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Analysis of the Terpene and Sesquiterpene Hydrocarbons in Some Citrus Oils

SUMMARY

The terpene and sesquiterpene hydrocarbons in various citrus essential oils are analyzed in a two-step procedure. During the first step, the oxygen-containing compounds are removed by column chromatography. In the second step, the terpenoid-free oil is analyzed by gas chromatography, using a small sample to obtain the terpene analysis and a larger sample to obtain the sesquiterpene analysis. It was found expedient to increase the temperature during the analysis to shorten the sesquiterpene elution time. This method has been applied to different types of orange and grapefruit oils, also to tangerine, lemon, and lime oils. It is now possible to obtain a rapid evaluation of the hydrocarbons in these essential oils. A semiquantitative relationship between the hydrocarbons in the individual oils and between various oils can be obtained directly from the chromatograms. The identity of the constituents was determined by infrared and mass spectroscopy, and the wide compositional variations between the oils are discussed.

INTRODUCTION

Methods for the analysis of the citrus hydrocarbons, which constitute the major fraction of these oils, have been concerned primarily with terpenes (Kirchner and Miller, 1952; Clark and Bernhard, 1960; Gouch *et al.*, 1961). Recently, Ikeda *et al.* (1962) described a method for quantitative determination of the total monoterpene hydrocarbon composition of citrus oils by gas chromatography. None of these procedures take the sesquiterpene hydrocarbons into consideration. This study was undertaken to provide further basic information on the composition of citrus essential oil.

The procedure herein described provides a means for rapid evaluation of the major hydrocarbon content of citrus oils. The general procedure is similar to that reported for orange and grapefruit oils (Hunter and Brogden, 1964a,b), except that it has been simplified, by obviating the need for distillation, and the sample requirements have been reduced to milliliter quantities. The material represented by each peak was trapped and examined by infrared and mass spectroscopy.

The sesquiterpene hydrocarbon composition of most of these citrus essential oils has not been reported in the literature, and correlations between the individual terpene and sesquiterpenes are now presented.

EXPERIMENTAL

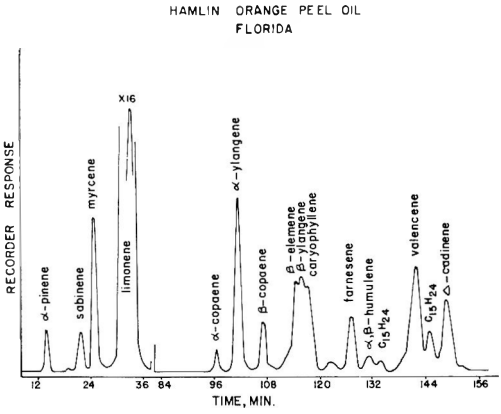
Citrus essential oils. The citrus oils were obtained from various commercial manufacturers. The peel oils were expressed with a screw press, flushed with water, and centrifuged. Juice oils were obtained by direct centrifugation of the juice, and the condensate oil was obtained from the first-stage condenser during the juice concentration process.

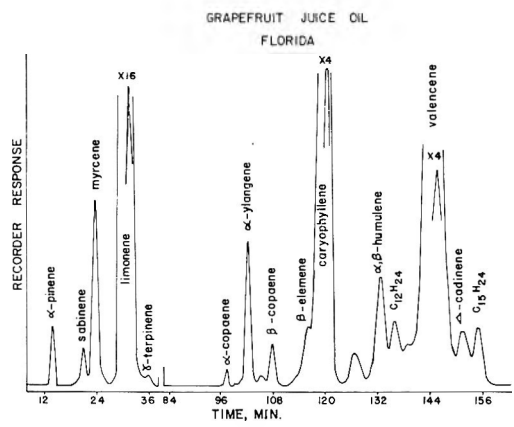
Separation of terpenes and sesquiterpenes. The oxygen-containing constituents in each oil were removed by percolation of 2 ml of oils through a ½ × 8-in. column containing basic alumina. The column was subjected to aspirator vacuum and prewashed with *n*-hexane. The oil hydrocarbons, upon elution from the column with 40 ml of hexane, were recovered upon removal of the hexane *in vacuo*. No variations could be discerned in the gas chromatographic curves upon repetition of the above procedure.

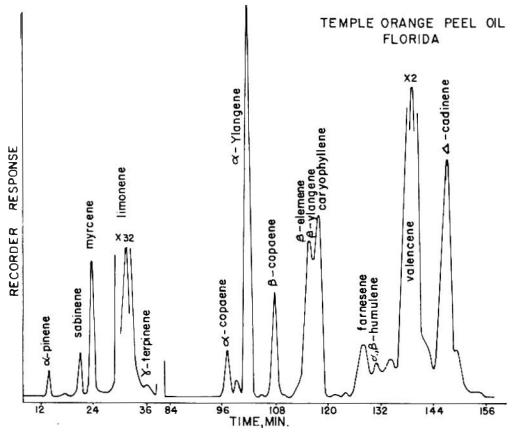
Gas Chromatography. Each of the chromatograms shown in the figures was obtained with a column consisting of 5% Carbowax 30M on Wilkens' 60–80-mesh acid-washed firebrick contained in a 20-ft length of ¼-inch aluminum tubing. The helium flowrate was maintained at 25 ml per min. The terpene analysis, in most cases using 2.5 μl of material, was obtained with a column temperature of 135°C. The sesquiterpene analysis, requiring 100 times as much (in this case 250 μl), was obtained by operating the column at 135°C for 78 min (elution of the terpenes) and then increasing the temperature to 175°C (requiring 1 min) to complete the analysis. Each of the curves reported herein is, therefore, a composite of two separate chromatograms, the terpene chromatogram obtained during the sesquiterpene analysis being replaced by one obtained using 1/100 of the material. In this way it is possible to display a semiquantitative relationship between the various hydrocarbons within the oil and from one oil to another.

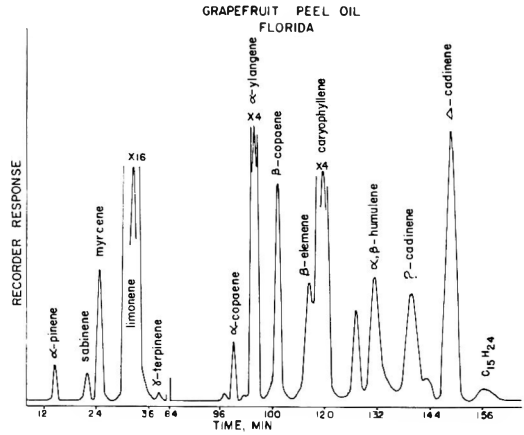
To determine the identities of the hydrocarbon

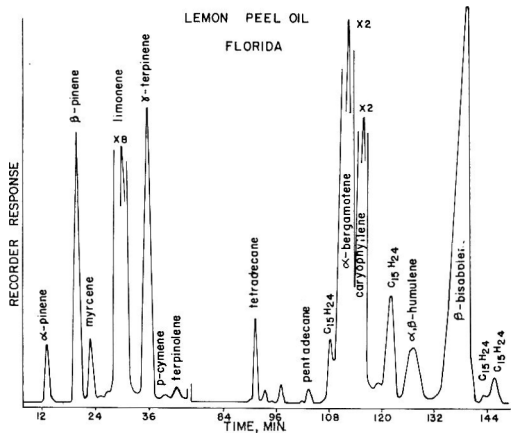
constituents, it was necessary to obtain larger amounts of material for chemical and spectral analyses. Separation, in this case, was carried out on a ¼-inch × 18-ft gas chromatographic column consisting of aluminum tubing containing 25% Carbowax 30M on 30–60-mesh Chromosorb P. The column was operated at 135°C for terpenes and 175°C for the sesquiterpenes analysis, with a helium flowrate of 60 ml per min in both cases. The individual constituents were collected in 0.3-ml vials cooled in liquid nitrogen (Hunter and Brogden, 1964a,b).

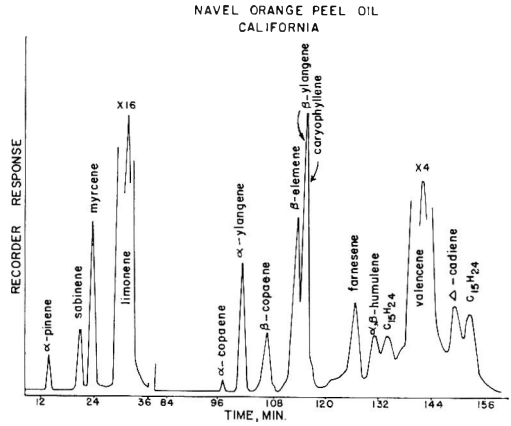












RESULTS AND DISCUSSION

The curves obtained by gas chromatography with the various essential oils and extracts are shown in the figures.

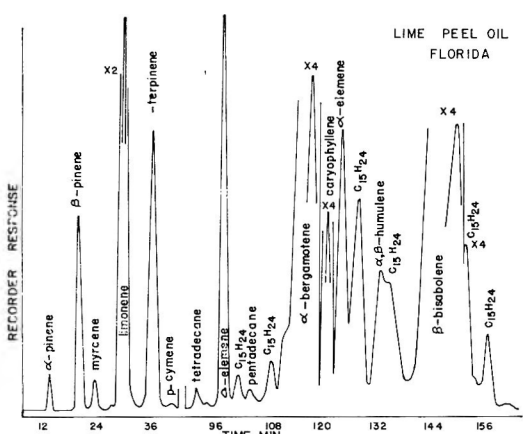
Florida Valencia orange peel oil. This analysis has been reported by Hunter and Brogden (1964a), but is included here for comparative purposes since it is obtained under standardized conditions.

California Valencia orange peel oil. Identities of materials represented by each peak are the same as in the Florida oil; however, there is an absence of α-copaene and a preponderance of valencene. In general, the total hydrocarbon concentration is higher in the California oil.

Florida Valencia orange condensate oil. The identities of the materials represented by each peak are the same as in orange peel oils; however, valencene is the predominating sesquiterpene. Δ-Cadinene, which is a constituent of Florida Valencia peel oil, did not appear in the condensate oil.

Florida Valencia orange centrifuged oil. The identity of the materials represented by each of the peaks is the same as that reported for Valencia cold-pressed orange oil; however, there is a twofold concentration of sesquiterpene hydrocarbons over that obtained from the first-stage condensate, which it otherwise resembles.

Florida Pineapple orange peel oil. This oil is characterized by the appearance of tetradecane and pentadecane, which were identified by mass spectroscopy through comparison of their cracking patterns with those published by the American Petroleum Institute. The identities of the materials represented by the remaining peaks are the same as those in Valencia orange oil. Pineapple orange oil is exceptionally rich in α-ylangene, valencene, and, in general, all of the sesquiterpene hydrocarbons.



Florida Temple orange peel oil. This oil is characterized by the relatively high concentration of sesquiterpenes. The constituents in Temple peel oil and in Valencia peel oil are identical. This oil is most similar to Pineapple orange oil except that the former oil contains less β-elemene and very much less β-ylangene.

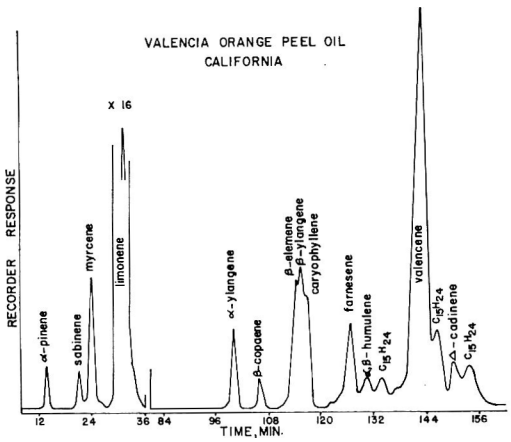
Florida Hamlin orange peel oil. The gas chromatographic curve is very similar to that obtained with Florida cold-pressed Valencia orange oil. The small difference in the unknown sesquiterpene peak between valencene and Δ-cadinene, and a larger peak Δ-cadinene, are noted.

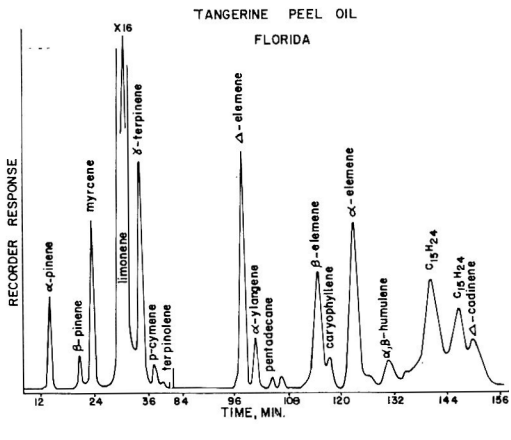
California Navel orange peel oil. This curve is characterized by the apparent absence of β-ylangene; however, it has actually been found to be present in small concentrations by mass spectral analysis. In addition, the concentration of the uncharacterized sesquiterpene between valencene and Δ-cadinene is high in this oil.

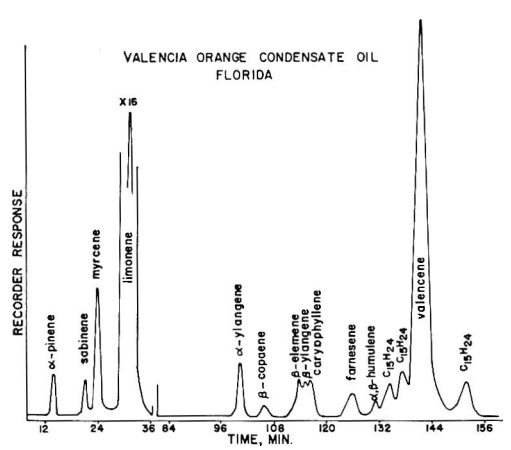
Florida Duncan grapefruit peel oil. The materials represented by each peak have been identified elsewhere (Hunter and Brogden, 1964b). It is included here as a reference for comparative purposes since it has been obtained under the same conditions as the other curves.

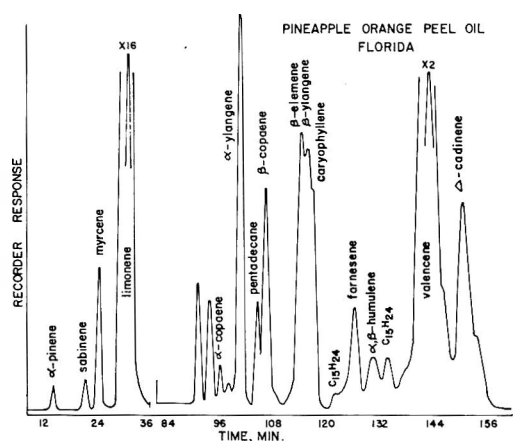
Florida Duncan grapefruit juice oil. The juice oil differs noticeably from the peel oil by the appearance of valencene in place of cadinene and there are lower concentrations of α-ylangene and β-copaene.

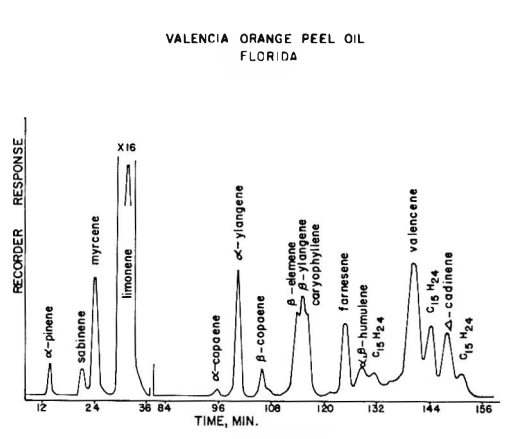
Florida tangerine peel oil. The terpenes were identified by obtaining infrared and mass spectra on the material represented by each peak and by comparison of the curves obtained with known terpenes. The sesquiterpene, Δ-elemene, was characterized by infrared spectroscopy through comparison with an infrared spectrum provided by Gouch *et al.* (1961). The material represented by the second-largest peak in the sesquiterpene area is now identified as α-ele-

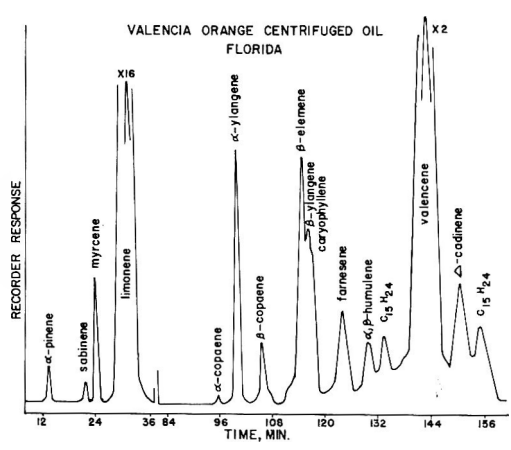












mene. The infrared spectrum obtained on this material was identical to that published by Pliva *et al.* (1960). Upon reduction, both Δ- and α-elemene gave elemane.

Florida lemon peel oil. This curve was obtained on Florida cold-pressed Avon lemon oil. The terpenes have been previously identified by Stanley (1961) and by Ikeda (1962). Tetradecane and pentadecane were identified by correlating their mass spectral cracking patterns with those published by the American Petroleum Institute. The compounds represented by the three largest peaks have previously been identified by MacLeod and Buigues (1964) and confirmed in the present work by infrared and

mass spectroscopy as α-bergamotene, caryophyllene, and β-bisabolene. In addition, α- and β-humulene were indicated and verified by infrared and mass spectral analyses.

Florida lime peel oil. The terpenes have been previously characterized by Ikeda *et al.* (1962), and were confirmed in the present work. Tetradecane and pentadecane were identified as described above for lemon peel oil. α-Bergamotene, caryophyllene and β-bisabolene were reported to be present by Kovats (1963) and were confirmed during this work. In addition, Δ-elemene, α-elemene, and α- and β-humulenes were also found to be present in lime oil and confirmed by infrared and mass spectroscopy. In this case the terpene and sesquiterpene analyses were obtained using 1.23 μl and 125 μl, respectively.

The gas chromatographic curves in the figures reveal very small differences in the terpene region within species. The relative sesquiterpenic concentrations, however, vary within species and differ in identity between species. The variations in sesquiterpenic concentrations within species is best exemplified by the analytical results obtained on Florida Valencia and Florida Pineapple orange cold-pressed (peel) oils as shown in the figures. In addition, a very noticeable higher concentration of valencene appears in the oil from the juice over that from the peel. The effect of processing on orange juice oils can be seen by the over-all decrease in total sesquiterpene concentration in condensate oil when compared to that of centrifuged oil.

Florida grapefruit peel oil and juice oil show variations both quantitatively and qualitatively. In particular, there is one-fourth as much ylangene in the juice oil as in the peel oil. Also, valencene, the principal sesquiterpene constituent of juice oil, was not noted in the peel oil.

Two new citrus sesquiterpenes, Δ- and α-elemene, have been isolated and identified in Florida tangerine oil. In addition, the sesquiterpenes α-ylangene, caryophyllene, humulenes, and Δ-cadinene, common to the citrus oils, are reported here for the first time to be constituents of tangerine oil.

Florida lemon oil contains, in addition to α-bergamotene, caryophyllene and β-bisabolene, which have previously been reported (MacLeod and Buigues, 1964), and four sesquiterpenes which could not be related to those of known structure. In addition, α- and β-humulene and two saturated straight-chain hydrocarbons, tetradecane and pentadecane, have been identified in this oil.

The peel oil of the Florida lime contains Δ- and α-elemene, which were characteristic of tangerine oil, in addition to α-bergamotene, caryophyllene, and β-bisabolene, which are characteristic of lemon oil. α- and β-humulene, and the two saturated straight-chain hydrocarbons, tetradecane and pentadecane, are also constituents of lime oil.

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Isolation and Identification of Volatile Components from High-Temperature-Cured Off-Flavor Peanuts

SUMMARY

This paper describes isolation, separation, and partial identification of 21 volatile components from high-temperature-cured off-flavor peanuts. Isolation was accomplished by vacuum distillation. Separation was achieved by gas chromatography, using diisodecylphthlate and polyethylene glycol 600. Identification was based upon relative retention volumes on the two columns used and functional group analysis. Eleven of the 21 compounds were identified: formaldehyde, acetaldehyde, ethanol, acetone isobutyraldehyde, ethyl acetate, butyraldehyde, isovaleraldehyde, 2-methyl valeraldehyde, methyl butyl ketone and hexaldehyde. Three others were partially identified.

INTRODUCTION

Since the inception of mechanized harvesting and curing processes for peanuts some 15 years ago, the problem of "off-flavors" in raw and processed peanuts and peanut products has been of increasing concern to the various segments of the industry. Extraneous and objectionable flavors in peanuts can arise from many sources and are not always attributable to the harvesting and curing process. There is a type of off-flavor, however, which has been shown to occur whenever uncured peanuts are subjected to high temperatures. This type of off-flavor, usually associated with improper mechanical curing (Beasley and Dickens, 1963), will be discussed exclusively in this paper.

The level of off-flavor in peanuts has been shown to be a function of curing temperature, time of exposure to the temperature, moisture content, and maturity stage of the kernels. The threshold temperature for off-flavor production is about 35°C. Levels of off-flavor increase with curing temperature up to about 58°C. For a given curing temperature, immature kernels have a much higher level of off-flavor than mature kernels. The immature kernels also have a lower threshold temperature for off-flavor production. The off-flavor production in peanut curing is produced more readily at about 25% moisture content (fresh-weight basis) than at either higher or lower moisture levels. Detectable concentrations of off-flavor can be produced in 1–2 hr under favorable conditions (Beasley and Dickens, 1963).

As peanuts approach normal dryness (5–10%), off-flavor production at high temperatures is greatly diminished. When dry peanuts, free of off-flavor, are moistened to 30% and subjected to a high temperature (58°C), off-flavor is produced just as in freshly harvested peanuts. Thus, enzyme systems which produce the off-flavor are not destroyed by normal curing. Normal-flavored dry peanuts which are autoclaved at 15 psi and moistened to 30% have little or no off-flavor when dried at a high temperature, thus indicating that the off-flavor is arising by an enzymatic and/or sporogenic process (Pattee, 1964).

Isolation and identification of the specific compounds which constitute the off-flavor in peanuts were undertaken as a means of defining the systems which produce the off-flavor and as a step toward developing an objective method of flavor measurement which can be incorporated into a quality-control system for better peanut evaluation.

EXPERIMENTAL PROCEDURE

Sample treatment. Off-flavored peanuts were produced by drying freshly harvested peanuts for 62 hr at 52°C and 50% relative humidity. Normal-

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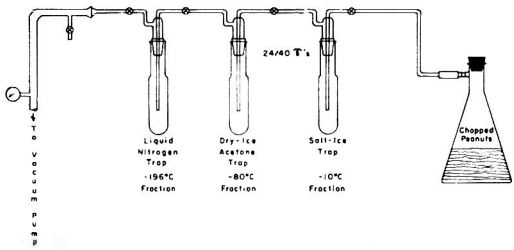

Fig. 1. Drawing of the 3-bulb vacuum manifold system used to collect volatile constituents from cured peanuts.

flavored peanuts were dried for 208 hr at 22°C and 50% relative humidity.

Preparation of sample for gas chromatography. Volatiles from peanut samples were removed by vacuum distillation. Fig. 1 shows the apparatus used to collect the desired fractions of condensate from peanuts. One thousand g of peanuts ground in a Waring blender for 15 sec were placed in a 1000-ml Buchner flask and attached to a 3-bulb vacuum manifold system. The air was pumped from the system to a final pressure of about 50 mm Hg. Distillation was allowed to proceed for approximately 15 hr. Three fractions were collected: 1) a salt-ice (—10°C) fraction, which contained predominantly water and consequently was not assayed; 2) a dry ice-acetone (—80°C) fraction, which contained the predominance of compounds both qualitatively and quantitatively; and 3) a liquid-nitrogen (—196°C) fraction, which contained the low-boiling components.

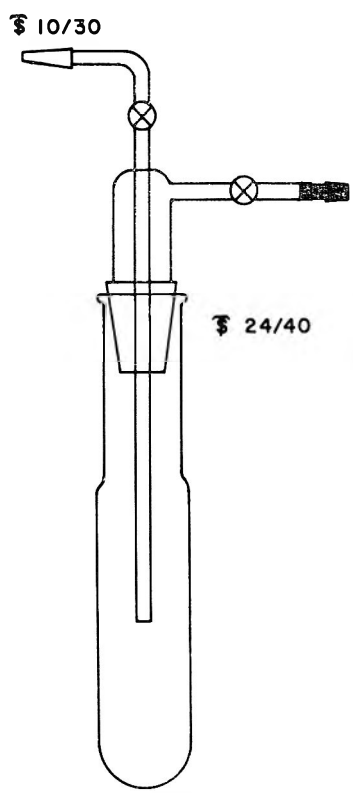

Fig. 2. Drawing of the transfer apparatus used to sample the —196°C fraction.

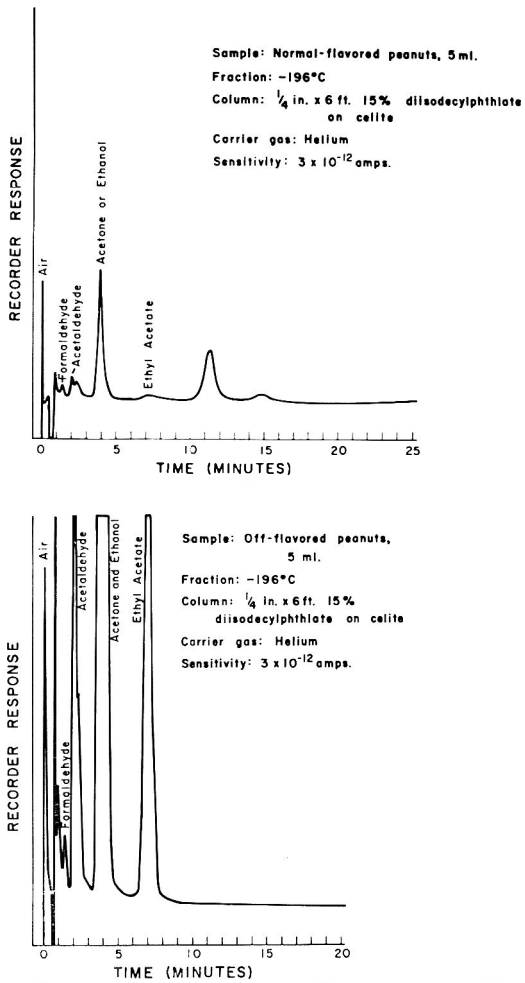

Fig. 3. Typical chromatograms of the —196°C fraction from normal and off-flavor peanuts.

Vapor analysis. Separation of the volatile constituents was accomplished with a Barber-Colman Model 10 gas chromatograph (no endorsement implied) equipped with two separate flame ionization detectors and columns. The columns were U-shaped, ¼-in × 6-ft heavy-walled glass tubing. Column A was packed with 15% diisodecylphthlate on Celite, 60–80-mesh, and Column B was packed with 15% polyethylene glycol 600 on firebrick super 22, 60–80-mesh. The operating conditions for Column A were as follows: temperature, 110°C; carrier gas, helium; and inlet pressure, 16 psig.

Operating conditions for Column B were: 105°C temperature; inlet pressure 16 psig, using helium as the carrier gas.

Sampling of the liquid nitrogen trap was accomplished by removing it from the distillation train, inserting the transfer apparatus (Fig. 2), and evacuating to approximately 200 mμ while maintaining its minimum temperature (—196°C). The trap was then allowed to warm to room temperature, and a 5-ml sample of the vapor was withdrawn from the trap with a hypodermic syringe and injected into the chromatograph for analysis.

The salting-out technique described by Bassette *et al.* (1962) was used, with minor modifications, to release the volatile components in the dry ice-acetone trap. A 2-ml sample of the liquid was drawn from the trap and placed in a 10-ml vial. After sodium sulfate was added to the vial (1.2 g/2 ml), it was sealed with a rubber septum, shaken for 5 min, and placed in a 68°C water bath for 3 min. A 5-ml vapor sample was then drawn from the vial and injected into the gas chromatography unit. Identification of compounds was based upon relative retention volumes from two separate columns and functional group tests. Functional group analysis was accomplished with the syringe technique as described by Hoff and Feit (1964). A slight modification was made in the sodium hydroxide reaction for esters. Two ml of 5% sodium hydroxide was added to the vial prior to the addition of the sodium sulfate, with subsequent vaporization and chromatographic analysis.

The 2,4-dinitrophenylhydrazone (2,4-DNP) derivatives of the carbonyl components were made as a further check on these functional groups. The 2,4-DNP derivatives were formed by placing 3 ml of a 2,4-dinitrophenylhydrazine solution (Shriner *et al.*, 1956) into liquid nitrogen and dry ice-acetone traps and collecting the respective fractions. The precipitates were collected, washed thoroughly with 2*N* HCl, rinsed with distilled water, and dried in a desiccator. The 2,4-DNP derivatives were assayed with flash exchange gas chromatography as described by Ralls (1964).

RESULTS AND DISCUSSION

Analysis of the volatile components in off-flavored peanuts, using gas chromatography, has revealed the presence of at least 21 components. Eleven of these were identified (Table 1). Of the remaining 10 components, one was identified as either 2 methyl-1-butanol or 3-methyl-1-butanol; a second was identified tentatively identified as furfural; a third was assigned a ketone functional

Table 1. Relative retention volumes and response of components of "off-flavor" peanuts to functional group reagents.

Compound	Relative retention volumes				Functional group class		
	Column A[a]		Column B[b]				
	Sample	Known	Sample	Known	Aldehyde	Ester	Carbonyl
Formaldehyde	0.11	0.11	0.16	0.16	+	—	+
Acetaldehyde	0.17	0.17	0.49	0.50	+	—	+
Ethanol	0.32	0.33	2.06	2.08	—	—	—
Acetone	0.34	0.34	1.00	1.00	—	—	+
Isobutyraldehyde	0.49	0.48	—	0.93	+	—	+
Ethyl acetate	0.58	0.61	1.39	1.39	—	+	—
Butyraldehyde	0.66	0.65	—	1.41	+	—	+
Isovaleraldehyde	1.00	1.00	1.77	1.77	+	—	+
2-Methyl valeraldehyde	1.34	1.29	2.59	2.58	+	—	+
Methyl butyl ketone	2.73	2.74	—	4.75	—	—	—
Hexaldehyde	2.90	2.86	5.05	5.18	+	—	—
Ketone functional group	2.01	—	3.36	—	—	—	+
2-Methyl-1-butanol	2.29	2.34	10.76	10.48	partial	—	—
or							
3-Methyl-1-butanol	2.29	2.37	10.76	10.61			
Furfural (tentative)	6.06	6.20	—	10.42	+	—	—
							Red hydrazone

[a] 15% diisodecylphthlate; relative to isovaleraldehyde.
[b] 15% polyethylene glycol 600; relative to acetone.

group; and the seven remaining components remain unidentified.

Fig. 3 shows two typical chromatograms of the volatile components in the −196°C fraction isolated from normal and off-flavored peanuts. A comparison of volatile components from normal and off-flavored peanuts indicates both qualitative and quantitative differences between them. What effect these differences have on flavor is not known. It has been shown, however, that when ground normal-flavored peanuts were mixed with the −196°C fraction from off-flavored peanuts, the flavor of the normal-flavored peanuts was changed to that characteristic of the off-flavored peanuts as judged by a panel of 3 judges trained to measure off-flavor. These tests indicate that the compounds contributing to off-flavor were present in the −196°C fraction. Comparison of the relative concentration of comparable compounds, such as acetaldehyde and ethyl acetate, from normal and off-flavored peanuts suggest that off-flavor might be due in part to an increase in amounts of the volatile components.

Chromatograms of volatile components found in the −80°C fraction of normal and off-flavored peanuts (Fig. 4) show that similar components are found in both peanut samples; however, a comparison of retention times on the polyethylene glycol 600 column indicated that qualitative differences do exist. What effect these differences have on flavor is not known. The aroma from the −80°C fraction was judged by 3 judges to be dissimilar to the off-flavor aroma of off-flavor peanuts.

Table 1 shows the relative retention volumes and the results of functional group tests for the off-flavored peanuts. The results of functional group analysis must be interpreted with care, as pointed out by Hoff and Feit (1964), since partial reactions or peak reductions may occur with compounds other than those for which the analysis was intended. For example, incomplete oxidation of the component identified as either 2-methyl-1-butanol or 3-methyl-1-butanol reduces peak height and would lead one to suspect an aldehyde; however, the increased retention volume of the component on the polyethylene glycol 600 column indicates it to be an alcohol. This increased retention volume of alcohols on the polyethylene glycol column was also of value in separating acetone and ethanol, which occur as a single

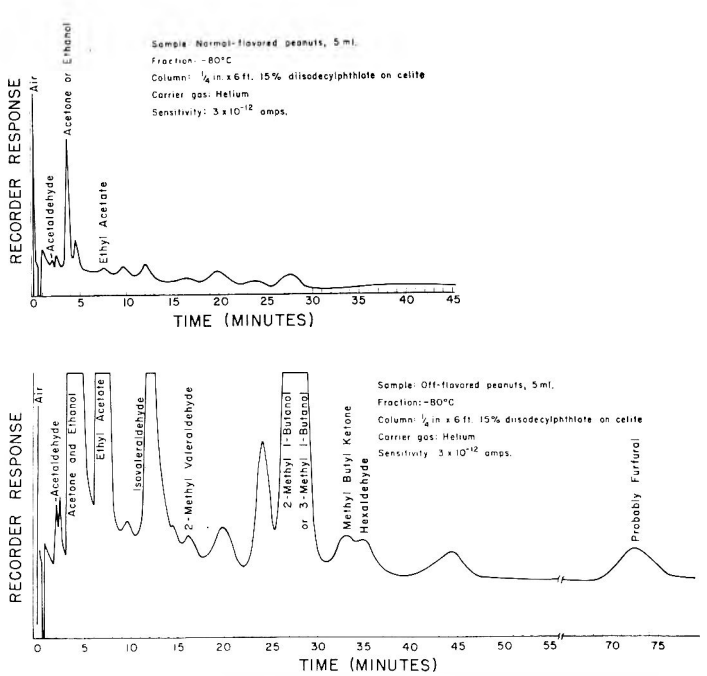

Fig. 4. Typical chromatograms of the −80°C fraction from normal and off-flavor peanuts.

peak on the diisodecylphthlate column.

The tentative identification of furfural was based upon the production of a red-colored hydrazone and the fact that no peak with a relative retention volume characteristic of heptaldehyde, the other aldehyde possibility, appeared on the polyethylene glycol column. The furfural peak would be masked by the 2-methyl-1-butanol or 3-methyl-1-butanol peak on the polyethylene glycol column.

As confirming evidence for identity, hydrazones from off-flavor peanuts were chromatogramed and six compounds are identified

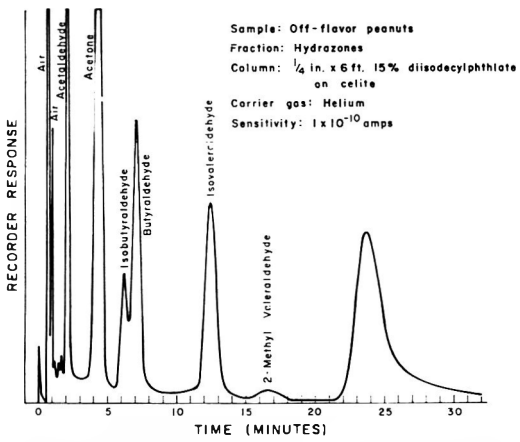

Fig. 5. Typical chromatogram of the hydrazones from off-flavor peanuts.

(Fig. 5). The failure of furfural, hexaldehyde, and methyl butyl ketone to appear on this chromatogram would be anticipated since Ralls (1964) reports the flash-exchange method to be of use only in the analysis of lower-molecular-weight aldehydes and ketones.

A comparison of volatile components from normal and off-flavored peanuts (Figs. 3 and 4) indicated a significant increase in all comparable components in the –80° and –196°C fractions from off-flavored peanuts. This increase was anticipated from the effect of temperature on the metabolic processes. Whitaker and Dickens (1964) have shown that high-temperature (58°C) curing increases respiration, and they hypothesized that the increased respiration causes a decrease in available oxygen, causing anaerobic respiration to occur. They have established a significant correlation between an anaerobic respiration index and the degree of off-flavor. This would also suggest that off-flavor might be due to an increase in amounts of the volatile components found in the –196°C fraction. Further work is under way to relate specific compounds to the off-flavor and to complete identification of the volatile components in normal peanuts.

