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CONTENTS

Effect of Stress Prior to Slaughter on the Chemical Composition of Raw and Cooked Pork [a]

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SUMMARY

Data are presented showing that stress from periodic electric sitimulation prior to slaughter significantly affected the chemical composition and cooking losses of certain chemical components of the longissimus dorsi, psoas major, and quadriceps femoris muscles of swine.

INTRODUCTION

Lewis *et al.* (1961) observed that stress prior to slaughter decreased the dry matter lost in cooking samples of the longissimus dorsi, psoas major, and quadriceps femoris muscles of fresh pork. Therefore, it seemed desirable to determine what chemical components in the dry matter lost in cooking fresh pork are affected by stress prior to slaughter. The various stresses referred to herein are, of course, administered to the live animal before slaughter.

EXPERIMENTAL

After an 18-hr shrink, twelve U. S. choice No. 1 crossbred hogs weighing 182–225 lb were sorted by sex and paired for less than a 10-lb difference within pairs. One pig in each pair was then allotted at random to either one of 2 lots. One lot of pigs received periodic electrical stimulation (three electric shocks every 10 min from an "electric hotshot") for 5 hr, and the other lot was placed in a pen without feed and water and not disturbed during this 5-hr period. Twenty-four hours after slaughter, tissue samples were taken from the longissimus dorsi, psoas major, quadriceps femoris, and the liver. Samples of the above muscles (50–60 g) were cooked for 1 hr at 300°F. The raw and cooked samples were frozen until moisture, protein, and ash determinations were made. These samples were then freeze dried until the other chemical analyses were determined. The following areas of the tissues were taken for chemical analysis: longissimus dorsi, the muscle between the 7th and 13th ribs; psoas major, the entire muscle in the loin; quadriceps femoris, the entire muscle in the ham; and liver, the entire left lateral lobe. Moisture, protein, and ash were analyzed by accepted methods of AOAC (1960). Glycogen was extracted with 30% potassium hydroxide and precipitated by adding 1.2 volumes of 95% ethyl alcohol as described by Pflüger (1905). The resulting precipitate was determined photometrically by the anthrone method of Colvin *et al.* (1961). Lactic acid was determined by the method of Barker and Summerson (1941). Inorganic phosphorus, sugar phosphorus, and total phosphorus were analyzed photometrically by methods described by Hawk *et al.* (1954). Sugar phosphorus was designated as the amount of organic phosphorus extracted by 10% trichloroacetic acid. Meat samples were ashed by the method of Toth *et al.* (1948). Sodium, potassium, calcium, and magnesium were determined on a Beckman DU flame spectrophotometer with an electronic power-supply attachment (Beckman, 1957; Close *et al.*, 1953). Corrections were made for the interference of potassium on the magnesium determination, and for the interference of phosphate on the calcium determination, by adding the amount of these interfering materials present in the samples.

Moisture was expressed on a fat-free, carbohydrate-free basis; lactic acid and glycogen on a fat-free, dry-matter basis; and protein, ash, inorganic phosphorus, sugar phosphorus, total phosphorus, potassium, sodium, calcium, and magnesium on a fat-free, carbohydrate-free basis.

The pH determinations were made 24 and 48 hr after slaughter, with a Beckman model G pH meter. The pH of the freeze-dried tissues was determined on a mixture of 4 ml of distilled water and 0.4 g of dried tissue that was allowed to stand for 1 hr.

Expressible water was determined on both the raw and cooked samples by the method of Grau and Hamm (1953) with the following alterations: samples weighing 250–350 mg were pressed in a

[a] Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

Carver press at 4,000 lb/sq in. for 5 min on a 11-cm no. 40 Whatman filter paper that had been brought to a constant water content. The following constants were used for the calculation of expressible water: 28.85 and 35.44 mg H_2O/sq in. of wetted filter paper for raw and cooked samples, respectively. The results are expressed as the percent of water pressed from the sample. The results were analyzed by analysis of variance, and all percentages were transformed to the arcsin before statistical analysis. This paper is concerned only with mean differences that were statistically significant.

RESULTS AND DISCUSSION

Table 1 shows the effect of stress on cooking losses and the chemical composition of uncooked pork. Stress increased the protein concentration in the raw psoas major but decreased the concentrations of lactic acid in the raw psoas major and raw quadriceps femoris, and the glycogen and sugar phosphorus concentrations in the raw psoas major. Stress had no effect on the concentration of other chemical components in the liver or muscles studied.

Briskey *et al.* (1959) reported that forced exercise had no effect on the glycogen, moisture, sodium, and potassium concentrations in the gluteus medius of hogs. Lewis *et al.* (1961) reported that stress decreased the glycogen concentration of the psoas major and the lactic acid concentration in

Table 1. Effect of stress prior to slaughter on the cooking losses and chemical composition of uncooked pork.[a]

	Liver		Psoas major		Longissimus dorsi		Quadriceps femoris	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Moisture %[b,e]	75.3	74.9	77.7	77.9	76.5	76.2	78.1	77.8
% loss			44.8	32.4*	51.6	45.8	49.3	36.7
Protein %[c,e]	93.7	93.6	94.7	95.0*	95.2	95.2	94.7	95.0
% loss			6.6	3.0	10.0	6.9	4.4	2.7
Ash %[c,e]	6.3	6.4	5.3	5.0*	4.8	4.8	5.3	5.0
% loss			40.1	21.7**	44.4	40.7	42.6	26.4**
Lactic acid mg/g[d,e]	7.2	16.2	31.7	16.5*	37.7	31.7	27.2	15.4*
% loss			41.5	32.9	44.2	40.7	27.3	23.5
Glycogen mg/g[d,e]	4.9	4.9	0.5	0.1	3.6	0.2	2.0	0.1
% loss			0.0	0.0	59.9	0.0	31.9	0.0
Inorganic phosphorus mg/g[c,e]	5.0	5.2	5.8	6.2	5.8	6.1	6.0	6.2
% loss			41.3	24.4	47.5	41.2	40.7	23.3
Sugar phosphorus mg/g[c,e]	2.3	2.6	1.4	0.9**	1.1	1.0	1.3	0.8
% loss			0.0	0.0	0.0	1.6	70.3	0.0
Total phosphorus mg/g[c,e]	16.3	16.4	9.5	9.4	9.6	9.2	9.6	9.4
% loss			30.9	19.6**	37.3	33.7	26.3	18.5
Potassium mg/g[c,e]	12.6	12.7	14.8	16.0	16.8	16.3	17.0	15.6
% loss			38.0	27.2	45.1	42.5	40.3	14.1*
Sodium mg/g[c,e]	2.7	2.5	2.0	2.2	1.6	1.6	2.1	2.1
% loss			34.6	32.5	53.9	43.7	49.0	30.2*
Calcium mg/g[c,e]	0.2	0.2	0.3	0.3	0.4	0.4	0.2	0.2
% loss			20.3	15.1	52.5	58.4	27.9	20.8
Magnesium mg/g[c,e]	0.8	0.8	0.7	0.8	0.7	0.7	0.7	0.7
% loss			26.7	15.5	51.1	52.3	37.4	21.0

[a] Six animals per treatment.
[b] Fat-free, carbohydrate-free, fresh basis.
[c] Fat-free, carbohydrate-free, dry-matter basis.
[d] Fat-free, dry-matter basis.
[e] Raw muscle.
* $P < .05$ ** $P < .01$

Table 2. Effect of stress prior to slaughter on the pH and expressible water of raw and cooked pork.[a]

	Psoas major		Longissimus dorsi		Quadriceps femoris	
	Control	Stress	Control	Stress	Control	Stress
24-hr pH	5.8	6.3**	5.8	6.1	5.9	6.2*
48-hr pH	6.0	6.3*	5.9	6.1	5.9	6.5**
Freeze-dried pH						
Raw	5.5	6.3*	5.2	5.5	5.6	6.2**
Cooked	5.4	6.4*	5.7	6.0	5.6	6.4**
Expressible water %						
Raw	27.4	16.4*	24.1	19.2	27.0	17.0*
Cooked	34.0	37.0**	29.1	33.0	30.9	33.3

[a] Six animals per treatment.
* $P < 05$
** $P < .01$

the psoas major and quadriceps femoris of hogs. Gibbons and Rose (1950) suggested that fatigued pigs have livers lower in glycogen, and psoas major muscles containing less lactic acid, than rested pigs. Sayre *et al.* (1961) reported that cold-water stress decreased the lactic acid concentration of the gluteus medius of hogs. Rose and Peterson (1951) showed that exercise decreased the muscle lactic acid of rats. Hall *et al.* (1944) reported that dark-cutting beef had very little glycogen and large amounts of inorganic phosphate. These data agree with results in the literature.

Stress decreased the loss of moisture and ash during cooking in the psoas major and quadriceps femoris. Stress also decreased the loss of inorganic and total phosphorus in the psoas major, and the loss of potassium and sodium in the quadriceps femoris during cooking. This treatment had no significant effect on the cooking losses of other chemical constituents studied. When the concentration of each chemical constituent in the raw pork was held constant by analysis of covariance, the cooking loss of that constituent was unchanged except that the inorganic phosphorus and total phosphorus cooking losses were significantly reduced by stress in the quadriceps femoris. These results show that cooked pork from stressed animals contains higher concentrations of certain chemical components than pork from rested animals. Therefore, cooked pork from stressed animals would contain more food nutrients per lb.

Table 2 shows the effect of stress on the pH and expressible water in raw and cooked pork. Stress increased the pH of raw psoas major and quadriceps femoris 24 and 48 hr after slaughter. The pH determined on the freeze-dried raw muscles gave similar results. Stress significantly increased the pH of freeze-dried cooked psoas major and quadriceps femoris. Other experiments have shown that stress would increase the pH of the muscle of hogs (Briskey *et al.*, 1959; Lewis *et al.*, 1961; Sayre *et al.*, 1961).

Stress decreased expressible water in raw psoas major and quadriceps femoris and increased the expressible water in the cooked psoas major. When the expressible water was expressed as the percent of the total moisture of the sample, stress decreased the expressible water in the raw psoas major and quadriceps femoris and had no effect on the expressible water in the cooked muscles. The results on the raw muscles are in agreement with the report of Briskey *et al.* (1960), who stated that exercise decreased the expressible water of the raw gluteus medius and biceps femoris. However, Sayre *et al.* (1961) showed that cold-water stress had no effect on the expressible water of the gluteus medius.

The results on the cooked psoas major can be explained since it has been shown that stress increases the moisture concentration of this muscle (Lewis *et al.*, 1961). Therefore, more water would be available for pressing out in the muscle from stressed animals.

These data suggest that the cooked muscles of stressed hogs contain more nutrients per lb than the cooked muscles of rested hogs. It has been previously shown that stress increased the tenderness and juciness of certain muscles of fresh pork (Lewis *et al.*, 1959). If these observations are true, fatigued animals would yield a more tender, jucier pork containing more food nutrients per lb than animals slaughtered in a rested condition.

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RESEARCH NOTE

Changes in the Total Sulfhydryl Group Content and Histochemical Demonstration of Sulfonates in Excised Chicken Muscle Aged in Air [a]

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A previous communication from this laboratory (Chajuss and Spencer, 1962) suggested that the formation and cleavage or reorientation of intermolecular or intramolecular disulfide bonds may play an important role in relation to the tenderness of poultry meat during onset and resolution of rigor mortis. The present paper reports investigation of changes in the total sulfhydryl group and demonstration of sulfonates in excised chicken muscle subjected to air oxidation.

CHANGES IN SULFHYDRYL GROUP CONTENT

Two adult chickens were slaughtered and allowed to bleed for 1 min. The right pectoralis major muscle was then excised, and eight sections weighing 1.00 g each were cut from the middle of the muscle. All sections were frozen in an acetone-dry ice mixture, the first section immediately, and the remaining sections after aging at room temperature (25–27°C) in a covered Petri dish for 1, 2, 4, 6, 24, 48, or 72 hr.

The method of Grunert and Phillips (1951) developed for determination of reduced glutathione was modified for chemical analysis. Each 1.00-g sample of frozen muscle tissue was ground with 3–5 volumes of dry ice in a pre-cooled mortar, and the mixture was transferred to a 10-kc Raytheon sonic oscillator chamber. Twenty ml of 8*M* urea were added, the air in the chamber of the oscillator was replaced with nitrogen, and the chamber was tightly sealed. The tissue was oscillated for 20 min with maximum power output. During the oscillation, the chamber was continuously cooled with running water at 10°C. The tissue-urea homogenate was immediately filtered through Whatman No. 1 paper. To 4.0 ml of 8*M* urea, 4.0 ml of the filtrate were added. Then, successively, 1.0 ml of 0.067*M* sodium nitroferricyanide, and 1.0 ml of 0.067*M* sodium cyanide in 1.5*M* sodium carbonate, were added. The optical density was read at 520 mμ with a Bausch and Lomb Spectronic 20 colorimeter against a blank containing 4.0 ml 8*M* urea, 4.0 ml filtrate, 1.0 ml water, and 1.0 ml 0.067*M* sodium cyanide in 1.5*M* sodium carbonate. Readings were taken 15 sec after the addition of the sodium nitroferricyanide reagent. A standard curve was prepared as above, with known amounts of cysteine · HCl dissolved in 8*M* urea in place of the tissue-urea homogenate filtrate.

The results (Table 1) show that aging muscle tissues subjected to air oxidation

Table 1. Changes in total sulfhydryl group content in muscle tissues subjected to air oxidation.

Aging time (hr)	Moles —SH ($\times 10^{-6}$) per gram tissue	
	Trial I	Trial II
0	9.9	8.4
1	7.3	6.8
2	6.9	6.4
4	6.7	6.2
6	6.4	6.1
24	6.6	6.1
48	6.2	5.9
72	6.0	5.8

[a] Scientific paper 2200, Washington Agricultural Experiment Stations, Pullman. Project 1515.

would undergo a rapid decrease in total sulfhydryl group content. This decrease was very rapid during the first 2 hr of aging, and leveled off after approximately 4–6 hr. De Fremery and Pool (1960), working on excised chicken pectoralis major muscle, showed that the onset of rigor was 2–4.5 hr after death. The rapid decrease in sulfhydryl group content during the development of rigor as shown in the present study supports the hypothesis that the formation of a contracted or strained three-dimensional network of protein stabilized by intermolecular and/or intramolecular disulfide bonds may be involved in the formation of the rigor state. Mobility and relaxation of such a strained network could be obtained through disulfide-sulfhydryl exchange reactions, which would therefore be regarded as rate-limiting steps in determining relaxation of the muscle (resolution of rigor mortis).

DEMONSTRATION OF SULFONATES

The pectoralis major muscles of 2 adult chickens were used. The muscle was excised immediately after slaughter, and rectangular sections (1 cm^2 cross section) were cut parallel to the muscle fibers. One section was immediately put into a formol-saline fixative; the other sections were allowed to age at 7–10°C in a Petri dish (covered with moist filter paper) for 1, 2, 6, 24, 48, or 96 hr. The samples were fixed for 24 hr in formol-saline and were brought to paraffin. Ten-micron transverse sections were made by cutting across the muscle fibers, and 12-micron longitudinal sections were made by cutting parallel to the muscle fibers. The sections were brought to water and stained for 1 hr with freshly prepared Alcian Blue stain. The stain was made as 1% solution of Alcian Blue in 2*N* sulfuric acid. The dye was added to the acid, and the mixture was heated to 70°C and filtered. The stained sections were observed under a microscope and the relative amount of sulfonates was estimated visually by the degree of dye sorption.

Staining was slight in sections of muscle fixed at death (unaged). The sections subjected to air oxidation (aged) showed staining to a greater extent, visible mainly between bundles of muscle fibers. Owing to the high similarity in the amount of staining in the aged sections, no attempts were made to rank the aged section slides. The ability of Alcian Blue dye to react with sulfonates at a low pH was utilized by Adams and Sloper (1956) who used the dye for histochemical demonstration of sulfonate radicals of cysteic acid, derived from cysteine or cystine after performic acid oxidation. Other substances present in the tissue likely to react with Alcian Blue were the sulfate radicals of acidic mucopolysaccharides. The initial staining observed in the nonaged sections could be due to metabolic sulfonate radicals or to sulfuric acid mucopolysaccharides present in the tissue ground substance. The increase in staining observed in aged muscle may be attributed to oxidative conversion of sulfhydryl or disulfide groups to sulfonates. Probably involved in the latter case were disulfide-sulfhydryl interchange reactions. The additional, though less likely, possibility that the increase in staining is due to increased availability and reactivity of acid mucopolysaccharides during aging should also be considered.

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A Spectrophotometric Investigation of Dihydroxyacetone-Arginine Browning

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SUMMARY

Color development was maximum at a 2:1 stoichiometric ratio of dihydroxyacetone to arginine when both the molar-ratio and Job's methods were applied to solutions maintained at a pH 6.3–6.5. The reactants, which produced the red-brown color through a series of chemical changes, gave reproducible absorbance data at 300 mμ for solutions containing a final concentration of 2–120 ppm of dihydroxyacetone.

Because of an increased interest in the triose sugar dihydroxyacetone, occasioned by its ability to form a brown-colored complex with protein matter, this compound was studied. It has been known for some time that sugars will react with protein matter to produce colored products (Maillard, 1912a,b, 1913, 1915a,b, 1916, 1917). Some investigators have determined stoichiometric ratios for the non-enzymatic browning reaction between amino acids and hexose or pentose sugars (Bauminger, 1957; Petit, 1959; Tan, 1950; Wolfrom, 1953). Literature on this Maillard reaction was recently reviewed extensively (Ellis, 1959). More specifically, it has been observed that dihydroxyacetone produced a brown color in the caries of the teeth (Dreizen, 1957), and that it imparted color to the human skin (Hecht, 1928; von Noorden, 1927). Because protein structures are often complicated and ill-defined, the red-brown color produced by the reaction of dihydroxyacetone with a definite amino acid was investigated. Arginine was selected for this study since it proved to be one of the most sensitive amino acids for the development of a red-brown color with dihydroxyacetone at the pH values used. A spectrophotometric study was made of the stoichiometric ratio of the reactants developing this color maximum.

EXPERIMENTAL

Apparatus. A Beckman Model DU spectrophotometer with 1-in. silica cells was used. A timer clock measured identical reaction-time intervals, and pH values were determined with a Beckman Model G-H meter.

Procedure. Because of its hygroscopic nature, USP-grade dihydroxyacetone (specially prepared by Chas. Pfizer and Co.) was dried at 40°C for 48 hr before use. Standard solutions of 0.10–6.00% dihydroxyacetone were prepared by weighing out 0.1000- to 6.000-g samples and diluting to 100 ml with distilled water in volumetric flasks. Fresh standard solutions were prepared periodically since dihydroxyacetone is subject to air oxidation and bacterial attack. A 1.00% solution of arginine was prepared from CP l-arginine (Eastman Chemical Company) with dilution to a final volume of 100 ml. The 0.1000*M* solutions for the stoichiometric studies were prepared by diluting 0.9008 g of dihydroxyacetone and 2.1067 g of arginine monohydrochloride to 100 ml with distilled water. The pH of the solutions was controlled within 6.3–6.5 during all measurements in order to minimize possible chemical changes in the dihydroxyacetone structure (Lento, 1960a,b; Neuberg, 1915).

The complex of dihydroxyacetone and arginine monohydrochloride was developed by pipetting 1.00-ml portions of the standard solutions into 100-ml beakers containing 5.00 ml of the 1.00% arginine solution, and digesting the mixture on a boiling-water bath at 96°C for exactly 1 hr. The evaporation of water left a red-brown residue, which was dissolved in distilled water, transferred quantitatively to 500-ml volumetric flasks, and diluted to the mark. The absorbance of these solutions was measured with a Beckman spectrophotometer against a solvent blank within 1 hr of the completion of color development.

The most suitable wavelength for absorption was found by determining absorbance values for

the complex from 250 to 1000 mμ at successive 1-hr intervals. Standard curves were obtained by measuring absorbances of water solutions of the complex developed from 0.100 to 6.00% dihydroxyacetone solutions.

Molar-ratio data were obtained (Yoe, 1944) by developing the complex in solutions containing varying molar ratios of the two components. Different molar-ratio solutions were prepared according to the standardized method described, and diluted to 500 ml with distilled water. Data obtained with Job's method of continuous variations (Job, 1928; Vosburgh, 1941) were taken on solutions of the complex developed in the standardized manner. The solutions were made up so that the total volume remained constant at 5.00 ml, and the volumes of 0.1*M* dihydroxyacetone were varied continuously, with the 0.1*M* arginine making up the remainder of the volume.

RESULTS AND DISCUSSION

From an absorbance-stability plot it was found that a maximum absorbance with time occurred at a wavelength of 300 mμ, which was selected for measurement of the stoichiometric ratio (Fig. 1). Reproducibility was greatest when the unknown samples were measured at exactly the same time interval as the standards. Results obtained by following the development of the red-brown color obeyed Beer's Law in concentrations ranging from 2 to 120 ppm of dihydroxyacetone. The absorbance values obtained were sufficiently reproducible in this range to serve successfully as a quantitative determination for dihydroxyacetone. Color development was maximum at a 2:1 stoichiometric ratio of dihydroxyacetone to arginine when both the molar-ratio method and Job's method were applied.

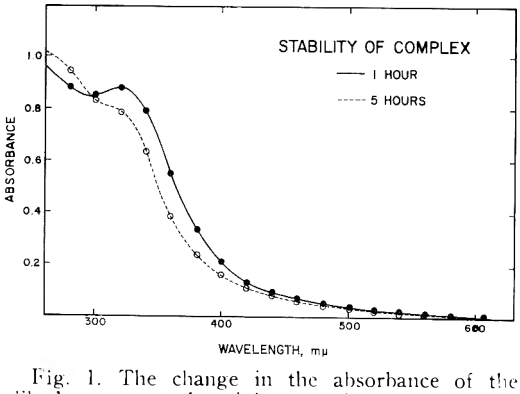

Fig. 1. The change in the absorbance of the dihydroxyacetone-l-arginine complex with time.

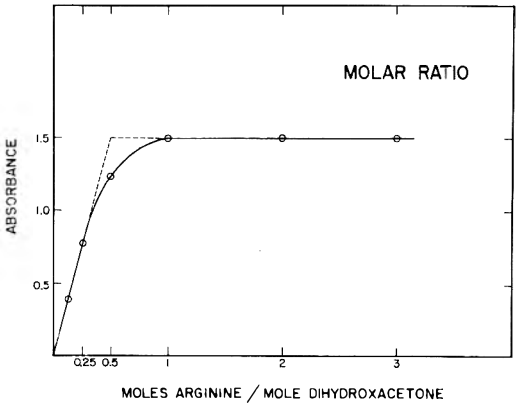

Fig. 2. The change in the absorbance of the complex with altering molar ratios.

In the application of the molar-ratio method for a determination of stoichiometric values, dissociation or equilibrium shifts are indicated when the curve does not break sharply but approaches the maximum absorbance asymptotically. The stoichiometric composition of the complex can be determined from such a graph by extrapolating the straight-line portions to a common intersection. The point where these two lines intersect represents the stoichiometric ratio. A degree of dissociation or equilibrium variation can be calculated as follows, where A_m is maximum absorbance and A_s is absorbance at the stoichiometric ratio of the complex:

$$a = A_m - A_s / A_m \times 100$$

When the molar concentration data were plotted, a ratio of 2 moles of dihydroxyacetone to 1 mole of arginine was obtained. On the basis of the above discussion it also appears likely that some dissociation or equilibrium shift occurred (Fig. 2).

Job's method makes use of the variation of equimolar concentrations of the reactants where x and $1 - x$ are the components. By finding the experimental values of x for which the curve is at a minimum or maximum, the number of moles of reagent to each mole of compound present can be determined. Data taken at 300 mμ further confirm that a 2:1 molar ratio of dihydroxy-

acetone to l-arginine is involved in the development of the color maximum (Fig. 3).

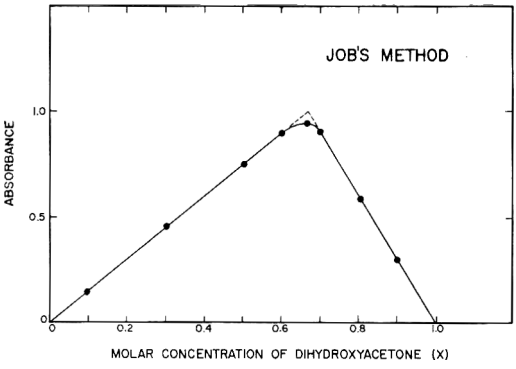

Fig. 3. The stoichiometric composition of dihydroxyacetone to l-arginine using Job's method of continuous variation, where $x/(1 - x)$ represents the ratio.

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Isolation and Description of the Pectinase-Inhibiting Tannins of Grape Leaves

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SUMMARY

Studies were continued on the tannin in Muscadine grape leaves previously reported to have inhibitory activity toward pectinase and cellulase. The tannin was isolated and purified by precipitation with caffeine and recovery by chloroform extraction. The material is a light tan, and in the freeze-dried form is quite bulky. It is a condensed tannin of high molecular weight, producing a large percentage of phlobaphenes upon acid treatment. The soluble portion after acid treatment was shown to contain gallic acid and glucose in small amounts.

INTRODUCTION

The importance of obtaining a source of inhibitor for enzymes responsible for the softening of cucumbers during brining was discussed by Bell and Etchells (1958), Etchells *et al.* (1958a,b), Bell *et al.* (1960), and Porter *et al.* (1961). Several plants were reported by Bell *et al.* (1962) to contain varying amounts of the inhibitor active against pectinase and cellulase. Grape leaf extracts were shown by Veldhuis (1961) to have inhibitory activity against pectin esterase. The active principle was identified by Porter *et al.* (1961) as a tannin or tannin-like material. Continued work toward isolation and identification has shown the active material to be a tannin of the condensed type.

MATERIALS AND METHODS

Plant materials. Muscadine grape leaves, Scuppernong variety, collected in North Carolina in late July, 1960, were washed with water, surface dried, placed in polyethylene bags, and frozen. The samples were stored at −15°C or below.

Extractions. The frozen leaves (80 g) were divided into 40-g portions, cut with scissors, and placed in a Waring blender (no endorsement implied). Distilled water (300 ml) was added to each 40 g of leaves, and the blender was run at low speed for 30 sec and then at high speed for 2.5 min. The slurry was filtered through four layers of cheesecloth. The filtrate was refiltered several times through the filter cake produced by the coarse fibers of the leaves, until essentially clear. The two extracts were combined, yielding about 600 ml.

Isolation of tannin fraction. The tannins were isolated by the method of Barnes (1956) slightly modified. The extract was cooled to 5°C using an ice-salt bath. Caffeine (6 g) was dissolved in 400 ml of water at 55°C, and the solution temperature was lowered slightly. The caffeine solution was added slowly, with stirring, to the extract. Addition was discontinued if the temperature approached 14°C, and was continued only when the temperature was lowered to less than 7 or 8°C. After all caffeine had been added, the mixture was cooled to 5°C and allowed to stand until the precipitate had settled (about 30 min). The clear supernatant solution was decanted, and the suspension of the caffeine-tannin complex was transferred to two 250-ml centrifuge bottles. The suspension was centrifuged for 20 min at 1850–2000 rpm in an International Centrifuge, type SB, size no. 1. The supernatant solution was decanted, and the precipitate resuspended in 25 ml of water and recentrifuged. The wash solution was discarded. The two precipitates were combined and transferred to a liquid-liquid extracter using a total of 125 ml of water. The suspension was continuously extracted with chloroform until the caffeine was removed and the tannin was redissolved in the water phase (about 10 hr). The tannin solution was filtered through Whatman no. 5 paper with suction, evaporated twice to a small volume (to remove chloroform) in a rotary evaporator at a bath temperature of 35°C. The resulting solution was freeze-dried and stored in tightly stoppered bottles.

Measuring enzyme inhibitor activity. The viscometric method of Bell *et al.* (1955), as modified by Porter *et al.* (1961), was still further modified to measure accurately enzyme preparations of high

activity and to correct for the decrease in enzyme activity that occurs during the analysis.

This decrease in enzyme activity was reduced by making up the enzyme to 0.0001 g/100 ml with 0.8% NaCl solution. The enzyme preparation used was Pectinol 10M (Rohm and Haas). Viscometric measurements were made at 5-min intervals in series of twelve. Standard enzyme-activity measurements (enzyme and substrate only) were run at the beginning and end of each series. An average of the two activity values was used, or values were interpolated for the appropriate 5-min intervals.

A further modification of the method was adoption of a half-hour period between the mixing of enzyme and inhibitor and their addition to the substrate, in order to allow the development of full inhibition. This was done because experimental tests indicated that the percentage inhibition increased from zero mixing time to 30 min prior to addition to substrate. The inhibitory power remained constant from 30 min to 3 hr.

To compare the activity of the isolated tannins with the activity of the original extract, each preparation from an 80-g sample was dissolved in water and made to 1000 ml. The water extracts, which had not been precipitated with caffeine, were also made to 1 L. Dilutions were prepared to give concentrations of each equivalent to 4 mg leaf/ml. The inhibitions obtained at this concentration were compared. The dilution was such that, at this concentration, any decrease in the activity of the preparation would produce large changes in the calculated inhibitory activity, as shown by Porter *et al.* (1961).

Paper chromatographic analysis. Many solvent variations were employed, using butanol, benzene, cresol, and toluene as the organic phase, water as the immobile phase, and acetic or *p*-toluene-sulfonic acid as the swamping acid. Ascending chromatography was on a small scale, using 6-inch squares of S & S no. 589 Blue Ribbon filter paper formed into cylinders. Dynel, polyvinylchloride, and glass-fiber supports, with butanol–acetic acid–water (4:1:5) as eluting solvent, were also employed. Ferric chloride, ultraviolet light, and ammoniacal silver nitrate were used for spot identification.

Hydrolytic methods. Samples of the isolated tannin were hydrolyzed by a method similar to that of Jurd (1956). The samples (0.5 g) were heated 22 hr at 100°C with 8 ml of 5% H_2SO_4. After cooling, the insoluble material was filtered and the filtrate extracted with ether.

RESULTS AND DISCUSSION

Description of isolated tannin. The solid obtained through the caffeine isolation and freeze-drying was pale tan and very light and bulky, became electrified on handling, and was surprisingly easy to keep dry. The yield (average of three 80-g portions) was 1.6% on a fresh basis. The fresh leaf contained 4.06% tannin as determined by the official hide-powder method of the American Leather Chemists' Association (1957). Recovery was 39.4%. Since the ALCA method measures all soluble materials removed by hide-powder under empirical conditions, it cannot be estimated what percentage would be precipitated by caffeine. The isolation is assumed to give relatively good recoveries of the tannin.

Activity of tannin isolates. All preparations, at concentrations equivalent to 4 mg leaf/ml, gave 97–98% inhibition, compared to 98–100% for the original extract. Since this concentration figure is on the slope of the activity-concentration curve, results of this order indicate that, in addition to fairly good recovery of the tannin, the isolation procedure also recovered 100% of the activity.

Stability of the inhibitor. The tannin inhibitor is quite stable. Measurements, by the viscometric method, on water extracts stored in a refrigerator for 1½ months still gave inhibitions of 98–100% at low dilutions. Solutions of the isolated tannins and of redissolved freeze-dried material, stored in a refrigerator for one month, also gave 100% inhibition.

Chemical and physical characteristics of the isolated tannins. Dialysis experiments using a cellophane membrane indicate a minimum molecular weight on the order of 10,000. Sedimentation studies show that there is a broad range of molecular weights. Light-scattering measurements gave an average molecular weight of 250,000. Therefore, the range is probably from about 10,000 to somewhere in the millions. Whether these high values are due to a range of discrete molecules or due to aggregation cannot be determined at this time. However, this may well explain the rather unique inhibitory power of these grape leaf tannins over other tannins of commercial value as hide tanning agents. According to White (1956), the

latter have molecular weights ranging between 600 and 2,000–3,000.

The original extracts behave essentially the same when chromatographed on paper using all of the solvents described and with the different support materials. One spot, containing essentially all of the material, remained at the starting position. Two other spots moved somewhat but were in very low concentration. A broad fluorescent streak was apparent in all chromatograms. The isolated tannins produced only one spot, which remained at the origin and produced a trace of streaking that fluoresced under ultraviolet light.

Samples of the isolated tannin produced red, insoluble phlobaphenes, almost equal to the weight of the original sample, when H_2SO_4 hydrolysis was attempted. The ether extract of the filtrate gave one strong spot and one weak spot when chromatographed on paper. The strong spot had the same R_f as gallic acid in all solvents, and produced the same color reactions with $FeCl_3$ and with ammoniacal $AgNO_3$. The ether extract also gave strong positive color tests for gallic acid with KCN. The aqueous layer from the ether extraction chromatographed to give one spot with the same R_f as glucose. Reaction on paper with ammoniacal $AgNO_3$ and *p*-anisidine hydrochloride gave identical results with those of pure glucose.

The tannin in Scuppernong grape leaves responsible for the inhibition of pectinase is a condensed type of extremely high average molecular weight. Acid treatment produces a high proportion of an extremely insoluble material (phlobaphenes). The presence of gallic acid and glucose in acid hydrolysates may be due to a slight hydrolysis of the condensed tannin, but may also be due to the presence of a trace of hydrolyzable tannin not easily separable from the major component.

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Mention of trade names in this paper does not imply endorsement by the U. S. Department of Agriculture.

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Seasonal Changes Occurring in the Pectinesterase Activity and Pectic Constituents of the Component Parts of Citrus Fruits. I. Valencia Oranges[a]

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SUMMARY

Pectinesterase activity, 3 pectic fractions, and other characteristics were determined periodically on 5 component parts of Valencia oranges during a 7-month maturation cycle for 2 seasons. Usually, PE activity for peel, membrane, and juice sacs was least in December, when the Brix/acid ratio was low, and highest in June, when this ratio was greatest. The order of component parts for PE in most cases, from highest to lowest activity, was juice sacs, membrane, peel, seeds, and juice. Water-soluble pectin generally remained constant in peel and juice sacs, increased slightly and then remained constant in the membrane, and was irregular throughout the cycle in the seeds. The trend of ammonium-oxalate-soluble pectin in the components was to increase during maturation. Protopectin in the component parts usually increased to a peak and then gradually decreased for the remainder of the season, except that protopectin in the juice sacs decreased throughout the sampling period. In this component, protopectin evidently was at its maximum by the first picking in December. Total pectin remained constant in the juice and seeds, and slowly declined in the other 3 components with maturation. Membrane contained the highest source of protopectin and total pectin throughout the season.

INTRODUCTION

Much emphasis in research has already been placed on studies of soluble solids, acid, Brix/acid ratio, color, etc., during maturation of the orange. State of Florida citrus fruit laws (1961) are mainly dependent upon these characteristics. These tests are standard procedures for quality selection of fruit, but additional information would be useful by determining the enzymic activity and pectic contents of the various component parts at successive stages of growth.

Orange pectinesterase (PE), which catalyzes the hydrolysis of methyl alcohol from pectin, was first isolated and characterized by MacDonnell *et al.* (1945). They determined the enzymic activity in the flavedo, albedo, and cell sacs of Navel and Valencia oranges. The distribution of PE in 6 component parts of 7 varieties of mature citrus fruits was reported by Rouse (1953). Information is lacking on PE activity of the various component parts during maturation of the orange.

Changes in water- and acid-extracted pectins from the albedo, pulp, and juice of Florida Valencia oranges during a 7-month growth period have been reported by Gaddum (1934). The purity of the alcohol-precipitated extracts was expressed as pectic acid. Total pectin as calcium pectate was published by Rouse (1953) for the flavedo, albedo, membrane or rag, juice sacs, seeds, and juice from 7 varieties of mature citrus fruits. Seasonal changes in water-soluble and

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total pectins were recently reported by Sinclair and Jolliffe (1958, 1961) in peel, pulp, and juices of California Valencia oranges. Their results on pectin are expressed as anhydrogalacturonic acid. Pectic fractions as determined by Rouse and Atkins (1955) identify each for solubility in water, ammonium oxalate, and sodium hydroxide, and the changes in the fractions are usually caused by enzymic or acid hydrolysis.

The primary purpose of this study is to determine seasonal changes in pectinesterase activity and in the 3 pectic fractions of component parts of Florida Valencia oranges. These pectic fractions are water-soluble, ammonium-oxalate-soluble (which are the insoluble pectates and pectinates), and sodium-hydroxide-soluble, or protopectin. Changes in weight, total solids of the whole orange, percentage by weight of the component parts, and soluble solids/acid ratio of the juices were also determined during maturation of the fruit. The component parts consisted of the peel, membrane, juice sacs, seeds, and juice.

EXPERIMENTAL PROCEDURE

Preparation of samples. Valencia oranges of the Reasoner variety from rough lemon rootstock were picked the first of each month (December through June) for 2 years from the same trees, washed, dried, and separated by hand into peel, membrane, juice sacs, seeds, and juice. These components are briefly described as follows:

1) Peel consists of the flavedo or epicarp, which is the outer colored portion of the peel, and the albedo or the white spongy portion known as the inner mesocarp. This also includes the central core.

2) Membrane is the segment membrane or wall tissue enclosing the juice sacs. This is sometimes referred to as the rag.

3) Juice sacs are the vesicles that hold the juice.

4) Seeds.

5) Juice is the liquid expressed from the sacs.

From 15 to 24 oranges, depending upon fruit size, were used in preparing the samples of component parts. The peel, membrane, and seeds were comminuted in an Osterizer, whereas the juice was expressed from the juice sacs by a Watson-Stillman hydraulic press using 40 psig.

To 50 g of comminuted peel, membrane, or juice sacs were added 100 g of 2% sodium chloride solution, and the mixture was comminuted 3 min to further size component particles and extract the PE. Samples of the slurry mixture, ranging from 2 to 5 g, depending on enzymic activity, and 5 g each of juice and comminuted seeds were assayed for PE activity.

Pectic fractions were determined directly on a 15-g sample of juice. Weighed quantities of the other components were added to 80% isopropyl alcohol, except for the seeds, where 99% alcohol was used because of the high oil content. These preparations were allowed to stand overnight, filtered, washed 2 successive times with their respective percentage of alcohol, and once with 99% alcohol, and finally dried 15 hr in a vacuum oven at 60°C. The dried alcohol-insoluble solids (AIS) were ground to pass a 20-mesh screen. Pectic fractions were extracted from the AIS of the various components.

Methods of analysis. PE activity was measured essentially by the method of MacDonnell *et al.* (1945) as modified by Rouse and Atkins (1955). Units of activity are expressed by the symbol (PE.u.)g of dry solids and multiplied by 1000 for easy interpretation.

