractability of salt soluble proteins from the muscles of birds which had been scalded and chilled intact was slightly lower than that from muscles which had been excised without scalding and chilled as isolated strips. These data are consistent with the finding of Goll *et al.* (1964) that detached muscle, chilled separately from the carcass, had a higher protein extractability than attached muscle despite shortening and consequent higher shear values in the excised muscle. Goll *et al.* (1964) attributed the higher protein extractability to more rapid cooling of the excised muscle. The important point to observe is that the time course of changes in the various myofibrillar fractions was essentially the same for both groups of birds.

The lack of change in NPN during aging agrees with findings by other investigators. Wierbicki *et al.* (1956) found no increase in NPN of bovine muscle during an aging period of several days. Weinberg *et al.* (1960) did not observe changes of NPN in chicken muscle between 30 min and 24 hr post-mortem. A more detailed investigation of the NPN fraction of the chicken muscle by Khan *et al.* (1964) revealed only small changes in the amount of NPN, but changes were noted in the composition of the fraction. These observations led the above authors to conclude that proteolysis is limited or quite specific.

Wierbicki *et al.* (1956) concluded that dissociation of actomyosin was not responsible for tenderization in bovine muscle. The decreased myosin and increased actomyosin extracted from muscle after aging 24 hr led Weinberg *et al.* (1960) to suggest that a specific cleavage of actin, allowing its extraction and binding to myosin in solution might account for post-mortem tenderization. The results of this study are consistent with the above views. The rapid initial decline in extractable myosin indicated that salt-insoluble actomyosin was being formed extensively during rigor mortis development. Subsequent to the development of rigor mortis, actomyosin became increasingly extractable although myosin extractability continued to decline throughout the first 24 hr post-mortem. Other work has shown that essentially all of the post-rigor tenderization takes place during this same 24 hr period (Koonz *et al.*, 1954; Pool *et al.*, 1959).

Haga *et al.* (1965) made continuous extractions of unaged rabbit muscle with Weber-Edsall solution (phosphate-free) for various periods of time up to 48 hr and found that actomyosin did not appear in the extract prior to 8–10 hr. These workers observed that actomyosin particles 1–2μ in length appeared at the beginning of actomyosin extraction and increased in number, but not in size, as more actomyosin was extracted. Mihalyi *et al.* (1966) found that ATP, pyrophosphate and orthophosphate prevented actomyosin extraction from unaged rabbit muscle. They postulated that these phosphate compounds stabilized the actin filaments or their attachment to the Z membrane. The phosphate buffer used in this study may have stabilized the actin filaments at the conditions existing when extraction was started. Thus the length of extraction had little affect on increasing the amount of actin extracted, but those thin filaments which were already detached from the matrix or depolymerized at the initiation of extraction were solubilized.

The initial post-mortem toughening of muscle reaches a maximum at 3 hr (de Fremery, private communication) which corresponds roughly in time to the period of maximum myosin binding prior to release of actomyosin into solution. However, post-rigor tenderization is essentially complete by 12 hr post-mortem (Pool *et al.*, 1959) which is only in the beginning stages of actomyosin extraction. However, tenderness, as measured by gross means with a shear apparatus or taste panel, may be appreciably affected by a relatively few breaks along the myofibrils.

Increased hydration of the salt-insoluble myofibrillar proteins, as indicated by swelling of the salt residue, may contribute somewhat to tenderization, particularly after some breaks begin to form in the myofibrils. Hydration seemed to accompany the formation of actomyosin, and continued to increase during actomyosin extraction.

As ATP was broken down post-mortem and myosin was bound to actin, the salt-soluble proteins decreased due to the non-extractability of actin. After 4–6 hr of aging, actin slowly started to be extracted, resulting in the appearance of increasing amounts of actomyosin in solution. There was not further change of total salt-soluble myofibrillar protein after 4 hr, but free myosin continued to decrease in the extract with a concomitant increase of actomyosin. These changes must have been due to an alteration of the muscle structure which allowed the thin filaments either to break or become detached from the Z membrane. Thus, they support the findings of Weinberg *et al.* (1960), Haga *et al.* (1965), and Mihalyi *et al.* (1966) which link changes in the thin actin filaments to post-mortem tenderization.

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Ms. rec'd 6/26/67; revised 8/20/68; accepted 9/5/68.

The author thanks I. V. Streeter for technical assistance and D. de Fremery for advice on the experimental design.

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Tenderness and Maturity in Relation to Certain Muscle Components of White Leghorn Fowl

SUMMARY—Tenderness of roasted and stewed pectoralis major and biceps femoris muscles (estimated by shear values) from White Leghorn female birds was studied in relation to maturity and composition at 4, 8, 12 and 18 months of age. Shear values of pectoralis major muscles did not change significantly with age or differ significantly between cooking methods. Shear values of biceps femoris muscles increased significantly with age of roasted specimens. No similar significant change occurred in stewed specimens. Roasted biceps femoris had significantly higher ($P < 0.01$) shear values than stewed samples at 12 months and more markedly at 18 months of age. No association was noticed between either intramuscular fat or moisture content and shear value trends. Hydroxyproline content (HOP) was determined in biceps femoris samples only. Total HOP content was significantly higher in more mature samples with a significant difference only at 12 months of age between cooking methods. Residual HOP content as a measure of collagen not converted to gelatin during cooking, showed a tendency to increase with age and differed significantly between cooking method only at 18 months of age. Total and residual HOP content of roasted biceps femoris were significantly correlated with shear values ($r = 0.64$ and 0.79, $P < 0.01$). The marked increase in residual HOP content at 18 months seemed to explain more satisfactorily the divergence in shear values.

INTRODUCTION

VARIABLE TENDERNESS and inconvenient methods of preparation are associated with a decreasing demand for stewing hens, and thus are related to the declining prices for farm fowl in the last 20 years. Processing problems, declining acceptance and depressed prices for laying fowl have suggested their use in pet foods and mink rations, which, though an outlet, represent a valuable protein loss and contribute to economic loss in the egg industry. This depressed value of laying fowl merits a study of the factors responsible for these problems.

Decreasing tenderness has generally been shown to be associated with advancing maturity in bovine animals (Goll *et al.*, 1963; Herring *et al.*, 1967; Kim *et al.*, 1967; Webb *et al.*, 1967) and likewise in chickens by Lowe (1948), May *et al.* (1962) and Wells *et al.* (1962).

Among the complex factors influencing tenderness, it has usually been assumed that collagen plays a major role in the decrease of tenderness with maturity. The importance of total intramuscular collagen per se in this area is not entirely clear. Goll *et al.* (1963) and Hill (1966) reported no trend between total collagen and maturity in bovine, swine or sheep muscles. Frohberg (1963) likewise found no increase in total collagen in raw fowl muscles from birds 5 to 15 months of age. Wilson *et al.* (1954) reported higher total collagen values for veal than for steers or cows. Herring *et al.* (1967) and Kim *et al.* (1967) each found total collagen to increase with age in only one of the two muscles being studied.

Although Goll *et al.* (1963), Herring *et al.* (1967) and Kim *et al.* 1967) found decreased tenderness in mature specimens, this was not universally related to corresponding increases in total collagen. Total intramuscular collagen has been shown to be negatively related to tenderness in studies by Husaini *et al.* (1950), Loyd *et al.* (1959), Adams *et al.* (1960) and Parrish *et al.* (1962) in studies in which maturity was not a primary factor.

The change in solubility of collagen with age was deemed important by Hill (1966), who noted solubility decreased with age of the muscle specimens and suggested that total collagen was not an adequate basis for explaining decreasing tenderness with maturity. Herring *et al.* (1967) found a decrease in intramuscular collagen solubility with advancing age and found significant correlations between this factor and panel judgment of tenderness. Ritchey *et al.* (1964) could find no definite relationship between age, tenderness and collagen remaining after cooking of various beef muscles.

The present study was undertaken to investigate the relationship between tenderness (as estimated by shear press values) and maturity in White Leghorn fowl, and to relate any possible differences to compositional factors including collagen, moisture and fat.

EXPERIMENTAL

Sample stock and processing

A total of 64 White Leghorn females representing a single commercial strain were obtained from commercial flocks, 16 for each age group of 4, 8, 12 and 18 months. The latter age group would possibly represent the earliest age at which mature layers would normally be marketed (12 months of production). All birds had been cage reared from an early age. The birds were stunned, exsanguinated for 1 min, scalded in a rotary scalder (1 min at 53°C), hand-picked, eviscerated and chilled in ice slush for 24 hr. An earnest effort was made to keep processing steps standard to minimize variable tenderness due to these factors. After chilling, carcasses were bagged (polyvinyl chloride), frozen, split and stored at −23°C until further treatment.

Cooking procedures

Eight carcass halves were cooked by a stewing and eight halves by a roasting method. The stewing procedure consisted of immersing carcass halves in boiling water.

After internal temperatures reached 85°C (185°F), as indicated by thermocouples located in the breast and thigh, cooking was continued for an additional hr to ensure maximum tenderization. The total cooking time was approximately 90 min. The roasting method consisted of roasting carcass halves in an oven at 177°C (350°F) without basting to obtain an internal temperature of 85°C (185°F), as indicated by thermocouples in breast and thigh, usually reached in 30–45 min. In both instances, cooked samples were allowed to cool 30 min before the biceps femoris and pectoralis major muscles were excised. A series of separate cookings was necessary to finish all samples. Specimens from different age groups were simultaneously cooked to avoid procedure induced bias.

Shear values

Shear values were obtained by using an Allo-Kramer shear press adjusted for a 30 sec ram speed, and equipped with a 2500 lb proving ring with a maximum indicating gauge.

Moisture and fat determinations

Meat samples were dried in a 103°C ± 2° oven for 24 hr. Percent moisture was calculated from weight loss. Crude fat was estimated by extracting the dried sample for 24 hr with ether in Bailey-Walker apparatus. Percent fat was calculated on the basis of the fat residue in the flasks divided by the weight of the wet sample.

Estimated values of total and residual collagen

The presence of hydroxyproline can quantitatively indicate the presence of collagen (Gross *et al.*, 1960). The sheared tissues were freeze-dried for 24 hr to a moisture content of 10 ± 2% and pulverized for subsequent analysis. Samples of 100 mg of accurately weighed, freeze-dried specimen were analyzed for total hydroxyproline by the Woessner (1961) method as modified by Goll *et al.* (1963). Samples of 600 mg of accurately weighed freeze-dried specimen were fractionated for residual hydroxyproline using the procedure of Ritchey *et al.* (1963); and the subsequent alkali-insoluble, autoclave-soluble hydroxyproline was quantitatively determined, again using the Woessner method with Goll's modifications. Values obtained for total and residual hydroxyproline were converted to the equivalent for dry tissue in proportion to the determined moisture content of freeze-dried powders, before calculation on the basis of cooked tissue.

Statistical analysis

Duncan's new multiple range test and correlation analysis were used to evaluate data, as outlined by Steel *et al.* (1960).

RESULTS AND DISCUSSION

The shear values for roasted and stewed muscles expressed as pounds force per gram of cooked tissue are presented in Table 1. Within age groups, the pectoralis major shear values were significantly lower ($P < 0.01$) than those of the biceps femoris regardless of the cooking method, with the exception of the roasted thigh muscle from the 4 month group. Hanson *et al.* (1942) and Gainer *et al.* (1951) reported a similar trend in tenderness scores in Leghorn broilers and roasting chickens, respectively. Mickelberry *et al.* (1962) reported a finding "partially contrary" to the foregoing in a study of White Rock broilers in which biceps femoris was intermediate in shear value between the outer 3 mm and next 3mm layer of pectoralis major.

There was no significant difference in shear values of the breast muscle with regard to either cooking method or age of fowl. Turning to the biceps femoris, there was a general increase in shear values of the roasted muscle with age, although no significant difference was noticed between samples from the 8 and 12 month birds. No significant differences were noted in stewed thigh muscle with respect to age. Differences between cooking methods were significant ($P < 0.01$) at 12 and 18 months. While a difference was noted at 12 months of age, the difference of greatest practical significance seems to be in the 18 month maturity group.

The results of analyses for moisture and fat content of cooked tissues are shown in Table 2. Moisture content of stewed breast muscle from 18 month old birds showed a significant decrease compared with other age groups. At all ages the roasted breast muscles had significantly higher moisture contents than the corresponding stewed samples. A significant increase in fat content of the biceps femoris was noted between 12 and 18 months of age regardless of cooking method. There was a similar trend, though not significant in all respects with regard to fat content of pectoralis major samples. The trends in moisture and fat do not seem to relate at all to the shear values for the corresponding muscles and treatments.

The association between amount of intramuscular fat

Table 1. Shear press values (lb/gm cooked tissue) for breast and thigh muscle at various ages.[1,2]

Muscle	Cooking method	Maturity (months) 4	8	12	18
Pectoralis major	Roasted	7.5 ± 0.5^{a}	9.3 ± 1.3^{abc}	9.5 ± 0.4^{abc}	9.8 ± 1.2^{abc}
	Stewed	7.6 ± 0.4^{a}	6.9 ± 0.6^{a}	8.6 ± 0.6^{ab}	8.4 ± 0.7^{ab}
Biceps femoris	Roasted	11.0 ± 0.8^{abcd}	16.7 ± 1.1^{ef}	18.8 ± 1.9^{f}	24.6 ± 1.3^{g}
	Stewed	11.8 ± 0.8^{bcd}	13.2 ± 0.8^{cde}	14.7 ± 1.2^{de}	14.2 ± 0.7^{de}

[1] Means with the same superscript are not significantly different at $P < 0.01$. Comparisons are valid among ages and between muscles and cooking method.
[2] Values are mean ± standard error.

Table 2. Moisture and fat content of cooked breast and thigh muscle at various ages.[1,2]

Muscle	Component	Cooking method	Maturity (months) 4	8	12	18
Pectoralis major	% Moisture	Stewed	64.76 ± 0.47^{a}	64.35 ± 0.42^{a}	64.99 ± 1.20^{a}	62.38 ± 0.47^{b}
		Roasted	68.64 ± 0.42^{c}	68.49 ± 0.44^{c}	68.96 ± 0.34^{c}	68.46 ± 0.42^{c}
	% Fat	Stewed	1.20 ± 0.49^{ab}	1.06 ± 0.07^{ab}	1.07 ± 0.14^{ab}	1.47 ± 0.10^{b}
		Roasted	0.86 ± 0.05^{a}	0.90 ± 0.36^{a}	0.84 ± 0.13^{a}	1.22 ± 0.14^{ab}
Biceps femoris	% Moisture	Stewed	66.69 ± 0.39^{ab}	66.90 ± 0.42^{ab}	66.61 ± 0.94^{ab}	65.87 ± 0.52^{a}
		Roasted	68.61 ± 0.37^{b}	68.22 ± 0.45^{b}	67.48 ± 0.38^{ab}	66.57 ± 0.30^{ab}
	% Fat	Stewed	1.53 ± 0.39^{a}	1.42 ± 0.10^{a}	1.71 ± 0.28^{ab}	2.49 ± 0.13^{c}
		Roasted	1.14 ± 0.13^{a}	1.70 ± 0.15^{ab}	1.46 ± 0.18^{a}	2.21 ± 0.15^{bc}

[1] Means with the same superscript are not significantly different at $P < 0.01$. Comparisons are valid only between cooking method and among ages by component (moisture or fat) and individual muscles.
[2] Values are mean ± standard error.

Table 3. Hydroxyproline content of cooked biceps femoris (mg/100 gm cooked tissue) at various ages and correlations with shear values.[1,2]

Hydroxyproline measurement	Cooking method	Maturity (months) 4	8	12	18	Correlation (r) with shear values[3]
Total	Stewed	180.5 ± 14.8^{a}	182.9 ± 2.2^{a}	237.8 ± 10.5^{b}	260.4 ± 2.0^{b}	0.24
	Roasted	156.6 ± 6.0^{a}	157.5 ± 1.2^{a}	188.2 ± 9.2^{a}	248.1 ± 2.4^{b}	0.64**
Residual	Stewed	7.54 ± 0.39^{a}	5.61 ± 0.25^{a}	31.08 ± 0.49^{a}	29.53 ± 0.67^{a}	0.38*
	Roasted	6.82 ± 0.17^{a}	9.13 ± 0.32^{a}	22.27 ± 0.68^{a}	86.52 ± 0.69^{b}	0.79**
% residual of total	Stewed	4.18	3.07	13.07	11.34	.....
	Roasted	4.36	5.80	11.83	34.87	.....

[1] Means with the same superscript are not significantly different at $P < 0.01$. Comparisons are valid only between cooking method and among ages by measurement (total or residual hydroxyproline).
[2] Values are mean ± standard error, where available.
[3] Significance of r at $P < 0.05$ indicated by *, at $P < 0.01$ by **.

and tenderness has been reported to vary from positive in pork loins (Harrington *et al.*, 1962; Kauffman *et al.*, 1964) to insignificant in beef muscles (Cover *et al.*, 1958; and Cover *et al.*, 1960). While intramuscular fat may affect juiciness and be associated with subjective tenderness judgment (American Meat Institute Foundation, 1960), the effect on shear values is uncertain. Strandine *et al.* (1949) failed to find a correlation between tenderness and moisture or fat content of twelve chicken muscles.

Because of the lack of change with maturity in shear values of the pectoralis major specimens, only the biceps femoris was studied in relation to hydroxyproline (Table 3) as an indication of collagen content. If connective tissue is a primary factor in relation to decreasing tenderness of maturing fowl, it would be more logical to expect differences in the biceps femoris as indicated by the histological observations and classifications reported by Strandine *et al.* (1949) and the collagen studies of Frohberg (1963) and Khan (1962) which indicated greater amounts of collagen in thigh than in breast muscle.

In the present study, there was a general increase in total hydroxyproline content (and presumably collagen) with advancing maturity. This agrees with the findings of Herring *et al.* and Kim *et al.* for one of the two beef muscles studied in each case. It does not necessarily contrast with Frohberg's (1963) observations on fowl muscles where maximum age was 15 months, since the major increase in the present study did not develop until 18 months. Khan (1962) has also demonstrated an increase in the stroma component of chicken breast and thigh muscle from 4 months and 10 weeks, respectively, to 1 year of age. While there was a highly significant positive correlation (Table 3) between shear values and total hydroxyproline in roasted specimens this does not adequately relate to the divergence in shear values between cooking methods noticed at 18 months of age, at which time there was no difference in total hydroxyproline contents.

Consideration was then given to the effect of cooking method on solubilization of collagen as indicated by residual hydroxyproline content (Table 3) of cooked tissue after elimination of gelatin (Ritchey *et al.*, 1963). There was a general increase in residual hydroxyproline values with increasing maturity. The values for 12 month stewed and roasted, and for 18 month stewed specimens (31.08, 22.27 and 29.53 mg/100 gm cooked tissue) differed significantly from other means at $P < 0.05$ ($P < 0.01$ used in Table 3), but not from each other. Ritchey *et al.* (1964) did not notice any definite trend in residual hydroxyproline in a variety of beef muscles representing three maturities. The trend with respect to maturity presented here is in agreement with the previously mentioned direct estimates of collagen solubility (Hill, 1966; Herring *et al.*, 1967). The only significant difference ($P < 0.01$) between cooking methods in residual hydroxyproline content was noticed in the 18 month maturity group. Correlations between

shear values and residual hydroxyproline were significant and positive for both cooking methods (Table 3) but at a higher confidence level for roasted specimens. Herring *et al.* (1967) has reported significant positive correlations between panel tenderness and collagen solubility.

The underlying motive of this study was to determine at what maturity a meaningful difference in shear value estimation of relative tenderness might arise which would affect the feasibility of cooking Leghorn fowl by convenient dry methods (roasting) as opposed to moist cookery (stewing) and to attempt to relate this difference to collagen content. The marked difference between cooking methods in shear values (biceps femoris) at 18 mouths of age appears to be most related to a corresponding decrease in the solubility of collagen (based on residual hydroxyproline values) noticed at this age.

Because of the maturities used, this decrease in solubility can only be assumed to begin between 8 and 12 months of age and accelerate somewhat between 12 and 18 months. Hill (1966), who also reported a decrease in solubilization of collagen when meat from older animals was compared with younger, suggested that this was most likely due to increased cross-linking or strength of cross-links in intramuscular collagen. The percent residual of total hydroxyproline (Table 3) figures indicate that a marked increase in collagen unaffected by cooking occurred in the 18 month roasted samples, presumably due to molecular changes in collagen structure which the roasting method could not alter. In contrast to this, the lower value for the 18 month stewed samples most likely is a result of the additional 60 min of moist cooking they received beyond the 85°C end-point temperature used for roasted samples.

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Ms. rec'd 2/16/68; revised 5/2/68; accepted 9/5/68.

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Partial Purification of Salmon Muscle Cathepsins

SUMMARY—Salmon muscle cathepsins hydrolyzed denatured hemoglobin optimally at pH 3.7 with two minor pH optima noted at pH 7.0 and 8.5. Cathepsins optimally active at pH 3.7 and extracted with 0.2 *N* KCl, were partially purified by (1) precipitation of inactive protein at pH 5.5, (2) $(NH_4)_2SO_4$ fractionation, and (3) column chromatography on DEAE-cellulose. Two cathepsins appeared to be separated by this procedure; one was purified 27-fold with 17% recovery and the other was purified 116-fold with 6.8% recovery.

INTRODUCTION

MOST INFORMATION AVAILABLE on catheptic enzymes stems from investigations on spleen, kidney, and liver, while those of muscle tissue have received comparatively little attention (Fruton, 1960). This situation is probably due to the relatively low activity levels found in muscle tissue as compared to organ tissues. Although Zender *et al.* (1958) reported that the proteolytic activity of lamb and rabbit psoas muscle was only 1/40 that of the lung, Siebert (1958) found that the catheptic activity of fish muscle was about ten-fold greater than that of mammalian muscle. Fish muscle cathepsins have been observed to play a role in the spoilage of fish prior to processing (Siebert, 1962). Sinnhuber *et al.* (1964) reported that heat treatment was required prior to irradiation to inactivate the enzymes that cause release of amino nitrogen in cod muscle.

Groninger (1964) purified a proteinase from albacore muscle which was similar to cathepsin E. Muscle cathepsins have recently been purified from porcine (Parrish *et al.*, 1966) and bovine (Parrish *et al.*, 1967) muscle. Both of these cathepsins appear to be similar to cathepsin D. In the present study a purified preparation of salmon muscle cathepsins was obtained.

EXPERIMENTAL

Samples

One-year old Chinook salmon *(Oncorhynchus tshawytscha)* were sacrificed by a blow on the head, eviscerated, sealed in polyethylene bags, frozen and stored at −28°C. The muscles were thawed at room temperature, carefully excised from skin and bone to avoid microbial contamination, diced, and homogenized with two parts extracting solution for 3 min with a Virtis "45" homogenizer at a setting of 75. Homogenates were used as soon as possible (generally within 15 min) after preparation. All operations, except the assay, were performed in the cold room (3°C).

Assay for catheptic activity

The assay procedure used was a modification of that of Tallan *et al.* (1952). A 10% urea denatured hemoglobin (Nutritional Biochemicals) solution was dialyzed against several changes of distilled water for 48 hr. After dialysis, the hemoglobin solution was diluted to 2.5% and 1 mg of Thimersal, N.F. (Mann Research Laboratories) was added per 40 ml of solution. This solution was stored at 3°C until used. Assays were carried out in a water bath at 37°C in 25-ml Erlenmeyer flasks which were continuously shaken 120 times per min through an oscillation of 1½ in. Assay mixtures contained 4 ml of hemoglobin solution, 1 ml of 0.4 *M* acetate (pH 3.2), 1 ml of muscle homogenate or enzyme solution and sufficient 0.5 *N* HCl or 0.5 *N* NaOH to adjust the pH to the desired level. Water was added to make the total volume 7 ml.