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The Carotenoids of Certain Fruits (Apple, Pear, Cherry, Strawberry)

SUMMARY

The carotenoids of nine varieties of apple, four varieties of pear, two of strawberry, and one of cherry were examined. The first three fruits are low in carotenoid (0.3–5.0 ppm on a fresh-fruit basis), the pears exceptionally so (0.3–1.2 ppm). The cherry ranks somewhat higher (5–11 ppm). With the possible exception of the cherry, no new polyene synthesis appears to occur during ripening.

In many fully ripened fruits, the carotenoid concentration is low, below 10 ppm, frequently within the range of 0.1–5 ppm. At such levels, the carotenoid contribution to the color is negligible, and in these cases it is of some interest to determine whether the carotenoids present in the ripe fruit are residual carotenoids characteristic of the chloroplasts when the fruit was green, or whether they are different.

To the extent that chlorophyll persists in the ripe fruit, one would anticipate a carotenoid distribution as follows: the diol lutein *ca.* 40–45%; the hydrocarbon β-carotene *ca.* 25–30%; the polyol neoxanthin, *ca.* 10%; the diepoxy diol-violaxanthin, up to 20%; and numerous other minor components (*cf.* Strain, 1938; Curl and Bailey, 1957).

This report is concerned mostly with the carotenoids of apples and pears, and to a lesser extent with those of strawberry and cherry. It therefore supplements the work of Curl (1964) on other fruits low in carotenoid.

Apart from Curl's work, relatively little has been reported. There are possibly five or six references to original work on the apple. From such work, values are recorded in compilations of the Medical Research Council (1960) and the USDA Agriculture Handbook (1950). Values were obtained by estimation either of epiphasic carotenoid (De, 1935; Fujita and Ajisako, 1941) or of β-carotene (De Felice and Fellers, 1938), or by bio-assay (Todhunter, 1939; Randoin and Boisselot, 1941). The most recent work appears to be by Cravioto *et al.* (1945) and Fraps (1947). The range in carotene (or vitamin A) content is from 0.1 to 0.84 μg per gram of edible portion. As noted by Todhunter, apple peel can contain up to five times as much carotene as the flesh. The disparity can be even greater with pears.

Estimates for pears, strawberries, and cherries are also found in some of the above references.

Pears have a range of 0.05–0.3 μg per gram, strawberries of 0.3–0.6. Cherries are markedly higher, 1.2–9.5 μg per gram.

Such figures may be contrasted with comparable figures for carrots, tomatoes, or leafy vegetables, such as spinach, which may be higher by at least one order of magnitude, sometimes by as much as two. It is therefore understandable that so few investigations have been made of the carotenoids of these fruits.

EXPERIMENTAL

Materials. The following fruit varieties were examined:

Apples—Roman Beauty, Red and Golden Delicious, Winesap, Pippin (Oregon and California), Gravenstein, McIntosh, Jonathan
Pears—Bartlett, Comice, D'Anjou, Winter Nelis
Strawberries—Marshall, Shasta
Cherries—Sweet Bing.

Procedures. Samples varying in size from 0.1 to 2 kg (depending on the amount required) were blended with acetone and transferred to petroleum ether solution following the usual procedures. Small samples were used for estimating the total carotenoid content, the extent of esterification of the xanthophyll fraction, differences between peel and flesh, etc. Large samples were used for countercurrent fractionation with the Post-Craig apparatus. Absorption spectra were measured on a Cary recording spectrophotometer, Model 15.

RESULTS

Apples —*Total carotenoid.* More than 30 analyses were made, from midsummer to midwinter. The carotenoid content for peeled mesocarp varied

irregularly from 0.9 to 5.4 ppm. Comparisons between unpeeled and peeled edible portions showed values 6–20% higher in the unpeeled. The highest value for peel itself, 5.60 ppm, was found in Golden Delicious. Chlorophyll varied from traces up to 2.3 ppm in Pippin varieties.

Simple partition. By partition between petroleum ether and 85% methanol, before and after saponification, the bulk of the pigment was shown to be esterified xanthophyll. Typical results are as follows:

	Hydrocarbons and Monols	Diol-polyol	
		Esterified	Free
Red Delicious 1	17.0	79.0	4.0
2	20.0	75.0	5.0
Oregon Pippin	30.0	67.5	2.5

Counter-current partition. Two solvent systems were used, petroleum ether—98% methanol, and petroleum ether—73.5% methanol. These permit determination of the distribution into groups as follows (*cf.* Curl, 1964):

	Hydrocarbon	Monol	Diol	Monoepoxy diol	Diepoxy diol	Polyol
Delicious	13.2	5.3	10.6	2.4	42.0	26.5
Pippin	31.8	1.4	35.7	not detected	21.1	10.0

The distribution for Delicious is typical of varieties low in chlorophyll. The Pippin values represent the extreme in greenness, resembling leaves in this respect (*cf.* Curl and Bailey, 1957).

Identification of carotenoids. Each group separated by counter-current partition was chromatographed and the spectra of individual components were determined. Routine recognition of phytofluene (traces), α-carotene, β-carotene, cryptoxanthin, and lutein needs no comment. Trace amounts of other components were also present in these groups. The main pigment groups were the diepoxy diols and polyols, in which violaxanthin and neoxanthin were respectively the predominant pigments.

The violaxanthin was compared with its counterpart from the yellow pansy, *Viola tricolor*, the neoxanthin with material isolated from leaves. In chromatographic behavior, both on adsorbent columns and on paper, spectroscopic constants, partition behavior, and in blue color with hydrochloric acid no differences were detected, so it is reasonable to assume their identity. In some runs, a *cis*-violaxanthin predominated, as shown by a 2-mμ shift in the maxima and by the presence of a small *cis* peak.

Traces of auroxanthin and a possible isomer were tentatively identified in the diepoxy diol group, as was luteoxanthin and a *cis*-isomer, on the basis of their absorption maxima, 422-398-374 and 418-393-373 for auroxanthin, and 445-419-396 for luteoxanthin (solvent, ethanol). The monoepoxy diol group on similar evidence would appear to consist largely of antheraxanthin.

In the varieties tested, four pigments predominate, none exclusively in all-*trans* form. The percentage distribution varies from that approximating a green leaf, shown to a considerable extent in Pippin, to the following for Golden Delicious:

Violaxanthin	45–50%
Neoxanthin	10–30%
β-carotene	*ca.* 10%
Lutein	*ca.* 10%

Pears. *Total carotenoid.* The peeled mesocarp contains appreciably less carotenoid than the apple, varying from 0.3 ppm for D'Anjou, analyzed in December, to 1.28 ppm for Bartlett in August. The peel itself is comparable with that of the apple, e.g., 5.6 ppm for Comice.

Counter-current partition. By use of petroleum ether–98% methanol, the following distribution was determined, after a 100-tube transfer:

Hydrocarbons	(peak at tube 96)	4.2%
Monols	(peak at tube 60)	2.5%
Diols-polyols	(peak at tube 12)	93.3%

Identification of components. Unlike apple extracts, pears gave only weak blue reactions in the hydrochloric acid test. The major components appeared to be lutein (nonreacting) and neoxanthin and its isomers (weakly reacting). They were identified by their absorption spectra and tube number in the counter-current partition. Phytoene, phytofluence, β-carotene, and cryptoxanthin were also detected.

Where components are present in trace amounts, variation in the proportions of each one present may be anticipated. Analyses in December, 1963, of D'Anjou indicated significantly higher proportions of phytofluene and phytoene in relation to colored carotenoid than was the case for Bartlett, Comice, or Winter Nelis in that year. In September, 1964, however, the Bartlett extract was not distinguishable from that of the D'Anjou.

Strawberry. The strawberry is also low in carotenoid, *ca.* 1.5 ppm, *cf.* 0.64 reported by Curl (1964). The distribution is clearly comparable in the two cases:

	I	II	III
	Hydrocarbons	Monols	Diols-polyols
This laboratory	13.8	5.2	81.0
Curl (1964)	14.1	1.5	84.3

The following carotenoids were identified: I, β-carotene, ζ-carotene; II, cryptoxanthin; III, lutein, antheraxanthin, luteoxanthin, auroxanthin.

Cherry. The cherry extracts contained between 5 and 11 ppm; cherry differs significantly from the other fruits considered here. After 100-tube transfer, petroleum ether–98% methanol, two approximately equal zones were found, with peaks at tubes 8 and 90. A small monol fraction, peak tube 55, was also observed.

Only the hydrocarbon fraction was examined in detail. Phytoene and phytofluene were present in amounts permitting some degree of accuracy in estimation, the former 0.7, the latter 0.14 ppm. The major component was β-carotene, *ca.* 3 ppm, and also present in significant amount were ζ-carotene and β-zeacarotene, while α-carotene was detected in trace amount.

The xanthophyll fraction was not investigated in any detail, but epoxy derivatives predominated. Two major components resembled antheraxanthin and luteoxanthin.

DISCUSSION AND CONCLUSIONS

If one compares the proportions of the different carotenoid fractions of apple, pear, and strawberry with those found in green leaves, there is a markedly lower proportion of hydrocarbon, seemingly dependent upon the extent to which chlorophyll has disappeared. On the same basis, the xanthophyll fraction is no longer dominated by lutein, but by esterified diepoxy diols and polyols. Differences in patterns are discernible, not only between apples and pears but between varieties of each. Such differences may be expected. However, the extent to which violaxanthin, neoxanthin, or lutein may predominate in a given case becomes inconsequential in view of the low total carotenoid content, from 0.5 to 5 ppm, on a fresh-fruit basis.

It is perhaps surprising that lutein-5,6-epoxide was not detected, although it is probably present in trace amounts. It has been suggested to us that lutein either is degraded more rapidly or possibly may be converted to a carotenoid whose structure is still uncertain, such as neoxanthin.

The cherry is somewhat anomalous because of the presence in significant proportions of the first three members of the aliphatic series, which can give rise to lycopene (not, however, found in Sweet Bing). The xanthophylls follow the pattern of the apple xanthophylls, and the β-carotene content is significantly higher.

It may therefore be concluded that, with the possible exception of the cherry, no significant quantity of new polyene is synthesized during ripening.

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Cranberry Anthocyanins

SUMMARY

The red pigments from Early Black cranberries were extracted with methanol, purified by lead acetate precipitation and polyamide columns, separated on silicic acid columns, and crystallized. The four pigments were identified as cyanidin-3-monogalactoside, peonidin-3-monogalactoside, cyanidin-3-monoarabinoside, and peonidin-3-monoarabinoside based on the following evidence: R_f data in three solvents, fluorescence, sugar-aglycone ratios, formic acid and hydrogen peroxide hydrolysis, and spectral data. The absorption coefficients in ethanol-0.1*N* hydrochloric acid (85:15) were also determined.

INTRODUCTION

Previous work (Sakamura and Francis, 1961) established that the four red pigments in cranberries, in order of increasing speed of movement on a silicic acid column, were cyanidin-3-monogalactoside, peonidin-3-monogalactoside, a cyanidin-monoglycoside, and a peonidin-monoglycoside. The above identifications were based mainly on R_f values in four solvents and visual color and fluorescence data. The present work was undertaken to complete the identification of the 3rd and 4th pigments and to provide more chemical evidence on the identity of the 1st and 2nd pigments.

MATERIALS AND METHODS

Source of berries. The cranberries used (*Vaccinium macrocarpon* Ait, variety Early Black) were grown at the University of Massachusetts Agricultural Experiment Station, Wareham, Mass., and stored at 0°F.

Extraction and purification of crude pigment. Two methods of extraction were employed. In the first, 500 g of cranberries were blended with 400 ml of *n*-butanol and filtered after standing overnight. The residue was extracted twice more, and the filtrates were combined. After adding twice the volume of petroleum ether, a lower aqueous phase containing the red pigments separated. The red solution was added to a resin column (Amberlite CG-50, Type I, Rohm and Haas, Philadelphia, Pa.). The pigments were eluted from the column with 95% ETOH, evaporated to dryness in a rotary evaporator, and taken up again with 2% conc. HCl in MeOH. After evaporation to dryness, the dry powder was stored in sealed tubes.

The second method of purification was essentially that of Chandler and Harper (1961, 1962). Five hundred g of cranberries were blended with 1 L of 1% HCl in MeOH, and filtered. After the residue was extracted 6 times, the filtrates were combined and evaporated at 40°C to 1 L in a rotary evaporator at atmospheric pressure. Saturated lead acetate was added, and the precipitate was centrifuged and washed with 500 ml ethanol. The precipitate was dissolved in 5% HCl in MeOH, the lead chloride ppt filtered off and the filtrate evaporated to dryness. One hundred ml of MeOH were added, and the pigments were precipitated by adding 500 ml anhydrous ether. The solution and precipitation was repeated five times, and the final powder was dried under vacuum. A polyamide (Ultramid EM, Badische Anilin and Soda Fabrik, Germany) column was prepared by dissolving 20 g of polyamide in 200 ml of formic acid at 85–95°F. After cooling to 60°C, 400 ml of ETOH and 40 g of Celite (100–200-mesh) were added. Two additional 200-ml portions of ETOH were added with stirring, and after the free formic acid was removed by decantation with distilled water, the slurry was packed into a column. The pigment powder was dissolved in the upper phase of a butanol-2*N* HCl mixture, containing 0.5% methanol, and added to the column. Developing with butanol-2*N* HCl and evaporating the eluate to dryness yielded a fine red powder.

Separation of pigments. The crude pigment obtained from both methods was dissolved in 1% HCl in MeOH, and dry cellulose powder was added (4 g powder for 35–100 mg pigment). The mixture was dried under vacuum and added to the top of a silicic acid (100-mesh, Mallinckrodt Chemical Works, N. Y.) column prepared by mixing 350 g of dry silicic acid with 193 ml of distilled water and packing the semidry powder into a 5 × 38-cm column (Spaeth and Rosenblatt, 1950). A layer of silicic acid was added above the thin cellulose-pigment layer, and the column was developed for 2–3 hr with the upper phase of an *n*-butanol–acetic acid–water benzene (4:1:5:2) mixture. After extrusion, the column was sectioned and the pigments eluted with 1% HCl in MeOH. After evaporation to dryness, each pigment was taken up in 95% ethanol-0.5*N* HCl (1:) and repurified on a polyamide column. The eluates were evaporated to dryness, taken up with 2% HCl in MeOH or with 2*N* HCl, and left to crystallize in the cold. The crystals were washed with

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2*N* HCl, dried over P_2O_5, and stored in sealed glass tubes.

Analytical methods. *Paper chromatography.* Whatman No. 1 paper was used with the following descending solvent systems at 20±1°C in the dark.

BAW	*n*-butanol–acetic acid–water, 4:1:5, upper phase aged 3 days
BuHCl	*n*-butanol–2*N* hydrochloric acid, 1:1, upper phase
1% HCl	water–12*N* hydrochloric acid, 97:3
HAc-HCl	water–acetic acid–12*N* hydrochloric acid, 82:15:3
Forestal	water–acetic acid–12*N* hydrochloric acid, 10:30:3
Acet-pyridine	ethyl acetate–pyridine–water, 3.6:1:1.15
Acet-HAc	ethyl acetate–water–acetic acid, 3:3:1

Spectral curves. All curves were obtained with a Beckman DU spectrophotometer. The shifts with $AlCl_3$ were determined by measuring the spectra after the addition of 3 drops of a solution of anhydrous aluminum chloride in 95% ethanol (5% w/v) to the cuvette.

Acyl groups. One mg of pigment was dissolved in 1 ml of 2*N* NaOH and heated to boiling. After addition of 0.3 ml conc. HCl, half of the solution was extracted three times with 0.5-ml portions of amyl alcohol. Three hundred μl were spotted on paper and developed with BAW, BuHCl, and HAc-HCl. The other half of the above solution was extracted three times with diethyl ether, and 300 μl were spotted on paper. After developing with BAW and BuHCl for 15 hr, the R_f values were measured.

Aglycones. One mg of pigment was refluxed with 0.1 ml 10*N* HCl and 0.2 ml ethanol under nitrogen in the dark for 30 min. One hundred μl were chromatographed for 15 hr in BAW and BuHCl. A second 1-mg portion was hydrolyzed as above and diluted to 100 ml with 98% EtOH containing 1% HCl for determination of the aglycone concentration, using the absorption coefficients provided by Ribereau-Gayon (1959). The aglycone concentrations were calculated using molecular weights of 323 and 337, respectively, for cyanidin and peonidin.

Sugars. For identification of the sugars, one mg of each pigment was refluxed for 30 min with 0.3 ml of 85% formic acid and 0.7 ml water. Three hundred μl were spotted, along with standard solutions of sugars, and developed with BAW, Acet-pyridine, and Acet-HAc. The papers were dried, sprayed twice with aniline phosphate solution (Ash and Reynolds, 1954), and heated to 105–110°C.

For the quantitative sugar determinations, 150 μl were spotted with a similarly refluxed standard sugar solution and developed with BAW. After air drying, spraying with aniline phosphate, and heating, the colored spots were cut out and eluted for 70 min in ethyl alcohol–water–10*N* HCl (8:13:7). Using a blank from a portion of the paper treated in the same manner, the optical densities were measured at 365 mμ for galactose and 360 mμ for arabinose (Chandler and Harper, 1962). In the present work, 100 μg of arabinose and galactose in 3.5 ml gave optical densities of 0.748 and 0.542.

For determination of the position of the sugar moiety, one mg of pigment was dissolved in 0.2 ml of methanol, and 40 μl of 30% hydrogen peroxide were added. After 4 hr, a few grains of palladium catalyst were added to decompose the excess peroxide. After 20 hr, 50 μl of 0.880*N* ammonium hydroxide were added and the solution was heated on a boiling-water bath for 5 min. After cooling, the sugar solutions were chromatographed with BAW.

Absorption coefficients. The crystals of each pigment which had been dried over phosphorus pentoxide were weighed, dissolved in 95% ETOH–0.1*N* HCl (85:15), and measured, without dilution, in a Beckman DU spectrophotometer. The absorption coefficients were expressed as 1%, 1 cm at the wavelength of maximum absorption.

RESULTS AND DISCUSSION

Purification of pigment. The procedure described in the preceding section resulted in the preparation of pigment solutions which would readily yield crystals of pure pigments. Method 2 is to be preferred over the first because the crude pigment was much purer as judged by the amount of impurities left on the silicic acid column. The silicic acid mixture in a 0.55:1 ratio of water to absorbent provided an efficient means of separating 30–100 mg of crude pigment per column in 2–3 hr when used with suction. The use of cellulose powder to absorb the pigment provided an effective means of concentrating the pigment in a thin layer on top of the column. The addition of silicic acid on top of the pigment layer prevented the pigment from moving upward by dissolving in the added solvent. Benzene was added to the developing solution because trials with thin-layer chromatography had shown that the solutions traveled faster and the anthocyanins appeared in different shades of bluish red.

Only one of the four pigments (peonidin-3-monogalactoside) would crystallize readily from 2% HCl in MeOH after separation on the silicic acid column, in spite of the previous purification. A second purification on a polyamide-Celite column was necessary to promote crystallization of the other three pigments.

Spectral data. A shoulder at 440 mμ on the absorption curve is useful for distinguishing anthocyanins substituted at the 3- and 3,5-positions (Harborne, 1958). The ratios A_{440}/A_{max} (Table 1) in the present work were suggestive of monosubstituted compounds. The corresponding 3,5-diglycosides have ratios of the order of 12. The slight shoulder at 410–450 mμ also suggests that the 5-position is free. The lack of fluorescence under ultraviolet light was also suggestive of monosubstituted compounds in the 3-position.

The spectral shift with addition of aluminum chloride is characteristic of pigments with free ortho-dihydroxy groups. The data in Table 1 are compatible with the previous identification (Sakamura and Francis, 1960) of the aglycones of pigments 1 and 3 as cyanidin and 2 and 4 as peonidin.

Table 1. Spectral analysis of cranberry anthocyanins.

Pigment[a]	Absorption max. in MeOH:0.1% HCl (mμ)	Absorption ratio $\frac{A_{440}}{A_{max.}}$ (%)	Absorption shift with aluminum chloride
1) Cyanidin-3-monogalactoside	526	23	+
2) Peonidin-3-monogalactoside	524	20	none
3) Cyanidin-3-monoarabinoside	528	23	+
4) Peonidin-3-monoarabinoside	527	29	none

[a] In order of increasing speed of movement on a silicic acid column.

The absorption curves revealed only one peak in the ultraviolet region. If acyl groups were present, two peaks would be evident because of the superposition of the acyl group absorbance. The absence of acyl groups was confirmed by alkaline hydrolysis prior to paper chromatography of the anthocyanins. The R_f values for all four pigments were unchanged after alkaline hydrolysis.

Paper chromatography of anthocyanins. Data on R_f values for the anthocyanins and anthocyanidins are present in Table 2. The values are slightly different from those previously published (Sakamura and Francis, 1960), probably because the crystallized pigments were purer. The R_f values for cyanidin-3-monogalactoside and cyanidin-3-monoarabinoside were similar to those published by Harborne (1959, 1962). Values for peonidin-3-monogalactoside and peonidin-3-monoarabinoside were comparable to those expected by analogy with similar pigments.

Analysis of sugar components. The sugar solutions from formic acid hydrolysis of the anthocyanins were chromatographed in three solvents together with standard sugars. R_f and color data (Table 3) suggested that the sugars in Bands 1 and 2 were galactose and in 3 and 4 were arabinose.

The sugars from the formic acid hydrolysis were also determined quantitatively along with the anthocyanidins in order to determine the sugar-aglycone ratio. The data in Table 4 indicate that all four pigments have one molecule of sugar in each molecule of pigment.

The anthocyanins were also degraded with hydrogen peroxide, which is specific for the removal of sugars in the 3-positions (Karrer and de Meuron, 1932). The sugar-aglycone ratios (Table 4) were almost

Table 2. R_f values of cranberry anthocyanins and aglycones.

Solvent system	Anthocyanin[a]				Aglycone			
	1	2	3	4	1	2	3	4
BAW	0.37	0.39	0.43	0.44	0.68	0.69	0.67	0.70
HAc-HCl	0.25	0.30	0.24	0.29				
BuHCl	0.23	0.28	0.32	0.34				
Forestal					0.50	0.62	0.50	0.63

[a] See Table 1 for name of each band.

Table 3. R_f values of sugar moieties from cranberry anthocyanins.

Sugar	Solvent system[a] BAW	Acet-pyridine	Acet-HAc	Color developed by aniline phosphate spray
Moieties	(R_f)[a]	(R_f)[a]	(cm)[b]	
Band 1	0.91	0.75		Brown
Band 2	0.91	0.75		Brown
Band 3	1.16		24.3	Pink-brown
Band 4	1.16		24.3	Pink-brown
Standards				
Glucose	1.00	1.00	16.6	Brown
Galactose	0.92	0.75	14.6	Brown
Arabinose	1.16	1.12	24.3	Pink-brown
Mannose	1.16	1.11	19.7	Brown
Xylose	1.13	2.00	30.0	Pink-brown
Rhamnose	1.77	3.00		Brown

[a] R_f referred to glucose as unity after 22 hr of irrigation.
[b] Distance traveled in cm after 39 hr of irrigation.

Table 4. Sugar-aglycone ratios for cranberry anthocyanins after hydrolysis.

Hydrolysis	Pigment bands 1	2	3	4
Formic acid	1:1.01	1:0.99	1:1.11	1:1.08
Hydrogen peroxide	1:1.02	1:0.96	1:1.10	1:1.09

identical with those from the formic acid hydrolyses, indicating that all four pigments were monosubstituted in the 3-position.

Absorption coefficients. Absorption data for the four anthocyanins in 95% ethanol–0.1*N* HCl (85:15) are presented in Table 5. These data will be used to replace

Table 5. Absorption coefficients for cranberry anthocyanins in 95% ETOH–0.1*N* HCl.

Pigment	Abs. max (mμ)	A(1 cm, 1%, Amax.)
1	535	920
2	532	936
3	538	941
4	532	947

the Congo Red method for total pigment determination proposed for cultural and processing studies on cranberries (Francis, 1957).

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Changes in the Anthocyanin Pigments of Raspberries During Processing and Storage

SUMMARY

The effect of ingoing sirup concentration, various headspace atmospheres, and time and temperature of storage on the retention of the anthocyanin pigments in canned red and black raspberries was determined. The four anthocyanins of red raspberries and the three anthocyanins of black raspberries were separated by column chromatography and analyzed spectrophotometrically. The anthocyanins appeared to be similar in the two species as determined by paper chromatography. The absorption maximum of the pigments shifted during storage. Prolonged times and higher temperatures of storage both significantly reduced the recoverable anthocyanins. Increased concentration of ingoing sirup and the presence of oxygen resulted in greater pigment destruction.

INTRODUCTION

The water-soluble red pigments in raspberries and other fruits are anthocyanins. Willstätter and Bolton (1916) reported a cyanidin bioside extracted with 1% HCl in MeOH from raspberries. Harib and Brown (1956) reported four anthocyanins in raspberries, and Lamort (1959a) confirmed this for the Newburgh variety. Karrer and Strong (1936), Parkinson (1954), and Lukton *et al.* (1955) used column chromatography, with different support and solvent systems, for the separation of anthocyanins from fruits. Paper chromatography based on the method of Bate-Smith and Westall (1950) has been used extensively. Markakis (1960) approached the separation of anthocyanins by paper zone electrophoresis.

The degradation of anthocyanin pigments during the storage of processed fruits is a function of many factors. Kertesz and Sondheimer (1948) found that high temperatures were detrimental to the stability of anthocyanins in strawberry preserves during storage. Meschter (1953) showed that increased levels of sugars decreased the stability of the pigment in strawberries and that pH and ascorbic acid affected the rate of destruction. Markakis *et al.* (1957) showed that oxygen in the headspace of canned raspberries contributed to color destruction. Time and temperature during processing were stated by Joslyn (1942) to be important in color retention.

This experiment was conducted to study the effect of time and temperature of storage on the stability of the anthocyanin pigments of canned Willamette red raspberries and of an unidentified variety of black raspberries. The effects of added levels of sucrose and of the nature of the gaseous headspace were also investigated by chromatographically separating the pigments on columns packed with cellulose. Variables imposed were: 1) storage time, 0, 30, 60, and 90 days; 2) storage temperature, 34, 70, and 100°F; 3) sirup concentration, 0, 25, and 50°Brix; and 4) headspace gas, air, vacuum, and nitrogen. Tentative identification of the anthocyanins of the two species was made by paper chromatography.

EXPERIMENTAL

Enameled fruit cans (303 × 406) were filled with 275 g of washed and drained berries; then 170 g of water (0° Brix), 25 or 50° Brix sucrose sirups, heated to 170°F, were added, then the cans sealed and processed at 212°F in water for 10 min. Headspace gas was adjusted, for the second experiment, in the following manner: 1) atmospheric, the cans were closed after addition of 50° Brix sucrose sirup at 170°F; 2) vacuum, the berry-filled cans were evacuated 2 min to 25 inches Hg; 50° Brix sirup, cooled to room temperature, was applied under vacuum, the vacuum broken and the weight of the ingoing sirup adjusted to 170 g, the vacuum reapplied for 1 min and the cans closed at 25 inches; and 3) nitrogen, the berry-filled cans were twice evacuated for 2 min at 25 inches and the vacuum broken with nitrogen, they were then siruped under vacuum, the vacuum broken with nitrogen, the 50° Brix sucrose sirup was adjusted to the proper weight (170 g), the vacuum reapplied, broken with nitrogen, and the cans sealed. The sealed cans were processed at 212°F in water for 10 min, water-cooled, and stored.

Storage temperatures were 34, 70, and 100°F. Periods of examination of the anthocyanin pig-

ments were immediately after processing (0 time) and at periods of 30, 60, and 90 days storage at the indicated temperatures.

The anthocyanin pigments were obtained in the following manner: 60 × 3-cm columns were packed with Whatman standard-grade cellulose powder. The cellulose was washed with distilled water, then with 0.5% HCl in MeOH, and then dried with a suction of warm dry air. The contents of the cans were blended for 3 min, then 12.5 g of the homogenate was mixed with 15 ml of 1% HCl in MeOH and left overnight. Following filtration, 5 ml of the pigment solution were adsorbed on 3 g of the washed column packing and dried under vacuum. The adsorbed and dried pigment was packed on the top of the column and covered with dry cellulose. The column was then inverted into the solvent, which was allowed to ascend one-third the column length. It was then inverted and the solvent applied to the top of the column. When the solvent front reached the column orifice, the R_f values were determined by measurement. Reproducibility of the R_f values was ±0.02. For red raspberries, *n*-butanol-acetic acid–water (5:1:4 v/v) was applied as the solvent system (Garber *et al.*, 1962). For black raspberries, a mixture of acetic acid–water (15:85 v/v) was used successfully.

The various fractions of red raspberry anthocyanins were eluted with the developing solvent, freed from *n*-butanol by selective extraction with petroleum ether and then each fraction divided into two equal volumes which were vacuum evaporated to dryness at 54°F. Sorensen's citrate buffers of pH 3.4 and 2.0 were used to dissolve the dried pigments. The reading of the optical density at the wave length shown in Table 1 was used to measure the anthocyanins according to the method of Sondheimer and Kertesz (1948) using the respective citrate buffers in the reference cells.

For paper chromatography, the method of Bate-Smith and Westall (1950) was used in ascending systems. The aglycon was obtained by total hydrolysis of a methanolic solution of the pigment. This was accomplished by heating the solution with 12*N* HCl (2:1 v/v) in a water bath at 100°C. The reflux was continued for 1 hr under nitrogen atmosphere with constant stirring. The mixture was then cooled and filtered, and the anthocyanidin portion was extracted with *n*-amyl alcohol. Partial hydrolysis was performed according to the method of Abe and Hayashi (1956).

Cyanidin 3,5-diglucoside obtained from the Chrysler Imperial rose was used for comparative purposes. The extracted pigment was used in its natural state and was partially and completely hydrolyzed as suggested by Abe and Hayashi (1956).

RESULTS AND DISCUSSION

The red and black raspberry pigments were respectively separated by column chromatography into four and three distinct bands. Table 1 gives the R_f values obtained on the column as a measure of the position of the front of the band from the origin; the absorption maximum in citrate buffer at pH 2.0; the concentration of the pigment in the fresh fruit; and the loss of the pigment due to thermal processing. Table 2 gives the corresponding R_f values of the pigments of both red and black raspberries obtained with paper chromatography using four solvents.

From Table 2 it is evident that the aglycon

Table 1. R_f value, absorption maximum, concentration and distribution in fresh fruit, and percent loss due to processing of the anthocyanin pigments of raspberries.

Band	R_f value	Absorption maximum (mμ)	Mg pigment/100 g fresh fruit	% distribution total pigment	Percent loss due to processing (10 min @ 212°F)
a) Red raspberries[a]					
I	0.69	497	1.1	2.8	7.2
II	0.59	507	2.2	5.5	14.3
III	0.52	510	35.1	86.8	26.1
IV	0.39	511	2.0	4.9	10.9
b) Black raspberries					
I	0.65	515	103.5	23.2	12.5
II	0.52	511	315.1	71.0	20.2
III	0.40	510	26.6	5.8	21.9

[a] R_f values on cellulose columns with BAW (5:1:4 v/v) as solvent.
[b] R_f values on cellulose columns with HAc:H_2O (15:85 v/v) as solvent.

Table 2. R_f values of the resolved individual pigments of red and black raspberries by paper chromatography in ascending systems (temperature 21°C).

Compound	1% HCl in H_2O		HAc:H_2O (15:85 v/v)		HAc:HCl:H_2O (15:3:82 v/v/v)		BAW (5:1:4 v/v)	
	Red	Black	Red	Black	Red	Black	Red	Black
Hydrolyzed total fruit pigment	0.01	0.01	0.13	0.13	0.09	0.09	0.68	0.68
Resolved spots, fruit pigment								
1	.....	.....	.....	.....	0.09	0.09	0.68 I	0.68
2	0.09	0.09	0.35	0.39 III	0.27	0.28	0.41 II	0.41
3	.....	0.19	0.57	0.57 II	.....	0.43	0.25 III	0.23
4	0.39	0.39	.....	0.68 I	0.61	0.61	0.20 IV	.....
5	0.57	0.58	0.84	0.84 I	0.78	0.78	0.14 IV	0.14
Chrysler rose cyanin								
3,5-diglucoside	0.19		0.55		0.43		0.22	
3-glucoside	0.09		0.36		0.27		0.38	
5-glucoside	0.09		0.36		0.27		0.30	
aglycone	0.01		0.13		0.09		0.68	

I, II, III, IV, band number on cellulose column.

part of the total fruit pigment is cyanidin (Lamort, 1959a,b; Fuassin, 1956). The R_f values obtained with the hydrolyzed individual black and red raspberry pigments were the same as that of the cyanidin from the rose. Therefore the individual pigments of both varieties are glycosides of the same aglycon-cyanidin. The R_f value with BAW is in agreement with that reported by Bate-Smith and Westall (1950) for cyanidin.

Paper chromatography of the anthocyanins and anthocyanidins revealed that Band I of the red raspberries obtained from column chromatography may consist of cyanidin resulting from a partial hydrolysis of fruit pigment.

The main pigments of both varieties appear to be similar, differing only in the concentration. When one compares the R_f value to that of cyanidin 3-monoglucoside, in the respective solvents, column Bands III and II, respectively of the black and red raspberries, seem to contain monoglycosides, probably 3-monoglucoside. Bands II and III of the black and red raspberries also seem to correspond to 3,5-diglycoside, probably 3,5-diglucoside. The pigments represented by spots 4 and 5 in Table 2 were not separated by column chromatography. According to Harborne's theories (1959) they evidently possess a higher degree of glycosidation and apparently occur in the I and IV column Bands of black and red raspberry pigments, respectively. The pigments of Bands I and IV were not distinctly separated by paper chromatography, but nevertheless were present.

In paper chromatography with BAW, an additional spot was observed in both red and black raspberries. This spot, R_f 0.31, appears to correspond to the 5-glucoside of the Chrysler rose (R_f 0.30). In the other solvent systems, the corresponding spot was not resolved.

A slight shift in the absorption maximum (hypsochromic effect) occurred after processing and became more pronounced after storage, increasing with time until, at 90 days of storage, all values ranged between 494 and 498. It is probable that changes

occurred in the composition of the bands, but these were not determined.

The effect of the main variables on the anthocyanins of black raspberries was the same as with those of the red raspberries. Table 1 gives the concentration of the three anthocyanins in black raspberries; however, only the effects of the variables on the red raspberry anthocyanins will be discussed.

Increasing the concentration of the ingoing sirup resulted in a decrease in the recoverable pigments in canned red raspberries as shown in Table 3. Each increase in sirup concentration resulted in a significant reduction of the Band I pigment. The pigment of Bands II and IV were affected in a similar manner by increasing sirup concentrations. While significant reductions in the amount of the recoverable pigment were shown between 0 and 25° Brix, the use of 50° Brix sirup did not result in further statistically significant reductions of the anthocyanins. No statistically significant differences could be shown in the concentration of the anthocyanin pigment of Band III, the pigment of greatest concentration in red raspberries.

Table 3. Mean concentration of anthocyanin pigments of canned red raspberries as affected by sirup concentration, storage temperature and time (mg/100 g of fruit).