Pectin was divided into water-, ammonium-oxalate-, and sodium-hydroxide-soluble fractions, and each of these fractions was determined as anhydrogalacturonic acid (AGA) by an adaptation of a colorimetric method (Dische, 1947; Rouse and Atkins 1955). The pectic fractions were prepared from the AIS of each component, except for the juice, by placing 1 g of the material in a 250-ml centrifuge bottle, and extracting 3 consecutive times for 15 min each with 160 ml of distilled water at 30°C. The water extracts were combined and made to 500 g. The residue was again extracted 3 times with like amounts of 0.5% ammonium oxalate at 30°C, and the extracts made to 500 g. Finally, the residue was extracted 2 times with 225 ml 0.05*N* sodium hydroxide for 20 min each at 30°C, and the extracts made to 500 g. Centrifugation was used after each extraction to separate the liquid extract from the residue, and the liquid extract was filtered through a Buchner funnel with gentle suction using S & S No. 589 black ribbon analytical filter paper. The extracts were analyzed for AGA and reported as percentage pectin on a dry-weight basis.

Soluble solids as degrees Brix by spindle at 17.5°C, total acid as citric, and moisture contents were determined by conventional methods. Ratios of total soluble solids to acid were calculated. Also by calculation, total solids of the whole orange were obtained. This was accomplished by using the percentage of each component in the orange (Table 2) and the total solids of each component (data obtained but not presented in this manuscript).

Table 1. Comparison of Valencia orange characteristics in relation to maturity during 2 citrus seasons.

Sampling time months	1959-60 season				1960-61 season			
	Orange average wt. (g)	Total solids of av. whole orange (%)	Degrees Brix of juice	Brix/acid ratio of juice	Orange average wt. (g)	Total solids of av. whole orange (%)	Degrees Brix of juice	Brix/acid ratio of juice
December	200	14.6	9.00	6.77	184	17.6	9.80	5.98
January	244	15.0	9.95	9.21	215	16.4	9.80	7.97
February	235	15.7	10.40	10.51	212	16.6	10.75	9.68
March	258	15.5	10.65	12.38	227	16.1	11.05	11.88
April	239	15.2	11.05	13.00	241	16.4	11.27	13.26
May	240	15.5	11.15	16.64	264	15.9	10.80	16.88
June	231	16.7	11.75	18.65	270	14.3	10.00	18.87

RESULTS AND DISCUSSION

The 22 trees from which the Valencia oranges were picked and examined for this study were from Block II at the Citrus Experiment Station. They had received normal fertilizer and spray practices. Seasonal differences were found in some characteristics of Valencia oranges during the 2 growth seasons, and these will be discussed.

Table 1 presents some general characteristics of Valencia oranges for a 7-month period during the 1959–60 and 1960–61 seasons. The weight of an average orange was 200 g in December 1959, 258 g in March, and 231 g in June 1960. Average total solids of the whole orange during this growth period increased from 14.6 to 16.7%. During the 1960–61 season the average weight of the orange was 184 g in December and increased to a maximum of 270 g in June, whereas the average total solids of the whole orange decreased from 17.6 to 14.3%, the opposite of that found for the previous season. Total soluble solids in the juice were respectively 9.00–11.75 and 9.80–10.00° Brix in 1959–60 and 1960–61. However, during the second season the maximum of 11.27° Brix was reached in April. The Brix/acid ratios of the extracted juices for each corresponding month were similar for the 2 maturation periods, respectively 6.77–18.65 and 5.98–18.87.

Component parts of the fruit. The percentages of component parts in Valencia oranges examined during this 2-year study are presented in Table 2 for each month, December through June. Actually, the percentage of an individual component did not vary greatly during this part of the maturation period. The order of least to greatest quantity of component parts was seeds, membrane, peel, juice sacs, and juice. The respective average percentage values for each of these component parts were 0.8, 9.0, 19.2, 19.9, and 51.1 in 1959–60, and 0.8, 12.0, 20.8, 23.7, and 42.7 in 1960–61. There was appreciably less juice in the oranges the second year, and relatively more membrane and juice sacs.

Pectinesterase activity. Table 3 shows the distribution of PE activity, on a dry-weight basis, in the component parts of Valencia oranges for 2 seasons. The peel, membrane, and juice sacs were the most active in PE. In most instances these 3 components had less activity in December, the first month of this maturation study, when the Brix/acid ratio was least, than during the following 6 months. They had their greatest PE activity in June, when the Brix/acid ratio was highest.

The order of component parts for PE, from lowest to highest activity, was juice, seeds, peel, membrane, and juice sacs. The respective average PE values for these components were 2.2, 3.8, 44.8, 52.2, and 362.3 in 1959–60, and 1.0, 5.0, 53.7, 69.5, and 344.7 in 1960–61. The highest PE value reported by Rouse (1953) in mature Valencia oranges was 474.3 units for the juice sacs, whereas the highest value for this component was found to be 626.2 units during this 2-season study.

Pectic substances. Individual values of the water-, ammonium-oxalate-, sodium-hydroxide-soluble, and total pectins, for each

Table 2. Distribution of the component parts in Valencia oranges during 2 citrus seasons.

Sampling time months	1959-60 season					1960-61 season				
	Peel (%)	Membrane (%)	Juice sacs (%)	Seeds (%)	Juice (%)	Peel (%)	Membrane (%)	Juice sacs (%)	Seeds (%)	Juice (%)
December	18.2	8.7	21.1	1.2	50.8	20.7	15.8	22.8	1.1	39.6
January	18.8	8.3	23.3	0.7	48.9	23.1	11.0	20.0	0.8	45.1
February	19.0	10.8	16.7	0.7	52.8	21.6	11.6	26.5	0.9	39.4
March	19.7	9.7	17.8	0.7	52.1	20.2	11.1	22.7	0.9	45.1
April	19.1	8.3	21.6	0.7	50.3	20.5	11.7	26.2	0.7	40.9
May	18.9	8.8	18.5	0.7	53.1	20.2	11.5	22.0	0.7	45.6
June	20.6	8.2	20.0	0.9	50.3	19.0	11.4	25.8	0.5	43.3
Av.	19.2	9.0	19.9	0.8	51.1	20.8	12.0	23.7	0.8	42.7

Table 3. Distribution of pectinesterase activity in the component parts of Valencia oranges during 2 citrus seasons.

Sampling time	1959-60 season (PE.u.)g total solids × 1000					1960-61 season (PE.u.)g total solids × 1000				
	Peel	Membrane	Juice sacs	Seeds	Juice	Peel	Membrane	Juice sacs	Seeds	Juice
December	41.4	36.2	219.8	3.3	3.5	26.1	35.6	199.1	5.4	1.1
January	59.5	50.3	319.8	5.0	2.1	41.7	45.7	263.4	5.4	1.1
February	36.9	44.2	435.3	5.0	2.1	73.7	81.7	407.5	7.1	1.0
March	27.5	40.5	284.8	2.1	1.0	58.4	64.8	360.2	5.0	1.0
April	45.1	43.6	290.6	3.9	1.9	51.9	70.3	319.4	3.2	0.9
May	45.3	57.4	359.8	3.7	1.0	47.8	79.2	346.5	4.5	1.0
June	57.9	93.1	626.2	3.8	3.6	76.6	109.0	517.1	4.7	1.1
Av.	44.8	52.2	362.3	3.8	2.2	53.7	69.5	344.7	5.0	1.0

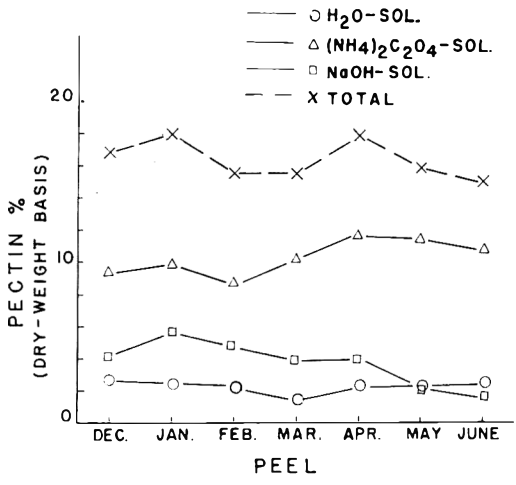

Fig. 1. Changes in the pectic fractions of the peel during maturation of the Valencia orange. Each curve represents the mean values for 1959–60 and 1960–61 seasons.

component part for each month during 2 seasons, were averaged together (Fig. 1–4). Because of the low concentration of pectic fractions in the juices, their average amounts are not shown. Total pectin ranged from 0.30 to 0.43%, of which the water-soluble pectins corresponded to 71.3–83.3%.

Changes in pectin during growth of the Valencia orange found in this study were not always in agreement with those found by Gaddum (1934). Similar differences were also pointed out by Sinclair and Jolliffe (1958) in their study of the California Valencia orange during growth and development. The differences are thought to be due mainly to techniques in extractions and methods of calculation. Trends in water-soluble pectin of the peel and juice sacs, during maturation of the Valencia orange, were similar to those found by Sinclair and Jolliffe (1961). Those authors referred to pulp rather than juice sacs, since a large portion of the pulp consisted of juice sacs plus some membrane.

Peel. Fig. 1 shows changes in pectic fractions on a dry-weight basis for the peel of Valencia orange. Water-soluble pectin remained almost constant from December (2.58%) through June (2.59%) except for a decrease for March (1.56%). This decrease occurred in both 1959–60 and 1960–61. Quantity of the ammonium-oxalate-soluble fraction was greatest in this component, and varied from 9.23% in December to a high of 11.67% in April, and then declined slightly to 10.84% in June. Sodium-hydroxide-soluble pectin, protopectin, increased from 4.17% in December to 5.66% in January, and decreased to 1.83% in June. Total pectin, of course, resulted from the sum of the 3 pectic fractions, the highest (18.05%) in January and the lowest (15.28%) in June.

Membrane. Fig. 2 shows percentages of the pectic fractions in membrane. Total pectin is most abundant in the membrane because of its excellent source of water-soluble pectin, and particularly of the sodium-hydroxide-soluble pectin present. Water-soluble pectin gradually increased during December through February (2.93–4.33%), decreased in March, and then generally remained constant to June (4.24%). There was a general increase in the percentage of ammonium-oxalate-soluble pectin throughout the maturation season (5.42–7.71%). The immature fruit contained 11.35 and 12.81%, respectively, in December and January of the sodium-hydroxide-soluble fraction, which then continued to diminish during maturity to 5.18%, in June.

Juice sacs. This component is of great interest to the citrus processor of frozen concentrated orange juice because it contains a high potential of PE activity per g of

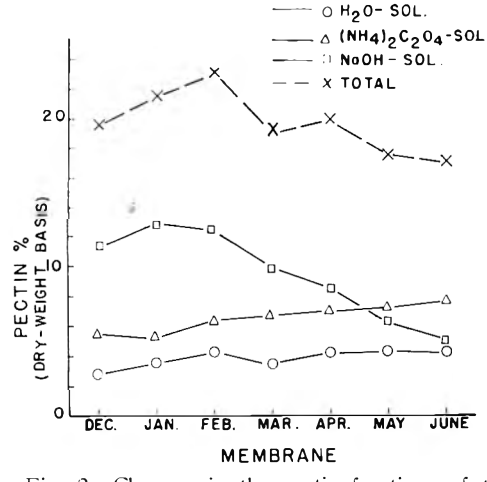

Fig. 2. Changes in the pectic fractions of the membrane during maturation of the Valencia orange. Each curve represents the mean values for 1959–60 and 1960–61 seasons.

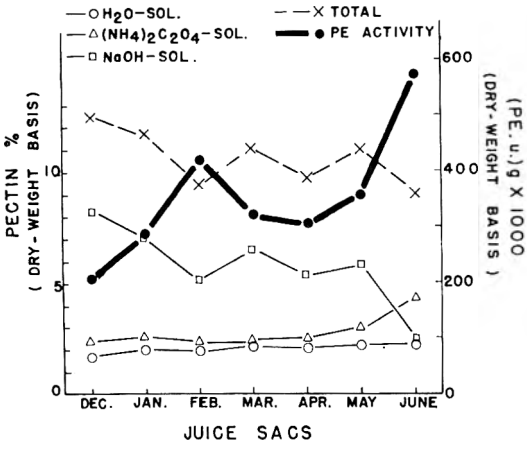

Fig. 3. Changes in the pectic fractions and PE activity of the juice sacs during maturation of the Valencia orange. Each curve represents the mean values for 1959–60 and 1960–61 seasons.

tissue and a large portion of the pulp in citrus juice consists of juice sacs. Thus, Fig. 3 not only shows the changes in the pectic fractions but also changes in PE activity. This PE curve represents the average values of the juice sacs for each month during the 2 maturation seasons (Table 3). For these 2 seasons, PE activity in this component rose to a high point (av. 421.4) in February and decreased and remained somewhat constant (av. 326.9) for March, April, and May, after which rate of activity was maximum (av. 571.6) for June. If these 2 periods of high PE activity are confirmed not only for the Valencia orange but for other citrus varieties, the peaks of activity would serve as criteria for the processor to heed so that proper procedure could be taken to protect the water-soluble pectin from demethylation.

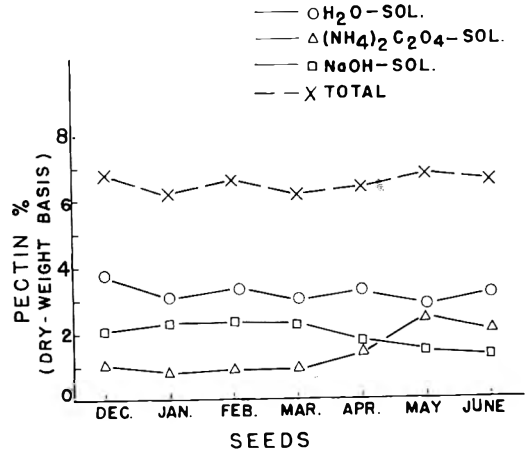

Fig. 4. Changes in the pectic fractions of the seeds during maturation of the Valencia orange. Each curve represents the mean values for 1959–60 and 1960–61 seasons.

Water-soluble pectin in juice sacs remained fairly constant from December (1.77%) through June (2.22%), whereas the oxalate-soluble fraction showed little change between December (2.44%) and April (2.42%) but definitely increased in May and June (4.32%). There was a general decline in the sodium-hydroxide-soluble pectin during this period, from 8.34 to 2.46%. Total pectin in the juice sacs is the primary source of pectin in citrus juice, varying from 12.55 to 9.00%.

Seeds. Seeds account for only approximately 1% (Table 2) of the whole Valencia orange. However, the total solids of the seeds varied between 44.0 and 50.0%, the lower percentage when the Brix/acid ratio was low, and the higher percentage when this ratio was greater. Note that the water-soluble pectin (Fig. 4) was irregular during this growth period (2.81–3.74%). The general pattern for the oxalate-soluble fraction was low, ranging from 0.84% in January to 2.50% in May, after the orange reached maturity. Sodium-hydroxide-soluble pectin rose to a slight plateau from December through February (2.11–2.35%), and then continued to decline through June (1.37%). Thus, total pectin (6.21–6.95%) remained almost constant for the maturation cycle of the orange.

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Volatile Ester Equilibria in Plant Tissue[a]

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SUMMARY

Variations in the apparent dissociation constants of ethyl acetate for several different samples of banana pulp are noted. It is hypothesized that the variations might be due to the involvement of coupled reactions that supply or delete one or more of the reacting species at a rate faster than that at which the tissue esterases function to establish equilibrium. In the same samples, the constants for methyl acetate and iso-amyl acetate remained relatively constant.

Enzymes responsible for the hydrolysis of carboxylic ester linkages have been found in a wide variety of materials. The aliesterases, which act on aliphatic esters of low molecular weight, are widely distributed in the animal body and in the higher and lower plants. Although it is often assumed that the aliesterases catalyze both the synthesis and hydrolysis of the simple esters, evidence has been presented that ethyl acetate is produced in fermentations by the yeast *Hansenula anomala* by a different mechanism (Baba, 1956; Cantarelli, 1955; Peel, 1951; Tabachnick and Joslyn, 1953). Baba (1956) observed that the amount of ethyl acetate formed varied in no consistent pattern with the accumulation of ethanol and acetate, indicating that something more than a simple condensation of these substances is involved in the metabolic production of ethyl acetate. It has been postulated that ethyl acetate is produced by means of the formation of an active two-carbon group such as acetylcoenzyme A, probably from ethanol, followed by a condensation reaction with ethanol to the acetate ester (Cantarelli, 1955; Peel, 1951). Calcium pantothenate, a precursor of coenzyme A, has been shown to enhance the ethyl acetate synthesis by yeast (Cantarelli, 1955). There is no evidence that the acid moiety of the ester is derived from acetate (Peel, 1951). The ethyl acetate synthesis in yeast requires oxygen; is not inhibited by low pH's (as are most esterase-catalyzed reactions) or by the general esterase inhibitor diisopropylfluorophosphate; and gives much higher yields than would be expected if the ester were formed by a simple condensation of ethanol and acetate by an esterase.

A quantitative analysis of some of the volatile compounds produced in banana pulp at different ripeness stages and under various conditions likely to affect the metabolism of the banana, such as the effect of various gases on the fruit during ripening and storage, has been recently reported (Hultin and Proctor, 1961). Table 1 shows data relating to the volatile acids, alcohols, and esters expressed in moles per kg of tissue.

The green, half-ripe, ripe, and overripe samples represent different ripeness stages of the banana. Ripening of the ethylene-ripened sample was hastened with ethylene stimulation. The scalded sample was also treated with ethylene, and in addition the carbon dioxide content of the storage atmosphere was allowed to increase above 10%. The coated sample was dipped in a mixture of acetylated monoglycerides and stored at a low temperature (13.3°C) for 4 days. The coated control was stored in the same manner but without the dip treatment. These samples and the methods used in the analyses were described in detail by Hultin and Proctor (1961).

[a] Contribution No. 1323, University of Massachusetts, College of Agriculture, Experiment Station, Amherst, Massachusetts.

Table 1. Some volatile acids, alcohols, and esters of banana pulp (moles per kg banana pulp).

	Acetic acid ($\times 10^5$)	Ethyl alcohol ($\times 10^4$)	Ethyl acetate ($\times 10^4$)	Methyl alcohol ($\times 10^4$)	Methyl acetate ($\times 10^4$)	Iso-amyl alcohol ($\times 10^4$)	Iso-amyl acetate ($\times 10^6$)
Green	4.3	2.4	25.4	6.9	6.1	0.3	1.5
Half-ripe	9.3	0.9	10.8	57.8	12.8	0.5	7.7
Ripe	8.0	1.1	[a]	4.7	1.8	0.1	1.5
Ethylene-ripened	8.3	2.8	18.0	12.5	5.4	0.5	7.7
Scalded	7.0	2.4	12.5	17.2	14.9	0.5	53.9
Overripe	26.2	49.1	19.2	17.5	6.8	6.4	92.4
Coated	10.2	162	13.5	9.4	8.1	0.8	15.4
Coated control	17.7	194	8.4	9.4	12.0	1.7	15.4

[a] No detectable ethyl ester in the ripe sample.

Apparent equilibrium constants (K) for the dissociation of the esters were calculated from the data in Table 1 as follows:

$$K = \frac{(ROH)\ \ (HOAc)}{(ROAc)\ \ (HOH)}$$

where ( ) indicates the concentration of each molecular species in moles/kg of banana pulp. The apparent equilibrium constants are presented in Table 2. In calculating these constants, it was assumed that the water content of each of the banana samples was 75%.

The calculated apparent equilibrium constants for the methyl and iso-amyl acetate esters did not vary greatly, indicating that in all samples there was a relatively constant relation between the concentrations of the ester and its component parts. This may indicate that an esterase-catalyzed reaction is possibly important in synthesis and degradation of these esters *in vivo*.

Table 2. Apparent dissociation constants (K) of volatile esters in various samples of banana pulp.

Sample	Ethyl acetate ($K \times 10^5$)	Methyl acetate ($K \times 10^6$)	Iso-amyl acetate ($K \times 10^5$)
Green	1.0	1.2	2.0
Half-ripe	1.9	10.1	1.4
Ripe	>1800[a]	5.0	1.3
Ethylene ripened	3.1	4.6	1.3
Scalded	3.2	1.9	0.2
Overripe	162	16.2	4.3
Coated	292	2.9	1.3
Coated control	427	1.5	2.2

[a] No detectable ethyl ester was present in the ripe banana pulp. On the basis of the sensitivity of the analytical methods employed, the equilibrium constant was calculated as being greater than 1800×10^{-5}.

On the other hand, the extremely large variations in the ethyl acetate dissociation constants among some of the samples led to the tentative conclusion that the formation of ethyl acetate does not necessarily depend only on the concentrations of ethanol and acetate in the tissue under all circumstances. There are three explanations that could account for this fact. The first is that equilibrium is not reached, because of an unfavorable reaction rate caused by lack of a catalyst, i.e., esterase. This is considered unlikely in view of the evidence cited above pertaining to the methyl acetate and iso-amyl acetate equilibrium data. Owing to the low specificity of the esterases (Ammon and Jaarma, 1951), it would be expected that if an esterase is present that can catalyze the formation and degradation of these esters from their respective alcohols and acetate, then it could also catalyze the formation and degradation of ethyl acetate from ethanol and acetate.

A second explanation is that the over-all concentrations of the reacting species are unrelated to the activities, or effective concentrations, in the tissue due to adsorption on cell particulate matter, concentration at interfaces or other physical phenomena that would tend to concentrate one or more of the reacting substances in one area in the cell. Again, however, the relative constancy of the equilibrium values of the other esters leads to the conclusion that this would probably not be the major factor for the large variations in the ethyl ester constant, i.e., if the methyl and iso-amyl alcohols and esters are not concentrated in one or more particular areas of the cell, there is no reason to

Table 3. Some dissociation constants (K)[a] of methyl, ethyl, and iso-amyl acetate.

Compound	K	Reference
Ethyl acetate	0.26	Taussig and Petreanu, 1956
	0.26	Glasstone, 1946
	0.23	Parks and Huffman, 1932
Methyl acetate	0.17	Taussig and Petreanu, 1956
	0.12	Friedman and Elmore, 1941
Iso-amyl acetate	2.7	Mardashev and Kuznetzoy, 1935

[a] $K = \frac{(\text{acid})\ (\text{alcohol})}{(\text{ester})\ (\text{water})}$

assume that ethanol or its acetate ester would be.

The other explanation is that, in some cases at least, a coupling reaction(s) is involved that is supplying or deleting one or more of the reactants or products of the hydrolysis at a rate that is considerably faster than the rate at which the esterases function to establish equilibrium. This would allow for the presence of at least one component of the reaction in excess of its equilibrium value, which would make the apparent equilibrium constants differ from the true dissociation equilibrium constant and might cause the variations noted among the several banana tissue samples. This seems the most probable explanation for the variability of the apparent ethyl acetate equilibrium constants. Similar anomalies for the yeast *Hansenula anomala*, as noted above, have been ascribed to an oxidative synthesis of ethyl acetate from ethanol that does not involve acetate.

One other fact that must be considered is that the apparent equilibrium constants obtained in this study showed very large variations from the true thermodynamic equilibrium constants, as calculated from approximately equimolar concentrations of all reacting species and reported by other investigators. Some of these latter constants are given in Table 3.

The major implication to be derived from the differences between the apparent equilibrium constants calculated from the data in Table 1 and the true thermodynamic equilibrium constants as expressed in Table 3 is that the calculated values may not represent a true equilibrium state. It is surprising, however, that the constants for the methyl and iso-amyl esters should be so constant under such varying conditions if a true equilibrium state is not represented. A possible explanation for these discrepancies is that in calculating the constants for the banana esters it was assumed that all the water in the tissue was free to take part in the reaction, which of course is not the actual situation, since much, if not most, of the water is bound to cellular protein or polysaccharides or is made unavailable at the reaction site by physical barriers.

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Extraction and Fractionation of Proteins in Fresh Chicken Muscle[a]

A. W. KHAN

(Manuscript received May 29, 1962)

SUMMARY

Different buffer systems were compared for efficiency of extraction of chicken muscle proteins, and a technique was developed for routine fractionation and estimation of major protein fractions in one operation. KCl-borate and KCl-phosphate buffers of pH 7.3–7.5 and $\Gamma/2 = 1.0$ gave maximum extraction, with a reproducibility of $\pm 2\%$. Protein fractionation in KCl-borate buffer showed that in one-year-old chicken meat, stroma-, myofibrillar-, and sarcoplasmic-protein nitrogen respectively contributed 13, 42, and 30% of total nitrogen in breast muscle, and 27, 30, and 22% in leg muscle. Results with different chickens showed that, with increase of age, stroma increased and myofibril decreased in both breast and leg muscle. These two protein fractions also differed for breast and leg muscle, and varied with the source of supply of the chickens. The difference was small between birds of the same flock and between left and right halves of the same bird.

INTRODUCTION

To follow changes in muscle proteins involved in quality deterioration of poultry during freezing and refrigerated storage, a routine method was required for separation and estimation of chicken muscle protein fractions. Although extensive work has been done on the extraction and fractionation of the muscle proteins of mammals (Bailey, 1954) and fish (Hamoir, 1955), little information is available on the applicability of these methods to chicken muscle and to routine estimation of stroma, myofibril, sarcoplasm, and non-protein nitrogenous materials in one operation. The work reported here compares different methods of extraction and fractionation of chicken breast and leg muscle from fresh-killed laboratory-raised 10-week-, 4-month-, and one-year-old birds, fresh-killed birds from local markets, and samples from left and right halves of the same bird.

EXPERIMENTAL METHODS

For extraction, meat samples were dispersed, made up to 100 ml volume with the extraction system under examination, and fractionated according to a scheme (Fig. 1) obtained by combining and modifying the techniques used by other workers (Bailey, 1954; Dyer *et al.*, 1950; Robinson, 1952a; Smith, 1935; Weinberg and Rose, 1960). After centrifuging the muscle suspensions, the supernatant was fractionated into non-protein and dialyzable nitrogen, actomyosin, myosin, and sarcoplasmic proteins, and the residue was fractionated into stroma (Lowry *et al.*, 1941) and denatured proteins.

Nitrogen estimations were made by a micro-Kjeldahl method (A.O.A.C., 1955). Total nitrogen was determined in 0.2–0.4-g samples of muscle. All tests were made in duplicate, and extraction, centrifugation, and fractionation were carried out at 0–5°C.

Preparation of sample. Most of the samples were obtained from chickens raised in a laboratory fieldhouse by methods comparable to good commercial practice, slaughtered in the laboratory (Weinberg and Rose, 1960), and stored in crushed ice for 24–48 hr. The breast and leg muscles were removed, freed from visible fat, tendons, and connective tissues, and minced with scissors (Robinson, 1952a).

Extraction systems. For extraction, salts known to have a depolymerizing or denaturing action on myofibrillar proteins (Dubuisson, 1950; Edsall and Mahl, 1940) were avoided. In most of the tests, buffer systems having a pH of 7.2–7.5 (Dubuisson, 1950; Edsall and Mahl, 1940) and an ionic

[a] Contribution from the Division of Applied Biology, National Research Council, Ottawa 2, Canada. Issued as N.R.C. 7016.

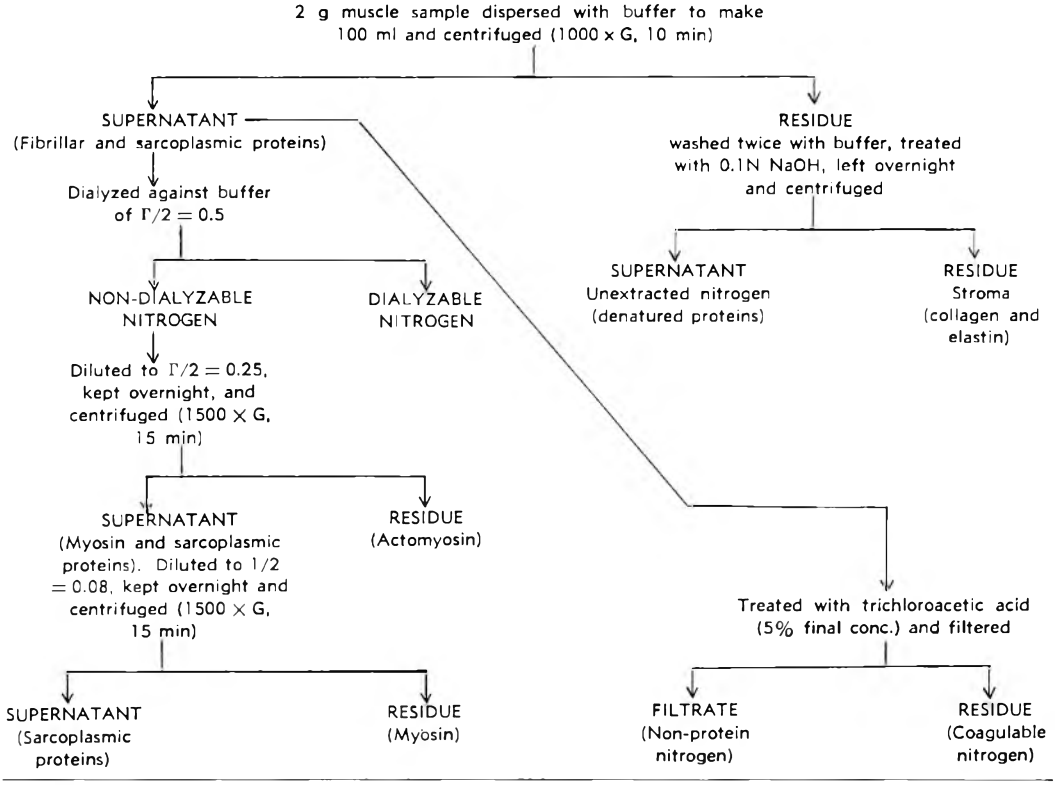

Fig. 1. Scheme for the fractionation of chicken muscle proteins.

strength ($\Gamma/2$) of 0.55 or 1.0 were used. The buffer systems selected were:

a) 0.065M H_3BO_3, 0.0013M $Na_2B_4O_7 \cdot 10\ H_2O$, KCl to give $\Gamma/2 = 1.0$, pH 7.5 (Robinson, 1952b)

b) 0.049M $Na_2HPO_4 \cdot 7\ H_2O$, 0.0045M $NaH_2PO_4 \cdot H_2O$, KCl to give $\Gamma/2 = 0.55$ or 1.0, pH 7.3 (Weinberg and Rose, 1960)

c) 0.02M $NaHCO_3$, KCl to give $\Gamma/2 = 0.55$ or 1.0, pH 7.4 (Greenstein and Edsall, 1940)

d) 0.05M sodium barbitone, KCl to give $\Gamma/2 = 1.0$, pH 7.5

e) 0.05M tris(hydroxymethyl amino methane), KCl to give $\Gamma/2 = 1.0$, pH 7.5 (Dingle and Hines, 1960)

f) 0.066M K_2HPO_4, KCl to give $\Gamma/2 = 1.45$, pH 8.5 (Robinson, 1952a)

Extraction procedure. In initial tests, conditions for maximum extraction of proteins were studied by varying: 1) weight of meat sample (1–5 g in a 100-ml suspension); 2) method of grinding (manual grinding with sand, Waring blender); and 3) ionic strength of buffers ($\Gamma/2 = 0.55$, 1.0, and > 1.0). In subsequent work, 2-g samples were ground with a pestle and 5 g of sand in a cooled mortar and made to 102 ml (including sand) with the buffer system under examination.

Fractionation procedure. For fractionation (Fig. 1), breast and leg-muscle extracts were used in KCl-borate, pH 7.5, $\Gamma/2 = 1.0$; and KCl-phosphate, pH 7.3, $\Gamma/2 = 1.0$, and pH 8.5, $\Gamma/2 = 1.45$. Dialysis was carried out against 20 volumes of a solution obtained by diluting the extractant buffer to a total ionic strength of 0.5. The solution was changed twice, and dialysis was continued with shaking for 18 hr. The difference in the amount of soluble nitrogen at $\Gamma/2 = 0.5$ and $\Gamma/2 = 0.25$ is reported as actomyosin nitrogen, and that at $\Gamma/2 = 0.25$ and $\Gamma/2 = 0.08$ as myosin nitrogen. These ionic strengths for the precipitation of actomyosin and myosin were determined in separate experiments.

RESULTS AND DISCUSSION

The amount of nitrogen extracted from chicken muscle with buffers of ionic strength 0.55 depended on the weight of the muscle sample per unit volume of buffer. With solutions of $\Gamma/2 = 1.0$ or > 1.0, however, extractable nitrogen was independent of sample weight. The nitrogen extracted (ex-

pressed as percent of total nitrogen), for example, was 54.4, 58.4, and 86.0%, respectively, when 1, 2.5, and 5 g of breast muscle were extracted with 100 ml of KCl-phosphate buffer ($\Gamma/2 = 0.55$, pH 7.3), whereas 86.7–88.4% of the total nitrogen was extracted with a similar solution at $\Gamma/2 = 1.0$, regardless of sample weight. These results are in agreement with the work of Helander (1957), who used solutions of $\Gamma/2 = >1.0$ for the extraction of myofibril from 1 g of sample, and the work of Weinberg and Rose (1960), who used 10-g samples for extraction at $\Gamma/2 = 0.55$.

The results from manual grinding of the muscle samples with sand were reproducible within $\pm 2\%$, indicating that this method was almost as effective as the exhaustive extraction procedure of Smith (1935). Dispersal of the sample with a Waring blender caused foaming and consequent protein denaturation, which was not eliminated by fitting the blender jar with a plastic plate (Dyer *et al.*, 1950). A similar attempt by Dingle *et al.* (1955) to solve this problem was also unsuccessful.

Table 1 shows the efficacy of the various buffer systems as chicken-muscle protein extractants. In breast muscle, KCl-borate buffer gave maximum extraction, whereas in leg muscle, both KCl-borate and KCl-phosphate buffers gave good results.

Table 1. Efficacy of various buffer systems as chicken muscle protein extractants ($\Gamma/2 = 1.0$, pH 7.5; muscle from 10-week-old birds).

Buffer system	Soluble nitrogen as % of total nitrogen	Stroma [a] as % of total nitrogen	Efficacy [b]
Breast muscle			
KCl–H_3BO_3–$Na_2B_4O_7$	92.4	4.9	.97
KCl–NaH_2PO_4–Na_2HPO_4	81.2		.86
KCl–$NaHCO_3$	82.8		.87
KCl–tris(hydroxymethyl) amino methane	69.0		.73
KCl–sodium barbitone	76.5		.81
Leg muscle			
KCl–H_3BO_3–$Na_2B_4O_7$	75.6	18.8	.93
KCl–NaH_2PO_4–Na_2HPO_4	74.7		.92
KCl–$NaHCO_3$	32.9		.40

[a] Average results.

[b] Efficacy = extracted nitrogen/(total nitrogen–stroma nitrogen).

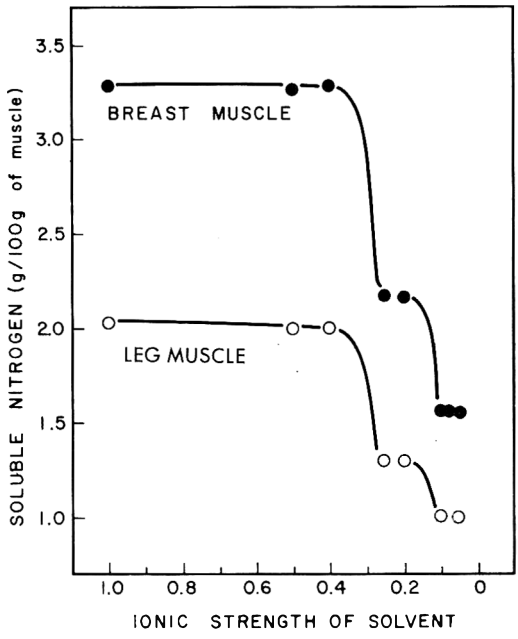

Fig. 2. Effect of gradual dilution on the solubility of chicken muscle proteins in KCl-borate buffer at pH 7.5.

The solubility-ionic strength relation of actomyosin and myosin was established by serial dilution of breast and leg muscle extracts from $\Gamma/2 = 0.50$ to $\Gamma/2 = 0.05$. The precipitation occurred in two steps (Fig. 2): in the first step the precipitation of actomyosin was completed at $\Gamma/2 = 0.25$, and in the second the precipitation of myosin was completed at $\Gamma/2 = 0.08$. These results are in good agreement with those Weinberg and Rose (1960) obtained with breast muscle. Fig. 2 also indicates a similarity of the two kinds of muscle in precipitation behavior.

Table 2 gives the results of extraction and fractionation of major protein fractions of chicken breast and leg muscle in: a) KCl-borate, $\Gamma/2 = 1.0$, pH 7.5; b) KCl-phosphate, $\Gamma/2 = 1.0$, pH 7.3, and c) KCl–phosphate, $\Gamma/2 = 1.45$, pH 8.5. Buffer c, used by Robinson (1952a) for measuring changes in the protein composition of chick muscle, was used for comparison. As would be expected (Dubuisson, 1950), the results depended to some extent on the buffer system

Table 2. Effect of buffer systems on the fractionation of chicken muscle extract.[a]

Buffer system	Extractable		Non-protein	Myofibrillar			Sarcoplasmic
	Weight (g/100 g)	% of total nitrogen		Total	Actomyosin	Myosin	
			(g nitrogen/100 g of muscle)				
Breast muscle							
KCl-borate $\Gamma/2 = 1.0$, pH 7.5	2.82	75.6	0.58	1.25	0.92	0.33	0.98
KCl-phosphate $\Gamma/2 = 1.0$, pH 7.3	2.80	75.0	0.58	1.27	0.88	0.39	0.95
KCl-phosphate $\Gamma/2 = 1.45$, pH 8.5	2.86	76.6	0.59	1.30	.....	.....	0.96
Leg muscle							
KCl-borate $\Gamma/2 = 1.0$, pH 7.5	2.16	67.7	0.35	1.11	0.90	0.21	0.69
KCl-phosphate $\Gamma/2 = 1.0$, pH 7.3	2.15	67.4	0.35	1.14	0.82	0.32	0.66
KCl-phosphate $\Gamma/2 = 1.45$, pH 8.5	2.12	66.4	0.35	1.11	.....	.....	0.66

[a] One-year-old chicken; weight after evisceration, 3400 g.

used, but the agreement of the data for the different systems was close enough to indicate that the fractionation and estimation technique would be satisfactory in more extensive investigations of muscle proteins. KCl-borate buffer was selected for further work because it gave maximum extractability, and the myofibrillar protein fractions obtained were suitable for the determination of adenosinetriphosphatase activity.

Using the conditions giving maximum extraction with KCl-borate buffer (pH 7.4–7.5, $\Gamma/2 = 1.0$), a study was made to determine variations between left and right halves of a bird, between birds from a single flock, effect of age and origin of bird, and amount of the various protein components in breast and leg muscle. Table 3 shows results for breast and leg muscle from birds of different ages but from the same flock. The variability between birds of one flock was small, and right and left halves of the same bird varied less than 3%. With increasing age, stroma increased and myofibril decreased, but non-protein nitrogen and sarcoplasm did not change appreciably. Depending on the age of the chickens, leg muscle had 2–3 times as much stroma nitrogen as breast meat, but less total, myofibrillar, sarcoplasmic, and non-protein nitrogen. Content of the different protein fractions of birds from different sources varied from 10 to 25%, presumably because of differences in breed, nutritional state, and pre- and post-slaughter conditions.