After a 1-hr incubation, 9 ml of 0.3 *M* trichloroacetic acid (TCA) were added, flasks were swirled by hand for 20 sec and replaced in the water bath for 20 min. The precipitate was removed by filtration through Whatman No. 1 filter paper. Blanks were run in the same manner, except the TCA was added immediately before the homogenate or enzyme solution. Activities were expressed as the differences in absorbance (280 mμ) of the TCA filtrates of the blanks and assay mixtures. In the pH studies, the extent of protein hydrolysis was also determined using Folin *et al.* (1927) reagent by the method of Anson (1938). Protein content of the fractions was estimated by absorbance of the solutions at 280 mμ (Dixon *et al.*, 1964).

Column chromatography on DEAE-cellulose

DEAE-cellulose (diethylaminoethyl-cellulose, type 20, 0.8 meq/g, Carl Schleicher & Schuell, Co.) was washed successively with 1 *N* NaOH, 1 *N* HCl, 1 *N* NaOH, and water and suspended in 500 ml of 0.1 *N* NaOH. After deaeration under reduced pressure, the agitated slurry was siphoned into a column (2.5 × 50 cm) containing 0.1 *N* NaOH. The stopcock was partially opened and the resin was allowed to settle by gravity flow. A column 35 cm in height was obtained from 30 g of the resin. The packed column was placed in a cold room and washed with water until the pH of the effluent was neutral.

Then 100 ml of 0.1 *M* phosphate (pH 6.5) were passed through the column, followed by starting buffer (0.005 *M* phosphate, pH 6.5) until the pH of the effluent was 6.5. After each chromatographic run, the resin was regenerated in the column by the method of Peterson *et al.* (1962) followed by 100 ml of 0.1 *M* phosphate (pH 6.5) and starting buffer until the pH of the effluent was 6.5.

The protein solution, generally 25 ml, was washed onto the column with several small portions of starting buffer. A constant flow micropump, adjusted to 80 ml/hr, was attached to the column and chromatography was initiated with starting buffer until the first protein peak had emerged.

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A concave gradient elution program was started with the aid of a nine-chambered Varigrad (Peterson *et al.*, 1959) to a limit buffer of 0.005 *M* phosphate containing 0.5 *N* NaCl. Volume percentages of limit buffer in chambers one through nine were 0, 0, 4, 0, 15, 0, 25, 40, and 100, respectively. A total of 2,250 ml of buffer was used in the Varigrad. Approximately 30 hr were required to complete a chromatographic run.

The absorbance at 280 mμ of the column effluent was monitored continuously by a Beckman DB recording spectophotometer equipped with a flow-through cell. The effluent was collected in 10-ml fractions.

RESULTS AND DISCUSSION

pH optimum

Fig. 1 shows the pH-activity curves of the catheptic activity of salmon muscle homogenate. Both curves show

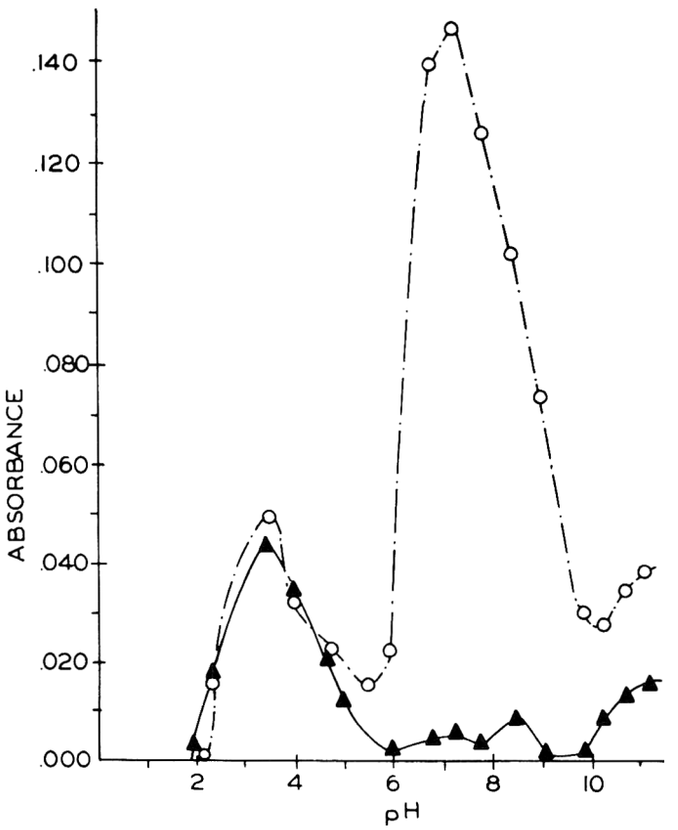

Fig. 1. Effect of pH on the catheptic activity of salmon muscle homogenate. Absorbance was determined at 280 mμ (○ - — - ○) and as determined by Folin's reagent (▲ — ▲).

a pH optimum between 3.6 and 3.8. The curve determined by absorption at 280 mμ reveals a high activity peak with an optimum at pH 7.3, while the curve determined by Folin's reagent indicates two relatively minor peaks at pH 6.9 and 8.5. Since Folin's reagent has been reported to be specific for tyrosine and tryptophan (Anson, 1938), the pH optimum observed at pH 7.3 by absorption at 280 mμ would appear to be due to the presence of substances other than tyrosine and trytophan that were released from the muscle homogenate at this pH. No evidence is available about the identity of thes subsances. Nucleic acids might have been responsible, since ribonuclease from animal tissues has a pH optimum of 7 to 8 (Anfinsen *et al.*, 1961).

Groninger (1964) showed that purified protease from albacore muscle has a pH optimum of 2.4 to 2.5. Siebert (1962) found cod muscle protease to have a pH optimum of 4.3. Hence, it would appear that salmon muscle protease may be more similar to that of cod muscle, but differs from albacore muscle protease on the basis of pH optimum. It was decided to attempt the purification of the enzyme which exhibited a pH optimum at 3.6 to 3.8.

Extraction of cathepsins

Homogenates of muscle tissue were prepared with various salt and buffer solutions and centrifuged at 34,000 × G for 20 min. For the control, a homogenate, prepared with distilled water, was used as the enzyme source. Effect of a holding period after homogenization and before centrifugation was not determined. Catheptic activity and protein content of the clear supernatants were determined. The data are summarized in Table 1.

The maximal specific activity was obtained by extracting with 0.15 *M* acetate buffer (pH 4.5) containing 0.1 *N* KCl; however, the recovery was low. Homogenates, prepared with 0.15 *M* phosphate (pH 7.5) and 0.15 *M* phosphate (pH 7.5) containing 0.1 *N* KCl, formed gels and could not be separated by centrifugation at 34,000 × G. Solutions of KCl appeared to show the greatest promise as extracting solutions.

Table 2 shows the results of a series of extractions made

Table 1. Extraction of salmon muscle cathepsins with various solutions.

Solution	Specific activity (activity/mg protein) × 10^3	Percent recovery
Homogenate		100
Water	1.73	60
0.1 *N* KCl	2.27	87
0.6 *N* KCl	1.67	67
0.1 *N* LiCl	0.94	37
Acetate (pH 4.5, 0.15 *M*)	2.46	37
Acetate (pH 4.5, 0.15 *M*) + 0.1 *N* KCl	2.70	46

Table 2. Effect of KCl concentration on extraction of salmon muscle cathepsins.

Solution	Specific activity (activity/mg protein) × 10^3	Percent recovery
Homogenate		100
Water	2.23	69
0.05 *N* KCl	1.96	76
0.1 *N* KCl	1.86	89
0.2 *N* KCl	1.97	95
0.3 *N* KCl	1.88	105

with various concentrations of KCl. Extracts obtained with 0.2 *N* KCl yielded high specific activity consistent with high recovery. Hence, 0.2 *N* KCl was adopted as the extracting solution in the following investigations. This crude extract was designated fraction A.

pH stability of salmon muscle cathepsins

To establish the pH of the buffers to be used in ion-exchange chromatography, it was necessary to define the stability of the cathepsins at various pH levels. Various quantities of 0.5 *N* HCl and 0.5 *N* NaOH were added to adjust the pH of fraction A. Sufficient water was added to maintain constant volumes. After storage in the cold room for 24 hr. the pH of fraction A was readjusted to the original pH and catheptic activity was determined.

Results presented in Table 3 reveal that as the pH of fraction A was varied from pH 6.5, catheptic activity decreased. Acidic conditions did not appear to be as harmful as those in the basic region. From these data, it was concluded that the pH would have to be maintained close to pH 6.5 to prevent loss of activity during ion-exchange chromatography.

Acid treatment

In the preceding experiment, precipitates formed in fraction A when the pH was reduced to 5.5 or 4.5. To determine if inactive proteins could be removed by this procedure, the pH of fraction A was reduced by the careful addition of 0.5 *N* HCl. Samples were allowed to stand for 10 min before centrifugation at 1,000 × G for 20 min. The pH of the clear supernatant was readjusted to the original pH with 0.5 *N* NaOH and the volumes were standardized with water. Data presented in Table 4 indicate that specific activity was increased 2.6-fold, with 86% recovery, when the pH was reduced to pH 5.5. The fraction resulting from this procedure was called fraction B.

Dialysis

In preparation for column chromatography, fraction B was dialyzed against 0.005 *M* phosphate buffer (pH 6.5) for 12 hr. A precipitate formed upon dialysis and was removed by centrifugation at 1,000 × G for 20 min. Activity and protein were determined on the supernatant and undialyzed portion. These data revealed that the specific activity was increased from 2.69×10^{-3} in the undialyzed portion to 5.45×10^{-3} in the dialyzed supernatant with 98.1% recovery. Purification waas increased 2-fold. This fraction was designated fraction C.

Several attempts were made to purify fraction C by DEAE-cellulose chromatography. However, the catheptic activity in the column effluent was too low to be determined. Therefore, $(NH_4)_2SO_4$ fractionation was employed in an attempt to concentrate the activity.

Ammonium sulfate fractionation

Various quantities of solid $(NH_4)_2SO_4$ were added to portions of fraction C. Precipitates were recovered by centrifugation at 34,000 × G for 10 min, dissolved in minimal amounts of 0.005 *M* phosphate (pH 6.5), and dialyzed against the same buffer for 12 hr. Precipitates which formed on dialysis were removed by centrifugation at 34,000 × G for 10 min. Catheptic activities of the supernatants were determined. Preliminary results showed that most of the activity was present in the 0.25 to 0.50 saturated fraction. This range of concentration was investigated in more detail and the data are presented in Table 6.

Similar amounts of activity were recovered in the 0.25 to 0.40 and 0.40 to 0.50 saturated fractions with somewhat higher purity in the 0.25 to 0.40 fraction. Since no separation between these two fractions was apparent, the 0.25 to 0.50 saturated fraction was used for chromatography on DEAE-cellulose. This fraction was called fraction D.

Table 3. Stability of salmon muscle cathepsins at various pH levels.

pH	Decrease in activity (%)
2.58	47
3.41	25
4.44	19
5.59	2
6.50	0
6.91	19
8.06	47
9.76	91

Table 4. Effect of acid treatment on catheptic activity of fraction A.

pH	Specific activity (activity/mg protein) × 10^3	Percent recovery	Purification (fold)
6.49 (control)	0.72	100	
5.49	1.84	86	2.6
4.45	1.45	86	2.0

Table 5. Effect of dialysis on catheptic activity of fraction B.

Treatment	Specific activity (activity/mg protein) × 10^3	Percent recovery	Purification (fold)
Dialyzed	2.69	100	
Not dialyzed	5.45	98.1	2.0

Table 6. Ammonium sulfate precipitation of catheptic activity from fraction C.

Fraction	Specific activity (activity/mg protein) × 10^3	Percent recovery	Purification (fold)
A	1.32	100	
B	3.37	85	2.5
C	5.40	56	4.1
0.25[1] to 0.40[2]	9.44	20	7.1
0.40 to 0.50[2]	6.03	19	4.6
0.50 to 0.60[2]	2.99	4	2.3
0.60 to 1.00[2]	0.52	15	0.4

[1] No precipitation appeared at 0.25 saturation.
[2] Saturation of ammonium sulfate.

Column chromatography

Results of chromatography of fraction D on DEAE-cellulose are presented in Fig. 2. Catheptic activities were determined on the fractions containing the highest protein concentration; however, activity levels were too low to be meaningful. To concentrate catheptic activity, fractions comprising protein peaks between vertical dashed lines were combined and lyophilized. The lyophilized fractions were dissolved in minimal amounts of water and dialyzed against starting buffer for 24 hr. A precipitate formed and was removed by centrifugation at 1,000 × G for 10 min. Catheptic activity and protein concentration were determined on the supernatants. The results are presented in Table 7.

An increase in purification of 5- and 21-fold over fraction D was noted in peaks 5 and 8 respectively. Maximal purification of 116-fold, with respect to fraction A (the crude extract), was obtained in peak 8. These data indicate that two catheptic enzymes were present in fraction D and have sufficiently different properties to be separated by column chromatography on DEAE-cellulose. This experiment was repeated with similar results.

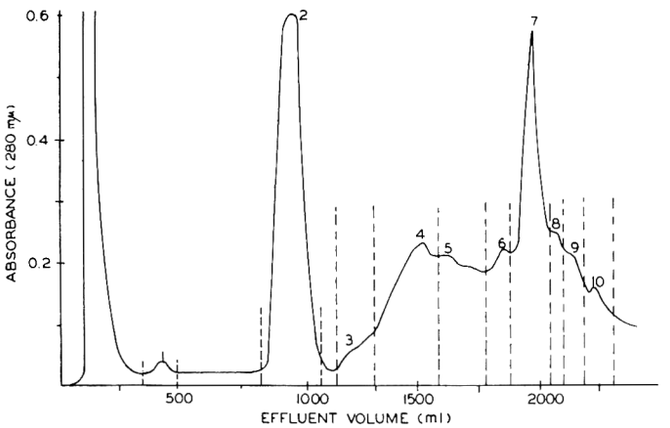

Fig. 2. Chromatography of fraction D on DEAE-cellulose. Sample size: 25 ml (1,215 mg protein) with activity of 0.514 absorbance unit/ml. Gradient started at 280 ml. The fractions between the vertical lines were combined.

Table 7. DEAE-cellulose chromatography of fraction D.

Fraction	Specific activity (activity/mg protein) × 10^3	Percent recovery	Purification (fold)
A	1.9	100	
B	3.4	88	1.8
C	3.9	74	2.0
D	10.6	56	5.6
peak			
1[1]	....	....	....
2[1]	....	....	....
3[1]	....	....	....
4	3.0	1.2	1.6
5	52.0	17	27.4
6	30.2	1.8	16.0
7	32.0	8.5	17.0
8	221	6.8	116
9	80.0	4.6	42.1
10	48.2	1.7	25.3

[1] Activity too low to be meaningful.

Purification procedures used in this study were similar to those used by Groninger (1964) for the purification of protease from albacore muscle. With DEAE-cellulose chromatography, he reported that the major portion of protease activity was eluted in one protein peak, followed by lesser quantities of protease activity in three other protein peaks. Parrish *et al.* (1966) reported the presence of only one peak of catheptic activity in the purification of porcine muscle cathepsin on DEAE-cellulose.

In the purification of cathepsin C by ion-exchange chromatography and gel filtration, Planta *et al.* (1964) reported that concentration of the fractions "by all methods tried" caused heavy losses in enzyme activity. However, Groninger (1964) was able to concentrate purified albacore muscle protease by ultrafiltration. During the present work, catheptic activity was lost when the column chromatographic fractions were concentrated by ultrafiltration, pervaporation, or by use of "Aquacide No. 2" (Calbiochem). In ultrafiltration, the catheptic activity was recovered in the filtrate. Since all of these procedures involved the movement of water through a semi-permeable membrane, it is possible that salmon muscle cathepsin is of sufficiently low molecular weight to pass through the membrane; i.e. less than 10,000. In dialysis, there is generally only a slight movement of water with a diffusion of ions through the membrane, therefore, a great loss of a low molecular weight protein would not be expected. This may be presumptive evidence that cathepsins of salmon muscle have low molecular weights.

In the chromatogram presented in Fig. 2, it would have been ideal to determine the activity on each fraction; however, since a laboratory lyophilizer was not available during the work and considering the time involved in performing catheptic assays, this was not considered practical. Higher purification may have resulted if each fraction had been analyzed or if the fractions had been combined in a different manner.

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Ms. accepted 5/20/68.

Technical Paper No. 2403, Oregon Agricultural Experiment Station.

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Adsorption of Volatile Organic Compounds in Dehydrated Food Systems. 1. Measurement of Sorption Isotherms at Low Water Activities

SUMMARY—Removal of water vapor during food dehydration results in losses of volatile compounds that contribute to flavor. The relative importance of diffusion and adsorption to retention of volatile components by solid food constituents during dehydration processes is not well known.

A frontal analysis gas chromatographic apparatus has been developed to measure vapor-solid adsorption at low adsorbate concentrations. Volatile compound is sorbed from the gas stream by an adsorbent in a system designed to minimize diffusion and kinetic effects. The system includes saturator chambers, which maintain constant concentration of adsorbate in the carrier gas stream, and a flame ionization detector.

Data collected are presented as sorption isotherms. With microcrystalline cellulose powder as adsorbent and hexane and acetone vapors as adsorbates partial pressures in the range of 1 millitorr were produced and maintained. Resulting concentrations measured were in the range of micrograms adsorbate per gram adsorbent (ppm), orders of magnitude encountered in real dehydrated food systems.

INTRODUCTION

ALL DEHYDRATION processes that remove water as a vapor cause some loss of volatile organic constituents as well as a modification of flavor. In some cases this change is not objectionable. The objective of dehydration of fruits and vegetables is, of course, to produce a product resembling as closely as possible the raw materials employed. Of dehydration methods currently in commercial use, freeze-drying maintains the structure and flavor of the finished product at the highest level of acceptability.

In principle, freeze-drying is an effective distillation procedure. In the absence of any interactions with other food constituents, the volatile flavor components should be quantitatively distilled at the pressures and temperatures usual in commercial freeze-drying practice. Drying conditions that minimize the time necessary to reach a moisture content at which product quality and stability during storage is adequate are usually employed.

Temperatures are kept low enough to prevent thermal damage to the product. Although retention of volatile components was not generally given specific attention during development of the process, high retention may be attained fortuitously by choosing processing conditions based on other considerations.

Diffusion and adsorption may be involved in reducing loss of volatile organic compounds with water removed during dehydration. Rey *et al.* (1962) found that in a freeze-dried model system containing 5 to 25% glucose prior to dehydration, retention of acetone increased with increasing glucose concentration. As much as 45% of the original 0.1% added acetone was retained. Since, under the freeze-drying conditions employed in their experiments, pure acetone would have distilled almost instantaneously, it was clear that the acetone interacted with glucose. It was not clear, however, whether acetone retention was increased by sorption on the glucose or by reduction in the rate of diffusion through the dry surface layer at high glucose concentration.

Menting *et al.* (1967) investigated retention of acetone

Presented at the 27th Annual Meeting of the IFT in Minneapolis, Minnesota, May 17, 1967.

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during dehydration of aqueous carbohydrate droplets. They concluded that in dehydration of droplets containing 100 to 500 g maltodextrin per liter of water, acetone retention was caused by formation of an impermeable membrane rather than by adsorption.

Saravacos *et al.* (1968) investigated retention of acetic acid, ethyl acetate, ethyl butyrate, and methyl anthranilate in a variety of freeze-dried food gels and in apple slices. Retention of 93% of 500 ppm methyl anthranilate in a freeze-dried cellulose gel was observed even though the volatility of methyl anthranilate was 3.3 relative to water. It was impossible in their experiments to separate the effects of adsorption and resistance to diffusion in the dry surface layer. They measured equilibrium sorption of volatile compounds by conventional gravimetric methods. To obtain measurable weight differences, it was necessary to work at concentration levels between 0.5 and 5%. These concentrations are much higher than those present in real foods.

The degree of sorption of volatile organic compounds at very low (parts per million) concentrations—those naturally occurring in foods—may be quite different from that at higher concentrations. A small number of very active sites may be available for sorption of small quantities of volatile compounds. Thus, sorption isotherms measured at high concentration may not be valid at the low levels found in nature. Water activity may also influence sorption of flavor components through such mechanisms as steam distillation and competition for active sites at the food surface. This paper describes a frontal analysis gas chromatographic system for measurement of sorption of volatile organic compounds by solid food components at concentrations in the parts per million range.

A system was designed capable of measuring sorption isotherms for a variety of flavor components with a wide range of volatilities on sorbents typical of food components such as cellulose, starch, and protein. Provision was made for varying sorbate concentration and sorbent temperature. Also included in the design, but not yet executed experimentally, is the ability to vary water activity of the sorbent.

It is possible to measure concentrations of volatile flavor components in foods by conventional elution gas chromatography with ionization detectors. The sorbent can be equilibrated with the vapor of interest in a closed container and then analyzed to determine the amount sorbed. But such analyses are cumbersome and subject to errors from evaporation of sorbate during preparation for analysis. Reproducible partial pressures and concentrations of sorbate in the range of interest are difficult to establish and maintain.

Gas chromatography is an appropriate method for investigating interactions between volatile compounds and solids. Gale *et al.* (1964) used a method based on conventional elution chromatography for determination of heats of adsorption of gases on carbon. The sorbent was contained in a chromatographic column and known volumes of gas injected as pulses. The slope of the initial linear portion of the isotherm was computed from the measured retention volume and weight of sorbent. This method is appropriate, however, only for systems providing linear isotherms and symmetrical chromatographic peaks. The requirement for extremely low sorbate concentrations (ppm) for examination of food components imposes an additional limitation on the application of the eluted pulse technique. If quantities of a few grams of solid are to be employed, then samples of volatile materials must be of the order of micrograms. Since any solvent might be adsorbed, severe sampling problems result.

Eberly (1961) measured adsorption isotherms and surface areas by frontal analysis gas chromatography. Beebe *et al.* (1966) evaluated this technique and compared it with the eluted pulse method for determination of isotherms and heats of adsorption of gases. Malamud *et al.* (1967) used frontal analysis for measurement of adsorption isotherms of xylene isomers at relative vapor pressures (p/p_o) in the range 0.01 to 0.977 on zinc oxide. The lowest relative vapor pressure employed corresponded to a concentration of 0.185 mg p-xylene/g of solid. Thermal conductivity detectors provided adequate sensitivity for measurements in this range.

Constant partial pressures of sorbate entering the column may be provided by saturating the carrier gas with a pure liquid maintained at constant temperature. But more sensitive detectors and a different approach to control of sorbate partial pressure are required for adsorption studies in the concentration range of interest for volatile food components.