	Band I	Band II	Band III	Band IV
Sirup concentration (°Brix				
0	0.84	1.58	18.38	1.41
25	0.76	1.38	16.76	1.28
50	0.66	1.31	15.80	1.22
LSD (5%)	0.07	0.12	NSD	0.11
Storage temperature (°F)				
34	0.92	1.68	23.10	1.58
70	0.79	1.49	17.15	1.46
100	0.54	1.12	10.03	0.87
LSD (5%)	0.07	0.12	3.90	0.11
Storage time (days)				
0	1.04	1.92	25.90	1.80
30	0.80	1.56	17.12	1.42
60	0.67	1.24	13.80	1.07
90	0.49	1.00	11.10	0.92
LSD (5%)	0.08	0.14	4.51	0.12

The effect of increasing concentration of sirup resulting in lowering the amount of recoverable pigments substantiates the research by Tinsley and Bockian (1960), who showed that, in model systems, an increase in the concentration of sugar lowered the amount of extractable anthocyanins in strawberries. The loss of the pigment was noted to be partially dependent upon the formation of degradation products. An increase in the concentration of the ingoing sirup in the presence of a low pH would be expected to result in more degradation products and high losses of the pigment. The fact that a variation in the sirup concentration of 0–50° Brix did not significantly affect the degradation of Band III pigment may be explained by the fact that the quantity of pigment in the band was approximately 20× that of the other bands. Therefore, with the same degree of sugar degradation and formation of active products but with a pigment concentration of 20-fold, the effect of a amount of active products was not sufficient to result in a statistically significant lowering of the pigment concentration. Another possible explanation may be that certain of the anthocyanins are more susceptible to degradation than others.

The effect of increasing storage temperatures and duration of storage in significantly reducing the anthocyanin pigments of canned red raspberries may be observed in Table 3. The analysis of variance indicated that there were no significant interactions of the main effects other than that of time × temperature of storage.

The second experiment was designed to show the effects of headspace gases on the individual anthocyanins of canned red raspberries in 50° Brix sucrose sirup. As evidenced in Table 4, the nature of the headspace affected the destruction of the pigments. The effects of time and temperature of storage showed the same pattern of changes as before (Table 3). The effect of using nitrogen in the headspace on the retention of the anthocyanins was not always significantly superior to the retention under vacuum closing. This is most likely due to traces of oxygen in the headspace and/or entrapped in the cavity of the berries.

Table 4. Mean concentration of the anthocyanin of canned red raspberries as affected by headspace gas, storage temperature and time (mg/100 g fruit).

	Band I	Band II	Band III	Band IV
Headspace gas				
Air	0.51	1.26	15.53	1.20
Vacuum	0.65	1.29	16.12	1.23
Nitrogen	0.67	1.39	16.20	1.30
LSD (5%)	0.02	0.09	NSD	0.06
Storage temperature (°F)				
34	0.74	1.64	22.28	1.54
70	0.66	1.43	16.21	1.38
100	0.43	0.95	9.38	0.80
LSD (5%)	0.02	0.09	0.62	0.06
Storage time (days)				
0	1.04	1.92	25.90	1.80
30	0.61	1.61	16.00	1.49
60	0.49	1.06	11.87	1.03
90	0.23	0.78	10.03	0.78
LSD (5%)	0.26	0.10	0.71	0.07

During extraction and concentration of the pigment from the canned raspberries, varying amounts of an insoluble brownish-red precipitate were observed. More of the precipitate was found under conditions of high-temperature storage and when the berries were packed with air in the can headspace. The loss of the anthocyanins and the development of the precipitate may serve to indicate the direct and indirect effect of oxygen on the anthocyanins. Lukton *et al.* (1956) suggests that the precipitate forms from direct oxidation of the pseudobase of strawberry anthocyanin followed by its hydrolysis and development of the precipitate. Sondheimer and Kertesz (1953) and Markakis *et al.* (1957) pointed to the oxidation of ascorbic acid as being involved in the loss of anthocyanins and development of the precipitate.

The fact that protection of the individual anthocyanins was greater with nitrogen used as the closing gas confirms the results by Tinsley and Bockian (1960), who showed that the rate of destruction of strawberry pelargonidin-3-monoglucoside, in model systems, was markedly lower in the presence of nitrogen than under oxygen.

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Free Amino Acid Content of Chicken Muscle from Broilers and Hens

SUMMARY

Free amino acid analyses were conducted on 24 muscle tissue samples of chicken, both young and old, fresh and aged, in an attempt to show some properties or constituents of the meat which might be related to quality factors, such as tenderness. In general, ammonia nitrogen remained fairly constant throughout the study. Storage resulted in increases in free amino acids, with proline being a major exception. Light meat showed less free amino acids than dark meat, with major exceptions being lysine and histidine. In most cases, broilers had more free amino acids than hens. Taurine concentration was much higher in the dark meat of both broilers and hens. No relation was found between tenderness and the general pattern of free amino acid concentration or between tenderness and the concentration of any single free amino acid.

INTRODUCTION

Knowledge concerning the chemical composition of chicken meat is important for many reasons. Meat is consumed primarily for its nutritive value, and additional information about the amino acid composition is essential for calculating accurate nutrient values from such meat. Muscle composition may also contribute to other quality attributes such as flavor, storage stability, and tenderness. The last will receive considerable emphasis in this discussion since Pearson (1963) suggested that free amino nitrogen might be used as an indicator of tenderness in beef muscle.

Meat from young birds is more tender than meat from old birds (Peterson *et al.*, 1959; Dodge and Stadelman, 1959; Shrimpton, 1960; and May *et al.*, 1962). Poultry aged after slaughter also results in more tender meat as reported by Stewart *et al.* (1945), Hanson *et al.* (1942), and Dawson *et al.* (1958). Present evidence indicates that differences in tenderness between breast and thigh muscle may depend on the method used for such determinations or other unknown variables. Breast meat was found to be more tender than thigh meat by Strandine *et al.* (1949) (panel scores) and Goodwin *et al.* (1962) (shear), and less tender than thigh by Carlson *et al.* (1962) (shear), Goertz *et al.* (1961) (shear), and Dawson *et al.* (1958) (panel).

It has been known for many years that the aging or ripening process for meat is associated with tenderization. It is well known also that certain changes occur in the muscle proteins during the aging of meat. In a review of the literature before 1952, however, Swanson and Sloan (1953) showed that research involving studies of chemical changes in stored frozen meats, fish, and poultry were rather limited, and the results reported were quite contradictory in nature. This was especially true, they found, in the area concerned with protein changes.

A review of the literature since that report revealed a continuing lack of information in this area, especially as far as poultry is concerned. Many contradictions apparently have not been resolved.

With this information in mind, it was thought that a study of free amino acids in the muscles of chickens both young and old, fresh and aged, might show some properties or constituents of the meat that would reflect quality as represented by tenderness. In a similar study by Ma *et al.* (1961), analysis of 11 cuts representing nine muscles from a cow showed a variation in the content of certain free amino acids. The more tender cuts contained more leucine-isoleucine than the less tender cuts.

Specifically, this study was made to determine the relative amounts of free amino acids in the light and dark muscles from

broilers and hens before and after a post-slaughter aging period. Differences, if found, could then be related to tenderness.

LITERATURE REVIEW

Changes brought about by post-mortem aging can usually be associated with either one or a combination of the following factors: 1) changes in the connective tissue; 2) dissolution of actomyosin; 3) increased hydration of the proteins; and 4) proteolysis (Whitaker, 1959).

After the death of the animal, the enzymes of the muscle are still quite active. Proteolytic enzymes in tissues hydrolyze the peptide bonds of the proteins. These enzymes are called cathepsins to distinguish them from those of the digestive tract. They are amply supplied with substrate, and at least one has been found which is active in frozen meat and has a pH optimum approximately 4.1 (Balls, 1938). The exact role played by these enzymes in the increase in tenderness associated with aging meat is rather obscure (Whitaker, 1959).

Classical approaches to get evidence on the effect of cathepsin in the proteolytic aging process have yielded negative or, at best, inconclusive results (Wierbicki *et al.*, 1954). The increases in non-protein nitrogen that should occur during proteolysis or autolysis have not always been found, though increases in free amino acids have been reported (Locker, 1960). Many of the reported changes in the various nitrogen fractions were obtained with meat held longer than the normal aging period. In most of the work, proteolysis was determined by measuring the amino acids produced. It is apparent that a protein can be extensively degraded before many amino acids are liberated. Also, extensive release of a few amino acids could occur with little total protein breakdown.

The changes that occur when cod muscle is allowed to spoil during storage in ice are different from those produced by autolysis alone (Hodgkiss and Jones, 1955; Shewan and Jones, 1957). With spoilage, there are two factors to be considered: 1) leaching losses due to the ice-water melt; and 2) the transformation by both bacterial and autolytic enzymes.

The possibility of using free amino nitrogen as an indicator of tenderness was suggested by Pearson (1963) since it increases as proteolysis proceeds.

EXPERIMENTAL METHODS

Free amino acids analyses were conducted on samples from the breast and thigh meat of fresh-chilled and refrigerator-stored broilers and hens. In this study, broilers were considered more tender than hens and aged meat more tender than fresh meat.

Six broilers and six hens of the same strain and sex were divided into two equal groups, each consisting of three broilers and three hens. All broilers were fed a commercial broiler ration and were approximately the same age and weight. All hens were fed a commercial laying ration and were approximately the same age and weight.

All birds were hung on a killing wheel for 2 min, bled by the so-called "Kosher" method (outside cut), placed in a Roto-matic scalder containing water maintained at 138±2°F, scalded for 20 sec, and machine-picked in an automatic rubber-fingered picker. The birds were then hung on shackles, pinned, eviscerated, washed, and placed in slush ice for 18 hr.

Samples from birds of Group I were prepared at the end of the 18-hr chill period. Birds of Group II were removed from the slush ice after the 18-hr chill period, vacuum packaged in Cryovac S-303 clear bags (Cryovac Division of W. R. Grace & Co., Duncan, South Carolina), and refrigerated at 35±2°F for 1 week prior to sample preparation.

Sample preparation was done essentially according to the method of Spackman (1960). Samples of fat-free and connective tissue free muscle of 12.50±0.02 g were taken from the right sides only. Breast meat samples were prepared by taking a cross-section of the pectoralis major and minor muscles; thigh meat, by taking the same representative muscles in every case.

After weighing, the samples were cut into 1–3-g portions and ground for 2 min in a Waring blender with 125 ml of 1% picric acid. The resulting protein precipitate was promptly removed by centrifugation for 30 min at 30,000 rpm. The supernatant liquid was passed through a Dowex 2-X10 (chloride form) resin bed. The resin was packed 4 cm high in a 2 × 20-cm chromatography tube and covered with glass wool. Prior to use, the resin bed was washed with five 6-ml portions of 1*N* hydrochloric acid and then with water until the effluent was neutral.

Once the supernatant fluid was passed through the resin bed, the walls of the tube and the bed were washed with five 6-ml portions of 0.02*N* hydrochloric acid. The clear effluent and washings were concentrated in a rotary evaporator under vacuum to about 5 ml and made up to 25 ml with water. A 5-ml portion was removed, diluted to 15 ml, and frozen until the night preceding analysis.

At that time, the sample was brought to room temperature and the solution adjusted to pH 7.2–7.5 with 1*N* sodium hydroxide. One ml of a freshly prepared 0.5*M* solution of sodium sulfite was added, and pH was again adjusted to the above range.

The solution was allowed to stand, open to the air, for 4 hr and stirred periodically. Glutathione and cysteine interfere in this chromatography method in that they emerge in peaks which underlie several of the essential amino acids. This sulfiting procedure was necessary to convert these compounds to forms which emerge at a small effluent volume and thus do not interfere. The pH of the sample was then adjusted to 2.0–2.2 with 1*N* HCl, the sample was diluted to 25 ml and frozen overnight. Two 2-ml portions were used after thawing for analysis on a Beckman Spinco amino acid analyzer, model 120. One aliquot was used for the determination of basic free amino acids and ammonia, the other for the neutral and acidic free amino acids and taurine. Ammonia and taurine are ninhydrin-positive compounds which are analyzed by the same reaction as the amino acids.

RESULTS AND DISCUSSION

The Beckman amino acid analyzer provides for complete 24-hr separation and quantitative analysis of the amino acid content of unknown mixtures. The basic principles underlying the analysis performed by that model are elution chromatography from buffered columns of ion-exchange resin followed by colorimetric determination of the separated components by the ninhydrin reaction. The amount of each component amino acid in a sample analyzed by the model 120 is determined by measuring the area enclosed by its corresponding peak on a chromatogram and comparing it to that of a standard. Fig. 1 shows a typical chromatogram of the basic amino acids and ammonia.

Tables 1 and 2 list the average concentration in micromoles of free amino acids found in the 24 samples analyzed. In comparing the amounts of free amino acids in light and dark broiler meat (Table 1), lysine, histidine, methionine, and isoleucine were found

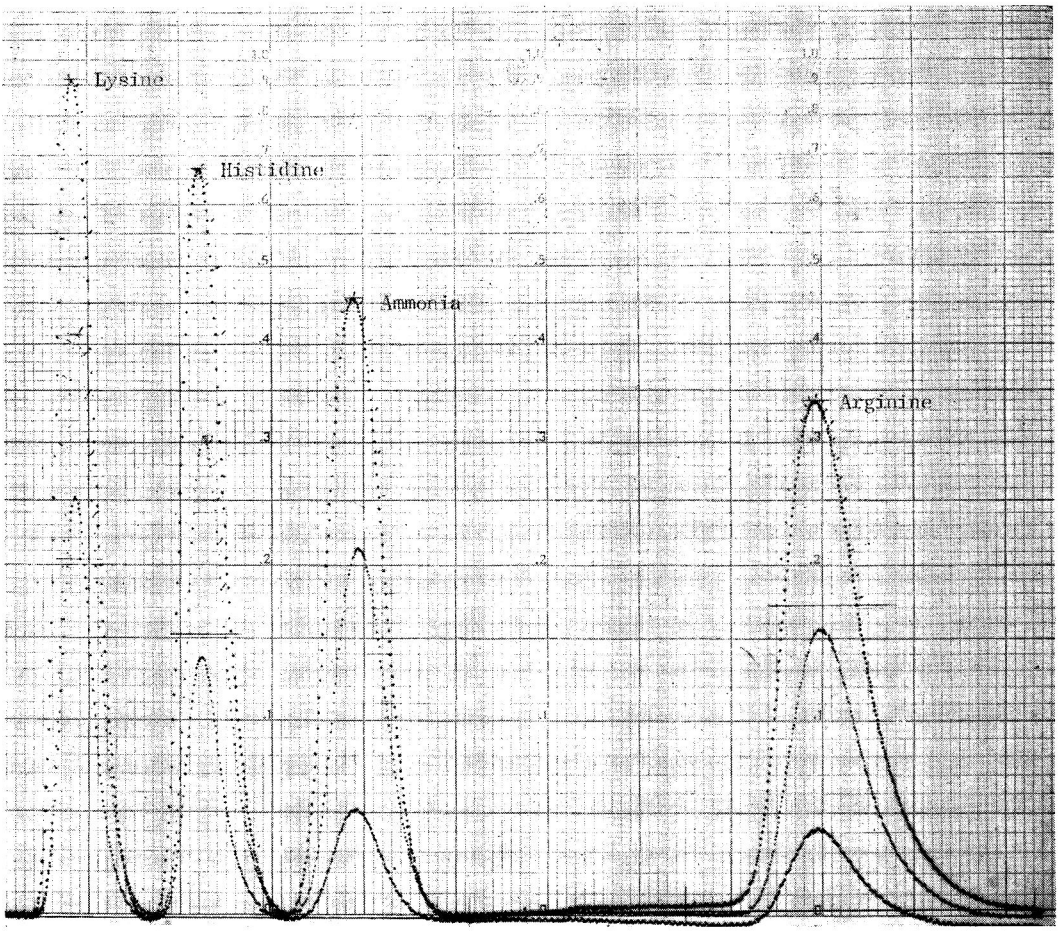

Fig. 1. Typical chromatogram of the basic amino acids and ammonia.

Table 1. Average free amino acid concentration[a] of fresh-chilled broilers and hens (Group I).

	Broilers				Hens			
	Dark meat		Light meat		Dark meat		Light meat	
Lysine	6.05	(5.35–6.50)[b]	16.2	(13.5–17.6)	3.55	(2.30–4.26)	17.2	(15.1–20.3)
Histidine	2.42	(2.09–2.63)	11.5	(8.95–14.00)	2.75	(1.03–4.17)	15.5	(12.2–18.2)
Ammonia nitrogen	6.35	(5.95–6.85)	7.75	(7.30–8.10)	6.35	(5.15–7.10)	7.40	(7.25–7.55)
Arginine	0.451	(.325–.530)	0.277	(.217–4.00)	0.299	(.199–.433)	0.147	(.083–.194)
Taurine	14.1	(11.65–15.65)	0.510	(.390–.745)	21.3	(20.2–23.1)	0.232	(.061–.344)
Aspartic acid	0.248	(.216–.313)	0.125	(.066–.169)	0.373	(.242–.475)	0.0580	(.057–.061)
Threonine	0.715	(.535–.950)	0.424	(.298–.500)	0.575	(.399–.930)	0.312	(.190–.483)
Serine	4.87	(3.89–5.45)	0.870	(.810–.970)	3.06	(2.25–3.83)	0.550	(.478–.665)
Glutamic acid	1.53	(1.17–1.97)	0.635	(.560–.775)	1.97	(1.63–2.27)	0.750	(.655–.930)
Proline	0.605	(.585–.620)	0.499	(.381–.690)	0.555	(.530–.595)	0.379	(.254–.600)
Glycine	1.42	(1.34–1.52)	0.595	(.535–.685)	1.05	(.60–1.32)	0.363	(.324–.388)
Alanine	2.13	(1.96–2.36)	0.775	(.680–.875)	2.16	(1.68–2.51)	0.500	(.474–.537)
Valine	0.209	(.202–.217)	0.206	(.173–.248)	0.219	(.176–.258)	0.204	(.177–.224)
Methionine[c]	0.172	(.133–.240)	0.243	(.145–.412)	0.178	(.102–.303)	0.096	(.077–.122)
Isoleucine	0.098	(.086–.116)	0.119	(.109–.133)	0.139	(.105–.194)	0.079	(.066–.088)
Leucine	0.226	(.204–.247)	0.209	(.182–.249)	0.240	(.227–.247)	0.156	(.142–.172)
Tyrosine	0.196	(.170–.214)	0.189	(.133–.293)	0.107	(.090–.120)	0.141	(.100–.196)
Phenylalanine	0.111	(.085–.127)	0.108	(.040–.205)	0.118	(.075–.157)	0.0695	(.062–.074)

[a] Average concentration in micromoles of three 1-g samples.
[b] Numbers enclosed by parentheses represent range of values.
[c] Total free methionine and free methionine sulfoxide concentration.

to be higher in light meat, and the others were lower. In hens, lysine, histidine, and tyrosine were higher in the light meat than in dark meat. After storage (Table 2), lysine and histidine remained higher in the light meat of both broilers and hens; and valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine were also higher. The biggest difference in composition in the light and dark meat of both broilers and

Table 2. Average free amino acid concentration[a] of refrigerator-stored broilers and hens (Group II).

	Broilers				Hens			
Free amino acid	Dark meat		Light meat		Dark meat		Light meat	
Lysine	5.50	(4.10–6.65)[b]	14.5	(13.2–16.3)	5.30	(4.05–6.80)	18.4	(17.3–20.0)
Histidine	3.19	(2.43–4.22)	8.00[c]	(7.1–8.9)	4.71	(3.29–6.65)	19.1	(15.5–23.3)
Ammonia nitrogen	6.80	(6.60–6.95)	6.30	(5.80–7.05)	6.65	(6.25–6.85)	8.65	(8.25–9.20)
Arginine	0.560	(.354–.720)	0.353	(.234–.830)	0.438	(.417–.471)	0.396	(.338–.438)
Taurine	13.1	(10.8–17.6)	1.33	(.90–1.82)	17.4	(14.3–22.3)	0.995	(.595–1.52)
Aspartic acid	0.585	(.362–.805)	0.337	(.205–.555)	0.430	(.347–.498)	0.201	(.178–.230)
Threonine	0.840	(.805–.885)	0.705	(.550–1.01)	0.390	(.375–.409)	0.420	(.373–.461)
Serine	4.81	(4.56–5.05)	1.78	(1.37–2.52)	3.94	(3.30–4.83)	1.29	(1.22–1.34)
Glutamic acid	2.88	(1.76–3.83)	1.29	(.93–1.92)	2.28	(1.41–2.93)	1.26	(1.06–1.45)
Proline	0.575	(.510–.640)	0.371	(.272–.454)	0.945	(.600–1.610)	0.377	(.314–.488)
Glycine	1.79	(1.50–2.01)	1.04	(.76–1.58)	1.53	(1.32–1.91)	0.635	(.600–.685)
Alanine	2.61	(2.38–2.82)	1.56	(1.12–2.35)	2.27	(2.12–2.42)	1.05	(.99–1.13)
Valine	0.432	(.299–.590)	0.530	(.375–.835)	0.294	(.257–.341)	0.406	(.395–.427)
Methionine[d]	0.235	(.119–.322)	0.448	(.348–.649)	0.164	(.132–.196)	0.332	(.277–.385)
Isoleucine	0.253	(.148–.387)	0.361	(.243–.570)	0.153	(.141–.175)	0.239	(.222–.261)
Leucine	0.472	(.330–.600)	0.675	(.500–.995)	0.314	(.286–.361)	0.494	(.468–.540)
Tyrosine	0.263	(.168–.324)	0.380	(.287–.525)	0.134	(.107–.153)	0.279	(.228–.318)
Phenylalanine	0.180	(.136–.230)	0.275	(.200–.408)	0.108	(.101–.111)	0.202	(.177–.216)

[a] Average concentration in micromoles of three 1-g samples.
[b] Numbers enclosed by parentheses represent range of values.
[c] Average of 2 samples only.
[d] Total free methionine and free methionine sulfoxide concentration.

hens was in the taurine concentration. It was much higher in dark meat.

Generally, the amount of free amino acids was higher in broilers than in hens; however, many exceptions were noted. In the dark meat of fresh-chilled birds (Table 1), the concentrations of many of the amino acids were higher in hens; in the light meat of both fresh-chilled and refrigerator-stored birds (Tables 1 and 2), the concentrations of lysine and histidine were higher in hens; and in refrigerated birds (Table 2) the proline concentration was higher in hens.

Variations between birds were expected and observed. The variations were more pronounced in hens than in broilers. The fact that some of the hens used in this study were in production while others were not, may explain some of the variation.

Increases in free amino acids during storage (comparison of results in Table 1 and Table 2) were noted in this study, with few exceptions, the main ones in broilers being that taurine decreased slightly in dark meat, proline showed a decrease in both light and dark meat, and lysine and histidine decreased in the light meat only; in hens, taurine again decreased in the dark meat while proline remained fairly constant. These expected increases are generally in agreement with previous reports by Ginger *et al.* (1954), Colombo and Gervasini (1954; 1955a,b; 1956), Niewiarowicz (1956), and Thompson *et al.* (1961). Ammonia nitrogen remained fairly constant throughout this study, with little difference between broilers and hens, fresh-chilled or refrigerator-stored.

Poor resolution or incomplete separation between lysine and histidine and between threonine and serine proved to be a problem. In one case, the histidine concentration could not be calculated. Although the concentration of these amino acids in the other runs was calculable, the poor resolution may explain some of the variations noted among these compounds. The values found for histidine may represent artifacts. Anserine or carnosine, being structurally similar to histidine, may have been eluted along with histidine and could have been included in these calculations. Some histidine might also have been obtained by hydrolysis of carnosine. The only unknown peak of any significance occurred prior to taurine.

No relationship could be found between tenderness and the general pattern of free amino acid concentration or between tenderness and the concentration of any single free amino acid.

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Research Note

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Volatiles of Apples During Storage and Ripening

In an earlier communication Grevers and Doesburg (1962) reviewed investigations of nonethylenic "odorous" emanations from apples during storage. It was concluded the apples can produce aliphatic alcohols and carboxylic acids containing 1–6 carbon atoms and a C-8 acid. Various esters derived from these alcohols and acids and carbonyl compounds have also been detected. Until now, the C-8 acid (*n*-caprylic) has been found only as an ester component (Buchloh, 1956; Henze *et al*, 1953).

It was also concluded (Grevers and Doesburg, 1962) that there is a good correlation between the qualitative composition of volatile products of living fruits and the volatiles found in apple juices. However, several factors can be mentioned which account for the quantitative differences in the composition of the mixture of nonethylenic volatiles in apple juices and gaseous emanations of these fruits.

Since the palatability of stored and ripened fruits is intimately associated with the amount of volatiles, studies have been made on the behavior of these fruits during storage and ripening.

MATERIALS AND METHODS

In the 1962 season, Golden Delicious and Belle de Boskoop apples were picked at the time of commercial harvesting and stored at 3, 6, 10, and 15°C, whereas in the 1963 harvest, Golden Delicious and Cox Orange Pippin apples were stored at 3 and 6°C only.

Two weeks after storage at different temperatures, a part (45 kg) of these fruits were kept for two weeks at 15°C and subsequently put into 100-L containers at 15°C for two weeks.

To collect the volatiles, pure air (purified by passage through activated carbon) was passed at a rate of 450 L/hr over the fruits in the containers. Thereafter the air was led through a cold trap provided with melting ice for removal of excess of water, and finally through a glass cylinder with 100 g of carbon (Desodorex II/III, Bayer).

The ripening at 15°C of fruits from storage at different temperatures and the collection of volatiles were repeated every month.

From the fruits stored at 3, 6, and 10°C, monthly samples were also taken. These were placed for two weeks in 100-L containers and stored at the same temperature. During these periods volatiles were collected as described above.

The volatiles given off by fruits stored at different temperatures or during ripening at 15° were removed from the carbon by high-vacuum desorption.

The principles of this desorption have been described by Turk and Messer (1953). The details of the modification used have been reported by Grevers and Doesburg (1962).

The amounts of volatiles produced during each treatment were estimated by weighing. Prior to weighing, traces of water were expelled by placing the volatile samples at —20°C, which enabled the removal of small ice crystals. The samples of volatiles were then submitted to gas chromatographic analysis. A Becker gas chromatograph with double hydrogen flame detector was used.

Column packing: Poly (propylene glycol). UCON-Oil-LB-550-X on Chromosorb R (60/80-mesh).

Column size: length 2 m, 4 mm ID.

Column temperature: 60–120°C, increasing at a a rate of 100°C/30 min.

Injector temperature: 200°C.

Recorder: 1-mv full-scale Philips recorder.

Chart speed: 30 cm/hr.

Carrier gas and flow rate: nitrogen at 50 ml/min.

Hydrogen gas flow rate: 36 ml/min.

Air flow rate: 340 ml/min. All the flow rates were measured separately at the outlet of the burner and at room temperature. All volatiles of the injected samples were eluted off the column within 45 min.

Injected amount of samples: 0.3 μl.

Used for identification of individual components were gas-liquid chromatography retention times. The retention times of 33 substances, reported in literature as the components of "odorous" apple volatile mixtures, were determined (Grevers and Doesburg, 1962). Pure substances were added to check the identification of compounds correspond-

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Table 1. Amounts of volatiles in g, emanated by Golden Delicious apples during storage at 3, 6, 10, or 15°C and after ripening at 15°C.

Storage period (months)	Year 1962–63						Year 1963–64			
	Storage temperature			After ripening at 15° after removal from different storage temperatures			Storage temperature		After ripening at 15° C after removal from different storage temperatures	
	3°C	6°C	10°C	15°C	3°C	6°C	10°C	3°C	6°C	3°C	6°C
1	0.7	1.2	3.0	1.9	6.4	6.9	4.9	0.9	1.1	6.4	7.5
2	0.4	1.8	1.9	2.6	4.5	2.3	3.5	1.2	1.6	5.9	6.1
3	0.3	1.6	2.5	1.4	4.0	2.4	1.7	0.9	0.5	6.2	4.0
4	0.2	1.6	1.5	--	4.7	3.1	--	0.3	0.5	4.0	1.4

ing with individual peaks. The results of this identification of the main peaks, mentioned below, are in perfect correspondence with the work of Strackenbrock (1961), who also applied other chemical identification methods.

RESULTS

The amount of volatiles produced by the Belle de Boskoop apples during storage and ripening was extremely small. In all cases, after desorption a small quantity of a watery sample of volatiles was obtained which could not be used for reliable gas chromatographical analyses and determination of the amount of emanated volatiles by weighing, because the water could not be separated out by freezing.

For these reasons, results are reported only for Golden Delicious apples in 1962 and 1963, and for Cox's Orange Pippin in 1963.

Table 1 shows the amount of volatiles produced by 45 kg Golden Delicious apples in two weeks after 1–4 months at different temperatures, and the amounts of volatiles collected in two weeks after 14 days of ripening at 15°C subsequent to removal from storage at different temperatures after increased storage periods. Similar results obtained with Cox's Orange Pippin in 1963–64 are given in Table 2.

From the 61 samples of volatiles mentioned in Tables 1 and 2, gas chromatograms were made and compared. Only some chromatograms are presented here, and only the main trends of the individual compounds in the mixtures of volatiles produced during storage at 3 and 6°C and during ripening are mentioned.

For this purpose the components shown in the chromatograms have been divided in groups *A* and *D*, whereas the individual compounds ethyl-n-butyrate, butyl acetate, *n*-butyl-n-butyrate, and hexyl acetate (as indicated by Strackenbrock, 1961; and Grevers and Doesburg, 1962) are marked by *B*, *C*, *E*, and *F* (see Figs. 1, 2).

The main component of the volatiles of Golden

Table 2. Amounts of volatiles in g, emanated by Cox's Orange Pippin apples during storage at 3 and 6°C and after ripening at 15°C (season 1963-64).

Storage period (months)	Storage temperature		After ripening at 15° after removal from storage at 3 and 6°C	
	3°C	6°C	3°C	6°C
1	0.9	1.5	4.5	1.9
2	1.0	2.1	3.8	4.7
3	0.3	1.3	2.1	3.8
4	0.2	1.2	1.9	3.3

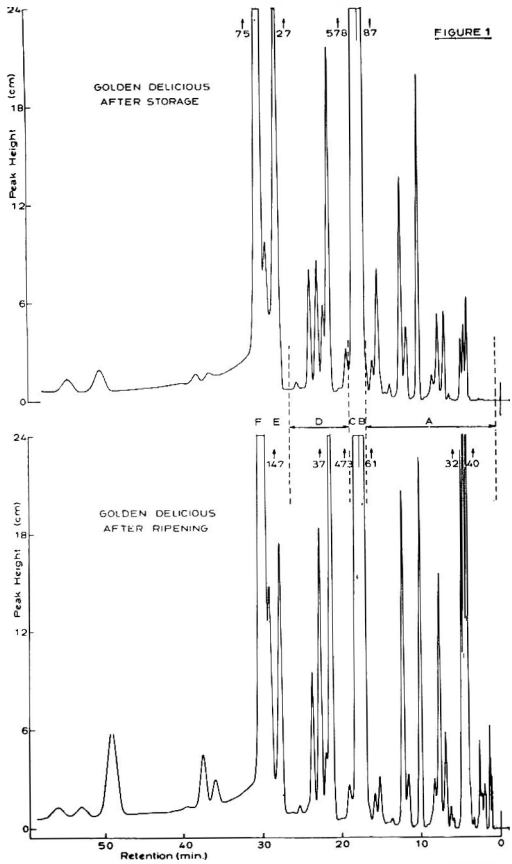

Fig. 1. Comparison of chromatograms of volatiles emanated by Golden Delicious apples during cold storage and after cold storage and two weeks of ripening at 15°C.

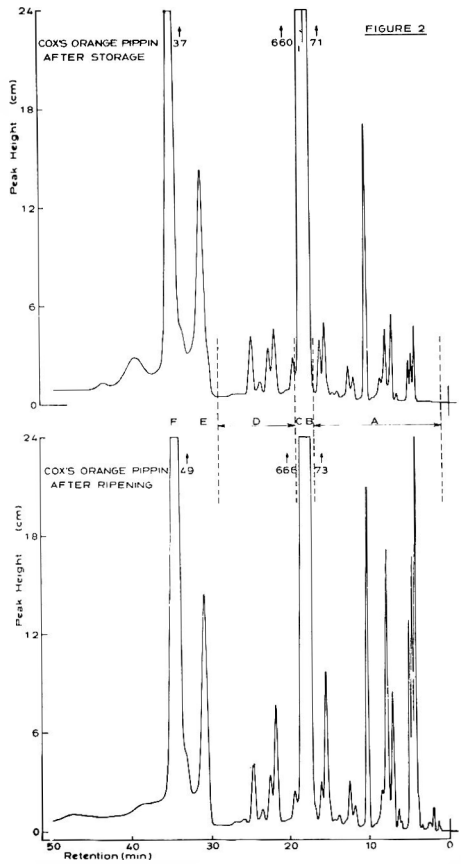

Fig. 2. Comparison of chromatograms of volatiles emanated by Cox's Orange Pippin apples during cold storage and after cold storage and two weeks of ripening at 15°C.

Delicious and Cox's Orange Pippin apples is shown to be butyl-acetate (*C*), which exhibited no quantitative trend in the volatiles of both varieties during storage at 3 and 6°C. Similar results have been found for the butyl butyrate (*E*) content of the volatiles collected in both years from Golden Delicious during storage at 3 and 6°C. In volatiles from Cox's Orange Pippin the amount of butyl butyrate increased during storage at 3°C, whereas it remained nearly the same at 6°C. The proportion of hexyl acetate (*F*) in the volatiles of both varieties showed no special trend and fluctuated somewhat during storage at 3 and 6°C.

When comparing the amounts of components of group *A* in the volatiles produced by both varieties during storage at 3 and 6°C it was found that these compounds generally formed a greater part of the volatile samples from 6°C than from 3°C. In both

Table 3. Effect of ripening at 15°C on the relative composition of volatiles produced by Golden Delicious and Cox's Orange Pippin apples after storage at low temperatures.

Constituents	Golden Delicious		Cox's Orange Pippin
	1962-63	1963-64	1963-64
Group A	Decreased	Strongly increased	Strongly increased
Ethyl butyrate (B)	No significant changes	Decreased	No significant changes
Butyl acetate (C)	No significant changes	Decreased	No significant changes
Group D	Slight increase	Strongly increased	Fluctuated
Butyl butyrate (E)	No significant changes	Strongly increased	No significant changes
Hexyl acetate (F)	Strongly increased	Strongly increased	Strongly increased

varieties the amount decreased during storage at 6°C.

Special attention has to be given to changes in composition of volatiles during ripening after cold storage since the palatability of fruits may depend largely on these changes. These alterations are summarized in Table 3.

Minor differences were found in the proportional changes of various constituents from both varieties as listed in Table 3 when the influence of ripening was compared after different periods of storage at 3 and 6°C.

The influence of storing and subsequent ripening at 15°C for Golden Delicious (1963–64) and Cox's Orange Pippin (1963–64) is illustrated in Figs. 1 and 2, respectively, showing the gas chromatograms of volatiles after cold storage and after treatment at 15°C. In order to produce these chromatograms, samples were used which were obtained by mixing the volatiles mentioned in Tables 1 and 2, which had been sampled after various times of storage at 3 and 6°C or after different periods of storage at these temperatures and subsequent ripening at 15°C. For the preparation of these mixtures the used amounts from the individual samples were proportional to their weights as reported in Tables 1 and 2.

DISCUSSION

As shown in Tables 1 and 2 the amount of volatiles given off by apples is greatly dependent on the storage temperature.

In most cases (except Golden Delicious 1962–63 stored at 3 or 10°C) the greatest amount of volatiles is produced at the end of a storage period of two months. After this period the amount of volatiles decreased. The quantity of volatiles produced is lower at 3°C than at 6 or 15°C. As shown in the experiment with Golden Delicious in 1962–63, the amount of volatiles produced at the beginning of the storage period at 6°C was also lower than the amount produced at 10 and 15°C. However, at these higher temperatures the emanations decreased greatly during storage, so that, finally, the quantity of volatiles produced at 6, 10, and 15°C was nearly the same.

As shown in Tables 1 and 2, after ripening, the amount of produced constituents decreased as the preceding storage time increased. This may account partially for the decrease of palatability of apples when they have been in cold storage for some months. From Table 1 it can be concluded that Golden Delicious apples produce a greater amount of volatiles during ripening when they have been stored 2–4 months at 3°C than after storage at 6°C. The reverse effect was found with Cox's Orange Pippin apples.

The influence of prolonged storage at 3 and 6°C upon the amount of volatiles produced by ripening apples is shown to be rather great. As far as volatile constituents affect the palatability of stored and ripened apples, it may be concluded that the decrease in the amount of volatiles is the main factor, since in the same season the composition of the mixtures of volatile constituents was found to be nearly the same for fruits ripened after different periods of storage at 3 or 6°C.