ACKNOWLEDGMENT

The author gratefully acknowledges the skilled technical assistance of Mr. G. W. Daechsel.

Table 3. Protein components of chicken muscle.[a]

Muscle	Breast		Leg	
	4 mo.	1 yr	10 wk	1 yr
Average weight after evisceration (g)	1650	2485	723	2485
Number of birds	6	3	3	3
Total nitrogen (g/100 g muscle)	3.77±0.08	3.93±0.11	3.23±0.06	3.39±0.06
Extractable N (g/100 g muscle)	3.29±0.07	3.20±0.15	2.45±0.04	2.30±0.11
Extractable N (% of total N)	87.2	81.4	75.5	67.8
Stroma (g/100 g muscle)	0.39±0.12	0.52±0.12	0.61±0.16	0.93±0.14
Stroma (% of total N)	10.3	13.2	18.8	27.4
Non-protein N (g/100 g muscle)	0.60±0.03	0.63±0.14	0.41±0.03	0.39±0.02
Non-dialyzable N (g/100 g muscle)	2.64±0.08	2.38±0.01	2.04±0.03	1.84±0.03
Myofibrillar N (g/100 g muscle)	1.70±0.06	1.52±0.05	1.45±0.10	1.34±0.16
Actomyosin N (g/100 g muscle)	1.19±0.06	1.29±0.14	1.04±0.02	1.06±0.14
Myosin N (g/100 g muscle)	0.51±0.10	0.23±0.10	0.41±0.08	0.27±0.05
Sarcoplasmic N (g/100 g muscle)	0.94±0.12	0.86±0.10	0.58±0.11	0.50±0.17

[a] Extractant, KCl-borate buffer, $\Gamma/2 = 1.0$, pH 7.4–7.5; all birds from one flock raised in the laboratory under conditions comparable to good commercial practice.

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The Flavor Spectrum of Apple Wine Volatiles. II. Volatile Fatty Acids[a]

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(Manuscript received April 8, 1962)

SUMMARY

This paper describes the separation and partial identification of 10 volatile fatty acids present in apple wine. The acidic fraction was prepared by "stripping" the wine in a rising film evaporator and separating the volatile acids from the strippings by appropriate pH adjustment, steam distillation and extraction. When the volatile acids of apple wine were examined by gas chromatography, using Ucon 50-HB-2000 and Ucon-nonpolar, ten distinct peaks were revealed. In addition the volatile acids were methylated with diazomethane, and the composition of the methyl ester fractions was studied by gas chromatography using diethylene glycol succinate and Carbowax-400.

The chemical composition of the volatile components of various food products has recently become a topic for many research investigations. The chief concern in this laboratory has been to define and differentiate flavors and off-flavors in apple wine. In previous work (Matthews *et al.*, 1962) we reported the isolation and identification of 18 compounds present in the volatile fraction of apple wine, but did not detect an acidic fraction when a column packing consisting of Carbowax-400 on firebrick C-22 was used. Subsequent to that investigation of the volatile ethyl-chloride-extractable components of apple wine, the possibility of the presence of volatile free acids was brought to our attention.

Similar studies have been conducted on the volatile compounds of beer (Jenard, 1959; Van der Kloot *et al.*, 1958), brandy (Bouthilet and Lowrey, 1959), grain spirits (Austin and Boruff, 1960), whiskey (Caroll and O'Brien, 1959), and other fermented food products (Baraud and Genevois, 1958; Mecke and deVries, 1959).

Volatile fatty acids in food have been examined by gas chromatography (Hunter *et al.*, 1961; Hughes, 1960; Hawke, 1957; Vorbeck *et al.*, 1961; Coffman *et al.*, 1960; Diemain and Schams, 1960).

In addition, several volatile acids that are components of apple juice volatiles have been identified by chemical methods (White, 1950; Willaman, 1950). However, because of varietal and cultural differences as well as changes taking place during fermentation it would not be of any significance to compare the results of Willaman and White with those obtained in the present study.

EXPERIMENTAL

Preparation of sample for gas chromatography. Apple wine was made from Duchess apples by the rapid method of Atkinson *et al.* (1959).

The volatiles were concentrated approximately fivefold by "stripping" in a rising film evaporator and condensing in a two-stage heat exchanger. The first stage was cooled with tap water, and the second with ice water. The wine residue, after stripping, had almost no odor. The condensate was held at 0°C until required for separation of the acidic fraction.

Before separation by gas chromatography the acids had to be concentrated and transferred to a non-aqueous medium. The most convenient method for separating the volatile acids from apple wine strippings is by steam distillation, the technique

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used in all the methods developed for estimating the volatile acidic fraction.

The volatile acidity of apple wine strippings was determined by the method described by Ruck (1956). Strippings analyzed 0.011% volatile acid, and the stripped portion 0.012%.

The volatility of fatty acids in steam increases with increasing molecular weight or carbon number. For the lower numbers, especially formic acid and acetic acid, steam distillation has to be continued for a considerable time and a large volume of distillate collected before a quantitative recovery of these acids can be achieved.

The possible contribution of ester saponification to the volatile fatty acid fraction during stripping and distillation was examined. Methyl and ethyl formate are hydrolyzed by boiling in water. However, neither of these two compounds or methyl acetate has been found in apple wine. Ethyl acetate, which is present in apple wine, was subjected to the following treatment: A 1% solution was adjusted to pH 8.1 with 10% Na_2CO_3 and steam distilled for 30 min. The residue was adjusted to pH 1.0 with 18*N* H_2SO_4, steam distilled, and extracted with ethyl chloride. Gas chromatography of the extract did not show any hydrolysis products.

The acidic fraction was prepared as follows: One liter of the apple wine strippings containing the neutral and acidic fractions was adjusted to pH 8.1 by addition of 10% Na_2CO_3. The neutral fraction was removed by steam distillation for 30 min.

The volatile acidic fraction was recovered from the alkaline aqueous solution by adjusting the pH to 1.0 with 18*N* H_2SO_4 and then distilling with steam. The condensate was saturated with NaCl, and the acids were extracted by the procedure described by Matthews *et al.* (1962), in which 150 ml of the aqueous solution of volatile acid was extracted with 15 ml of ethyl chloride at 0°C for 30 min on a mechanical shaker.

About 0.1 ml of a yellow-colored viscous liquid with a strong acidic odor was obtained from 1 L of apple wine strippings. This extract was used as the sample.

Gas chromatography. The concentrates of the acidic fraction were examined with a Beckman GC-2 gas chromatograph.

A 4-ft × ¼-in. column was used with four packings:

a) 10% Ucon 50-HB-2000 on firebrick C-22.
b) 10% Ucon-nonpolar on Chromosorb W.
c) 10% Carbowax-400 on firebrick C-22 pretreated with 2% phosphoric acid.
d) 10% Carbowax 20M on firebrick C-22 pretreated with 2% phosphoric acid.

Under these conditions, separation of mixtures of acids in the range C_1 to C_8 was achieved in 70 min as illustrated in Fig. 1 and Table 1. Results, in terms of maximum number of fractions and degree of separation, were good with Ucon 50-HB-2000 and Ucon-nonpolar at 160°C. The other columns could separate acids in the range C_1–C_5; however, it was necessary to increase the column temperatures to separate the acids of high

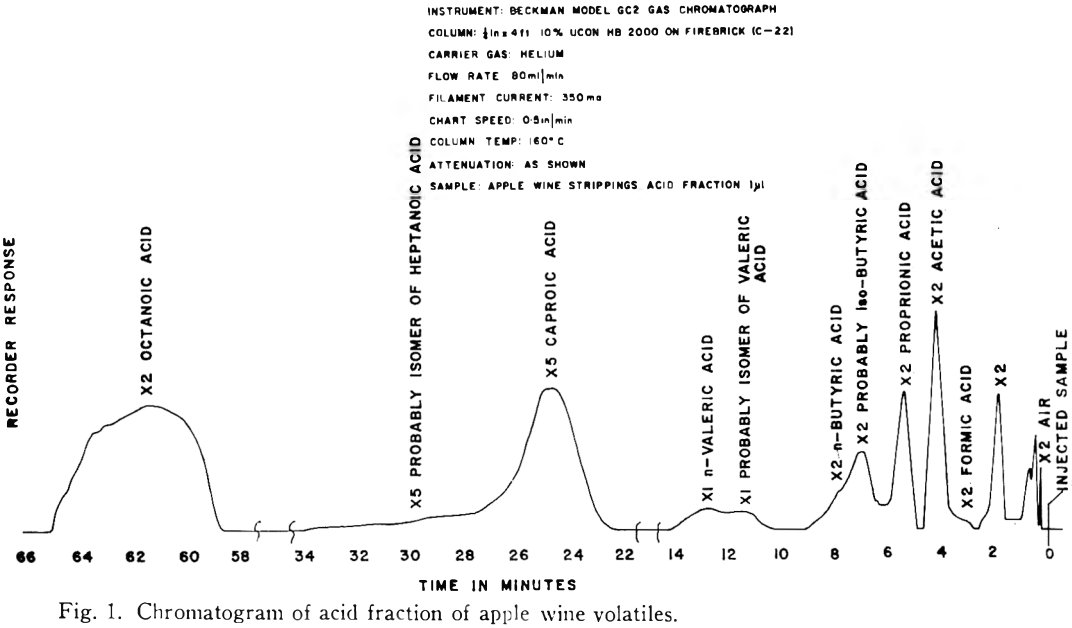

Fig. 1. Chromatogram of acid fraction of apple wine volatiles.

Table 1. Relative retention volumes[a] of standard fatty acids and components of acidic fraction of apple wine in two columns.

	Column A[b]		Column B[c]	
	Standard	Sample	Standard	Sample
Formic	0.61	0.61	0.37	0.37
Acetic	0.69	0.72	0.48	0.48
Propionic	1.00	1.00	1.00	1.00
iso-Butyric	.....	1.38	.....	1.26
n-Butyric	1.47	1.53	1.55	1.54
Isomer of valeric[d]	.....	2.10	.....	2.13
iso-valeric	2.06	.....	2.18	.....
n-Valeric	2.70	2.55	2.50	2.50
n-Caproic	4.85	4.76	4.80	4.81
Isomer of heptanoic[d]	.....	6.60	.....	6.60
n-Heptanoic	7.48	.....	7.60	.....
n-Octanoic	10.62	10.62	10.26	10.37

[a] Retention volumes relative to propionic acid.

[b] A; 4-ft × ¼-in. column, 10% Ucon 50-HB-2000 on firebrick C-22, mesh 30-60, 160°C, He 80 ml/min, 350 ma. Aerograph A-90-P, Thermistor detector.

[c] B; 4-ft × ¼-in. column, 10% Ucon-nonpolar on Chromosorb W, acid washed, mesh 60-80, 160°C, He 40 ml/min, 350 ma. Aerograph A-90-P, Thermistor detector.

[d] Boiling point indicates probably iso- form.

molecular weight. Carbowax-400 could not be used above 150°C, because of excessive "bleeding."

The exact experimental parameters of operation, i.e., temperature, flow rate of carrier gas, etc., accompany the figures and tables (1 and 2).

Fatty acids are usually identified as their esters by gas chromatography. This technique was also used on the methyl esters of the acidic fraction to confirm this work. After removing the remaining neutral fraction, the fatty acids from apple wine volatiles were methylated with diazomethane using the ether solution of diazomethane prepared from nitrosomethylurethan in the usual manner.

The ether solution of the methylated sample was injected into the gas chromatograph, and the composition of the methyl ester fraction was studied with an Aerograph A-600-B Hydrogen Flame Ionization Detector. Column packings were of the two types: 10% diethyleneglycol succinate on Chromosorb W, and 10% Carbowax-400 on Chromosorb W. A chromatogram of the methyl esters is shown in Fig. 2.

RESULTS AND DISCUSSION

The main portion of the neutral components was removed by a 30-min steam distillation, but a small amount still remained

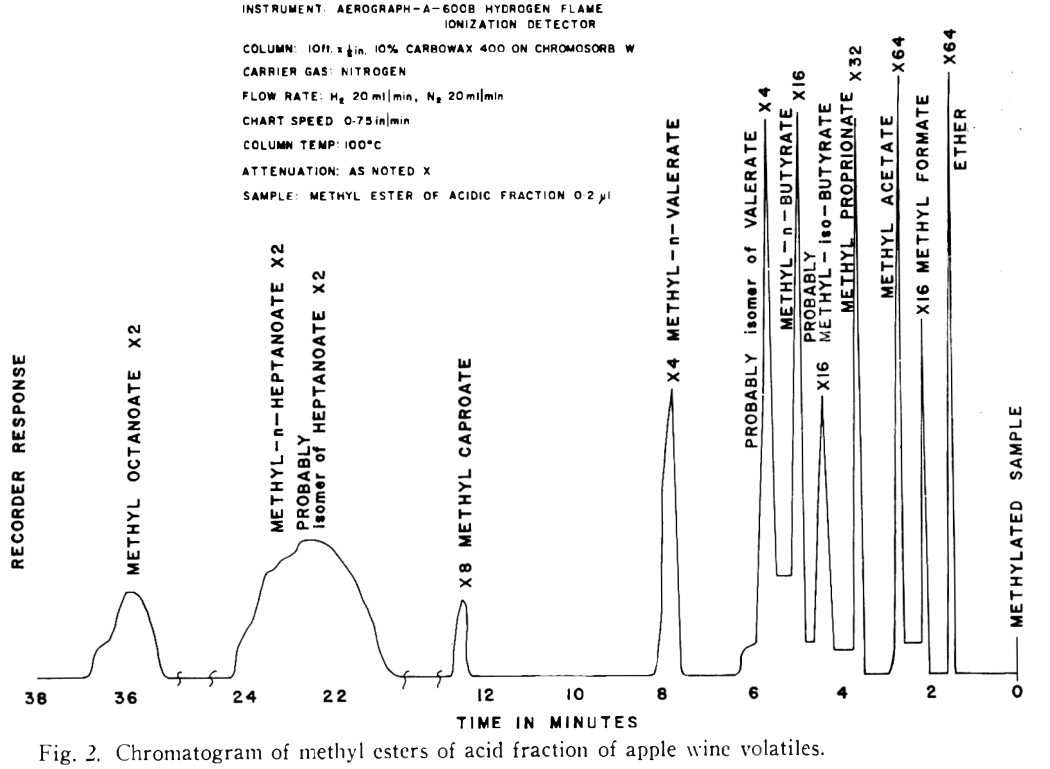

Fig. 2. Chromatogram of methyl esters of acid fraction of apple wine volatiles.

in the residue. The gas chromatographs of neutral and acidic fractions were examined under the same conditions, and their chromatographic patterns compared. The neutral components that remained were eluted faster than formic and acetic acid in these columns.

Examination of the volatile-acid fraction in apple wine strippings revealed that there are 10 distinct peaks in the chromatograms when separated by Ucon 50-HB-2000 on firebrick C-22 and Ucon-nonpolar on Chromosorb W. In previous work (Matthews *et al.*, 1962) the components of the neutral fractions were isolated individually and identified by their retention times and at least one or more of three techniques, i.e., infrared spectrum, paper partition chromatography, or derivative formation.

Volatile acids could not be obtained in sufficient amounts for identification by any of the above three methods; consequently, they were identified by determining the corrected retention times and volumes of known fatty acids and comparing these with those of the unknown peaks. These results are shown in Table 1. The retention volumes were confirmed by determining them with two different columns, and also by examining the correlation between retention times, boiling point, and carbon number, as shown in Figs. 3 and 4. If the stationary phase has a low or no polarity and the sample components have a relatively high polarity, the components are separated on the basis of boiling-point difference rather than differences in polarity, as described by Theron (1959).

Attention may be directed to the peaks of the isomers of valeric and heptanoic acid. Because of their many isomeric forms, only iso-valeric acid was examined by the columns described above. These compounds were estimated by using their boiling points derived from the relation between retention times and boiling points. They agreed well with those of the knowns. However, the boiling points determined from the correlation line are approximate values, and consequently are noted as probable.

For the separation of lower fatty acids, a column packing coated with phosphoric acid can be used. Metcalf (1961) reported that a column packing consisting of a polar sub-

Table 2. Relative retention volumes[a] of the methyl esters of standard fatty acids and methyl esters of components of acidic fraction of apple wine.

	Column C[b]		Column D[c]	
	Standard	Sample	Standard	Sample
Formic	0.62	0.59	0.45	0.45
Acetic	9.76	0.76	0.70	0.70
Propionic	1.00	1.00	1.00	1.00
iso-Butyric	.....	1.24	.....	1.25
n-Butyric	1.38	1.38	1.45	1.50
Isomer of valeric[d]	.....	1.60	.....	1.75
iso-valeric	1.57	.....	1.75	.....
n-Valeric	2.19	2.19	2.60	2.65
n-Caproic	3.61	3.58	4.80	4.80
Isomer of heptanoic[d]	.....	5.85	.....	6.40
n-Heptanoic	6.28	6.30	8.00	.....
n-Octanoic	11.50	11.40	17.10	17.00

[a] Retention volumes (cc N_2) relative to methyl propionate.

[b] C; 10-ft × ⅛-in. column, 10% Carbowax-400 on Chromosorb W, acid washed, mesh 60–80, 100°C, H_2 20 ml/min, N_2 20 ml/min: Aerograph A-600-B, Hydrogen Flame Ionization Detector.

[c] D; 5-ft × ⅛-in. column, 10% DEGS on Chromosorb W, acid washed, mesh 60–80, 50°C, H_2 20 ml/min, N_2 20 ml/min: Aerograph A-600-B, Hydrogen Flame Ionization Detector.

[d] Boiling point indicates probably iso- form.

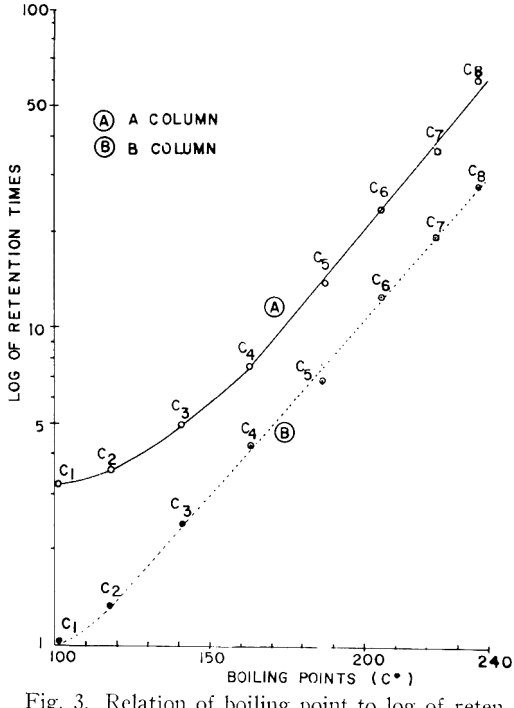

Fig. 3. Relation of boiling point to log of retention times for C_1 to C_8 normal fatty acids.

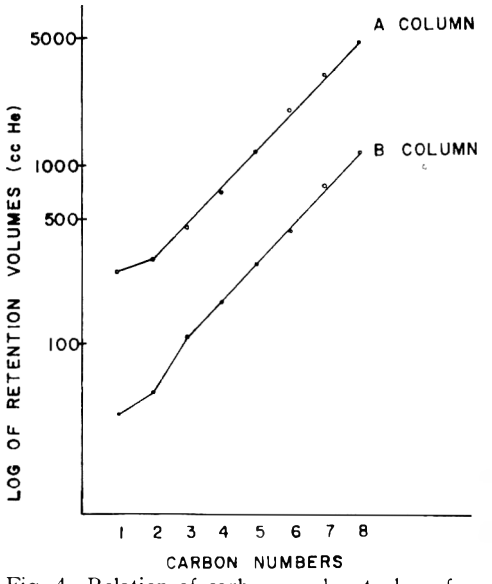

Fig. 4. Relation of carbon number to log of retention volume for C_1 to C_8 normal fatty acids.

stance and phosphoric acid were excellent, and a good quantitative analysis could be made. Jowett and Harrocks (1961) reported good separation of fatty acids on a column packing consisting of glass beads, polyethyleneglycol, and phosphoric acid.

The acidic fraction was examined using a stationary phase consisting of Carbowax-400 or 20M and phosphoric acid. Acids in the range of C_2–C_5 were separated at 160°C, but those of C_6 or over moved through the column much more slowly than had been expected. Cross plotting or two-dimensional plotting is one of the simplest ways of obtaining a second parameter for peak identification. A cross-plot was made by plotting the log of the relative retention times of a sample run on one column against the log of the relative retention times of the same sample on a second column.

The cross-plot (Fig. 5) related 11 compounds on Columns A and B.

If two points line up on a horizontal line, it means that these compounds cannot be separated by column B. Similarly, if two points line up on a vertical line these two cannot be separated by column A. If two compounds form a single point on the cross-plot, it is likely that the compounds are identical or very closely related chemically. From the cross-plot (Fig. 5) it is possible to use the results obtained by the two columns for identification of fatty acids.

Good separation of fatty acids at least to C_8 was obtained by simple steam distillation without the added step of methylation. Comparison of results with free fatty acids and their methylated counterparts (Fig. 2 and Table 2) showed the same peaks on various packings except that methyl-*n*-heptanoate was found only on column C. Plotting of the log of retention times on two different columns or against carbon number of the acid gave essentially straight lines, and the location of a peak on this line is helpful in identifying unknown acids.

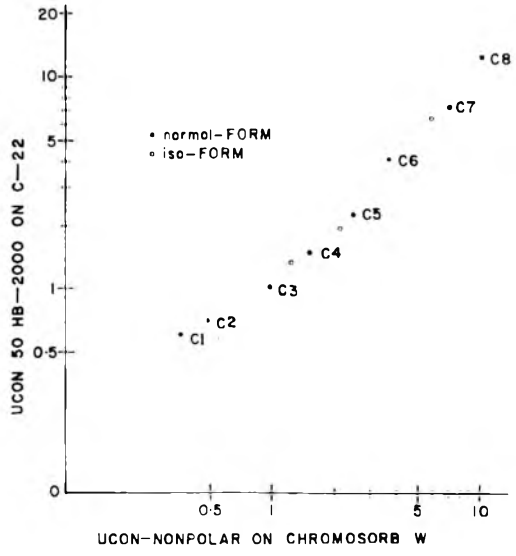

Fig. 5. Cross plot of log retention times of fatty acids on two different columns.

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Infrared Spectra of Some Pectic Substances[a]

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(Manuscript received May 4, 1962)

SUMMARY

Infrared spectra for a number of commercial types of pectic substances were determined with a film technique used elsewhere on gums and pectin. It is possible to distinguish between pectins with high methoxyl and with low methoxyl and polygalacturonic acid. Although the spectrum for the only apple pectin examined differed in some respects, particularly at longer wavelengths, from citrus pectin spectra, additional samples should be examined with higher resolution before conclusions are made about fruit sources. Results now reported should be of use in general qualitative identification of pectic substances. Improvements in equipment and techniques are suggested.

INTRODUCTION

During the century or more that the pectic substances have been known and studied as important constituents of fruits and vegetables, investigators have sought new and improved methods for their characterization. Many gravimetric and colorimetric chemical methods are now available for the quantitative estimation of these substances. Certain combinations of these methods have proved effective in identifying pectic substances, but they usually require much time and rather large samples.

Because of the increasing use of pectic substances in foods and pharmaceuticals and the commercial appearance of several modifications of pectin, rapid optical methods for specific identification and possible quantitation are currently of considerable interest. Measurements of the index of refraction and of streaming birefringence of pectin sols have been of little value. Studies of the Tyndall effect in pectin sols have been useful for estimating molecular-weight averages, and X-ray studies have given an insight into the spatial structure of the pectin molecule.

The widespread availability of spectrophotometers suitable for use in recording infrared radiation in the range of 2–15 μ made possible a new technique for the identification of many water-soluble gums and related natural colloids that are capable of forming films. This film-forming property is especially fortunate since these materials are insoluble in the usual solvents used in infrared spectrometry. Newburger *et al.* (1952, 1953), who developed the film technique, applied it to more than 40 samples of gums and gum-like materials found in foods, and also studied one pectin sample designated as "Pectin, Citrus Practical," and another as "Pectin, Pure Citrus" of 1942.

Solms *et al.* (1954) published infrared spectra obtained on Nujol mulls of monomeric and polymeric galacturonic acids and their methyl esters and amides. The nature of the pectin particle, however, is such that it is not possible to utilize the mull technique or the potassium bromide disc technique. McNulty (1960), using the film technique of Newburger *et al.* (1952, 1953), devised a method for the isolation and detection of gums in frozen desserts. He found an ice cream mix in which pectin was present, and was able to identify it by comparison with the infrared absorption curves published earlier (Newburger *et al.*, 1952, 1953).

Presented herein are the infrared spectra of current commercially available pectins, citrus and apple, slow and rapid set, and

[a] Prepared for presentation at the 22d Annual Meeting of the Institute of Food Technologists, at Miami Beach, Florida, June, 1962.

[b] Present address is Orland, California.

of some commercial derivatives or modifications of pectin. Investigators relying entirely upon the single infrared spectrum for commercial pectin published by McNulty (1960) and for spectra of the two pectin samples now 20 years old, used by Newburger *et al.* (1952, 1953), as "standards" for comparison in identifying film-forming substances found in foods, might fail to classify as "pectin" one of the newer modifications whose infrared spectrum differs in the 5.5–6.5-μ region from the Newburger, Solms, or McNulty published spectra.

EXPERIMENTAL METHODS

The method used to prepare the films for infrared examination was essentially as described by Newburger *et al.* (1952, 1953) and by McNulty (1960). The only deviations were in the amounts of pectin used and the technique used in preparing the sols from which the films were made. With unstandardized pectin, 200 mg of material were dissolved in 33 ml of water, and with standardized pectins, 300 mg of material were dissolved in 33 ml of water. (Commercial pectin is considered "standardized" when its jelly-forming ability has been reduced to some fixed value, generally by blending with sugar.) The sols were prepared as follows: the dry pectin was sprinkled or dusted slowly into the water during vigorous stirring. The dispersion was allowed to stand 2 hr with occasional stirring to ensure complete hydration of the pectin.

The films made from the sols prepared in this manner, after drying were placed between sodium chloride plates, and their spectra were determined with a Beckman Model IR-5 infrared spectrophotometer. Spectra were determined on the commercial pectins listed in Table 1, and on some of them after applying various treatments such as

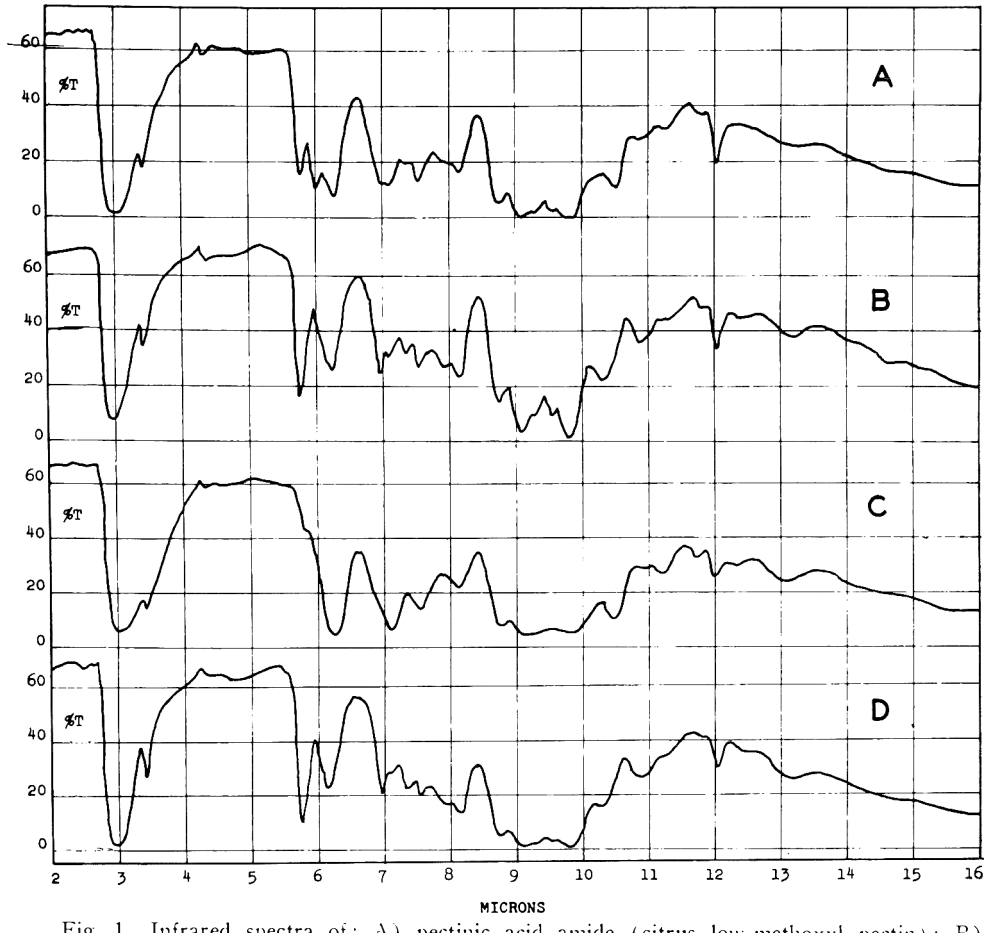

Fig. 1. Infrared spectra of: A) pectinic acid amide (citrus low-methoxyl pectin); B) citrus pectin, N.F. quality; C) sodium polypectate; D) apple pectin.

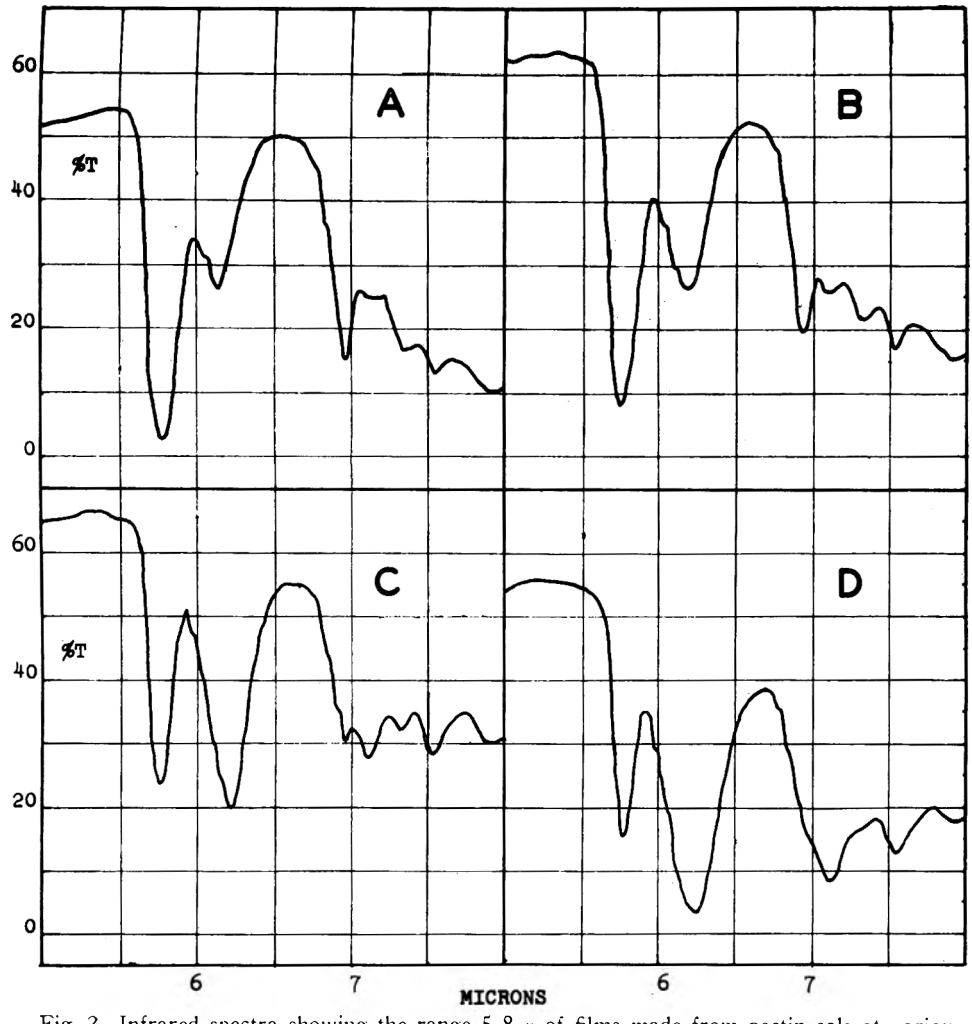

Fig. 2. Infrared spectra showing the range 5–8 μ of films made from pectin sols at various pH levels. A) pH 2.92; B) pH 3.48; C) pH 8.3; D) pH 10.2

alcohol washing, acid-alcohol washing, addition of standardizing sugar, and prolonged heating of the films. Spectra were also determined on films made from sols at various pH levels, prepared from acid-alcohol-washed N.F. pectin (National Formulary, 11th Edition, 1960).

RESULTS AND DISCUSSION

The first three spectra in Fig. 1 are for a pectinic acid amide (citrus low-methoxyl pectin), citrus pectin N.F. quality, and a sodium salt of pectic acid (sodium polypectate). The fourth spectrum of Fig. 1 is for a commercial apple pectin. These spectra cover the range of 2–16 μ. Fig. 2 shows the spectra over the range of 5–8 μ of an acid-alcohol-washed citrus pectin (N.F. quality); the films were prepared from sols at different pH levels. The spectra of some pectins with different setting times are shown in Fig. 3.

Examination of these spectra reveals that they are identical for most pectins. The absorption bands most common to all of the materials are the OH-stretching vibrations at about 3 μ, the C=O-stretching vibration of the free and esterified carboxylic acid at 5.75 μ, the two bands at 6.25 and 7.10 μ, which correspond to the asymmetrical and symmetrical vibrations of the COO^- structure, and the band at 6.95 μ due to the symmetrical bend of the carbomethoxy methyl group.

The absorption at 6.25 μ has been attributed mainly to bound water, although it was surmised that the amide linkage as well as metallic carboxylic salts may play a role (Newburger *et al.*, 1953). The infrared spectrum of a pectin film was found to be unchanged after heating the film for 2 hr at 105°C. This seems to indicate that bound water may not be responsible for the band at 6.25 μ, although further work will be necessary to ascertain the identity of this absorption peak.

Table 1. List of pectic materials examined.

Pectic material	Source [a]
Pectin, citrus, N.F.	A
Pectin, citrus, low methoxyl	A
Pectin, citrus, very rapid set	A
Pectin, citrus, rapid set	A
Pectin, citrus, slow set	A
Pectin, citrus, rapid set [b]	A
Pectin, citrus, slow set [b]	A
Pectin, citrus, dispersible	B
Pectin, citrus, rapid set	B
Pectin, citrus, rapid set	C
Pectin, citrus, medium set	D
Pectin, apple, rapid set	E
Sodium polypectate, citrus	A

[a] The various manufacturers or distributors from whom the samples originated.
[b] Blend of alcohol-precipitated and metal-salt-precipitated pectins.

Low-methoxyl pectins prepared according to Bryant (1949) show three absorption bands in the 5.75–6.25-μ region. The bands at 5.75 and 6.25 μ have already been mentioned. The third band is due to the primary amide C=O-stretching vibration. The absorption band due to the carbomethoxy methyl group, as is to be expected, is considerably less in low-methoxyl pectins than in the rest of the pectins.

The spectrum of sodium polypectate reveals only three major bands: the hydroxyl-stretching vibration at about 3 μ, and the asymmetrical and symmetrical vibrations of the carboxylate ion at 6.25 and 7.10 μ.

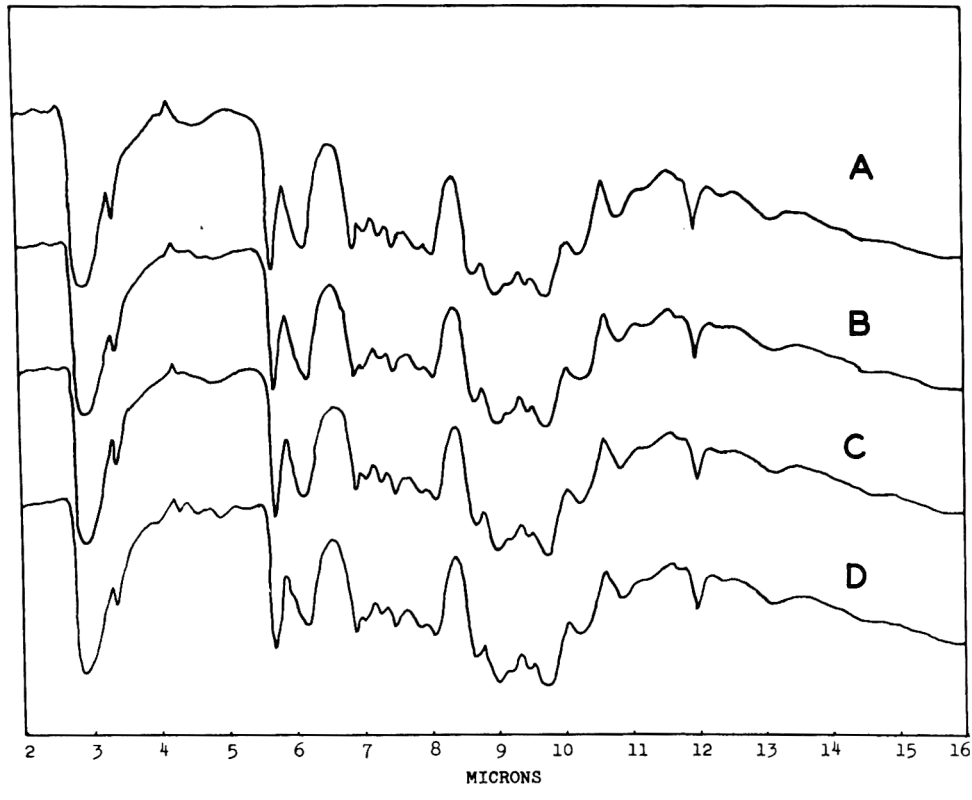

Fig. 3. Infrared spectra of pectins with different setting times. A) very rapid set; B) rapid set; C) medium set; D) slow set.

The spectra of alcohol-washed pectins were virtually identical with those of untreated pectin. Addition of standardizing sugar to N.F. pectin caused no appreciable differences in the major absorption bands.