Fig. 1 is a schematic frontal analysis curve. Prior to time t_o, pure carrier gas passes through the sorbent sample. At t_o, carrier gas containing a constant known concentration of sorbate is introduced. The detector signal remains equal to that observed prior to sample introduction until the sorbent approaches equilibrium with the carrier gas stream. At this point, t_b, the sorbate breaks through and appears at the detector. At t_e, the sorbate is in equilibrium with the sorbent. Detector output after t_e corresponds to

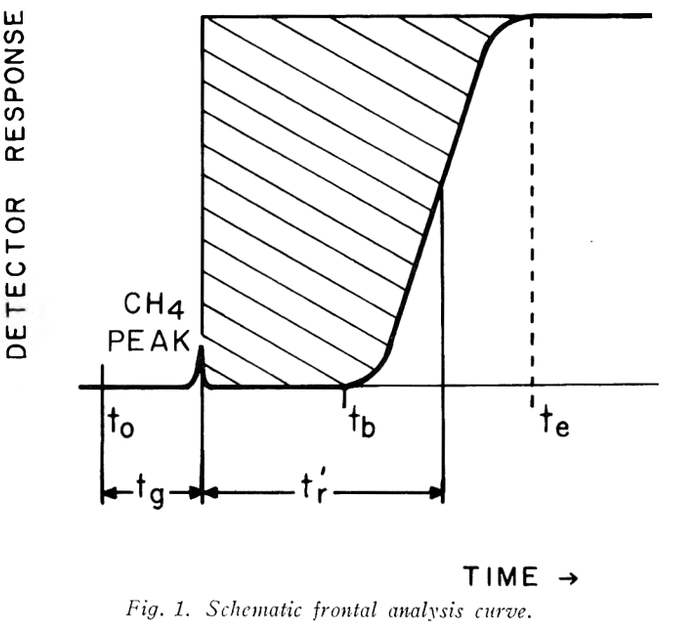

Fig. 1. Schematic frontal analysis curve.

the value that would be observed for carrier plus sorbate before entering the column. Observed retention volume can be corrected for sample interstitial volume and instrument dead volume by injecting a non-sorbed gas and measuring the volume of carrier required for elution. The time corresponding to this volume is t_g. Desorption data may be obtained simply by switching to pure carrier gas at any time after equilibrium is attained.

All data required to establish a single point on an isotherm are displayed on the recorder chart. Amplitude of the signal at equilibrium is proportional to partial pressure of sorbate in the carrier gas stream. This signal is measured as a current and may be converted to mass flow rate of sorbate by calibration of the detector with each sorbate. Partial pressure of sorbate, p_{ads}, may be expressed as:

$$p_{ads} = \frac{m_{ads}}{m_c} \times \frac{MW_c}{MW_{ads}} \times \bar{p} \qquad [1]$$

Average pressure in the column, $\bar{p}$, is given by:

$$\bar{p} = p_o \times \frac{1}{f_p} = p_o \times \frac{2}{3} \frac{\left[\left(\frac{p_i}{p_o}\right)^3 - 1\right]}{\left[\left(\frac{p_i}{p_o}\right)^2 - 1\right]} \qquad [2]$$

where f_p is the compressibility correction factor, derived by James *et al.* (1952).

Mass flow rate of carrier gas, m_c, may be calculated from its measured volumetric flow rate, F_R, corrected to column temperature:

$$m_c = F_R \times \frac{T_C}{T_R} \times \rho_c \qquad [3]$$

The amount of volatile compound, w_{ads}, removed from the carrier (shaded area in Fig. 1) is the product of adsorbate mass flow rate, m_{ads}, and true retention time, t:

$$w_{ads} = m_{ads} \times t = m_{ads} \times t_r' \times f_p \qquad [4]$$

If the weight of sorbent in the column, w_s, is known, concentration, c, can be calculated:

$$c = \frac{w_{ads}}{w_s} = \frac{m_{ads} \times t_r' \times f_p}{w_s} \qquad [5]$$

EXPERIMENTAL

Apparatus requirements for measuring frontal analysis curves are shown in simplified form in Fig. 2. Prepurified nitrogen was used as carrier gas. Commercial flow controllers are adequate for control to better than 1% in the flow rate range (about 50 cc/min) required. Columns were simply copper tubes packed with weighed amounts of sorbent. The flame ionization detector currently employed is part of a conventional gas chromatograph (Model F-11, Perkin-Elmer Corp., Norwalk, Connecticut).

The saturator must maintain concentrations of volatile compounds in the carrier gas constant in the parts per million range for the time required to complete a measurement. This criterion is difficult to satisfy with a pure compound in a gas washing bottle. At temperatures low enough to produce vapor pressures on the order of 10^{-3} torr, most compounds of interest as flavor components are solids. Therefore, we employed dilute solutions of these compounds in a non-volatile solvent.

Dow-Corning-550 silicone fluid was purified for use as a solvent by heating to 130°C at 1 torr while purging with

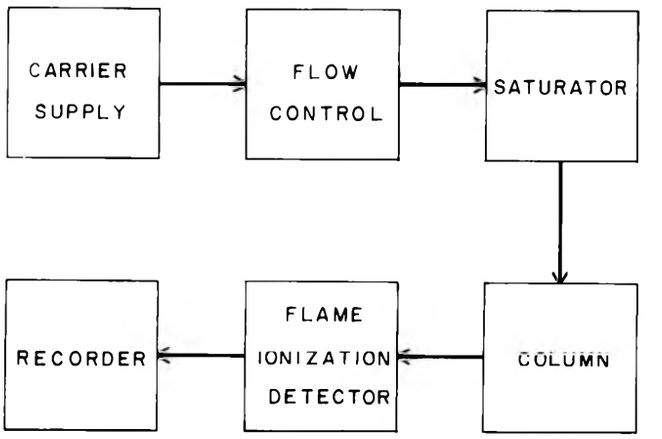

Fig. 2. Block diagram of frontal analysis chromatographic apparatus.

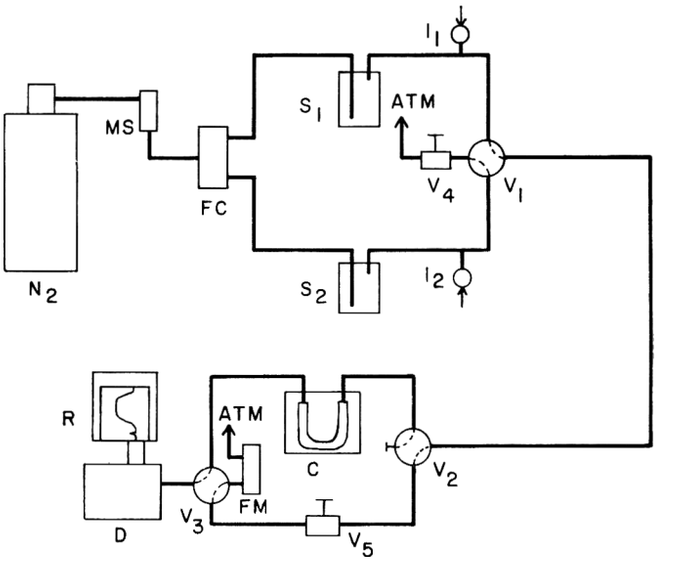

Fig. 3. Diagram of frontal analysis gas chromatographic apparatus. N_2, pre-purified nitrogen gas supply; MS, molecular sieve, Linde 18 ×; FC, dual flow controller with rotameter (Varian-Aerograph); S_1 and S_2, glass saturator chambers (~200 ml); I_1 and I_2, injection ports; V_1, V_2 and V_3, four port switching valves (Model 4 SW, Perkin-Elmer Corp., Norwalk, Conn.); V_4, metering valve (Nupro Model 2S); V_5, metering valve (Nupro Model 2SA); ATM, atmospheric outlets; C, column; FM, soap-film flowmeter; D, flame ionization detector (Perkin-Elmer Corp. Model F-11); R, recorder, 1 mV Speedomax H with disc integrator (Leeds and Northrup Co., Philadelphia, Pa.)

dry nitrogen for 72 hr. This treatment reduced the flame background current when carrier was passed through the silicone fluid from 4×10^{-9} amp for unpurified material to about 1×10^{-11} amp after treatment. This background did not differ significantly from values recorded for pure nitrogen. Concentrations of 50 ppm of hexane and acetone in silicone fluid resulted in initial partial pressures with the saturator at room temperature on the order of 3×10^{-2} and 2.5×10^{-2} torr, respectively.

The initial partial pressure corresponding to 100 ppm ethanol in silicone oil at room temperature was 4.5×10^{-2} torr. These partial pressures decrease less than 1% during the time required to perform a single analysis. Since a measurement of partial pressure is included in each frontal

analysis experiment, long-term depletion does not cause significant error.

A diagram of the apparatus currently employed for studies of sorption of volatile flavor compounds is shown in Fig. 3. A number of refinements have been made to simplify the measurement procedure and provide control of experimental variables. Pre-purified nitrogen was further purified by the molecular sieve column (MS). Flow to the saturators was regulated and stabilized by a dual flow controller (FC). Two saturators (S_1 and S_2) were arranged symmetrically to prevent pressure surges during stream switching and to equalize the system dead volumes during passage of pure carrier and sample gas through the column. To further minimize switching transients, needle valve, V_4, was adjusted to provide a pressure drop equal to that of the column. Flow rate of the inactive stream may be measured at the outlet of V_4 and changed if necessary while the other stream is connected to the column portion of the instrument.

V_1, V_2 and V_3 are low volume valves (Perkin-Elmer Corp., Norwalk, Connecticut) employed for gas stream switching. Normal gas flow is through the column, C, containing from 0.5 to 10 g of solid material, and flame ionization detector. Alternatively, the stream can be diverted through needle valve V_5 (set to provide a pressure drop equal to that of the column) to the detector for checking sorbate concentration. The two injection ports (I_1 and I_2) were employed for introduction of small volumes of methane allowing measurement of interstitial volume, V_g.

Temperatures of column and saturators were controlled by use of appropriate cryostat baths. We employed room temperature (23°C), 0°C (ice water), and −17°C (frozen ethylene glycol slurry) in our preliminary studies.

The sorbent was purged overnight with pure nitrogen to remove volatile impurities and water. After this treatment, one of the saturators was filled with a dilute solution of sorbate in silicone oil and placed in the constant temperature bath. Saturator effluent was monitored by the detector until a constant concentration was attained, usually 10 to 15 min. The sample saturator output was then vented to the atmosphere through V_4 while pure carrier was passed through the column. When a steady baseline was observed, V_1 was switched rapidly and pure nitrogen was replaced by sample gas.

After the frontal curve reached a plateau, indicating that equilibrium had been attained, V_1 was again switched and desorption begun. During the time required for desorption, the temperature of the sample saturator can be changed to establish a new partial pressure of sorbate for the next isotherm point. Since the new partial pressure can be computed from the height of the frontal curve, it is not necessary to measure it each time a change is made. The procedure was repeated until the number of points required to establish the isotherm had been measured.

RESULTS AND DISCUSSION

A FRONTAL ANALYSIS curve for hexane on 5.8 g of Whatman CC-31 cellulose is shown in Fig. 4. Column temperature was 0°C; the saturator was maintained at

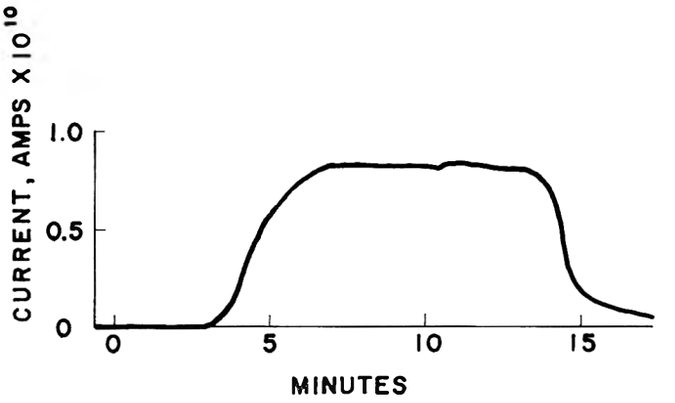

Fig. 4. Example of frontal analysis curve. Sorbate, hexane, saturator chamber (50 ppm) at 0°C; sorbent, Whatman CC 31 microcrystalline cellulose powder, 5.8 g, column at 0°C; nitrogen flow, 33 ml/min; chart speed, 0.5 in./min.

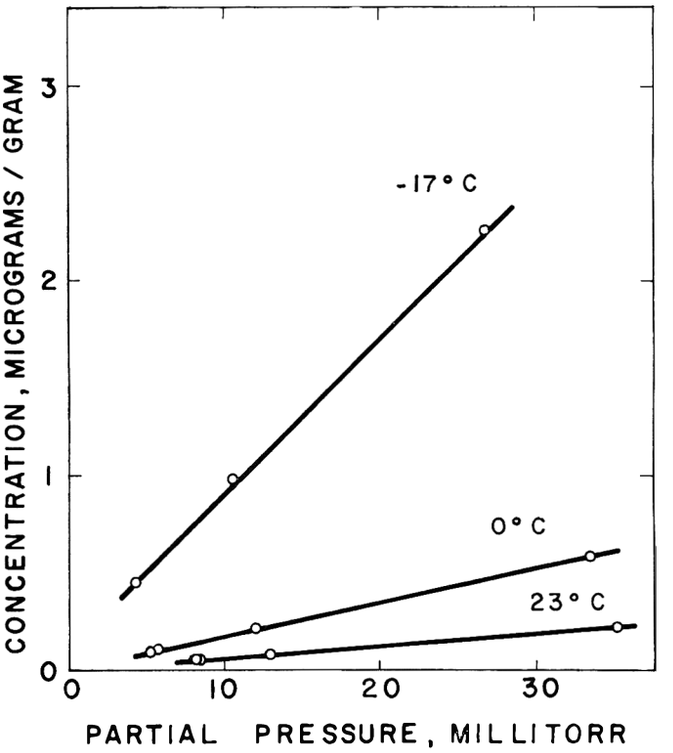

Fig. 5. Sorption isotherms of hexane vapor on cellulose (5.8 g column).

0°C. The height of the curve represents a partial pressure of 12.2 millitorr and the amount sorbed was 0.23 μg. Both adsorption and desorption are shown in this curve. The slight increase in signal level when V_1 was switched was probably due to a small difference in flow rate between the two gas streams.

Portions of isotherms for low concentrations of hexane on 5.8 g of cellulose at three different temperatures are shown in Fig. 5. The number of points was limited by the inconvenience of changing saturator temperature in the present apparatus configuration. Addition of automatic temperature control baths should improve flexibility. The point corresponding to hexane partial pressure of 8.6 millitorr on the 23°C isotherm represents the lower limit of

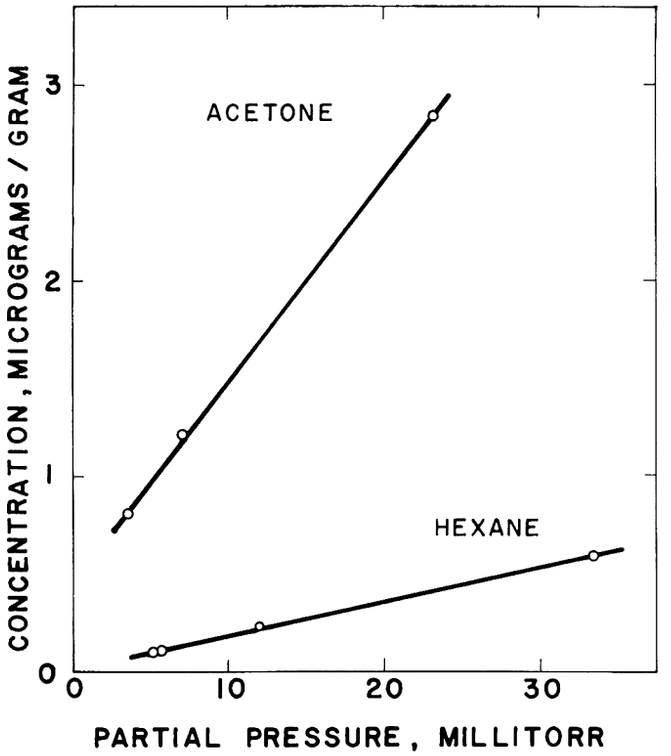

Fig. 6. Comparison of 0°C sorption isotherms of hexane and acetone vapors on cellulose (5.8 g column).

reliable measurement for the present instrument configuration. Measured retention time was 2.4 min, approximately twice the retention time of methane on the same column. The corresponding concentration was 56 parts per billion.

Fig. 6 shows a comparison of adsorption of hexane and acetone on microcrystalline cellulose at 0°C. Sorbate activities do not differ greatly. At 15 millitorr partial pressure, activity of hexane (p/p_o is 3.3×10^{-4} while for acetone, p/p_o is 2.2×10^{-4}. However, the amount of acetone sorbed is 7.5 times greater than that of hexane. This result emphasizes the need to study interactions of a variety of volatile food components with non-volatile constituents to formulate reasonable concepts of the mechanisms of retention of volatile compounds in dehydrated food systems.

The methods described provide direct measurement of interactions of volatile with non-volatile food constituents. We believe these measurements can contribute to our understanding of factors affecting retention of flavor components during dehydration and storage of foods.

LIST OF SYMBOLS

(Subscripts ads, c, s, and C refer to adsorbate, carrier gas, adsorbent, and column, respectively.)

c	concentration of volatile compound $\left[\frac{g}{g}\right]$.
F_R	volumetric flow rate of carrier gas at room temperature $\left[\frac{ml}{min}\right]$.
f_p	correction factor for compressibility of carrier gas.
m_{ads}	mass flow rate of adsorbate $\left[\frac{g}{min}\right]$.
m_c	mass flow rate of carrier gas $\left[\frac{g}{min}\right]$.
MW_{ads}	molecular weight of adsorbate.
MW_c	molecular weight of carrier gas.
p_i	pressure at column inlet.
p_o	pressure at column outlet (atmospheric).
p_{ads}	partial pressure of adsorbate [torr].
T_C, T_R	column and room temperature, respectively [°K].
t_r'	apparent retention time, measured from inert gas peak [min].
t	corrected retention time [min]; $t = f_p \times t_r'$.
w_{ads}	amount adsorbed [g].
w_s	amount of adsorbent in the column [g].
ρ_c	density of carrier gas at column temperature $\left[\frac{g}{ml}\right]$.

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Ms. rec'd 1/22/68; revised 3/18/68,; accepted 6/12/68.

We are grateful to Mrs. Susan Udin for her contributions to the experimental work. This work was supported by NASA and M.I.T. Center for Space Research (NsG-496) and a USPHS Training Grant UI-01033 (formerly ES-00022) from the National Center for Urban and Industrial Health.

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Polycyclic Hydrocarbon Composition of Wood Smoke

SUMMARY—Eleven polycyclic hydrocarbons derived from predominantly hard maple sawdust smoke have been separated and identified. The hydrocarbons were isolated and separated stepwise by a combination of liquid-liquid extraction, chromatography on silicic acid, thin-layer chromatography with acetylated cellulose powder and chromatography on aluminum oxide. They were characterized by ultraviolet and fluorescence studies on the fractions thus obtained from the aluminum oxide column. The polycyclic hydrocarbons found in the hardwood sawdust smoke include naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, 1,2-benzanthracene, chrysene, 3,4-benzopyrene and 1,2-benzopyrene. Analysis of whole wood smoke and the vapor phase obtained by an electrostatic air filter showed only quantitative differences.

INTRODUCTION

OF ALL THE carcinogens, which include organic and inorganic compounds, virus types and high energy radiation, the polycyclic hydrocarbons may be the most abundant in the human environment. Numerous reports have been published on the polycyclic hydrocarbons, particularly 3,4-benzopyrene in cigarette-, cigar-, and tobacco-smoke condensate (Bently *et al.*, 1958; Campbell *et al.*, 1957; Cooper *et al.*, 1955; Lindsey, 1959; Lyons *et al.*, 1957; Van Duuren, 1958; Van Duuren *et al.*, 1958) and in polluted air (Dubois *et al.*, 1964; Grimmer, 1966; Katz *et al.*, 1964; Sawicki *et al.*, 1962a; and Sawicki *et al.*, 1962b).

Much less attention has been given to the analysis of foods for polycyclic hydrocarbons. Grimmer (1966) reported that his analyses, carried out for the first time with vegetable and grain samples, showed by comparison with other food stuffs a surprisingly high content of polycyclic hydrocarbons. The content appeared to depend on the field location, whether from heavily industrialized or industry-remote regions, pointing out that the vegetables and grain become polluted with hydrocarbons through air dust and soot.

Lijinsky *et al.* (1964) identified many polycyclic hydrocarbons in charcoal-broiled meat. Grimmer (1966) found little 3,4-benzopyrine in roasted and grilled meat. Bailey *et al.* (1958) analyzed Icelandic smoked fish for their content of various polycyclic hydrocarbons. The only major carcinogen found was 3,4-benzopyrene, its quantity appreciable in the heavily smoked fish. In contrast, Grimmer (1966) found that ordinary smoked foods (smoked fish, ham and sausage) had a small amount of 3,4-benzopyrene.

No extensive analytical work has been done on wood smoke *per se* in relation to polycyclic hydrocarbons. Reports have been made on the measurable levels of 3,4-benzopyrene in wood smoke or wood tar (Beránková *et al.*, 1953; Hollenbeck *et al.*, 1963; Tilgner, 1958a,b). Hollenbeck *et al.* (1963) could identify 3,4-benzopyrene even in a smoke flavor solution, called "Char Sol," which was prepared by absorbing the vaporous components of wood smoke in water and removing all of the insoluble materials. The smoke solution they prepared, however, cannot be considered as the true vapor phase of wood smoke.

There have been no simple methods developed for the analyzing of various polycyclic hydrocarbons in complex samples, such as polluted air, tobacco tars and foodstuffs. The earlier work in this field employed mostly repeated paper chromatography or alumina column chromatography techinques which had been developed by Wedgwood *et al.* (1953), followed by spectrophotometric and/or fluorometric determination. Studies continue on various modifications of chromatographic, spectrophotometric and fluorescent procedures for separating and analyzing 3,4-benzopyrene and other polycyclic hydrocarbons. Recently, the use of gas chromatography has been introduced to separate and analyze many polycyclic hydrocarbons (Cantuti *et al.*, 1965; DeMaio *et al.*, 1966; Lijinsky *et al.*, 1963).

By combining all the improved procedures of extraction, separation and characterization, except gas chromatography, the present study was undertaken to determine the polycyclic hydrocarbon composition of whole wood smoke and the vapor phase.

MATERIALS AND METHODS

Solvents

The solvents used in this study included cyclohexane, iso-octane, benzene, methanol, acetone, diethyl ether, ethanol and toluene. Benzene and diethyl ether were purchased as spectro grade (Eastman Organic Chemicals). Methanol, ethanol, toluene and acetone (reagent grade, Baker Chemical Co.) were redistilled. Cyclohexane and iso-octane (practical grade, Eastman Organic Chemicals) were purified by percolation through activated charcoal followed by distillation (Crosby *et al.*, 1966).

Collection of wood smoke

Wood smoke was generated and collected from the smoke-generating unit described by Porter *et al.* (1965). Both whole smoke and vapor phase were collected with a hot plate temperature of 440 to 460°C. At this hot plate temperature the actual smouldering temperature was around 750–800°C. The vapor phase of smoke was obtained by separating the particle phase from the whole smoke using an electrostatic air filter (Trion electronic air cleaner model 24–102). The filter contained thirty-one 23 × 10¾ in. negatively charged aluminum collection

plates and thirty-two 21¼ × 9¾ in. positively charged aluminum plates.

The plates were spaced 0.3 in. apart. The ionizing section contained ten 0.0065 in. diameter ionizing wires 2 in. apart midway between nine 2½ in.-wide electrodes. The wires and electrodes were 22 in. long. Voltage across the collection plates and in the ionizing section was 7900 volts DC; the current flow was <.01 amperes. Hardwood sawdust, predominantly maple, which could be sifted through ¼-in. but not ⅛-in. mesh screen was used. Moisture content of the sawdust averaged 6.1%.