It has been shown that ripening does not always have the same effect on the composition of emanated constituents when results from different years are compared. This is shown for two different lots of Golden Delicious apples used in 1962–63 and 1963–64 (Table 3). However, in all cases the relative amount of hexyl acetate in the mixture of volatiles of Golden Delicious and Cox's Orange Pippin apples strongly increased after ripening of the fruit. The same effect was found by Strackenbrock (1961) for Golden Delicious apples.

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The Precursors of Chocolate Aroma: The distribution of free amino acids in different commercial varieties of cocoa beans

SUMMARY

A study of the distribution of the free amino acids in different commercial varieties of cocoa beans revealed differences which might account. at least in part, for the acknowledged variations in aroma and flavor of the products obtained from these raw materials.

INTRODUCTION

In a previous publication in this series (Rohan, 1964) the importance of the amino acids in the production of the primary characteristic aroma of chocolate was demonstrated. There is now some evidence, from recent work in these laboratories, that the primary aroma of chocolate comprises a very small number of components of which not more than two are carbonyl compounds. These are the only two components which could conceivably have originated from the oxidative deamination of amino acids. The remaining 18 or so amino acids present in fermented cocoa beans may also undergo Strecker degradations during the roasting process, with the production of a range of carbonyl compounds, and it is likely that the latter contribute to the over-all aroma of the final product. The chocolate industry has long recognized the existence of characteristic differences in the "bouquet" of chocolates prepared from different commercial types of cocoa beans. Numerous suggestions have been made to account for this lack of organoleptic uniformity. These include such effects as variations in microflora on the products of pulp fermentation, variations in soil types, climatic variations, and genetic variations. Although some of these factors may be contributory in the present context, no experimental evidence has yet been presented in support of any one. It occurred to the authors, after comparison of the amino acid distribution in Trinidad (De Witt, 1957), Brazilian (Biehl, 1961), and Accra (Rohan, 1963) cocoa beans, that the differences observed might have some significance. Consequently the present investigation was undertaken, and extended the field of observation to 9 different commercial varieties of cocoa beans, in order to determine if the free amino acid pattern appears to be sufficiently variable to permit the tentative hypothesis that this is one factor contributing to the flavor and aroma differences already mentioned. Simplified techniques were employed for this exploratory work, and a more detailed investigation was planned if the results of the present work justified one.

EXPERIMENTAL

Materials used. Commercially fermented unroasted cocoa beans of the following marks were employed: Bahia (Brazil), Trinidad, Grenada, Sanchez (Dominican Republic), Carupano (Venezuela), New Guinea, and Accra (Ghana). A specially prepared sample of unfermented Accra cocoa beans was also examined.

Extraction of free amino acids. Finely ground shell-free cocoa beans (20 g) were blended with water (200 ml) in a paladin blender for 5 min, and the resultant suspension was filtered. An aliquot (100 ml) of the filtrate was passed through a column (20 × 1 cm) of Amberlite IR.120.H resin to remove the amino acids. The resin was washed with 50 ml of a 20% solution of iso-propanol in water followed by 50 ml of an 80% solution of the same alcohol in water, and finally with distilled water. This treatment freed the resin from the bulk of adsorbed flavonoids, and the amino acids were then eluted with a 4N aqueous solution of ammonia. The effluent, which was collected until the pH was 10.0, was evaporated to dryness on a rotary evaporator, and the powdery residue was used for subsequent paper chromatography.

Group separation and semi-quantitative estimation of free amino acids. The amino acid concentrates from the various commercial cocoas were each taken up in 1 ml of water containing one drop of ammonia. An aliquot (10 μl) of the amino acid solution was applied as a series of spots to a sheet of Whatman No. 1 paper which was irrigated, by ascending chromatography, with butanol–acetic acid–water (60:15:25 v/v) prepared 2 hr before

use. After 24 hr, when the solvent front had risen 40 cm, the papers were air dried and traces of residual solvent removed by immersing the papers in ether, after which the amino acids were revealed with ninhydrin. The chromatogram was arbitrarily divided into 9 zones, and the colored ninhydrin complexes eluted from each strip with water-acetone (50:50 v/v). The solutions were each made up to 10 ml with water, and the optical densities determined with a Unicam spectrophotometer at 570 mμ in a 1-cm cell.

Identification of individual amino acids. The amino acids in each of the nine fractions, described in the previous paragraph, were identified by two-way chromatography on Whatman No. 1 paper using butanol–acetic acid–water (60:15:25 v/v) for the first dimension, and phenol–water (100:25 v/v) for the second. Specific chromogenic reagents were used to confirm the presence of certain amino acids, and chromatograms were run in ethanol–ammonia–water (180:10:10 v/v) and in tert. butanol–methyl ethyl ketone–water (80:40:40 v/v) for additional confirmation.

RESULTS AND DISCUSSION

The results of this investigation are condensed in a series of bar charts (Fig. 1) which show the optical densities of the colored ninhydrin reaction products of each of the eight fractions into which the amino acids were arbitrarily divided by paper chromatography. The ninth fraction, proline, was not examined spectrophotometrically. Appreciable variations in amino acid distribution were observed among the different commercial cocoas examined. The precision of chromatographic and colorimetric methods used was confirmed by running replicate analyses on one of the samples of cocoa beans. Bailey *et al.* (1962) have already drawn attention to the importance of the volatile carbonyl compounds formed by the Strecker degradation of amino acids when heated with reducing sugars, a reaction which they suggested took place in the cocoa bean, and Rohan (1964) has shown that the free amino acids and reducing sugars are important components of the precursors of chocolate aroma. Variations in the distribution of the free amino acids in different varieties of cocoa beans would therefore be expected to result in variations in distribution of the carbonyl compounds formed during roasting, and in variations in the aroma

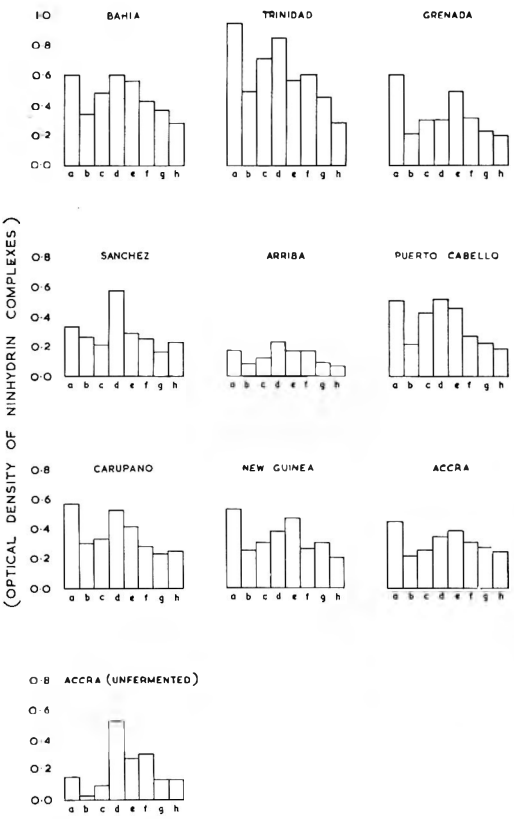

Fig. 1. Amino acid distribution in different commercial varieties of cocoa beans.

of the ultimate products. Thus, differences would be expected not only in the strength of the primary aroma of chocolate, but also in the auxiliary aroma. The application of gas chromatography in another part of this study has already revealed significant differences in the amounts of primary characteristic chocolate aroma produced from equal amounts of the different commercial cocoas used in this investigation (Rohan, unpublished).

The identities of the individual components of the nine fractions into which the free amino acids have been subdivided are shown in Table 1, together with approximations of their relative concentrations based on visual observation of their ninhydrin reaction products on two-way paper chromatograms. The latter results were intended to facilitate interpretation of the more precise semiquantitative results illustrated in Fig. 1, and to permit

e	a	100(100)	100(100)	100(100)	100(100)	4	4	4	2	2	3	3	4	4
ine		98	95			–	4	–	2	2	2	2	2	2
alanine	b	90(87)	98(99)	94(96)	97(97)	4	4	2	2	–	2	2	3	2
	c	80(80)	88(90)	93(95)	86(86)	4	4	3	3	2	3	3	3	3
phane		82(83)	91(90)		90(93)	–	2	2	1	–	1	–	–	–
ine	d	61(61)	85(84)	54(52)	70(70)	4	5	3	5	4	3	4	3	3
ne		70(73)	69(70)	75(75)	83(82)	2	2	2	2	1	2	2	2	2
e		60(55)	107(110)			2	2	2	2	2	2	2	2	2
e	e	58(59)	65(67)	65(65)	73(73)	4	4	4	3	4	4	4	4	4
tified						3	3	2	–	–	2	1	3	2
nine	f	52(52)	57(56)	61(61)	73(73)	3	3	2	–	–	2	1	3	2
nic Acid		51(52)	37(39)	8(8)	58(62)	3	3	3	4	3	3	2	3	4
ic Acid	g	40(42)	24(24)	7(7)	49(53)	2	1	1	3	2	2	2	3	3
		40(42)	40(41)	42(40)	64(60)	2	2	2	2	2	2	2	3	2
e	h	15(19)	15(19)	7(7)	40(46)	2	2	2	2	2	2	2	3	–
tified		23	30	27		–	–	–	–	–	2	1	3	3
gine		32(30)	46(46)	15(17)	51(50)	2	2	2	2	1	2	2	3	2
tified		32	53			–	–	1	–	1	1	2	2	2
ne		28(22)	76(76)	42(44)	57(57)	3	3	1	1	–	2	1	2	2

Fig. 1.
anol–acetic acid–water, 60 :15 :25 v/v.

1, weak; 2, moderate; 3, strong; 4, very strong; 5, inten
B, Bahia; T, Trinidad; G, Grenada; S, Sanchez;

a breakdown of the nine groups into individual acids. The influence of fermentation on the free amino acid concentration has already been established by De Witt (1957), and the effect on the free amino acid distribution of variations in fermenting procedure in different countries must therefore not be overlooked. This effect was amply demonstrated in the two samples of Accra cocoa beans, fermented and unfermented. The unfermented sample was characterized by a relatively large proportion of fraction *D* (Fig. 1). The only other cocoa which showed a large excess of this fraction was Sanchez, which was judged, on the evidence of the large proportion of "slaty" (unfermented) beans in the sample, to have been "underfermented." Arriba cocoa, which normally receives hardly any fermentation (Lipscomb, 1949), had a very low total concentration of free amino acids, of which group *D* was, again, relatively large. All samples, apart from Sanchez and Arriba, seemed to be well fermented; nevertheless the amino acid distribution varied considerably. These variations could be reflected in the composition of the carbonyl material produced as a result of roasting, and thus in the characteristic differences in over-all aroma and flavor which exist in the final products. The results therefore permit the tentative hypothesis that amino acid distribution is a contributory factor in flavor and aroma differences in commercial cocoas, and they also justify a more detailed investigation.

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The Effect of Boron on the Lipid Content and Discoloration of Potatoes

SUMMARY

Ontario, Katahdin, and Pontiac potatoes grown with and without boron foliar spray were examined for discoloration and lipid content. The lipid was fractionated into free fatty acids, neutral fat, and phospholipids. The free fatty acids from each of the three fractions were esterified and determined quantitatively by gas chromatography. Discoloration was measured with a Hunter color-difference meter.

The total lipid of all three varieties was higher in potatoes treated with boron than in the control potatoes. Cortex tissue was significantly higher in lipid content that center tissue. Pontiac potatoes, the variety most resistant to discoloration, had the highest lipid content. The phospholipid fraction of all three varieties was higher in the potatoes treated with boron, and the neutral-fat fraction was higher in Pontiac potatoes treated with boron than in untreated potatoes.

Treatment of potatoes with boron tended to increase the amount of unsaturated and decrease the amount of saturated fatty acids. The free fatty acid fractions of potatoes receiving boron treatment were lower in palmitic and higher in linoleic acid than the control, the neutral fat fraction was higher in linolenic acid, and the phospholipid fraction was lower in palmitic acid. Potatoes receiving boron discolored less than control potatoes.

INTRODUCTION

Although much work has been done on the essentiality of boron for plant life, there is still some controversy as to its function.

Gauch and Dugger (1953, 1954) suggested that boron facilitates the movement of sugar in the plant by forming a more freely permeable boron-sugar complex or by joining with the cell membrane in a way that makes it more permeable to sugar. Dugger *et al.* (1957) found that boron affected the starch-sugar balance of bean leaf discs. These same workers, using potato starch phosphorylase, showed that boron inhibited the reaction

glucose-1-phosphate ⟶ starch + inorganic phosphate and concluded that boron affects the rate of translocation of sugar through the effect on the sugar-starch balance of the leaf.

Odhnoff (1957) and McIlrath and Skok (1958) proposed that boron exerts an influence on cell development and concluded that this is brought about by the effect of boron on polysaccharide synthesis. The first pathologic effects of boron deficiency are physiological and tend to speed up cell division and growth. Cell wall formation and cell differentiation are concurrently interrupted. Cell walls remain thin, and parenchyma tissue increases at the expense of conductive tissue.

Spurr (1957), in studying the effect of boron on celery, showed that boron had a pronounced effect on cell-wall thickness. He found that under boron deficiency most of the collenchyma cell walls were thinner, but other tissue in the same sections showed an opposite response, for the cell walls of phloem parenchyma and ground parenchyma became thicker.

Fatty compounds such as cutin, suberin, and waxes occur in varying amounts in the walls of many types of cells, especially those located on the periphery (Street, 1963). The surface of contact between the cytoplasm and the central vacuole appears from staining reactions to be relatively rich in lipid material. The fatty material at the vacuolar-cytoplasmic interface appears to be phospholipid. Many solutes can diffuse into cells, but substances with a high solubility in lipids penetrate most rapidly. Mitochondria, living components of the protoplast, are seen

by the electron microscope to be enclosed by an outer membrane and within this an inner membrane which is infolded, and the phospholipid content seems to be concentrated in these membranes. Plastids, like mitochondria, are rich in lipoprotein. Since lipids are a vital part of the plant cell, it seemed desirable to study the effect of boron on the lipid content of potatoes.

Sawyer and Collins (1960) found that potato varieties differ in firmness and black spot susceptibility because of inherent differences in lenticel and periderm structure. The lenticel opening of Pontiac potatoes, a variety resistant to darkening, varied from 30 to 60 μ in diameter, and the lenticel opening of the Ontario, a variety susceptible to darkening, was approximately 200 μ in diameter. Periderm is frequently associated with suberized primary cortical cells, and the principal function of the periderm is protection from drying-out. Cork layers, when of considerable thickness, may afford a certain amount of protection against mechanical injury to the tissue beneath. The concentration of lipids is known to be highest in the periderm, and the Pontiac variety, which is known to be resistant to bruising, was shown to have the thickest periderm. Bruising of the tissue accelerates the darkening reaction. Susceptibility to bruising may be due to abnormal cell development and changes in the chemical structure of the cell wall or cellular membranes. Possibly, a weakening of the cellular membranes may cause oxidative enzymes to be brought in contact with substrate more easily than in normal cells and thus accelerate the darkening reaction.

Previous work in this laboratory, Bond (1961) showed that the application of boron foliar spray to Ontario potatoes decreased their tendency to exhibit black spot. Other work in this laboratory (Mondy *et al.*, 1963) has shown that two varieties of potatoes, differing widely in their susceptibility to discoloration, also differ in their fatty acid composition. It therefore seemed desirable to study the relationship of boron to lipid content and to the tendency of potatoes to discolor.

EXPERIMENTAL

Three varieties of potatoes (Ontario, Pontiac, and Katahdin) were grown at the Cornell Vegetable Research Farm at Riverhead, Long Island. Each variety was divided into two plots, the control and a plot to which boron foliar spray was applied at the level of 3 lb per acre at 10 weeks and again at 13 weeks after planting. Potatoes were harvested 20 weeks after planting, and crude lipid was determined in the potatoes 3 weeks after harvest.

The potatoes were divided into cortex (including the peel) and pith sections. The sections were frozen, lyophilized in a Stokes freeze-dryer, and ground in a Wiley mill through a 40-mesh screen.

Total crude lipid. The method of Lee and Mattick (1961) for the extraction of lipids in peas was adapted for use with potatoes. A 40-g sample of dehydrated potatoes was combined with a solvent consisting of chloroform and methanol (2:1) and stirred for 3 hr under nitrogen with a magnetic stirrer. The solution was then filtered through sintered glass and the residue taken up in fresh solvent and stirred again for 1½ hours and filtered. The filtrates from the two extractions were then combined. Ten ml of solvent per gram of sample was used for the first extraction, and 5 ml per gram for the second.

The filtrate was freed of water-soluble impurities by a modification of the method of Folch *et al.* (1957). A solution of 0.034% magnesium chloride was added to the filtrate so that the ratio of chloroform, methanol, and magnesium chloride was 8:4:3 (v/v). The solutions were shaken together and stored under nitrogen overnight in a freezer. The following day the upper phase was drawn off by suction and discarded, and the interface was washed twice with small amounts of "pure solvents" upper phase. This was obtained by shaking together chloroform, methanol, and magnesium chloride in the proportions 8:4:3 (v/v) and collecting the upper phase.

The solvent was removed in a rotary evaporator, and the samples were stored under vacuum over phosphorus pentoxide until they reached constant weight.

Lipid fractionation. Crude lipid was separated into free fatty acid, neutral fat, and phospholipids by a modification of the method of Wagenknecht (1957) for the fractionation of lipids in peas. The scheme for the fractionation is shown in Fig. 1.

Phospholipid fraction. Crude lipid was extracted with acetone under nitrogen for 1½ hr and the supernatant decanted and filtered. The residue was extracted with 10 additional portions of acetone, and the acetone-soluble material was pooled for free fatty acid and neutral fat analyses.

The acetone-insoluble fraction containing the

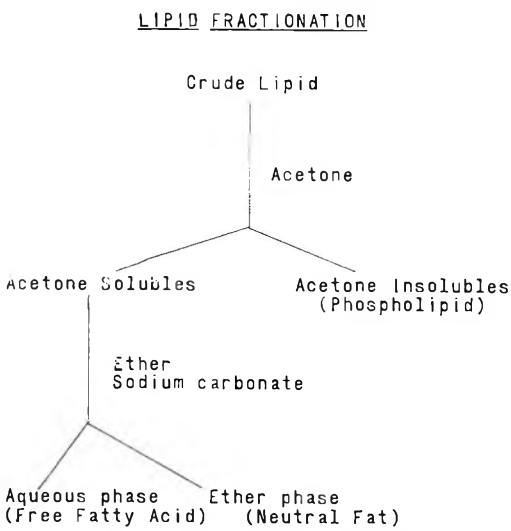

Fig. 1. Flow diagram for lipid fractionation.

phospholipids was dissolved in a methanol-chloroform mixture, filtered, evaporated, and dried over phosphorus pentoxide. The sample was refluxed with hydrochloric acid for 20 hr, water was added, and the sample was transferred to a separatory funnel and shaken with ether. Sodium carbonate solution was added and the aqueous phase drawn off and acidified with sulfuric acid. The residue was extracted with ether three times, and the ether extracts pooled. Anhydrous sodium sulfate was added, and the extracts were evaporated and dried over phosphorus pentoxide.

Free fatty acids and neutral fats. The acetone solubles were evaporated, extracted with ether, sodium carbonate, ethyl alcohol and water added, and the ether phase saved for the neutral fat determinations.

The aqueous phase which contained the free fatty acids was acidified with sulfuric acid and the free fatty acids extracted with ether and dried to constant weight according to the method described by Mattick and Lee (1959).

The ether phase containing the neutral fat was evaporated to constant weight, transferred to a reflux flask, and refluxed with potassium hydroxide for 3 hr. The sample was then extracted with ether and acidified with sulfuric acid, and the free fatty acids extracted with ether and dried to constant weight. The free fatty acids from each of the three fractions were esterified according to the method of Roper and Ma (1957) as modified by Vorbeck *et al.* (1961).

A Barber-Colman gas chromatograph, model 10, employing a β-ray ionization detection cell containing 56 μc of radium-226, was used to separate the methyl esters of the fatty acids. The stationary phases used were apiezon L, a nonpolar saturated paraffin hydrocarbon, and diethylene glycol succinate, a polar polyester of succinic acid. The preparation of the columns and the operating parameters for the instrument have been described in detail by Vorbeck *et al.* (1961). The techniques described by James (1959) and Hawke *et al.* (1959), using the relative retention volume on a polar and nonpolar stationary phase and establishing a grid using known acids, were employed to identify the fatty acids. The acids were quantitated by peak-area integration.

Determination of potato discoloration. Color measurements were made on 200 g of ground potato tissue with a Hunter color-difference meter. The standard gray tile used for standardization of the instrument had the value: *Rd* 39.0, *a* —1.1, and *b* —3.3. Only *Rd* values are reported here, since these were most closely associated with potato discoloration as observed visually.

Potatoes were sampled by cutting longitudinally from bud to stem end. The cortex section was separated from the center or pith. Approximately 200 g of tissue were ground in a food chopper and allowed to stand in air for 20 min before readings were taken. Approximately 4 tubers were sampled for each determination, and 4 determinations were made on each variety of potato.

RESULTS AND DISCUSSION

Total lipids. A comparison of the amount of total lipid extracted from control potatoes and those treated with boron is given in Fig. 2. In each of the varieties studied (Pontiac, Ontario, and Katahdin) the amount of total lipid was higher in potatoes treated with boron. These differences were highly significant. The cortex was considerably higher than the center tissue in

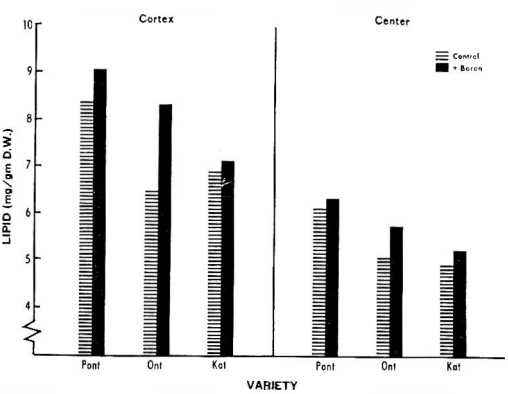

Fig. 2. The effect of boron on the total lipid content of three varieties of potatoes (Pont., Pontiac; Ont., Ontario; Kat., Katahdin).

lipid content in all the varieties studied. Pontiac potatoes, the variety most resistant to discoloration, had the highest lipid content.

Lipid fractions. A comparison of the lipid fractions from the three varieties of potatoes (Fig. 3) showed that the Pontiac variety was highest in free fatty acids and in phospholipids in both the control and boron-treated potatoes. In all three varieties the potatoes treated with boron were higher in phospholipid fraction than the control potatoes. These differences were highly significant. The neutral fat fraction in the Pontiac variety was higher in the boron-treated potatoes. In the Pontiac and Katahdin varieties the free fatty acid fraction was lower in the potatoes receiving boron treatment.

The results obtained in this study indicate that boron may be involved in some way in lipid synthesis in the potato, particularly in the synthesis of phospholipids. Gauch and Dugger (1953, 1954) suggested that boron facilitates the movement of sugar in the plant by forming a more freely permeable boron-sugar complex or by joining with the

Table 1. The effect of boron on three fractions of the cortex tissue of three varieties of potatoes.[a]

	Pontiac		Ontario		Katahdin	
Acids[b]	Control	Boron	Control	Boron	Control	Boron
Free fatty acid						
16:0	21.5	17.0	25.7	16.8	23.7	18.0
16:1	—	2.1	1.7	1.1	1.3	—
18:0	2.0	2.0	4.4	2.9	3.2	2.7
18:1	1.5	1.4	2.6	—	2.0	1.2
18:2	40.9	25.2	42.4	42.5	39.2	43.9
18:3	29.2	40.4	20.1	32.5	25.3	30.2
19:1	—	6.2	—	—	1.9	—
19:2	—	4.6	—	—	—	—
20:0	—	—	—	1.4	1.8	1.2
Neutral fat						
16:0	25.7	29.3	35.9	25.1	25.7	37.1
16:1	—	1.0	2.8	—	9.6	1.7
18.0	2.3	2.7	6.0	5.7	4.6	4.7
18:1	1.2	1.3	2.7	2.5	3.7	1.9
18:2	45.0	42.1	26.7	31.3	21.9	31.8
18:3	18.9	22.3	10.6	23.5	9.6	16.1
19:1	1.6	—	5.2	3.4	13.4	1.5
19:2	1.4	—	—	—	—	—
20:0	1.5	—	5.1	4.8	7.7	1.7
Phospholipid						
16:0	30.1	16.2	35.9	27.9	27.9	33.3
16:1	—	—	2.8	—	—	—
18:0	2.4	3.5	6.0	3.3	4.3	4.6
18:1	—	—	2.7	—	1.2	1.2
18:2	50.7	46.1	26.7	56.3	45.3	44.2
18:3	14.0	29.8	10.6	10.4	17.6	13.9
19:1	—	1.6	5.2	—	—	—
19:2	—	—	—	—	—	—
20:0	1.0	—	5.1	1.3	2.8	1.9

[a] Values expressed as mole percent based on peak-area integration. Anything less than 1% was not included.

[b] The first number refers to the number of carbon atoms in the fatty acid, the second number to the number of double bonds.

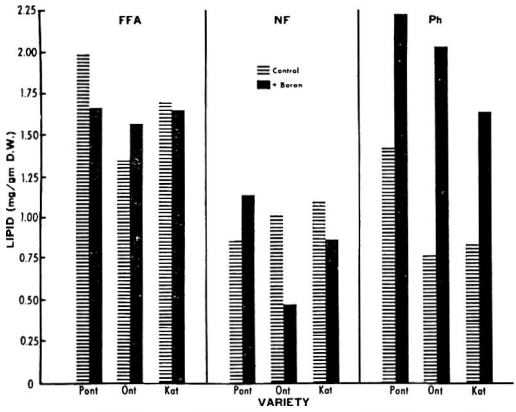

Fig. 3. The effect of boron on the lipid fractions of potato cortex tissue. (FFA, free fatty acids; NF, neutral fat; Ph, phospholipid. Pont., Pontiac; Ont., Ontario; Kat., Katahdin).

cell membrane in a way that makes it more permeable to sugar. It has been shown previously that cell wall formation and cell differentiation are concurrently interrupted in boron deficiency. Fatty compounds occur in varying amounts in the walls of many types of cells. The lipid components of the cell walls may function in some way in the movement of sugar in the plant and may eventually be involved in the final storage products which influence the specific gravity of the potato. Previous work in this laboratory (Bond, 1961) showed that the specific gravity of potatoes was decreased by the addition of boron foliar spray.

Free fatty acids. The influence of boron foliar spray on the free fatty acid fraction of potatoes is given in Table 1. In each of the three varieties studied, the application of boron decreased the amount of palmitic and increased the amount of linolenic acid present in the free fatty acid fraction. In the Ontario and Katahdin varieties boron treatment decreased the stearic acid and increased the linoleic acid content although this trend was not consistent in the Pontiac variety. In general, boron tended to increase the amount of unsaturated and decrease the amount of saturated fatty acids in the free fatty acid fraction of potatoes.

Neutral fat. The effect of boron on the neutral fat fraction is also given in Table 1. Linolenic acid increased in all three varieties when boron was applied. No consistent effect of boron on the other fatty acids of the neutral fat fraction was observed.

Phospholipids. The fatty acid composition of the phospholipid fraction of potatoes is also given in Table 1. Palmitic acid decreased in Pontiac and Ontario potatoes when boron was applied, and a marked increase in linoleic acid occurred in the Ontario variety. No consistent trend was observed in the other fatty acids of the phospholipid fraction.

Discoloration. The influence of boron foliar spray on potato discoloration is given in Table 2. Potatoes treated with boron showed less tendency to discolor than the control potatoes. These results confirmed earlier findings in this laboratory (Bond, 1961). The effect of boron foliar spray on the discoloration of Ontario potatoes is shown in Fig. 4. Scudder (1951) and Massey (1952) found a positive correlation between black spot and specific gravity. Bond (1961) found that, within a given variety,

Table 2. Effect of boron on potato discoloration.[a]

Variety	*Rd*[b]	
	Control	Boron-treated
Pontiac	20.6	23.1
Ontario	15.9	17.3
Katahdin	15.2	16.1

[a] Cortex tissue was used since this section of the potato that exhibits the greatest degree of discoloration.

[b] *Rd* values decrease as blackening increases. Each value is the mean of 4 determinations on 16 potatoes.

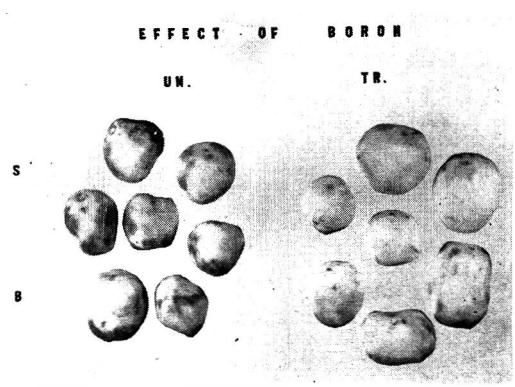

Fig. 4. Ontario potatoes treated with boron foliar spray (S, stem end; B, bud end; UN, untreated; TR, treated).

the specific gravity was lower in potatoes treated with boron. The effect of boron on potato discoloration may be due to its effect on the lipid content or other components which may affect the specific gravity of the potato.

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Structure of the Carotenoid Neoxanthin

SUMMARY

The products formed on reaction of neoxanthin with hydrochloric acid in methanol and in acetone indicate that neoxanthin is 3,3′,5′-trihydroxy-5′6′-dihydro-5,6-epoxy-beta-carotene. The tertiary 5′-hydroxyl group reacts in a similar manner to the allylic 3′-hydroxyl group in lutein.

The major carotenoid constituents of green leaves are beta-carotene, lutein, violaxanthin, and neoxanthin. The structure of neoxanthin has not been completely elucidated. It was discovered by Strain (1938), who assigned to it the formula $C_{40}H_{56}O_4$. The spectral absorption curve was similar to that of violaxanthin, but the maxima were several mμ shorter in wave length. Strain (1938) reported that neoxanthin gave a deep blue color in the hydrochloric acid-ether test, but Strain *et al.* (1944) later stated that neoxanthin did not give a blue color.

Curl and Bailey (1957) separated the carotenoid polyol fraction of leaves by countercurrent distribution, and from it obtained two neoxanthin-like bands, which were termed neoxanthins *a* and *b*. The former was eluted first from a magnesia column (Westvaco 2642 plus Celite) and was considerably greater in amount. Both of these bands gave a pale-blue color in the hydrochloric acid-ether test, indicating the probable presence of one epoxide group. On treatment with iodine in light, the wave length of the spectral absorption maxima of both substances increased 2–3 mμ, indicating these compounds to be *cis*-isomers. Violaxanthin (3,3′-dihydroxy-5,6,5′,6′-diepoxy-beta-carotene), which was found in a less polar fraction than neoxanthin on countercurrent distribution, gave a very deep blue color in the hydrochloric acid-ether test. When violaxanthin was treated with iodine in light the spectral absorption maxima decreased slightly in wave length. The resulting product had spectral absorption maxima almost identical with those obtained from the neoxanthins, indicating that the latter had the same conjugated double-bond system as violaxanthin, which has 9 conjugated double bonds, all in the central chain. Curl (1960b) reported the isolation of the all-trans isomer of neoxanthin from cling peaches; it has not been obtained from leaves.

On treatment with hydrochloric acid in methanol, neoxanthin *a* was converted to a pair of substances with spectral absorption maxima *ca.* 18 mμ shorter in wave length (5,8-epoxide isomers) (Fig. 1), confirming

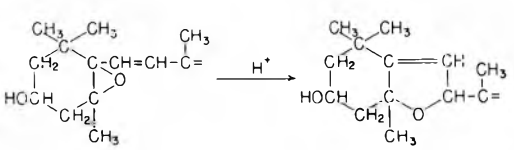

Fig. 1. Conversion of carotenoid 5,6-epoxide to 5,8-epoxide, as in neoxanthin to neochrome.

neoxanthin *a* as a 5,6-monoepoxide (Curl and Bailey, 1957); the names neochrome *a* and neochrome *b* were proposed for these 5,8-epoxide isomers (Curl, 1960a).

Goldsmith and Krinsky (1960) reported on the epoxide nature of neoxanthin and suggested that neoxanthin was 3,3′,5′-(or 6′) trihydroxy-5′,6′-dihydro-5,6-epoxy-beta-carotene. Krinsky (1963) stated that neoxanthin contained only nonallylic hydroxyl groups as evidenced by the lack of change in the partition coefficient on treatment with acidic methanol. Petracek and Zechmeister (1956) showed that in carotenoids acidic methanol readily converts allylic hydroxyl groups to methyl ethers.

Some years ago the author treated neoxanthin with hydrochloric acid in methanol and found that it was readily converted into a complex mixture of substances, most of which were less polar than neoxanthin; more recently, the action of hydrochloric acid in acetone was investigated. These studies indicate that the 3,3′,5′-trihydroxy structure

proposed by Goldsmith and Krinsky (1960) is correct; apparently a hydroxyl group on a tertiary carbon (5′) is quite reactive and behaves in a similar manner to an allylic hydroxyl.

EXPERIMENTAL

The neoxanthin and neochrome preparations used in this work were obtained from elm and apricot leaves as previously described (Curl and Bailey, 1957). The all-trans neoxanthin was obtained from cling peaches (Curl, 1960b).

Acid treatment. Carotenoid solutions were evaporated *in vacuo* and the residues dissolved in: a) a mixture of 9 ml methanol and 1 ml concentrated hydrochloric acid; b) 10 ml of acetone to which 1 ml of concentrated hydrochloric acid was then added; or c) a mixture of 15 ml each of ethyl ether and methanol, to which 2 ml of concentrated hydrochloric acid was then added. After standing at room temperature for the specified time, the solutions were neutralized by potassium hydroxide in methanol diluted with water and ether, and the carotenoids recovered as previously described (Curl and Bailey, 1954).

Countercurrent distribution. Runs were carried out as previously described (Curl, 1953, 1960b) using three solvent systems: I, hexane–99% methanol, 1.8:1 by volume; II, hexane–benzene–87% methanol, 1:1:1.15 by volume; III, hexane–acetone–13.5% methanol, 5:4:3 by volume.

Chromatography was carried out on columns of magnesia (Westvaco 2642) plus Celite (1:1 by volume), using solutions of ethanol in hexane as eluants (Curl, 1959). Spectral absorption data were obtained with a Cary recording spectrophotometer.

RESULTS AND DISCUSSION

Neoxanthin is known to contain 4 oxygen atoms, one of which occurs as a 5,6-epoxide. Violaxanthin also has 4 oxygen atoms, two of which are in hydroxyl groups and two in 5,6-epoxide groups (Karrer and Jucker, 1945). Neoxanthin on countercurrent distribution is considerably more polar than violaxanthin, which indicates that all three of the nonepoxide oxygen atoms are in hydroxyl groups. In plant carotenoids the hydroxyl group is not known to occur on any of the methyl groups, nor on any of the CH groups in the central chain. When one or two hydroxyl groups are present, they occur on the 3 and 3′ carbon atoms; it is assumed here that 2 of the 3 hydroxyl groups in neoxanthin are in these positions. Supporting evidence for this is presented below. The remaining hydroxyl group could be on the 2 or 4 carbons of the ring containing the epoxide group, or on the 2′,4′,5′, or 6′ carbons of the other ring. It is shown below that neoxanthin is quite reactive toward hydrochloric acid in methanol, indicating that it contains either an allylic hydroxyl group, or another very reactive group such as a tertiary alcohol. The products formed preclude the third hydroxyl group's being on the 2 or 4 carbons of the ring containing the epoxide group.