The 9–10-μ region shows some fairly well defined bands for N.F. pectin. This region could not be resolved for other pectins. Thinner films is not the answer to this, since they become opaque when removed from the glass plate on which they are prepared. A possible solution to this problem is to form a thin film directly on the window of a demountable cell, made of "Irrtran–2" (Eastman Kodak) plastic material.

In a series of preliminary experiments to see whether the film technique could be used for semi-quantitative determination of methoxyl content, amide content, salt content, and percentage esterification of pectic materials, films were prepared at various pH levels from sols made from acid-alcohol-washed N.F. pectin. Fig. 2 shows the spectra of these films covering the region of 5–8 μ.

The carbonyl band of the free carboxylic acid and ester is large at low pH, decreasing as the pH is increased. The two bands due to the carboxylate ion increase at higher pH, and the band due to the carbomethoxy methyl group decreases. These spectra show very clearly the decrease of free acid and ester and the increase of the pectinate anion. This points out the fact that it may be possible to obtain certain quantitative or semi-quantitative values from infrared spectroscopic data of films of pectic materials. Unfortunately the authors were not able at this time to correlate the data with actual chemically determined values. The samples were too small for chemical analyses, and not all the films were of equal thickness. It will be necessary to find a suitable technique to measure the thickness of the films or to find a suitable material for internal compensation before an attempt can be made to correlate infrared data with chemically determined values.

Aside from the fact that this method may be used for quantitative work, it is clear that previously published spectra of "pectin" are not sufficient for qualitative work. It has become necessary to determine spectra of the newer modifications of pectin if one wishes to identify an unknown as being a gum or pectin by direct comparison of the spectra.

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Phenolic Compounds of Commercial Wheat Germ

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SUMMARY

Two related glycoflavones (flavonoids A and B) have been isolated from commercial wheat germ samples, where they occur to the extent of some 0.2–0.3%. Each consists of an apigenin nucleus to which is attached a highly hydroxylated glycosyl-type side chain. Evidence from ultraviolet spectrophotometric studies and from similarities with known glycoflavones suggests that the side chain is in the 8-position. Short hydrolysis of flavonoid A splits off a molecule of sinapic acid, leaving flavonoid B, while more prolonged mineral acid treatment of both flavonoids leads to partial formation of a third glycoflavone. In addition to the glycoflavones, ferulic acid and vanillic acid have been found in wheat germ in the free form. The presence of a methoxy hydroquinone glycoside has also been indicated.

INTRODUCTION

The lack of much definite information about the phenolic components of cereals is due mainly to the occurrence of only small amounts of such compounds, making it difficult to isolate quantities of these substances sufficient for detailed investigation. In the last few years, however, advances have been made with studies of the phenolic compounds of barley, both in the plant and in the grain. Harris and Ricketts (1958) have isolated vanillic acid, ferulic acid and *p*-coumaric acid from crude malt husk, and have shown that the bulk of the polyphenols of malt, located in the pericarp, consist essentially of flavone derivatives. More recently, Wall *et al.* (1961) have shown the presence of the above phenolic acids and *p*-hydroxybenzoic acid, concentrated in the husk fraction of pearled barley. Van Sumere *et al.* (1958) reported that appreciable amounts of sinapic acid occur in the husks of winter barley, and Cook and Pollock (1954) showed that certain aromatic acids exert an inhibitory effect on the germination of barley grain. Vanillic acid is known to be one such compound.

The presence of flavonoid compounds in wheat was reported first by Markley and Bailey (1935).

The only flavone so far characterized from wheat is tricin (5,7,4′-trihydroxy 3′,5′-dimethoxy flavone) isolated from *Triticum dicoccum* (var. Khapli) by Anderson and Perkin (1931) to the extent of 1.3 g from 26.5 lb of leaves. Tricin is insoluble in water, but Anderson also reported that a water-soluble pigment of the flavone class exists in the variety investigated. He suggested that this pigment may be a mixture of two or more flavone glucosides. Later, Anderson (1932) found the same flavone in var. Marquis, though in lesser amount: 0.06 g of pure tricin was isolated from 30 lb of leaves. Lewicki (1929) studied the composition of boiling-water extracts of various wheat species and concluded that flavones were present in the free state in the glumes of red varieties. He also reported the presence of flavone glucosides in white glumes. Both free flavones and flavone glucosides were present in black glumes. Anthocyanin pigments were claimed to be of widespread distribution and seemed to be related to the origin of the wheat. The pigments occurred only in the cell contents of the outer epidermis, and when much pigment was present it was in the parenchymatous tissue of the glume and in the inner bundle. The above identifications were put

forward on the basis of qualitative tests, and no one compound was identified. Seikel and Geissman (1957) showed that the leaves of barley seedlings contained saponarin, the 7-glucoside of an unusual type of flavone, vitexin. The structure of vitexin has been established by Evans *et al.* (1957) and Briggs and Cambie (1958) as having an apigenin nucleus to which is attached at the 8-position an hydroxylated tetrahydrofuran ring. Further examination of barley by Seikel and Bushnell (1959) has revealed the luteolin analog of saponarin, lutonarin, in the leaves.

The presence of flavonoid compounds other than tricin in wheat was observed by Daniels (1955) in this laboratory, working with commercial wheat germ. He separated two water-soluble pigments, both giving bright-yellow indicator solutions with alkalis, and he suggested that the pigments were flavonoids. The isolation of these compounds has now been repeated and their chemistry examined in detail, confirming their essentially flavone-like nature. A preliminary communication (King, 1961) has reported the method of their isolation and qualitative behavior to various diagnostic reagents, and has suggested that they belong to the "glycoflavone" class, typified by vitexin.

METHODS

Materials. Isolation of the flavonoids was carried out with samples of commercial wheat germ. These were derived from a mixed grist containing 60% Manitoba wheat, 40% English wheat. It is not claimed that the embryo was entirely uncontaminated with endosperm or bran, but this does not invalidate the results. Various endosperm and bran samples have been examined separately for flavonoid components, and in no case has any been found. On the other hand, no sample of commercial wheat germ has been examined that does not contain both flavonoids, although their proportions vary from sample to sample.

Bran was obtained from samples of wheat milled at the Cereals Research Station.

Fractions containing saponarin and saponaretin were obtained from the leaves of soapwort (*Saponaria officinalis*) grown in the grounds of the Cereals Research Station.

Elementary, methoxyl, and acetyl analyses were carried out by Drs. Weiler and Strauss, Oxford, England.

UV absorption spectra were measured on a "Uvispek" spectrophotometer (Hilger and Watts Ltd., London).

RESULTS AND DISCUSSION

Isolation of flavonoids A and B. Wheat germ [moisture content 11.5%, 1275 g after defatting with petroleum ether 40–60° by cold percolation] was percolated in a large glass column with ether (4L), and the ether extract (I) was taken down to a brown syrup (7.3 g) on the water bath. The residual germ was percolated with 5 L of acetone-water (8:2). Acetone and water were removed from the percolate by distillation under vacuum until the residual volume was 800 ml. The cloudy residue was then extracted with *n*-butanol (3 × 250 ml), and the combined *n*-butanol extracts were taken down to a brown syrup (II). The aqueous residue from the extraction was set aside for further examination (III).

The residual wheat germ was extracted by stirring with 3 L of water. Paper chromatograms of the extract gave no reactions for flavonoids or other phenolic components.

Fraction I (ether extract). The brown syrup was dissolved in ether (100 ml), and the solution was extracted with 10% aqueous sodium bicarbonate solution (2 × 10 ml). The combined alkaline extracts were acidified (conc. HCl) and re-extracted with ether (2 × 5 ml). The ether extracts were combined, and spots of the resulting solution were compared with standards of typical phenolic acids on Whatman No. 1 paper using the following solvent systems: a) 6% acetic acid; b) the upper phase of toluene–acetic acid–water (4:1:5) and the upper phase of benzene–methyl ethyl ketone–2% formic acid (9:1:1). Papers were examined under uv light for fluorescences, and then sprayed with diazotized *p*-nitroaniline solution and oversprayed with aqueous sodium carbonate solution. The presence of ferulic acid and vanillic acid was established.

Fraction II (*n*-butanol extract). The syrup was dissolved in water (200 ml) with warming, and freed from proteins and other polypeptide material by passage down a column of Zeo Karb 225 ion-exchange resin (H^+ form). The crude, relatively uncontaminated flavonoid effluent was taken

down to a brown glass (16 g). This was warmed with water (100 ml), the suspension was cooled and filtered, and the volume of filtrate reduced to 50 ml and chromatographed on a column of Perlon (polyamide) powder (38 × 4 cm diam.) with water as solvent. Flavonoid B was collected in the first 2 L. When it had cleared the column (seen by examination under uv light), the solvent was changed to 50% methanol, and flavonoid A was eluted. The solvent was removed by distillation under vacuum, and the almost pure flavonoids were left as pale-yellow or cream amorphous powder: Yields, flavonoid A, 0.5 g; flavonoid B, 0.7 g. Purification was effected by refluxing the compounds in ethanol and allowing the solution to cool. The highest points obtained for the non-crystalline products were: flavonoid A, 199° (d); flavonoid B, 222–225° (d). Found for products dried at 100°: flavonoid A: C, 53.61, 53.67; H, 6.20, 5.70; −OMe = 7.65%, 6.98%. Calc. for $C_{33}H_{32}O_{16}$ $(OCH_3)_2$ $2H_2O$; C, 53.7; H, 5.37; −OMe = 7.95%. Flavonoid B: C, 49.99, 49.29; H, 5.45, 5.65. Calc. for $C_{24}H_{28}O_{14}$ $2H_2O$; C, 50.0; H, 5.55.

Acetyl derivatives. Flavonoid A (242 mg) was acetylated by refluxing it for 3 hr with acetic anhydride (10 ml) and anhyd. sodium acetate (0.5 g). The product was isolated by pouring the cooled reaction mixture into water (100 ml), filtering off the derivative when solid, washing with cold water and recrystallizing from ethanol.

The pure product (125 mg) was white, m.p. 220–225°. It reacted with $FeCl_3$ to give a red-brown color. Found: C, 55.90, 56.60; H, 5.31, 4.99; −OAc = 40.52%; −OMe = 6.18%, 5.94%. Calc for $C_{33}H_{23}O_{16}$ $(OCH_3)_2$ $(COCH_3)_{10}$; C, 56.5; H, 5.02; −OAc = 36.8%; −OMe = 5.30%. UV absorption maxima: 260 mμ, ($E_{1\,cm}^{1\%}$ = 240) and 298–299 mμ ($E_{1\,cm}^{1\%}$ = 330).

Flavonoid B (250 mg) acetylated as above, gave 147 mg pure product, m.p. 245°. It reacted with alc. $FeCl_3$ to give a red-brown color. Found: C, 54.91; H, 5.17; −OAc = 43.75%; calc. for $C_{24}H_{18}O_{14}$ $(COCH_3)_{10}$; C, 54.9; H, 5.00; −(10)OAc = 44.8%. uv absorption maxima: 257–259 mμ ($E_{1\,cm}^{1\%}$ = 193); 299 mμ ($E_{1\,cm}^{1\%}$ = 205).

Methoxyl derivative of flavonoid B. Flavonoid B (0.3 g) was suspended in methanol (10 ml), and a solution (excess) of ethereal diazomethane was added. A voluminous orange precipitate was produced immediately. The reaction mixture was allowed to stand at 0° overnight. Excess diazomethane was destroyed with glacial acetic acid and the suspended product was filtered off and dried over P_2O_5 (vac.) at room temperature. Yield: 0.17 g, dark, orange-red amorphous solid that was recrystallized from ethanol (20 ml)/methanol (10 ml) to give an orange-colored product, m.p. 213°, having a negative reaction to alcoholic $FeCl_3$. The methoxyl derivative was difficultly soluble in alcohol but very soluble in water. It fluoresced bright light-blue under UV light, becoming bright blue when exposed to ammonia vapor. It did not become yellow (visible light) after this treatment. Its R_F in 6% acetic acid was 0.75. Uv absorption maxima: 266 mμ ($E_{1\,cm}^{1\%}$ = 201) and 325 mμ ($E_{1\,cm}^{1\%}$ = 290) in 95% ethanol; 260–265 mμ ($E_{1\,cm}^{1\%}$ = 222) and 315 mμ ($E_{1\,cm}^{1\%}$ = 270) in 0.002*M* sodium ethoxide. Found: C, 53.86; H, 6.43; −OMe = 15.25%. Calc. for $C_{24}H_{25}O_{11}(OCH_3)_3 \cdot H_2O$; C, 54.0; H, 6.00; −OMe = 15.5%.

Methoxy acetyl derivative of Flavonoid B. Acetyl flavonoid B (0.3 g) was dis-

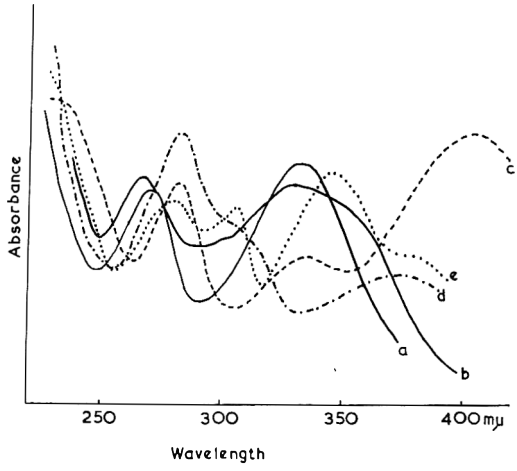


Fig. 1. UV absorption spectra of: (a) apigenin in ethanol; and both flavonoids, (b) in ethanol, (c) in 0.002*M* sodium ethoxide (----), (d) in excess ethanolic sodium acetate (-.-.), and (e) in 0.2% ethanolic aluminum chloride (....). The spectra are draw for comparison only, and the absorbancies shown are not necessarily to the same scale.

solved in methanol (10 ml), and excess ethereal diazomethane was added. The clear yellow solution was allowed to stand overnight at 0°, and excess diazomethane was then destroyed with glacial acetic acid. The solvent was distilled off, and the remaining off-white solid was dried over P_2O_5 (vac.) at room temperature. Yield: 0.20 g. The product was recrystallized from methanol (4 ml)/water (2 ml), yielding a white product, m.p. 260° (d), shrinking at 155°. The product had R_F 0.42 in 6% acetic acid, giving a blue spot (uv light), unchanged on exposure to ammonia vapor. A positive red-brown reaction was given with alcoholic $FeCl_3$. Uv absorption maxima: 254–255 mμ ($E_{1\,cm}^{1\%}$ = 131), 316 mμ ($E_{1\,cm}^{1\%}$ = 191), Found: C, 55.04; H, 5.38; −OAc = 40.57%; −OMe = 4.38%. Calc. for $C_{24}H_{18}O_{13}$ $(COCH_3)_9$ (OCH_3); C, 55.3; H, 5.13; −OAc = 41.5%; −OMe = 3.32%.

Hydrolysis of flavonoids. *With alkali.* Five mg of each flavonoid was hydrolyzed with 20 % aq. NaOH (0.2 ml) for 10 min in an open tube on a boiling-water bath. The hydrolysate was just acidified with conc. HCl, and the resulting solution chromatographed on paper at once. Sinapic acid, flavonoid B, and the new flavonoid component (C) were present in the hydrolysate from flavonoid A. Flavonoid B yielded a small amount of flavonoid C, with much unchanged starting material.

With acid. A solution of flavonoid A (10% in aqueous ethanol) was first chromatographed on paper in the solvent system *tert*-butanol–methylethyl ketone–water (12:5:3) to check whether free reducing sugars were present. No reaction for such compounds was given with the aniline hydrogen phthalate spray. Accordingly, flavonoid A (0.48 g) was refluxed for 3 hr with *2N* HCl (25 ml). The solution, initially slightly cloudy, became a clear, bright-yellow color after 1½ hr, and deposited a trace of tarry material in the flask. The hydrolysate was decanted off and again examined by paper chromatography for sugars, a negative result again being obtained. The presence of sinapic acid in the hydrolysate was confirmed by: a) its R_F values in several solvent systems, b) its characteristic uv fluorescence before and after exposure to ammonia vapor, and c) its reactions with the diazotized *p*-nitroanaline spray, alone and when oversprayed with Na_2CO_3 solution. Further examination by paper chromatography showed that no flavonoid A remained but that flavonoid B and a new flavonoid C (R_{FS} 0.41 and 0.18, respectively, in 6% acetic acid) had been formed. The hydrolysate was taken to dryness, with small additions of water from time to time to remove HCl, and was dried finally over P_2O_5-NaOH (vac.) at room temperature. The product, a bright-yellow amorphous powder (0.30 g), melted at 144° (d) after shrinking at 91°. Recrystallization from ethanol afforded flavonoid B, m.p. 220–225° (d); uv absorption max., 274 mμ ($E_{1\,cm}^{1\%}$ = 324) and 336–340 mμ ($E_{1\,cm}^{1\%}$ = 366). Found: C, 51.71; H, 6.10; Calc. for $C_{24}H_{28}O_{14}H_2O$; C, 51.8; H, 5.39.

Acetylation of this product (60 mg) with acetic anhydride (2 ml) under reflux for 5 hr and isolation of the acetyl derivative in the usual way afforded an off-white solid (34 mg), m.p. 185–190°. Analyses for this compound indicate substitution of all the available −OH groups by acetyl, thus: Found: C, 54.45; H, 5.38; −OAc = 51.00%; Calc. for $C_{24}H_{16}O_{14}(COCH_3)_{12}$; C, 54.3; H, 5.10; −OAc = 50.7%.

The mother liquors from the crystallization of flavonoid B were evaporated to small volume and banded on to Whatman No. 3 papers for chromatography in 6% acetic acid. The bands corresponding to flavonoid C were cut out and eluted with ethanol. Uv absorption spectral data are given in Table 1. It has not yet been found possible to obtain sufficient material for melting-point determinations and preparation of derivatives.

Fraction III (aqueous residue from *n*-butanol extraction). The volume was reduced to 200 ml by distillation under vacuum, and the resultant brown solution was passed down a column of Solka Floc powdered cellulose (BW grade) and eluted with water. Carbohydrates and salts ran out before the phenolic compounds. Paper chromatography of the main effluent showed that flavonoid B and a spot having the same R_F and qualitative reactions as a synthetic

Table 1. UV absorption spectral maxima for flavonoids A and B and related apigenin derivatives.

Flavone	λ max. mμ ethanol	λ max. mμ 0.002 *M* sodium ethoxide	λ max. mμ 0.2% ethanolic AlCl$_3$	λ max. mμ excess ethanolic NaOAc
Apigenin [a]	269, 336	277, 329, 398	278, 303, 342, 382	277, 300 (inflex) 379
Vitexin [a]	269, 334	280, 331, 399	279, 303, 341, 382	280, 300, 388
Saponaretin [a]	272, 336	279, 333, 402	280, 301, 345, 379	–
Saponarin [a]	272, 335	394	280, 301, 345, 380	271, 336, 400
Flavonoid A	274–275, 335	284–285, 338, 405	282, 305, 346, 375–385 (inflex)	282, 295–305 (inflex) 380
Flavonoid B	274, 335	284–285, 332, 404–405	281, 306, 345, 375–385 (inflex)	283, 300–310 (inflex) 380
Hydrolysis product (C) from flavonoid A	272–273, 339	288, 331–332, 397–398	–	–

[a] Data of Jurd, L., Geissman, T. A., and Seikel, M. (*Arch Biochem. Biophys.*, 1957, **67**, 284), for the flavone constituents of *Spirodela oligorrhiza*.

sample methoxy hydroquinone glucoside were present. The solvent was removed as above, leaving a light-colored hygroscopic solid (44 g) that gave a strong ninhydrin reaction. The solid was dissolved in water (250 ml), and the polypeptide material was removed by passage of the solution down a Zeo Karb 225 column. The effluent, now negative to ninhydrin, gave a golden-yellow color with NaOH, and a purple color with acidified KIO_3/aniline. The solution was taken down to a brown glass (37 g) and dissolved in water (100 ml). Three portions of equal volume of this solution were chromatographed on a column of Solka Floc (40 × 4-cm. diam) with water as solvent. A brown band moved down the column quickly, followed by flavonoid B. The fractions were collected, combined, and taken to dryness. The fraction in which the methoxyhydroquinone glucoside had been detected yielded 29 g of an amorphous hygroscopic dark-brown glass. It did not give the expected reaction for the glucoside when chromatographed on paper, but gave a strong aniline phthalate reaction for sugars. Hydrolysis of a portion of the fraction with 2*N* HCl for 1 hr under reflux failed to produce methoxy hydroquinone.

The fraction containing flavonoid B gave 0.7 g of a cream-colored product, m.p. 220–225° (d), pure by paper chromatography.

Isolation of saponarin- and saponaretin-containing fractions from soapwort (Saponaria officinalis). The leaves were harvested from six mature plants of *Saponaria officinalis*, each plant having several stems. The leaves were macerated in a Waring blender with water, and extraction was allowed to proceed overnight at room temperature. The aqueous extract was filtered through cloth, chlorophyll was removed by ether extraction, and the remaining solution was extracted with *n*-butanol as in the case of wheat germ extracts. Isolation of fractions rich in saponarin and saponaretin was carried out by chromatographing an aqueous solution of the *n*-butanol extract on Solka Floc with 2% acetic acid as solvent. The required substances were isolated as amorphous light-brown powders sufficiently pure for use as chromatographic markers.

Examination of wheat bran for flavonoids. Wheat bran (100 g, moisture content 12.9%) was extracted under reflux with petroleum ether (40–60°, 500 ml) for 2 hr, allowed to stand overnight, and filtered. The residual bran was dried in air and percolated with 80% aqueous acetone (1 L). The percolate was taken to dryness. Paper chromatography of the extract did not reveal any concentration of hydroxycinnamic acids or simple phenolic acids. The extract was dissolved in water (100 ml), and the solution was extracted with *n*-butanol (3 × 50 ml). The *n*-butanol extracts were combined, and the solvent was distilled off, leaving a brown syrup (0.34 g). Paper chromatographic examination of this fraction did not reveal any compound of a flavonoid nature. The extract was very soluble in chloroform and soluble in ethanol.

DISCUSSION

Flavonoids A and B constitute the major part of the phenolic components of commercial wheat germ, being present to the extent of approximately 0.2–0.3%. The proportion of each varies from sample to sample, and in samples so far examined both flavonoids have been present. The noncrystalline nature of some glycoflavones has been noted previously (Seikel and Geissman, 1957; Barger, 1906), especially in cases where the 8-substituent is considered to be of the straight-chain form (saponarin, saponaretin). Flavonoids A and B are also noncrystalline, making purification difficult. The characteristic qualitative reactions of the two flavonoids, the production of phloroglucinol and *p*-hydroxy benzoic acid on microalkaline fusion, their uv absorption spectra, and their behavior on paper chromatograms to uv light and to diagnostic spray reagents are indicative, in the main, of the apigenin nucleus only, and are not characteristics of glycoflavones. It appears reasonable by analogy, however, that flavonoids A and B are also 8-C-substituted compounds.

Both flavonoids are noncrystalline water-soluble pale-yellow solids, capable of purification from ethanol, flavonoid A being the more soluble. The melting points are somewhat variable from sample to sample, the highest values obtained being: flavonoid A, 199° (d); flavonoid B, 222–225° (d). Their solubility in water and relative insolubility in organic solvents make determinations of molecular weight difficult.

Elementary analyses are also somewhat variable, but indicate that the flavonoids can be satisfactorily represented by the following empirical formulae: flavonoid A, $C_{24}H_{27}O_{14}(C_9H_5O_2)\ (OCH_3)_2 \cdot 2H_2O$; flavonoid B, $C_{24}H_{28}O_{14} \cdot 2H_2O$. The experimental evidence suggests that the nonflavone substituent consists of a chain of 9 C atoms, each carrying an —OH group. Such a structure, if of a glycosidic nature, is at present unknown. Simple sugars of more than six C atoms are rare, although synthetic polyols exist of 8, 9, and 10 C atoms.

The uv absorption spectra of flavonoids A and B and other glycoflavones are shown in Table 1.

It is interesting to compare the optical densities of the two flavonoids at the spectral maxima with those of apigenin. The optical densities are assumed to be proportional to the amount of apigenin structure in each flavonoid.

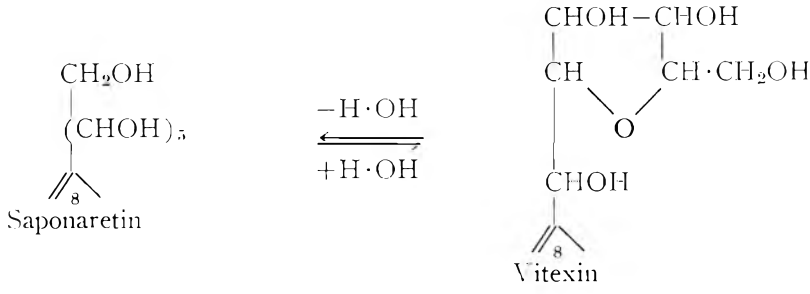
	mμ (max.)	$E_{1cm}^{1\%}$	M.W.	mμ (max.)	$E_{1cm}^{1\%}$	M.W.
Apigenin	269	753	270	336	772	270
Flavonoid A	274	249	820 (calc.)	335	404	517 (calc.)
Flavonoid B	274	325	628 (calc.)	335	375	557 (calc.)

Acetic anhydride and sodium acetate (reflux, 2 hr) acetylate only ten of the available twelve hydroxyls, the 5-OH position apparently remaining free in both cases. A longer period of acetylation with acetic anhydride alone (5 hr), applied to flavonoid B (isolated from the hydrolysis of flavonoid A), results in the production of an acetyl derivative, the analysis for which indicates substitution of twelve —OH groups.

The uv absorption spectrum of each acetyl derivative is similar to that of acetyl vitexin, having maxima at 257–260 and 298–299 mμ. Deacetylation of flavonoid A with aqueous barium or sodium hydroxide liberates sinapic acid and restores the spec-

trum to its original form, with maxima at 274 and 332 mμ.

The presence of the sinapoyl residue in the flavonoid A molecule is clearly indicated by the results of hydrolysis experiments, by the characteristic fluorescence under uv light, and by its typical color reactions, but the uv absorption spectra of flavonoid A (Table 1) do not reveal whether the sinapoyl residue is attached to the flavone nucleus or to the side chain.



Methylation of acetyl flavonoid B with diazomethane appears to be effective in replacing one acetyl group only, the 5-OH position remaining free, as shown by a positive $FeCl_3$ reaction. The derivative is insoluble in water, however, in contrast to that formed by the action of diazomethane alone on flavonoid B. Methoxy flavonoid B is an orange-colored compound, sparingly soluble in ethanol but easily soluble in water and alkalis. Methoxyl analyses indicate the substitution of three hydroxyl groups, probably in the 5, 7, and 4′-positions. The compound gives no reaction with alcoholic $FeCl_3$. The uv absorption spectrum ($E_{max.}$ at 266 mμ and 325 mμ), and the bright-blue appearance of a spot of the compound in uv light, unchanged by exposure to ammonia vapor, bear a marked resemblance to the corresponding properties of 5,7,4′-tri-*O*-methyl apigenin.

Acid hydrolysis of flavonoid A liberates sinapic acid to form flavonoid B and a small amount of a third flavonoid (C) of the same glycoflavone type. The transformation of flavonoid A by the above treatment resembles: a) the acid hydrolysis of saponarin to give saponaretin and vitexin, and b) the interchange undergone by these aglycones. Seikel and Geissman (1957) suggest that the saponaretin⟷vitexin change may be represented by a loss or gain of the elements of water between the ring-opened and the ring-closed forms:

The R_Fs of the transformation flavonoid C in aqueous solvents are identical with those of vitexin but differ considerably on changing to predominantly organic solvents. Flavonoid C is not, therefore, vitexin, and its precursors A and B have also been shown to differ from either saponaretin or saponarin by comparison with these substances isolated from the leaves of *Saponaria officinalis*. It is interesting, however, that Geissman and Kranen-Fiedler (1956) were able to isolate glycosides of vitexin that do not give saponaretin as a hydrolysis product. Nakaoki (1944) also found that when saponarin is hydrolyzed by emulsin the aglycone was neither vitexin nor saponaretin. The behavior of the wheat germ flavonoids on paper chromatograms is compared with that of known glycoflavones in Table 2.

Examination by paper chromatography of the extracts of wheat germ during isolation of the flavonoids reveals that small quantities of phenolic acids are also present. The ether extract contains ferulic acid and vanillic acid, but not sinapic acid in the free state. Both acids are present only to a small extent, and in the ether extract are heavily contaminated with oily, non-phenolic compounds. Their presence and that of sinapic acid, in bound form in flavonoid A, is interesting in view of the earlier work of Bungenberg de Jong *et al.* (1953) on the isolation of methoxy hydroquinone glucoside from wheat germ, and with the results of Cosgrove *et al.* 1952), who isolated both methoxy benzoquinone and 2,6-dimethoxy

Table 2. R_f values (to center of spot) of flavonoids A and B and related apigenin derivatives.

Solvent	6% acetic acid	33% acetic acid	*n*-Butanol/ 27% acetic acid (1:1)	*n*-Butanol/ acetic-acid/water (6:1:2)	Ethyl acetate/ 90% formic acid/ water (10:2:3)
Flavone					
Apigenin	0.00	0.30	0.89	0.87	0.86
Vitexin	0.18	0.67	0.53	0.43	0.57
Saponaretin	0.32	0.82	0.68	0.63	0.66
Saponarin	0.54	0.87	0.45	0.33	0.73
iso-Saponarin	0.67 [a]	0.80 [a]	0.64 [a]	–	–
Flavonoid A	0.55	0.91	0.49	0.43	0.82
Flavonoid B	0.41	0.76	0.34	0.24	0.65
Hydrolysis product (C) from flavonoids A and B	0.18	0.67	0.41	0.21	0.49

[a] From Jurd *et al.* (See Table 1) (iso-saponarin is the 4'-glycoside).

p-benzoquinone from fermented wheat germ. Although methoxy hydroquinone glucoside has not been isolated, there is strong evidence that it is present since paper chromatograms show a spot with the same R_f value, uv fluorescence, and color reactions as those given by a synthetic sample of methoxy hydroquinone glucoside. It would be expected that the precursor of the 2,6-dimethoxy quinone is present in wheat germ, although this has not been found yet.

Simpson (1935) reported the presence of flavones in wheat bran, but his claims were not substantiated in the present work. Extracts of bran, subjected to the same scheme of extraction as for the wheat germ flavonoids, fail to reveal any flavones or glycoflavones when chromatographed on paper; neither are there any peaks characteristic of flavones in the uv absorption spectra measured in ethanol or sodium ethoxide. In ethanol, absorption is low throughout the range 220–400 mμ, with a broad flat peak from 270 to 280 mμ, ($E_{1\,cm}^{1\%} = 30$). Addition of sodium ethoxide serves only to raise the general level of absorption. There appears to be no concentration of hydroxy cinnamic acids in the bran.

The other phenolic components of commercial wheat germ constitute but a very small fraction of the whole. The occurrence of phenolic acids also substituted with methoxyl groups (e.g., vanillic acid, ferulic acid, sinapic acid) is by no means unexpected considering the widespread occurrence and distribution of this type of compound in Gramineae, but the presence of a hydroxy quinone so substituted (Bungenberg de Jong *et al.*, 1953) is interesting.

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The Monoterpene Hydrocarbon Composition of Some Essential Oils

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SUMMARY

The monoterpene hydrocarbon composition of 29 non-citrus essential oils was determined with silicic acid chromatostrips and gas-liquid chromatography. Data are presented showing the total monoterpene hydrocarbon content and the relative proportions of 19 of these compounds in each oil.

In the analysis of citrus oils for their monoterpene hydrocarbons, difficulties were encountered with compounds present in quantities so small that it was possible to recover only enough material for their infrared spectra. It was therefore of interest to survey the monoterpene hydrocarbon content of a number of commercially available non-citrus essential oils to determine whether any of them contained the same compounds found in citrus oils, and whether quantities were sufficient to permit isolation and identification. The general procedure was to examine these essential oils for the major monoterpene hydrocarbons having gas chromatographic retention times similar to those in the citrus oils. Compounds were collected from the gas column, and their infrared spectra were determined. If the spectrum of a compound was identical to that of a minor monoterpene hydrocarbon compound in citrus oils, then further attempts were made to collect enough of the compound from the non-citrus essential oil to permit identification by conventional procedures. In this connection, it is desirable to use an oil that does not contain other hydrocarbons with retention times on gas-liquid chromatography (GLC) so close to the desired compound that separation is impractical.

The monoterpene hydrocarbon composition of some of these essential oils has not been reported in the literature in detail, and most of the work reported does not contain quantitative data; much of this work has been reviewed by Guenther (1949, 1950, 1952a,b).

MATERIALS AND METHODS

Essential oils. Half-ounce samples of essential oils were obtained from a chemical essential-oil house.

Separation of monoterpene hydrocarbons. The oils were deterpenated by the downward chromatostrip procedure, followed by gas chromatographic analysis (Stanley *et al.*, 1961). For quantitative estimation of total monoterpene hydrocarbon content, *n*-butylbenzene was weighed into a known quantity of each oil as an internal standard before the sample was applied to the chromatostrip. (This method will be reported in detail in a future publication.)

Gas chromatography. The total monoterpene hydrocarbon content of individual oil samples was determined by injecting 50–90 μl of the material eluted from chromatostrips into a gas chromatographic apparatus equipped with a ¼-in. × 10-ft stainless-steel column packed with 25% (w/w) diethyleneglycol succinate (DEGS) on 60–80-mesh firebrick and a 4-filament thermal conductivity detector. Analyses were run at 75–80°C with a helium flow rate of 120 ml/min. For determination of the relative concentrations of individual monoterpene hydrocarbons, 1–4 μl of the eluted material was analyzed in the gas chromatographic apparatus using a ⅛-in. × 10-ft aluminum column

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packed with 2% (w/w) DEGS on 80–100-mesh firebrick and a flame ionization detector. Analyses were made at 70°C with a flow rate of 10 ml nitrogen/min and 20 ml hydrogen/min.

Two gas chromatographic analyses were made of each sample of oil on the ⅛-in. column. For estimation of relative concentrations of individual monoterpene hydrocarbons, one run was made so that all peaks remained on scale with no added suppression. A second run was made at higher sensitivity to detect trace quantities of compounds not detected at the higher suppression.

The chromatograms were recorded on a 5-mv 2-sec pen-response strip-chart recorder equipped with an integrator to determine the area under each peak.

RESULTS AND DISCUSSION

The total monoterpene hydrocarbon content and the relative concentrations of individual monoterpene hydrocarbons found in each of the 29 essential oils are shown in Table 1. Also included are relative retention times (based on *n*-butylbenzene as 100) for the various monoterpene hydrocarbons on the ⅛-in. column using 2% DEGS and the ¼-in. column using 25% DEGS.

Separations were better with the ⅛-in. column than with the ¼-in. column. Consequently, the ⅛-in. column was used for determining relative concentrations of the individual monoterpene hydrocarbons and the ¼-in. column for determining total monoterpene hydrocarbon content. For comparison, Figs. 1 and 2 show the chromatograms of the monoterpene hydrocarbons of black pepper oil on both columns. This oil contained most of the monoterpene hydrocarbons found in this survey. One disadvantage with the ⅛-in. column was that myrcene and limonene could not be separated. These two compounds were separated on the ¼-inch column, which made it possible to calculate the amount of myrcene in the limonene peak. Also, the separation of *p*-cymene from terpinolene was poor with the ⅛-inch column. In some cases, two peaks were observed on the chromatogram, whereas in others only one broad peak could be seen. The difference in relative retention times observed for the two columns probably reflects differences in the relative importance of adsorption on the solid carrier as compared to partition between the gas and liquid phases.

Stanley *et al.* (1961) previously reported that α-pinene, β-pinene, *d*-limonene, and γ-terpinene have similar detector responses on both the flame ionization and thermal conductivity detectors. It is reasonable to assume that the detector response of the other monoterpene hydrocarbons reported in this survey are similar because of the similarity in their basic structures.

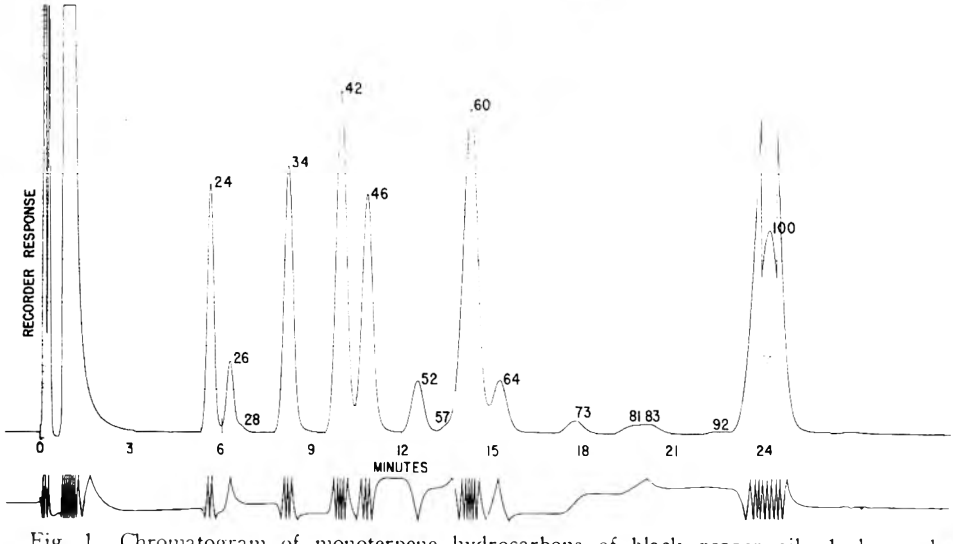

Fig. 1. Chromatogram of monoterpene hydrocarbons of black pepper oil. 1-μl sample, ⅛-in. × 10-ft column, 2% DEGS on 80–100-mesh firebrick, 68°C, 10 ml N_2/min, flame ionization detector. Peaks identified at bottom of Table 1 by reference to their relative retention times.

Table 1. Monoterpene hydrocarbon composition of some non-citrus essential oils.