The smoke was drawn through six suction flasks at a rate of 5–7 L per min by connecting the flasks to a vacuum line (¼ in. I.D.). The first two suction flasks connected to the smoke unit were charged with a layer of cyclohexane with the smoke aerosol bubbling through the solvent (Cooper *et al.*, 1955). The first flask was kept at room temperature and the second one kept in an ice bath to slow the evaporation of cyclohexane. Both flasks had to be recharged periodically. The third flask at room temperature contained glass wool (washed with distilled water and acetone, and dried) to collect the major portion of tars. The fourth, fifth and sixth flasks (empty) were in dry ice-ethanol baths (below −60°C) to collect any hydrocarbons still in the smoke and the evaporated cyclohexane. Smoke from 10 lb of sawdust was collected for both whole smoke and the vapor phase samples.

Extraction

The pale yellow cyclohexane solution in flasks 1, 2, 4, 5 and 6 was separated from a reddish-brown hydrophillic condensate. The hydrophillic phase was extracted twice with fresh cyclohexane. All cyclohexane solutions were combined and reduced to a volume of about 200 ml (Solution 1). The evaporation of all solvents and concentration of all solutions were done, throughout the study by aspiration at about 30°C using a rotating flask apparatus (Rinco) unless otherwise indicated.

The tar collected in the flask walls and bottoms was dissolved by the acetone used to rinse the glass wool and the tubings between flasks. Acetone was evaporated from the solution and the tarry residue was extracted three times with benzene (100 ml each) and twice with methanol (100 ml each) by refluxing for 30 min for each extraction. The tar extract (benzene plus methanol) was concentrated to about 2 ml, and distributed between cyclohexane (100 ml) and 90% methanol (110 ml). The methanol phase was again shaken with fresh cyclohexane (100 ml). The two cyclohexane extracts were combined to form Solution 2. The procedure used for extraction of the tarry residue was adapted from the method of Grimmer *et al.* (1965).

Solutions 1 and 2 were washed with 2N H_2SO_4 (three times), water, 2N NaOH (three times) and water (Cooper *et al.*, 1955), and dried over anhydrous sodium sulfate. The two solutions were then combined and concentrated to about 0.5 ml. The concentrate was dissolved in about 5 ml of iso-octane and again evaporated to about 0.5 ml to eliminate any remaining cyclohexane.

Silicic acid column chromatography

Silicic acid (Mallinckrodt) was washed with acid and activated as described by Grimmer *et al.* (1965). The smoke extract in a small volume of iso-octane was applied to an activated silicic acid column (15 g, 1 × 20 cm) packed with iso-octane. Aliphatic hydrocarbons were eluted first with 400 ml of iso-octane and the aromatic fraction containing polycyclic hydrocarbons was eluted with 150 ml of benzene (Rosen *et al.*, 1955). The aromatic fraction in benzene was concentrated to about 0.5 ml by aspiration, and the remainder of the benzene was carefully evaporated under a weak stream of nitrogen at room temperature. The residue was taken up in a small volume of cyclohexane (about 0.5 ml) for further removal of impurities by thin-layer chromatography (TLC).

Thin-layer chromatography

The aromatic fraction from the silicic acid column was applied by streaking with a capillary pipette on TLC plates (thickness 500 μ) of acetylated cellulose powder (MN cellulose powder 300 G/Ac from Macherey, Nagel & Co., distributed by Brinkman Instruments, Inc.). The plates were developed with ethanol-toluene-water (17:4:4, v/v).

The chromatoplates were coated with a 1:4 (w/v) slurry of the acetylated cellulose and 95% ethanol. The slurry was prepared by blending in a Waring blendor for 1 min. The coated plates were dried at room temperature.

The developed plates were examined under both long (3660Å) and short (2537Å) wavelength ultraviolet (U.V.) light using a Chromato-Vue cabinet (Kensington Scientific Corp.), while plates were still moist. All fluorescent bands below the oily front band were scraped from the moist plates with a zone collector (Brinkman). Polycyclic hydrocarbons were extracted with cyclohexane and methanol, and the extract was filtered through a fine sintered glass funnel to remove the adsorbent, followed by concentration to about 0.5 ml. The remaining solvents were carefully evaporated under a weak stream of nitrogen at room temperature for fractionation into individual components by column chromatography on aluminum oxide.

Alumina column chromatography

Alumina (Fisher Scientific, 100–200 mesh) was washed and activated in the same way as silicic acid and partially deactivated by addition of distilled water. For partial deactivation, the alumina was covered with sufficient cyclohexane to give a supernatant depth of about 0.5 in., distilled water equal in weight to 1.8% of the alumina was added to the alumina-cyclohexane slowly and the mixture shaken thoroughly. The alumina was then equilibrated by allowing to stand overnight.

The extract residue from TLC was dissolved in a small volume of cyclohexane and applied to a partially deactivated alumina column (15 g, 1 × 17 cm) packed with cyclohexane. The column was eluted with successive 100 ml volumes of cyclohexane containing 0, 3, 6, 9, 12 and 15% of diethyl ether. During the elution, the column was protected from light by aluminum foil. Fractions of 15 ml were collected in graduated centrifuge tubes and concentrated to 2–3 ml in a vacuum desiccator connected to an aspirator, at room temperature and in the dark. The final volume of each fraction was adjusted to 3 ml with cyclohexane.

Ultraviolet absorption spectrophotometry and fluorometry

The U.V. absorption spectra of the successive fractions were measured from 240 to 450 mμ by a Beckman DK-2A recording spectrophotometer and compared with those of authentic polycyclic hydrocarbons. For many later fractions, it was necessary to increase the total volume from 3 ml to 6, 9, 12 and even up to 15 ml to reduce the background absorption. The identification was confirmed, where possible, by determining the fluorescence spectra in cyclohexane and H_2SO_4 (for 3,4-benzopyrene) using an Aminco-Bowman spectrophotofluorometer with one centimeter cells. The settings used in the spectrophotofluorometer were: sensitivity 50, slit arrangement No. 2 (where the narrowest slit width is 1/32 in.), meter multiplier (MM) from 0.3 to 0.003 as necessary, and phototube IP 21 (the maximum sensitivity of which occurs at 500 mμ).

With some background absorption still present, the concentration of each hydrocarbon was calculated from the U.V. absorption spectra by the "base line" method (Commins, 1958; Cooper, 1954).

RESULTS AND DISCUSSION

MANY FRAGMENTS of the major wood components, namely, cellulose, hemicellulose and lignin, give rise to numerous compounds in smoke during pyrolysis and oxidation. It would be conceivable, then, that all five steps, i.e., liquid-liquid distribution, washing with acid and alkali, silicic acid column chromatography, TLC on acetylated cellulose powder and alumina column chromatography, were necessary to purify polycyclic hydrocarbons in wood smoke and remove the substances which contributed to high background absorption in U.V. absorption and fluorescence spectra. Even after the third step of purification, silicic acid column chromatography, the smoke extract was still oily and yellow when concentrated to a small volume.

Much of this oily yellow material was removed by the TLC. The scheme of separation used in this study is very similar to that of Grimmer *et al.* (1965) except that we included one more purification step, washing the extract with acid and alkali and that we used TLC on acetylated cellulose powder whereas they employed chromatography on dimethylformide-impregnated paper following silicic acid column chromatography.

For concentration of extracts or evaporation of solvents, caution should be taken not to lose certain polycyclic hydrocarbons (see methods). Several hydrocarbons are markedly volatile in evaporating to dryness under certain conditions. Grimmer *et al.* (1965) found that while evaporating solvent from a 100 ml-round flask at 30°C bath temperature and 12 Torr vacuum on a rotary evaporator, half of the added phenanthrene (5 μg) was volatilized. Anthracene, pyrene, fluoranthene and 1,2-benzanthracene were reported to be slightly volatile, but show a great evaporation loss at a higher bath temperature (50°C). No drying loss was found during evaporation for all other hydrocarbons.

To identify the polycyclic hydrocarbons from the U.V. spectra of the successive fractions, relative height and shape of the peaks, their relative rise and fall, chromatographic sequence, and position must all be taken into account (Wedgwood *et al.*, 1953). Some fractions still showed a high background absorption even after diluting back the fractions to 15 ml, making the identification difficult or impossible. In this case the fraction was rechromatographed on the plate of acetylated cellulose powder, and the fluorescent zones of polycyclic hydrocarbons following the oily front band were again extracted for determining U.V. absorption or fluorescence spectra.

The U.V. absorption peaks which served best for identification of polycyclic hydrocarbons of wood smoke, in the presence of some background absorption and accompanying hydrocarbons, are listed in Table 1. The best peaks of a pure authentic polycyclic hydrocarbon solution were not always the best ones in the smoke fraction containing that hydrocarbon, due to the background absorption by unknown impurities.

Table 1. The U. V. absorption peaks used for identifying polycyclic hydrocarbons in wood smoke.

Polycyclic hydrocarbon	U.V. absorption peak (mμ)[1]
Naphthalene	266, 276
Acenaphthene	289, 321 (300)
Fluorene	300, 272 (261)
Phenanthrene	292, 280, 274
Anthracene	373, 354 (337)
Pyrene	333, 317 (272)
Fluoranthene	286, 357 (281, 276)
1,2-Benzanthracene (Benz(a)anthracene)	287 (299, 276)
Chrysene	267 (319)
3,4-Benzopyrene (Benzo(a)pyrene)	296, 282 (381, 362, 345)
1,2-Benzopyrene (Benzo(e)pyrene)	288, 330, 315

[1] The peaks in parentheses represent those of secondary value for identification.

The hydrocarbons were eluted from the partially deactivated alumina column in the following order: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, 1,2-benzanthracene, chrysene, 3,4-benzopyrene and 1,2-benzopyrene. Upon eluting the column with cyclohexane containing increasing concentrations of diethyl ether, unknown variables sometimes caused the fractions to elute sooner or later than expected, although the relative location of the fractions was always the same.

The analysis of whole wood smoke and the vapor phase showed no qualitative but quantitative differences. It should be mentioned, however, that some tar was collected, although a very small amount, in the vapor phase. It is not known whether a small portion of smoke tar passed through the electrostatic precipitator or the condensation of hot smoke vapor in the collection flasks, outside the generator, resulted in some tar. Therefore, this small amount of tar was included in analyzing the vapor phase smoke.

In Table 2 the polycyclic hydrocarbons of whole wood smoke and the vapor phase are presented in the relative order in which they were eluted from the alumina column.

Table 2. Polycyclic hydrocarbons in whole wood smoke and the vapor phase.

Polycyclic hydrocarbon	μg/4.5 kg sawdust	
	Whole smoke	Vapor phase
Phenanthrene	51.5	28.4
Anthracene	3.8	1.9
Pyrene	5.5	4.1
Fluoranthene	5.7	4.2
1,2-Benzanthracene	7.0	4.3
Chrysene	2.6	0.3
3,4-Benzopyrene	1.2	0.4
1,2-Benzopyrene	0.9	Trace

Of the three trials of smoke collection and analysis, the results of the last trial are shown in Table 2. Thus, the fraction or tube numbers specified in later discussion denote those of the same last trial. In the first and second trials, the hydrocarbons were found in the whole smoke and the vapor phase in similar proportions as in the last trial. Whole smoke contained a higher quantity of each hydrocarbon than the vapor phase.

In addition to the hydrocarbons shown in Table 2, naphthalene, acenaphthene and fluorene were also identified in both whole smoke and the vapor phase, these being eluted from the alumina column prior to phenanthrene. Among the polycyclic hydrocarbons identified in wood smoke only 3,4-benzopyrene and 1,2-benzopyrene are known to be major carcinogens.

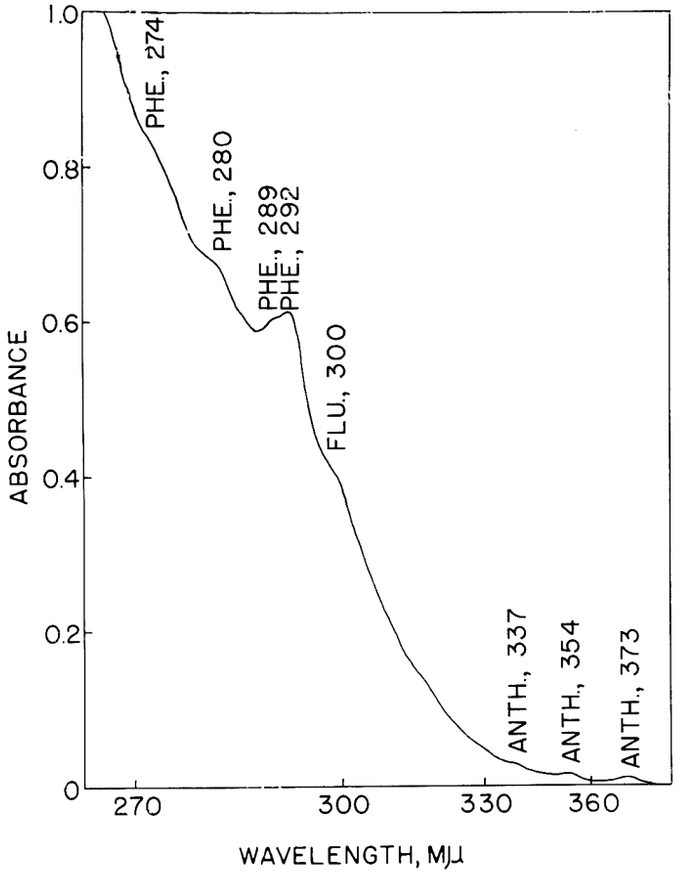

Fig. 1. Ultraviolet absorption spectrum in cyclohexane; No. 5 fraction of the vapor phase (3 ml). PHE: Phenanthrene; FLU: Fluorene; ANTH: Anthracene.

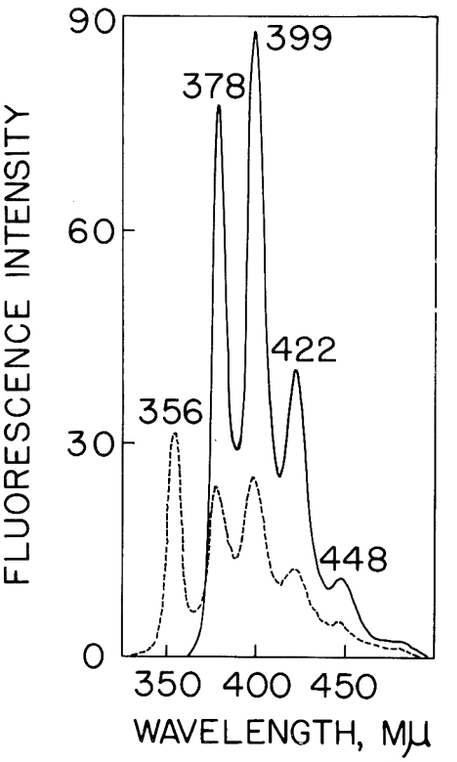

Fig. 2. Fluorescence specrta in cyclohexane at activating wavelength 356 mμ. Pure anthracene, 1 μg/ml, MM = 0.1 (———); No. 5 fraction of the vapor phase, 3 ml, MM = 0.01 (- - - -).

Of the polycyclic hydrocarbon fractions of wood smoke, those of anthracene and 3,4-benzopyrene showed very good fluorescence spectra with very small quantities when proper activation (excitation) wavelengths were used, even in the presence of some impurities and accompanying hydrocarbons. The fluorescence spectrum of every sample fraction, however, showed Rayleigh scattering, which can be easily distinguished from fluorescence. Rayleigh scattering of solvent, scattering from colloidal particles, and scatter from container surfaces occur at the wavelength of activation.

Figs. 1 and 2 show the U.V. absorption and fluorescence spectra in cyclohexane of one anthracene fraction of the vapor phase smoke (No. 5 tube from the alumina column). In this fraction the quantity of phenanthrene (10.8 μg) was much higher than that of anthracene (0.70 μg) as can be seen in the U.V. absorption spectrum of this fraction (Fig. 1). However, the fraction appeared as if it contained only anthracene when the fluorescence spectrum was measured at activation wavelength 356 mμ (Fig. 2). Absence of interference by phenanthrene and fluorene in the fluorescence spectrum of anthracene is due to the differences in activation wavelengths of these compounds. Phenanthrene and fluorene do not have any activation wavelengths beyond 350 mμ, whereas anthracene is highly activated at 356 mμ. Furthermore, anthracene is strongly fluorescent.

A similar situation also applies to 3,4-benzopyrene. Fig. 3 presents the U.V. absorption spectrum in cyclohexane of No. 16 fraction of whole smoke, in which chrysene and 3,4-benzopyrene are present in small concentrations. Fig. 4 shows the fluorescence spectrum in cyclohexane of the same fraction at activating wavelength 381 mμ, which resembles that of pure 3,4-benzopyrene. Sawicki *et al.* (1960) recommended the use of activation and fluorescence spectra of 3,4-benzopyrene in H_2SO_4 solution as an integral part in the detection of this hydrocarbon.

In our study, the presence of 3,4-benzopyrene in any fraction also was confirmed by this procedure. As an example, Figs. 5 and 6 illustrate the activation and fluorescence spectra in H_2SO_4 of No. 16 fraction of whole smoke for 3,4-benzopyrene. Sawicki *et al.* (1960) reported that 3,4-benzopyrene was one of the few hydrocarbons that had an activating wavelength at 520 mμ (at 523 mμ in our

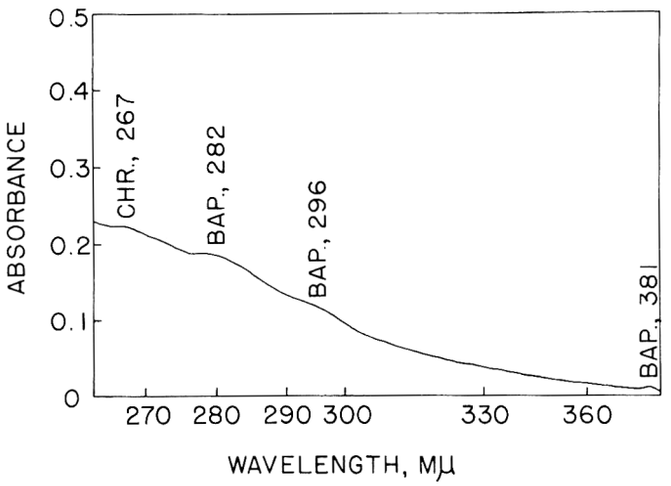

Fig. 3. Ultraviolet absorption spectrum in cyclohexane; No. 16 fraction of whole smoke (9 ml). CHR: Chrysene; BAP: Benzo-(a) pyrene (3,4-benzopyrene).

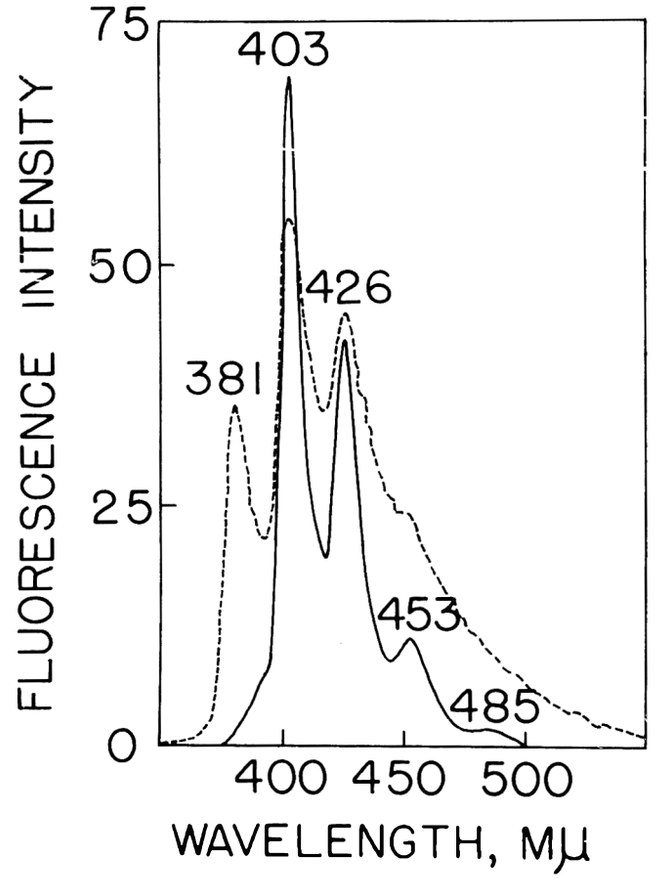

Fig. 4. Fluorescence spectra in cyclohexane at activating wavelength 381 mμ. Pure 3,4-benzopyrene, 2 μg/ml, MM = 0.3 (———); No. 16 fraction of whole smoke, 3 ml, MM = 0.01 (- - - -)

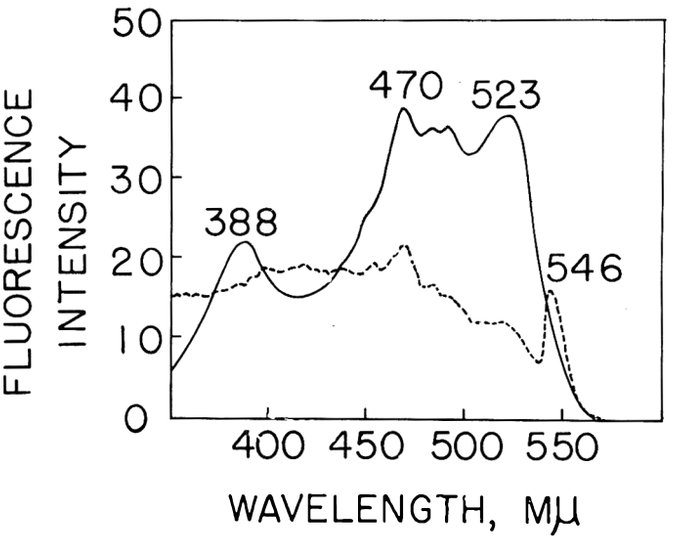

Fig. 5. Activation spectra in H_2SO_4 at fluorescent wavelength 546 mμ. Activation spectrum of pure 3,4-benzopyrene, 5 μg/ml, MM = 0.3 (———); activation spectrum of No. 16 fraction of whole smoke, 3 ml, MM = 0.1 (- - - -).

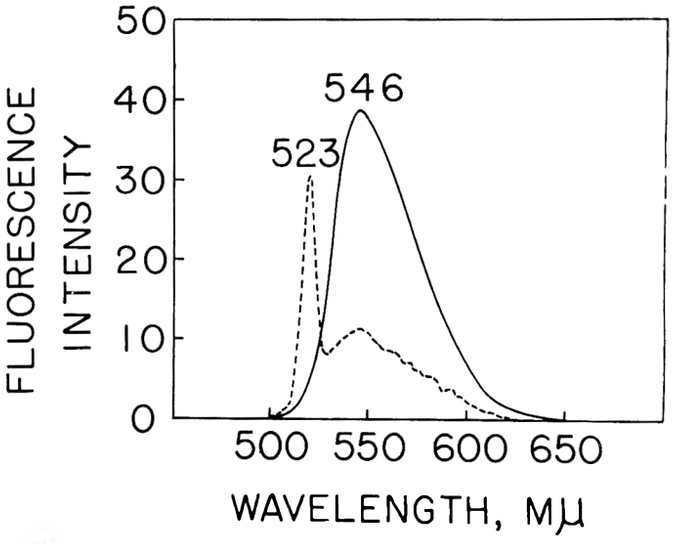

Fig. 6. Fluorescence spectra in H_2SO_4 at activating wavelength 523 mμ. Fluorescence spectrum of pure 3,4-benzopyrene, 5 μg/ml, MM = 0.3 (———); fluorescence spectrum of No. 16 fraction of whole smoke, 3 ml, MM = 0.1 (- - - -).

study) in H_2SO_4. When they compared the fluorescence spectra in H_2SO_4 of the different hydrocarbons, 3,4-benzopyrene was the only one that had a band near 545 mμ. At the activating wavelengths of 470 and 520 mμ, a fluorescence wavelength maximum near 540–550 mμ was obtained if 1% 3,4-benzopyrene was present in the organic fraction. For lower concentrations of 3, 4-benzopyrene, the fluorescence wavelength maximum obtained at activating wavelengths of 470 and 520 mμ was below 540 mμ and above 550 mμ, respectively.