Four possible positions of the third hydroxyl group are shown in Fig. 2. It is

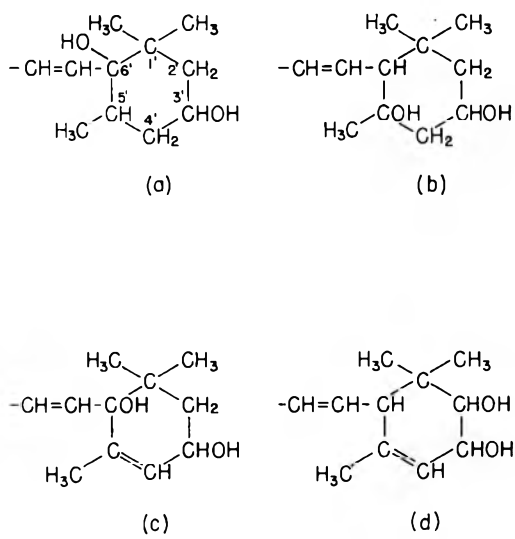

Fig. 2. Possible structures of the non-epoxide ring of neoxanthin.

uncertain whether neoxanthin contains 56 or 58 hydrogen atoms; Goldsmith and Krinsky (1960) showed that 58 is quite possible. If 58 is the correct number, structures *a* and *b* are possible; if 56, *c* and *d*; all of these possibilities are considered here. The third hydroxyl on either carbons 2′ or 4′ of the saturated ring would be secondary, and hence not very reactive. Structure *c* is that proposed by Lippert *et al.* (1955) for trollixanthin, a triol monoepoxide from the flowers of *Trollius europaeus*.

The effect of hydrochloric acid in methanol on neoxanthin. Neoxanthin *a* was treated with hydrochloric acid in methanol

for 30 sec and the recovered carotenoids chromatographed. It was anticipated that neoxanthin *a* would convert very rapidly to the isomeric 5,8-epoxide (Fig. 1). Such epoxides are often found as pairs of bands with practically identical spectral absorption maxima. About 85% of the product consisted of neochromes *a* and *b;* three minor bands were eluted from the column much more rapidly than the neochromes. These had spectral absorption maxima close to those of the neochromes and appeared to have fewer hydroxyl groups.

Neochromes *a* and *b* were each treated similarly with hydrochloric acid in methanol for 10 min. On chromatography, 9 bands were obtained from each product, 7 of which were spectrally similar to the neochromes. Five of these, comprising about two-thirds of the total, were eluted from the column more rapidly than the neochromes. The other two minor bands were mutatoxanthin-like; the spectral absorption curves indicated that an additional double bond had formed in the 5′-6′ position (Fig. 4a). These results indicated the presence of an allylic hydroxyl, or other reactive hydroxyl group, in neoxanthin.

Two portions of neoxanthin *a* were treated with hydrochloric acid in methanol for 30 sec and 10 min, respectively. The products were subjected to countercurrent distribution with solvent system II (Fig. 3). In 30 sec about 25% of the carotenoids had been converted to products containing fewer hydroxyl groups, and in 10 min, 97%. Two new fractions were formed in both cases. The major one had an N_{100} value of 48–49, intermediate between the values for monoepoxide diols (55) and diepoxide diols (40); the minor one had a value of 90–91, intermediate between those of monols (96) and diols (69).

The three fractions from the two runs were combined and chromatographed. The products obtained from fractions IV and III are given in Table 1. The polyol fraction (IV) consisted mainly of neochromes *a* and *b*, together with a much smaller amount of a hydroxy-lutein-like substance. The neochromes had practically all disappeared at the end of the 10-min treatment (Fig. 3). 5,6-Epoxides on treatment with hydrochloric acid are converted to a small extent to the corresponding compound with a double bond in the 5,6-position (Karrer, 1945). The hydroxy-lutein-like substances may be derived from neoxanthin by replacement of the 5,6-epoxide group with a double bond.

Table 1. Products formed by the action of hydrochloric acid in methanol on neoxanthin.

Apparent identity	Spectral absorption maxima in benzene (mμ)	Percentage of fraction
Polyol fraction (IV)		
Hydroxy-lutein-like	483,453,430	6
Neochrome *a*	458,431,406	52
Neochrome *b*	456,428,405	42
Diol fraction (III)		
Hydroxy-lutein-like methyl ether[a]	484,456,430	5
Neochrome *a* methyl ether	459,431,406	47
Neochrome *b* methyl ether	458,429,406	28
Mutatoxanthin *a*	465,436,415	9
Mutatoxanthin *b*	464,436,415	10
Monol fraction (II)	459,431,406	—

[a] Or lutein.

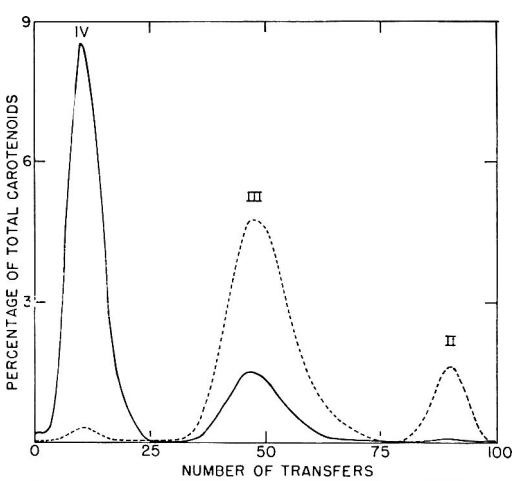

Fig. 3. Countercurrent distribution with solvent system II of neoxanthin *a* treated with hydrochloric acid in methanol: 30 sec ——; 10 min - - - - - -.

The major part of fraction III consisted of two bands with an absorption spectrum similar to that of the neochromes, but the much higher N_{100} value indicates the loss of one hydroxyl group. The increase in fraction III with duration of the acid treatment indicates that a substance or substances reason-

ably stable to acid were formed. It could have a methyl ether group at carbon 6′ (Fig. 2a), 5′ (Fig. 2b), or 3′ (Fig. 2d). In Fig. 2c, however, there are two allylic hydroxyl groups, so further reaction would be expected. These two bands appear to be monomethoxy-mono-5,8-epoxy-diols.

Fraction III also contained two minor bands which were apparently mutatoxanthins *a* and *b*. These could have been formed by the loss of a molecule of water from carbons 5′ and 6′ in either Fig. 2a or 2b with the addition of a double bond to the conjugated system; the formation of a double bond in Fig. 2c or 2d would be considerably more complicated. The mutatoxanthins in solvent system II have N_{100} values of about 55. In Fig. 3, the curves for fraction III are rather asymmetrical, indicating the presence of substances with higher N_{100} values, such as the mutatoxanthins. Also present in fraction III was a minor, lutein-like component which may have been formed from the hydroxy-lutein-like substance in fraction IV by the loss of a molecule of water from carbon 4′ and 5′ in Fig. 2b to form lutein, or which might have a methyl ether group on either carbons 6′ (Fig. 2a) or 5′ (Fig. 2b).

Chromatography of fraction II showed the presence of 3 minor bands which were collected together because of the very small amount present. The spectral absorption maxima of the combined bands were almost the same as those of the neochromes, indicating no change in the conjugated double-bond system. When the recombined fraction was subjected to countercurrent distribution with solvent system I, almost all of the material occurred as a fraction with an N_{100} value of 32. This value is quite close to that of cryptoxanthin-5,6,5′,6′-diepoxide (32–33) or the 3′-methyl ether of lutein-5,8-epoxide (33). Both of the latter have one hydroxyl group and in addition either two epoxide groups or else one epoxide group and one 3′-methyl ether group, which would affect the N_{100} value in almost the same way. The substances in fraction II are apparently formed from the neochromes by dehydration at carbons 4′ and 5′ to form an allylic hydroxyl group like that in lutein, followed by the formation of a methyl ether at carbon 3′. The resulting substances would be the 3′-methyl ether of lutein-5,8-epoxide, which would be expected to occur in several stereoisomeric forms.

The formation of fraction II indicates that the third hydroxyl group in neoxanthin is on carbon 5′ (Fig. 2b). This group could form a methyl ether at carbon 5′, or dehydrate at carbons 5′ and 6′ to form mutatoxanthins, or at 5′ and 4′ to form lutein-5,8-epoxide which would then be methylated at the 3′-hydroxyl to form the methyl ether.

The effect of hydrochloric acid in methanol on all-trans neoxanthin. A substance identified as the all-trans isomer of neoxanthin was isolated from peaches (Curl, 1960b); it had an N_{100} value in solvent system III very close to that of neoxanthin *a* (62 and 61, respectively). This substance was treated with hydrochloric acid in methanol for 10 min as above, and the products subjected to a countercurrent distribution with solvent system II. The distribution curve was quite similar to that obtained with neoxanthin *a* (Fig. 3), except for the presence of a minor maximum at N_{100} of 67 which was probably due to lutein; the curve of neoxanthin *a* had a slight inflection at N_{100} of about 67. The quantitative distribution of the various fractions and their N_{100} values are in quite close agreement (Table 2), confirming the peach carotenoid as the all-trans isomer of neoxanthin.

The effect of hydrochloric acid in acetone on neoxanthin a. Preliminary work

Table 2. Fractions obtained on countercurrent distribution with solvent system II [a] of the products formed by the action for 10 min of hydrochloric acid in methanol on neoxanthin *a* from leaves and on all-trans-neoxanthin from cling peaches.

	Leaf neoxanthin		Peach neoxanthin	
Fraction	N_{100} [b]	% of fraction	N_{100}	% of fraction
Polyols (IV)	11	3	10	4
(III)	49	82	48	79
Lutein	(67) [c]	—	67	3
(II)	91	15	91	14

[a] Benzene–hexane–87% methanol (1:1:1.15 by volume).

[b] Position of maximum on countercurrent distribution for 100 transfers.

[c] Shoulder on distribution curve, % included in fraction III.

on the effect of hydrochloric acid in acetone on lutein indicated that the main reaction was dehydration in the alpha-ionone ring. Neoxanthin *a* was treated with hydrochloric acid in acetone for 12 min, and the product subjected to a countercurrent distribution with solvent system II. The results are summarized in Table 3.

Table 3. Fractionation by countercurrent distribution of the reaction products of neoxanthin *a* with hydrochloric acid in acetone and of neoxanthin *b* with hydrochloric acid in ethyl ether–methanol.

	Neoxanthin a		Neoxanthin b	
Fraction	N_{100}	% of fraction	N_{100}	% of fraction
Polyols (IV)	0.28	1.3	0.8,27	0.7
Monoepoxide diols (III)	56	47.5	54	50.2
(II)	95	51.2	93	49.1

About half of the recovered material occurred as a fraction with N_{100} of 56, and half with an N_{100} of 95; both values are appreciably higher than the N_{100} values of the corresponding fractions obtained with hydrochloric acid in methanol—49 and 91, respectively (Table 2). The polyol fraction (IV) (in which neoxanthin occurs) had practically disappeared.

The N_{100} value of fraction III corresponded closely to that of the monoepoxide diol fraction found in many fruits. On chromatography, about 95% of the recovered material consisted of two bands which appeared to be mutatoxanthins (Fig. 4a), the 5,8-epoxide isomers of antheraxanthin (zeaxanthin-5,6-epoxide). This supports the assumption that two of the three hydroxyl groups in neoxanthin are on the 3- and 3′-carbon atoms; if they were on other carbons, such as 4 and 4′, the N_{100} value would be quite different. Similar products were obtained in the treatment of neoxanthin *a* with hydrochloric acid in methanol, but to a much smaller extent. In the hydrochloric acid-acetone mixture, dehydration in the 5′-6′-position was a major reaction, compatible with either Fig. 2a or 2b, but not 2c or 2d. Since the 3′-hydroxyl in the mutatoxanthins is not allylic, or tertiary, little further reaction would be expected, if any.

Fraction II was more complicated and contained at least 5 bands. The major band (41%) had spectral absorption maxima in hexane of 446, 419, and 395 mμ, indicating the presence of the same conjugated double-bond system as the neochromes. The N_{100} value of this band in solvent system 1 was 42, whereas that of the 3′-methyl ether of lutein-5,8-epoxide was 33, and 3′-methyl ether of lutein was 38, and the main dehydration product of lutein with hydrochloric acid in acetone was 44. The product in the present work apparently differed from the last by having a 5,8-epoxide group, and was formed from neochrome by the loss of two molecules of water on carbons 4′-5′ and 2′-3′. Two minor bands amounting to a total of 23% of fraction II had similar absorption maxima, but the N_{100} value of the combined bands in system I was 34, close to that of a monol diepoxide. This pair of substances had the same conjugated double-bond system as the major band, but were more polar. Another pair of minor bands had maxima in hexane at 449, 423, and 401mμ, and appeared to have the same conjugated double-bond system as the mutatoxanthins; these may have been formed from the mutatoxanthins by dehydration, perhaps in the 2′-3′-position. It might be expected that the secondary hydroxyl groups would undergo dehydration, but to a much smaller degree than tertiary or allylic hydroxyl groups.

It appears that in fraction II, dehydration of the neochromes had taken place at carbons 4′–5′ to form lutein–5,8-epoxide (Fig. 4b), the hydroxyl on carbon 3′ becoming allylic. This allylic hydroxyl then underwent further

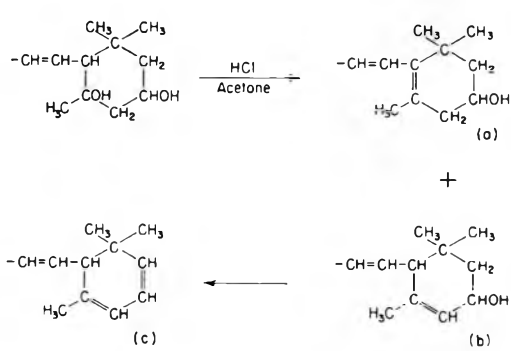

Fig. 4. Dehydration of neochrome to mutatoxanthin (a) plus lutein-5,8-epoxide (b), and further dehydration of the latter (c).

dehydration (Fig. 4c) or other reaction to eliminate the 3′-hydroxyl group. The loss of water from carbons 5′-6′ and 5′-4′ apparently occurred at about the same rate.

The effect of hydrochloric acid in ether-methanol on neoxanthin b. In another connection, neoxanthin *b* was treated with hydrochloric acid in ethyl ether-methanol in an almost completely filled bottle in the dark at room temperature for 25 hr. The product was subjected to countercurrent distribution with solvent system II. The results (Table 3) agreed closely with those of the reaction of hydrochloric acid in acetone on neoxanthin *a*. The products obtained on chromatography were also very similar, qualitatively and quantitatively. The results of this experiment indicate that neoxanthins *a* and *b* are very similar chemically, probably being stereoisomers. They may have a cis-configuration on different double-bonds in the central chain, or they may differ in the configuration of the hydroxyl group on carbon 5′.

The complete structural formula of neoxanthin given in Fig. 5 is in best accord

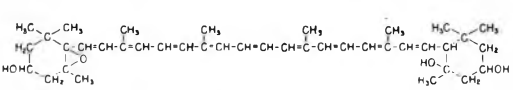

Fig. 5. Tentative structure of neoxanthin.

with the experimental data reported here. From the proposed structure it appears that neoxanthin may be formed from violaxanthin *in vivo* by the addition of two hydrogen atoms to one of the 5,6-epoxide rings.

Of the numerous naturally occurring carotenoid polyols which have been reported, a definite structure has been assigned only to trollixanthin (Lippert *et al.*, 1955). The structure differs from Fig. 5 in that the configuration of the right ring is that in Fig. 2c. The data presented here indicate that neoxanthin does not have the structure in Fig. 2c. Another trihydroxy carotenoid 5,6-epoxide is taraxanthin (Eugster and Karrer, 1957), which has been reported in various plants, especially in flowers, mainly as a minor constituent. It is possible that taraxanthin and trans-neoxanthin are identical.

Similar studies to the above were carried out on the non-epoxide polyols trolleins *a* and *b* and on two other 5,6-epoxide polyols obtained from peaches and oranges (Curl, 1960b). In all cases no hydroxyl groups reactive to acid methanol were found. It appears, then, that in these substances the third (and fourth?) hydroxyl group was not present on carbons 5′ and 6′ but may have been on carbons 4 or 4′ (or 2 or 2′?).

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Spectral absorption data were obtained by Glen F. Bailey.

Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

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Effect of Storage at Ambient Temperature on the Volatile Components of Irradiated Beef

SUMMARY

Volatile components of beef irradiated at 5 megarad by concurrent radiation-distillation, by radiation just prior to distillation, and by radiation and storage at ambient temperature for 6 months before distillation, were isolated and characterized. The total yield of odor isolate from stored beef was approximately 4.4 mg per lb (about 10 ppm), or about one-third of the yield obtained from freshly irradiated beef or from unirradiated beef. The *n*-alkanals and methional, major volatile components of freshly irradiated beef, are minor components of stored irradiated beef. *n*-Alkanols are present in relatively greater amount in odor concentrates of stored beef. *n*-Alkanes and 1-alkenes are volatile components of both stored and freshly irradiated beef, but are present in much smaller quantity in the stored product.

INTRODUCTION

A problem associated with radiation-sterilization of beef is the production of an unpleasant flavor and odor. Though progress has been made in isolation and identification of the volatile components of raw and enzyme-inactivated irradiated beef (Burks *et al.*, 1959; Merritt *et al.*, 1959; Stahl, 1957; Batzer and Doty, 1955; Wick, 1963; Wick *et al.*, 1961a, 1961b; Batzer *et al.*, 1962), the relationship of these components to the sensory properties of irradiation-processed beef is essentially unknown. Such a correlation requires accumulation of detailed knowledge of the volatile components of unirradiated as well as irradiated beef. Otherwise, no determination can be made of which components are produced by irradiation (and thus may be responsible for the off-odor) and which components are normally present in beef.

An investigation was therefore made of enzyme-inactivated irradiated beef and of unirradiated beef before and after 6 months of storage at ambient temperature to further characterize and identify the substances responsible for the unpleasant irradiation off-odor.

Alcohols, *n*-alkanals, methional, benzaldehyde, and a series of *n*-alkanes and *n*-1-alkenes have been shown (Wick, 1963) to be components of beef irradiated both prior to and concurrent with removal of the volatiles from aqueous slurries by distillation. This paper describes the characterization of additional volatile components of irradiated beef processed by both concurrent and non-concurrent procedures and of irradiated beef stored six months at ambient temperatures.

EXPERIMENTAL PROCEDURES AND RESULTS

Enzyme inactivation and irradiation. Approximately 15-lb batches of ground raw lean beef were vacuum packed in sardine cans and enzyme-inactivated (Chiambalero *et al.*, 1959) by bringing the beef to an internal temperature of 175°F for 9 min.

Irradiation of canned enzyme-inactivated beef at 5 megarad was carried out in the MIT cobalt-60 source, or concurrent radiation-distillation at 5 megarad was carried out by procedures previously described (Wick *et al.*, 1961a).

Preparation and investigation of beef odor distillates. Aqueous slurries (8 L) of very finely ground lean beef in distilled water (1:3) were circulated through an irradiation chamber beneath the electron tube of a 1-Mev GE Resonant Transformer, to a flash evaporator for the removal of volatile components, and back through the irradiation chamber. In this manner, slurries containing 10–15 lb (4.5–6.8 kg) of beef were irradiated at 5-megarad doses, and at almost the same time volatile components were distilled from the slurry at pressures of about 25 mm Hg. The average temperature of the circulating mixture was 32–36°C, and the rate of distillation was approximately 6 liters per hour. When radiation was carried out prior to distillation, cans of enzyme-inactivated beef were subjected to 5 megarad in the MIT cobalt-60

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Table 1. Carbon content of distillates and yield of odor concentrates.

Preparation	Quantity (kg)	Mg C/kg	Total mg odor concentrate	Yield (ppm)
A. 1. Unirradiated	6.8	9.0	170.8	25.1
2. Unirradiated	5.0	32.4	182	36.4
				Average 30.8 ± 7.9
B. Radiation prior to distillation				
1. Irradiated	6.8	10.6	204	30.0
2. Irradiated	4.1	31.0	213.1	52.0
3. Irradiated	6.8	—	266.6	39.2
4. Irradiated	7.0	—	165.9	23.7
Water blank	0.0	—	30	—
				Average 36.2 ± 12.8
C. Stored 6 months				
1. Irradiated	5.4	15.8	53.3	9.9
2. Irradiated	14.1	—	135.8	9.6
				Average 9.7 ± 0.2
D. Concurrent radiation-distillation				
1. Irradiated	5.4	32.2	184	34.1
2. Irradiated	4.1	—	201.3	49.1
3. Irradiated	6.4	—	298.4	46.6
Water blank	0.0	—	7.7	—
				Average 43.3 ± 7.8

source. They were then opened either immediately or after six months of storage at ambient temperature, the beef was slurried, and the distillation carried out as previously described (Wick, 1963).

Unirradiated beef slurries were processed in exactly the same manner as were periodic "blank" distillations of distilled water to allow detection of contaminants or artifacts contributed by the distillation apparatus.

The oxidizable-carbon content of aliquots of the total aqueous distillates (approximately 8 L) was determined (Gertner and Ivecovic, 1954). The distillates were saturated with sodium chloride and extracted with diethyl ether (2.9 volumes of distillate to 1 volume of ether). The ether extracts were dried over anhydrous sodium sulfate and then concentrated by careful distillation to the minimum practical volume (about 1 ml). The yields of the resulting odor concentrates on an ether-free basis were estimated gas chromatographically. Table 1 gives these yields and the oxidizable carbon content of the aqueous distillates from which they were derived.

Informal odor evaluation of the odor concentrates and distillates from freshly irradiated beef confirmed that they exhibited typical irradiation off-odor. The same fractions from six-month-stored irradiated beef were bland in odor. Analogous unirradiated beef fractions had typical bland meatlike odors.

The odor concentrates were stored at 0°C in the dark and in ether solution for 3-4 weeks without deterioration [as judged by gas chromatographic analysis under standard conditions (see below)].

Gas chromatographic separation of odor concentrates and examination of the resulting fractions. The GC apparatus was constructed in this laboratory. The detector was a thermal-conductivity cell employing a matched pair of 100,000-ohm thermistors (type A-177, Victory Engineering Corp., Union, N. J.). It was operated at 12 ma. bridge current and maintained at 202°C.

Preparative separations of 40- or 50-μl samples were obtained on a 2-meter 4-mm-ID stainless-steel column packed with 20% Carbowax 20M on Chromosorb P (60–65-mesh). The column was programmed from approximately 70 to 196°C at a rate of 2.4 degrees per min. It was operated at an initial back pressure of 7.8 psig and a helium flow of 48 ml per min.

Fractions were trapped at the detector outlet in glass U-tubes chilled in liquid nitrogen. Contents of the tubes were transferred on a vacuum manifold to 4 × 80-mm glass tubes, sealed under nitrogen, and stored in a freezer until infrared spectra could be determined.

Infrared spectra of individual fractions were determined with a Beckman IR-5 spectrophotometer equipped with a 5 × KBr lens-type beam condenser. Fractions were transferred to a type D sodium chloride cavity cell (Connecticut Instrument Co., Wilton, Conn.) of nominal path length 0.05 mm. When quantities permitted, pure liquid as well as carbon tetrachloride solution spectra were obtained. Infrared spectra of selected reference compounds were obtained from samples purified by chromatography.

The purity of all fractions collected was evaluated by separation of the infrared samples on 2-meter 2-mm-ID stainless-steel columns packed with either 5% Ucon 50HB-2000 on 100–120-mesh Gas-Chrom A or 5% *tris* (2-cyanoethoxy) propane on 100–200-mesh Gas-Chrom A. Flame ionization detectors in either a Research Specialties Model 60-1A or in a HyFy Model 500 instrument were used.

DISCUSSION

Table 1 shows that the quantity of beef processed in any given investigation varied from 5 to about 14 kg (11–31 lb). Odor concentrates from unirradiated beef were isolated in yields ranging from 25.1 to 36.4 ppm. Radiation prior to distillation gave odor concentrates in quantities averaging 36.2 ± 12.8 ppm. Odor concentrates from concurrent radiation-distillation averaged 43.3 ± 7.8 ppm in yield. In all cases concentrates from irradiated beef strongly exhibited typical irradiation off-odor while concentrates from unirradiated beef had normal beef odor.

Variations in the yields isolated are believed to result from experimental difficulties inherent in measuring, on the one hand, kilograms and liters of materials and, on the other hand, very small volumes of volatile ether solutions.

A significantly smaller yield of odor concentrate was obtained from irradiated beef that had been stored 6 months at ambient temperature. Only about 10 ppm (roughly one-third of the amount obtainable from freshly irradiated beef) was isolated. In addition, informal sensory evaluation showed that the intensity of irradiation off-odor was significantly less in the stored beef as well as in the concentrates derived from it, than in freshly irradiated beef.

As indicated in Table 1, blank preparations in which only water was processed were carried out along with the concurrent and nonconcurrent procedures. In each case, an ether-soluble concentrate of odorless organic material was isolated and analyzed by the same procedures used with the meat odor concentrates. In this manner the presence and relative importance of artifacts introduced during processing was assessed.

Separation of the odor concentrates was carried out by temperature-programmed gas chromatography on a 20% Carbowax 20M column. Fig. 1 shows typical chromatograms

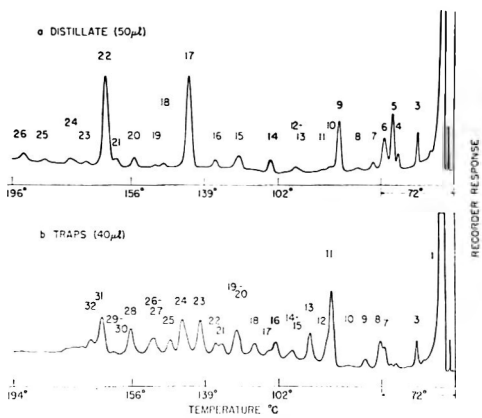

Fig. 1. Temperature-programmed separation of irradiation flavor isolates on a 20% Carbowax 20M column.

obtained from the distillate, i.e., the odor concentrate isolated from the material condensed at 0°C during distillation, and from the traps, i.e., the odor concentrate isolated from the condensate collected at —78°C. The beef had been irradiated at the cobalt-60 source and then distilled. The distribution of components is quite different in each isolate, thus simplifying the separation and isolation of individual components.

The instrument used to obtain these chromatograms and those in Figs. 2–5 was fitted with a katharometer detector and a 6-foot-¼-inch-ID column. If a capillary column and an ionization detector had been used, many additional minor components would

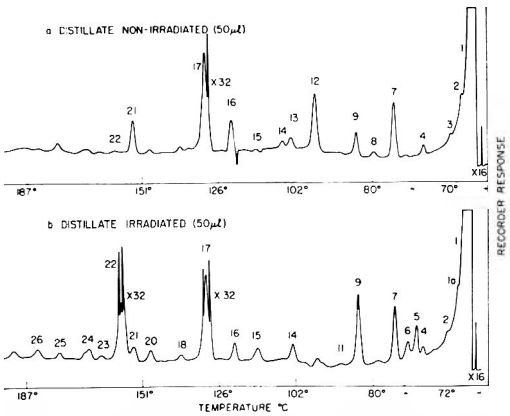

Fig. 2. Temperature-programmed separation of isolates from unirradiated and irradiated beef on a 20% Carbowax 20M column.

undoubtedly have been detected. However, because of the minute concentrations of such minor substances, even if detected, they could not have been identified by infrared spectra determination, the method available in our laboratory.

The chromatograms in Fig. 1 represent components isolated from irradiated beef. Many of them could, however, be components of unirradiated beef which had survived the irradiation process. To determine which substances were products of irradiation (and therefore contributors to irradiation off-odor) unirradiated beef odor concentrates were separated under the same chromatographic conditions. The results obtained with an unirradiated distillate concentrate are compared in Fig. 2 with the analogous irradiated isolate. Some major differences exist, even though at first glance the chromatograms seem quite similar.

The detailed nature of the observed differences was determined by examination of individual fractions. The identifications in Table 2 are based on the identity of infrared spectra and retention data with those of authentic reference compounds.

It is evident that methional is not present in significant quantities in unirradiated beef although it is a major component of irradiated beef. *n*-Pentanol (Fraction 16) and *n*-hexanal (Fraction 12) are present in larger amounts in unirradiated than irradiated beef, but ethyl acetate (Fraction 4), *n*-hexanol (Fraction 18), and phenylacetal-

Table 2. Components of irradiated and unirradiated beef.

Irradiated distillate Fig. 2 (curve *b*)	Unirradiated distillate Fig. 2 (curve *a*)
Fraction	*Fraction*
4. Ethyl acetate	—
5. 2-Butanone	4. 2-Butanone[a]
6. Ethanol	5–6. ?
7. Benzene (artifact?)	7. Benzene (artifact?)
8. *n*-Pentanal	8. *n*-Pentanal[a]
9. 2-Butanol	9. 2-Butanol[a]
12. *n*-Hexanal	12. *n*-Hexanal
14. *n*-Butanol	13. *n*-Butanol
15. *n*-Heptanal, *iso*-Pentanol, unknown carbonyl	14. Unknown 15. *n*-Heptanal
16. *n*-Pentanol	16. *n*-Pentanol
17. Acetoin, *n*-Octanal	17. Acetoin, *n*-Octanal
18. *n*-Hexanol	18. —
20. *n*-Nonanal	20. *n*-Nonanal
21. *n*-Heptanol	21. *n*-Heptanol[a]
22. Methional	22. —
23. ?	23. ?
24. Benzaldehyde, *n*-Octanol	24. Benzaldehyde[a] *n*-Octanol[a]
25. *n*-Undecanal or 2-Undecanone	25. *n*-Undecanal or 2-Undecanone
26. Phenylacetaldehyde	26. —

[a] Identity based on retention data alone.

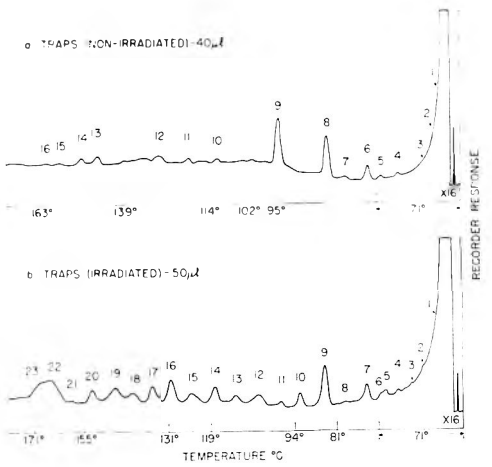

Fig. 3. Temperature-programmed separation of isolates from unirradiated and irradiated beef on a 20% Carbowax 20M column.

dehyde (Fraction 26) appear to be absent.

Fig. 3 compares the distribution of irradiated and unirradiated beef components isolated in the —78°C traps. Since the yield of the unirradiated odor concentrate (curve *a*) was only about 13% (3.2 ppm) of the total yield (25 ppm), the amount of each fraction isolated was very small. The irradiated isolate from the —78°C traps contained numerous additional compounds. The fractions shown in Fig. 3 are identified in Table 3. A series of *n*-alkanes and 1-alkenes was apparently produced by irradiation. On the basis of these results and those in Fig. 2 and Table 2, methional and the hydrocarbons appear to be unique constituents of irradiated beef. The *n*-alkanals and alkanols are components of both unirradiated and irradiated beef.

The effect of storage for 6 months on the volatile components of irradiated beef is shown in Fig. 4. Of greatest significance is the almost complete absence of methional (Fraction 22) in the stored distillate (curve *a*) and the greatly diminished quantity of all components in the chromatogram of the stored traps (curve *b*). Detailed investigation of each fraction in curve *a* showed that fractions which had contained *n*-alkanals as major components in freshly irradiated beef now contained *n*-alkanols primarily, and very small quantities of aldehydes. For example, Fraction 12–13 contained *iso*-butanol and a trace of *n*-hexanal, Fraction 15 contained *iso*-pentanol and a trace of *n*-heptanal, and Fraction 19 contained acetoin and a trace of *n*-octanal. In addition, the quantities of *n*-heptanol (Fraction 21), *n*-hexanol (Fraction 18), *n*-pentanol (Fraction 16), and *n*-butanol (Fraction 14) had increased. These results, when considered in conjunction with the sensory observation that stored irradiated beef exhibited only weak irradiation odor, indicated that methional, hydrocarbons, and *n*-alkanals are important contributors to irradiation off-odor.

Fig. 5 illustrates the effect of concurrent radiation-distillation on the volatile components of enzyme-inactivated beef. The upper

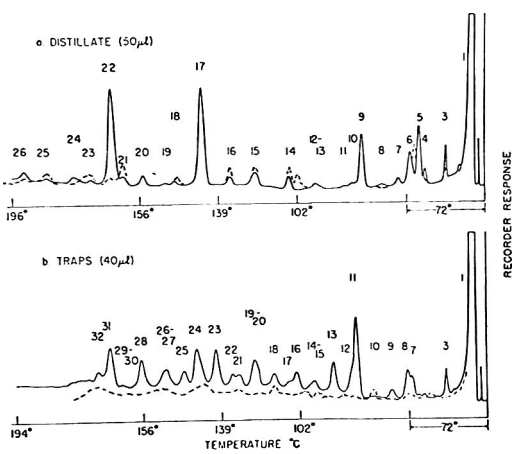

Fig. 4. Temperature-programmed separation of irradiation-flavor isolates on a 20% Carbowax 20M column. Not stored ——; stored - - - - -.

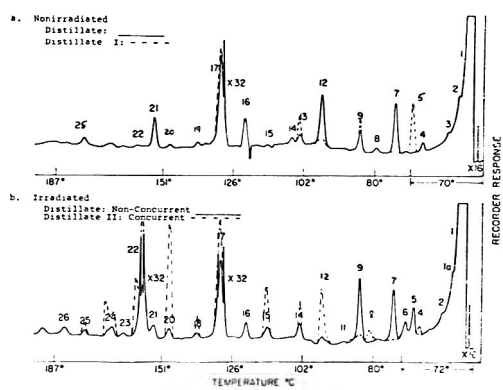

Fig. 5. Temperature-programmed separation of isolates from unirradiated and irradiated beef on a 20% Carbowax 20M column.

Table 3. Components of irradiated and unirradiated beef.

Irradiated traps Fig. 3 (curve *b*)	Unirradiated traps Fig. 3 (curve *a*)
Fraction	*Fraction*
4. 2-Butanone[a]	4. 2-Butanone[a]
5. *n*-Nonane	5–6. Ethanol
6. Ethanol	7. *n*-Pentanal
7. Not detected	8. Unknown (2-Butanol?)
8. Not detected	9. *n*-Hexanal
9. *n*-Decane	10. *n*-Heptanal
10. 1-Decene	11. *n*-Pentanol
11. *n*-Hexanal	12. Acetoin, *n*-Octanol (?)
12. *n*-Undecane	13. *n*-Nonanal
13. 1-Undecene	14. *n*-Heptanol
14. *n*-Heptanal *n*-Pentanol[a]	15. Unknown
15. *n*-Dodecane[a] An unknown	16. Benzaldehyde
16. 1-Dodecene	
17. *n*-Octanal Acetoin	
18. *n*-Hexanol	
19. n-Tridecane[a] 1-Tridecene[a]	
20. *n*-Nonanal	
21. *n*-Heptanol	
22–24. Unknown mixtures	

[a] Identity based on retention data alone.

chromatogram (curve *a*) compares the distribution of components in an unirradiated distillate with those found in Distillate 1, the condensate collected before radiation is started in the concurrent processing procedure. Except for Fraction 5, which is unknown and may be an artifact, the fractions have been identified as those listed in Table 2 as components of unirradiated beef. Similarly, the components isolated from the distillate obtained by the concurrent procedure (curve *b*) were identified as those listed in Table 2. Thus, concurrent radiation-distillation appeared to affect the quantity of, but not the qualitative nature of, irradiated beef components. Comparison of concurrent and nonconcurrent irradiation methods had been of interest since, if components produced by the concurrent method were unique, they might be intermediates in the formation of substances isolated by the other processing procedures. This was not the case. The only difference noted was the larger quantity of saturated aldehydes produced during the concurrent procedure. This quantitative difference in aldehyde concentration apparently had little effect on sensory quality, however, since the intensity and quality of irradiation off-odor of isolates was about the same for both the concurrent and nonconcurrent procedures.

All the compounds identified in this study are listed in Table 4. These substances are the major components of the odor concentrates studied. Additional minor components could undoubtedly be detected if separation were carried out on a capillary column and if mass spectrometric analysis of fractions were accomplished. The relative importance of the compounds listed to typical irradiation off-odor is under current investigation. The fact that irradiation off-odor is easily recognized when all the fractions [except ether (fraction 1)] shown in a chromatogram (Figs. 1–5) of an irradiated odor concentrate are collected together in a single trap, indicates that the substances essential to production of off-odor are isolatable. Future work will determine whether the compounds already identified are responsible, or whether

Table 4. Volatile components of enzyme-inactivated irradiated beef.