Essential oils and total monoterpene hydrocarbon content (%)	% relative concentrations of individual monoterpene hydrocarbons																				
	?	?	?	α-Pinene	α-Thujene	Camphene	β-Pinene	Sabinene	Δ³-Carene	?	α-Phellandrene	α-Terpinene	Myrcene	d-Limonene	β-Phellandrene	γ-Terpinene	p-Cymene		Terpinolene	Ocimene	n-Butylbenzene
Abies alba (89.3)	....	....	....	16.5	....	Tr	2.2	....	....	....	....	....	77.3	4.0	....	....	....		....	....	
Bay N.F. (25.7)	....	....	....	2.8	....	....	Tr	....	....	....	2.8	....	70.0	20.2	2.3	....	1.1		....	1.0	
Bergamot N.F. (32.2)	....	....	....	1.9	0.2	....	10.8	1.5	....	....	....	....	1.2	69.2	....	12.6	2.2		....	0.4	
Caraway (38.0)	....	....	....	Tr	Tr	....	Tr	Tr	Tr	Tr	Tr	....	....	100	....	....	....		....	....	
Cardamom N.F. (20.6)	....	....	....	12.3	1.1	....	2.8	38.4	....	....	....	0.7	18.6	21.4	....	3.3	0.7		....	....	
Cascarilla (12.6)	....	....	0.3	11.2	14.8	2.0	2.5	1.6	....	....	1.5	2.6	7.9	7.9	0.7	4.8	42.2		....	....	
Cedar Leaf (6.7)	....	0.4	0.2	15.6	13.0	12.5	2.3	15.0	0.3	....	0.4	3.3	10.5	8.8	1.3	7.6	8.0		....	....	
Celery Seed (56.1)	....	....	....	....	....	....	0.3	Tr	....	Tr	....	....	0.9	98.8	....	....	....		....	....	
Citronella Ceylon (15.2)	....	....	4.2	8.1	....	31.0	0.2	1.3	Tr	Tr	....	....	2.4	44.5	3.4	....	....	(4.4)[a]	....	0.7	
Coriander (10.9)	....	....	....	18.7	....	2.6	2.1	1.8	0.2	0.2	....	....	3.2	19.0	0.7	30.5	21.0		....	....	
Cubeb (9.5)	....	....	....	12.1	13.2	....	2.3	47.1	....	....	1.9	1.1	4.5	0.9	12.7	1.5	....	(2.6)[a]	....	0.1	
Eucalyptus N.F., Rect. (15.9)	....	....	....	44.8	....	0.4	2.7	....	....	....	1.2	....	....	36.3	....	0.4	14.0		....	....	
Eucalyptus dives (39.8)	....	....	....	1.2	7.7	....	....	....	....	....	37.4	2.1	....	5.2	5.6	1.7	....	(38.9)[a]	....	0.2	
Galbanum (62.7)	....	....	....	13.8	0.4	0.1	59.9	Tr	19.6	....	0.3	....	....	4.5	0.1	....	....		1.4	Tr	
Hemlock (42.0)	4.3	Tr	3.1	23.5	....	36.5	3.4	....	7.3	....	0.8	....	....	16.7	2.2	....	....		2.0	....	
Laurel Leaf (36.0)	....	....	....	21.3	2.1	1.4	15.9	36.6	0.5	0.2	1.4	0.9	6.9	4.6	1.8	1.6	4.8		....	....	
Sweet Marjoram (36.4)	....	....	....	3.0	3.1	0.2	1.1	11.0	....	....	1.8	18.1	5.5	4.9	5.0	28.1	....	(17.2)[a]	....	1.0	
Nutmeg (65.8)	....	....	....	16.3	3.0	0.4	13.3	39.8	2.4	0.2	0.9	2.9	3.8	4.2	3.4	5.9	....	(3.6)[a]	....	....	
Opoponax (17.9)	....	....	....	Tr	....	....	....	Tr	Tr	....	....	....	Tr	Tr	....	....	....		Tr	100.0	
Origanum (19.1)	....	....	....	5.6	4.0	0.4	0.5	....	0.2	Tr	0.4	5.6	7.2	1.0	0.6	35.1	39.3		....	....	
Parsley Seed (16.6)	....	....	....	44.4	0.7	0.5	37.8	1.7	Tr	Tr	0.1	....	2.4	2.1	7.1	0.5	....	(2.5)[a]	....	....	
Black Pepper (57.2)	....	....	....	9.4	2.9	0.1	13.8	20.4	15.1	....	3.6	0.3	6.4	21.6	4.1	1.0	....	(1.3)[a]	....	0.1	
Pinus pumilio (70.4)	0.2	....	0.3	18.4	....	4.3	8.1	Tr	11.5	....	0.1	0.6	3.6	42.1	8.0	0.3	....	(2.5)[a]	....	....	
Pinus sylvestris (68.9)	....	....	0.4	65.8	....	2.9	9.5	0.1	11.1	....	....	....	3.6	4.1	1.2	Tr	....		0.9	0.3	
Rosemary (60.8)	....	....	0.2	41.0	....	18.4	10.1	....	1.7	0.4	0.7	0.4	8.7	9.8	1.5	1.9	....	(5.3)[a]	....	....	
Savin (33.4)	....	....	....	7.0	4.2	....	0.3	59.4	1.4	0.4	....	2.5	10.8	2.9	....	5.9	....	(4.3)[a]	....	1.3	
Snake Root, Canada (10.8)	....	....	0.1	19.1	0.2	7.9	16.6	7.2	1.1	0.1	2.4	0.7	16.4	6.3	1.3	1.9	....	(10.9)[a]	....	7.8	
Thyme N.F., Rect. (29.7)	....	....	....	3.7	2.3	1.5	1.2	....	0.2	0.2	0.4	4.4	6.3	1.3	....	19.4	59.0		....	....	
Wormwood (4.3)	....	5.0	....	8.0	3.3	0.3	3.5	43.5	....	....	5.0	0.2	20.9	2.4	1.3	1.7	....	(4.5)[a]	....	0.4	
Relative retention times 1/8 in. column	*14*	*18*	*20*	*24*	*26*	*28*	*34*	*42*	*46*	*47*	*52*	*57*	*60*	*60*	*64*	*73*	*81*		*83*	*92*	*100*
Relative retention times 1/4 in. column	*13*			*17*	*19*	*24*	*29*	*32*	*37*		*42*	*44*	*40*	*50*	*54*	*63*	*79*		*56*	*69*	*100*

[a] When *p*-cymene and terpinolene occur together separation is not sufficient to determine percent of each.

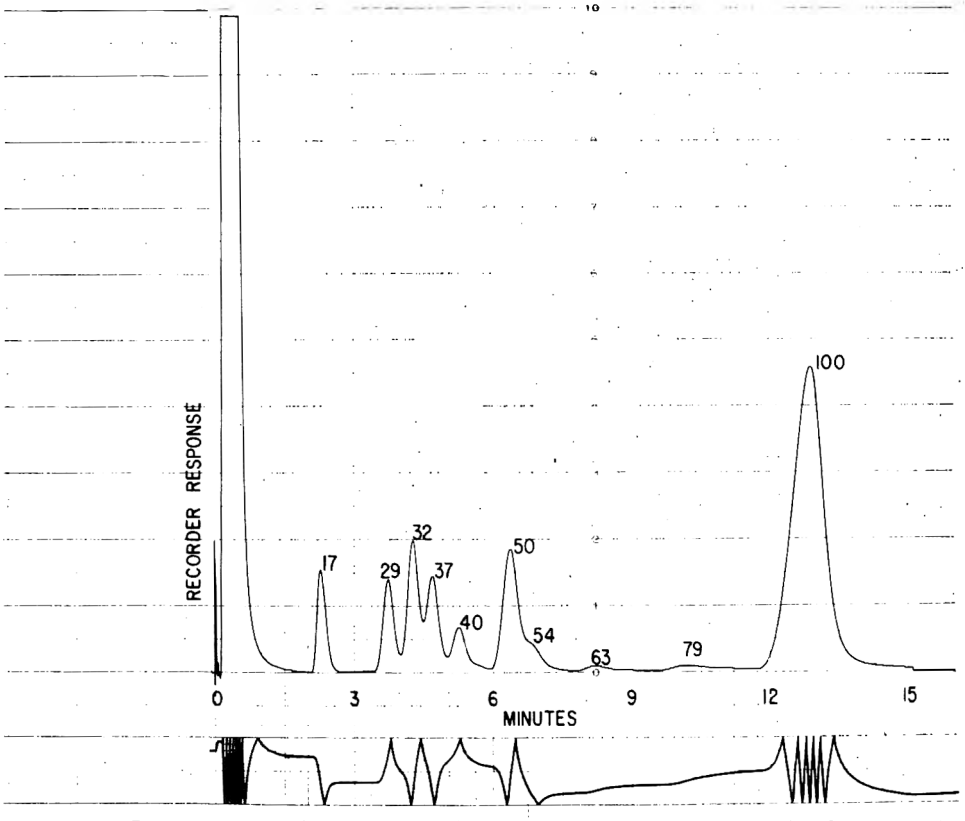

Fig. 2. Chromatogram of monoterpene hydrocarbons of black pepper oil. 80-μl sample, ¼-in. × 10-ft column, 25% DEGS on 60–80-mesh firebrick, 80°C, 120 ml helium/min, thermal conductivity detector. Peaks identified at bottom of Table 1 by reference to their relative retention times.

Tentative identification of the monoterpene hydrocarbons of the 29 essential oils is based on comparisons of retention times with known compounds. Monoterpene hydrocarbons not available were isolated from essential oils in which they have been reported, and their structures were confirmed by nuclear magnetic resonance spectra (NMR). Sabinene was isolated from oil of savin, and α-thujene from *Eucalyptus dives*. This work was reported previously (Stanley *et al.*, 1961). The major compound detected in oil of opoponax did not correspond in retention time to any of the available monoterpene hydrocarbons. Nimmo and Haagen-Smit (1961) found that one of the products from the synthesis of ocimene from linalool had a retention time identical with that of the monoterpene hydrocarbon in the oil of opoponax. Those workers isolated this material from oil of opoponax and found that its NMR spectrum corresponded to the structure of ocimene. Thus, oil of opoponax is a good source of ocimene, since it contains approximately 18% of this compound.

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Nutrient Content of Variety Meats. I. Vitamin A, Vitamin C, Iron, and Proximate Composition[a, b]

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SUMMARY

Analyses are presented of the vitamin A, vitamin C, and iron content and proximate composition of raw variety meats from beef, calf, lamb, and pork. Vitamin A content of liver varied considerably from reported values. Extreme variations within different liver samples were also observed. Lamb liver contained the largest amounts of vitamin A of any of the samples tested. Small amounts were found in kidney and lamb lung. Vitamin C content was greatest in calf and beef thymus.

Previous studies have provided considerable information on the nutrient content of meats (Schweigert and Payne, 1956; Leverton and Odell, 1959; Hopkins *et al.*, 1961). However, data on a number of nutrients in variety meats are inadequate or need re-evaluation.

In the initial studies reported here, the vitamin A, vitamin C, and iron content of variety meats from lamb, calf, beef, and pork were determined. In addition, proximate composition was also obtained on all the samples analyzed.

MATERIALS AND METHODS

Samples were from local markets or a local meat packing house, and were trimmed of the fat normally discarded. The samples were ground, mixed, and stored at −20°C under a nitrogen atmosphere until the analyses were completed. Vitamin A content was determined prior to storage.

Vitamin C was determined by the method of Schaffert and Kingsley (1955). The procedure of Pohle *et al.* (1947) was used for the iron analyses.

Vitamin A was determined using a modification of the method described by Ames *et al.* (1954). Anhydrous magnesium oxide was substituted for sodium sulfate in the vitamin A extraction procedure because it was found to yield clear antimony trichloride color complex solutions for most samples. It is believed that the magnesium oxide in the presence of the water of the samples caused a partial saponification of fat, with subsequent removal of much of the fat via the relatively insoluble magnesium salts. In the vitamin A analyses of several of the variety meats, additional modifications consisting of saponification and/or chromatography were necessary in order to obtain clean antimony trichloride color complex solutions. Saponification was carried out as described in "Methods of Vitamin Assay" (1947). Yellow-colored extracts were also tested for carotene content by chromatographic procedures (Schaeffer, 1950); however, the amounts of beta- or alpha-carotene detected in the samples were only negligible.

Preliminary evaluations of the analytical methods used indicated that they were adequate. A minimum of 5 individual samples were analyzed in duplicate for determining the vitamin C content of each variety of meat tested. Because of the high variation of liver vitamin A observed, a minimum of 8 individual samples were analyzed in duplicate. Iron determinations and proximate analyses (AOAC, 1955) were performed on a minimum of 3 individual samples. The proximate analyses were performed by the Service Laboratory of the American Meat Institute Foundation.

RESULTS AND DISCUSSION

Tables 1–4 show the mean values for the analytical data obtained. The standard deviation from the mean is included as an index of the range of values obtained.

Kidney was the only variety meat other than liver that contained appreciable amounts of vitamin A. The amounts ranged from

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[b] Journal paper No. 227, American Meat Institute Foundation.

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Table 1. Average[a] proximate composition, vitamin A, vitamin C, and iron content of variety meats from beef (fresh-wt basis).

Sample	Vitamin A (USP units/100 g)	Vitamin C (mg/100 g)	Iron (mg/100 g)	Moisture (%)	Ether-extractable fat (%)	Protein (%)	Ash (%)
Heart	traces	7.0±1.1	5.0±0.3	77.3±0.2	4.1±0.3	16.9±0.3	1.0±0
Liver	12,709±3,660[b]	22.4±1.7	9.1±0.9	71.3±0.3	4.1±0.3	20.3±0.3	1.4±0
Brain	nil	19.2±1.6	2.1±0.3	78.9±0.3	8.9±0.4	10.2±0.5	1.4±<0.1
Pancreas	nil	13.7±1.3	2.9±0.1	65.2±3.6	18.6±3.9	15.7±0.8	1.3±<0.1
Tongue	nil	3.3±0.3	3.2±0.4	67.8±0.8	13.9±0.7	17.5±0.9	0.8±0
Lung	nil	38.5±1.9	8.4±0.7	78.9±0.5	3.1±0.3	15.6±0.5	1.0±0
Kidney	880±100	10.4±0.7	8.5±0.6	76.5±1.5	3.3±0.3	17.4±0.2	1.2±<0.1
Thymus	nil	34.0±3.3	2.1±0.5	65.0±0.7	21.8±0.8	13.0±0.3	1.3±<0.1
Tripe	nil	3.4±0.9	1.2±0.2	79.0±0.4	6.6±0.3	13.6±0.6	0.4±0
Spleen	nil	45.5±2.9	75.0±2.3	78.4±0.2	3.0±0.2	17.4±0.3	1.4±0

[a] Average value ± standard deviation of the mean.
[b] Values obtained from 12 individual livers.

Table 2. Average[a] proximate composition, vitamin A, vitamin C, and iron content of variety meats from calf (fresh-wt basis).

Sample	Vitamin A (USP units/100 g)	Vitamin C (mg/100 g)	Iron (mg/100 g)	Moisture (%)	Ether-extractable fat (%)	Protein (%)	Ash (%)
Heart	nil	7.9±0.8	4.3±0.4	76.9±1.3	4.8±1.4	17.1±0.1	1.1±<0.1
Liver	13,530±4,400[b]	26.3±1.0	7.5±1.8	72.0±0.7	6.2±1.1	18.6±0.4	1.3±0
Brain	nil	26.1±1.3	2.9±1.2	80.9±0.3	6.9±0.2	10.2±0.1	1.3±0
Spleen	nil	41.0±1.2	8.7±1.1	78.3±0.7	2.2±0.2	18.3±0.3	1.3±<0.1
Pancreas	nil	15.9±0.8	2.1±0.5	70.6±1.4	13.1±0.5	15.0±0.4	1.3±<0.1
Tongue	nil	5.5±0.5	3.1±0.9	74.9±0.7	4.0±1.3	17.9±0.8	0.9±<0.1
Lung	nil	39.3±4.1	5.0±0.8	80.2±0.8	2.3±<0.1	16.3±0.7	1.1±<0.1
Kidney	trace	19.0±0.4	2.7±0.3	77.7±1.1	4.7±1.0	15.5±0.3	1.2±<0.1
Thymus	nil	56.0±2.6	2.0±0.4	79.3±0.1	2.9±0.6	18.0±0.9	1.9±0.2
Tripe	nil	9.4±0.5	10.0±1.7	74.4±0.8	10.4±0.4	12.7±0.4	0.6±<0.1

[a] Average value ± standard deviation of the mean.
[b] Values obtained from 8 individual livers.

Table 3. Average[a] proximate composition, vitamin A, vitamin C, and iron content of variety meats from pork (fresh-wt basis).

Sample	Vitamin A (USP units/ 100 g)	Vitamin C (mg/100 g)	Iron (mg/100 g)	Moisture (%)	Ether-extractable fat (%)	Protein (%)	Ash (%)
Heart	traces	5.0±0.4	5.3±0.4	77.2±1.2	5.0±1.6	17.0±0.3	0.9±0
Liver	15,142±4,500[b]	21.6±2.5	30.3±2.7	71.1±0.9	4.3±0.4	21.3±1.0	1.5±<0.1
Brain	nil	13.5±1.8	1.8±0.1	78.7±0.5	8.6±0.3	11.2±0.2	1.5±<0.1
Spleen	nil	30.0±4.7	19.4±2.6	79.3±0.1	2.4±<0.1	17.1±0.2	1.4±<0.1
Pancreas	nil	15.3±1.5	2.5±0.2	66.7±1.1	12.5±1.6	20.1±0.4	1.3±<0.1
Tongue	nil	4.4±0.7	3.7±1.1	65.9±0.8	17.2±0.8	16.3±0.2	0.9±<0.1
Lung	nil	13.1±1.2	18.9±0.2	82.0±2.1	3.3±0.8	14.9±2.2	0.8±<0.1
Chitterlings	nil	7.1±0.9	2.3±0.5	69.2±2.8	20.3±3.3	9.9±0.5	0.5±<0.1
Kidney	230±114	14.2±1.9	9.6±1.8	77.4±0.3	3.4±0.2	17.7±0.4	1.2±0

[a] Average value ± standard deviation of the mean.
[b] Values obtained from 8 individual livers.

Table 4. Average[a] proximate composition, vitamin A, vitamin C, and iron content of variety meats from lamb (fresh-wt basis).

Sample	Vitamin A (USP units/ 100 g)	Vitamin C (mg/100 g)	Iron (mg/100 g)	Moisture (%)	Ether-extractable fat (%)	Protein (%)	Ash (%)
Heart	trace	7.3±1.9	4.7± 0.2	75.8±0.5	6.7±0.3	16.3±0.2	1.0±<0.1
Liver	76,756±14,900[b]	25.0±4.2	11.9± 0.5	70.4±0.5	5.6±0.4	21.7±0.2	1.5±<0.1
Brain	trace	19.4±1.8	2.0± 0.2	78.9±0.1	8.1±0.1	10.7±0.1	1.4±0
Spleen	nil	23.2±2.1	60.1±27.8	78.4±0.6	3.1±0.2	17.2±0.7	1.3±0.1
Pancreas	nil	17.5±1.4	2.5± 0.5	73.2±1.0	10.5±1.4	14.7±0.4	1.4±0.1
Tongue	trace	6.8±1.0	3.1± 0.3	65.7±0.4	19.0±0.6	14.6±0.3	0.8±<0.1
Lung	89±28.8	31.4±1.4	6.4± 0.9	79.7±0.5	2.6±0.1	16.7±0.4	1.1±<0.1
Kidney	279±110	12.9±1.1	5.8± 1.4	79.3±1.0	3.8±0.6	15.7±0.4	1.2±<0.1
Tripe	nil	6.6±0.4	2.4± 0.7	79.7±0.9	6.6±0.6	12.9±0.3	0.5±<0.1

[a] Average value ± standard deviation of the mean.
[b] Values obtained from 8 individual livers.

approximately 100 to 1000 USP units/100 g fresh weight, relatively small compared to those found in liver. Surprisingly, lamb lung contained small but measurable amounts of vitamin A (60–120 USP units/100 g fresh weight).

Calf, beef, and pork liver varied considerably in vitamin A content between individual livers from the same type of meat animal—from 700 to 40,000 USP units/100 g fresh weight. The average values were considerably lower than those reported in the literature cited previously, probably reflecting differences in feeding practices. For lamb liver, however, the values are extremely high, 30,000–140,000 USP units/100 g fresh weight. Lamb liver under current feeding practices is therefore a rich source of vitamin A.

The largest values for vitamin C found in the samples tested were in calf thymus and beef thymus— 40–60 mg/100 g fresh weight of thymus. Liver, lung, spleen, and brain also showed a relatively high vitamin C content. The values obtained for vitamin C content of liver, kidney, and heart are in excellent agreement with those reported in the USDA Agriculture Handbook No. 8, (1950).

Pork liver contained larger amounts of iron (25–35 mg/100 g) than the other variety meats (1–19 mg/100 g) except spleen, which in lamb showed a high degree of variation (11–125 mg/100 g). This high variability may be owing to variations in slaughter procedures, since the spleen is known to respond to hemorrhagic stress by depleting itself of iron.

Iron content in this study compared favorably with values for certain variety meats reported by Hopkins *et al.* (1961), which were slightly but consistently lower than our results. Differences in method may be responsible: Hopkins *et al.* used flame spectroscopy, not the colorimetric method used by this laboratory.

The values for the proximate analyses of the variety meats are in excellent agreement with those reported by Hopkins *et al.* (1961).

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Studies on the Composition of Egg Lipid [a]

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SUMMARY

Studies were carried out on the fractionation and analysis of egg lipid by several new techniques employing thin-layer chromatography. The fatty acid composition of the major lipid classes and subfractions thereof was determined by a combination of alkali-isomerization and gas-liquid chromatography. The positional arrangements of the fatty acids in the glycerides and lecithin fraction were determined. The fatty acids in lecithin were distributed mainly in one type, the α-saturated β-unsaturated type, in contrast to the fatty acids of triglycerides.

Although the lipid of chicken eggs has been investigated extensively, particularly the fatty acid composition (Fisher and Leveille, 1957; Feigenbaum and Fisher, 1959; Rhodes and Lea, 1957; Reiser 1951a,b), new methodology permits further insight into the lipid of this nutritionally important food. Gas-liquid chromatography (GLC), which has been generally adopted for the determination of fatty acid composition, has been applied to egg lipids by several investigators (Evans *et al.*, 1960; Fisher and Leveille, 1957). The fractionation and analysis of egg lipid was investigated extensively by Rhodes, Lea, and Stoll, 1955; Rhodes and Lea, 1956a.; Rhodes and Lea, 1956b.; Rhodes and Lea, 1957; and their techniques, in various modifications, are widely used. Van Beers *et al.* (1958) demonstrated that the neutral and phospholipids can be separated quantitatively by dialysis with a rubber membrane, and this technique has been used on egg lipid (Hawke, 1959). Enzymatic methods of analysis have been applied, particularly to egg lecithin (Hanahan *et al.*, 1960; Hanahan *et al.*, 1954; Hanahan, 1957; Inouye and Noda, 1958; Long and Penny, 1954; Marinetti *et al.*, 1959; Tattrie, 1959; Zeller, 1952).

Described here is the fractionation and analysis of egg lipid by methods employing thin-layer chromatography (TLC).

EXPERIMENTAL

Preparation and analysis of crude egg lipid. A blend of spray-dried yolk and whole egg powder was prepared for this study by the Poultry Laboratory of the USDA, Albany, California, from eggs obtained from a commercial producer. This producer fed a commercial laying mash of the following analysis: crude protein, not less than 16.0%; crude fat, not less than 3.5%; crude fiber, not less than 6.0%; ash, not more than 5.0%.

The powder was shipped to our laboratory in vacuum-tight cans and stored at −18°C until used. The fat was extracted from both the egg powders and the mash with chloroform-methanol (2:1 v/v) at room temperature in a Servall Omni-Mixer under an atmosphere of nitrogen. The yield of lipid from the egg powders was 47% calculated on a moisture-free basis, and the mash contained 6.9% fat on a moisture-free basis.

The fatty acid composition of the dietary fat and the crude egg lipid was determined by both alkali isomerization (Herb and Riemenschneider, 1953) and gas-liquid chromatography (Table 1). A small sample of each fat was converted to methyl esters by interesterification using H_2SO_4 as a catalyst for the GLC analyses.

Alkali-isomerization was used primarily for analysis of polyunsaturated fatty acids, which were present in only minor amounts. Oleic, linoleic, and the saturated fatty acids were determined by GLC. The values for linoleic acid by the two methods agreed very well. The GLC analyses

[a] A report of work done under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act of 1946; it was supervised by the Western Utilization Research and Development Division, Albany, California.

were carried out with an F & M model 500 instrument, employing a hot-wire thermal-conductivity detector with a six-foot-¼-in. column packed with Gaschrom P containing 15% ethylene glycol succinate polyester as the stationary phase. Temperature of the column was 200°C and the flow rate of helium was 90 ml per min. The percent compositions were calculated on the basis of the ratios of the peak areas. Quantitative analysis of the NIH standard mixtures of saturated methyl esters showed that the instrument exhibited good linearity under these conditions.

Table 1. Fatty acid composition of crude egg lipid and dietary fat.

Fatty acid	Crude egg lipid	Dietary fat
	%	%
Palmitic	23.5	14.0
Stearic	14.0	2.4
Palmitoleic	3.8	2.7
Oleic	38.4	29.1
Linoleic	16.4	44.4
Linolenic	1.4	3.2
Arachidonic	1.3	0.8
Eicosapentaenoic / Docosapentaenoic	0.4	0.8
Docosahexaenoic	0.8	1.3

Fractionation of egg lipid. The crude egg lipid was fractionated into cholesteryl esters, cholesterol, triglycerides, and phospholipids by TLC on 20×20-cm plates, using as an adsorbent, silicic acid containing 5% added calcium sulphate for a binder. The silicic acid was prepared in our laboratory to ensure its freedom from organic matter. The lipid was placed on the base of the plate in a row of about 10 spots of approximately 200 μg each and chromatographed with 1:10:90 (v/v/v) acetic acid, diethyl ether, and petroleum ether (bp 30–60°C). The separation of the lipid classes is demonstrated in Fig. 1 by a photograph of a plate in which the spots are charred. The charring was carried out by heating the plate at 280°C after spraying it with 50% sulfuric acid. The compounds giving the two small spots between the cholesterol and phospholipid spots (Fig. 1) were not identified specifically. They appeared to be due to minor constituents in the oil, however. Since they did not belong in any of the major lipid classes, they were not isolated.

Since the lipid classes could not be located by charring for their isolation, a small pilot plate (2×8 in.) spotted with a sample of a standard mixture consisting of cholesterol, cholesteryl palmitate, tripalmitin, and purified egg lecithin, as well as the crude egg lipid, was developed under conditions identical to those used in developing the large plate. Each lipid class was located on the large plate by reference to the small plate, and was scraped off in a band of silicic acid with a razor blade. Cholesterol, cholesteryl esters, and triglycerides were recovered from the adsorbent by extraction with freshly distilled diethyl ether.

The phospholipid class was extracted with 10% pyridine in methanol. Then this solution was added to a mixture of 1:1 diethyl ether and petroleum ether in a separatory funnel and washed first with 5% aqueous hydrochloric acid, then with water. There is a slight loss of phospholipid by this technique, because of the solubility of these compounds in water as evidenced by the results of the recovery of egg lecithin in the standard mixtures shown in Table 2. Thus, the washings with aqueous phases should be held to a minimum in regard to both the number of extractions and the volumes used. Nevertheless, the yields are about

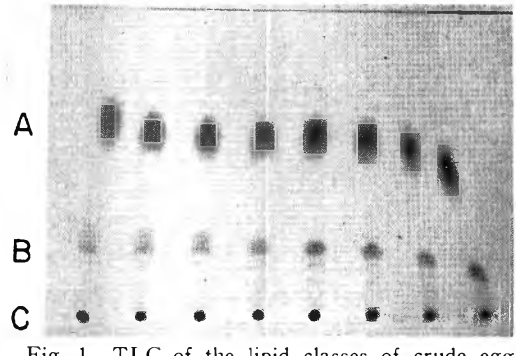

Fig. 1. TLC of the lipid classes of crude egg lipid with 10% diethyl ether in petroleum ether (bp 35–60°C), 1% acetic acid. A) triglyceride; B) cholesterol; C) phospholipid.

Table 2. Analysis of lipid class.

Standard mixture			Egg lipid TLC	
Compounds	Known (wt. %)	TLC (wt. %)	Compounds	Wt. %
Cholesterol	23.8	24.2	Cholesterol	5.2
Cholesteryl oleate	25.2	26.1	Cholesteryl esters	Trace
Triolein	23.3	23.2	Triglycerides	65.5
Egg lecithin	27.4	25.1	Phospholipids	28.3

Table 3. Percent fatty acid composition of lipid classes.

	Triglycerides	Lecithin	Cephalin	Neutral lipid[b] minor constituents
% weight[a]	64.2	22.9	4.7	3.5
Palmitic	22.5	37.0	21.6	18.0
Stearic	7.5	12.4	32.5	7.9
Palmitoleic	7.3	0.6	Trace	5.6
Oleic	44.7	31.4	17.3	46.5
Linoleic	15.4	12.0	7.0	15.6
Linolenic	1.3	1.0	2.0	3.0
Arachidonic	0.5	2.7	10.2	1.7
Eicosapentaenoic / Docosapentaenoic	0.2	0.8	3.0	0.4
Docosahexaenoic	0.6	2.1	6.4	1.3

[a] Determined by conventional methods (minor constituent phosphatides not included).
[b] Contained 34% unsaponifiable matter.

90%, and it is doubtful that the slight loss is accompanied by a fractionation. Since the phospholipids are prone to alteration, they must be scraped from the plate while it is still wet with solvent. Each lipid class was recovered by evaporation of the solvent (after it was dried with anhydrous Na_2SO_4) and weighed on a semimicro analytical balance. The results of the analysis of egg lipid, as well as the standard mixture, are presented in Table 2.

The cholesterol, cholesteryl palmitate, and tripalmitin used in the standard mixture were obtained from The Hormel Foundation. No impurities could be detected by TLC in these preparations. The purified egg lecithin was prepared as described below.

The fractionation of egg lipid was also carried out by a combination of dialysis (van Beers *et al.*, 1958), molecular distillation (Privett *et al.*, 1961), and column chromatography (Rhodes and Lea, 1956b.). Ten grams of the lipid was dialyzed in a small rubber-finger cot with petroleum ether (bp 35–60°C) until separation of the phospholipids and neutral lipids was complete as monitored by TLC. The undialyzed fraction was recovered and weighed as phospholipid. The neutral lipid fraction was further fractionated by molecular distillation at 220°C (Privett *et al.*, 1961) into the triglycerides and a minor constituent fraction consisting mostly of cholesterol (Table 3).

Fractionation of the phospholipids. The phospholipids were also fractionated by TLC on 20×20-cm plates as well as by column chromatography. In the latter, a 2-g sample was placed on the top of a 2.75×30-cm column of 100–200-mesh silicic acid and eluted successively with various ratios of chloroform and methanol, starting with 100% chloroform and finishing with 100% methanol. The fractionation was monitored by TLC analysis of the eluate, which was collected in 20-ml portions. Four fractions were collected: pure cephalin and lecithin, a mixture of lecithin and cephalin, and a highly polar fraction, which was eluted last. The cephalin-lecithin intermediate fraction was rechromatographed to permit a quantitative analysis of these components (Table 3). The molar N/P ratios of the lecithin and cephalin preparations were very close to unity (1.01), cephalin containing 1.70% nitrogen and 3.72% phosphorus, and lecithin 1.78% nitrogen and 3.90% phosphorus. On the basis of these data, and since no impurities could be detected in these preparations by TLC, they were judged to be pure.

The fractionation of the phospholipids by TLC was carried out with 70:30:3 chloroform, methanol, water. Fig. 2 shows the curve obtained by densitometry (obtained with a Photovolt Densitometer model 52C and 501A photometer equipped with a semiautomatic stage) of the charred spots on the pilot plate used to locate the separated components on the large plate. As in the previous

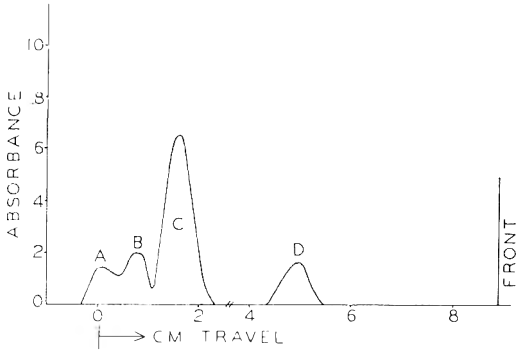

Fig. 2. Analysis of egg phospholipids by adsorption TLC, 70:30:3 chloroform-methanol-water. A and B are unidentified; C) lecithin; D) cephalin.

analysis (Fig. 1) these spots were visualized by heating the plate at 280°C after spraying it with 50% concentrated sulfuric acid. The lecithin and cephalin spots, respectively peaks C and D (Fig. 2), were identified from the R_f values of the purified compounds isolated as described above. Peaks A and B (Fig. 2) were not identified. These are presumably due to sphingomyelin and lysophosphatides, both of which have been detected in egg phosphatides (Rhodes and Lea, 1956b.; Hanahan *et al.*, 1954).

Fatty acid composition. Table 3 shows the fatty acid composition of the lipid classes and subfractions thereof. Alkali-isomerization (Herb and Riemenschneider, 1953) was used to calculate the highly unsaturated acids, and GLC the monoethenoid, linoleic, and individual saturated fatty acids.

Structural analysis of triglycerides and lecithins. The analysis of the triglycerides was carried out by the general procedure previously described by Privett and Blank (1961). This method has also been applied to lecithin (Privett and Blank, 1962) using the reverse-phase system of TLC of Malins and Mangold (1960) to separate the lecithin "cores." In both of these analyses, the separated "cores" were charred and analyzed by densitometry as previously described (Privett and Blank, 1961). Figs. 3 and 4 show the curves given by the triglycerides, and Fig. 5 the curve given by lecithin.

Quantitative analysis of the triglycerides and lecithin types (Table 4) was made on the basis of the areas of their peaks, assuming an average chain length for the saturated fatty acids of 16.5 and that the unsaturated fatty acids were converted to 9 carbon omega aldehyde moieties in the core. This assumption is based on the fatty acid composition of the triglycerides and lecithin (Table 3). Although this assumption is not strictly true, especially for lecithin, which contains about 5% highly unsaturated acids, the magnitude of the error from this source does not change the results significantly.

The peaks representing the various types of triglycerides in Figs. 3 and 4 were identified on the basis of the analyses of pure standards as previously described (Privett and Blank, 1961). Since no reference lecithins were available for analysis, the peaks representing the various lecithin types had to be identified by other means. Peak 1, having the lowest R_f value, represented the lecithin type with the least polarity, namely, the fully saturated type. This was confirmed by the analysis of fully hydrogenated lecithin, which gave a single spot having the same R_f value. Analysis of this preparation by IR as well as TLC also showed that the basic lecithin molecule passed through the reductive-ozonolysis procedure without any alteration in its structure. The "core" giving peak II, which made up the bulk of the sample, was attributed to the α-saturated β-unsaturated type on the basis of the enzymatic studies on egg lecithin by Tattrie (1959) and Hanahan *et al.* (1960). Peak III was designated as being due to the "core" from the α-unsaturated β-saturated type. Although both these types contain one saturated and one unsaturated fatty acid, infrared analysis showed that the structure of the "core" given by the type with the unsaturated fatty acid in the β-position was associated as compared with the "core" with the saturated fatty acid in the β-position. This explained the difference in polarity that made it possible to separate these two types by TLC.

Since the "core" giving peak IV was the most polar and contained none of the constituent satu-

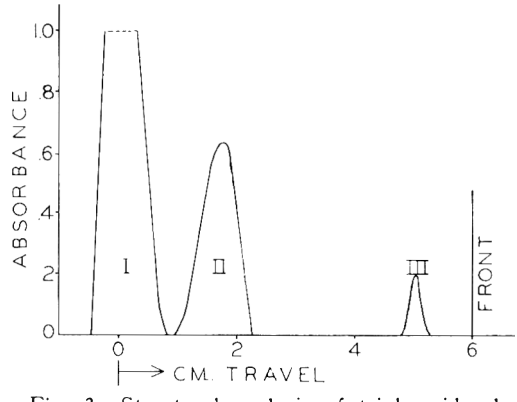

Fig. 3. Structural analysis of triglycerides by adsorption TLC of the cores of the different types with 15% diethyl ether in petroleum ether (35–60°C). Peak I represents a mixture of GU_3 and Gu_2S, peak II represents GU_1S_2, and peak III represents GS_2.

Table 4. Structural analysis of egg lecithin and triglycerides.

Lecithin		Triglyceride	
Type	%	Type	%
α-saturated β-saturated	4.5	Trisaturated	2.0
α-saturated β-unsaturated	87.6	Monounsaturated-disaturated (GU_1S_2)	27.8
α-unsaturated β-saturated	4.9	Monosaturated-diunsaturated (GS_1U_2)	30.9
α-unsaturated β-unsaturated	3.0	Triunsaturated (GU_3)	39.3

rated fatty acids of egg lipid, it obviously represented the fully unsaturated type.

DISCUSSION

This study demonstrates that TLC in conjunction with supplementary methods, particularly GLC, provides a simple and relatively fast means for the analysis of egg lipid on a semimicro scale.

Alkali-isomerization was used for the calculation of the highly unsaturated fatty acids since it seemed to give a more accurate analysis of minor amounts (<5%) of these acids than did GLC. The values for linoleic acid by both methods were in close agreement, however.

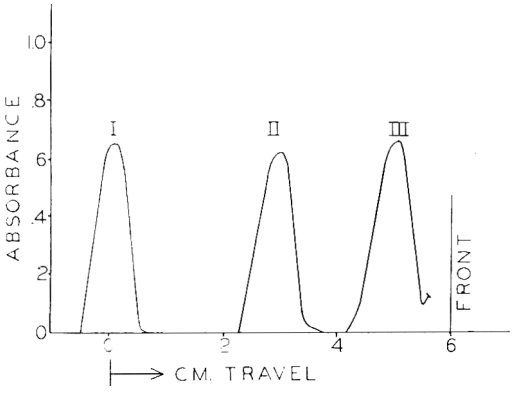

Fig. 4. Structural analysis of triglycerides by adsorption TLC of the cores of the different types with 15% ethyl ether in petroleum ether (bp 35–60°C). Peak I represents GU_3, peak II GU_2S, and peak III GUS_2.

The fatty acid composition of the crude egg lipid was about what might be expected from a commercial-type diet. Since the dietary fat had a more or less normal fatty acid composition, it was significant that the pattern of the fatty acid composition of lecithin and cephalin was the same as that observed by Hawke (1959) in that the lecithin contained a higher concentration of linoleic acid and lower proportions of highly polyunsaturated acids than cephalin. Stearic acid was the dominant saturated fatty acid in cephalin, whereas palmitic acid was the dominant saturated fatty acid in lecithin. These results also are in accord with the findings of Hawke (1959).

The positional arrangement of the fatty acids in lecithin has been of great interest in connection with the action of lecithinase A. In accordance with these findings, the results presented here show that egg lecithin consists primarily of one type. On the basis of studies of Tattrie (1959) and Hanahan *et al.* (1960) and other considerations discussed herein, this appears to be the α-saturated β-unsaturated type. Our data also show the presence of small but significant amounts of the other three lecithin types. The evidence for the presence of the fully saturated and fully unsaturated types is unequivocal and indicates that the method employed here is more sensitive than enzymatic methods for the determination of the lecithin types. Standards representing the various types of lecithins were not available for analysis. However, since the "cores" of the different types of lecithin have the same basic structure, it is believed that the relative areas of curves obtained by densitometry of the charred spots provide a fairly accurate analysis of the different types.