The significance of the amount of carcinogenic polycyclic hydrocarbons in wood smoke for actual carcinogenesis is unknown. However, it should be noted that some tar accumulated on the interior chamber walls of the generator before the smoke reached the collection flasks. If all smoke had been collected, the amount of hydrocarbons given in Table 2 may have been somewhat higher. Also to be noted is that the phenolic fraction of cigarette smoke condensate had a synergistic effect on carcinogenesis, even though the phenolic fraction alone did not produce tumors (Roe *et al.*, 1959; Boutwell *et al.*, 1959). In wood smoke, the concentration of phenols is much higher than that of polycyclic hydrocarbons.

It has been found by many investigators in Europe that the temperature of smoke generation exerts a great influence upon the production of polycyclic hydrocarbons in wood smoke. Tilgner (1958a) suggested that formation of carcinogenic polycyclic hydrocarbons can be minimized by controlling the temperature of smoke to below 300°C.

Miler (1962) produced smoke from completely dry beechwood in a two-stage laboratory smoke generator. The wood was decomposed in a stream of heated nitrogen. The volatile destruction products were oxidized in a heated stream of air in the second chamber of the smoke generator. He tested the wood destruction and oxidation temperatures of up to 425 and 375°C, respectively, for production of 3,4-benzopyrene and 1,2,5,6-dibenzanthracene. Neither carcinogenic hydrocarbon could be found in any of the smokes produced under the above conditions.

On the contrary, Dikun *et al.* (1965) identified 3,4-benzopyrene in the products obtained by fractional pyrolysis of wood at 300–600°C. Wynder *et al.* (1958) reported that, upon pyrolyzing the base-free material by hexane extraction of cigarettes, carcinogens were formed chiefly at temperatures of 800°C and above; below 700°C little carcinogenic material was formed. From all these findings it is likely that the temperature of smoke generation in our experiments, a hot plate temperature of 440–460°C or >750°C of actual smouldering temperature, could be a favorable condition for production of the carcinogenic polynuclear aromatic hydrocarbons.

This study indicates that the use of an electrostatic precipitator in food smoking may reduce the amount of polycyclic hydrocarbons. Tilgner (1958b) also stated that good smoke curing could be attained by applying the gas phase to the exclusion of the particle phase, whereas Sikorski (1965) found no influence of the high voltage processing upon the content of polycyclic hydrocarbons in smoke. Nevertheless, it remains to be determined whether foods smoked with the vapor phase would have the same acceptability and/or storage characteristics as those smoked with whole wood smoke, although Foster *et al.* (1961) reported that the principal flavoring effect of smoking was in the vapor phase.

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Ms. rec'd 10/31/67; revised 2/15/68; accepted 6/11/68.

Journal Article 4202, Michigan Agricultural Experiment Station. This study was supported in part by Public Health Service Research Grant No. UI 00120-04 from the National Center for Urban and Industrial Health.

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The Volatile Alcohols of Ripe Bananas

SUMMARY—The volatile alcohols in ripe bananas were identified in preparation for the study of their biosynthesis. The following 13 alcohols were identified by gas chromatography-mass spectrometry supported in some cases by IR spectral data: ethanol, propan-1-ol, 2-methylpropan-1-ol, butan-1-ol, pentan-2-ol, 3-methylbutan-1-ol, hexan-1-ol, heptan-2-ol, *cis* and *trans* hex-3-en-1-ol, *cis* and *trans* hex-4-en-1-ol, and *cis* pent-2-en-1-ol (tentative). 2-Methylbutan-1-ol was shown to be associated with 3-methylbutan-1-ol in a ratio of 1:200.

INTRODUCTION

ISOAMYL AND ISOBUTYL alcohols and their esters are prominent constituents of the volatiles of ripe bananas (Issenberg *et al.*, 1963; McCarthy *et al.*, 1964; Wick *et al.*, 1966). These constituents are synthesized during ripening and contribute to the aroma and flavor of bananas (McCarthy *et al.*, 1963). In preliminary studies with ^{14}C-labeled compounds evidence was obtained that the branched-chain amino acids valine, leucine and isoleucine are precursors of isobutyl and isoamyl alcohols (Wyman, H., Buckley, E. H., McCarthy, A. I. and Palmer, J. K., unpublished data). However the ^{14}C-labeled peaks were not positively identified and the ^{14}C-activity may have resided in some minor constituent hidden by the main peaks.

For further biosynthetic studies, it was essential to fully identify the alcohols present and to estimate their relative amounts. This information was not available from published data as gas chromatographic separation of the alcohols was in many cases incomplete, largely due to interference of esters.

In the present work the separation and identification of the alcohols has been greatly simplified by prefractionation of the alcohols from other classes of constituents by liquid chromatography on silica gel (Murray *et al.*, 1968a). The separated alcohol fraction was then found to contain relatively few components, which were readily identified by combined gas chromatography-mass spectrometry.

EXPERIMENTAL

Isolation of the volatile concentrate

Bananas, "Cavendish" variety, ripened with ethylene to a full yellow, were peeled and the pulp immediately frozen in liquid nitrogen. The frozen pulp (6 kg) and the analytical grade sodium chloride (2.1 kg) were rapidly minced together into a container where the purée was held below −20°C under an atmosphere of nitrogen. Still protected by nitrogen the purée was slowly admitted in batches to the flask (10 l) of a distillation assembly evacuated to about 1 mm pressure. In so doing it was partly degassed which greatly reduced the problem of foaming during the initial stages of the distillation. The flask was then warmed by a water bath at 20°C and the volatiles distilled off at 8 mm pressure through reflux condensers at 0°C, the dis-

tillate being collected in a liquid nitrogen-cooled trap. During distillation (5 hr) the temperature of the continuously stirred purée rose from −20 to 15°C.

The distillate (60 ml) was saturated with sodium chloride and fractionated by a previously described procedure (Shipton *et al.*, 1966) to separate most of the water. The major part of the volatiles was recovered as an oil from the aqueous distillate (ca. 2 ml) collected in the U-tube separator after saturation with ammonium sulphate. This was combined with the lower boiling fraction from the liquid nitrogen-cooled trap to give an essentially water-free concentrate (0.6 ml) with the characteristic fresh banana aroma.

Group separation of the alcohols. The volatile concentrate (30 μl) was chromatographed on silica gel at 1°C according to the dry column-ascending solvent front technique already described (Murray *et al.*, 1968a). Previous experience had indicated that under the conditions used the major part of the alcohols could be obtained as a single fraction (Fraction 1) entirely free of other classes of constituents, by recovering the volatiles from the first inch of packing measured from the point of application of the concentrate. To account for the remainder of the alcohols Fraction 2 was recovered from the next ½-inch of the packing.

Identification of the alcohols. The technique and conditions used for gas chromatography, combined gas chromatography-mass spectrometry and infra-red spectroscopy have been described in detail (Murray *et al.*, 1968a).

Fractions 1 and 2 above were first examined by gas chromatography on the 67-ft × ⅛-in. FFAP column previously employed. Fraction 1 showed a few major peaks, which were well resolved, and a number of minor components also well resolved. Their mass spectra were then scanned with the same column coupled to the mass spectrometer. Multiple scans were made of the peaks of the six major alcohols to establish that each represented a single compound. Valid spectra were obtained on all but several very minor components, the spectra of which were of little value due to the tailing of major peaks or interference from column phase bleed.

Fraction 2, similarly examined, showed the presence of the same alcohols along with several aldehydes and ketones. However certain of the minor alcohols of Fraction 1, notably heptan-2-ol and two hexenols, were present in higher concentration and this permitted their mass spectra to be recorded completely free of interference from phase bleed.

Fraction 1 was re-examined by GC-MS using a coupled capillary column (1000 ft, 0.030 in. I.D.) coated with Carbowax 1540. The superior resolution of this column allowed stronger spectra to be obtained particularly of the four hexenols.

Separation of 2-methyl and 3-methylbutan-1-ol

The major alcohol peak identified as 3-methylbutan-1-ol was examined for the presence of the unresolved 2-methyl isomer (active amyl alcohol). Fraction 1 was gas chromatographed on a 6 ft, ¼ in. Carbowax 20M column, and the 3-methylbutanol peak was trapped and transferred to a 5% Carbowax 200 column which had previously been used to completely resolve the two isomers (Singer, 1966; Murray *et al.*, 1968b).

Table 1. Alcohols of ripe bananas.

GC Peak No.	GC Retention t_R (3-methylbutanol)	MS Evidence[3]	IR Evidence	Amount rel. to 3-methylbutanol	Conclusion or comment
1	0.402	—	—	trace	Unknown
2	0.441	—	—	trace	Unknown
3	0.469	+	Complete spectrum	330	Ethanol
4	0.570	—	—	0.05	GC retention butan-2-ol
5	0.598	+	—	4.2	Propan-1-ol
6	0.682	+	Complete spectrum	106	2-methylpropan-1-ol
7	0.737	+	Complete spectrum	54	Pentan-2-ol
8	0.804	+	Prim. OH, CH_3, CH_2	26	Butan-1-ol
9	0.888	m/e 86, M^+	—	0.15	MS suggests a pentanol—not pent-1-en-3-ol
10	1.00[2]	+	Complete spectrum	100	3-methylbutan-1-ol with 0.5% 2-methylbutan-1-ol
11	1.16	—	—	0.15	GC retention pentan-1-ol
12	1.20	m/e 86,68,67	—	0.3	MS suggests a pentenol
13	1.26	—	—	0.05	Unknown
14	1.49	+	—	0.6	Heptan-2-ol
15	1.53	MS weak	—	0.4	*cis*-pent-2-en-1-ol (tentative)
16	1.74	+	Prim. OH, CH_3, CH_2	6.4	Hexan-1-ol
17	1.84	+	—	0.4	*trans* hex-3-en-1-ol
18	2.03	+	—	0.1	*cis* hex-3-en-1-ol
19	2.21	+	—	0.15	*trans* hex-4-en-1-ol
20	2.39	+	—	1.1	*cis* hex-4-en-1-ol
21[1]	3.62	—	—	0.15	Unknown

[1] No further significant peaks up to a t_R = 7.20.
[2] Retention time = 42.3 min.
[3] + Denotes agreement of MS with those of authentic specimens.

RESULTS

THE RESULTS are summarized in Table 1. Twelve alcohols have been identified by the agreement of their mass spectra and gas chromatographic retention values with those of authentic compounds examined under identical conditions. Infra-red evidence supports the identification of the six most abundant alcohols. The estimate of the relative amounts of the alcohols was calculated from the gas chromatographic peak areas of Fraction 1 and does not therefore account for the smaller amounts of alcohols in the mixed Fraction 2.

Of the remaining minor and trace components cis-pent-2-en-1-ol has been tentatively identified while fragmentary mass spectral evidence suggests that two other pentenols are present. Retention values of two trace components correspond to those of butan-2-ol and pentan-1-ol.

The gas chromatographic examination of the 3-methylbutan-1-ol peak on Carbowax 200 indicated the presence of 2-methylbutan-1-ol as a small, well-resolved peak of the correct retention value. Its mass spectrum could not however be recorded due to the excessive bleed rate of the phase but since it has correct retention values on two phases the evidence for its presence is strong. A ratio of the 2 methyl/3 methyl of 1:200 was calculated from the relative peak areas.

DISCUSSION

OF THE 10 saturated alcohols previously reported in bananas (Issenberg *et al.*, 1963; McCarthy *et al.*, 1964; Wick *et al.*, 1966) only 2-methylpropan-1-ol (isobutyl alcohol), butan-1-ol and 3-methylbutan-1-ol (isoamyl alcohol) appear to have been rigorously identified. The occurrence of these alcohols has been confirmed and in addition the following have been identified in "Cavendish" bananas: ethanol, propan-1-ol, pentan-2-ol, hexan-1-ol, heptan-2-ol, *cis* and *trans* hex-3-en-1-ol, *cis* and *trans* hex-4-en-1-ol.

The presence of the hexenols is of considerable interest. The low relative concentration of *cis* hex-3-en-1-ol is noteworthy in view of its widespread occurrence, often as a major component of volatiles from plant sources. The hex-4-en-1-ols appear to have been identified for the first time as naturally occurring. The possibility that they are artifacts from the isomerization of the 3-enols is unlikely, since parallel work on the alcohols of green peas (Murray *et al.*, 1968b) performed in an identical manner, showed no evidence for the presence of the 4-enols, even though *cis* hex-3-en-1-ol was one of the major alcohols present. It is clear that the identification of the hexenols and other minor components would have been much less clear cut, or impossible, if the alcohols had not been separated from other classes of constituents.

Another interesting finding is the low concentration of 2-methylbutan-1-ol relative to 3-methylbutan-1-ol since the 2-methyl isomer usually forms an appreciable proportion of the "isoamyl" alcohol complex isolated from other sources (Singer, 1966; Murray *et al.*, 1968b). In the alkan-1-ol series pentan-1-ol was indicated only as a trace amount while members above C_6 were clearly absent. Under the conditions of isolation alkan-1-ols up to C_{10}, if present, would have been recovered in the concentrate. The C_5 and C_7 chain lengths are represented as 2-anols.

The results of this work make it possible to proceed with biosynthetic studies. Preliminary separation of the alcohols as described gives a clear-cut alcohol fraction which can be readily resolved by gas chromatography and few problems should therefore be encountered in locating the ^{14}C-label on an alcohol. The work could readily be extended to the examination of the ^{14}C-labeling on the ester and carbonyl constituents present in other fractions obtained from the chromatography of the volatiles on silica gel.

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Ms. rec'd 2/26/68; accepted /31/68.

The authors gratefully acknowledge the gift of specimens of *cis* and *trans* hex-4-en-1-ol from Dr. E. Honkanen, Biochemical Research Institute, Helsinki.

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Petals of Aztec Marigold, Tagetes erecta, as a Source of Pigment for Avian Species

SUMMARY—The deposition of the pigments of Aztec marigold petal was determined in the egg and tissue of laying hens fed 33–66 and 99 mg of the pigments per kg of low pigment diet. Thirty-three mg pigment per kg of feed produced yolk color which is considered acceptable to the consumer. With an increase in the amount of pigment in the feed, color deposition in the organs increased, but efficiency of utilization was lowered.

INTRODUCTION

THE LOW ENERGY content of certain pigmenting feed ingredients have made it necessary to eliminate or drastically reduce the amount of them used in poultry feeds. This fact necessitates exploration of concentrated sources of pigments that are readily utilized by poultry. The consumers in various regions of the United States show a preference for adequately pigmented broilers and egg yolks and thus the pigmentation of broilers and egg yolks has become an economically important factor.

Palmer (1915) showed that the yellow color of chicken skin is imparted by the xanthophylls present in certain feedstuffs. Most xanthophylls have no nutritive value and are deposited or stored in different organs of the body. Palmer *et al.* (1919) and Peterson *et al.* (1939) showed that xanthophylls are deposited in the body fat, shanks, skin and egg yolk of laying hens. Consequently, pigmentation is a problem of supplying sufficient xanthophyll in the feed. However, providing a satisfactory pigment source for use in poultry feed is still a problem and continued investigation for economical pigmenting agents is necessary.

Aztec marigold *(Tagetes erecta)* is a bright yellow flower widely cultivated in Mexico and the United States for decorative purposes. Analytical studies by Alam and co-workers in 1967 showed that the Aztec marigold contains a large amount of xanthophylls. The present study was undertaken to evaluate the utilization of these pigments by the avian species.

PROCEDURE

AT THE START of the experiment, white leghorn pullets reared on a practical type diet for 27 weeks were placed in individual, raised, wire cages. The hens were then put on a low-pigment milo diet (Table 1). After 3 weeks on the milo diet the hens were distributed into four groups of six hens each. The distribution was on the basis of egg production so that all groups had approximately equal pre-experimental production.

At the start of the experimental period, hens in group I were fed the same low-pigment milo basal diet. The remaining three groups (II, III and IV) were fed the basal diet containing 33, 66 and 99 mg/kg, respectively, of Aztec marigold petal pigments (dehydrated Aztec marigold petal meal obtained from Special Nutrients, Bay Harbor, Fla.). No caloric or protein adjustment was made in the diet for this addition of marigold meal since the total weight of the petals did not exceed 50g/25kg of diet at the highest level fed.

At the end of the 3 week experimental period the eggs were collected and blood samples were collected by heart puncture from all birds for estimation of xanthophyll content. Abdominal adipose tissue, lived tissue and a skin sample from the breast area were collected for analysis.

Serum carotenoids, yolk and adipose tissue were analyzed by the method of Kimble (1939). Liver was analyzed by the method of Ames *et al.* (1954) and skin was analyzed by the method of Wilgus (1954).

RESULTS AND DISCUSSION

DEPLETION OF THE PIGMENT content of tissue and eggs of laying birds was adequately obtained by feeding a low-pigment milo diet. The feeding of 33 mg of pigments per kg of diet maintained what was considered a normal range of pigments (25–30 mcg/gm) in the egg yolk. The pigment content of the yolks and other organs increased as the amount of the pigment in the diet was increased. However, when graded levels of pigment from Aztec marigold were added to the feed, a diminishing efficiency in the efficiency of utilization was obtained (Fig. 1). These findings suggest that when added to a low-pigment diet, a

Table 1. Composition of low-pigment basal diet.

Ingredient	Percent
Milo	73.5
Soybean oil meal (44%)	15.0
Fish meal	5.0
Oyster shell	3.5
Pollyphos	2.25
Vitamin mix[1]	0.5
Salt	0.25
Total	100.00

[1] The diet was supplemented with the following vitamins and minerals per kg: 4.4 mg riboflavin, 11 mg D-calcium pantothenate, 27.5 mg niacin, 13.2 mcg Vit. B_{12}, 8800 I.U. Vit. A, 2200 I.C.U. Vit. D_3, 550 mg choline chloride, 2.2 mg Menadione sodium bisulfite, 5.5 I.U. Vit. E, 11 mg santoquin (antioxidant), 220 mg ZnO, 1100 mg $MnSO_4$, H_2O.

Table 2. Deposition of marigold pigments in organs of avian species.

Pigment supplement to basal diet	Egg yolk mcg/g[1]	Liver mcg/g[1]	Serum mcg/g[1]	Adipose tissue mcg/g[1]	Skin mcg/cm²[1]
None	4.0 (0.2)[2]	2.1 (0.3)	not detect.	not detect.	0.03 (0.01)
15 mg/lbs	27.9 (2.06)	16.7 (0.9)	5.3 (0.1)	0.27 (0.03)	0.11 (0.02)
30 mg/lbs	41.3 (3.3)	23.2 (1.8)	7.0 (0.7)	0.41 (0.02)	0.21 (0.01)
45 mg/lbs	56.4 (3.5)	32.1 (1.2)	7.6 (0.5)	0.57 (0.01)	0.26 (0.01)

[1] β-carotene equivalent.
[2] Parentheses represent mean deviation.

smaller quantity of pigments would be utilized more efficiently than a higher level, even though deposition was greater with the higher levels.

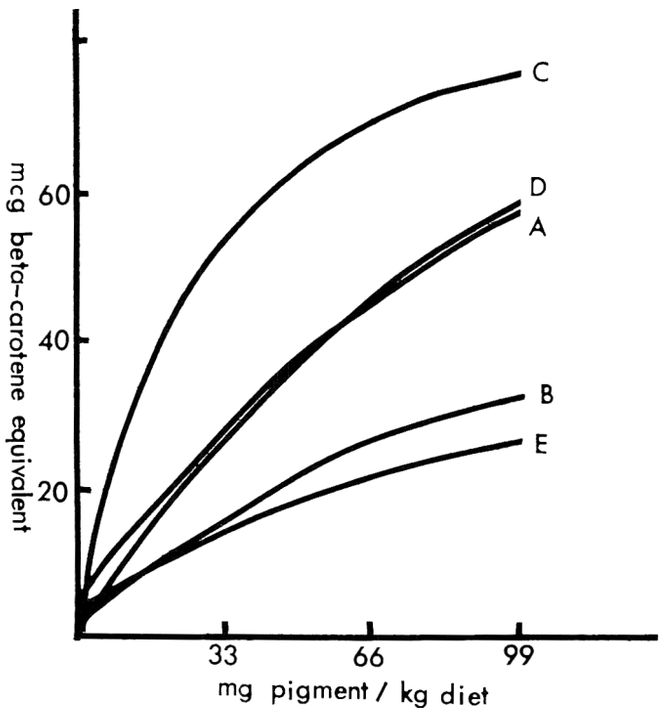

Fig. 1. Deposition of pigments in eggs and various tissue when fed at increasing levels in the diet of the laying hen. A—Egg yolk pigment mcg/g; B—Liver pigment mcg/g; C—Serum pigment mcg × 10/ml; D—Adipose tissue pigment mcg × 10²/g; E—Skin pigment mcg × 10²/cm².

In this study egg yolk, serum, skin, liver and adipose tissue were analyzed to determine pigment deposition. Yolk contained the greatest amount of pigment, followed by liver, adipose tissue and skin. Although serum contained a considerable amount, serum pigments are considered to be in a transitory stage (Table 2).

To determine the effect of storage condition on the stability of the petal pigments, weekly analyses of the mixed diet were carried out. The results indicated that pigment was stable for the entire period of the experiment. The experiment was carried out during December when the temperature range was 30–70°F.

The experiment strongly suggested that Aztec marigold petals can be effectively utilized as a concentrated source of xanthophyll pigments in poultry feed.

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Ms. rec'd 7/3/67; revised 7/18/67; accepted 8/1/68.

A contribution of the Texas Agricultural Experiment Station.

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Role of Flavin Adenine Dinucleotide in Stabilization of Cytoskeleton of Chicken Muscle Cell

SUMMARY—Aging of muscle had previously been shown in our laboratory to increase the propensity of properly treated muscle cell segments to empty on extraction with water. It has been suggested that this emptying is caused by breakdown of a cytoskeleton, and, further, that this cytoskeleton is stabilized by flavin adenine dinucleotide (FAD). Due to the possible relationship of cytoskeletal breakdown to quality changes in meat post-mortem, the role of FAD in the preservation of cytoskeletal structure in chicken breast muscle was studied. No significant differences in FAD decomposition or extraction were found between samples handled in a manner such as to produce very large differences in the extent of emptying, the measure of cytoskeletal breakdown. Similarly, adding FAD to suspensions of muscle cell segments could not inhibit emptying under conditions where the supernatant fraction of a muscle homogenate could. It was concluded that FAD plays no role in the stabilization of the cytoskeleton of chicken breast muscle.

INTRODUCTION

IN SKELETAL MUSCLE, the plasma membrane is of great interest because its invaginations are the transverse tubules (or T-system) that allow the exterior milieu to penetrate deep into the muscle cell causing the whole cell to react rapidly to stimuli. The interior endings of these transverse tubules are located in juxtaposition to terminal sacs of the sarcoplasmic reticulum. It has been suggested that contraction of muscle is initiated by depolarization of the T-system with a concomitant release of calcium from the terminal sacs of the sarcoplasmic reticulum (Constantin *et al.*, 1965).

Several workers have reported on methods useful in the preparation of sarcolemmae from muscle cells (Abood *et al.*, 1966; Kono *et al.*, 1961; McCollester, 1962; Rosenthal *et al.*, 1965; Westort *et al.*, 1966). Methods of preparation have been of two types. In one, strong salt solutions are used to extract the contractile elements (Kono *et al.*, 1961). In an attempt to use a procedure more nearly physiological, McCollester (1962) employed dilute salt solutions and near neutral pH values with an incubation of the cell segments at 37°C or a longer one at 4°C. Modifications of this procedure have been made (Rosenthal *et al.*, 1965; Westort *et al.*, 1966).