Ethyl acetate[b]	*n*-Undecanal (?)
Ethanol[a, b]	Benzaldehyde[a, b]
iso-Propanol (?)[a, b]	Phenylacetaldehyde[b]
n-Butanol[a, b]	Methional[b]
iso-Butanol	Dimethyl sulfide (?)
2-Butanol[a, b]	Acetone
n-Pentanol[a]	*2*-Butanone[a, b]
iso-Pentanol	Benzene[a]
n-Hexanol[b]	*n*-Nonane
n-Heptanol[a, b]	*n*-Decane[b]
n-Octanol	1-Decene[b]
n-Nonanol (?)[b]	*n*-Undecane[b]
Acetoin[a, b]	1-Undecene[b]
Diacetyl (?)	*n*-Dodecane[b]
n-Butanal[b]	1-Dodecene[b]
n-Pentanal (?)	*n*-Tridecane
iso-Pentanal (?)[a, b]	1-Tridecene
n-Hexanal[a, b]	*n*-Tetradecane (?)
n-Heptanal[a, b]	
n-Octanal[a, b]	
n-Nonanal[a, b]	
n-Decanal (?)	

Three unknown carbonyl compounds (1690 cm^{-1}) which may be members of a homologous series

Additional sulfur compound(s)

[a] Also present in unirradiated odor isolates.

[b] Detected in isolates prepared by either concurrent or nonconcurrent irradiation-distillation procedures.

identification of the probable additional minor components will be necessary.

The presence of C_2 to C_9 *n*-alkanes and 1-alkenes in irradiated beef has been previously reported (Merritt, 1961, 1964), and it has been suggested that *n*-hexane, 1-hexene, and dimethyl sulfide are important contributors to irradiation off-odor. These particular compounds were probably present in the odor distillates studied in this investigation but lost during solvent extraction and isolation of odor concentrates. In similar fashion the very volatile mercaptans and sulfides reported by others (Merritt *et al.*, 1959; Batzer and Doty, 1955) are too volatile to have been isolated. The fact that such compounds would not be detected was recognized from the beginning of this work. It was not considered a limitation, because the odor distillates obtained and the odor concentrates isolated from them clearly exhibited typical irradiation off-odor. Subsequent results supported this belief since irradiation off-odor was exhibited when all chromatographic fractions (after ether) from any irradiated odor concentrate were collected together in aqueous solution in a single trap.

The identification of methional [3-(methylthio)-propionaldehyde] as an irradiation flavor component is unique to this work. Its presence had been postulated by others (Witting and Batzer, 1957), but it was not isolated. Its absence in the isolates of Merritt (1961, 1964) and Merritt *et al.* (1959) and its presence in the current isolates may result from differences in irradiation and analytical procedures. There is no doubt that it is an important component of irradiated beef as prepared in this investigation.

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The Ionization Behavior of Some Poly (Meta) Phosphates Used in Food Products

SUMMARY

The degree of ionization of some sodium polyphosphates used in the food industry was determined with sodium ion electrodes. The effect of the presence of the calcium ion on the ionization of these phosphates was also studied. Sodium orthophosphate completely dissociated in solution, with the degree of dissociation of the polyphosphates inversely proportional to the number of P atoms in the chain or the ring. The addition of calcium enhanced the dissociation of the polyphosphate and lowered the pH in each case.

INTRODUCTION

Polyphosphates are being used in various food products for obtaining certain desirable qualities, such as diminishing cooking losses by water expulsion in ham and poultry, obtaining a better consistency in the manufacture of sausages, extracting fruit juices, and preventing gelation of concentrated milk during storage.

Although various explanations have been offered in the literature, the mechanism by which the phosphates bring about the desired effect, especially with respect to meat hydration, has not been established. Hellendoorn (1962) has reviewed the various explanations available and summed them up into two opinions as expressed by Bendall (1954, 1958) and Hamm (1955). The latter explained the effect as due to the complexing of calcium by the various phosphates. However, it should be realized that only very limited information is available on the ionization behavior of these condensed phosphates in solution, and on their cation-complexing ability, in spite of their widespread industrial applications. The present study was undertaken to obtain such information.

In the past, attempts have been made to determine the dissociation of some of the ring and chain polyphosphates. Davies and Monk (1949) and Gross and Cryder (1955) studied the ionization of certain sodium polyphosphates, respectively using conductivity and potentiometric methods, but their results do not seem to be in agreement with each other. Van Wazer and Callis (1958), who have published a number of papers on the chemistry of polyphosphates, consider the results of Davies and Monk (1949) superior to those of Gross and Cryder (1955), although in both cases an indirect method was used.

With the availability of specific ion electrodes, it was decided to study the dissociation of some of the polyphosphates important in the food industry.

The sodium ion electrodes (Beckman) are glass electrodes with sensitive tips that are specific to the sodium ion. The electrode paired with reference electrode (fiber junction) can be used for the direct measurements of sodium ion concentration.

The measurement of sodium ion activity with these electrodes is based on the fact that the potential that is developed between the test sample and the special filling solution inside the body of the electrode is proportional to the sodium ion concentration of the test sample.

An expanded-scale pH meter (Beckman Model 76) was used with the sodium ion electrode.

EXPERIMENTAL METHODS

Sodium hexametaphosphate and sodium tetraphosphate were commercial products generously supplied by Rumford Chemical Works, Rumford, R.I. Sodium tripolyphosphate (food grade) was a gift of Calgon Inc., Pittsburgh, Pa. All other chemicals were reagent grade.

Solutions of five phosphates shown in Table 1 were made to contain .1, .01, and .001*N* sodium, and the actual concentration of the sodium ion was then determined with the sodium ion electrode (Beckman specific ion electrodes, Bulletin 7017). The degree of ionization of the phosphate was calculated from the concentration of sodium

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Table 1. Degree of dissociation of sodium phosphates at different concentration at 25°C.

Electrolyte	Concentration (mole/L)	% dissociation (d)
Sodium orthophosphate (secondary)	.05	100
	.005	100
	.0005	100
Sodium pyrophosphate	.025	62
	.0025	80
	.00025	90
Sodium tripolyphosphate	.020	60
	.0020	78
	.00020	86
Sodium tetraphosphate	.016	48
	.0016	58
	.00016	70
Sodium hexametaphosphate	.016	44
	.0016	52
	.00016	61

ion dissolved in solution and the actual concentration measured by the electrode at the above three concentrations.

The calibration curve for the determination of the sodium ion concentration is shown in Fig. 1, which conforms to the original Beckman calibration curve (Bulletin 7017). The curve is plotted on a semilogarithmic scale.

In the second part of the experiment varying amounts of calcium as calcium chloride were added to the solutions of the intermediate concentration of the different phosphates in Table 1, and the degree of ionization and the pH determined in Table 2.

RESULTS AND DISCUSSION

The degree of dissociation of the solution of the various sodium phosphates at three different concentrations is shown in Table 1. There appears to be an inverse relation between the degree of dissociation and the number of P atoms in the chain or ring of the phosphate compound as indicated by the results in Table 1, which supports the findings of Wall and Doremus (1954).

Sodium orthophosphates (secondary) dissociated completely in solution at the concentrations shown in Table 1 since its degree of dissociation was found to be equal to that of sodium chloride solutions. This is contrary to what has been reported by Smith and Alberty (1956), who calculated the stability constants of the sodium complexes with orthophosphate ion from the pH measurements after making certain assumptions.

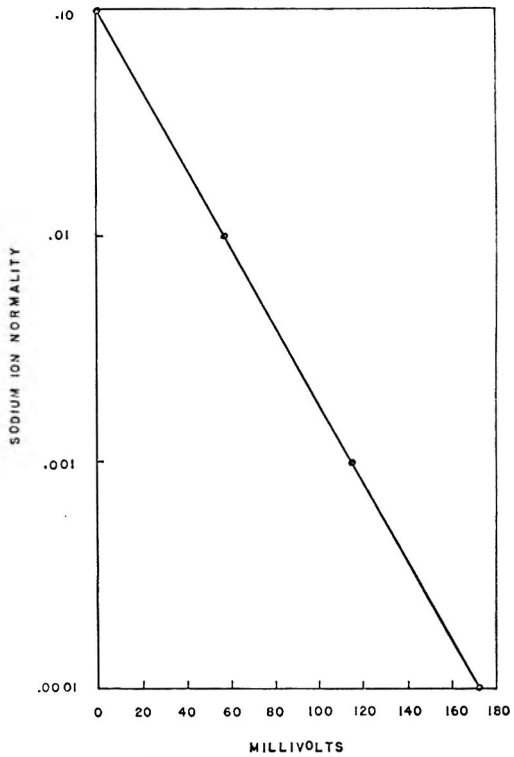

Fig. 1. Calibration curve for the determination of the sodium ion concentration.

It is possible to calculate the value for the dissociation constants of these sodium phosphates, but, because of certain uncertainties involved such as hydrolysis and the presence of uncombined constituents (Monk, 1949), it is not worthwhile to evaluate these calculations, and the results are expressed as percent dissociation.

On the addition of calcium to the solutions of various sodium phosphates in Table 1, sodium is readily exchanged by calcium, as indicated by the data in Table 2, and in most cases all of the sodium is dissociated in solution by the successive addition of calcium. This is of great interest from the viewpoint of the dissociation behavior of the various sodium phosphates in the presence of other cations, especially di- and trivalent cations.

Some of the phosphates were precipitated

Table 2. The effect of the addition of calcium on the degree of dissociation and pH of sodium phosphates at 25°C.

Phosphate	Concentration moles/l	Ca^{++} added in moles/l	% dissociation	pH	Precipitation[a]
Orthophosphate	.005	0	100	8.60	....
		0.00125	100	8.20	T
		0.00250	100	7.40	P
		0.00500	100	6.70	P
Pyrophosphate	.0025	0	80	9.90	....
		0.00125	90	9.60	....
		0.00250	96	9.20	T
		0.00500	100	8.25	P
Tripolyphosphate	.002	0	78	9.75	....
		0.00125	86	9.45	....
		0.00250	94	8.4	....
		0.00500	100	7.6	P
Tetraphosphate	.0016	0	58	8.55	....
		0.00125	78	7.8	....
		0.00250	87	7.05	....
		0.00500	96	6.15	T
Hexametaphosphate	.0016	0	52	7.55	....
		0.00125	67	7.05	....
		0.00250	85	6.3	....
		0.00500	95	5.45	....

[a] T—Turbid.
P—Precipitated.

with the addition of a certain minimum amount of calcium, and the observations are recorded in the last column of Table 2.

It will be seen from these results that, in general, the higher the number of P atoms in the phosphate compound, the greater is the quantity of calcium required to form a precipitate. In other words, most of the sodium is released in solution before calcium forms a precipitate with the phosphate ion. Thus it would appear that the lower the degree of dissociation of the sodium phosphate, the greater is its capacity to complex not only its component ion, but other divalent ions (calcium in this case) at the expense of the sodium ion, and this supports previous work done in this laboratory by Batra and deMan (1964) on the calcium-complexing ability of these phosphates. The calcium-complexing ability of sodium hexametaphosphate was found to be the highest, and that of orthophosphate was nil.

The pH in every case, as seen in Table 2, was lowered by the addition of calcium, as would be expected. The pH was brought up within 7–10 by triethanolamine for the measurement of sodium ion concentration in these solutions.

The results confirm that the higher the number of P atoms in the chain or the ring of the sodium polyphsophates, the lower is its dissociation in solution, and that sodium orthophosphate dissociated completely in solution under the present set of conditions. Evidence is also presented to show that the dissociation of the sodium polyphosphates generally increases with the presence of calcium and presumably other divalent ions in solution as they are preferentially complexed over the monovalent ion. This is now being investigated in this laboratory.

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Influence of Temperature and Salt Addends on Vapor Equilibration of Headspace

SUMMARY

The effects and interrelationships of time, temperature, and solute concentration on the vapor space equilibration of a dilute ester system is described. The results, which are of interest to those engaged in gas chromatographic headspace analysis or in sniff-test methodology, show that dilute solutions behave in a predictable manner, even though the data do not fit the Clausius-Clapeyron equation.

Headspace compositions and the factors that affect them are of interest to both the flavor chemist and the sensory analyst. The chemist is concerned with the volatile composition of a canned or bottled product, as well as with comparison of the volatiles emanating from fresh plant tissues in closed containers (e.g. Buttery and Teranishi, 1961). In evaluating the aroma of volatiles (e.g. Heinz *et al.*, 1964), the sensory analyst is concerned with the significance of the time lag between sample preparation and judgment, in addition to solute and temperature effects.

In either case, intersample comparisons require that the concentration and composition of the vapor sample be representative of (or, ideally, equilibrated with) the solution. The chemist sometimes adds nonvolatile solutes to increase the partial pressures of the volatile constituents and so increase their concentration in the vapor phase (e.g. Bassette *et al.*, 1962). Sugars and salts are frequently added to processed foods to improve taste, but it is not known whether volatile flavor is also increased.

Saturated aqueous solutions of selected volatile compounds have been investigated intensively, and the field was reviewed by Long and McDevit (1962). It has been reported that most salts increase the escaping tendency of dissolved volatiles such as benzene (Saylor *et al.*, 1952; McDevit and Long, 1952a), carbon dioxide and nitrous oxide (Geffcken, 1904; Markham and Kobe, 1941), ammonia (Dawson and McCrae, 1901), trimethylamine (Herz and Stanner, 1927), aniline (Glasstone *et al.*, 1927), acetone (Gross and Iser, 1930), and various organic acids (Linderstrom-Lang, 1923; Rivett and Rosenblum, 1914; Bockris *et al.*, 1951; Kolthoff and Bosch, 1932; Kendall and Andrews, 1921; McDevit and Long, 1952b; Endo, 1927; Herz and Stanner, 1927), aniline (Glasstone *et al.*, 1927), acecentrated solution, however, are not necessarily applicable to solutions whose volatile concentrations are a few parts per million or less, which are of more concern to the flavor chemist and sensory analyst. This work was directed toward determining the time required for vapor equilibration of dilute solutions in a model system and the effect of temperature and nonvolatile solutes on the equilibrated vapor composition.

METHODS AND MATERIALS

Ethyl acetate-1-C^{14}. One mc of high-activity ethyl acetate (1.77 mc/mM) was obtained from Tracerlab, Inc.

Counting apparatus. The Geiger phenomenon is adversely affected by the presence of water vapor in the sensitive zone. Consequently, if a Geiger tube or adaptation thereof is to be used as a sensing device, it is necessary to use an end window that is reasonably impervious to both water vapor and ethyl acetate. At the same time, the low energy level of C^{14} emission (.158 mev) limits both the material of which the end window can be made and its density. After several preliminary experiments, Mylar (Du Pont polyester film), .001 inch, aluminized both sides, was used (obtained from White and Company, Skokie, Illinois).

It is important for the purpose of this study to differentiate between active molecules in the gas phase and active molecules in solution. Because a relatively thin film of water would serve as an effective shield for this low-energy radiation, it might be permissible for the Geiger tube to be positioned directly over the solution. However, to ensure that counting was restricted to gas-phase molecules, an offset design was adopted. This intro-

duces a disadvantage in that the gas over the solution must diffuse into the sensitive zone for counting. However, because a period is required for equilibrium to be established between the concentration of ethyl acetate in solution and the concentration of ethyl acetate in the vapor, it was felt that the offset design would probably not be a limiting factor.

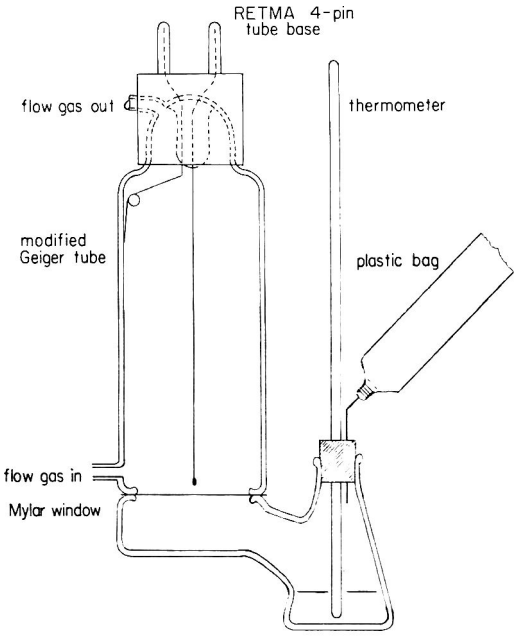

Fig. 1. Modified counting chamber.

Because thin Mylar, of which the end window was constructed, is slightly permeable to both water vapor and helium, it was necessary to modify the Geiger tube for flow conditions. This was accomplished by blowing a gas inlet tube near the window end, and an outlet tube near the opposite end.

The reaction vessel, coupled to the sensing device (Fig. 1), was constructed from two salvaged glass end-window Geiger tubes (Tracerlab type TG C-2). One was fitted with sidearms and an aluminized Mylar window as discussed above. The shell of the other was cleaned in concentrated nitric acid, and approximately ½ inch of the open end was drawn off and sealed. A sidearm of 12-mm glass tubing was used to attach this to a 25-cc Erlenmeyer at a point just below the neck. The vessel was closed with a rubber stopper containing a thermometer. To achieve isobaric conditions, a hypodermic needle was inserted through the stopper, and a deflated plastic bag tied securely over the exposed end. This compensated for pressure changes that would have been induced by the volume changes brought about by solute additions. The flanged ends of the two tube shells were abutted and separated by the Mylar window, and the joint sealed by building up epoxy resin on the outside.

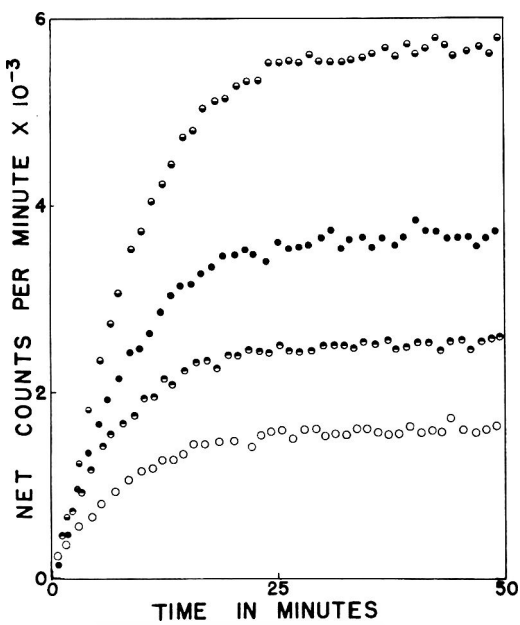

Fig. 2. Counts per minute as a function of time, headspace over solution containing 5 ppm C^{14}-labeled ethyl acetate at 25°C [NaCl], from top, 3.9*M*, 2.8*M*, 1.5*M*, 0*M*.

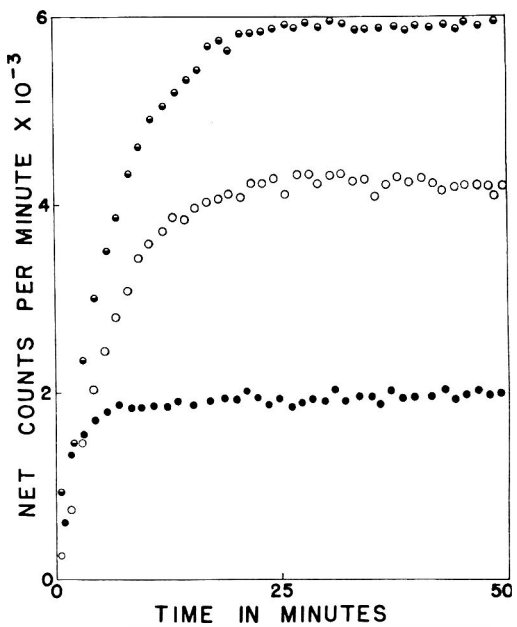

Fig. 3. Counts per minute as a function of time, headspace over solution containing 5 ppm C^{14}-labeled ethyl acetate at 25°C [NaCl], from top, 4.5*M*, 3.4*M*, 0.8*M*.

A magnet, sealed in glass, was placed in the flask, and the assembly was suspended so that the reaction vessel was in a bath of tempered water. By using thin-walled aluminum for the bath container, sufficient magnetic flux was passed to stir both the bath and the reaction vessel. A short length of shielded coaxial cable was used to connect the cathode and anode of the sensing tube to the input of a Tracerlab Versa/Matic II Scaler. Background counts, determined before and after each run, usually approximated 30 counts per minute (cpm).

The appropriate weight of NaCl (Bakers C.P.) was placed in the Erlenmeyer, and the apparatus immersed in a tempered water bath to a depth just short of the Mylar window. The temperature was allowed to equilibrate, 3.0 cc of a tempered solution of 5 ppm labeled ethyl acetate in double distilled water was quickly added, and the stopper replaced. One-min counts were commenced immediately; 15-sec intervals were usually sufficient to permit recording the result and resetting the scaler.

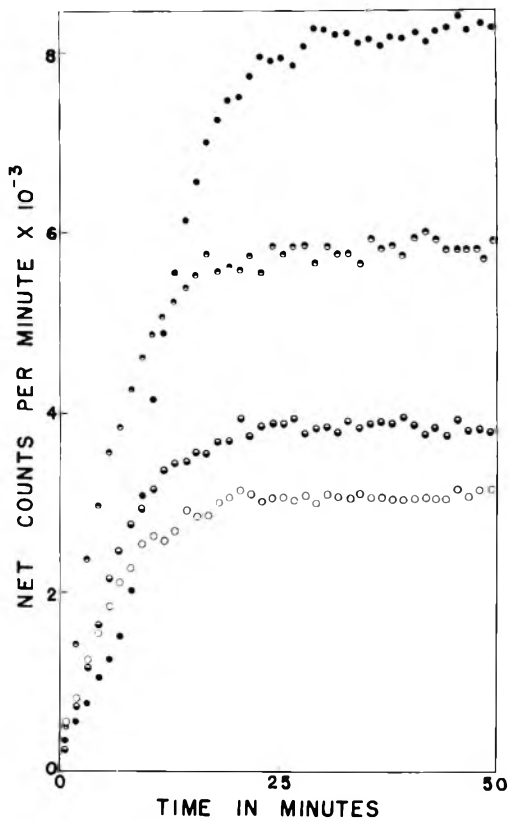

Fig. 4. Counts per minute as a function of time, headspace over solution containing 5 ppm C^{14} labeled ethyl acetate at 40°C [NaCl], from top, 3.9*M*, 2.2*M*, 0.8*M*, 0*M*.

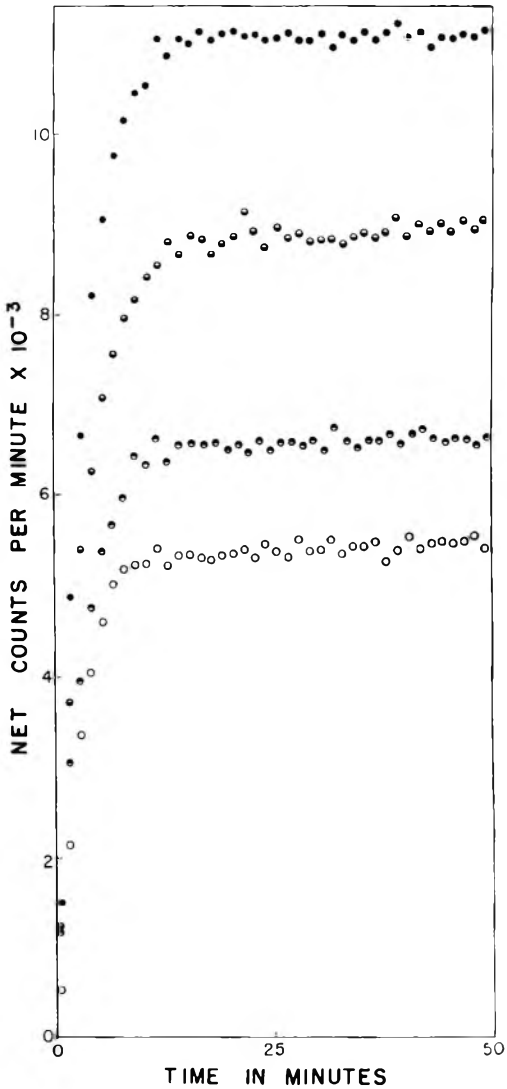

Fig. 5. Counts per minute as a function of time, headspace over solution containing 5 ppm C^{14}-labeled ethyl acetate at 60°C [NaCl], from top, 3.9*M*, 2.2*M*, 0.8*M*, 0*M*.

RESULTS AND DISCUSSION

Inasmuch as the ethyl acetate represents the only radioactive species in the reaction vessel, it seems reasonable that the equilibrium count rate can be taken as a function of the vapor pressure of ethyl acetate. The activity of ethyl acetate should be close to unity under these very dilute concentrations, but the same assumption is not necessarily true for the solvent, water.

Figs. 2 and 3 show plots of the cpm as a function of time at various sodium chloride

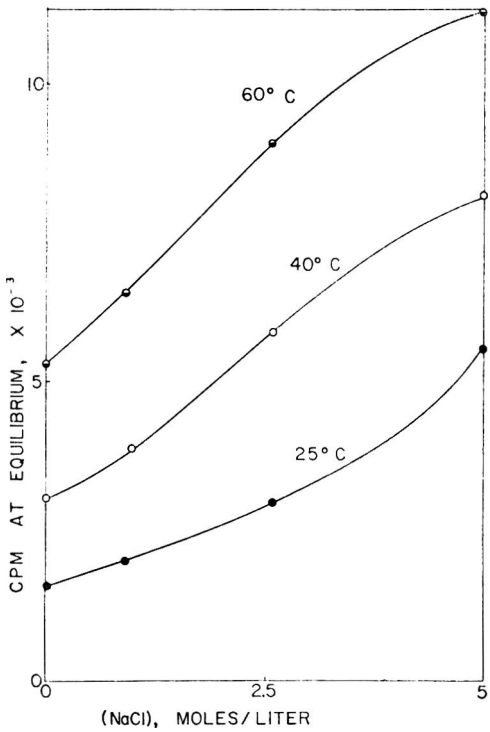

Fig. 6. Effect of NaCl concentration on equilibrium level of ethyl acetate in headspace.

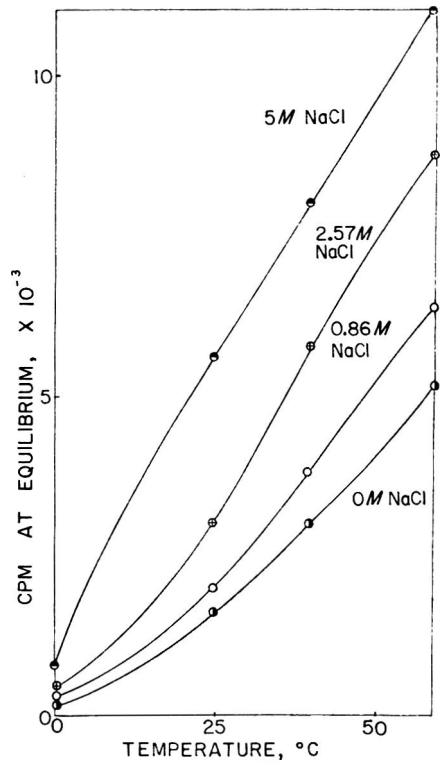

Fig. 7. Effect of temperature on equilibrium level of ethyl acetate in headspace.

concentrations and 25°C. Scatter is apparent, which can probably be attributed to counting statistics, but the general shape of the curve is evident. It appears that vapor equilibration in this model system requires about 25 min under these conditions. Both the equilibrium concentration of ethyl acetate in the headspace, and the rate of attaining equilibrium show a marked dependence on the solute concentration. Figs. 4 and 5 show that these same conclusions can be applied at higher temperatures, but as the temperature is increased, vapor equilibration occurs more rapidly and the effect of solute concentration on the rate of attaining equilibrium is less marked. This is probably due to the higher kinetic energy of the individual molecules at higher temperatures.

Fig. 6 shows the effect of NaCl concentration on the equilibrium headspace concentration of ethyl acetate at several temperatures, and Fig. 7 shows the effect of temperature on headspace composition for several solute concentrations. The data do not fit the Clausius-Clapeyron equation, but it is of interest that these dilute solutions do behave in a predictable manner. The slopes of the pre-equilibrated portions of the curves emphasize that intersample comparisons would require sampling after equilibrium had been achieved. In samples containing intact cellular materials, diffusion processes would require considerably longer for equilibrium to be attained.

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Studies on the Gelatinization of Starch
I. Competition for water by protein and cellulose derivatives

SUMMARY

Some effects of egg albumen, gelatin, methyl cellulose (MC) and carboxymethyl cellulose (CMC) on the gelatinization of unmodified corn and waxy maize starches are reported. The effect of increasing concentrations of those four substances on gelatinization of the starch was determined at 70, 80, and 100°C, and with 40, 60, and 80% moisture. The main criterion for the amount of gelatinization was the susceptibility of the starch to beta-amylase.

Albumen, at the higher concentrations employed, inhibited gelatinization of the starches heated at 70°C with 60 and 80% moisture but did not affect degree of gelatinization at higher moisture levels or at higher temperatures. Gelatin inhibited gelatinization of the starches heated at 70°C more than did albumen, and inhibited to some degree the gelatinization of starches heated to 80°C with 60% moisture. At higher moisture levels and temperatures, gelatin had no effect on extent of starch gelatinization. Methyl cellulose significantly affected starch gelatinization at 70°C but had less and no effect at 80 and 100°C, respectively. Carboxymethyl cellulose had pronounced effect on starch gelatinization at all temperatures and moisture levels except the 80% moisture level system heated at 100°C.

In bread dough containing between 36 to 38% moisture, starch gelatinization increased with temperature in the center of the loaf. The rate of increase of starch gelatinization was less than the rate of increase of temperature during baking.

Processing most starchy foods requires gelatinization of starches in the presence of other substances that may compete for the available moisture. Protein, native or supplemental, is invariably present in most natural foods. Frequently, substances such as gums, mucilages or cellulose derivatives are added to control consistency of the cooked food. These substances, because of their high affinity for water, may alter the degree of starch gelatinization. The extent to which this happens in systems of low moisture content is relatively unknown.

The degree of gelatinization of starches, which occurs during cooking, depends on many factors such as temperature, duration of heating, presence of salts and fats, source of starch, available moisture and the nature and amount of all substances that may compete for the available moisture. The importance of knowledge on the effect of substances that compete for moisture during starch gelatinization is obvious.

Considerable research has been done on effects of salts, fats and surfactants on the gelatinization of starch (Caesar, 1944; Gortner, 1933; Hodge *et al.*, 1948; Leach, 1962; Mangels and Bailey, 1933; and Reychler, 1928). However, the literature reveals little regarding the effect of protein or cellulose derivatives on starch gelatinization, particularly as related to systems in which water is limited. Bechtel (1959) has described the relationship between starch gelatinization and protein during bread baking although he presents no evidence. He stated that proteins in bread dough are highly hydrated, whereas starch absorbs little water. However, as the temperature of the dough increases, the protein becomes denatured and loses its water-binding capacity. At a slightly higher temperature, the starch begins to gelatinize (swell) and water originally bound by the proteins becomes available for starch gelatinization.

If insufficient water is present during the gelatinization process, swelling of starch granules may be greatly inhibited. Katz (1928) and Katz and Rientma (1930) showed that wheat starch in the interior of a bread loaf subjected to temperatures of 90–100°C during baking swelled only to a degree normally attained at 70°C in excess water. The degree of starch gelatinization occurring during bread baking is still unknown.

Crossland and Favor (1948) showed that

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carboxymethyl cellulose (CMC) and sodium alginate caused certain starches to swell in stages during gelatinization. They found no interaction between starches and adjuncts, which suggested that the effects were physical. Two-stage swelling was observed only in starches that had an amylose-amylopectin ratio as found in wheat and corn. Sandstedt and Abbott (1964) have also studied the effect of CMC on starch gelatinization. Adding CMC (0.8%) to 4–5% corn or wheat starch suspensions caused two-stage swelling. This was observed by viscosity, light transmission, solubles, and water absorption measurement criteria of starch gelatinization. The effect of CMC on viscosity was attributed to increased swelling of starch.

Several methods have been used to study gelatinization of starch. Sandstedt and Abbott (1964), studying systems in which water was not limited, concluded that measurement of such diverse properties of starch by light transmission, soluble starch, water absorption and viscosity, represented different aspects of the same phenomenon.

Sullivan and Johnson (1964) showed that during starch gelatinization, a simultaneous increase in starch susceptibility to beta-amylase and loss of birefringence occurred. Both changes occurred over the same temperature range. Use of starch susceptibility to beta-amylase to measure degree of starch gelatinization has several advantages when applied to complex systems, particularly when the amount of water is limited. The mixture of starch, protein or other adjuncts may be heat-treated at any desired moisture or temperature level and then dispersed before taking a representative sample to measure susceptiblity to beta-amylase. Thus, the method does not depend on measuring effects over a relative narrow range of experimental conditions such as imposed by viscosity methods. The method does not require knowledge of the state of the starch prior to gelatinization because the measurement depends on the starch made available to beta-amylase after gelatinization. The method is relatively sensitive to small changes in starch gelatinization. Results are influenced by starch retrogradation which may occur in gelatinized starch gels.

This investigation was made to gain further insight into the competitive nature of proteins and cellulose derivatives for available moisture during starch gelatinization by heat.

EXPERIMENTAL

Unmodified corn and a waxy maize Regular Amioca Starch (American Maize Products, Roby, Ind.) were used. The gelatin was a bacto-gelatin prepararation (Difco Laboratories, Detroit, Mich.). Albumen was a commercial dried egg albumen (Seymour Foods, Topeka, Kans.). Methyl cellulose (4000 centipoise) (MC) was obtained from Fisher Scientific Company and the carboxylmethyl cellulose (CMC-7 HP) from the Dow Chemical Company.

A 5-g sample of starch, plus the approximate weight of albumen, gelatin, MC or CMC to give a 5, 10, 20, and 30% protein or cellulose derivative in the mixture on a total-dry-weight basis, was thoroughly blended and introduced into a 125-ml Erlenmeyer flask. Water was added to the mixtures to obtain systems containing 40, 60, or 80% moisture based on dry weight of total solids. Water was added to the samples of 40% moisture the night prior to heat treatment to allow the system to come to equilibrium. With the samples brought to 60 and 80% moisture, the water was added immediately prior to heat treatment. The samples were thoroughly mixed before and after water was added.

Gelatinization of the starches was performed at 70, 80, and 100°C for 30 min. An autoclave was used for heating at 100°C, with the Erlenmeyer flasks stoppered with cotton plugs while heating. A water bath was used for heating at 70 and 80°C with rubber stoppers instead of cotton plugs.

Heated samples were dispersed in water by passing through a 54-GG silk sieve and blending for 15 sec in a Waring blender. Waxy maize starch samples heated at 80 and 100°C were not passed through the silk sieve but were blended directly because of the starch's gelatinous nature. Samples were made up to 500 ml volume, and a 40-ml portion was taken for beta-amylolysis determinations. Susceptibility of the starches to beta-amylase was determined by the method of Sullivan and Johnson (1964). Maltose values were modified slightly in that table 22-18 in Cereal Laboratory Methods (1963) was used to express maltose equivalents.

Amounts of water held separately by the starches, albumen and MC after heating in an excess of water were determined in the following manner: A 2-g sample of starch, albumen or MC was weighed into a 125-ml Erlenmeyer flask containing 50 ml of water. The samples were heated 30 min at either 70, 80, or 100°C. The material was

centrifuged for 15 min at 900 × G. The supernatant was decanted, and percent moisture determined by drying the centrifugate overnight at 50°C and then heating for 1 hr at 100°C in a forced-air oven. This procedure may have compressed the starch sacs and thus released some of the water normally held. The procedure is similar to that used by Leach *et al.* (1959) to measure the swelling power of starch.