According to present concepts, the biosynthesis of triglycerides and lecithin involves a common diglyceride precursor (Kennedy, 1957; Weiss *et al.*, 1960). The data showing that egg lecithin is characterized mainly by one type, in contrast to the trigylcerides, are not necessarily in discord with this theory, but demonstrate, rather, the complexity of the biosyntheses of these compounds. In this connection, an interesting adjunct to

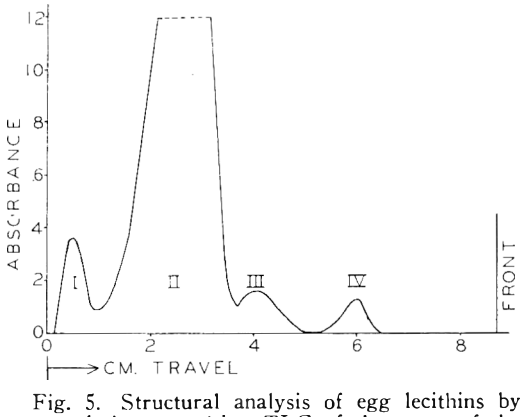

Fig. 5. Structural analysis of egg lecithins by reversed-phase partition TLC of the cores of the different types. Solvent system, 85% aqueous acetic acid as the mobile phase, and silicone as the stationary phase. Peaks I, II, III, and IV respectively represent the disaturated, α-saturated β-unsaturated, α-unsaturated β-saturated, and diunsaturated types.

the present study would be a study of the relation between the structures of lecithin and triglycerides of egg lipid, and changes in the fatty acid composition of the dietary fat.

ACKNOWLEDGMENT

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Tenderness of Beef. I. The Connective-Tissue Component of Tenderness [a]

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(Manuscript received February 19, 1962)

SUMMARY

Panel scores for juiciness and six components of tenderness were considered in relation to possible multiple causes of tenderness or toughness and to certain histological, chemical, and physical data. One-inch steaks from longissimus dorsi and biceps femoris from 180 young cattle in nine lots were cooked to 61°C (rare) and to 80°C (well-done) by dry heat, and to 100°C by moist heat. Connective tissue in longissimus dorsi was scored tender at 61°C, and was tendered only slightly by increases in temperature, whereas in biceps femoris it was scored tough at 61°C and became progressively more tender at 80 and 100°C. Collagen content was greater in biceps femoris than in longissimus dorsi at 61 and 80°C, but there was little difference in the two muscles at 100°C; the losses in both muscles increased with increasing meat temperatures. The relation of panel scores for tenderness of connective tissue to collagen content and to the loss of collagen on heating is discussed.

INTRODUCTION

Perhaps one reason that the causes of tenderness or toughness are not understood is that tenderness of meat is not a simple one-component system. It has long been known that two structural components—muscle fibers and connective tissues—are involved. Chemical and histological means of isolating connective tissue from muscle fibers are available, but little has been done until recently toward separating these two components in a scoring procedure. There is an additional problem: Juiciness appears to be related positively to tenderness in some instances but negatively in others. The possible causes underlying these differences seem impossible to determine with one score for tenderness.

At the Texas Station, attempts at characterization began when one score for tenderness was found inadequate to account for certain variations in chemical and physical data. With the various cooking conditions rigidly standardized, and with animals obtained from various breeds and crosses, differences among animals, muscles, and conditions of cooking were slowly characterized into components of tenderness—first into two (Cover *et al.*, 1957), then into three (Cover, 1959; Cover and Hostetler, 1960), and finally into six. These particular six components may be applicable only to this particular situation, but similar attempts at independent characterization appear to have been advantageous in other experiments with tenderness (Blumer, 1961; Ginger and Weir, 1958; Wilson *et al.*, 1960). Lowe and Kastelic (1961) noted the current confusion about causes of tenderness in meat such as age and fatness, and suggested that new approaches to the problem should be developed. The use of components of tenderness provides a new theoretical framework within which to approach the physical and chemical causes of variations in tenderness.

This paper is concerned with the connective-tissue component of tenderness; the second paper with juiciness and the two softness components; and the third paper with the three muscle-fiber components. The main purpose is to note differences in

[a] This study was supported in part through a contract sponsored by the Animal Husbandry Research Division, Agricultural Research Service, United States Department of Agriculture.

Table 1. Brief description and number of animals within 9 lots.

Lot and no. of animals	Source	Year/season/sex[a]	Age in days
I (39)	McGregor	1959/spr/st	377–507
II (17)	McGregor	1959/spr/hf	318–458
III (18)	McGregor	1959/sum/st	430–489
IV (13)	McGregor	1959/sum/hf	424–503
V (23)	McGregor	1960/spr/st	388–436
VI (10)	McGregor	1960/spr/hf	400–443
VII (17)	McGregor	1960/sum/st	441–529
VIII (16)	College Sta. (Angus breeders)	1960/spr/st	unknown
IX (27)	Brazos Bottom	1960/spr/st	521–588

[a] Spr, spring; sum, summer; st, steers; hf, heifers.

panel scores for juiciness and the six components of tenderness that are associated with differences in muscles or in final meat temperatures, and to attempt to identify the physical and chemical causes of such differences in the scores. A fourth paper will deal with the shear-force values and the extensibility of muscle fibers, and attempt to determine which of the six components of tenderness are most closely related to these physical tests.

EXPERIMENTAL

The meat was obtained from 180 young animals in nine unequal lots (Table 1). Details of processing and storage of the experimental meat were the same as those given by Cover (1959). One-inch steaks were obtained from two muscles—longissimus dorsi (LD) from the anterior end of the short loin, and biceps femoris (BF) from the dorsal end of the round. Five steaks were obtained from each muscle on each side and allotted as shown in Table 2. In 1959 the steaks for the panel were trimmed to the muscle sheath before cooking, but in 1960 the muscle sheath on three edges was removed, leaving only the outer layer of fat on the fourth edge. For chemical analyses, the steaks were trimmed free of the muscle sheath on all sides.

The two methods of cooking, dry and moist heat, were described by Cover and Hostetler (1960). Browning was omitted from the procedure for moist heat. Thermometers were inserted into the frozen steaks at about −2°C. Cooking began when the steak temperature reached 1°C. The three final steak temperatures were 61°C (rare) and 80°C (well-done) by dry heat, and 100°C plus holding there for 25 min. by moist heat.

An additional steak from all of the animals in lots 1 through 4 had been cooked by moist heat to 85°C (Table 2). No significant differences were observed in panel scores or shear-force values between cooking to 80°C by dry heat and to 85°C by moist heat. Such results seemed to confirm the conclusion of Cover and Hostetler (1960) that the important consideration in tenderness and juiciness is steak temperature rather than moist or dry heat. The 85°C temperature was therefore omitted from lots 5 through 9, and is not mentioned further.

Steaks from all of the animals were scored by a trained panel. Samples for the panel were taken from the central portion of each steak. The samples were cut with the grain of the meat, and were *ca.* ¾ × ¾ × ⅜ in. thick (Fig. 1 of Hostetler and Cover, 1961). Each panel member received his samples from the same relative position in each steak. The six samples of meat from one animal were tested at one judging session. Order of scoring the samples was determined from a

Table 2. Plan for utilization of steaks from the two muscles.[a]

	Steak No. 1	Steak No. 2	Steak No. 3	Steak No. 4	Steak No. 5
Plan for lots 1, 2, 3, and 4					
	Panel	Panel	Chem.	Chem.	Chem.
Left	80°C	100°C	Raw	61°C	100°C
	Panel	Panel	Chem.	Chem.	Chem.
Right	61°C	85°C	100°C	61°C	Raw
Plan for lots 5, 6, 7, 8, and 9					
	Panel	Panel	Chem.	Chem.	Chem.
Left	80°C	100°C	Raw	61°C	80°C
	Panel	Moisture	Chem.	Chem.	Chem.
Right	61°C	100°C	80°C	61°C	Raw

[a] Steaks were cut in order from anterior end of loin or dorsal end of round.

Table 3. Scoring sheet used by panel.

Juiciness		Tenderness								
		Softness			Muscle fibers					
		To tongue & cheek		To tooth pressure	Ease of fragmentation across the grain		Mealiness	Apparent adhesion between fibers		Connective tissue
V. juicy	9	V. soft	9	V. soft	V.easy	9	V. mealy	V. slight	9	Tiny amt. soft
	8		8			8			8	
Juicy	7	Soft	7	Soft	Easy	7	Mealy	Slight	7	Small amt. soft
	6		6			6			6	
Sl. juicy	5	Firm	5	Firm	Mod. easy	5	Sl. mealy	Moderate	5	Small amt. firm
	4		4			4			4	
Dry	3	Hard	3	Hard	Difficult	3	V. sl. mealy	Tight	3	Small amt. hard
	2		2			4			2	
V. dry	1	V. hard	1	V. hard	V. diff.	1	None	V. tight	1	Large amt. hard

table of random numbers. A person not on the panel arranged the samples on the plate. The trained panel consisted of four technical persons for lots 1–4, but one of them was absent for lots 5–9. The mean of the panel scores was used as the score for the steak. Steak scores were averaged to obtain treatment means.

The score card used is shown in Table 3. The six components of tenderness were weighted so that the highest score would indicate the greatest tenderness. Softness to tongue and cheek was judged by feel. Softness to tooth pressure was judged by muscular force exerted; cutting was not necessary. Ease of fragmentation required cutting or breaking the muscle fibers across the grain. Mealiness was a special kind of fragmentation: the fragments were tiny, dry, and hard, and clung to cheek, gums, and tongue. Scores for lack of apparent adhesion were lowest when the muscle fibers seemed almost felted together. Connective tissue was scored on amount and hardness of connective tissue. The most tender connective tissue received the highest score.

Chemical analyses for collagen by the hydroxyproline procedure of Ritchey and Cover (1962) were made on raw steaks and on steaks cooked to 61, 80, and 100°C (Table 2). These data were collected on some of the animals in lots 1, 5, and 6 (Table 1).

Separating the variation caused by muscles from that caused by internal temperature, though desirable, was not possible with the usual form of the analysis of variance (Table 4, analysis 1). A significant interaction (muscles × temperatures) was obtained for all variables analyzed except for juiciness in lot 2. When this interaction is significant, use of analyses 2 and 3 is permissible. But analysis 2 did not distinguish among the three temperatures, and therefore Tukey's test for dif-

Table 4. Outline of analyses of variance.

Variables	Degrees of freedom	Error terms
Analysis 1		
Animals	(a)	A × T
Treatments	5	A × T
Muscles	(1)	M × T
Temperatures	(2)	M × T
Muscles × temperatures	(2)	A × T
Animals × treatments	(a)	
Total	(a)	
Analysis 2		
Muscles	1	Steaks
Steak temperatures within muscles	4	Steaks
Within longissimus dorsi	(2)	Steaks
Within biceps temoris	(2)	Steaks
Steaks within muscles and temperatures	(a)	
Total	(a)	
Analysis 3		
Steak temperatures	2	Steaks
Muscles within temperatures	3	Steaks
Steaks within muscles and temperatures	(a)	
Total	(a)	

(a) These degrees of freedom vary with the lot. Total is the same for the three analyses within a lot. In analyses 2 and 3 the error term (steaks within muscles and temperatures) is the same for both analyses within a lot.

Table 5. Collagen nitrogen content of steaks from two muscles.

Muscle[a]	Collagen nitrogen (g/100 g total nitrogen)[b] Final steak temperature Raw	61°C	80°C	100°C
10 steers in lot 1				
LD	1.32 ± .13	0.76 ± .12	[c]	[d]
BF	2.35 ± .21	1.32 ± .17	[c]	[d]
17 steers in lot 5				
LD	1.51 ± .07	0.93 ± .06	0.34 ± .03	[c]
BF	2.49 ± .12	1.58 ± .09	0.62 ± .06	[c]
10 heifers in lot 6				
LD	1.65 ± .12	1.00 ± .09	0.46 ± .06	[c]
BF	2.77 ± .20	1.82 ± .14	0.90 ± .13	[c]

[a] LD, longissimus dorsi; BF, biceps femoris.
[b] Mean and standard error.
[c] Not analyzed.
[d] Too small to measure.

ferences was used to separate: 1) the temperature effects within each muscle, and 2) the muscle effects within each temperature. The data were analyzed by lots. These data are presented in bar graphs to show the muscle-temperature effects within lots and also the differences in average scores among lots.

Interrelationships for each pair of variables within a muscle and condition of cooking for each lot were measured with simple correlation coefficients. There was no problem of interpretation when, for one pair of variables, the coefficients for all lots were either significant or nonsignificant. However, for some pairs of variables the coefficients were significant in some lots but not in others. To evaluate all correlations equally well, the within-lot variances and covariances were pooled to obtain a single coefficient for each pair of variables. Thus, the effects of differences among lots were bypassed and the linear association of one variable to another within lots was determined.

RESULTS AND DISCUSSION

Tenderness of connective tissue is the component that shows the greatest difference between the two muscles. In LD it was scored tender (Fig. 1). BF had much tougher connective tissue than LD at 61 and 80°C, but at 100°C the difference was significant only in lot 9. The connective tissue was significantly more tender at 80 than at 61°C in six lots for BF but in only three lots for LD. It was very much more tender (highly significant) at 100 than at 80°C in all lots for BF, but for LD this difference was small and not significant in any lot. Fig. 1 shows obvious differences in lot averages, which indicates the need of further study of production and heritability factors.

Connective tissue in muscle is made up of collagen, elastin, reticulin, and the ground substance. Of these constituents, the one present in the largest amount is collagen, which is heat-labile. Thus, collagen analyses seemed especially pertinent in a study of muscles, which differ in amounts of tough connective tissue, and of final meat temperatures that vary from 61°C (near the conversion temperature of some collagens) up to 100°C and holding there for 25 min. The collagen data are given in Tables 5 and 6.

Table 6. Losses in collagen nitrogen during cooking of steaks from two muscles.

Muscle[a]	Losses of collagen nitrogen (% from raw)[b] at different final steak temperatures 61°C	80°C	100°C
10 steers in lot 1			
LD	44.0 ± 4.3	[c]	[d]
BF	40.6 ± 5.0	[c]	[d]
17 steers in lot 5			
LD	38.4 ± 2.1	77.5 ± 1.7	[c]
BF	36.6 ± 2.0	75.1 ± 1.9	[c]
10 heifers in lot 6			
LD	37.6 ± 5.3	71.9 ± 3.8	[c]
BF	33.6 ± 3.1	67.6 ± 4.5	[c]

[a] LD, longissimus dorsi; BF, biceps femoris.
[b] Mean and standard error.
[c] Not analyzed.
[d] Not enough to measure.

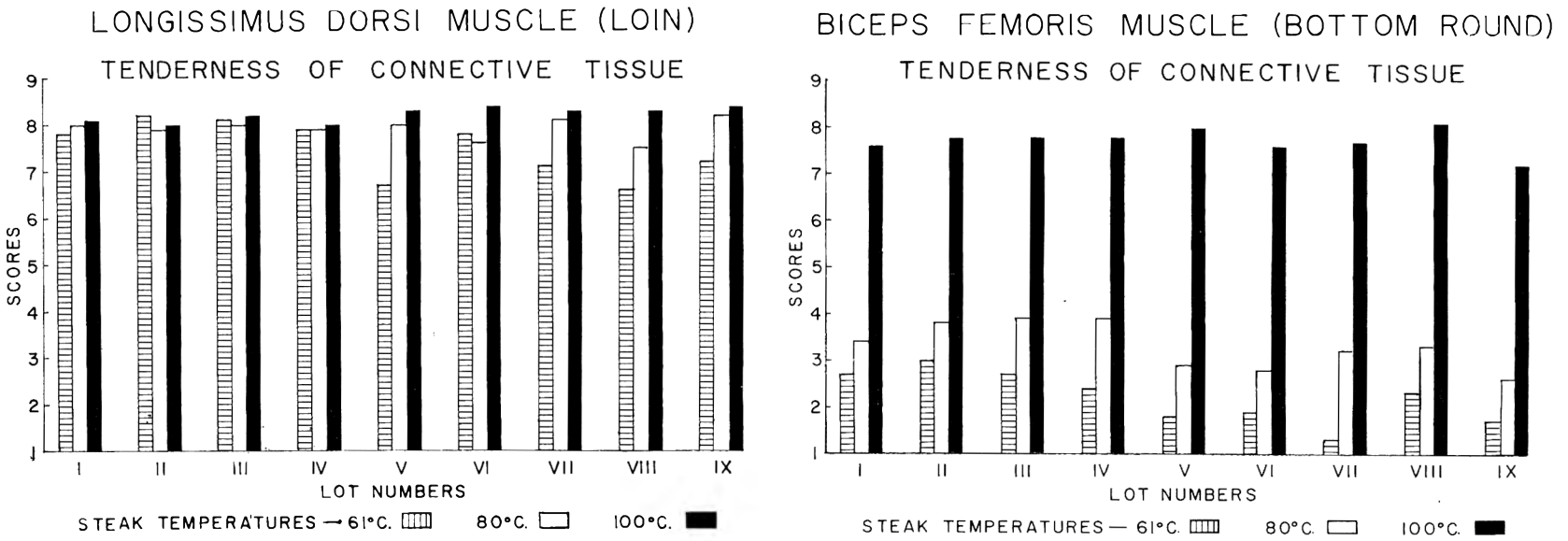

Fig. 1. Average scores for tenderness of connective tissue for steaks from two muscles cooked to three internal temperatures.

The relation of content of collagen (hydroxyproline method) to scores for tenderness of connective tissue may be summarized as follows:

1) Scores for tenderness of connective tissue were lower and collagen content was higher in BF than in LD at 61 and 80°C. There was little difference between the two muscles at 100°C in either scores or collagen content.

2) In BF, collagen content decreased and scores increased with increasing internal temperature.

3) In LD, collagen content also decreased with increasing internal temperatures but scores did not indicate much further tendering of the already tender connective tissue. This might indicate that the judges could not detect differences in collagen content as low as that in LD (below about 1.0 g collagen-nitrogen per 100 g total nitrogen). Other facts, given later, do not bear out this assumption.

4) Collagen content was lower (though the connective tissue was scored tougher) in BF at 80°C than in LD at 61°C. Perhaps a structural difference in the two muscles may influence scores but not collagen content. The low scores for tough connective tissue in BF at 61 and 80°C appeared to reflect strands or lumps of heavy connective tissue that were difficult to masticate. They formed a network that could be identified easily by the tongue. Such heavy strands were seldom encountered in LD.

If the collagen in BF were concentrated in these strands, the loss of collagen and the tendering of the strands with increasing temperatures could still occur. If the collagen in LD were distributed more evenly through the muscle, its detection as separate identifiable connective tissue would be difficult; yet loss of collagen with increasing temperatures could still occur.

It seems unlikely that collagen is in a form more resistant to heat in BF than in LD, for the losses (percent of total collagen in raw) were similar for the two muscles at each of the internal temperatures. This was true not only when collagen tests were based on the hydroxyproline method but also when they were based on alkali-insoluble autoclave-soluble nitrogen (Ritchey and Cover, 1962; Irvin and Cover, 1959). Moreover, when the collagen content in both muscles was too low to be measured accurately (at 100°C), the scores for both muscles were similar.

Cover and Smith (1956) used one score for tenderness, and found broiled steaks from LD to be tougher than steaks from BF although LD contained less collagen than BF. Use of scores for the connective-tissue component of tenderness has increased the precision of this phase of the attack on the multiple causes of tenderness.

The correlations were pooled within lots for each muscle-temperature combination. Tenderness of connective tissue was not really closely associated with any of the other scores. The closest (.55) was for softness to tooth pressure in BF at 61°C, but the variability in tenderness of connective tissue accounted for only 30% of the variability in softness to tooth pressure. In this cut cooked to this temperature, the toughness of connective tissue was most obvious.

No tests were made for the other connective-tissue fibers—elastin and reticulin—or for the ground substance. Neither their effects on tenderness of connective tissue within LD and BF nor their possible unequal distribution among muscles is known, but such possibilities should not be overlooked. Moreover, the reticular network around and within muscle fibers should be kept in mind in relation to possible effects on tenderness of muscle fibers. If this network is found to vary in predictable ways, perhaps a mouth sensation could be identified (by thoughtful and discriminating chewing) that would be suitable for detecting the variations in this factor and thus give subjective proof of its relation to tenderness.

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Tenderness of Beef. II. Juiciness and the Softness Components of Tenderness

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SUMMARY

This paper reports work related to scores for juiciness and two components of tenderness: softness to tongue and cheek, and softness to tooth pressure. As steak temperature increased from 61 to 80°C the meat became drier and harder, but more markedly in biceps femoris than longissimus dorsi. From 80 to 100°C, longissimus dorsi became still drier and harder; biceps femoris also became drier but did not change in softness to tongue and cheek and became softer to tooth pressure. These changes were studied in relation to cooking times and weight losses during cooking; to percent loss of water from raw meat; and to the size and possible hydration of muscle fibers. Correlations calculated on a lot-muscle-temperature basis indicated that juiciness was not closely associated with any of the six components of tenderness. The closest was softness to tongue and cheek, which accounted for about 35% of the variation in biceps femoris at 80°C. The results are discussed in relation to theories of tendering or toughening.

INTRODUCTION

The review of literature and general purpose of these studies were given in the first paper of the series (Cover *et al.*, 1962), which also covered the experimental procedures common to all of the work: a brief description of the animals, the plan for utilizing the meat from the two muscles [longissimus dorsi (LD) and biceps femoris (BF)], the scoring sheet used by the panel, outlines of the analyses of variance, and cooking and sampling methods. The connective-tissue component of tenderness was considered in the first paper. This paper reports the work on juiciness and two kinds of softness.

EXPERIMENTAL

Panel scores (Table 3 of Cover *et al.*, 1962) were weighted so that the highest score would indicate the greatest tenderness. Juiciness was tested by squeezing the meat between the teeth in the first few gentle chews. Softness to tongue and cheek was estimated by feeling with the tongue and cheek. Softness to tooth pressure was judged by the muscular force needed to sink the teeth into the meat during the first few gentle chews. Cutting with the teeth was not necessary.

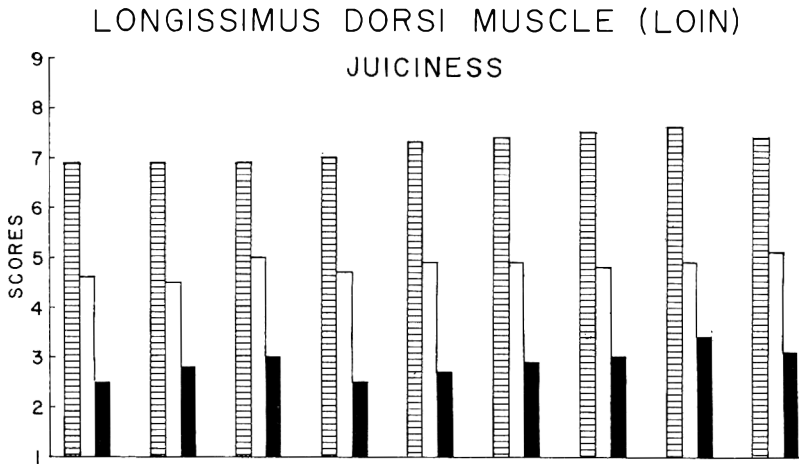
The number of muscle fibers per unit area was obtained for LD steaks cooked to 61 and 80°C. A Howard micrometer disk in 7.5× eyepiece was used with a 21× objective. One frozen section 25 μ thick was obtained from each of the four cores. Counts were made on three 0.27-sq-mm areas of each section, a total of 3.24 sq mm per steak.

Moisture was determined by the method of the A.O.A.C. (1955) on raw steaks and on those cooked to 61, 80, and 100°C. Moisture loss during cooking was calculated as percent loss from the amount in the raw or control steak. These data were collected on 18 steers in lot 5 and on 8 heifers in lot 6.

RESULTS AND DISCUSSION

The results from LD are reported first because LD is the muscle used more frequently for palatability and chemical and physical tests. Connective tissue does not appear to interfere to any large extent with juiciness and softness scores in LD, whereas it may in BF.

Trends with temperature within LD. The well-known toughening effect when LD is heated from rare (61°C) to well-done (80°C) was reflected in some of the tenderness components. At the higher temperature, this muscle was drier and harder to


LONGISSIMUS DORSI MUSCLE (LOIN)
JUICINESS
SCORES
9
8
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6
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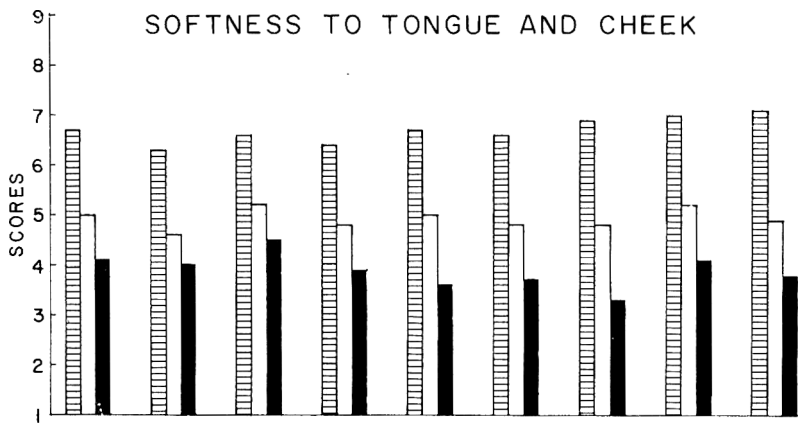
4
3
2
1


SOFTNESS TO TONGUE AND CHEEK
SCORES
9
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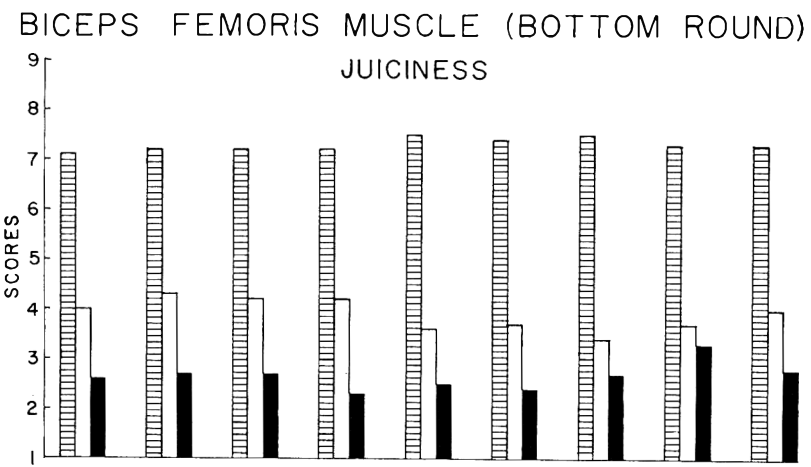
BICEPS FEMORIS MUSCLE (BOTTOM ROUND)
JUICINESS
SCORES
9
8
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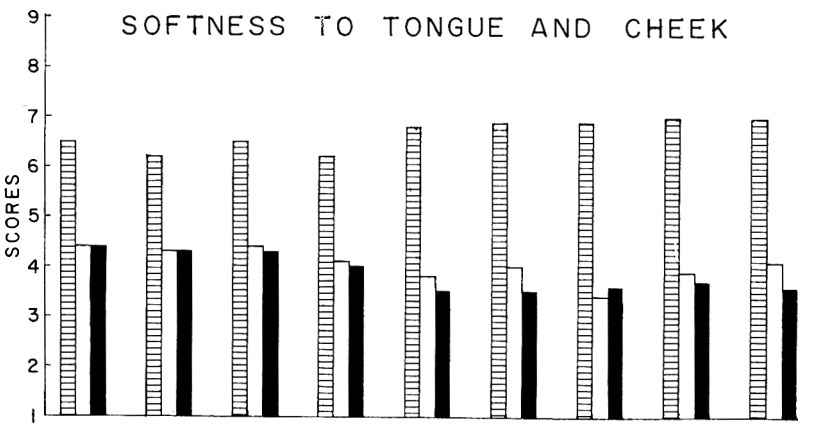
SOFTNESS TO TONGUE AND CHEEK
SCORES
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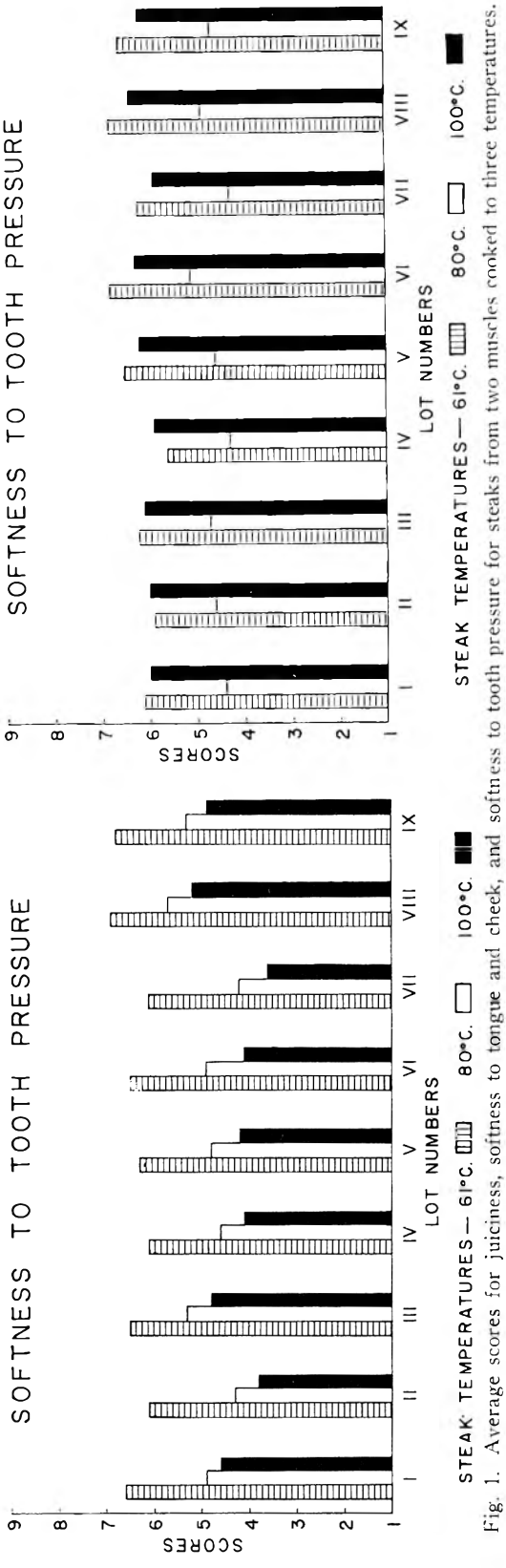

Fig. 1. Average scores for juiciness, softness to tongue and cheek, and softness to tooth pressure for steaks from two muscles cooked to three temperatures.

tongue and cheek and to tooth pressure (Fig. 1). These differences were highly significant in each lot. LD became drier and somewhat harder to tongue and cheek at 100°C than at 80°C (highly significant in all lots). It was also slightly, though not significantly, harder to tooth pressure in each lot. Since these trends are similar in all nine lots, LD from young animals is likely to respond in this way to heating. Increases in temperature within this range cause decreases in all of these scores, but this does not necessarily imply a causal relation between juiciness and softness. Because the two kinds of scores for softness were the only ones in which the toughening on heating was so uniform for all of the nine lots, they may be the components in LD that are most influenced in this direction by heat.

The scores for juiciness and softness in LD were associated with logical and real changes in the meat. It should be possible to associate these scores with non-subjective tests.

Cooking times were longer and weight losses greater at 80 than at 61°C, and these changes were associated with drier and harder meat. In this case, cooking conditions other than final meat temperature were the same (dry heat), but when 80 and 100°C were compared, a change in the conditions of cooking was involved (80°C dry heat vs. 100°C moist heat). In this case the steaks at the higher meat temperature had the shorter time of cooking and only slightly higher weight losses, but were associated with drier and harder meat. Thus, weight loss and cooking time were not the only factors that influenced juiciness and softness as internal meat temperature rose.

Moisture losses, as a percentage of that in raw, increased from 61 to 80°C and again from 80 to 100°C (Table 1), a trend followed inversely by juiciness scores. Although the data on moisture losses in LD are from only 18 steers in lot 5 and on only 8 heifers in lot 6, they are indicative that moisture is lost as the temperature rises in this range. Moist heat does not prevent moisture from being lost.

Weir (1960) considers that juiciness may be separated into two effects: the impres-

Table 1. Moisture loss from meat during heating to three temperatures.

Final meat temperature	Loss of moisture (%)			
	Longissimus dorsi		Biceps femoris	
	Mean	Range	Mean	Range
Lot 5 (18 steers)				
61°C	5.46	4.72– 6.54	4.93	4.41– 6.16
80°C	16.19	12.47–19.75	22.55	17.16–26.87
100°C	23.32	21.05–25.01	23.57	20.36–26.16
Lot 6 (8 heifers)				
61°C	6.48	5.66– 7.20	5.49	4.27– 6.17
80°C	15.13	13.69–17.74	21.40	16.43–28.82
100°C	25.15	23.68–27.18	26.32	23.83–29.60

sion of wetness during the first chews produced by the rapid release of meat fluids, and a sustained juiciness due to slow release of serum and to the stimulating effect of fat on salivary flow. Juiciness, as scored in the present study, may have been largely of the first type since it was scored during the first chews.

Two kinds of moisture are thought to be present in meat. One kind is relatively free to run or be pressed out as juice. Sources of this "juice" may be the lymph and any liquid remaining in the blood vessels and perhaps some of the "capillary" or "immobilized" water referred to by Hamm (1960). Scores for juiciness may be based on it. The other kind is adsorbed water, which varies greatly in degree of binding. Some of it is probably released during heating for the relatively short times employed in cooking. The larger amounts of bound water may be responsible for softness to tongue and cheek at low steak temperatures, whereas hardness at the higher steak temperatures may be associated with small amounts.

Softness to tooth pressure is related to the elasticity of meat. The elasticity of a material that can be pushed out of shape while still holding together is described in physics textbooks in terms of stress (the pushing force) and strain (the deformation, or change in dimension). Thus, the elasticity associated with high scores for softness to tooth pressure would have low stress (tooth pressure) and high strain (large deformation of the meat). Low scores would involve high stress (tooth pressure) and low strain (slight deformation of meat). However, scores for softness to tooth pressure may be high for a reason other than ease of deformation of the meat. Some well-done meat is shattered by slight tooth pressure; low stress carries the material beyond its elastic limits. These differences in elasticity and in scores for softness to tooth pressure may be associated with the relative amounts of bound water.

If loss of water of hydration during heating from 61 to 80°C is the cause of the hardening in meat, it would seem that muscle fibers ought to become smaller in size and also closer together (denser) as meat temperature rises. Such measurements of muscle fibers were attempted. Counts were made of the number of muscle fibers per unit area in frozen sections of LD from 15 animals. More fibers per unit area were found in steaks cooked to 80 than to 61°C (averages of 410 vs. 317 fibers per sq mm)—an increase of about 30%. This seems to indicate that the steak was more dense at 80°C and may imply that the fibers were smaller and less hydrated—a logical basis, perhaps, for the greater hardness. However, there seemed to be no good way to measure the sometimes large spaces around the fibers and to determine whether the shrinkage had been in the fibers or in the spaces or both. A better method was needed.

Measuring the "diameter" of individual muscle fibers that had been teased in the blender was attempted on LD from three animals. The average diameter was 52.9 μ at 61°C and 45.9 μ at 100°C—a decrease of about 12%. This downward trend was consistent among the three animals, as was

the downward trend in softness. However, the diameter appeared to change as the fibers were rotated under the microscope, indicating that they were not round.

Recently, a way has been found of following the changes in length of a small section of a muscle fiber or fibril as it is heated from 30 to 80°C. Such tests are in progress. The results should add considerable physical data about the temperature of shortening of muscle fibers and the association of this occurrence with harder and drier meat at 80 than at 61°C. This is one way in which separation of the components of tenderness is leading to new methods of attack on some of the multiple causes of tenderness.

It has already been suggested that hydration is a possible factor involved in the decreasing scores for softness to tooth pressure with increasing meat temperatures. The work of Hamm and Deatherage (1960) seems especially pertinent. The physical property of meat, which they called "rigidity," may be similar to the subjective evaluation called "softness to tooth pressure" in this study. The change in area of a weighed amount of ground meat after pressing was the measurement used by Hamm and Deatherage. This measurement indicated marked increases in rigidity between 30 and 50°C. No further increase with higher temperatures occurred in two of the four animals, but in at least one of the others considerable further increases were evident at 50, 60, and 80°C. They associated "rigidity" inversely with "bound" water, which decreased markedly between 30 and 50°C. However, they reported that from 60°C hydration decreased continuously with increasing temperature although the decrease was less pronounced than between 40 and 50°C. Thus, in steaks of this study, the temperatures between which the decrease in softness to tooth pressure was largest (61–80°C) were within the range of some increase in rigidity and considerable decrease in hydration but were above the temperatures at which these changes were greatest. Certain differences should be noted between the experimental conditions of the two studies. Each temperature used by Hamm and Deatherage was maintained for 30 min. Their time-temperature relationships were very different from those in the intact steaks, where heating ended as soon as the specified temperature was reached (61 or 80°C). The rapid rate of heat penetration in the range from 1 to 61°C (20–30 min as shown in scatter diagrams by Cover *et al.*, 1957) might not allow time for the completion of reactions that at lower temperatures would be possible with sufficient time. The slower rate of heat penetration from 61 to 80°C (25–45 min), together with the higher temperatures, should favor completion of some of these reactions. Thus, it may be that the temperatures at which the meat hardens and some of the "bound" water is released may be considerably higher under normal cooking conditions than under conditions designed to study the degree of possible completeness of a reaction at a certain temperature. The methods of Grau and Hamm (1953, 1957), Hamm (1959), and Asselbergs and Whitaker (1961) are being adapted to follow the relative amounts of "free" and "bound" water in meat from steaks cooked to various internal temperatures. These tests should provide physical data about the differences in water binding at 61 and 80°C and the relation of this change to differences in scores for softness to tooth pressure. This is another way in which separation of the components of tenderness is leading to new methods of attack on some of the multiple causes of tenderness.

Comparison of the two muscles. As the temperature increased from 61 to 80° C, BF became significantly drier, harder to tongue and cheek, and harder to tooth pressure (Fig. 1). These trends were less marked in LD. This was shown by no significant difference between the two muscles at 61°C but significantly greater decreases at 80°C in BF than LD. BF had a greater area of cut surface exposed than LD. This appeared to facilitate greater evaporation because losses in weight and moisture were greater in BF than LD during dry-heat cooking to 80°C (Table 1). These conditions may have been the reason why the panel found the differences in juiciness and softness more marked in BF than in LD.