The work of McCollester is noteworthy in its scope in the study of the emptying of muscle cells (McCollester, 1962; McCollester *et al.*, 1964, 1966). He observed that the contractile proteins of muscle cell segments, while initially insoluble and capable only of swelling on addition of water, would after proper treatment become soluble. This solubilization would empty the cell segments, taking out most of the intracellular contents. It was suggested that the restriction on the solubility of the contractile proteins was imparted by a "cytoskeleton" that somehow prevented the contractile proteins from behaving as if in free solution. On treatment this cytoskeleton broke down, and the contractile proteins then behaved as if in free solution. It was further suggested that the "cytoskeleton" was composed of elements of the sarcoplasmic reticulum and the Z membrane and that both ionic and enzymic effects were operative in its breakdown (McCollester *et al.*, 1964).

In a later study, McCollester *et al.* (1966) presented evidence that flavin adenine dinucleotide is involved in the stabilization of the cytoskeleton against breakdown in the case of rat skeletal muscle. This conclusion was based upon the observations that:

1. FAD could reverse the enhancement of emptying caused by certain crude enzyme preparations, viz., alkaline phosphatase and L-amino acid oxidase, which were considered to be contaminated by nucleotide pyrophosphatases, and
2. D-amino acid apo-oxidase, which specifically binds FAD, promoted cytoskeletal breakdown, presumably by removing FAD bound to the cytoskeleton, while several other enzymes specific for other nucleotide pyrophosphates had no effect.

We have observed an enhancement of emptying of chicken breast muscle segments with post-mortem aging. Significant effects are found within 3–4 hr (Westort *et al.*, 1966). If there is a correlation between emptying and cytoskeletal breakdown as McCollester has suggested, then it must be presumed that this breakdown can be brought about by the aging process. In chicken breast muscle, significant chemical and physical changes occur at 3–4 hr post-mortem (de Fremery, 1963). The rate and extent of these changes are of prime importance in determining the quality of the product. How these changes are related to cytoskeletal breakdown is unknown, but it is possible that rupture of this cytoskeleton is intimately connected with the post-mortem reactions of muscle. Understanding the causes of stabilization and breakdown of the cytoskeleton may be prerequisite to understanding post-mortem muscle changes. Therefore, we have studied in some detail the role played by FAD in the emptying reaction of cell segments from chicken breast muscle.

EXPERIMENTAL

Preparation of empty cell segments

Consult Westort *et al.* (1966) for details of the preparation of the empty cell segments. Briefly, domestic chickens with no prior fasting were sacrificed by injection of air into the heart. The breast muscle, *Pectoralis major,* was

excised immediately and placed in cold distilled water, and, if the muscle was to be aged prior to homogenization, held at 0–4°C for about 4 hr. After aging, 20 g of muscle was homogenized in 200 ml of cold 0.5 m*M* $CaCl_2$ solution for about 4 sec at high speed in one-half-pint glass jar fitted with a Polytron Model BEW Cutter (Will Corporation) powered by an Osterizer blender. The homogenate was strained through a double layer of cheesecloth and then examined under low power with a light microscope. Homogenates with more than 10% severely disrupted cells or more than 10% clumped cells were discarded at this point. Homogenates that passed the initial screening were centrifuged for 6 min at 600 × G in 250-ml bottles. This and all subsequent procedures were performed at room temperature (25–30°C).

The supernatant fraction was discarded and the residue taken up into four 50-ml centrifuge tubes with a solution of 25 m*M* NaCl–2.5 m*M* histidine, adjusted to pH 7.4–7.5 with tris [tris(hydroxymethyl)aminomethane]. The tubes were centrifuged in a clinical centrifuge for 10–12 sec at maximal speed. This washing procedure was repeated twice. The residue was then taken up in distilled deionized water buffered to pH 7.4–7.5 with tris, centrifuged as before, and the supernatant fraction discarded. The sediment was taken up in the tris-water again, shaken vigorously, and emptied rapidly into about 15 volumes of the tris-water stirred by means of a magnetic stirrer. In one set of experiments where it was desired to allow added FAD to have ample time to react (Table 4), a 30-min −38°C incubation was included prior to the salt washes.

Analytical methods

Flavins were determined after first extracting them from the tissue with cold trichloroacetic acid by a riboflavin fluorescence method (Yagi, 1962). FAD and FMN were purchased from Sigma, riboflavin from Wyeth.

Protein was estimated by the biuret reaction (Gornall *et al.*, 1949).

Measurement of cell emptying and statistical analysis of data

The percentage yield of membrane was assumed in this work, as it has been previously (McCollester *et al.*, 1964), to be indicative of the extent to which the cytoskeleton had been broken down. The data are presented as percentage of membranes following water extraction. To determine these figures samples of each preparation were examined using the low power objective of a light microscope. Differential counts of segments and membranes were made; at least 50 fields were counted for every sample. There was no correlation between length of segment and ability to empty. Therefore, counts were made without regard to size.

The percentage of membranes was calculated by dividing the number of membranes by the sum of segments and membranes and multiplying by 100. Counts were made directly on the extract without isolation of the cell segments. It is felt that this represents a truer picture of emptying since in the isolation procedure, either empty or full segments may be preferentially removed. To test statistical significance of the difference between treatment means, either the F variance ratio or, in case of only two means, Student's "t" test was applied (Steel *et al.*, 1960).

Table 1. Effect of aging on flavins and cell emptying.

Incubation, hr	Total flavin μg/g tissue	FAD μg/g tissue	FAD % of total flavins	% Empty cells
0	0.74	0.72	97	21
4	0.74	0.70	95	57
Standard deviation	0.03	0.03	2.2	6.9

RESULTS

Previous work (Westort *et al.*, 1966) showed that aging whole muscle prior to homogenization enhances emptying. This aging should, therefore, accelerate cytoskeleton breakdown, and if the cytoskeleton is maintained by FAD, then FAD should be liberated or broken down enzymically. To test this theory excised muscle was held in water (0–4°C) for various intervals. The muscle was homogenized in 0.5 m*M* $CaCl_2$ and a portion was assayed immediately for flavins. The rest of each sample was processed using the standard procedure for emptying. These results are presented in Table 1. It is apparent that a marked increase in emptying is not accompanied by a significant decrease in FAD content. If hydrolysis of FAD to FMN is involved in the emptying reaction, then only a very small part of the cellular FAD could be implicated in cytoskeletal stabilization.

A necessary step to produce empty cell segments is washing with salt, e.g., 25 m*M* NaCl–2.5 m*M* histidine, pH 7.4. To obtain good yields, homogenization in the presence of $CaCl_2$ is recommended. Since there appeared to be little breakdown of FAD with conditions leading to emptying (see Table 1), perhaps the FAD present initially was washed out with the salt treatments. Therefore, two treatments were examined on fresh muscle. In one, the muscle tissue was homogenized in cold distilled water followed by washing in water buffered to pH 7.4 with tris. This procedure produced no empty cell segments. In the other treatment the muscle was homogenized in 0.5 m*M* $CaCl_2$ and washed with salt in the usual way (Westort *et al.*, 1966). This produced significant quantities of empty segments.

Total flavins were determined on the whole homogenates, the supernatant fractions from the homogenizations, and the sediments from the first and last NaCl-histidine or tris-water washes. Total flavins were determined because it was earlier found that there was no significant hydrolysis of FAD to FMN with time (Table 1). Results of these experiments are given in Table 2. Although the differences in the percentage of empty cell segments between the two treatments were striking, there were no differences ($P > 0.10$) in flavin content in any fraction between the sample homogenized in 0.5 m*M* $CaCl_2$ and washed in salt and that treated with water (as determined by the "t" test). There was no significant difference in the extent to which flavins were removed from the muscle cell segments by procedures giving a large percentage of emptying and those giving none at all.

Table 2. Extractability of flavins from muscle cell segments by water and salt solutions.

	Homogenized in 0.5 m*M* $CaCl_2$	Homogenized in water
	(μg total flavins per mg protein $\times 10^3$)	
Fraction		
Whole homogenate	4.8	5.0
Homogenate supernatant	6.7	5.3
Sediment from first wash	4.4	4.9
Sediment from last wash	3.3	4.1
% Empty cell segments	63	0

[1] The samples homogenized in $CaCl_2$ were washed in 25 m*M* NaCl–2.5 m*M* histidine, pH 7.4; those homogenized in water were washed in water. Each figure represents the average of five replications.

Table 3. Effect of added flavins[1] on cell emptying.

Flavin added	Total μg flavin added	mμg flavin retained in final sediment per mg protein	% Empty cells
None	0	4	79
FAD	150	45	72
FMN	100	30	73
Riboflavin	60	19	73

[1] The flavin was added after the last salt wash. Each figure in the table represents the average of four determinations.

Since no obvious correlations in favor of the hypothesis that FAD stabilizes the cytoskeleton were observed in the above experiments, a new approach was attempted that could be effective even if the FAD involved in cytoskeletal stabilization was only a small fraction of total cellular FAD. If FAD is effective in stabilizing the muscle cytoskeleton, then it might be possible to prevent emptying by replacing FAD after it has been removed or destroyed. McCollester *et al.* (1966) reported that added FAD could reverse the enhancement of emptying by crude intestinal alkaline phosphates. These workers added the FAD with the enzymes during the incubation step.

We initially conducted our experiments by adding the FAD in tris-buffered water after the final wash and before the extractions with tris-buffered water. The tubes containing the segments and flavins were shaken gently at room temperature for 3–5 min. The samples were centrifuged, and a portion of the pellet removed for flavin analysis. The rest of the segments were then emptied in tris-water by the standard procedure. The results in Table 3 compare the effects with FAD, FMN and riboflavin. No differences in emptying (standard deviation = 6.9%) were found among these or with a control extracted immediately after the salt washes. In later experiments FAD was added during all steps of the procedure but no inhibition of emptying was found.

To confirm that flavin was bound to the muscle segments, another series of experiments was carried out to determine the amount of the retained FAD that was actually bound. To do this the apparent bound FAD was calculated as in the previous experiments, but subtracted from this figure were three corrections necessary to insure that the final figure would reflect only bound, added FAD. The first correction was to subtract the FAD content of a control sample, identical with the experimental sample except that no FAD had been added. The second correction was the "swell" factor, designed to correct for the phenomenon of swelling that accompanies the adding of tris-water to the final pellet. When this tris-water solution is spun down the pellet is seen to increase in volume 2 to 3 times.

The volume of the "swell" is calculated by the difference in volume between the swollen and unswollen pellets. This volume times the μg of flavin per ml in the supernatant fraction gives the total μg of FAD that could be trapped in the "swell." The final correction factor compensates for the FAD in the "free water" of the muscle cell. This "free water" is taken to be water not chemically bound within the cell. The remaining or "bound" water in the cell is bound to chemical constituents.

It was decided that only the FAD associated with the "bound" water fraction should be considered when calculating "bound" FAD. It was assumed that 75% of the water in a muscle cell is free water (Fisher, 1962) and that fresh chicken muscle contains about 72.5% total water (Watt *et al.*, 1963). Thus, about 54% of the weight of the cell is free water, viz., free to exchange with the added FAD solution through the open ends of the muscle segment. This value times the weight of the unswollen pellet times the FAD concentration in the supernatant fraction gave the factor used to correct for the error due to free water. To insure that the FAD added was given every opportunity to bind, it was added at all points during the procedure including 100 μg added during homogenization of the fresh muscle in 0.5 m*M* $CaCl_2$, 100 μg added prior to a 30 min-38°C incubation with shaking, and 800 μg added in the wash solutions. The results of these experiments are presented in Table 4 and show that even with the corrections applied there is greater than a two-fold increase in bound FAD over the control sample. It was concluded that even though a considerable portion of the added FAD does bind, it has no effect on the emptying reaction.

Table 4. Binding of FAD to fresh muscle segments and its effect on emptying.

Total μg FAD added	Total μg apparently bound	Total μg due to "swell"	Total μg in "free" water	Net μg bound	Net μg FAD bound per mg protein $\times 10^3$	% Empty cells
1000[1]	0.470	0.143	0.024	0.169	9	42
0	0.134	.....	.....	.....	4	42

[1] The FAD was added at all steps in the procedure, which included a 30 min —38° incubation period. All figures represent the average of 2 experiments.

DISCUSSION

THE RECOVERY of acid-extractable flavins in muscle cell segments on treatment with trichloroacetic acid appeared to be complete since extensive homogenization and treatment with detergent or ultrasonic vibrations did not cause any increase in yield of flavins. Quantitative recoveries of added FAD, FMN and riboflavin were routinely obtained. The flavin assays had to be completed immediately after sampling since hydrolysis of FAD to FMN takes place in the trichloroacetic acid extracts even when stored at −30°C.

One approach was to take samples of muscle treated in ways that would give very great differences in the amount of emptying and to determine how the various procedures affected the FAD contents of the samples. Aging of excised, whole muscle for 3–4 hr greatly increased the percentage of empty cell segments, especially with extractive procedures that restrict emptying such as low pH and ionic strength (Westort *et al.,* 1966). However, aging had little effect on FAD content. If the muscle segments were homogenized and washed in water instead of by the usual salt procedures, emptying was completely inhibited. Again, there was no apparent change in FAD content of samples treated with this technique versus the usual one where emptying will vary from 60–90%.

These results argue against the involvement of FAD in the emptying reaction. One has to consider, however, the possibility that total FAD is not important but only a very small portion of it not detectable with our experimental techniques. Presumably, only that FAD associated with the subcellular structure comprising the cytoskeleton need be affected to produce the results observed. McCollester *et al.* (1964) suggest that the sarcoplasmic reticulum and the Z line system form the cytoskeleton.

Although distribution data for FAD in the muscle cell are not available, it has been reported that the microsomal fraction of liver and kidney contains 7–27% (Kokawa, 1962; Muntwyler *et al.,* 1950) of the total flavins in the cell. Assuming that 10% of the FAD in our muscle is associated with the cytoskeleton, and noting from Table 1 that a 10% variation in the flavin assays is just slightly greater than two standard deviations, we could expect it might be difficult to detect a significant change in cytoskeletal FAD.

Another possibility must be considered. This is that there is little or no change in flavin content of the cell but that there is a redistribution of flavin during homogenization responsible for destabilization of the cytoskeleton. This would be difficult to detect experimentally in the muscle cell due to problems in obtaining good yields of reasonably pure subcellular fractions (Edelman *et al.,* 1965; Kleine, 1965).

To gain additional information a second approach was used. The muscle segments were brought to the point just before the two treatments with tris-water that result in emptying. The FAD was then added to determine if emptying could be inhibited. It could not; other experiments in which FAD was added at all stages also gave negative results. If FAD was stabilizing the cytoskeleton, one could expect to find it associated with the particulate fraction of the cell. It seemed necessary to be certain that the added FAD was in fact bound. This usually simple procedure is complicated in the case of muscle cell segments because the segments cannot be extensively washed with water at this stage to remove excess FAD without causing emptying, and the amount of nucleotide bound to the segments cannot then be ascertained.

Even one treatment with the tris-water causes considerable swelling of the segments. Washing with the NaCl-histidine solution will prevent swelling and emptying. However, if FAD is responsible for cytoskeletal stability and is removed by the salt washes, then it would not be possible to observe an effect of added FAD even if there was one. To solve this problem, assays for FAD were done after one treatment with tris-water (containing FAD) and correcting for the errors caused by swelling and the inability to wash extensively by the techniques described in the previous section. Again the results argue against a role for FAD since emptying was not inhibited even though considerable FAD was bound.

Once again, however, objections can be raised that such negative results do not rule out other possibilities. For example, the breakdown of the cytoskeleton may be irreversible, and even though FAD added at a later time will rebind to the cellular structure, it may no longer have a stabilizing effect. Or it may be that the added FAD binds to subcellular sites other than those involved in stabilization of the cytoskeleton.

No direct evidence for stabilization of the cytoskeleton of chicken breast muscle cell by FAD was found under our experimental conditions. However, conclusions based strictly on negative results are tenuous at best. To obtain positive results, we attempted to find some substances that when added back to muscle cell segments will inhibit emptying under the same conditions where FAD is not effective. The supernatant fractions from both the initial homogenate in $CaCl_2$, and the first NaCl-histidine wash will completely inhibit emptying when added to the muscle segments just before extraction with the tris-buffered water, indicating that some unknown inhibitor is present in these fractions. The significance of this to the present report is that it shows the technique of adding back FAD used in the experiments described here was apparently a correct one and should have prevented emptying if in fact FAD had this capability. The next step, obviously, is to identify the inhibitory factors in the supernatant fractions and to determine if they undergo changes on aging.

The correlation between cytoskeleton breakdown and aging of muscle implies that the one may have important implications in the other. Since the importance of changes in the muscle cell in the early post-mortem period to final meat quality is well established, it is of great significance to understand this disintegration of cytoskeleton that occurs with aging. Under our experimental conditions, FAD appears not to play a role in stabilizing the cytoskeleton of chicken breast muscle.

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Ms. rec'd 1/19/68; revised 7/23/68; accepted 8/1/68.

This work was supported in part by Grant GM-12064 from the National Institute of General Medical Sciences.

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Low Temperature Growth of Salmonella

SUMMARY—Salmonellae, normally grown at 35–37°C, can grow at much lower temperatures. Experimentally, minimum growth temperatures were obtained when salmonellae were grown on the surface of agar in a temperature-gradient incubator over a temperature range of 1.1 to 12.3°C. These minimum temperatures, as determined by visible growth for 7 serotypes, ranged from 5.5 to 6.8°C.

The pattern of survival or growth of *S. derby, S. heidelberg,* and *S. typhimurium* was followed by inoculating the organisms into tubes of broth and incubating the tubes in a polythermostat over a temperature range of 1.1 to 12.3°C. Minimum growth temperature obtained for *S. heidelberg* after 19 days' incubation was 5.3°C. The minimum growth temperature for the same length of time for *S. typhimurium* and *S. derby* were 6.2 and 6.9°C respectively.

The results indicate a growth temperature shift during extended incubation of *Salmonella* at low temperatures. This phenomenon and the low temperature growth capability of *Salmonella* could be significant in foods stored for long periods of time at temperatures above 5°C.

INTRODUCTION

ALTHOUGH *Salmonella* normally have an optimum growth temperature of 37°C, they can survive and even increase in numbers at much lower temperatures. This was discussed in the review of growth of organisms at low temperatures by Michener *et al.* (1964). Differences in minimum growth temperatures were observed for salmonellae in several foods by Angelotti *et al.* (1961). No growth was obtained in custard or ham salad between 40 and 50°F (4.4 and 10°C); however, in chicken á la king, growth of salmonellae occurred at temperatures of 44°F (6.7°C) and above after 4 days' incubation. These authors concluded that growth of salmonellae in perishable foods was prevented when the internal temperature was at or below 42°F (5.6°C). This further confirms the observations of Prescott *et al.* (1936, 1938) that 41°F (5°C) or less was required to prevent the growth of salmonellae in refrigerated foods.

Salmonella have been shown to grow competitively in crab meat at 22°C, but not at 11°C or lower (Matches *et al.*, 1968). However, on English sole, tissue serotypes grew out rapidly at temperatures as low as 8°C even with inoculum levels as low as 10^1 cells/g and in the presence of 10–100-fold higher numbers of saprophytes. At these lower temperatures, the saprophytes greatly influenced the growth of salmonellae. The saprophytes, having a lower optimum growth temperature, grew more rapidly and competed for nutrients more effectively than the salmonellae.

Elliott (1963), using a temperature-gradient incubator, recorded visible growth of *S. typhimurium* on an agar medium after 14 days at 8.0°C. Some growth occurred at a temperature at least as low as 7°C after 9 days; however, the amount was insufficient to be seen by eye.

The purpose of this study was to determine the lowest temperature permitting growth of salmonellae on agar and

in broth. To determine these minimum temperatures of growth, pure cultures of salmonellae serotypes were grown on an agar surface in a temperature-gradient incubator and in broth in a polythermostat over a temperature range of 1.1 to 12.3°C. Over this range, the minimum growth temperature as well as the temperature at which the number of viable cells declined could be measured.

EXPERIMENTAL

Test organisms

Salmonella serotypes *heidelberg,* ATCC 8326; *typhimurium,* ATCC 6994; and *derby,* ATCC 6966, were studied because they were among the serotypes most frequently isolated from human sources (Morbidity and Mortality Reports, 1965). The other serotypes, *S. aertrycke; S. montevideo,* ATCC 8387; *S. newport,* ATCC 6962; and *S. thompson* were available in the laboratory collection.

Inoculum

The inoculum for the polythermostat was standardized by adding 18-hr Trypticase Soy broth (BBL) culture of the organisms to 0.1% peptone water to obtain 55% transmittency at 660 mμ in a Bausch and Lomb spectrophotometer, yielding 10^8 cells/ml. Appropriate serial decimal dilutions for inoculation were prepared in 0.1% peptone water, chilled in an ice bath and added to flasks of chilled sterile Trypticase Soy broth. The chilled suspension was added to 26 × 55 mm vials (12 ml/vial) and held chilled until placed in the polythermostat.

The inoculum for the temperature-gradient incubator was 18-hr Trypticase Soy broth cultures, which were spread on the agar surface by means of a sterile cotton swab.

The minimum growth temperature on the temperature-gradient incubator was determined by visible growth as viewed by oblique lighting.

Temperature-Gradient Incubator

The temperature-gradient incubator consists of a 12″ × 36″ aluminum block 1.5-in. thick, similar to the one described by Elliott (1963). Six longitudinal channels (1″ × 1¼″ × 30″) machined into the block are filled with agar and the surface inoculated with the test organism. The block is chilled at one end by circulating a 50% methanol solution from a reservoir through a hole drilled transversely 1 in. from the end of the block. The temperature at the other end of the block is controlled by circulating water from a reservoir held at the desired temperature with a heating element and a cooling coil through which is circulated the methanol solution from the cold reservoir.

Polythermostat

The temperature-gradient incubator was converted into a polythermostat by placing tubes into the longitudinal channels and filling the channels with 3% agar in water. When the agar had solidified, the tubes were removed and the surface of the polythermostat was covered with Saran wrap to prevent desiccation of the agar during operation. The Saran wrap effectively controlled moisture loss for the duration of the incubation periods of 35 days or more. When the polythermostat had stabilized at the desired temperature, a cross (+) incision was made in the Saran wrap over each cavity in the agar and the chilled inoculated tubes were inserted for incubation.

Enumeration and detection

Salmonellae counts were determined by surface inoculations of Trypticase Soy agar with 0.1 or 0.01 ml volumes of the appropriate dilutions of the medium from each tube in the polythermostat. Plates were incubated at room temperatures (about 22–23°C).

Temperature control

A plexiglass cover extending over the entire aluminum block was used on both the temperature-gradient incubator and the polythermostat. The plexiglas cover and the aluminum block were insulated with 2-in. polystyrene to prevent rapid heat transfer. A partition was placed across the center of the plexiglass cover, which extended half way into the agar in the channels, forming two chambers over the temperature-gradient incubator or polythermostat. Prior to use, the plexiglass cover was wiped with 70% alcohol to reduce contamination and the temperature-gradient incubator was wrapped in aluminum foil and sterilized.

During operation, thermocouples were inserted into the agar in the temperature-gradient incubator and polythermostat, and the temperature was monitored with a six-point Brown potentiometer. The refrigeration unit was operated constantly without cycling, and a temperature fluctuation of only 0.6° was obtained on any area of the block. This fluctuation represents the difference between the minimum and maximum temperatures recorded during the time span of each experiment.