Increasing susceptibility of starch in bread crumb to beta-amylase during baking at 430°F for various periods was determined in the following manner: temperature of the inside of the loaf was noted by inserting a thermometer into the center of the loaf when it was removed from the oven. The loaves were removed from the oven at 5-min intervals. Forty-gram pieces, representing a cross section of the loaf, ¼ inch from the crust, were removed from the inside and the gelatinized starch was measured by beta-amylolysis (Sullivan and Johnson, 1964). The bread crumb was dispersed in 200 ml of water in a Waring blender. A 50-ml portion of the suspension was diluted to 500 ml and 40-ml samples of the dilute suspension were used for beta-amylolysis.

RESULTS

Figs. 1 and 2 show the effect of egg albumen on gelatinization of both unmodified corn and waxy maize starches as measured by beta-amylolysis. Increasing concentrations of albumen progressively inhibited gelatinization of the starch heated at 70°C. This was evident by a decrease in susceptibility of starch to beta-amylase. Decreasing starch gelatinization with increasing amounts of albumen was attributed to the greater affinity of albumen than of starch for water. Heating at 70°C did not fully gelatinize the starch so the attraction of starch for water did not overcome the forces of attraction of the albumen for the water.

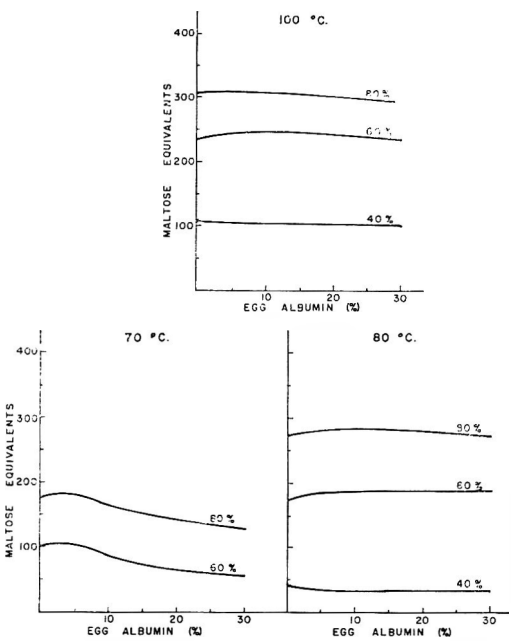

Fig. 1. Effect of egg albumen on the gelatinization of unmodified corn starch heated 30 min with 40, 60, or 80% moisture, as measured by beta-amylolysis.

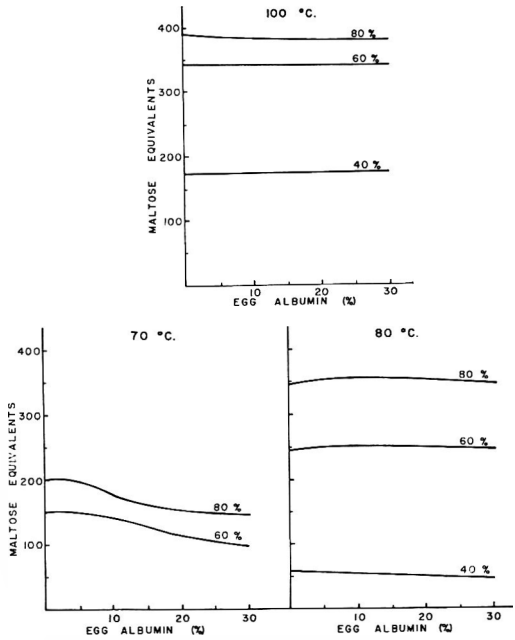

Fig. 2. Effect of egg albumen on the gelatinization of waxy maize starch heated 30 min with 40, 60, or 80% moisture, as measured by beta-amylolysis.

Albumen did not inhibit gelatinization of the starch when heated at 80 or 100°C. Adding as little as 5% albumen slightly increased the maltose equivalents at the 60 and 80% moisture levels. The slight increase was attributed to albumen holding the water early in the gelatinization process, which resulted in more uniform distribution of the water throughout the mass. The sample without albumen had a dry core of only slightly gelatinized starch in the center, particularly in samples heated at 100°C with 60% moisture. Gelatinization of the starches at 80 and 100°C provided sufficient energy to gelatinize the starches more completely and to fully coagulate the albumen. This created an attraction for the water which was greater for starch than for the albumen. The gelatinization of both starches was influenced similarly although the waxy maize starch was

generally more available than the unmodified starch to beta-amylase.

The effects of gelatin on gelatinization of unmodified and waxy maize starches are shown in Figs. 3 and 4. These data represent triplicate determinations for each condition. Values expressed as maltose equivalents agreed within 4% of each other for a given set of experimental conditions. At 70°C, for both starches only the 60 and 80% moisture systems exhibited measurable amounts of starch gelatinization. Even when the 40% moisture system was heated to 80°C, the extent of starch gelatinization, while measurable, was minor. Systems with 60% moisture, heated at 80°C, exhibited reduced amounts of starch gelatinization

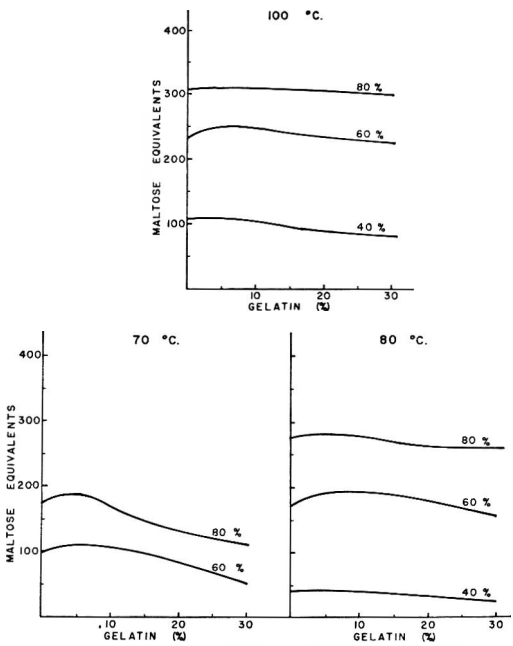

Fig. 3. Effect of gelatin on the gelatinization of unmodified corn starch heated 30 min with 40, 60, or 80% moisture, as measured by beta-amylolysis.

when the gelatin concentration was increased. The slight rise in maltose equivalents with 5–10% gelatin present was attributed to a more uniform distribution of water throughout the mass as was observed for experiments with albumen-starch mixtures. When the system was heated at 80°C with 80% moisture, gelatinization of the unmodified corn starch was slightly influenced by 20 or 30% concentration of the gelatin. When the systems were heated at 100°C, the degree of starch gelatinization increased more at the 40% moisture level than systems heated at 70 or 80°C. Increase in the amount of gelatin present in the 40% moisture level mixture reduced the degree of starch gelatinization. Even with 60% moisture at 100°C,

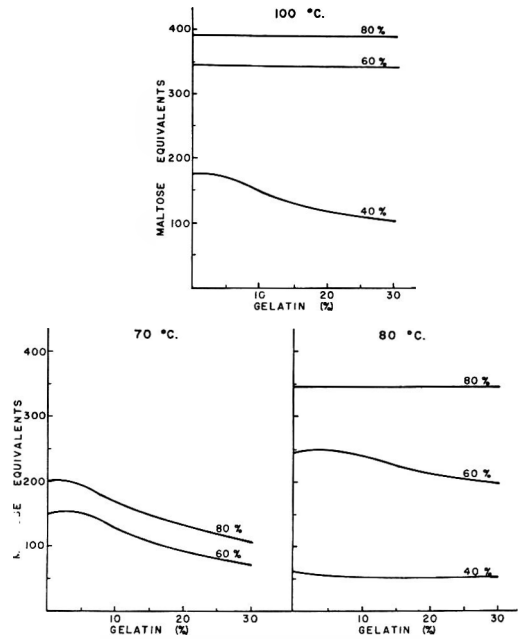

Fig. 4. Effect of gelatin on the gelatinization of waxy maize starch heated 30 min with 40, 60, or 80% moisture, as measured by beta-amylolysis.

unmodified corn starch was only slightly influenced by the gelatin concentration. With 80% moisture and heating at 100°C, the gelatin concentration exhibited no influence. The results show that both

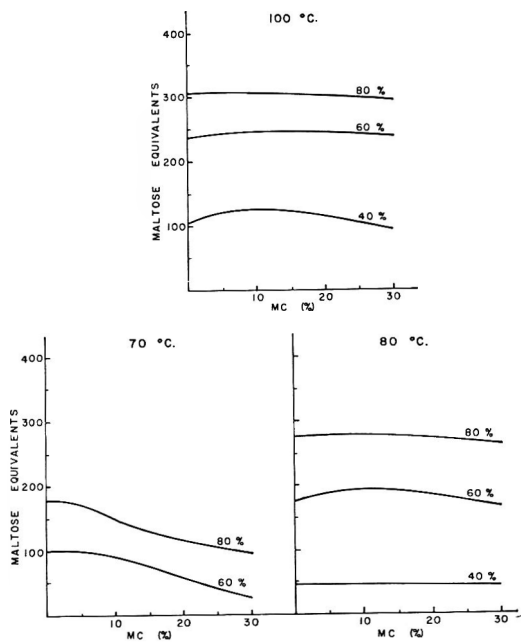

Fig. 5. Effect of MC on the gelatinization of unmodified corn starch heated 30 min with 40, 60, and 80% moisture, as measured by beta-amylolysis.

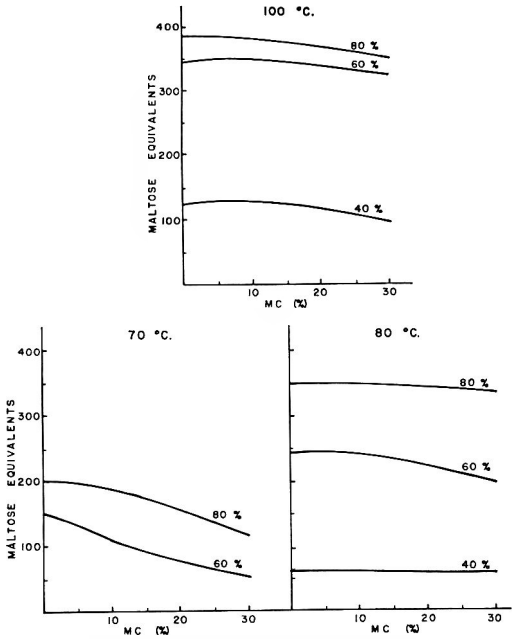

Fig. 6. Effect of MC on the gelatinization of waxy maize starch heated for 30 min with 40, 60, and 80% moisture, as measured by beta-amylolysis.

albumen and gelatin may influence starch gelatinization, and the effect which each has on the process depends on their concentrations, the temperature and the amount of water present.

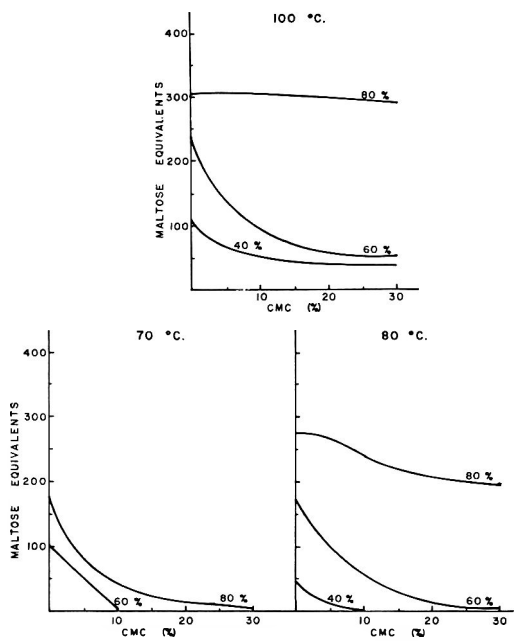

Fig. 7. Effect of CMC on the gelatinization of unmodified corn starch heated 30 min with 40, 60, and 80% moisture, as measured by beta-amylolysis.

Effects of MC on inhibition of gelatinization of unmodified corn and waxy maize starches are shown by Figs. 5 and 6. Increasing concentration of MC caused progressive inhibition of gelatinization of starches heated at 70°C at both 60 and 80% moisture levels, but with 40% moisture total gelatinization was insignificant. As the temperature increased to 80°C, inhibition of gelatinization of both starches occurred at a 15% concentration of the MC and was most noticeable at the 60% moisture level. At 100°C, with 60 and 80% moisture, inhibition of gelatinization of starches was slight.

Effects of CMC on gelatinization of starches are shown in Figs. 7 and 8. CMC was particularly effective as a competitive agent for water during

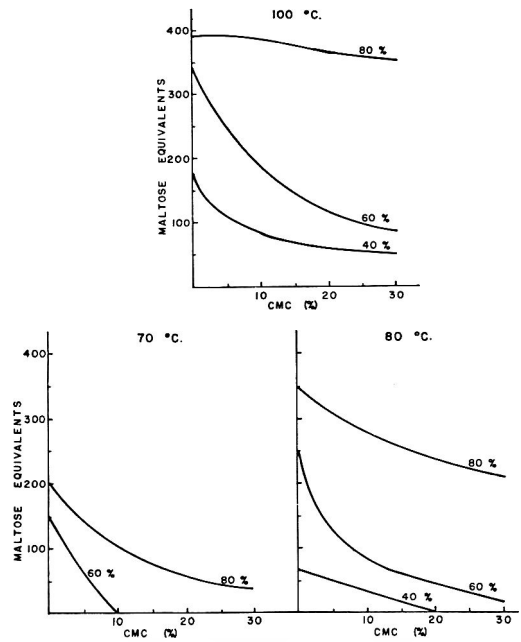

Fig. 8. Effect of CMC on the gelatinization of waxy maize starch heated 30 min with 40, 60, and 80% moisture, as measured by beta-amylolysis.

gelatinization of starches. Increasing concentrations of CMC reduced gelatinization of both starches at all temperatures and moisture levels except systems heated at 100°C with 80% moisture present, where the effect of CMC was slight. In systems heated at temperatures below 100°C and with limited moisture, as little as 5% CMC markedly reduced availability of starches to beta-amylase. The lower the available moisture and the lower the heating temperature, the greater was the inhibitory effect of CMC on starch gelatinization.

Table 1 shows the amount of water retained after centrifugation at 900 × G by unmodified corn and waxy maize starches, albumen, and MC when heated for 30 min at 70, 80, or 100°C with excess

water. Each value represents four determinations made in duplicate two different days. Duplicates checked within 1.5% moisture. Values could not be determined for systems with limited water because in such systems all water was held against centrifugation. Likewise, for systems containing gelatin or CMC, values could not be determined by this method because gelatin and CMC were completely dispersible in water.

As the temperature was increased from 70 to 100°C, the water absorption of both starches increased but albumen and MC showed no significant change. The data suggest that the absorptive capacity of starch increases with gelatinization but that the absorptive capacity of albumen or MC remains essentially constant when heated under similar conditions.

Table 1. Effect of heating on water retention by various substances against centrifugation at 900 × G.

Substance	Temperature (°C)		
	70	80	90
Unmodified corn	83.4	89.1	95.6
Waxy maize	89.9	91.6	94.5
Albumen	88.4	89.7	87.6
Methyl cellulose	94.5	94.5	94.2

It was of interest to observe the relationship of starch gelatinization to temperature during bread baking. In this system the moisture ranged from 36–38% and temperature never exceeded 100°C. Susceptibility of starch to beta-amylase in bread crumb as a function of baking time is illustrated in Fig. 9. Temperature at the center of loaves increased exponentially with time and attained a maximum after 20 min of baking. Starch showed a rapid increase in susceptibility to beta-amylase as the temperature inside the loaf reached gelatinization levels. Susceptibility of starch to beta-amylase after 30 min of baking indicated that the starch had undergone maximum gelatinization at the temperature attained and with the moisture available in the bread.

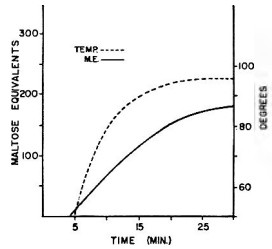

Fig. 9. Increase in susceptibility of starch in bread crumb when baked at 430°F.

DISCUSSION

The data indicate that any substance having great affinity for water may compete for available moisture during starch gelatinization. However, the degree of affinity of such substances and that of starch for water may be altered by varying the amount of water and temperature of the system. Specifically, CMC inhibited gelatinization of starches more and over a greater range of temperature and moisture levels than did MC, egg albumen or gelatin. CMC has a very high affinity for water and does not lose this ability to any great extent at elevated temperatures.

Sufficient energy must be supplied to a water-starch system to break the internal starch-granule bonds before gelatinization occurs (Caesar, 1944). After the internal bonds are broken, the granule of starch will expand and expose its hydroxyl groups. The exposed hydroxyl groups become free to form hydrogen bonds and swell as water is absorbed. However, if a substance in the presence of starch undergoing gelatinization should lose its affinity for water during gelatinization, as albumen does when it coagulates, then this substance will no longer compete so strongly for available water. Also, if sufficient water is present and the temperature is sufficiently high, the affinity for water may be higher for starch than for other substances, and the starch may subsequently fully gelatinize.

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Analysis of Pigments at the Surface of Fresh Beef With Reflectance Spectrophotometry

SUMMARY

There is need for a nondestructive method of measuring the proportions of myoglobin derivatives in meat samples. Such a method was developed in which reflectance spectra are recorded on the absorbancy scale for samples of fresh beef containing predominantly myoglobin (Mb), oxymyoglobin (MbO_2), or metmyoglobin (Mb^+) at the surface. The spectra are adjusted so that reflectance measured on the absorbancy scale (R_A), is 1.0 at 525 mμ, an isobestic point for the three derivatives. With this adjustment, the isobestic point for MbO_2 and Mb^+ at 474 mμ and the isobestic point for Mb and MbO_2 at 571 mμ are reproducible, and the R_A values at 474 mμ and 571 mμ are less variable. In that way, 100% reflectance values were established, but a model system was needed to determine intermediate reflectance values due to mixtures of myoglobin derivatives. The model system, containing known amounts of MbO_2 and Mb^+, indicated that Beer's law was not followed, but the deviation was small. Some of the difficulties inherent in this type of study are discussed, and a hypothesis is proposed to explain the peculiarities of reflectance spectra.

To better understand meat discoloration due to oxidation of myoglobin, it would be helpful to be able to measure the amounts of various forms of myoglobin present at the surface of the meat. Two possible methods are available: the absorption ratio method of Broumand *et al.* (1958) applied to extracts of meat taken from the surface of beef cuts; and the reflectance ratio method of Dean and Ball (1960).

Absorption spectrophotometry and the absorption ratio method are theoretically capable of excellent results. Practical problems arise, however, in extracting the pigments from meat to give a clear solution, in selecting the surface volume of meat to be analyzed, and in changes in the form of the pigment (mainly Mb $\longrightarrow$ MbO_2) during extraction and analysis (Mb = reduced myoglobin, MbO_2 = oxymyoglobin, Mb^+ = metmyoglobin).

The reflectance ratio method excludes errors due to extraction since the analysis is done on an intact or ground sample of meat, and no extraction is necessary. However, there is no sound theoretical basis relating reflectance spectra and quantities of pigments.

Dean and Ball (1960) attempted to surmount this difficulty by obtaining absorption coefficients from a table given by Judd (1952, page 358) which relates absorption coefficients to reflectivity. Broumand *et al.* (1958) had prepared graphs relating ratios of absorption coefficients (obtained by transmission measurements) to percent Mb, MbO_2, and Mb^+, and, in the method of Dean and Ball, ratios of absorption coefficients obtained by reflectance are applied to these graphs to give percent Mb, MbO_2, and Mb^+.

The reflectance ratio method outlined above suffers from several shortcomings. The percent reflectance will depend upon the concentration of pigment, the amount of intramuscular fat, and the amount of moisture at the surface of the meat as well as the oxidation or oxygenation state of the pigment. Consequently, absorption coefficients based only on percent reflectance will not be correct. In obtaining absorption coefficients from Table D, page 382 (Judd, 1952), Dean and Ball assumed that scattering coefficients canceled, leaving a ratio of absorption coefficients. This is an unjustified assumption. The scattering of light is known to vary with the reciprocal of the fourth power of the wave length.

Nevertheless, it appears that reflectance spectrophotometry could be a powerful tool for measuring myoglobin derivatives at the surface of a piece of meat. Naughton *et al.* (1957) showed that reflected light measured on an absorbancy scale is directly proportional to copper concentration when dilute $CuSO_4$ is added to a nonabsorbing diluent powder. Naughton *et al.* (1958) claimed

that reflected light measured on an absorbancy scale is directly proportional to amounts of Mb, MbO_2, and Mb^+ at the surface of tuna samples.

In the present study, meat samples were prepared to give predominantly Mb, MbO_2, and Mb^+ at the surface, and reflectance spectra were obtained using the absorbancy scale (R_A). The purpose was to develop a method of quantitative analysis of meat pigments based on reflectance spectrophotometry.

EXPERIMENTAL

The meat used throughout these experiments was fresh beef purchased from a local market. Round steak was purchased and cut from an intact U. S. Choice round. In the laboratory, the round steaks were cut into samples measuring 1½ × 2 × ⅛ inches and placed in small plastic boxes of the same size with the top left open. The samples were immediately packaged in Saran (allowing them to bloom as little as possible) and left at room temperature for 4 hr. During this time, reflectance spectra were obtained, and, at the end of 4 hr the spectra indicated predominantly Mb, and the spectra were no longer changing. These samples were then measured as representing 100% Mb.

Next the samples were unwrapped, left 2 hr at 0°C, packaged in transparent cellulose film 195-MSAD-80, and stored overnight at 0°C. The next day, these samples were measured as representing 100% MbO_2. The same samples were then unwrapped, and the surface was painted with 1% $K_3Fe(CN)_6$. Samples were stored at 0°C and treated three times with $K_3Fe(CN)_6$ at 1-hr intervals. The samples were then overwrapped with transparent cellulose film 195-MSAD-80 and stored overnight at 0°C. The next day reflectance spectra were obtained for the samples, which were assumed to be 100% Mb^+ at the surface.

Reflectance spectra were recorded in the range 400–700 mμ on a Beckman DK2A spectroreflectometer with a tungsten lamp and photomultiplier. The reference material was MgO, and sample holders were set for total reflectance. The time constant was 0.2, scale 10 mμ/cm, and sensitivity control 12. Care was taken to place the samples in the sample holder so that the same portion of the surface was scanned for each myoglobin derivative.

Reflectance spectra are usually obtained by using the percent-transmission scale, and are reported as percent reflectance. Since the purpose of this study was to relate reflectance spectra to quantities of myoglobin derivatives, and since, in transmission measurements, only the absorbancy scale is directly related to quantities of absorbing materials, it was decided to use the absorbancy scale for recording reflectance spectra. The symbol used to denote reflectance recorded on the absorbancy scale is R_A.

RESULTS

The R_A spectra were recorded for 18 meat samples taken from two adjacent slices of beef round treated to contain predominantly Mb, MbO_2, and Mb^+ at the surface. Fig. 1 shows R_A plotted against wave length for three myoglobin derivatives from one meat sample. Qualitatively, the spectra are typical of those expected for Mb, MbO_2, and Mb^+ based on transmission measurements, but it is evident that the R_A curves are distorted. For example, in transmission measurements, the Soret peak in the 400–450-mμ region has approximately ten times the absorbancy of the peaks at 500-600 mμ. Such is not true for these reflectance spectra. Nevertheless, the fact that R_A maxima for each myoglobin derivative were at the same wave lengths as found in transmission measurements was helpful in interpreting the spectra.

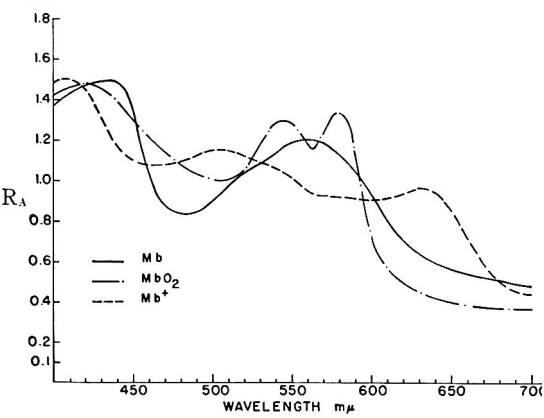

Fig. 1. Reflectance (R_A) spectra for fresh beef samples treated to contain predominantly Mb, MbO_2, or Mb^+ at the surface.

For quantitative analysis of these results, it is necessary that isobestic points (the wave length at which absorbancy indices of two or more pigments are identical) be established which are reproducible. The R_A curves as recorded do not satisfy this condition because there is too much scatter in the data, but, by a simple adjustment of the R_A curves, it was found that good reproducibility of isobestic points was achieved. Each R_A curve was adjusted to have an R_A of 1.0 at

525 mμ (an isobestic point for Mb, MbO_2, and Mb^+ according to transmission measurements). That is, the entire curve was moved upward or downward the amount necessary to give an R_A reading of 1.0 at 525 mμ. This adjustment is necessary to eliminate uncontrolled variables such as intramuscular fat or wrinkles in the film used to wrap the samples. Such variables affect the amount of light reflected by the sample but are not related to amounts and kinds of pigment present. Fig. 2 shows the data of Fig. 1 after adjustment.

The adjustment of R_A spectra leads to isobestic points which are relatively stable and reproducible. As shown in Fig. 2, there is an isobestic point for Mb^+ and MbO_2 at approximately 474 mμ, and for Mb and MbO_2 at approximately 571 mμ. These isobestic points were chosen for further work because the rate of change of R_A for the three forms of pigment seems less at these points than at other isobestic points which might have been chosen. Also, the difference in R_A due to pigment changes was greatest at these two isobestic points. For the 18 meat samples, the wave length was measured at which the isobestic points actually occurred in the vicinity of 474 and 571 mμ. The average wave lengths (± the standard error) were found to be 473.9 ± 0.6 mμ and 571.1 ± 0.2 mμ.

With the method of Broumand *et al.* (1958) it was necessary to select two isobestic points for each combination of pigments and to obtain a ratio of absorbancies, so that differences in absorbancies due to the amount of pigment extracted could be eliminated. Dean and Ball (1960) also used ratios calculated at two different isobestic points for each combination of pigments. After making the adjustment of R_A to 1.0 at 525 mμ for all forms of the pigment, it appeared that R_A at the isobestic points was characteristic for any one form of the pigment. As shown in Table 1, the variation in R_A at an isobestic point is relatively small. Consequently, it appeared possible to measure the amount of pigment present based only on reflectance, R_A, at two wave lengths. For example, 100% Mb^+ or MbO_2 would have a reflectance, R_A, of 0.987 at 474 mμ, whereas the R_A at 474 mμ would be 0.781 for 100% Mb. From this information and similar data for the isobestic point at 571 mμ, it is possible to construct graphs relating R_A to percent of the three myoglobin derivatives.

To determine how reproducible the isobestic points and 100% R_A values are, a second set of 18 meat samples was treated the same as the first set, and the R_A spectra recorded for Mb, MbO_2, and Mb^+ at the surface of the samples. For the second set of data (after adjustment to R_A of 1.0 at 525 mμ), the average isobestic points were at 472.9 ± 0.7 mμ for Mb^+ and MbO_2 and at 570.9 ± 0.4 mμ for Mb and MbO_2. Table 1 shows the R_A values for the second set of data. Using the *t* test, it was shown that the average R_A values for MbO_2 and Mb at 571 mμ were different from those of the first set at the 0.001 level. This difference could be attributed to the presence of Mb^+ in the Mb and MbO_2 samples. Consequently, the second set of data was not pooled with the first set. It seems reasonable to assume that, if 100% R_A values for any one pigment vary significantly in the direction of contamination with another pigment, these data should be discarded. If new R_A values vary significantly in the direction of higher 100% values, the new data should be substituted for the old 100% values. Nevertheless, it is apparent, from a comparison of the two sets of data, that the method described gives consistent results for 100% values of the three pigment forms at two isobestic points. Care has to be taken in preparing the samples containing MbO_2 and Mb

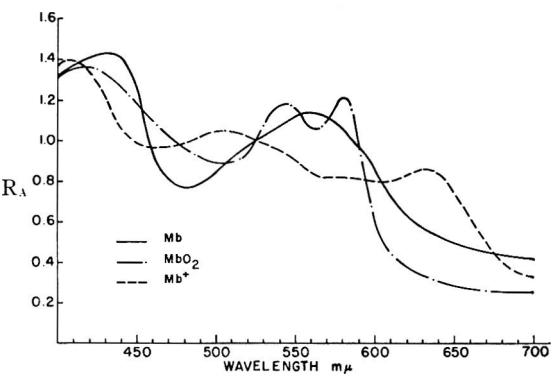

Fig. 2. Reflectance (R_A) spectra of Fig. 1 adjusted so that R_A at 525 mμ is 1.0 for all three myoglobin derivatives.

to minimize contamination with Mb^+. The samples containing predominantly Mb^+ at the surface are easily prepared. Also, one must adjust the R_A spectra so that they have values of 1.0 at 525 mμ to minimize scatter in the data due to uncontrolled variables. Since the method seeks to provide information only on percentage values of myoglobin derivatives and not absolute amounts of myoglobin, the adjustment of the R_A spectrum is valid.

With R_A values established for beef samples containing 100% Mb^+, MbO_2, or Mb at the surface, it remains to be determined how R_A values vary as intermediate amounts of pigment form. To prepare meat samples with known mixtures of the pigments would be virtually impossible, so a model system was devised whereby light could be reflected from a liquid system to which known amounts of the pigments could be added. A sus-

pension of 5% nonfat dry milk was found to be satisfactory for reflecting light, and known mixtures of MbO_2 and Mb^+ were added to the suspension. Nonfat dry milk was used as a blank, and the recorded spectra are shown in Fig. 3. These spectra are similar to those obtained from meat samples in that the peaks are flattened in comparison to transmission spectra. If the percent MbO_2 or Mb^+ is plotted versus the reflectance, R_A, the relation is seen to be nonlinear (Fig. 4). The nonlinearity increases with the difference in R_A between 100% MbO_2 and 100% Mb^+. For example, at 500 mμ, where there is relatively little difference, the relation is close to linear, whereas, at 580 mμ the deviation is considerable.

A second method used to measure R_A values intermediate to 100% MbO_2 and 100% Mb^+, was the combination in one package of two pieces of meat. One piece of meat was treated to contain predominantly MbO_2 and the other to contain predominantly Mb^+. These two pieces of meat

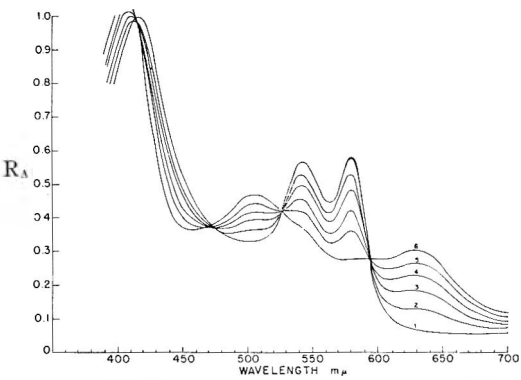

Fig. 3. Reflectance (R_A) spectra of solutions of mixtures of MbO_2 and Mb^+ in suspensions of 5% nonfat dry milk. 1 = 100% MbO_2; 2 = 80% MbO_2 and 20% Mb^+; 3 = 60% MbO_2 and 40% Mb^+; 4 = 40% MbO_2 and 60% Mb^+; 5 = 20% MbO_2 and 80% Mb^+; 6 = 100% Mb^+.

were then positioned in the sample holder of the Beckman DK-2A spectroreflectometer so that varying proportions of each could be exposed to the light beam and reflectance spectra obtained. The reflectance spectra were then adjusted to an R_A of 1.0 at 525 mμ, and the R_A's at 571 mμ were measured. A plot of R_A at 571 mμ against position of the samples (which should have corresponded to percent MbO_2 or Mb^+) yielded an S-shaped curve. The reason for this type of curve was not determined, but it was concluded that varying proportions of MbO_2 and Mb^+ in a single sample of meat could not be approximated by exposing varying proportions of 100% Mb^+ and 100% MbO_2 to the light beam of the spectrophotometer.

Reflectance spectra and Gardner a values were

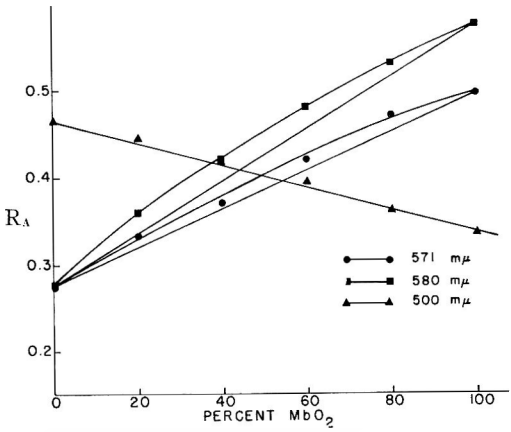

Fig. 4. Relationship between R_A and percent MbO_2 or Mb^+ at 580, 571, and 500 mμ. These data are taken from the spectra of Fig. 3.

obtained for stored beef samples as they deteriorated in color at 0°C. The spectra were then adjusted to R_A of 1.0 at 525 mμ and R_A values at 571 mμ and 474 mμ plotted against time. These data are shown in Fig. 5, along with Gardner a values. The Gardner a values start changing immediately upon storage, confirming earlier observations (Snyder, 1964). However, R_A at 571 mμ decreases very slowly at first and then faster as storage time increases. R_A at 474 mμ changes very little, and, from Table 1, this indicates that changes from MbO_2 or Mb^+ to Mb are not involved in the initial 8 days of experiment.

DISCUSSION

Reflectance spectrophotometry shows promise for determining relative amounts

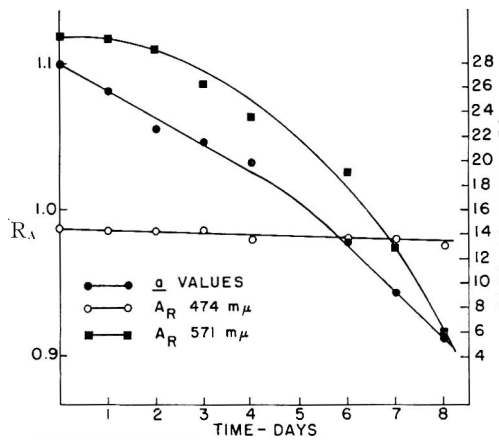

Fig. 5. Change in R_A at 571 mμ, R_A at 474 mμ, and Gardner a values as samples of fresh beef round change in color during storage in air at 2°C. Each point represents an average of 20 samples.

of Mb, Mb^+, or MbO_2 at the surface of a piece of meat. The method outlined above is an improvement over earlier methods since it takes into account reflectance changes due to factors other than myoglobin and minimizes these changes by adjustment of the reflectance spectrum so that R_A at 525 mμ is 1.0. Also, data are presented for the first time on reflectance values for meat samples containing predominantly Mb, MbO_2, or Mb^+ at the surface.

In this study, emphasis was placed on quantitative measurements of meat pigments. As a result, observations were made which otherwise may have gone unnoticed. Fig. 5 indicates that R_A at 571 mμ for meat stored at 0°C changes very slowly for the first few days in comparison with Gardner *a* values. This difference can be attributed to the fact that the initial change in fresh beef color is beneath the surface. Gardner *a* values result from irradiation of the sample with bright white light, and measurement of the portion of light which is reflected and passes a red filter. Reflectance absorbancies result from illumination of the sample with very low-intensity monochromatic light and measurement of all light that is reflected. One would expect the results of Gardner *a* values to be more indicative of changes beneath the surface of the meat than reflectance absorbancies, inasmuch as higher-intensity light would penetrate further. If this supposition is true, two procedures could be useful in making reflectance values more sensitive to color changes beneath the surface of fresh beef. The light source and detector can be exchanged in position in the Beckman DK-2A spectroreflectometer to operate under direct-sphere illumination. This technique allows for direct illumination of the sample with bright white light, and the reflected light is directed through the monochromator and detected. A second possibility is to use the reflectance technique but to concentrate on wave lengths in the red region (particularly 630 mμ) to detect changes from MbO_2 to Mb^+. During these experiments, it was observed that, as stored fresh beef changes in color, changes are much larger at 630 mμ than at 571 mμ. This is probably a result of the greater penetrating power (less scattering) of long-wave-length light.