With the temperature increase from 80 to 100°C, the trends in BF varied considerably from those in LD (Fig. 1). Although BF was drier at 100 than at 80°C (highly significant for all except lot 8), the amount of the change is much less than in LD (except in lot 2, where the two muscles had similar increases in dryness). Scores for softness to tongue and cheek in BF did not change significantly between 80 and 100°C, whereas in LD they were significantly lower at 100°C. Scores for softness to tooth pressure in BF showed a marked and highly significant increase in softening at 100°C over 80°C, which is in marked contrast to the slight (non-significant) hardening in LD under these conditions. The softening to tooth pressure in BF appeared to have been influenced by somewhat greater ease of fragmentation, greater mealiness, and very much greater tenderness of connective tissue at 100°C. This combination of factors tends to result in shattering or breaking from tooth pressure. At 100°C the elastic limit appeared to be lower in BF than in LD. Thus, in BF, marked decreases in hydration are accompanied by increases in tenderness.

Hamm (1960) listed a series of conditions under which hydration is related to tenderness in a positive manner, but concluded that "tenderness is not only a matter of muscle hydration. Splitting of protein chains during aging and the influence of connective tissue may also be important factors determining the degree of tenderness. Therefore, it is conceivable that a correlation between water-holding capacity and tenderness will not be found in all cases." Scores for the components of tenderness should provide a way of studying the effect of these specific factors separately.

The lot means in Fig. 1 indicate considerable variation among the lots in these meat characteristics. Production and heredity aspects of these characteristics should be investigated.

Interrelationships from linear correlations. Linear correlations were calculated for each lot-muscle-temperature combination, and then the lots were pooled for each muscle-temperature combination. The pooled coefficients are given in Table 2. In neither muscle was juiciness significantly associated with any of the components of tenderness at 61°C. At 80 and 100°C in both muscles, the highest association of juiciness with other scores was with softness to tongue and cheek, although the variability in juiciness accounted for less than 40% of the variability in softness to tongue and cheek. Though none of the coefficients can be called really high, they indicate that softness to tongue and cheek may be the component most concerned in the juiciness-tenderness relationship. This does not necessarily indicate that juiciness is the "cause" of softness to tongue and cheek.

Weight loss was negatively correlated with juiciness and with both measures of

Table 2. Pooled correlations calculated within lots.

	Pooled coefficients [b]					
	Longissimus dorsi			Biceps femoris		
Variables [a]	61°C	80°C	100°C	61°C	80°C	100°C
Juiciness vs:						
Softness to t./c.	.10	.48	.31	.08	.61	.20
Softness to t.p.	−.04	.26	.16	.12	.32	.10
Ease of fragmentation	−.08	.22	.10	.11	.19	.02
Mealiness	....	.02	−.10	....	−.15	−.12
Adhesion	−.11	.22	.07	.15	.17	.08
Tenderness of c.t.	−.06	.05	.01	.03	−.19	−.01
Weight loss	−.15	−.52	−.17	−.11	−.65	−.08
Cooking time	−.17	−.40	.15	.03	−.58	.10

[a] t./c., tongue and cheek; t.p., tooth pressure; c.t., connective tissue.
[b] Coefficients below .16 not significant; between .16 and .21 significant at 5% level; above .21 significant at 1% level.

softness for each cut-temperature combination. Weight losses and juiciness scores were most closely related at 80°C, although none of the coefficients could be called high. At 80°C the two kinds of softness scores were less closely associated with weight losses than were juiciness scores.

Cooking time and juiciness scores also were most closely related at 80°C, but the coefficients were not high. The softness scores were even less closely associated with cooking time at 80°C than were the juiciness scores.

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Tenderness of Beef. III. The Muscle-Fiber Components of Tenderness

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(Manuscript received February 21, 1962)

SUMMARY

With increase in temperature (61, 80, and 100°C), scores for ease of fragmentation and lack of adhesion between muscle fibers trended toward greater toughness in longissimus dorsi (LD) and toward greater tenderness in biceps femoris (BF). Of the two muscles, BF was the more easily fragmented and had less adhesion between muscle fibers at 80 and 100°C. Mealiness was not present at 61°C, but tended to be greater at 100 than at 80°C. BF was considerably more mealy than LD. These changes in tenderness are considered in relation to chemical and histological changes during aging reported by others.

INTRODUCTION

The review of literature and the general purpose of these studies are given in the first paper of the series (Cover *et al.*, 1962a). Also given were the experimental procedures common to all of the work: a brief description of the animals, the plan for utilizing the meat from the two muscles—longissimus dorsi (LD) and biceps femoris (BF)—the scoring sheet used by the panel, outlines of the analyses of variance, and descriptions of the cooking and sampling methods. Considered in the first paper was the connective-tissue component of tenderness. The second paper (Cover *et al.*, 1962b) reported work on juiciness and two softness components of tenderness. This paper considers the three muscle-fiber components of tenderness.

EXPERIMENTAL

Panel scores (Table 3 of Cover *et al.*, 1962a) were weighted so that the highest score indicated greatest tenderness. Ease of fragmentation was scored on how easily the muscle fibers could be cut with the teeth. Apparent adhesion between muscle fibers was judged by ease of separation of muscle fibers. Mealiness was a special kind of fragmentation: the fragments were tiny, dry, and hard, and clung to cheeks, gums, and tongue. High scores indicated a high degree of mealiness.

RESULTS AND DISCUSSION

Ease of fragmentation and adhesion. In LD the muscle fibers appear to fragment less easily and to adhere (or cohere) more tightly with increasing internal temperature (Fig. 1). This is a trend toward toughness. However, differences between 61 and 80°C were not significant in any lot. Differences between 80 and 100°C were significant only in lot 6. Even between 61 and 100°C the differences were not significant for all lots. Thus, the toughening of these two components with increase in steak temperature is neither as great nor as uniform among the lots as the components previously reported (softness to tongue and cheek, and softness to tooth pressure).

In BF the trend with increasing temperatures was toward greater ease of fragmentation and less adhesion between muscle fibers (Fig. 1), although the differences with temperature were seldom significant. This is a trend toward tendering, not toughening, with heat. Thus, the trends in the two muscles move in opposite directions. As proof for the opposing nature of these trends, it may be noted that at 61°C BF was slightly but seldom significantly more tender than LD, whereas at 100°C BF was uniformly and highly significantly the more tender. This divergence of trends (differential response) of the two muscles at the different temperatures was also measured statistically by the significant muscle × temperature interaction.

No single change or reaction is known in

muscle fibers that would account for both the slight toughening in LD and the slight tendering in BF. Perhaps several changes or reactions are involved, with those that cause toughening predominating in one muscle and those that cause tendering predominating in the other muscle. Further considerations are presented later.

Mealiness. Mealiness was not present at 61°C, because any particles that might have been present were moist, not dry, and did not cling to cheeks, gums, and tongue. Mealiness in LD steaks seemed to increase slightly with an increase in steak temperatures from 80 to 100°C (Fig. 1), but the differences were significant only in lot 1.

In BF, mealiness also increased from 80 to 100°C (Fig. 1), and this difference was significant except in lots 5, 7, and 9. At 80 and 100°C, BF was uniformly and highly significantly the more mealy of the two muscles. The increase in mealiness from 80 to 100°C was greater in BF than in LD.

The trends in mealiness are toward a tendering instead of a toughening response to heat. This is because the fibers separate readily into such tiny particles. The tendering changes responsible for mealiness are more prominent in BF than in LD.

Discussion of the muscle-fiber components. A toughening reaction in muscle fibers to heat could be the tightening of the network of protein structure during denaturation as new stable cross linkages are formed between the peptide chains (Hamm and Deatherage, 1960). This probably occurs in all muscles and has been discussed under softness to tooth pressure. One way of decreasing ease of fragmentation might be for the tightening effect to occur along the chain to make breaking across it more difficult. No clear evidence of this type of change in protein structure has been found in the literature. Perhaps all intact muscle fibers resist breaking across the grain.

An increase in ease of fragmentation and mealiness would involve separations across the fiber to allow it to come apart into shorter lengths. Peptide bonds are unaffected by heat alone, and if the fibril is a long chain of peptide bonds these bonds must have been weakened previously in some way. Weakening prior to cooking is suggested by the high animal or carcass variability of scores for ease of fragmentation and mealiness. Some peptide bonds are thought to be broken as a result of "aging" after rigor (Hamm 1960, p. 392). Paul *et al.* (1952) reported that histological examination of semitendinosus muscle revealed cracks in the fibers after rigor had diminished (48-53 hr of storage), and that on longer storage (6 days) these cracks developed into breaks and even into granulated areas in the fibers. If similar happenings take place in LD and BF during the standardized 7-day storage period, the meat of this study could have had not only some weakened peptide bonds but some cracks and breaks in the fibers, which would have made fragmentation across the grain easy. It is not known whether these were accentuated by higher temperature, thus accounting for the slight increase in ease of fragmentation of BF. The granulated areas might have supplied preformed tiny particles. These tiny particles could have become hard and dry during heat denaturation, contributing to the mealy sensation in the mouth.

Paul *et al.* (1952) did not report measurements of their particles. Locker (1959, 1960) reported sarcomeres 0.7–3.7 μ long in beef muscle. The mealy particles that seem tiny to the tongue may be large in terms of lengths of amino acids or of segments in the histological structure of the fibrils (sarcomeres).

A few measurements were made of the lengths of the fiber particles obtained after mastication by one of the Texas judges. In steaks with high scores for mealiness there were many particles below 400 μ, with some around 100 μ, and considerable debris. In steaks with low scores for mealiness there appeared to be fewer such small particles. Further evidence of shorter fibers in steaks with high scores for mealiness will be presented in the fourth paper of this series, which will discuss the availability in these steaks of muscle fibers for extensibility determinations.

Further experiments are needed to relate differences in scores of the muscle-fiber

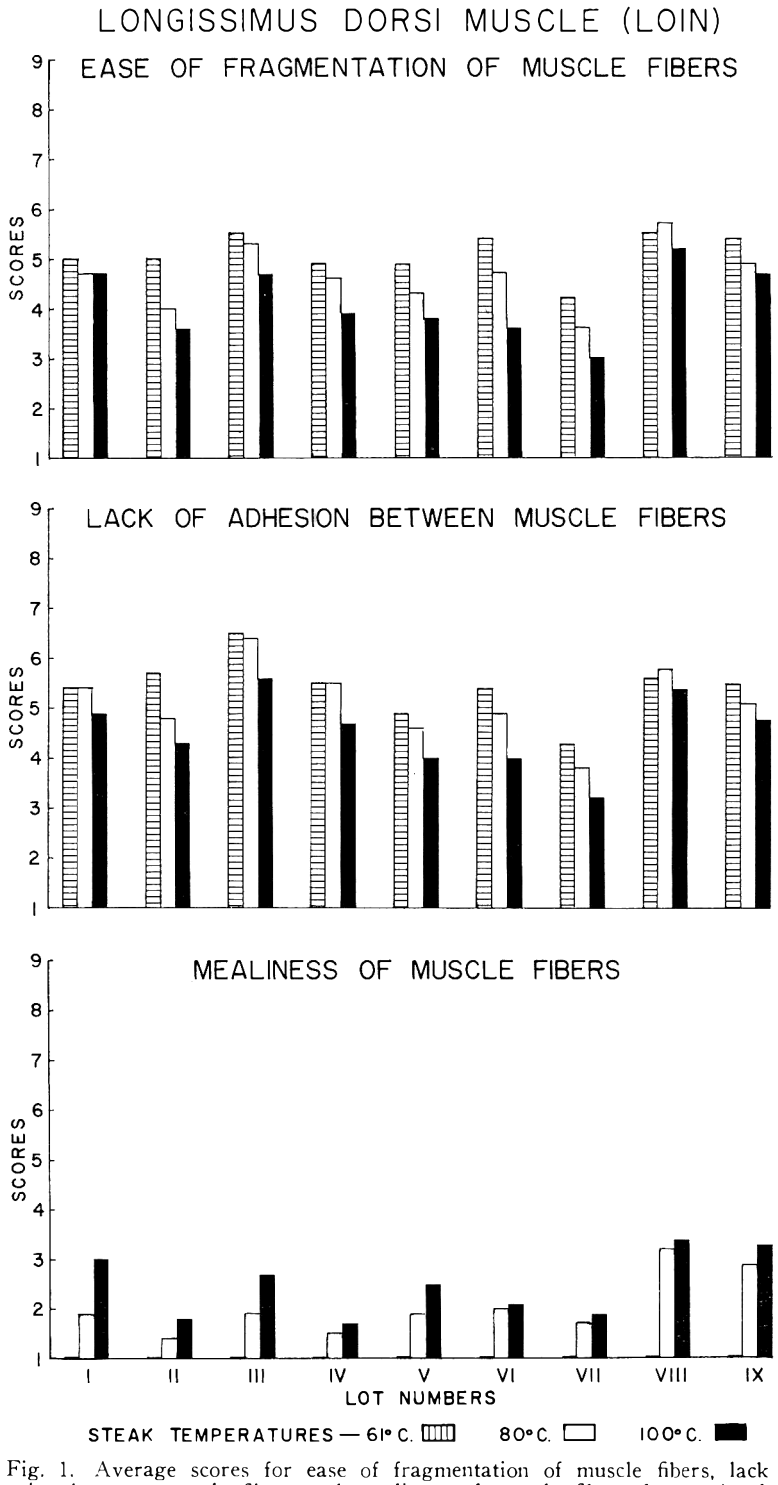
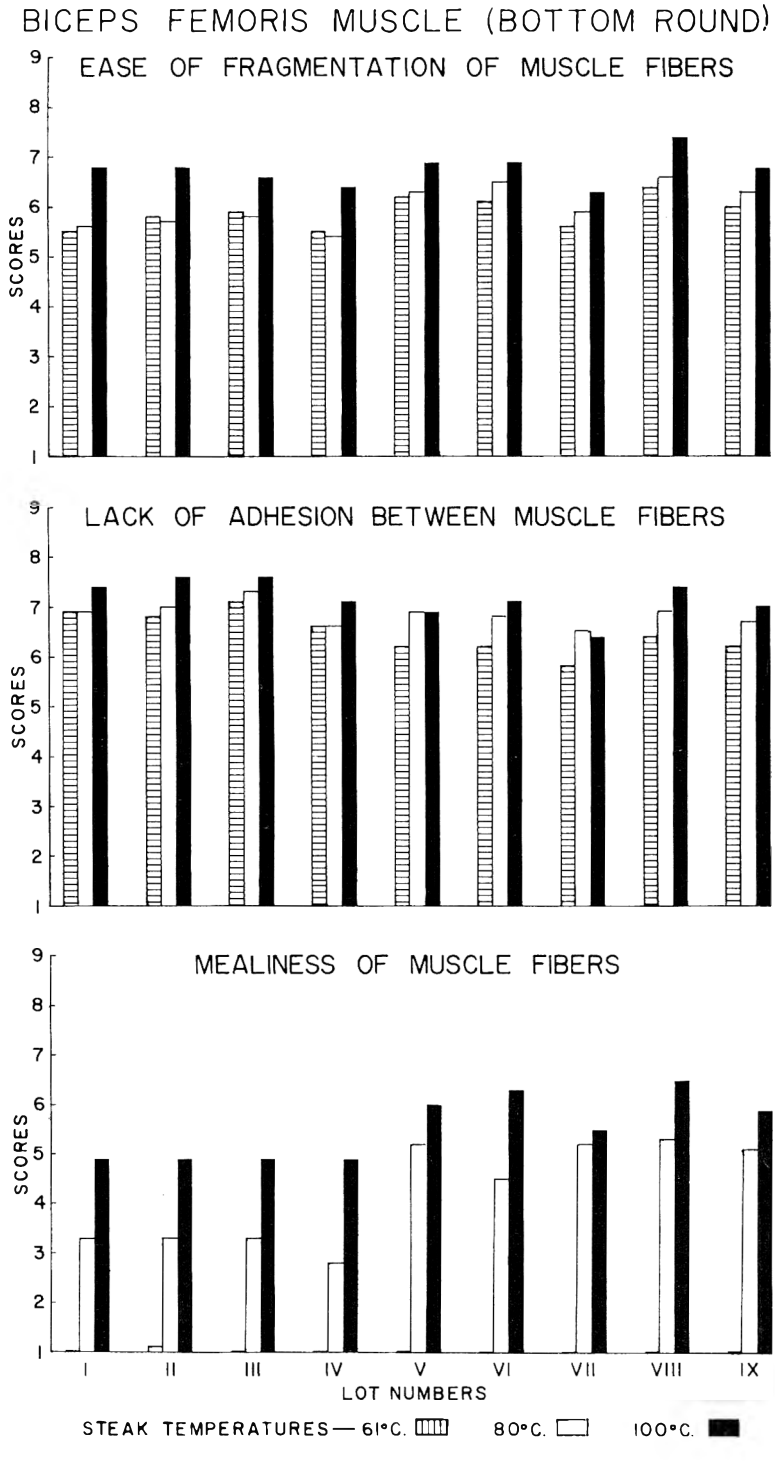


Fig. 1. Average scores for ease of fragmentation of muscle fibers, lack of adhesion between muscle fibers and mealiness of muscle fibers for steaks from two muscles cooked to three internal temperatures.


BICEPS FEMORIS MUSCLE (BOTTOM ROUND)
EASE OF FRAGMENTATION OF MUSCLE FIBERS
LACK OF ADHESION BETWEEN MUSCLE FIBERS
MEALINESS OF MUSCLE FIBERS
SCORES
1
2
3
4
5
6
7
8
9
I
II
III
IV
V
VI
VII
VIII
IX
LOT NUMBERS
STEAK TEMPERATURES — 61°C. 80°C. 100°C.

components to the physical and chemical changes in meat cooked to different internal temperatures.

Adhesion is a remarkable property. It is not an outstanding characteristic in all meat, but it can be very objectionable, especially in some LD steaks. The apparent felting that takes place on mastication is difficult to explain. Barbs (minute projections from the fiber) might cause such felting (cohesion), but no such structures are known. Nor has any separate substance been demonstrated that would bind the fibers closer together at the higher temperatures (adhesion). This property shows high variability among animals, but there is no indication whether the changes responsible for differences in adhesion occurred before or after slaughter.

Fig. 1 shows considerable variation among lot means. This indicates the need for investigation of the influence of production and heredity factors on these components of tenderness.

Interrelations from linear correlations. The correlations were pooled within lots for each muscle-temperature combination (Table 1).

Ease of fragmentation was not closely associated with scores for juiciness or tenderness of connective tissue or with weight loss or cooking time. This gives added emphasis to the separateness of the characteristics on which are based these scores for juiciness, ease of fragmentation, and tenderness of connective tissue. On the other hand, ease of fragmentation was related most closely to lack of adhesion. This means that muscle fibers that were most difficult to fragment across the grain also tended to felt together most strongly. Subjectively, these characteristics seem quite distinct. Much further

Table 1. Pooled correlations calculated within lots.

	Pooled coefficients [b]					
	Longissimus dorsi			Biceps femoris		
Variables [a]	61°C	80°C	100°C	61°C	80°C	100°C
Ease of fragmentation vs:						
Juiciness	—.08	.22	.10	.11	.19	.02
Softness to t./c.	.51	.61	.64	.31	.35	.34
Softness to t.p.	.76	.89	.91	.37	.57	.84
Adhesion	.90	.92	.94	.75	.86	.88
Mealiness	....	.70	.83	....	.67	.83
Tenderness of c.t.	.24	.14	.36	.22	—.20	.20
Weight loss	—.03	—.24	—.33	—.11	—.29	—.32
Cooking time	.11	—.07	—.12	.01	—.29	—.09
Adhesion vs:						
Juiciness	—.11	.22	.07	.15	.17	.08
Softness to t./c.	.48	.62	.65	.30	.28	.30
Softness to t.p.	.70	.86	.90	.36	.46	.79
Mealiness	....	.65	.82	....	.69	.75
Tenderness of c.t.	.21	.14	.41	.19	—.24	.24
Weight loss	—.02	—.26	—.37	—.16	—.21	—.32
Cooking time	.02	—.07	—.08	.00	—.21	—.11
Mealiness vs:						
Juiciness	....	.02	—.10	....	—.15	—.12
Softness to t./c.	....	.31	.51	....	.14	.27
Softness to t.p.	....	.61	.75	....	.36	.72
Tenderness of c.t.	....	.04	.32	....	—.08	.21
Weight loss	....	—.07	—.28	....	—.04	—.26
Cooking time	....	.10	—.01	....	—.05	—.13

[a] t./c., tongue and cheek; t.p., tooth pressure; c.t., connective tissue.
[b] Coefficients below .16 not significant; between .16 and .21 significant at 5% level; above .21 significant at 1% level.

work is needed to explain these observations.

The component next-most closely associated with ease of fragmentation is softness to tooth pressure. This association becomes progressively higher as temperatures increase, especially in BF, where the correlation at 61°C is low. Hardness to tooth pressure and difficulty of fragmentation appear to be distinct subjective impressions.

Lack of adhesion was not closely associated with juiciness or tenderness of connective tissue or with weight loss or cooking time. It was closely associated with softness to tooth pressure. This indicated that meat that felted most tightly also tended to be the hardest to tooth pressure. This relation was closer in LD than in BF. The subjective impressions for the two components seemed quite distinct.

Mealiness was not closely associated with scores for juiciness or tenderness of connective tissue, or with weight loss or cooking time. It seems to be associated most closely with ease of fragmentation, lack of adhesion, and softness to tooth pressure at 100°C.

Physical tests suitable for the muscle-fiber components appear to be those involving cutting or breaking across the grain, such as shear-force values and extensibility of muscle fibers. The relation of these physical tests to scores will be discussed in the next paper.

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Confusion in Sensory Scoring Induced by Experimental Design

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SUMMARY

Replicated scorings of 4 series of wines were made by 8 tasters with two different designs of presentation. Design 1 consisted of different wines with additives (each wine with four concentrations of additives was tasted as a block) and design 2 consisted of randomization of tastings over all wines and additives. In any one design, tasters did not react the same for any given series. The differences showed up in both mean scorings and in the variability of the scorings. When the design was broadened to include more diverse categories in a randomized fashion, the scorings became biased in rather unpredictable ways and the variability of the scorings increased in general, in some cases.

INTRODUCTION

Commercial and experimental wineries frequently have large numbers of wines to taste. Included are many types of wines, such as red, white, dry and sweet from different regions. Some people advocate randomizing all wines to be tasted, whereas others think it better to taste wines in smaller groups that are more nearly alike—for instance, dry wines from one region made from a single variety of grapes.

It has been the general belief that tasting wines by randomizing the orders for all wine types and groups was better except that the order of presentation should be restricted to: 1) dry white, 2) dry red, 3) white sweet, and 4) red sweet. Work by Ough and Baker (unpublished and 1961), however, indicated that such wide randomization was not desirable if variations within types within regions were of interest. Very large interactions of taster × region and wine × taster were found, and, further, the residual error variances, when compared to the duplicate error variance, were found, in general, to he significantly larger. These findings do not prove conclusively that the design of the experiment was at fault, but they do indicate that the design of such experiments should be investigated further.

In view of these facts an experiment was planned to test design differences with 4 groups of wines with predetermined and significant quality differences.

EXPERIMENT

The wines used were standard quality with respect to chemical analysis. Previous tastings had shown no outstanding defects or extraordinary qualities. Acetic acid was added to series I in varying amounts to give concentrations that would allow the separation of levels. Series II and III were treated likewise, but with acetaldehyde and sulfur dioxide. Series IV was treated with sorbic acid. Sorbic acid, at the levels used, would normally be detectable in wine after the treated samples had been stored for a short time. However, the fact that no differences were shown in levels is probably because the wines for each test were freshly made up and tasted within a several-hour period each day. All treatments were selected so as to be familiar to the panel and to decrease quality, thus partially eliminating 2-tail effects.

Samples, in wine glasses coded with three-digit numbers, were presented individually to 8 tasters over a 16-day period. The 4 samples of series I were presented in duplicate the 1st day to the panel as a complete randomized block, and on the 9th day in the same manner (design 1). On the second day 8 wines of series I, II, III, and IV were presented to the tasters. [The 16 wines were randomized into two 16×16 blocks, and each taster assigned 2 rows from each block. The 1st 8 wines of the 1st 8 rows of the 1st block were given on the 2nd day, and the last 8 of a row on the 4th day, etc. (design 2)]. Wines of series II were presented on the 3rd and 11th days as described for series I. Wines of series III and IV were given similarly on the 5th and 13th and on the 7th and 15th days. Thus the wines were presented in design 1 on the odd days and in design 2 on the even days. Scoring was by a 20-point quality scoring system discussed by Ough and

Baker (1961). The scores are given in Table 1. Mean scores for the four replicates of each taster for each of the 4 levels of the different series are given in Table 2.

The panel members had no individual who could be classed in the novice rank. Tasters 2 and 8 had panel experience in scoring wines in excess of 15 years; tasters 3, 4, and 6 had more than 4 years; and tasters 1, 5, and 7 had two years of experience.

Table 3 indicates the squariances of each series level for each design.

ANALYSIS AND DISCUSSION OF DATA

Although the standard analysis of variance is not strictly applicable to these data, because of variances that are significantly different by the F-test as indicated in Table 3 (see Anscombe, 1961), such analyses were made as an exploratory technique since the procedure of breaking sums of squares into parts and assessing the relative importance of the parts is known to be robust and usually leads to further analysis that is closer to the real structure of the situation being examined. The analyses indicated differences between tasters, treatments, designs. Interactions were important. The error terms were uniformly higher for the combined design than for the wines presented in more homogeneous groups.

In order to make clear the nature of the differences in mean scorings given in Table 2, consider Figs. 1–4, which group the tasters on the basis of changes in average

Table 1. Four replicated scorings for 4 series of wines in 4 categories presented in 2 designs to 8 tasters.

	Series I								Series II							
	A Designs		B Designs		C Designs		D Designs		A Designs		B Designs		C Designs		D Designs	
Tasters	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	12	11	10	11	12	10	7	10	13	11	12	12	12	10	12	10
	13	11	10	13	13	11	12	12	14	13	13	12	13	10	12	10
	15	12	12	13	14	13	14	12	14	13	14	12	14	10	12	12
	15	14	13	13	14	13	14	13	15	14	15	15	14	12	12	12
2	13	13	13	12	12	10	12	10	13	8	9	4	6	4	4	4
	13	13	13	12	13	12	13	10	13	9	11	5	6	6	4	6
	13	13	14	13	14	12	14	12	13	13	12	6	6	10	4	8
	14	14	14	13	14	12	14	13	14	14	13	8	8	12	6	8
3	11	10	11	9	9	7	8	8	12	12	10	8	10	9	8	5
	12	10	11	10	10	8	8	8	13	13	12	10	11	11	9	7
	12	10	12	12	10	9	10	9	14	13	13	12	12	12	10	8
	13	12	13	12	10	9	10	11	15	15	14	13	14	12	11	8
4	12	5	10	5	10	8	8	7	15	14	14	10	12	6	11	5
	12	11	13	11	11	9	10	8	15	14	14	12	13	12	12	11
	13	12	13	11	13	10	11	9	15	15	14	14	13	13	12	12
	13	13	14	12	13	11	13	10	15	17	14	14	14	13	13	13
5	11	5	12	5	6	5	6	5	11	8	9	5	8	6	4	5
	11	8	12	7	10	6	8	5	14	11	10	8	9	8	8	11
	12	11	12	10	11	12	8	6	14	13	11	12	10	10	9	12
	12	11	12	11	12	14	11	7	15	15	13	13	11	12	9	12
6	13	8	11	13	13	6	10	6	11	8	11	10	11	10	10	10
	15	12	12	13	14	7	12	8	12	11	12	11	12	11	10	10
	15	12	12	14	14	8	13	12	15	12	13	12	14	11	11	10
	15	14	13	16	15	13	14	14	16	12	13	13	14	11	12	10
7	10	11	10	8	8	6	4	4	13	9	7	9	8	8	4	8
	10	11	11	9	13	6	8	6	14	13	13	10	10	8	4	8
	12	12	12	10	14	8	11	9	14	13	14	12	12	8	4	9
	13	13	14	12	14	9	12	11	14	16	14	12	13	12	6	12
8	14	8	8	4	7	5	7	3	11	11	10	8	8	5	6	4
	14	10	12	4	8	9	10	4	14	12	10	9	8	6	6	5
	14	13	12	7	8	9	12	8	15	14	10	9	12	7	6	6
	14	14	14	9	9	9	12	10	15	15	15	12	12	11	7	7

Table 1 (continued)

	Series III								Series IV							
	A Designs		B Designs		C Designs		D Designs		A Designs		B Designs		C Designs		D Designs	
Taster	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	14	13	13	11	12	10	12	9	14	12	13	12	14	12	15	12
	14	13	13	15	12	10	12	10	15	13	16	14	14	14	15	13
	15	13	14	15	13	14	12	12	15	13	16	14	15	15	15	13
	15	15	15	15	14	15	13	13	16	16	16	15	16	15	16	13
2	10	10	11	10	12	5	12	9	8	13	8	12	12	9	9	12
	12	12	12	11	12	10	12	11	10	13	9	13	12	13	11	13
	12	13	13	12	14	11	12	11	13	14	10	13	13	14	11	14
	14	13	13	13	14	12	12	12	13	14	12	13	13	15	13	14
3	9	9	9	11	8	8	8	9	10	10	8	10	11	10	10	13
	12	12	10	11	9	10	11	9	12	11	10	12	12	10	10	13
	12	13	10	11	10	12	12	13	13	12	12	14	14	13	11	13
	13	14	13	13	12	12	13	13	13	13	12	15	14	14	13	14
4	13	10	12	14	12	12	12	11	12	13	12	13	13	10	12	9
	13	13	12	14	12	12	13	12	13	13	13	13	13	12	13	10
	13	13	14	15	13	13	14	12	14	14	14	14	14	13	14	12
	14	15	14	15	14	14	14	13	15	14	14	14	14	14	15	12
5	13	12	13	13	12	11	12	10	8	10	5	11	5	13	7	8
	14	12	14	13	12	12	12	11	13	12	7	12	5	14	11	10
	14	14	15	14	12	13	13	12	14	12	8	14	7	14	12	14
	14	14	15	15	13	14	14	13	14	13	13	15	11	14	12	14
6	12	11	11	10	12	13	8	8	12	12	8	8	8	11	10	7
	13	13	12	10	13	13	10	8	12	12	11	10	13	11	10	10
	14	14	12	14	14	14	11	11	13	12	12	11	14	12	11	10
	15	14	15	15	14	14	12	13	13	15	15	11	14	13	13	12
7	12	8	10	12	6	10	6	6	10	10	8	8	12	9	12	6
	12	12	13	12	12	12	8	12	12	10	9	10	13	10	12	8
	14	12	13	12	13	13	12	12	12	14	9	12	13	13	12	12
	14	15	13	12	13	14	12	15	13	14	12	12	14	13	14	12
8	10	12	14	10	6	10	6	9	13	14	15	14	14	14	14	14
	11	14	14	14	6	13	8	9	14	14	15	14	14	14	15	14
	14	14	14	14	12	13	8	10	15	15	15	15	14	14	15	15
	14	15	15	15	12	14	10	11	15	15	15	15	15	15	15	15

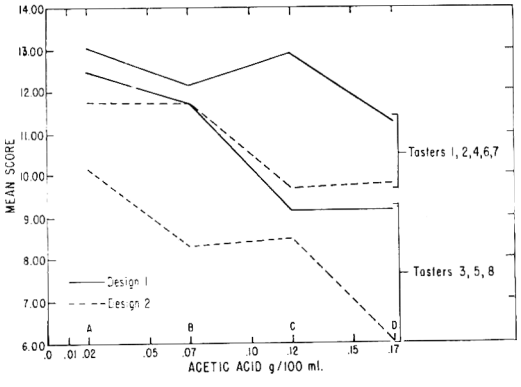

Fig. 1. Series I means for tasters 1, 2, 4, 6, and 7, and tasters 3, 5, and 8, for designs 1 and 2.

scores from A to D for homogeneous (design 1) tastings and compare the designs for such groups. These figures also indicate important interactions of taster × design.

The reduced sums of squares of Table 3 indicate definitely that some tasters are very confused by design 2 (completely randomized presentation). This confusion is not consistent from series to series. Some tasters are much more variable for both designs than are others. Tests for significance can be made on the basis of the usual F-test, and are not presented in detail since the reader can easily make any pertinent test in which he is interested.

Table 2. Mean scorings for 4 series of wines in 4 categories presented in two designs to 8 tasters.

Taster	Design	Series I				Series II				Series III				Series IV			
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1	1	13.75	11.25	13.25	11.75	14.00	13.50	13.25	12.00	14.50	13.75	12.75	12.25	15.00	15.25	14.75	15.25
	2	12.00	12.50	11.75	11.75	12.75	12.75	10.50	11.00	13.50	14.00	12.25	11.00	13.50	13.75	14.00	12.75
2	1	13.25	13.50	13.25	13.25	13.25	11.25	6.50	4.50	12.00	12.25	13.00	12.00	11.00	9.75	12.50	11.00
	2	13.25	12.50	11.50	11.25	11.00	5.25	8.00	6.50	12.00	11.50	9.50	10.75	13.50	12.75	12.75	13.25
1	1	12.00	11.75	9.75	9.00	13.50	12.25	11.75	9.50	11.50	10.50	9.75	11.00	12.00	10.50	12.75	11.00
	2	10.50	10.75	8.25	9.00	13.25	10.75	11.00	7.00	12.00	11.50	10.50	11.00	11.50	12.75	11.75	13.25
4	1	12.50	12.50	11.75	10.50	15.00	14.00	13.00	12.00	13.25	13.00	12.75	13.25	13.50	13.25	13.50	13.50
	2	10.25	9.75	9.50	8.50	15.00	12.50	11.00	10.25	12.75	14.50	12.75	12.00	13.50	13.50	12.25	10.75
5	1	11.50	12.00	9.75	8.25	13.50	10.75	9.50	7.50	13.75	14.25	12.25	12.75	12.25	8.25	7.50	10.50
	2	8.75	8.25	9.25	5.75	11.75	9.50	9.00	10.00	13.00	13.75	12.50	11.50	11.75	13.00	13.75	11.50
6	1	14.50	12.00	14.00	12.25	13.50	12.25	12.75	10.75	13.50	12.50	13.25	10.25	12.50	11.50	12.25	11.00
	2	11.50	14.50	8.50	10.00	10.75	11.50	10.75	10.00	13.00	12.25	13.50	10.00	12.75	10.00	11.75	9.75
7	1	11.25	11.75	12.25	8.75	13.75	12.00	10.75	4.50	13.00	12.25	11.00	9.50	11.75	9.50	13.00	12.50
	2	11.75	9.75	7.25	7.50	12.75	10.75	9.00	9.25	13.00	12.00	12.25	11.25	12.00	10.50	11.25	9.50
8	1	14.00	11.50	8.00	10.25	13.75	11.25	10.00	6.25	12.25	14.25	9.00	8.00	14.25	15.00	14.25	14.75
	2	11.25	6.00	8.00	6.25	13.00	9.50	7.25	5.50	13.75	13.25	12.50	9.75	14.50	14.50	14.25	14.50
Averages for Design																	
	1	12.84	12.03	11.50	10.50	13.78	12.16	10.94	8.38	12.97	12.84	11.72	11.12	12.78	11.62	12.56	12.44
	2	11.16	10.44	9.25	8.75	12.53	10.31	9.56	8.69	12.88	12.84	11.97	10.91	12.88	12.59	12.72	11.91
Average Total		12.00	11.23	10.38	9.62	13.16	11.23	10.25	8.53	12.92	12.84	11.84	11.02	12.83	12.11	12.64	12.17

Table 3. Squariances for 4 series of wines in 4 categories presented in two designs to 8 tasters.

		Series I				Series II				Series III				Series IV			
Taster	Design	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1	1	6.75	6.75	2.75	32.75	2.00	5.00	2.75	0.00	1.00	2.75	2.75	0.75	2.00	6.75	2.00	0.75
	2	6.00	3.00	6.75	4.75	4.75	6.75	3.00	4.00	3.00	12.00	20.75	10.00	9.00	4.75	6.00	0.75
2	1	0.75	1.00	2.75	2.75	0.75	8.75	3.00	3.00	8.00	2.75	4.00	0.00	18.00	8.75	1.00	8.00
	2	0.75	1.00	3.00	6.75	26.00	2.75	40.00	11.00	6.00	5.00	29.00	4.75	1.00	0.75	20.75	2.75
4	1	2.00	2.75	0.75	4.00	5.00	8.75	8.75	5.00	9.00	9.00	8.75	14.00	6.00	11.00	6.75	6.00
	2	3.00	6.75	2.75	6.00	4.75	14.75	6.00	6.00	14.00	3.00	11.00	16.00	5.00	14.75	12.75	0.75
1	1	1.00	9.00	6.75	13.00	0.00	0.00	2.00	2.00	0.75	4.00	2.75	2.75	5.00	2.75	1.00	5.00
	2	38.75	30.75	5.00	5.00	6.00	11.00	34.00	38.75	12.75	1.00	2.75	2.00	1.00	1.00	8.75	6.75
5	1	1.00	0.00	20.75	12.75	9.00	8.75	5.00	17.00	0.75	2.75	0.75	2.75	24.75	34.75	24.00	17.00
	2	24.75	22.75	58.75	2.75	26.75	41.00	20.00	34.00	4.00	2.75	5.00	5.00	4.75	10.00	0.75	27.00
6	1	3.00	2.00	2.00	8.75	17.00	2.75	6.75	2.75	5.00	9.00	2.75	8.75	1.00	25.00	24.75	6.00
	2	19.00	6.00	29.00	40.00	10.75	5.00	0.75	0.00	6.00	20.75	1.00	18.00	6.75	6.00	2.75	12.75
7	1	6.75	8.75	24.75	38.75	0.75	34.00	14.75	3.00	4.00	6.75	34.00	27.00	4.75	9.00	2.00	3.00
	2	52.75	8.75	6.75	29.00	24.75	6.75	12.00	10.75	24.75	0.00	8.75	42.75	16.00	11.00	12.75	27.00
8	1	0.00	19.00	2.00	16.75	10.75	18.75	16.00	0.75	12.75	0.75	36.00	8.00	2.75	0.00	0.75	0.75
	2	22.75	18.00	12.00	32.75	20.00	9.00	20.75	5.00	4.75	14.75	9.00	2.75	1.00	1.00	0.75	1.00

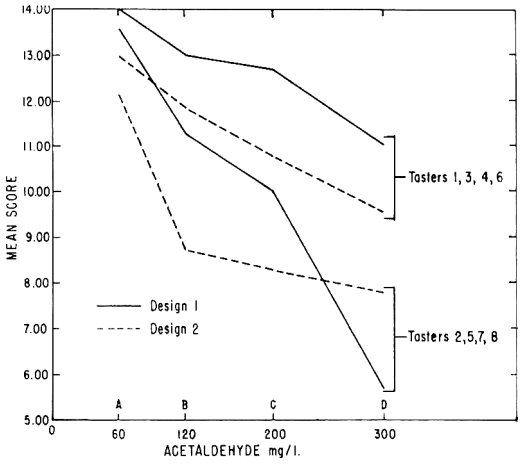

Fig. 2. Series II means for tasters 1, 3, 4, and 6, and for tasters 2, 5, 7, and 8, for designs 1 and 2.