RESULTS

Salmonella SEROTYPES were grown on the surface of Trypticase Soy agar in the temperature-gradient incubator. The minimum growth temperatures were determined after 7 days' incubation by recording the temperature on the temperature-gradient incubator where visible growth could no longer be seen (Table 1). A distinction was made between heavy growth as found on the warm end and light growth as found near the minimum growth temperature. The end of heavy growth was taken as the division between a heavy solid smear of growth and small isolated

Table 1. Growth of salmonellae on agar surface in the temperature-gradient incubator over a temperature range of 1.1 to 12.3°C.

Test organism	Minimum growth temperature	
	Heavy growth °C	Slight growth °C
S. heidelberg ATCC 8326	6.7	5.5
S. derby ATCC 6966	6.8	6.1
S. typhimurium ATCC 6994	6.1	6.1
S. aertrycke	6.7	6.1
S. montevidea ATCC 8387	6.1	5.5
S. newport ATCC 6962	6.8	6.5
S. thompson ATCC 8391	6.7	6.4

colonies. The minimum temperatures obtained for heavy growth varied between 6.1 and 6.8°C, and the minimum temperatures obtained for slight growth varied between 5.5 and 6.5°C.

The growth of salmonellae on the surface of agar in the temperature-gradient incubator was complicated by occasional contamination and also by desiccation of the medium. For experiments of approximately 7 days' duration, the movement of moisture from the warm end and the condensation on the cold end was controlled by the barriers imbedded into the agar and extending up to the cover. For experiments of longer duration, the temperature-gradient incubator was converted into a polythermostat with 15 tubes placed in each channel at 1¼-in. intervals.

During operation, a linear temperature-gradient was obtained across the polythermostat. This temperature-gradient was monitored during operation and a fluctuation of only 0.6°C was obtained at any given point (Fig. 1). As the growth temperature approaches the minimum for an organism, growth presumably takes place only during the upper part of the temperature fluctuations. For this reason, the remaining temperatures given in this paper will be those from the upper part of this range.

Counts were made on all 15 tubes to follow the increase or decrease in numbers of cells during each experiment. Although 15 tubes were used in each channel, the critical temperature zone for *Salmonella* was between tubes number 6 and 10. Very rapid growth was obtained at the higher temperatures in tubes 1 through 5, representing a temperature range between 12.3 and 9.1°C. At the other end of the incubator, no growth was obtained in tubes 11 through 14, representing a temperature range between 4.3 and 1.1°C. A decline in numbers of viable cells was in fact obtained at these lower temperatures.

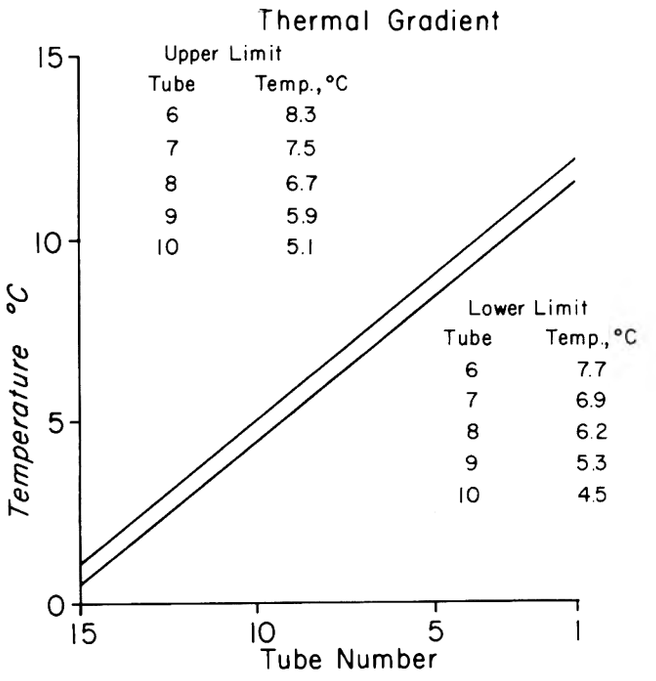

Fig. 1. Upper and lower temperature in culture tubes along temperature-gradient incubator.

The minimum growth temperature and rates of growth of the three serotypes are shown in Figs. 2, 3 and 4. *S. heidelberg* increased in numbers at 5.9°C and above during 35 days' incubation (Fig. 2). At 5.1°C and lower, growth was not obtained and an actual decline in numbers of viable cells took place. At incubation temperatures above 5.9°C, an increase in numbers of cells took place after 5 days, and this increase continued throughout the incubation period. In the tubes incubated at the higher temperatures, the numbers of bacteria increased more rapidly, reaching maximums by about 20 days.

S. derby (Fig. 3) has a higher minimum growth temperature than *S. heidelberg*. Growth was obtained at 8.3°C after 5 days' incubation, but only after 12 days when incubated at 7.5°C. At temperatures of 6.7°C and lower, the cells decreased in numbers throughout the test period.

The minimum growth temperature for *S. typhimurium* (Fig. 4) falls between the minimums for *derby* and *heidelberg*. When the organisms were incubated at lower temperatures, the length of the lags were increased. At 8.3°C, the lag is short, but at the lower incubation temperatures of 7.5 and 6.7°C, the lag is extended to 12 days. This lag is further extended to 26 days when the organisms are incubated at 5.9°C. The slopes of the curves obtained when the organism is grown at 8.3, 7.5, 6.7, and 5.9°C are very similar at all four incubation temperatures even though the lag period increases with a decrease in temperature. The results appear to indicate that a growth temperature shift takes place during extended incubation at low temperatures.

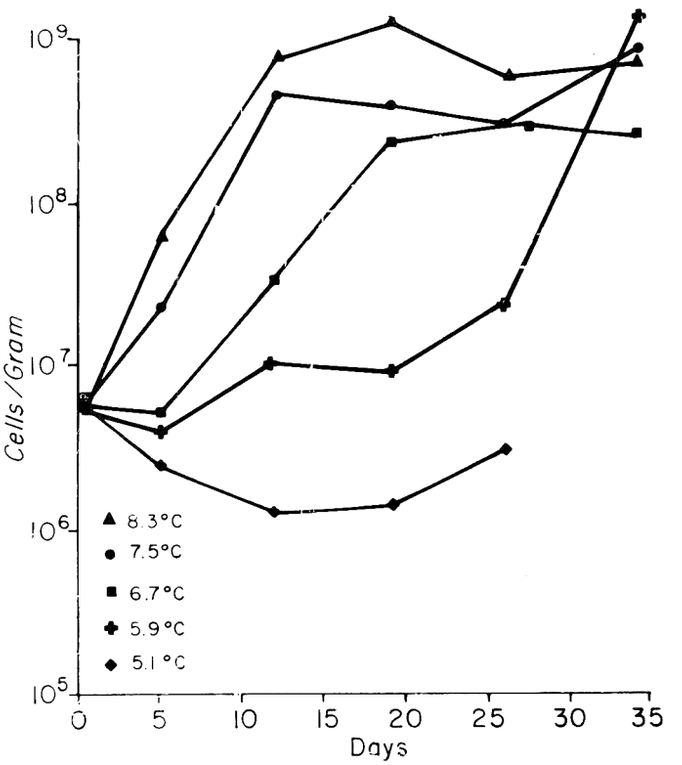

Fig. 2. Growth of S. heidelberg *at low temperatures.*

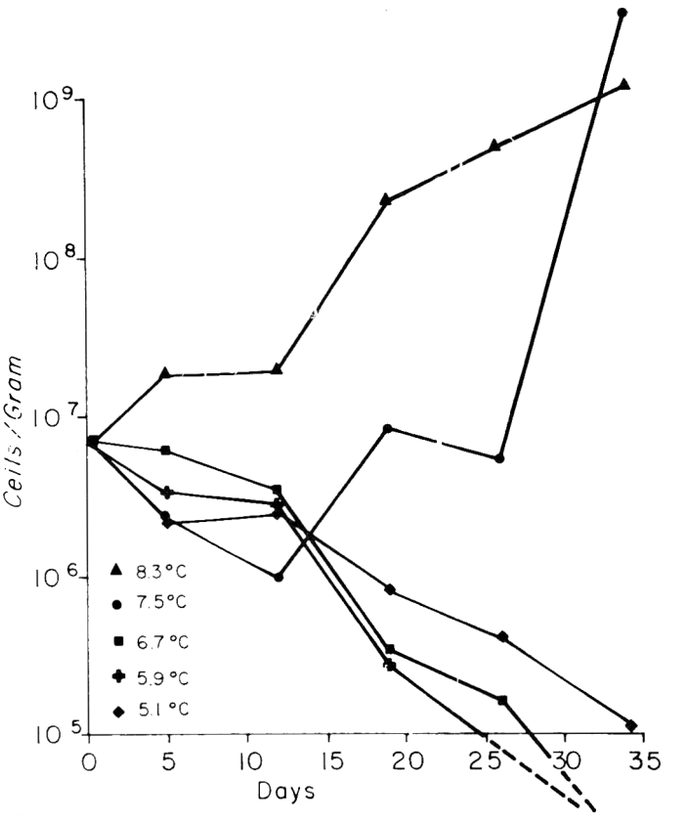

Fig. 3. Growth of S. derby *at low temperatures.*

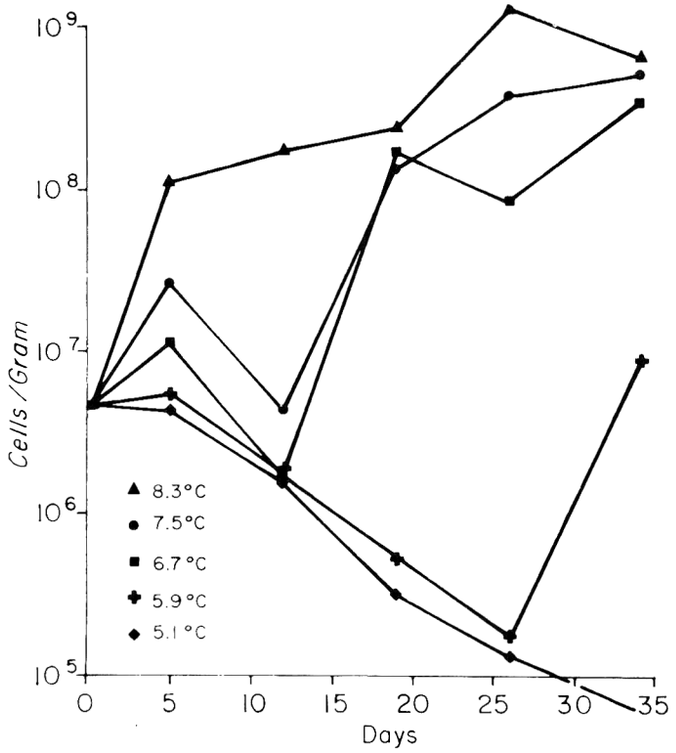

Fig. 4. Growth of S. typhimurium *at low temperatures.*

Table 2. Minimum growth temperature of salmonellae in relation to incubation time.

Time	Minimum growth temperature (°C)		
(Days)	*S. heidelberg*	*S. typhimurium*	*S. derby*
5	7.5	7.5	8.3
12	5.9	7.5	8.3
19	5.9	6.7	7.5
26	5.9	6.7	7.5
34	5.9	6.7	7.5

A summary of the data for the 3 serotypes obtained during five experiments is shown in Table 2. The minimum growth temperature in which a change was recorded is given after 5, 12 and 19 days' incubation. After 5 days' incubation, *S. heidelberg* has a minimum growth temperature of 7.5°C. This minimum decreases to 5.9°C after 12 days, but does not change during the remaining incubation period. *S. typhimurium* has a minimum growth temperature of 7.5°C, which drops to 6.7°C after 19 days' incubation. A similar pattern was observed with *S. derby,* which possessed the highest minimum growth temperature of the 3 serotypes tested. In this case, the shift to a lower minimum growth temperature occurred after 12 days.

DISCUSSION

THE MINIMUM TEMPERATURE at which these salmonellae will grow and increase in number is influenced by the growth medium as well as by competing microorganisms. Salmonellae are capable of growing on seafoods and other foods at 8°C, competing with the normal spoilage bacteria and reaching high numbers. At lower temperatures approaching the minimum for salmonellae growth, psychrotrophic saprophytes can grow very rapidly, reaching high numbers before the growth of *Salmonella* is initiated (Matches *et al.,* 1968). In this manner, the growth of salmonellae appears to be suppressed. When grown in pure cultures on the temperature-gradient incubator and in the polythermostat, salmonellae are able to increase in numbers more rapidly and initiate growth at lower temperatures than when grown in competition with other microorganisms.

The minimum growth temperatures of *Salmonella* are difficult to determine on the agar surfaces in the temperature-gradient incubator. The growth apparent to the eye becomes decreasingly distinct at lower temperatures, as was also reported by Elliott (1963) and fades from heavy confluent growth to small isolated colonies. Special techniques can be used to detect the organisms, such as the reduction of tetrazolium dyes or removal of pieces of agar and making a direct count of the numbers of cells on a given area. These methods are either tedious or not particularly reliable.

The polythermostat is a more precise system that permits enumeration of viable cells in cultures held simultaneously over a range of temperatures. Because of the discontinuous temperature gradient provided by the polythermostat system, it is probably best to use the continuous gradient block first to establish the approximate minimum tempera-

ture and then set the polythermostat gradient to straddle the minimum temperature zone. The growth or decline of the inoculated bacteria populations may then be followed at particular temperatures over quite long periods of time. In this way the duration of the lag phase and the rate of growth can be determined at and near the growth minimum.

The long incubation times made possible by the polythermostat may account in part for the lower minimum temperatures found in this investigation than have been reported by previous authors (Michener *et al.*, 1964). However, similar minima were found in thermal-gradient incubator and polythermostat experiments. Different growth minima were observed with the various serotypes tested, indicating another possible reason for the differences in published reports. The principal reason, however, for the low growth temperatures observed was undoubtedly the absence of a competing flora of phychrophilic saprophytes, which normally tends to limit growth of salmonellae in foods.

As temperatures are decreased towards the minimum, the most obvious effect on the growth pattern of salmonellae is an extension of the lag phase. The rate of increase of population during lag phase growth is very similar down to the minimum. This was most clearly seen in the case of *S. typhimurium*, which showed a very short lag phase down to 8.3°C and then increasing lag periods at lower temperatures. This behavior suggests that some type of metabolic reorganization may be necessary for growth at the lower temperatures. Most probably this is due to a drastic drop in enzyme activity as the minimum temperature of activity of particular enzyme systems is reached.

It has been shown that a shift from a mesophilic enzyme system to a psychrophilic system takes place in a psychrotrophic pseudomonad when the organism is transferred from mesophilic to psychrophilic growth (Bannerjee *et al.*, 1968). The particular system involved in this case is amino acid oxidation, and it has been suggested that low temperature induction of a "psychrophilic" isoenzyme might occur. While it seems unlikely that a frank mesophile like *Salmonella* would possess such a system, it is possible that synthesis of enzymes with higher activity at temperatures below 8°C than the usual *Salmonella* does take place during the extended lag periods.

From the practical point of view, it is important to recognize that growth of *Salmonella* may occur at temperatures below 6°C after a relatively long period of time. Good temperature control is thus particularly important in the case of foodstuffs stored under refrigeration for extended periods. In such cases, food temperatures should be held below 5°C at all times.

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Ms. rec'd 2/19/68; revised 2/26/68; accepted 6/12/68.

Contribution No. 291, College of Fisheries, University of Washington, Seattle, Washington.

This investigation was supported in part by U. S. Public Health Service research grant 5 RO1 EF 00558-02 from the Environmental and Food Protection Division, and by Atomic Energy Commission Contract No. AT(45-1)1549/1730.

Presented at the 26th Annual Meeting, Institute of Food Technologists, Portland, Oregon.

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Dynamic Viscoelastic Characterization of Solid Food Materials

SUMMARY—There currently exists a large demand for objective methods to be used in the evaluation of food texture. A very logical approach to the evaluation of some of the parameters of texture would appear to be the use of engineering parameters for the characterization of mechanical response of materials used in processing or direct consumption. Considering the changeability of living materials, dynamic tests in which information is derived in a relatively short time appear to be highly desirable. Dynamic methods currently available include direct stress-strain measurements, transducer methods, resonance methods and wave propagation. Results from any of these tests may be presented in the form of storage and loss moduli. Interpretation of results is dependent upon the final use of the product. In most cases, a relation between the structural mechanics of the food material and the observed mechanical behavior appears to be desirable both as an aid in quality control and as a guide to the development of synthetic foods.

INTRODUCTION

THERE APPEARS to be little doubt about the need for a set of objective measurements that may be used for the evaluation of the physical properties of food materials. When considering the measurement of an undefined quality such as "texture" it may be necessary to evaluate several different parameters and then combine them in a manner most indicative of the complete texture concept. The authors have previously presented their approach and justification for evaluation of mechanical behavior in terms of well-defined engineering parameters (Morrow *et al.*, 1966). Such an evaluation is obviously not the complete profile of texture, but may afford a better description of some of the constitutive components, namely the mechanical parameters. Other factors have been proposed to be psychological and biological parameters (Mohsenin, 1968).

When evaluating the mechanical behavior of a biological system such as that of most food materials, it is essential that the fundamental nature of the material does not change during the time in which the test is being conducted. Consider, for example, a specimen of fresh muscle undergoing the physicochemical changes that will eventually lead to rigor mortis. If the mechanical response of such a prerigor sample is to be studied, test data must be obtained during a very short time. To obtain a maximum amount of information in this short period, it is desirable to use procedures known as dynamic testing, in which either cyclic stress or cyclic strain is imposed and the remaining quantity (strain or stress) is measured.

In addition to the quantity of information obtained, dynamic testing may be justified from similarity to field conditions. Practically all applications involving the mechanical behavior of food materials are dynamic whether they be mastication in the human mouth or industrial processing such as grinding and mixing. The behavior of a food under these conditions may therefore be best represented by a test that is itself dynamic in nature.

While a large number of dynamic tests have been proposed for conventional engineering materials, only a very few of them have been successfully utilized for food materials. This paper will review a number of techniques believed to be adaptable to foods for the purpose of obtaining viscoelastic response functions.

LITERATURE REVIEW

A PRESENTATION of the reported dynamic studies on engineering materials would be entirely too extensive to be of interest. A number of specific techniques will be discussed under the instrumentation section of this paper. Several good review sources are available that deal with dynamic techniques for materials classified according to their firmness.

Sharma (1965) classifies tests for viscoelastic materials according to the ratio between the wave-length of the vibrating source and the length of the specimen. He also presents some discussion of the accuracy and limitations of the various techniques.

Ferry (1961) has shown various experimental techniques for the study of both "soft" and "hard" viscoelastic solids. His descriptions are too brief to be very useful to experimental design although many of the methods described for "soft" solids would appear to be quite useful for food materials. Eirich (1956) also presents a textbook summary of methods that should be useful to food scientists.

Marvin (1952) discusses the measurement of dynamic properties of rubber based upon characteristic specimen lengths. He also shows methods for interconversion between the commonly used methods of expressing the results of dynamic measurements. Nolle (1948) shows a number of techniques suitable for measuring dynamic properties over the range of 10^{-1} to 10^{5} cycles per second. In a later paper (1949), he illustrates the application of these techniques to various materials.

Silberberg (1965) presents a number of instruments that may be used to study dynamic mechanical properties of polymers and classifies them according to the nature of the results obtained.

In reviewing dynamic studies conducted on food materials, it is necessary to emphasize two major points: (1) Only solid foods are considered, and (2) studies must be amenable to the evaluation of engineering parameters. When these objectives are considered, only a very few studies of interest may be located in the literature.

Drake (1962) performed some of the more recent pub-

lished work on the vibrational properties of foods. His techniques require the shaping of a uniform specimen for which resonant frequencies may be established. Having the complete resonance curves, he shows the manner in which internal friction and stiffness may be evaluated. Virgin (1955) also has used vibration techniques for the purpose of studying the turgor of plant tissues.

Recently, attempts have been made to apply the resonance technique for the evaluation of dynamic properties of fruits and vegetables in either their natural shape or in prepared specimens (Finney *et al.*, 1967; Abbott *et al.*, 1967). If prepared specimens are used it is possible to evaluate both damping and elastic properties of the material. The Nametre Company (Abbott *et al.*, 1967) related the resonant frequencies of natural fruits to their maturity. The workers also showed it is possible to relate the stiffness of fruit tissues to resonant characteristics of the composite fruit. A series of resonance curves was obtained for each specimen to determine which mode of vibration could be correlated to given qualities of the fruit. The square of frequency of these resonances multiplied by the mass of the apple was termed the "stiffness coefficient" of the apple. This is logical because the uniaxial elastic modulus is directly proportional to frequency squared and a constant that is dependent on the specimen's geometry and density.

Nybom (1962) placed soft fruits such as raspberries between the metallic diaphragm of two earphones and measured the intensity of vibrations transmitted through the fruit. Although he did not calculate engineering parameters, his experimental set-up should be amenable to such an analysis if uniform specimens were used.

Lebedeva (1965) has also used vibration transmission techniques to study the dynamic complex shear modulus of animal tissues. In this technique a plane shear wave is excited in a thin, flat tissue specimen and the vibrational phase and amplitude are measured on the surface of the specimen parallel to the shear wave front. As with any wave propagation method, this technique is limited to rather high frequencies of excitation.

Coulter *et al.* (1960) have evaluated the nonlinear passive mechanical properties of skeletal muscle. Their studies used a cam driven device for the direct application of strain. Such a technique had a frequency range of 0.5 to 25 cycles per second, a very interesting region insofar as food materials are concerned.

While a few more dynamic studies have been reported for foods, the above examples are believed to adequately illustrate the types of approaches that have been used. With few exceptions, these studies have not developed well-defined parameters and have therefore afforded something less than the optimum quantity of information from the available data.

EXPERIMENTAL METHODS

Type of testing

The authors have previously presented (Morrow *et al.*, 1966) a discussion of suitable loading patterns for materials when it is desired to derive uniaxial viscoelastic parameters. This manuscript shall discuss only techniques that lead directly to the establishment of uniaxial tensile or compressive moduli. In many instances quite similar techniques and instrumentation may be used to obtain hydrostatic (dilatation) or shear (deviatoric) moduli. The methods presented also apply only to solid food materials although in some instances a similar approach has been used for fluids.

There are several different schemes that may be used for classification of different dynamic testing techniques. One of the simplest seems to be a classification on the basis of the manner in which the measurement is made. Although each of the techniques studies the vibrational properties of the material, the nature of the response is considerably influenced by the magnitude and frequency of the generated motion. Basically the different methods may be classified as follows: (1) direct measurement of stress and strain, (2) transducer methods, (3) resonance methods, and (4) wave propagation.

Direct measurement of stress and strain

If the size of the specimen, the consistency of the material and the frequency range are such that inertia effects may be neglected, direct measurement of stress and strain is one of the most easily analyzed methods of obtaining complex dynamic moduli. Normally this is achieved by measuring both stress and strain as functions of time when the sample is subjected to a sinusoidal deformation.

This method may be used in tension, compression, and alternating tension and compression. The frequency range for this type of measurement is normally continuous over the region from 10^{-6} to 10^{2} cps. This range will probably be considerably reduced unless a high degree of sophistication is available for shielding and isolation of the mechanical and electrical circuits.

A typical arrangement for direct measurement of stress and strain is shown in Fig. 1. This arrangement is similar to that used on muscle by Coulter *et al.* (1960). When using such an arrangement the force exerted by the sample is monitored by a proof ring or load cell connected in series with the driving mechanism. The total deformation imposed on the sample is determined by the amount of eccentricity of the driving cam. Deformation as a function of time may be measured by means of a transducer attached to the driving mechanism.

If the deformations imposed by the system are sufficiently small that nominal stresses and strains may be

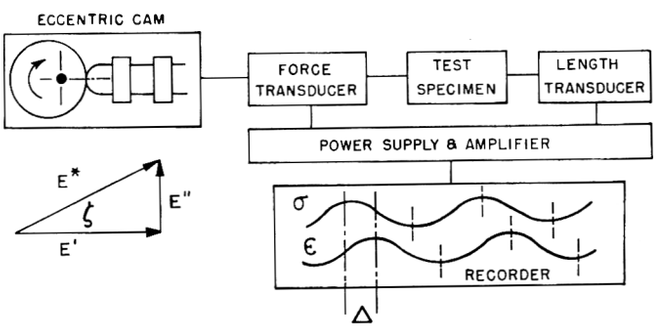

Fig. 1. Block diagram of apparatus for direct measurement of stress and strain.

assumed, interpretation of results is relatively simple. The stress, σ, may be defined as the ratio of load to original cross-sectional area, A. The strain, ϵ, is defined as the ratio of the deformation to the original length of the specimen, l. A complex modulus, E*, may now he defined as the ratio of maximum stress to maximum strain.

$$| E^* | = \sigma_m/\epsilon_m \quad [1]$$

A loss factor, ζ, may be defined by considering the phase difference in radians between the peak stress and the peak strain (Fig. 1).

$$\text{Tan } \zeta = \Delta/T = \Delta \cdot \omega \quad [2]$$

where: Δ = time lag of stress or strain
T = period of imposed strain
ω = circular frequency

Knowing the above quantities, it is possible to describe a storage modulus, E′, related to the elasticity of the material and a loss modulus, E″, related to viscosity or internal damping.

$$E' = | E^* | \cdot \cos \zeta \quad [3]$$

$$E'' = | E^* | \cdot \sin \zeta \quad [4]$$

By plotting both E′ and E″ as functions of frequency a large amount of information may be derived concerning the material behavior. Mohsenin (1968) presents typical curves for both storage and loss moduli.

An alternative method of performing direct stress and strain measurements is through the use of cyclic deformation in a universal testing machine such as the Instron. This method is not compatible with the frequency response of the normal technique, however, because a sawtooth rather than a harmonic loading pattern is used. It should also be possible to use a motion or vibration generator of the form commonly referred to as shakers for generating sample deformation. The difficulty with this approach is that the maximum amplitude would then be a function of frequency and could not be independently controlled.

Transducer methods

In many instances the nature of the material being tested may be such that direct measurements become difficult due to small response levels in soft flaccid specimens. In such a case, transducer methods appear to be necessary although the required instrumentation is quite elaborate. In general, transducer methods are considered applicable in the frequency range from 10 to 10^4 cps, which is termed the low audio and subaudio region.

A technique for measuring mechanical impedance was originally developed by Fitzgerald *et al.* (1953), in which they utilized a pair of matched electromagnetic coils for transmitting and receiving vibrations within the sample. Their original system was intended primarily for measurements in shear although it could have been modified to include uniaxial compression studies. The Franklin Institute later developed a single transducer system, as reported by Keiper (1962), that makes both compression and shear measurements.

The later instrument utilizes a gas bearing for support of the coil in the moving element. A similar coil, which

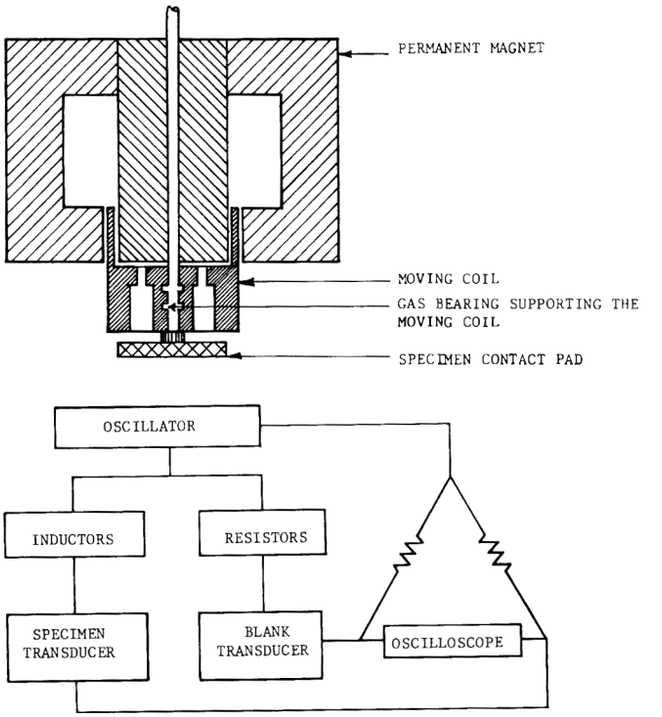

Fig. 2. Schematic diagram of transducer type apparatus for dynamic viscoelastic measurements.

is stationary, is used for electrical impedance matching. A schematic diagram of this apparatus is shown in Fig. 2. Basically this instrument measures the electrical impedance change that occurs when a coil is vibrated against a stationary specimen. The relation between the electrical impedance of the coil and the mechanical impedance of the specimen is of the form

$$Z_{mech} = A \cdot \frac{1}{Z_{mot}} \quad [5]$$

where Z_{mech} = mechanical impedance
Z_{mot} = electrical impedance change
A = system constant.

For uniaxial compression measurements the mechanical impedance may be related to complex modulus by the relation

$$|E^*| = i(2\pi\omega) \cdot Z_{mech} \cdot F \quad [6]$$

where i = complex operator
ω = frequency of moving coil
F = shape factor for the specimen.

The complex modulus may be further divided into storage and loss moduli by transformation moduli similar to those presented by Eirich (1956).

The application of transducer techniques to solid food materials has been quite limited. The Eastern Utilization Laboratory, USDA, Wyndmoor, Pa., owns an apparatus specifically designed for use with biological materials. However, very little is reported for this instrument. Mohsenin *et al.* (1967) have reported some data obtained with this instrument on single corn kernels, but these specimens were sufficiently rigid to make transducer techniques difficult to apply.

Resonance methods

A number of techniques have been developed that use resonance characteristics of the specimen to derive a complex modulus function. These techniques are often seen by the names of vibrating reed, torsional pendulum, and resonance devices.

Drake (1962) used a device in which the reed of material was caused to vibrate by means of an electromagnetic coil that attracted a piece of iron attached to the bottom of the reed. A recording galvanometer system followed the displacement of the reed as a function of time. Scherr (1966) has described another very convenient and accurate method for measuring the amplitude of a vibrating reed.

Finney *et al.* (1967) have developed a very convenient system for the measurement of resonance phenomena. A schematic diagram of their apparatus is depicted in Fig. 3. To evaluate complex modulus values when using a system of this form, it is necessary to have a uniformly shaped specimen. For a system in which forced vibrations are generated, the storage modulus, E′, may be defined as

$$E' = 4\rho f_1^2 L^2 \qquad [7]$$

where ρ = mass density of the sample
f_1 = fundamental longitudinal resonant frequency
L = length of the specimen.

If the wavelength, λ, of the generated vibration is not long compared to the diameter of the specimen, it may be necessary to apply a correction factor to equation 7.

The ratio between the loss and the storage modulus may be conveniently determined by measuring the response at a series of frequencies near resonance and constructing a response curve similar to that shown in Fig 4. The half width $\Delta\omega_{0.5}$ corresponding to the difference between frequencies at which the response is half its maximum value may be determined from this curve. Knowing these values, it is possible to obtain

$$E''/E' = \frac{\Delta\omega_{0.5}}{\omega_1\sqrt{3}} \qquad [8]$$

where ω_1 = forced resonant frequency = $2\pi f_1$
$\Delta\omega_{0.5}$ = half width frequency difference

from which E″ may be determined.

In some instances, as when using a reed, it may be more desirable to use free resonance techniques in which the specimen is displaced from its equilibrium position and then allowed to oscillate freely. The storage modulus may be determined to a close approximation by means of equation 7. The ratio between the storage and the loss modulus may be obtained by the relation:

$$E''/E' = \zeta^1/\pi = \frac{1}{\pi} \ln \frac{A^1}{A_2} \qquad [9]$$

where: ζ^1 = logarithmic decrement of the damped response curve given by the logarithm of the ratio of two successive amplitudes (Fig. 6).

Finney *et al.* (1967) and Abbott *et al.* (1967) have presented results in which whole fruits were used instead of prepared specimens. At the present time, results from studies of this sort are not readily amenable to interpretation of complex moduli functions. As the stress distributions within the fruits become more completely understood, it is possible that a shape factor may be derived for the entire fruit, which would allow establishment of absolute moduli

One of the primary difficulties encountered when using resonance methods is that the size and shape of specimens must be altered a number of times to obtain a frequency range of storage and loss moduli. In general, resonance techniques are reliable in the frequency range 1 to 10^4 cps, but for any particular sample the observable responses will be limited to the regions surrounding resonant frequencies.

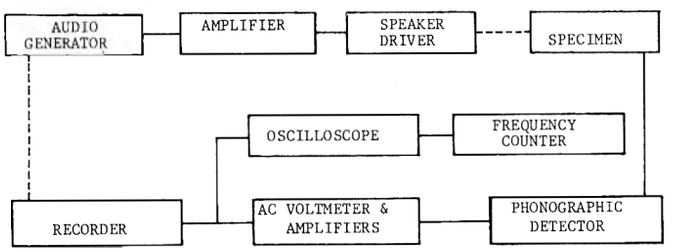

Fig. 3. Block diagram of instrumentation required for resonance measurements (fom Finney, 1967).

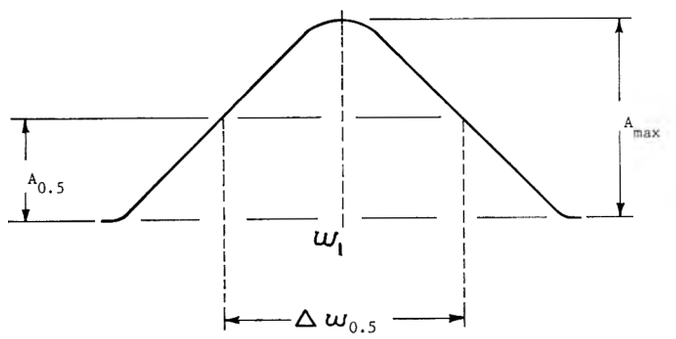

Fig. 4. Typical resonance curve used for obtaining damping factor in dynamic studies.

Wave propagation

When the critical dimension of a specimen is large compared to the wave length of the driving device, waves will propagate through the specimen rather than the entire sample vibrating as a unit. By observing the velocity and attenuation of these waves as they propagate through the specimen it is possible to derive viscoelastic coefficients for the material. Normally the frequency with which these waves are generated will be in the range of 10^2 to 10^4 cps, somewhat above the range of most of the previously described techniques.

The instrumentation required in a wave propagation test is such that the wave approximates only a one-dimensional disturbance. The driving and receiving devices normally used in wave propagation studies are some form of piezoelectric crystals that will transfer an electrical signal to a mechanical motion or a mechanical motion to an electrical signal. A schematic diagram of the additional equipment required is shown in Fig. 5

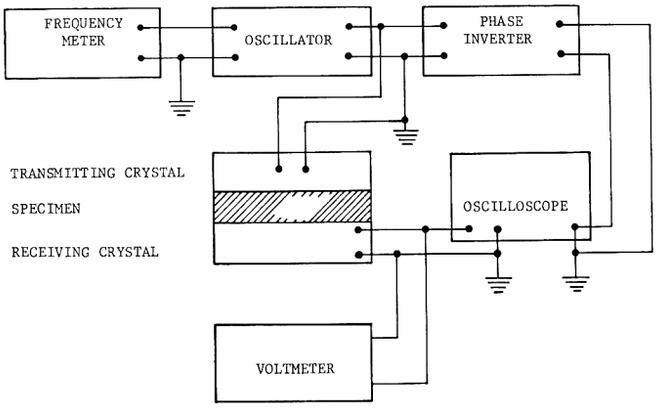

Fig. 5. Block diagram of instrumentation required for wave propagation.

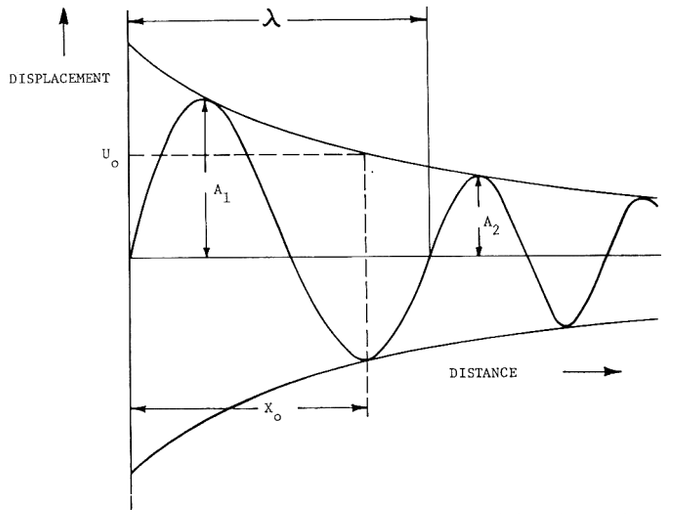

Fig. 6. Typical response curve used for obtaining logarithmic decrement in wave propagation studies.

The output curve from this apparatus will be in the form of an attenuated wave as shown in Fig. 6. Knowing the various parameters shown on the curve, it is possible to derive storage and loss moduli, as shown by Sharma (1965), to be of the form

$$E' = \frac{\omega^2\lambda^2\rho[4x^2 - (\lambda)^2]}{[4x^2 + (\lambda_o)^2]} \qquad [10]$$

$$E'' = \frac{4\pi\omega^2\lambda^2\rho\left(\frac{\lambda}{x_o}\right)}{\left[4\pi^2 + \left(\frac{\lambda}{x_o}\right)^2\right]^2} \qquad [11]$$

where x = thickness of specimen
ρ = mass density

The above equations assume that the cross section is small compared to the length of the specimen.

In many instances it is preferable to use pulsed forms rather than continuously generated waves. Leslie (1949) states that the velocity of a pulsed wave is unaffected by the size or shape of the specimen so the pulse technique

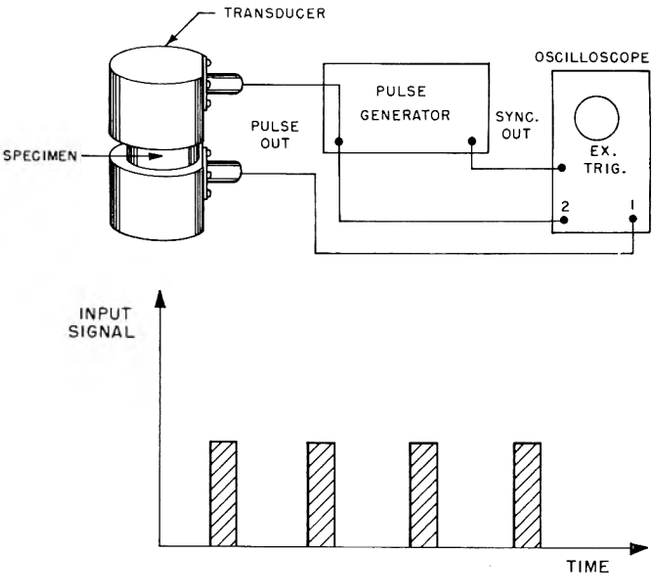

Fig. 7. Block diagram of instrumentation and waveforms used in pulsed wave propagation methods (from Mohsenin, 1968)

results in a better non-destructive test than does continuous generation methods. The instrumentation required in the pulse method is shown in schematic form in Fig. 7. An impulse generated in an extended solid can be shown to separate into three groups of waves, a compressional, a shear, and a surface wave, each travelling with a different velocity. The compressional waves travel with the greatest velocity and therefore are the first to reach the receiver. The arrival time can be determined accurately and from the resulting velocity the dynamic storage modulus is simply calculated using the following equation:

$$E' = \rho V^2 \frac{(1+\nu)(1-2\nu)}{(1-\nu)} \qquad [12]$$

where: ρ = mass density
V = compressional pulse velocity
ν = Poisson's ratio

Because variations in ν affect the result only to a minor degree the last term may be considered constant for a particular material, to a first approximation. Thus equation 12 becomes

$$E' = C\rho V^2 \qquad [13]$$

By consideration of damping that occurs as the wave passes through the material, it is possible to also derive a dynamic loss modulus. Note that in the frequency range considered, most materials will show very little damping effects.

INTERPRETATION OF RESULTS

QUITE OBVIOUSLY the mere existence of curves relating storage and loss moduli to frequency of excitation is of little significance unless correct interpretation of them can be derived. When conducting any dynamic test it is generally believed important that the frequency range being studied has some application to actual field conditions for the food material. Of course, there are instances

in which the behavior of a material may be of interest without having a specific application.

One of the most obvious areas of interest in mechanical responses appears to be in application to texture evaluation. However, it must be emphasized that texture itself is an undefined concept that certainly includes more than purely mechanical response. In some cases it is possible to establish a reliable correlation between sensory evaluations and purely objective mechanical evaluations. If additional components of texture such as temperature, flavor, etc., could be similarly correlated, it may be possible to eventually derive a texture description based on a series of variables. By means of modern computer techniques it would then be possible to derive a family of relations for the most acceptable combinations of textural characteristics.

A point often overlooked is the significance of mechanical measurements to processing applications. It is quite feasible to assume that, in many instances, storage and loss moduli curves may indicate regions of the most desirable characteristics for grinding, mixing and similar transformations of solid foods. For instance, in the dry milling of corn, it would be highly desirable to isolate a region in which the response of germ and endosperm are the most disperse to ensure maximum separation. By specifying material response in terms of engineering parameters, it is possible for the design engineer to more adequately prepare processing machinery to fit field conditions.

The significance of the internal structure of a material can best be evaluated by means of gradually complexing models that will have the same response forms as specified for the food material. For instance, engineers have a number of theories for mechanical response of open and closed cell foams that would appear to be applicable to many biological tissues. Similarly the behavior pattern for a fiber reinforced plastic is probably similar to that of a muscle specimen. By developing suitable structural theories, it may be possible to synthesize the mechanical behavior of certain foods. The most rapid progress toward this objective is believed possible through utilization of principles and techniques developed for engineering materials.

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Ms. rec'd 1/5/68; revised 3/21/68; accepted 7/15/68.

Journal Paper No. 3293 of the Pennsylvania Agricultural Experiment Station, University Park.

Development of some of the techniques described in this paper was supported by National Science Foundation Grant GK 15 to the Agricultural Engineering Department of the Pennsylvania State University.

THEME:

Gains, Goals & Guidelines

ADD THE THIRD DIMENSION . . . .

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

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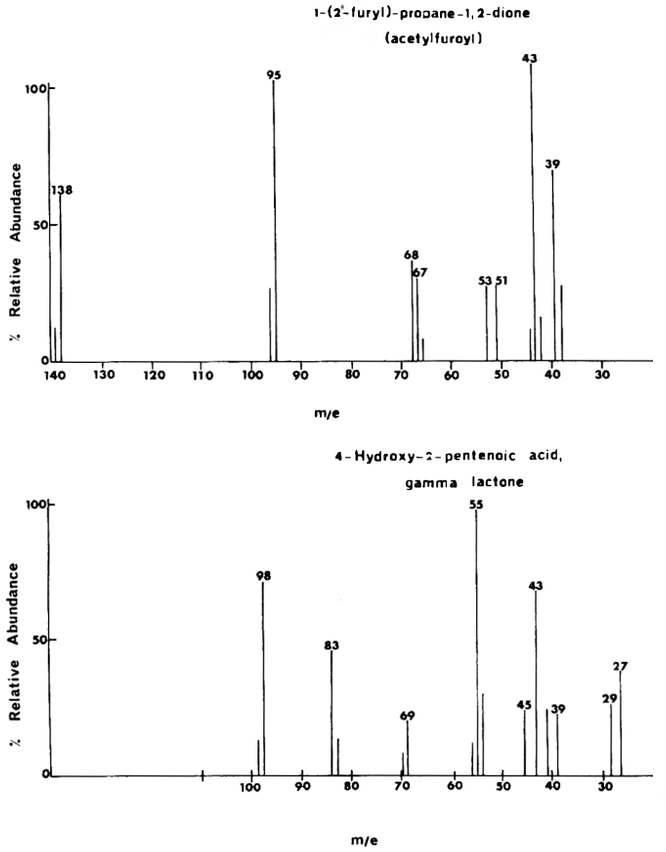
Erratum Notice

The following is the corrected Fig. 3 for the article entitled, "Volatile Compounds from Heated Glucose," by R. H. Walter and L. S. Fagerson appearing on page 297 volume 33 of the *Journal of Food Science*.



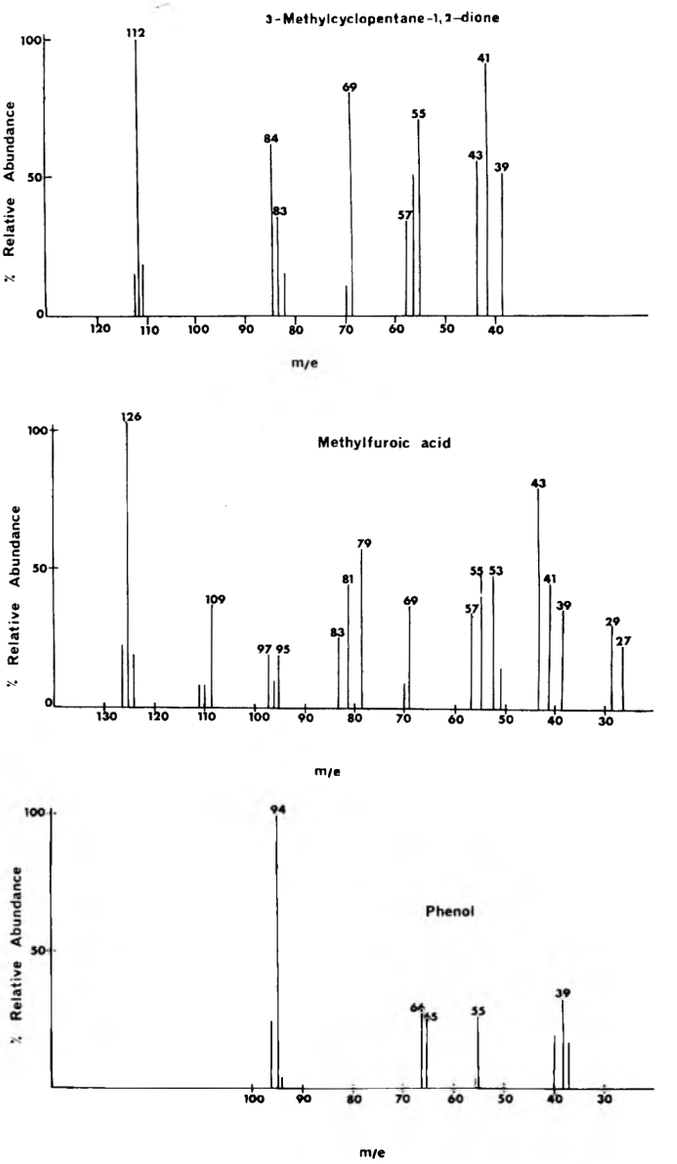


Fig. 3. Mass spectra of some compounds of intermediate volatility identified in the distillate of glucose heated at 250°C for 30 min.

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