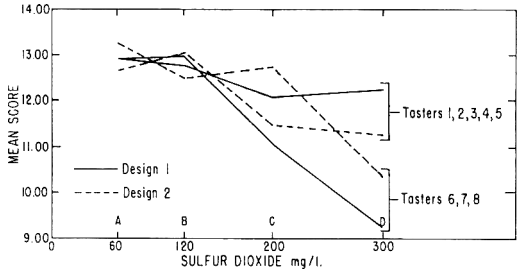

Fig. 3. Series III means for tasters 1, 2, 3, 4, and 5, and for tasters 6, 7, and 8 for designs 1 and 2.

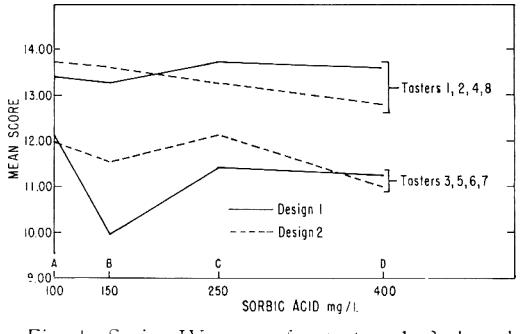

Fig. 4. Series IV means for tasters 1, 2, 4, and 8, and tasters 3, 5, 6, and 7, for designs 1 and 2.

RECOMMENDATIONS

Since no better method is available for evaluation of the quality of a large number of samples, it is assumed that scoring and analysis of variance will be used. Since it is also evident that the two designs presented do not yield the same magnitude of mean score (at least in series I and II), factors are operating that cause the tasters to change their concept of quality. One argument is that, in tasting each wine with additives as a block, no reference is available and the taster is oriented to his first sample score and must base the rest of his scores up or down from this score, depending on the level of the treatment. This argument is weak in that his first score could be either high or low; also, the two higher-treatment levels would appear first half of the time, leading to generally lower first scores than with design 2. The second argument is that, in the random sets of tasting, the general qualities of the wines of series III or IV or both were sufficiently superior to those of series I and II to cause the mean scores of I and II to be depressed; but conversely, the mean scores of series III and/or IV should have increased. None of these arguments satisfactorily explain the results.

Since the fact remains that design-2 tastings affect the mean scores, it would seem plausible to believe that tasting by design 1 would be more satisfactory in minimizing the confounding effects of other wines. Results should be more reliable if wines of the same quality type are judged as groups rather than a random mixing of different types, and the judgments should be more consistent and reflect the general quality within the type group more accurately year to year. The variabilities are more nearly equal for design 1 than for design 2, so that the usual analyses of variance are not disutrbed as much for the first type of design as for the second.

ACKNOWLEDGMENT

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Taste Interrelationships. III. Suprathreshold Solutions of Sucrose and Sodium Chloride

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SUMMARY

The taste interaction of suprathreshold solutions of sucrose and sodium chloride was determined by highly trained subjects using single- and paired-sample presentations. With both methods, the apparent saltiness of 0.12–3.24% sodium chloride was reduced by sucrose. In general, the sweetness of 0.75, 2.25, and 6.75% sucrose was enhanced by lower, and depressed by higher, salt additions. All levels of salt depressed the sweetness of 20.25% sucrose. Response to sweetness and saltiness varied with method of presentation. There was a large amount of variation in scoring attributable to differences between judges, but reproducibility of judgment was much greater in the paired- than in the single-stimulus method.

INTRODUCTION

When a solution of two distinct taste substances is tested, the compounds stimulate not only their specific receptors but other receptors in the oral cavity. As pointed out by Warren (1953), one does not think of a sweet compound as discharging a salt receptor, but it is possible that sugar can affect salt receptors in a manner that changes their sensitivity to sodium chloride. When, however, impulse frequency is measured in the afferent nerve of the chorda tympani, a combination of two taste components sums algebraically (Andersson *et al.* 1950). Although the interrelationship of tastes appears to be derived from the integrating action of the central nervous system rather than from the peripheral receptors on the tongue, peripheral interaction has not yet been entirely ruled out.

Several methods have been used in determining the effect of one taste on the apparent intensity of another. Three recent publications reported on multiple comparison (Pangborn, 1960), paired comparison (Pangborn, 1961) and single-sample presentation (Kamen *et al.*, 1961; Pangborn, 1961). The influence of salt on sweetness, and of sugar on saltiness, was not established conclusively with any of these methods.

The present paper describes the taste reactions of highly trained subjects to suprathreshold levels of sucrose and sodium chloride mixtures using single and paired presentations.

METHODS AND MATERIALS

Taste panel. Seven men and 3 women, with over 1 year of experience in tasting aqueous solutions, were selected on the basis of ability to recognize small differences in sweetness and saltiness in paired samples. Solutions were prepared daily in glass-stoppered volumetric flasks. All liquids were maintained at 70±2°F, and were served in 40-ml portions in coded beakers. Subjects were provided with distilled water for oral rinsing, and to avoid possible post-ingestion effects, were instructed not to swallow any sample. Evaluations were made between 2 and 3 P.M., Monday through Friday, in individual partitioned booths. Subjects were informed of their progress after each test session.

Single samples. The method of presentation and the score card used have been described (Pangborn, 1961). Ten of the 16 possible samples (Table 1) were presented at each session in a balanced, incomplete latin-square design (Cochran and Cox, 1957). Samples were judged for sweetness intensity in Study A, and for saltiness intensity in Study B. Sixteen test days were required to complete each study. Ten replications of each sample were obtained from each subject,

for a total of 100 evaluations per sample. Analysis of variance was applied to the individual intensity scores.

Paired samples. On the same 32 test days mentioned above, 10 paired sets were also evaluated, according to a method previously reported (Pangborn, 1961. Besides selecting the sweeter or saltier sample, the subject noted degree of difference in taste intensity within each pair on a 4-point scale of "slight, moderate, large, or extreme." Analysis of variance was applied to these difference scores. The order of presentation of the paired and single sets was alternated to offset any time-order effects. Although 30 solutions were tasted at each session, no physiological or psychological fatigue was indicated by the reproducibility of the responses or by oral or written comments of the subjects.

Table 1. Concentrations of sucrose[a] and sodium chloride.[b]

I. Single-sample presentation

A. Effect of sucrose on saltiness of sodium chloride

% sucrose	% NaCl			
	0.12	0.36	1.08	3.24
0	A	B	C	D
1.5	E	F	G	H
4.5	I	J	K	L
13.5	M	N	O	P

B. Effect of sodium chloride on sweetness of sucrose

% NaCl	% sucrose			
	0.75	2.25	6.75	20.25
0	A	B	C	D
0.12	E	F	G	H
0.36	I	J	K	L
1.08	M	N	O	P

II. Paired presentation

A. Effect of sucrose on saltiness of sodium chloride

% sucrose	% NaCl			
	0.12	0.36	1.08	3.24
0	A	B	C	D
0.5	E	F	G	H
1.5	I	J	K	L
4.5	M	N	O	P
13.5	Q	R	S	T

B. Effect of sodium chloride on sweetness of sucrose

% NaCl	% sucrose			
	0.75	2.25	6.75	20.25
0	A	B	C	D
0.04	E	F	G	H
0.12	I	J	K	L
0.36	M	N	O	P
1.08	Q	R	S	T
Pairs:	AE	BF	CG	DH
	AI	BJ	CK	DL
	AM	BN	CO	DP
	AQ	BR	CS	DT

[a] Baker analyzed, reagent grade.
[b] J. T. Baker, C. P. grade.

Table 1 lists the concentrations of sucrose and sodium chloride for both the single and paired presentations. The levels of both compounds were approximately logarithmically spaced, and ranged from slightly above threshold to very high concentrations.

RESULTS

Single samples. *Saltiness.* As indicated in Fig. 1, sucrose depressed the apparent saltiness of 3.24, 1.08, and 0.36% sodium chloride. The lowest salt level, 0.12%, was unaffected by the addition of sucrose. Analysis of variance of these data (Table 2) showed that salt, sucrose, judges, and replications significantly influenced saltiness scores, as did all the interactions.

Sweetness. The apparent sweetness of 2.25 and 0.75% sucrose was enhanced significantly by 0.12, 0.36, and 1.08% sodium chloride (Fig. 1). The sweetness of 6.75% sucrose was depressed slightly by sodium chloride, and that of 20.25% sucrose was reduced greatly. Variations in scoring attributable to the four main effects were highly significant (Table 2). The interactions of sodium chloride × replications and sucrose × replications were not significant.

Paired samples. *Saltiness.* Table 3 tabulates the frequency of selection of samples within a pair on the basis of intensity of saltiness. As sucrose

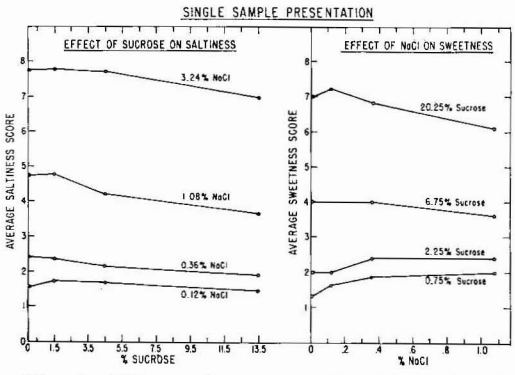

Fig. 1. Effects of sucrose and sodium chloride on average taste-intensity scores; single-sample presentation.

Table 2. Analysis of variance for single-sample presentation.

Source of variation	Degrees of freedom	Sucrose-saltiness [a]		NaCl-sweetness [b]	
		Mean square	F ratio	Mean square	F ratio
Total	1599				
NaCl (N)	3	2,882.914	3,520.042***	4.702	4.769**
Sucrose (S)	3	35.991	43.945***	2,089.244	2,118.909***
Judges (J)	9	16.535	20.189***	33.431	33.906***
Replications (R)	9	2.611	3.188**	4.135	4.194***
N × S	9	4.827	5.894***	11.911	12.080***
N × J	27	9.649	11.781***	6.905	7.003***
N × R	27	2.577	3.147***	0.811	0.823
S × J	27	2.194	2.679***	5.702	5.783***
S × R	27	1.333	1.628*	1.416	1.436
J × R	81	1.242	1.516**	1.288	1.306*
Remainder	1377	0.819		0.986	

[a] Effect of sucrose on saltiness scores for sodium chloride.
[b] Effect of sodium chloride on sweetness scores for sucrose.
* Sig. at P = 0.05.
** Sig. at P = 0.01.
*** Sig. at P = 0.001.

level increased, apparent saltiness decreased. Solutions containing 1.08% sodium chloride were depressed more than were higher or lower levels, an observation that is corroborated by the single-sample presentation. Plotting the difference scores (Fig. 2) further verifies these results. The saltiness of 0.12% sodium chloride was affected least by sucrose.

Sweetness. Concentrations of 0.04, 0.12, and 0.36% sodium chloride enhanced the apparent sweetness of the 0.75% sucrose solutions (Table 4). In addition, the lowest salt level increased the sweetness of 2.25% and 6.75% sucrose. All other combinations masked sweetness. It is of especial interest that this depression was of a greater magnitude at higher than at lower sucrose concentrations, an observation completely opposed to results obtained by adding acid to sucrose (Pangborn, 1961). The average difference scores plotted in Fig. 2 illustrate the magnitude of enhancement and masking that occurred at the concentrations tested. Whereas the single-sample presentation showed enhancement at 0.75 and 2.25% sucrose at all salt levels,

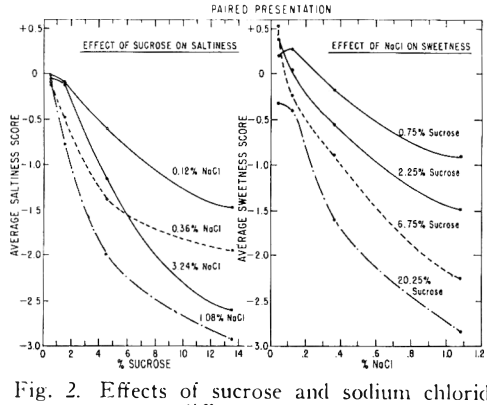

Fig. 2. Effects of sucrose and sodium chloride on average taste-difference scores; paired presentation.

Table 3. Effect of sucrose on saltiness of sodium chloride (values represent number of responses to saltiness within each pair).

% NaCl	I		II		III		IV		Totals	
	% sucrose									
	0	0.5	0	1.5	0	4.5	0	13.5	No sucrose	Added sucrose
0.12	51	49	56	44	63*	37	78***	22	248***	152
0.36	58	42	65**	35	86***	14	93***	7	302***	98
1.08	56	44	73***	27	93***	7	95***	5	317***	83
3.24	49	51	53	47	70***	30	89***	11	261***	139
Totals	214	186	247***	153	312***	88	355***	45	1128***	472

* Sig. at P = 0.05.
** Sig. at P = 0.01.
*** Sig. at P = 0.001.

Table 4. Effect of sodium chloride on sweetness of sucrose (values represent number of responses to sweetness within each pair).

% sucrose	I		II		III		IV		Totals	
	% sodium chloride									
	0	0.04	0	0.12	0	0.36	0	1.08	No NaCl	Added NaCl
0.75	44	56	41	59	49	51	60	40	194	206
2.25	38	62*	50	50	66**	34	76***	24	230**	170
6.75	43	57	63*	37	74***	26	90***	10	270***	130
20.25	59	41	68***	32	83***	17	96***	4	306***	94
Totals	184	216	222*	178	272***	128	322***	78	1000***	600

* Sig. at P = 0.05.
** Sig. at P = 0.01.
*** Sig. at P = 0.001.

paired evaluation showed these sucrose concentrations to be depressed in the presence of 0.36 and 1.08% salt. The only taste combination that resulted in an enhancement that differed statistically from the "0" point was the addition of 0.04% sodium chloride to 6.75% sucrose (P = 0.05).

Variance analyses for both sweetness and saltiness scores showed similar patterns (Table 5). No significant variation was due to replications, nor were there significant interactions of the 3 other main effects with replication. Thus it appears that the judges, though differing in opinion from each other, reproduced their responses during 10 replications.

DISCUSSION

The results lend further credence to the observation that the interaction of basic tastes is largely a function of concentration. Although that premise is recognized by most investigators, several experiments have involved one concentration only. Hahn and Ulbrich (1948) found that the threshold for sodium chloride was unaffected by sucrose whereas the threshold for sucrose was reduced by sodium chloride. Anderson (1950) reported that, at threshold levels, saltiness was inhibited by sucrose and sweetness was slightly enhanced by salt. Fabian and Blum (1943) observed that subthreshold levels of sodium chloride increased to varying degrees the sweetness of suprathreshold levels of sucrose, maltose, lactose, fructose, and dextrose. Subthreshold levels of these sugars consistently reduced the saltiness of suprathreshold levels of sodium chloride. Using the same methods as Fabian and Blum, Pangborn (1960) found similar results except that sodium chloride seemed to have little effect on the sweetness of

Table 5. Analysis of variance for paired presentation.

Source of variance	Degrees of freedom	Sucrose-saltiness [a]		NaCl-sweetness [b]	
		Mean-square	F ratio	Mean-square	F ratio
Total	1599				
NaCl (N)	3	54.633	23.640***	336.340	132.365***
Sucrose (S)	3	379.500	164.215***	94.947	37.366***
Judge (J)	9	50.860	22.008***	214.888	84.568***
Replications (R)	9	3.260	1.411	2.966	1.167
N × S	9	10.862	4.700***	11.536	4.540***
N × J	27	7.741	3.350***	20.905	8.227***
N × R	27	2.274	0.984	1.946	0.766
S × J	27	15.052	6.513***	9.526	3.749***
S × R	27	1.496	0.647	3.597	1.416
J × R	81	2.046	0.885	2.969	1.168
Remainder	1377	2.311		2.541	

[a] Effect of sucrose on saltiness scores for sodium chloride.
[b] Effect of sodium chloride on sweetness scores for sucrose.
*** Sig. at P = 0.001.

sucrose. In 1892 Zuntz reported that 1% sodium chloride increased the sweetness of sucrose. Two years later Kiesow (1894) found just the opposite. More recently, Sjöström and Cairncross (1955) indicated that 1% salt reduced the sweetness of 3–10% sucrose whereas 0.5% salt augmented the sweetness of 5–7% sucrose.

A wider range of concentrations has been studied recently. At levels ranging from threshold to solubility limit, Beebe-Center *et al.* (1959) observed mostly mutual masking of sucrose and sodium chloride, with some enhancement of sweetness in dilute solutions. At concentrations of 0.45–20.00% sucrose and 0.13–4.00% salt, Kamen *et al.* (1961) concluded that salt masked sweetness, primarily in solutions high in both compounds, but that sucrose had little effect on saltiness.

It is probable that the discrepancies in observations are attributable to differences in the methods and in the sensitivity of the tasters. In the experiment described herein, the single-sample presentation was almost identical to that used by Kamen *et al.* (1961) and the sweetness results agree very well. The results differ slightly in regard to the effect of the higher sucrose additions on the saltiness of the higher salt concentrations; Kamen *et al.* reported no effect, whereas the present results indicate a significant reduction in saltiness (Fig. 1).

The F-ratio for replications was lower for the paired-presentation than for the single-presentation, indicating greater precision of judgment for the former. In tests conducted by Gridgeman (1961), paired comparison, with scaling of degree of difference between pairs, was more discriminatory than rating. Helson (1954) pointed out that the comparative rating scale has an advantage over the method of absolute judgment in that it provides a stimulus standard that helps stabilize the adaptation level. The single stimulus becomes less of an absolute judgment, however, when samples are presented in series.

Barylko-Pikielna and Miler (1961) developed an equation adapted from Beidler (1954) to predict the response intensity of specific stimuli at fixed concentrations in pure water and in solutions of another stimulus. This formula needs extensive testing over a wide range of concentrations before its effectiveness can be established.

ACKNOWLEDGMENTS

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Studies on the Use of Tissue Culture for the Bioassay of Staphylococcal Enterotoxin [a]

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SUMMARY

An attempt was made to develop an assay procedure for staphylococcal enterotoxin based on the possible induction of cytopathogenic effects on several strains of tissue culture cells using enterotoxin preparations of varying degrees of purity produced in a laboratory medium. The results suggest that the cruder preparations contained a thermolabile cytotoxic material that is removed in whole or in part with progressive purification. No observable cytopathogenic effect could be attributed directly to the enterotoxin.

INTRODUCTION

The need for a simple, adequate and specific assay for staphylococcal enterotoxin remains acute (Casman, 1958) even though recently introduced serologic procedures offer some hope for a practical test for this toxin (Casman, 1960). Accordingly, we are investigating the use of tissue culture for this purpose.

Any investigation of staphylococcal enterotoxin is faced with a variety of complicating factors. Of the toxic moieties produced by staphylococci and given orally to rhesus monkeys, only enterotoxin causes vomiting (Bergdoll *et al.*, 1959). When administered parenterally, however, not only the enterotoxin but occasionally the alpha- and beta-hemolysins elicit emesis, necessitating the removal or neutralization of hemolysins when this method of enterotoxin assay is used (Casman, 1958).

Quantitative differences in enterotoxin production by different subcultures of the same strain of staphylococcus have been demonstrated (Sugiyama *et al.*, 1960). Worthy of note is the occurrence, in relatively small amounts, of a non-hemolytic heat-labile and dialyzable product of staphylococcal growth not producing heat-resistant enterotoxin but capable, after treatment with antialpha- and antibeta-hemolysins, of inducing emesis when injected into cats or ingested by monkeys (Casman, 1960). The frequently observed reduction in enterotoxicity of culture filtrates after heating may conceivably be due to selective destruction of this material or partial inactivation of the relatively heat-resistant enterotoxin (Casman, 1960).

There have been indications that some enterotoxins are more heat-susceptible than others, and they may also vary in potency. Of some concern is the finding that, using gel-diffusion technique, S6 enterotoxin produced in food materials did not react with antibody to laboratory-produced enterotoxin (Bergdoll, 1960a).

It has been reported (Vicari *et al.*, 1960) that several bacterial toxins inhibit the growth of tissue cells in culture and that this action can be neutralized by specific antitoxins. However, Lamanna (1959) observes that tissue cultures of mammalian cells are unaffected by botulinal toxins. He cautions that presumed effects of neurotoxins in tissue culture may be attributable to extraneous impurities, not to the neurotoxin itself, and that prevention through contact with antitoxin of an observed effect in tissue culture does not of itself constitute proof of toxin activity, since antitoxins as generally

[a] This paper was presented before the Laboratory Section of the American Public Health Association at the 89th Annual Meeting in Detroit, Michigan, November 16, 1961.

prepared contain antibodies against impurities.

Nevertheless, HeLa cells challenged with graded concentrations of crude filtrates obtained from both laboratory medium and custards seeded with enterotoxigenic and nonenterotoxigenic strains of staphylococci exhibited adverse reactions that appeared at significantly earlier incubation times with the enterotoxigenic strains (Allen, 1961).

Furthermore, staphylococcal alpha-, beta-, and delta-hemolysins and highly purified enterotoxin shown to be free of detectable enzymes and of cross-contamination with other lytic material, when added in graded concentrations, have been reported to affect embryonic heart fibroblasts cultivated in completely synthetic medium, producing characteristic cytopathogenic effects that were distinctly different for each toxin as indicated by the MLD, the shape of the toxcity response curve, and the morphology of the degenerating cells (Guerin *et al.*, 1961). Staphylococcal alpha-hemolysin has been reported to be extremely toxic to skin cultures (Lawrence, 1959).

OBJECTIVES

The immediate objective of this study was the elaboration of a test for detection and assay of staphylococcal enterotoxin. It was felt that the application of tissue-culture methods to the detection of enterotoxin produced in susceptible foods would be premature and unduly complicated. Hence, enterotoxin preparations produced in a laboratory medium of known composition have been used. Even though it is assumed that nonspecific substances in preparations made from selected foods might interfere with the demonstration and quantification of the enterotoxin, a method of assay for enterotoxin produced in a laboratory medium would permit the study of factors affecting enterotoxin production, an area where information is meager at this time.

The studies described include: 1) observation of the response of several selected cell lines to the action of enterotoxin preparations; 2) determination of the effect of serum in the culture fluid on enterotoxin preparation activity; 3) comparison of cytopathic effects of preparations made from several staphylococcal strains; 4) demonstration of the effect of heating solutions of the test preparations on cellular response; 5) attempted neutralization by "specific" antiserum of the tissue-culture reaction to enterotoxin; and 6) comparison of cellular response to the crude preparations with that of the more highly purified materials. It is admitted that many avenues of investigation were not explored, primarily because of the small quantities of preparations available.

MATERIALS AND METHODS

Enterotoxin preparations. With one exception (196E, derived from the 196E strain), all of the enterotoxin preparations were from the S6 strain. The preparations and pertinent information were provided by Drs. Merlin S. Bergdoll, Food Research Institute of the University of Chicago, and Dudley P. Glick and Edward J. Schantz, Chemical Corps Biological Laboratories at Fort Detrick, Maryland. The preparations are described by these investigators as follows:

196E. A crude preparation containing approximately 5% enterotoxin and with a dry weight of 10–90 μg per cat-vomiting dose. This preparation contained both alpha- and beta-hemolysins, but in very small amounts. Almost all of the enterotoxigenic staphylococci of food-poisoning origin studied have been found to produce only the 196E serologic type of enterotoxin (Casman, 1960).

PB8A. A relatively crude preparation containing approximately 20% enterotoxin, with little if any beta-hemolysin and only a small amount of alpha-hemolysin. Six of 6 monkeys vomited when orally fed 100 μg of this material, 8 of 12 vomited with 50 μg, and 2 of 12 with 30 μg. As assayed by intravenous injection, the emetic dose was 20–30 μg. It has been found to contain biologically active substances other than enterotoxin, such as fibrinolysin, apyrase, alpha-hemolysin, coagulase, and a hexabarbital hypnosis-potentiating factor. Many of these activities became considerably diminished with subsequent purification. Of considerable importance to the interpretation of the data obtained in these studies was the statement that further purification of the enterotoxin effected some loss of toxicity, apparently without otherwise affecting the toxin. This inactivation appeared to be related to the removal of some material that stabilized the toxin, which points to the desirability of using as few steps as possible in purification (Bergdoll *et al.*, 1959).

PB9B. A partially purified preparation from the PB8A material and containing approximately 80% enterotoxin. Five of 6 monkeys vomited with

25 μg fed orally, and with 10 μg injected intravenously. This preparation was reported to be 3–4 times as potent as PB8A as determined by monkey feeding tests (Bergdoll, 1960b).

D2. A highly purified preparation made directly from PB8A by high-speed centrifugation and chromatography containing an estimated 95% or more enterotoxin. Six-tenths μg, and in some instances 0.1 μg, produced emesis in monkeys on intravenous injection. On a relative basis, 10 μg of PB8A equals the hemolysin activity of 1000 μg of D2 (Schantz, 1961). This preparation contained 4% nitrogen, which is equivalent to about 25% protein, the remainder being phosphate buffer.

The indicated purity of these preparations should be viewed with caution since purity is dependent largely on the method used for its determination. All of these preparations contained demonstrable hemolysins although the tests indicated that alpha-hemolysins were greatly reduced in the highly purified materials. The enterotoxin materials were dissolved in sterile double-distilled water, with subsequent treatment in accordance with the experimental design used. Solutions were made in sterile double-distilled water just before use since on prolonged standing in solution the more highly purified preparations undergo changes that result in their precipitation as insoluble material. It seems plausible to assume that at least some of the precipitate formed is enterotoxin.

The usual tests for sterility gave no indications that the solutions were contaminated. Since Dr. Bergdoll found some apparent loss of toxicity when solutions are filtered through Millipore filter, the preparations used in this study were not filtered.

The serial twofold dilution schedules used were based on dry weight of the enterotoxin preparation irrespective of estimated enterotoxin content, for reasons that will become apparent later herein. Dilutions were made such that the required quantity of test substance was present in 1.0 ml of the tissue-culture maintenance medium.

Controls. It is unfortunate that control preparations derived from nonenterotoxigenic staphylococci were not available. Dr. Bergdoll informed us that he used one nonenterotoxigenic strain (184) carrying the material only through a partially purified state, i.e., through the first three steps of his procedure: acid precipitation, alumina adsorption, and alcohol precipitation. Approximately the same amount of material was obtained with this strain as with the enterotoxin-producing strains. We do not know if anything would have remained if the preparation had been taken through the complete purification procedure.

An unseeded staphylococcal-medium control would not appear to be of concern here since Dr. Bergdoll was unable to recover anything by extraction and concentration under these conditions.

Some control preparations with nonenterotoxigenic staphylococci were made by us with the method of Casman (1958). Tests with these materials, even with an excess of six times the largest amount of enterotoxin preparation used, had no adverse effects on the cell cultures.

Cell lines. The cell lines used were tissue-culture cell strains H.Ep#2 and HeLa, both derived from human carcinomas, and an epithelial strain derived from normal human heart cells. These three cell lines were selected because they gave the most satisfactory indication of toxic effect of the five lines tested, which included human embryonic intestinal mucosa (Henle) and mouse subcutaneous connective tissue cells (LLCM1).

In some of the experiments H.Ep#2 cells were maintained in Eagle's basal medium (EBM) containing 20% human serum or 20% calf serum. HeLa cells were carried in EBM containing 20% calf serum, and the human heart cells in EBM with 10% human serum. All media contained penicillin and streptomycin, respectively 100 units and 100 μg.

Stock solutions were prepared by inoculating milk dilution bottles with 10^6 cells in 10 ml of the appropriate medium. The medium was decanted and replaced with fresh medium every third or fourth day. Cells were passaged using 0.005*M* versene every 8–12 days. Test cultures were prepared in 16 × 120-mm screw-capped tubes as follows:

The cells were removed from the stock bottles with versene, centrifuged, washed in Hanks' balanced salt solution, and suspended in growth medium. After counting, the suspension was diluted in medium to approximately 6.0×10^4 cells per ml, and 1.0 ml of suspended cells was transferred to each tube. After the cultures were incubated for three days they were prepared for the tests by removing the growth medium and washing with Hanks' balanced salt solution. Dilutions of the test preparation in 1.0 ml of the appropriate medium were then added to the tube cultures. With few exceptions all tubes were set up in quadruplicate in the tests. Some of the experiments were conducted with Medium 199. For these, cells were propagated in Eagle's medium with serum whereas the titrations were performed with Medium 199.

RESULTS

Early in this investigation the metabolic inhibition test was used as indicator of effect. This rather simple approach tended to be sensitive to a multitude of factors, however, giving rise to

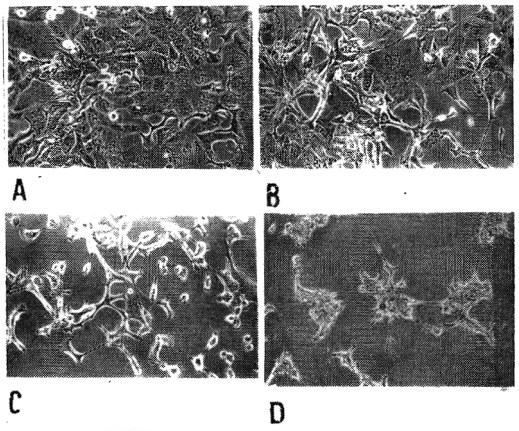

Fig. 1. Photomicrographs of cytopathogenic effect with time (× 125).

erratic and many times inconclusive results. It was determined that cytopathology as observed by light microscopy showed a more reproducible effect of the toxic preparations.

Cytopathogenic effect. Photomicrographs of H.Ep#2 cells exposed to the enterotoxic material for 3 days in a perfusion chamber, taken at 2- to 5-min intervals followed by longer periods toward the end of the exposure period, help characterize the cytopathogenic effects. Fig. 1-A shows the appearance of the cells in growth medium before any adverse effect is discernible. Fig. 1-B,C,D presents the evidence of progressive cytotoxic effects with time, culminating in the pronounced effect shown in D at the end of the 3-day exposure period. The action follows a pattern indicating retraction of cytoplasm followed by cell detachment from the substrate along with some clumping of what appear to be unhealthy cells. Near the end of the exposure period few rounded cells remain on the glass substrate. This residual fraction of the original cell population is inversely proportional to the amount of preparation added. There are indications of inhibition of cell division as well as the more overt cytopathic effects. In the early stages of incubation with the toxin preparation, dividing cells are present and as the toxic effect becomes manifest few if any dividing cells or mitotic figures are seen. Although the cultural control is not shown, all of the cells appeared normal throughout the 3-day exposure period.

Thermostability. To determine the heat stability of the toxin preparations, the cytotoxicity of heated and unheated aqueous solutions of these materials were compared. The samples were placed in a boiling-water bath for 30 min and then immediately cooled to room temperature. This procedure is commonly used to inactivate the hemolysins. H.Ep#2 cells were challenged with serial twofold dilutions of each preparation, with the highest concentration representing 200 μg of preparation per ml of culture fluid (EBM with 20% human serum). In all experiments, readings were made at the end of the 48-hour incubation period because when the incubation period was extended to 7 days with a change of medium on the third and fifth days the results were essentially similar. Since in all instances culture controls showed no adverse effects within this interval, any differences shown by the cultures inoculated with test preparations can be properly attributed to the factor(s) or substance(s) under study.

The data (Table 1) indicate that the cytotoxic material in each of the three preparations tested is thermolabile under the conditions of the experiment.

Table 1. Effect of heating and state of purification of enterotoxin preparations on the induction of cytopathology on H.Ep#2 cells.

Final concentration of preparation, μg/ml of culture fluid	PB8A		PB9B		D2	
	Heated	Not heated	Heated	Not heated	Heated	Not heated
200	+	++++	−	±	−	±
100	+	+++	−	±	−	±
50	−	++	−	−	−	−
25	−	+	−	−	−	−
12.5	−	−	−	−	−	−
6.3	−	−	−	−	−	−
3.1	−	−	−	−	−	−
1.6	−	−	−	−	−	−

Legend: ++++ = all cells damaged
+++ = 50% or more damaged, monolayer irregular
++ = cells appear degenerated, monolayer irregular, cells at edge retracted
+ = good monolayer, cells at edge retracted and degenerated
− = cells resemble control culture
± = doubtful reaction

Table 2. Cytopathogenic effect of enterotoxin preparations on H.Ep#2 cells in the presence and absence of serum.

Final concentration of preparation, μg/ml of culture fluid	PB8A				PB9B				D2	
	Heated		Not heated		Heated		Not heated		Serum absent	
	Serum		Serum		Serum		Serum			
	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Heated	Not heated
200	++	++++	++++	++++	−	±	−	++++	−	−
100	+	+++	+++	++	−	±	−	+++	−	−
50	−	++	++	+	−	−	−	−	−	−
25	−	+	−		−	−	−	−	−	−
12.5	−	−	−	−	−	−	−	−	−	−
6.3	−	−	−	−	−	−	−	−	−	−
3.1	−	−	−	−	−	−	−	−	−	−
1.6	−	−	−	−	−	−	−	−	−	−

State of purification. Preparation D2 is more highly purified than PB9B, and PB9B is purer than PB8A, both D2 and PB9B being made from the PB8A material. A comparison of the cytotoxicity of these three preparations in Table 1 indicates that loss of this activity occurs with purification, the loss of cytotoxicity being most marked between the PB8A and the PB9B preparations.

Effect of serum. Because of the thermolability of the cytotoxic material and its loss with purification of enterotoxin it is questionable that the material producing the cytotoxicity is the enterotoxin. In an attempt to increase the sensitivity of the test so as to detect the thermostable enterotoxin, serum was omitted from the tissue-culture maintenance medium. H.Ep#2 cells were propagated in EBM containing calf serum, and the toxin titrations were carried out in Medium 199 with and without calf serum. Under these conditions the cytotoxicity of the three enterotoxin preparations of varying degrees of purity, heated and unheated, is compared in Table 2. The more highly purified D2 preparation, because of its limited availability, was tested only in the absence of serum.

With PB8A preparation that was not heated, no significant difference in sensitivity is observed in either the presence or absence of serum. However, the heated material of the same preparation exerted a more marked effect in the absence of serum. With the more purified PB9B preparation, not heated, an increased sensitivity of the test in the absence of serum is indicated and a questionable increase in sensitivity is noted with the heated preparation in the absence of serum. An even more marked progressive decrease in cytotoxicity is observed with purification of the toxin preparation when no serum is used in the tissue-culture medium than has been shown with serum present.

Comparison of cell lines. Tables 1 and 2 present data obtained from experiments using H.Ep#2 cells. For comparison, Table 3 illustrates the effect of two of the toxin preparations on cell cultures of strain HeLa. HeLa cells were propagated in EBM with 20% human serum and changed to Medium 199 containing no serum for the test. As with the H.Ep#2 cells the cytotoxic component of the PB8A preparation is thermolabile whereas no toxic effect is noted with the more highly purified material PB9B. It should be pointed out that results were similar with human heart cells under conditions similar to those described here except that the titration was carried out in the presence of 10% human serum.

Attempted neutralization with enterotoxin "specific" antibody. Using a serial twofold dilution

Table 3. Cytopathogenic effect of enterotoxin preparations on HeLa cells on serum-free medium.

Final concentration of preparation, μg/ml of culture fluid	PB8A		PB9B	
	Heated	Not heated	Heated	Not heated
200	++++	++++	−	−
100	+	++++	−	−
50	−	+	−	−
25	−	−	−	−
12.5	−	−	−	−
6.3	−	−	−	−

schedule based on known antitoxic activity of the serum as tested in monkeys and a minimum cytotoxic dose of preparation PB8A, the toxin-antitoxin mixtures were incubated 6 hr at 37°C and overnight at 4°C. Cultures were then inoculated, incubated at 37°C, and read at 24, 48, and 72 hr.

Several experiments of this kind failed to show modification of the cytotoxic effect of the PB8A preparation by the serum. Several possible explanations may be offered. It is possible that the amount of antiserum used was too small, although concentrations were increased with each successive trial. The antiserum may not have contained antibody against the cytotoxin, which would suggest that the cytotoxin differs from the enterotoxin. Another possibility was that the antigen-antibody complex dissociated because of dilution when added to the culture or because it was unstable in the presence of the tissue-culture cells.

DISCUSSION

The multifactorial nature of the enterotoxin problem points to the possibility that a single factor may be responsible for more than one manifestation or that more than one factor may produce a single effect. In addition it is well known that the characterization of a substance may be limited by its sensitivity to adverse conditions, i.e., treatment with an acid, base, or organic solvent may produce a fundamental change resulting in a loss of original characteristics such as water solubility or specific biological activity.

On the basis of the reaction to heat the results presented suggest that the cytotoxic component of the preparations examined differs from the enterotoxin. Similarly it appears to be diminished or lost as purification of the enterotoxin proceeds. The failure to show neutralization of the cytotoxic principle by enterotoxin-"specific" antiserum, though inconclusive, supports the suggestion that the cytotoxin and enterotoxin are distinct entities. Based on the results of early experiments indicating that there may be differences in cytotoxicity between enterotoxigenic and nonenterotoxigenic strains of staphylococci, the tissue-culture system may be of some value if the cytotoxin can be shown to be common only to enterotoxigenic strains. It is mentioned that strain 196E enterotoxin preparation exerted a well-defined cytotoxic effect on human heart cells when the material was heated although no tests were made with the same preparation not heated.

Considering the possible role of the hemolysins in these experiments, the toxicity of alpha-hemolysin to skin cultures has been reported (Lawrence, 1959). The heat lability of cytotoxin implies that some, if not all, of the toxic effect might be attributed to a hemolysin-like material in the crude preparation. The fact that some activity is left after heating and that no demonstrable loss on heating occurs when titrated against H.Ep#2 cells in the absence of serum (Table 2), the possibility remains that an additional thermostable toxin is present in the relatively crude preparation.

A few preliminary experimental results obtained with fluorescent antibody technique imply that there is in the toxin preparation an antigenic component that localizes in the cytoplasm of the tissue cells. Thus, if the cytopathogenic effect parallels the amount of material in the cell it would be possible to state that the cytotoxic material has been fixed in the cell. There were indications that this may be the case. It is planned to repeat the experiment after attempting to adsorb out the nonspecific reactants from

the antiserum since the enterotoxin preparations used were relatively crude.

There is no ready explanation for the apparent differences between these studies and those reported in a similar study already cited (Guerin *et al.*, 1961). It is possible that these may be concerned with differences in technique in either the preparation of toxins or the use of tissue culture.

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