Table 1. Average R_A values[a] at isobestic points for myoglobin derivatives in meat treated to contain predominantly Mb, Mb^+ or MbO_2 at the surface.

	474 mμ	571 mμ
1st set		
Mb^+	0.986 ± 0.001	0.825 ± 0.004
MbO_2	0.989 ± 0.004	1.115 ± 0.004
Mb	0.781 ± 0.003	1.116 ± 0.002
$\frac{Mb^+ + MbO_2}{2}$	0.987 ± 0.002	
$\frac{MbO_2 + Mb}{2}$		1.115 ± 0.002
2nd set		
Mb^+	0.984 ± 0.002	0.825 ± 0.006
MbO_2	0.982 ± 0.002	1.100 ± 0.004
Mb	0.791 ± 0.007	1.089 ± 0.005
$\frac{Mb^+ + MbO_2}{2}$	0.983 ± 0.002	
$\frac{MbO_2 + Mb}{2}$		1.094 ± 0.004

[a] All values are ± the standard error.

As a result of this study, a need has arisen for a model system which approximates a piece of meat in light-scattering properties, but to which known amounts of MbO_2 and Mb^+ can be added. This model system would then be valuable in making interpolations between 100% MbO_2 and 100% Mb^+.

Although the reflectance values in this study were obtained with a recording instrument, it should be pointed out that preliminary experiments with the Beckman DU spectrophotometer and reflectance attachment indicate that it gives the same results as the recording instrument for meat samples with predominantly Mb, MbO_2, or Mb^+ at the surface. Consequently, further investigations on measurement of changes in myoglobin derivatives in intact meat samples with the method described herein do not depend upon the availability of a recording spectrophotometer with reflectance accessories.

One of the observations resulting from this series of experiments was the depression of the Soret peak of myoglobin in reflectance spectra in comparison with spectra ob-

tained by transmission spectrophotometry (see Figs. 1, 2). Normally, with transmission spectrophotometry the Soret peak is about 10 times the height of the peaks in the 500–600-mμ region. One possible explanation for the depression of the Soret peaks in reflectance spectrophotometry could be the positioning of samples for total reflectance rather than diffuse reflectance. With sample holders positioned for total reflectance, all reflected light, including that reflected from the first air-glass or air-film interface, is collected by the integration sphere and measured. Consequently, about 4% of the incident light is reflected regardless of the light-absorbing characteristics of the sample, and reflectance measured on the absorbancy scale would be limited to a maximum of 1.4 or 1.5. Although this limitation exists on the height of the Soret peak measured by total reflectance, the limitation does not serve as an explanation for the relative depression of the Soret peaks in relation to the 500-to-600-mμ peaks. A few reflectance measurements on meat and on model systems with the sample holders in the diffuse position showed that eliminating the light which was nonselectively reflected did not eliminate the relative depression of the Soret peaks.

A possible hypothesis for the observed relative depression of the Soret peaks in reflectance spectrophotometry is a decrease in path length of the light as one approaches an absorbancy peak. If one assumes that the myoglobin in meat is free in solution, as is indicated by its ease of extraction and qualitatively similar absorption spectra before and after extraction, then the usual equation relating absorbancy, concentration, and path length may be valid for reflectance measurements as well as for transmission measurements. That is: $R_A = acl$ where R_A = reflectance measured on the absorbancy scale, a = an absorbancy index, c = concentration, and l = path length. The one factor in this equation which conceivably would be quite different in reflectance as opposed to transmission measurements is the path length. In reflectance measurements, it is impossible to control path length, and one would expect that the reflection spectrum would result from light which has traversed many different path lengths through portions of the sample. The average path length could be expected to vary with the concentration and absorbancy index of the pigment. Qualitatively, if the product of absorbancy index and concentration is high, the average path length will be short. If the product of absorbancy index and concentration is low, the chances of any one photon being absorbed would be less, and average path length would increase. This hypothesis would explain why R_A spectra of myoglobin derivatives in meat samples are compressed or flattened in comparison with transmission spectra of the same pigments. The same conclusion was reached by Butler (1962) for transmission spectra obtained with turbid suspensions. For transmission measurements of clear solutions, the path length is held constant. For reflectance measurements, when an absorbancy peak is being approached, not only is more light being absorbed per centimeter of path length, but the average path length traveled is also shortened as a result of the increased chances of absorption. Tbe path length shortening would lead to a depressed Soret peak compared with the 500-to-600-mμ peaks. The same hypothesis would explain the observed deviation from linearity when R_A values are plotted against percent MbO_2 or Mb^+ (Fig. 4).

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The Use of Reflectance Spectrophotometry for the Assay of Raw Meat Pigments

SUMMARY

The literature on reflectance spectrophotometry, as it applies to meat pigments, is critically reviewed, and improved methods are suggested for determination of total pigments and of the percent metmyoglobin from reflectance data on raw meat. The suggested method for total pigment was based on reflectivity of the meat samples at 525 mμ, the isobestic point for myoglobin, oxymyoglobin, and metmyoglobin. The reflectivity data, when calculated as the corresponding ratios of the absorption coefficient *K* to the scattering coefficient *S* were linearly related to total pigment extract from the meat with acidified acetone. *K/S* values of pigment-free (peroxide-treated) samples were obtained as a base line. Lowering the pH of the meat decreased the *K/S* value. This was attributed to changes in texture which increased *S*. Metmyoglobin was determined from the ratio *K/S* 572 mμ/*K/S* 525 mμ. Limiting values for the ratio were established for meat containing 100% and 0% metmyoglobin, and a linear relation was assumed between the ratios and intermediate amounts of metmyoglobin.

INTRODUCTION

To follow rapid changes in pigment composition of fresh meats, it is highly desirable that measurements be made directly on the samples, without first extracting the meat pigments. Extraction procedures are cumbersome, destroy the sample analyzed, and are likely to result in changes in the relative proportions of the three pigments, myoglobin, oxymyoglobin, and metmyoglobin. The use of reflectance spectrophotometry for quantitative measurements of pigments in solid foods is still in a rather rudimentary stage; nevertheless, this appears to be the most promising tool available for such studies. An analysis of the literature on reflectance spectrophotometry and its application to the meat situation leads us to emphasize the following points as a basis for improving current procedures.

The layer of meat used for reflectance studies must be sufficiently thick that no change in reflectance occurs upon further increasing the thickness. The reflectance of such infinitely thick samples has been termed "reflectivity" and given the symbol R_∞ in standard treatments of opaque-colored materials (Judd and Wyszecki, 1963). Whether or not a given thickness of meat meets this condition can be determined most easily by measuring its reflectivity first against a white and then against a black background. There should be no difference in the two readings in any region of the spectrum.

Absorption peaks for the three meat pigments occur at the same wave lengths in reflectance studies on meat as in transmission studies with the pigments in solution. Naughton *et al.* (1957) called attention to the similarity in general form of the absorption curves of meat and of pigment solutions. However, as compared to clear solutions, the spectra of colored solutions in turbid media tend to be less definitive. Absorption peaks are partially flattened and valleys filled in (Butler, 1962).

Isobestic points are also identical in both situations. The isobestic points of myoglobin and metmyoglobin in ground beef are clearly shown (Stewart *et al.*, 1965) and coincide with isobestic points of the same pigments in pure solutions taken from the literature. Only one isobestic point, at 525 mμ, is common to all three pigments in the visible range.

The curves shown were obtained without removing the meat sample from its position on the spectrophotometer. Considerable fluctuations in absorbance at 525 mμ, and consequently in apparent isobestic points, are obtained when one works with different portions of the same lot of ground meat, or even the same portion after removing from the spectrophotometer and repacking. The lack of homogeneity of packed samples is undoubtedly a large factor in lowering the pre-

cision obtainable with reflectance studies on meat.

Although the spectra are qualitatively similar, quantitative evaluations of meat pigments from R_∞ data present unsolved problems. Earlier studies have attempted to use absorbance, i.e., the logarithmic function of "reflectivity" in percent ($2 - \log R_\infty$) in the same way that absorbance data are used in the analysis of solutions by application of Beer's law. Naughton *et al.* (1957) presented data which seemed to show a linear relationship between absorbance and concentration of copper sulfate solutions on crystalline alumina. However, their observations extended over only a very narrow absorbance range. Naughton *et al.* (1958), working with tuna fish, and Erdman and Watts (1957), with cured meats, used absorbance ratios from reflectance studies for rough determinations of proportion of ferric to ferrous pigments.

These studies failed to take into account the fact that a fraction of the incident light at each wave length is scattered in the opaque sample. The pigment solution surrounds a matrix of meat solids. The proportion of light absorbed by the pigment to that scattered by the matrix decreases with increasing R_∞.

The ratio of the absorption coefficient (K) to the scattering coefficient (S) varies with the total light reflected according to the expression:

$$K/S = \frac{(1 - R_\infty)^2}{2 R_\infty}$$

The derivation of this Kubelka-Munk equation, the tabulated values for K/S at all values of R_∞, and some examples of the usefulness of this expression in solving problems of matching colors in paints, papers, and textiles are described by Judd and Wyszecki (1963). It should be noted that the value of K, the absorption coefficient, is not obtained from R_∞ measurements alone. To determine K, it is necessary to first compute S, which involves measurements either of the transmittance of layers of known thickness, or of the reflectance of layers of known thickness against backgrounds of known reflectance. This has not been done for meat. If it could be assumed that S is reasonably constant under a given set of experimental conditions, then K/S values should be proportional to the amount of pigment and should be usable in much the same way that absorbance values are used in the analysis of clear solutions by transmitted light. However, it might be expected that differences in structure of the meat would change the scattering coefficient S, and consequently the K/S value, for a given pigment concentration.

Wodicka (1956) first called attention to the necessity of using K/S values rather than the absorbances in studying the pigment of cured meat. He did not attempt to make quantitative measurements of the meat pigments.

Dean and Ball (1960) employed ratios of K/S values at several wave lengths to estimate the proportion of myoglobin, oxymyoglobin, and metmyoglobin in raw meats. In setting up their ratios, however, they followed Broumand *et al.* (1958) in selecting wave lengths which would not be expected to give a linear relationship between the ratio and the proportion of pigments even with solutions of pure pigments. They then made the assumption that various proportions of the pigments in meat would give the same numerical values for the K/S ratios as those obtained for absorbance ratios of the pure pigments in solution, previously calculated by Broumand *et al.* (1958).

There does not seem to be a good theoretical or experimental basis for this assumption. It must be remembered that absorption of light by meats is only partially due to the heme pigments. Meat in which the heme pigments have been completely destroyed also absorbs and scatters light throughout the visible region. This situation is analagous to that of dyeing fibers—the total K/S values for the dyed fibers must be corrected for the K/S value of the undyed fiber to arrive at useful data for dye concentrations (Mersereau and Rainard, 1951). No absorbance data are available in the literature on pigment-free raw meats.

In view of these considerations, the experimental work reported here was undertaken in an effort to refine the reflectance method as a measure both of total pigment and of the proportion of metmyoglobin to ferrous pigments (myoglobin plus oxymyoglobin).

MATERIALS AND METHODS

Preparation of meat for spectral analysis. Locally purchased cuts of beef and pork were trimmed of any external fat, then ground twice with the grinder attachment to a Kitchen Aid Mixer, Model 3-C. All of the grinder surfaces in contact with meat were either aluminum or stainless steel.

To study the spectral characteristics of pigment-free meat, 12.5 ml of 30% hydrogen peroxide and 2.5 ml of water were added to 50-g samples of the ground meat. Preliminary experiments established that this rather large amount of peroxide was necessary to bleach the heme pigments, since much of the added peroxide was decomposed by catalase in the tissues. The decomposition was accompanied by a large evolution of gas, which was allowed to escape from the meat by stirring.

The meat pigment could be converted entirely to reduced myoglobin by the addition of 0.01 to 0.02 g of sodium hydrosulfite to 50 g of meat to which had been added 15 ml of water. Once the meat and the reducing agent had been mixed, the typical absorption spectrum of reduced myoglobin persisted for at least 25 min.

For conversion to metmyoglobin, potassium ferricyanide (0.05–0.1 g) in aqueous solution was added to 50 g meat. Again the total liquid added was 15 ml. This produced a typical metmyoglobin spectrum if examined immediately, but the ferric pigment is reduced more or less rapidly in different samples of meat (Stewart *et al.*, 1965).

Spectral analysis. All spectral curves were obtained on a Bausch and Lomb recording Spectrophotometer, Model 505, with reflectance attachment. The prepared meat samples were placed in aluminum cups 7/8 in. deep and wide enough to cover the sample ports. This thickness of meat was sufficient under all conditions to satisfy the requirements for R_x measurements. The meat was covered with a glass slide which was almost optically clear in the visible region. A similar slide was used to cover the reference standard.

The usual magnesium carbonate reference representing 100% reflectance could not be used conveniently for most meat samples because the absorption of the meat was so great that peaks often exceeded an absorbance of 1 and the scale had to be changed during the recording. For this reason, the meat spectra were actually recorded either against a hydrogen-peroxide-bleached sample of the same meat or, in later experiments, against a piece of gray cardboard. However, the absorbance of the bleached meat or cardboard against the magnesium carbonate standard was always added to that of the meat at each wave length under consideration so that the values presented throughout the paper represent the total absorption of the meat against magnesium carbonate.

The spectrophotometer was set to record in absorbance units rather than percent reflectance. To obtain K/S values, the readings were first converted to R_x from standard tables relating absorbance to percent transmittance. The K/S value corresponding to each R_∞ was then read from Table D in the appendix of Judd and Wyszecki (1963).

RESULTS AND DISCUSSION

Spectra of bleached meat. Spectra were obtained for 16 samples of bleached meat from rib, chuck, and round of beef and pork loin. All were qualitatively similar, showing no peaks but a gradual increase in absorption from longer to shorter wave lengths in the spectral region between 700 and 450 mμ. The absorbance at 525 mμ of the sixteen samples varied from 0.18 to 0.47, averaging 0.33. A typical curve having an absorbance of 0.30 at 525 mμ, had values of 0.20 at 700 mμ and 0.41 at 450 mμ. Although the absorbance of pork samples averaged less than that of beef, there were not sufficient samples to be sure that this was a consistent or significant difference. Considerable variation could be obtained even in the spectra of different portions of the same lot of meat, probably due largely to differences in air trapped in the samples. Bleached cooked meat showed spectra of the same general shape, but always with higher absorbances than the corresponding raw samples.

K/S at 525 mμ as a measure of total pigment. Since 525 mμ is isobestic for all three meat pigments, the reflectivity values at this wave length, expressed as the ratio K/S, might be expected to show some correlation with the total amount of heme pigments in the meat.

The total pigment from a number of samples of beef and pork was extracted with acidified acetone according to the method of Hornsey (1956). The assays were made in duplicate and averaged. The total pigment, expressed as parts per million of hematin, was plotted (Fig. 1) against the K/S values obtained from spectral curves of meat samples to which 15 ml water had been added to 50 g meat. This large amount of water was necessary in the original experimental work

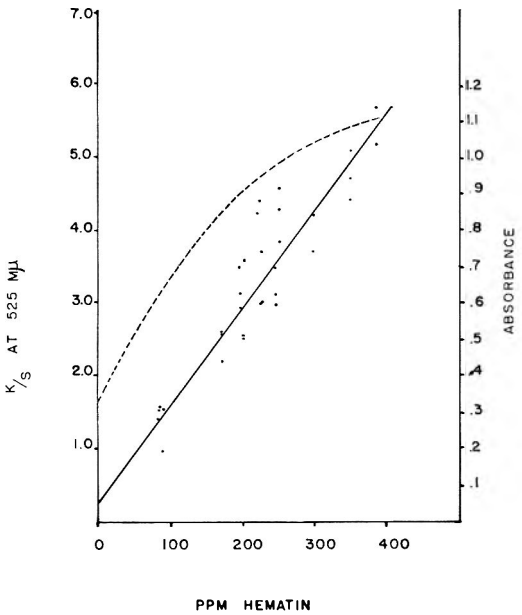

Fig. 1. Hematin concentration of pork and beef samples as related to K/S values at 525 mμ (—) and to absorbance values at 525 mμ (- - -).

to accommodate the amounts of peroxide, ferricyanide, etc., used to modify the pigments. However, it made the meat samples too soft to handle easily, and certainly would not be recommended for routine use. Some of the meat samples had been completely reduced, others oxidized; and in still others the pigment was largely oxymyoglobin. The distribution of points on the plot was not related to the oxidation state of the pigment; therefore, the pigment forms are not differentiated in Fig. 1. The K/S value plotted at zero hematin concentration was that obtained from the average absorbance of the sixteen bleached samples (absorbance 0.33, K/S = 0.30). Shown on the same figure (without experimental points) is the curve drawn through the same data when plotted as absorbance rather than K/S values.

Several conclusions can be drawn from these data. There is a positive correlation between the readings at 525 mμ and total pigment. Expressed as absorbance values, the relation is definitely nonlinear. When plotted as K/S, a straight line appears to best fit the data. The rather large scattering of experimental points is evidence of the lack of precision of the method. It must be remembered, however, that the samples represent a number of cuts, both of beef and pork, varying in pH, fat, connective tissue, etc., as well as in pigment.

It might be expected that any treatment or condition which significantly affects the texture of meat might also affect the scattering coefficient S and thus the relation between the K/S values at 525 and the actual concentration of pigment present. The large effect of changes of pH on meat texture is well known (Bate-Smith, 1948). As the pH is lowered, meat fibrils tend to shrink and scatter light. Meat of the same actual pigment content appears to be lighter in color.

Table 1. Effect of pH adjustment on K/S at 525 mμ.

pH	K/S 525 mμ
5.1	2.37
5.45	3.13
5.8	3.95
6.5	4.42
7.1	4.31

Table 1 shows the effect of varying the pH of a single sample of ground beef on the K/S values at 525 mμ. The original pH of the sample was 5.8; it was treated with 5N NaOH or 1N HCl to give the pH values recorded in Table 1. All samples had the same amount of total liquid added (5 ml to 100 g). Obviously, if there are large fluctuations in pH, unadjusted K/S values are not a useful measure of total pigment. Since pH measurements were made on only a few of the samples analyzed for total pigment (Fig. 1), it is not possible to say to what extent variations in pH were responsible for the scattering of experimental values. Normal variations in pH are not as wide as those shown in Table 1. The range in 20 samples of rib eye from different animals was from pH 5.5 to 5.8. Swift *et al.* (1960) reported that the pH of eight different muscles in one animal varied from 5.53 to 6.09. It is possible that a correction factor, based on more extensive data of the kind shown in Table 1, could be applied to adjust K/S to the expected value at some arbitrarily chosen pH.

In spite of the many possible sources of error, the method appears to offer sufficient

promise to warrant further testing on untreated ground meat or surfaces of whole cuts. It is possible that the precision could be improved if the method were applied to more uniform material, or if corrections were applied for pH variations.

Proportion of metmyoglobin to ferrous pigments. Examination of the absorption spectra of identical concentrations of the three meat pigments, reproduced from Bowen's original data by Dean and Ball (1960) and measured independently by Taylor (1961), show, in addition to the isobestic point for all three pigments at 525 mμ, an isobestic point for myoglobin and oxymyoglobin at 572 or 573 mμ. At these latter wave lengths, the absorption of metmyoglobin is very much less than that of the two ferrous pigments. The ratio of absorbances at 572/525, when plotted against percent of the total pigment as metmyoglobin, gives a straight line, in contrast to the curve obtained by plotting a similar ratio at 507/573, as was done by Broumand *et al.* (1958).

While it is not experimentally feasible to work with known mixtures of pigments in meats, it is easily possible to examine portions of the same meat in which the pigments have been converted entirely to metmyoglobin on the one hand or to reduced myoglobin on the other by treatment with ferricyanide and hydrosulfite, respectively.

Table 2. Ratios of K/S at 572 mμ to K/S at 525 mμ for known pigments.

Pigment	No. of Samples	Ratio		Standard deviation
		Range	Av.	
Metmyoglobin	19	0.51-0.65	0.56	.04
Reduced myoglobin	20	1.13-1.61	1.40	.16

Table 2 summarizes K/S ratios at 572 mμ/525 mμ calculated from the reflectance curves of a number of samples of ground beef. Again, there is a considerable spread of experimental points around the average values. The average values for the K/S ratios of reduced myoglobin are practically identical with the absorbance ratios of pure pigment solutions whereas the metmyoglobin K/S ratios are definitely higher than absorbance figures (calculating from Bowen's data, the ratios at 572 mμ/525 mμ are approximately 1.38 for the ferrous pigments and 0.40 for metmyoglobin; from Taylor's data, the corresponding ratios are 1.45 and 0.49). It should be emphasized that any agreement between the numerical values for the K/S and absorbance ratios is fortuitous. If the wave lengths selected for comparison had been nearer the maxima and minima of the absorption curves, the discrepancies would be much greater.

The K/S ratios for the same meat samples at the wave lengths used by Dean and Ball (1960), i.e., 507 mμ/573 mμ, gave an average value for reduced myoglobin of 0.50 (range 0.41–0.62). This average is close to the corresponding absorbance ratio, approximately 0.54, of reduced myoglobin in solution. On the other hand, the average K/S ratio for metmyoglobin at these wave lengths (close to the peak and valley of the metmyoglobin curve) was 1.86 (range 1.35–2.20) as compared to 3.3 for 100% metmyoglobin in solution. This large discrepancy emphasizes the danger of using absorbance ratios obtained on pure pigment solutions to predict the proportion of pigments corresponding to K/S ratios in meat.

In using the K/S ratios at 572 mμ/525 mμ, a linear relation is assumed between the ratios and the percent metmyoglobin in the meat sample (Fig. 2). A linear relation would be expected at this choice of wave

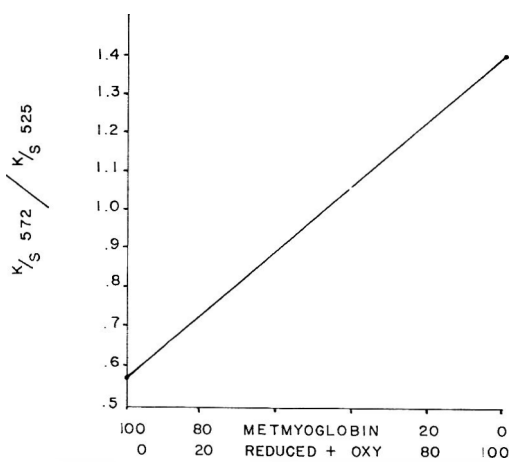

Fig. 2. Assumed linearity between limiting K/S ratios at 572 mμ/525 mμ for 0 and 100% metmyoglobin.

lengths, although the relationship cannot be experimentally verified in meats, nor have we been able to find in the literature a similar treatment of analogous situations. Snyder (1964) reports absorbance data on known mixtures of two heme pigments in milk. A comparison of K/S ratios in such a system would be enlightening.

In using this assumed relationship to determine metmyoglobin in meat, the wide spread of experimental points which have been averaged to obtain the limiting values in Fig. 2 must be kept in mind. Again, more precise results might be obtained on individual samples of meat by determining the limiting K/S ratios for 0 and 100% metmyoglobin for this particular meat, and calculating intermediate states of pigment reduction from these rather than from the averages of Fig. 2.

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Viscometric Behavior of Tomato Concentrates

SUMMARY

Tomato purées have a pseudoplastic non-Newtonian behavior which agrees reasonably well with the power-law model over substantial ranges of shear rates. Behavior is more consistent at higher shear rates (above 300 or 400 sec^{-1}), with values of the power-law exponent in the neighborhood of 0.4 over a wide range of concentrations and temperatures. Both concentration and temperature primarily affect the power-law coefficient rather than the exponent. Viscosity correlations in the lower shear-rate region are uncertain because of somewhat variable behavior, probably resulting from yield stresses, wall effects, and time dependency. Apparent viscosities are much higher in concentrates produced by direct evaporation than in concentrates made by centrifuging out the insoluble solids, concentrating the serum, and then reconstituting.

INTRODUCTION

There is a considerable volume of literature in which the viscosity or consistency characteristics of fruit and vegetable products are related to processing conditions or other physical properties (Hand *et al.*, 1955; Robinson *et al.*, 1956; Whittenberger and Nutting, 1957, 1958; Smit and Nortjé, 1958; Ezell, 1959). In most cases, however, the viscosities or consistencies reported are the results of single-point measurements; that is, a single viscosity or consistometer time is given for each sample. Although the investigators have generally recognized that single-point viscosity measurements have only limited value for non-Newtonian fluids such as tomato juices and concentrates, lack of time or suitable instrumentation has prevented more complete studies. Ideally, viscometric behavior of these fluids should be presented in the form of a curve of shear stress vs. shear rate for each sample. In this form, a Newtonian fluid will appear as a straight line having a slope equal to the viscosity. Most liquid food materials, including tomato products, fall into the pseudoplastic classification, which yields curves with slopes that decrease as shear rate increases. This behavior thus corresponds to lower apparent viscosities at higher shear rates (or flow rates).

If shear stress is plotted against shear rate on logarithmic coordinates, experimental data for many pseudoplastic fluids fall on substantially straight lines over a wide range of shear rates. Such lines can be represented by the power-law expression

$$\tau = K\gamma^n$$

in which τ is the shear stress, γ is the shear rate, and K and n are constants that are characteristic of the particular material. In the limiting case of a Newtonian fluid, n is equal to unity and K becomes the ordinary viscosity. The constant n is therefore a measure of the extent of departure from Newtonian behavior and is sometimes called the flow-behavior index. The constant K is more a measure of viscosity or consistency, and may be termed the fluid-consistency coefficient. Considerable literature has dealt with heat-transfer and flow problems for non-Newtonian fluids on the basis of the power-law model. Use of these results, either for process design or for analysis of processing plant operations, requires values of K and n for the material in question. Unfortunately, very little of the data published for tomato or other food products are complete enough for such purposes. Some data of this type have been presented by Charm and Merrill (1959), Charm (1960), and Harper (1960), along with more complete discussions of non-Newtonian phenomena.

More complete viscometric data, besides being applicable to engineering design of equipment, should have advantages over usual consistency measurements in characterizing product quality. No investigations have yet been reported on this subject, but developments in this direction are to be ex-

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pected from the increasing use of better viscosity measuring methods and the accumulation of published data. This paper presents the results of a study of the viscometric behavior of a series of tomato purées ranging from unconcentrated pulp up to 30% total solids. Also presented are the effects on viscosity of separating insoluble solids by centrifuging, followed by recombination.

EXPERIMENTAL

Material preparation. Fresh tomatoes of the VF6 variety were used, with the following raw-material analysis:

Degrees Brix at 20°C	5.27
pH	4.3
Total acids, %	0.379

The washed tomatoes were held 10 sec at 212°F in a hot-break operation, and were pumped through a paddle pulper with a 0.060-in. screen and a paddle finisher with a 0.033-in. screen. The resulting product was heated to 190°F in a scraped-wall heat exchanger and hot-filled into No. 2½ cans. The cans were cooked with steam for 12 min at 212°F, followed by cooling in running water. Four different concentrates, ranging from 12.8 to 30.0% total solids, were prepared from the juice in a conventional vacuum pan at 110°F. Total solids were determined by vacuum-oven drying according to the NCA procedure (1950). Another lot of the juice was centrifuged for separation of the insoluble solids; the serum obtained was concentrated to 62.5% total solids in the vacuum pan.

Viscosity measurements were made on the juice and concentrates and on samples of corresponding concentration reconstituted both from the 30% concentrate and from the pulp and serum. Concentrations of the reconstituted samples were adjusted by refractometer readings.

Viscometric measurements. The flow-behavior curves were obtained with a coaxial-cylinder viscometer, which is described in detail elsewhere (Harper, 1961). The cylinders are about 6 in. long by 3 in. in diameter. The speed of rotation of the outer cylinder can be varied continuously and automatically from zero to maximum and back to zero. Both the torque transmitted to the inner cylinder and the speed of rotation are given as electrical signals and recorded on an X–Y plotter. At the time the work reported here was done, the viscometer had not been completed in the form described above. As a result, the measurements on the tomatoes were made only with an 0.08-inch gap, and the useful range of shear rates lay between about 30 and 900 sec^{-1}. The tomato serum measurements were made with a 0.01-in. gap, and the shear-rate range was correspondingly larger. Flow-behavior curves were obtained at 90, 120, 150, and 180°F.

Thixotropy in a fluid will yield on the recorder a descending-speed curve that falls below the ascending-speed curve. But such behavior can also result from other causes, such as a temperature rise in the fluid from viscous dissipation or a permanent breakdown of fluid from the shearing action of the viscometer. Therefore, any assumption of thixotropy must be made with caution. To minimize effects of temperature rise and fluid breakdown, and also to ensure that the gap remained full at all times, each sample was introduced continuously into the viscometer at a low rate during the measurement period. Tests showed that this small flow of material through the gap did not, in itself, affect the torque reading.

The viscometer has an inherent accuracy of about one per cent. Because of uncertainties caused by temperature, fluid breakdown, and possible time dependency effects, errors as high as five per cent may be present in the measured viscosities.

RESULTS AND DISCUSSION

The flow-behavior curves all showed a strong pseudoplastic characteristic. In general, the ascending and descending curves were reasonably close together. In view of the uncertainties discussed above, no definite statement can be made regarding thixotropy. Many times, the ascending curves were somewhat erratic at the low-speed end with the more concentrated materials. This behavior is quite dependent on the rate at which the speed is increased, and subsequent measurements with a variety of materials have shown that it is related to the existence of a yield stress. Descending curves represented more nearly an equilibrium condition, and these were consequently used as a basis for analysis. No quantitative yield stress measurements were made. Again, subsequent measurements have shown that the effects of yield stresses on flow behavior curves are evident primarily at shear rates lower than those studied here.

Results for the unconcentrated pulp and the various concentrates are plotted on logarithmic coordinates in Fig. 1 as apparent viscosity against shear rate. Apparent viscosity, by analogy to Newtonian fluids, is

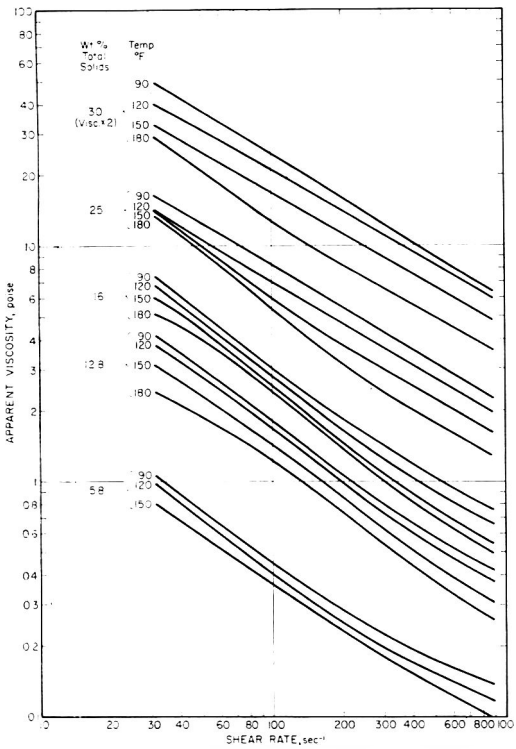

Fig. 1. Apparent viscosities of tomato concentrates.

defined as the ratio of shear stress to shear rate. For power-law fluids,

$$\mu_a = \tau/\gamma = K\gamma^{n-1}$$

A logarithmic plot of apparent viscosity against shear rate for a power-law fluid yields a straight line for which $n - 1$ is equal to the slope and K is equal to the apparent viscosity at a shear rate of 1 sec^{-1}. The lines in Fig. 1 show some curvature, although they can be approximated by straight lines over considerable portions of their lengths. At higher concentration, some of the lines are straight over the entire length. There is no consistent trend to the form of curvature of the various lines, and it does not appear possible to develop any more exact relationship to represent the behavior. Several equations other than the power law were examined, including those of Ree and Eyring (1955) and Casson (1959), but most such equations are more difficult to use and none give as good over-all agreement with the experimental data as the power law. It is obviously impossible to determine unique constants for any expression other than a power law to represent a straight line on logarithmic coordinates. The relatively small deviations from straight lines that occur in the present results are of such a varied nature that it is impossible to find any general representation other than the power law. Design and analysis methods based on the power-law model may be used for flow curves such as these if values of n and K are calculated for an appropriate range of shear rates.

In Fig. 1 the behavior appears to be much more consistent in the region of higher shear rates. This result has been confirmed by more recent results (Harper and Lebermann, 1962; Morrison, 1963) showing that behavior at low shear rates may be strongly influenced by yield phenomena, time dependency, and wall effects. Accordingly, analysis of the results is based on the region of high shear rate. Table 1 lists apparent viscosities at a shear rate of 500 sec^{-1} and and values of n and K computed from lines in the region of 500 to 800 sec^{-1}. As pointed out above, values of K are obtained by extrapolation of these lines to a shear rate of 1 sec^{-1}, and therefore are strongly influenced by small changes in slope. For several pairs of fluids, it will be observed that the one with a lower apparent viscosity at 500 sec^{-1} has a higher value of K. In order to compare the different fluids, it therefore appears desirable to use the apparent viscosity rather than K.

An appreciation may be gained of the magnitude of the shear-rate range used by consideration of a power-law fluid in a round tube. The pressure drop for laminar flow is given by the well-known equation

$$\frac{D\Delta P}{4L} = K\left(\frac{1+3n}{4n}\right)^n \left(\frac{8V}{D}\right)^n$$

where ΔP is the pressure drop, D the diameter, L the length, and V the average velocity. For n equal to unity, the equation reduces to Poiseuille's Law. The shear rate in such flow varies from a maximum at the wall to zero at the center. An effective shear rate might be taken as that corresponding to an apparent viscosity which would give

Table 1. Viscosity constants of tomato juice and concentrates.

Total solids wt. %	Temp. (°F)	μ_a, poise ($\gamma = 500$ sec^{-1})	n ($\gamma = 500 - 800$ sec^{-1})	K, dyne sec^n cm^{-2}
5.8	90	0.175	0.59	2.23
	120	0.153	0.54	2.7
	150	0.135	0.47	3.7
12.8	90	0.58	0.43	20
	120	0.52	0.425	18.8
	150	0.435	0.335	22.8
	180	0.37	0.345	21.2
16.0	90	1.03	0.45	31.6
	120	0.9	0.45	27.7
	150	0.76	0.40	31.8
	180	0.70	0.38	32.7
25.0	90	3.17	0.405	129
	120	2.75	0.415	105
	150	2.24	0.43	80
	180	1.77	0.43	61
30.0	90	4.5	0.40	187
	120	4.15	0.42	151
	150	3.35	0.43	117
	180	2.5	0.445	79

the correct pressure drop when substituted into the Poiseuille's Law expression. It is readily shown that this shear rate is given by the equation

$$\gamma = \left(\frac{1+3n}{4n}\right)^{\frac{n}{n-1}} \left(\frac{8V}{D}\right)$$

An example taken from actual cannery operation in production of tomato catsup shows a flow rate of from 40 to 100 gal. per min. in a 3-inch line. Using a value of n of 0.4, the above expression shows a corresponding range of shear rates of about 500 to 1100 sec^{-1}. The wall shear rates are about 70% higher. It thus appears that the shear-rate range selected for analysis of the experimental data is appropriate for application to typical flow situations. Common consistency measurements usually correspond to much lower shear rates, and these apparent viscosities might not correlate well, for example, with Bostwick consistency.

The results in Table 1 show that, except in the unconcentrated pulp, there is no regular trend of n with either temperature or concentration. A large part of the variation in n can be attributed to limitations in the accuracy of the experimental data. The viscometer is accurate to about 1% with Newtonian fluids, but, with food materials, time dependency, temperature effects, and irreversible breakdown lead to a higher order of uncertainty. In this regard, tomato purées appear to be much more troublesome than fruit purées. A relatively small change in the recorded experimental curve in the region of high shear rate has an important effect on n, and therefore on K. Apparent viscosities calculated for a shear rate in the range of interest are not nearly as sensitive to variations in experimental data, and form a better basis of comparison than values of K.

Apparent viscosities for the different concentrations are plotted in Fig. 2 against the reciprocal of the absolute temperature. The data agree well with straight lines of the same slope. It is believed that the deviations shown by the two higher concentrations more likely result from experimental error

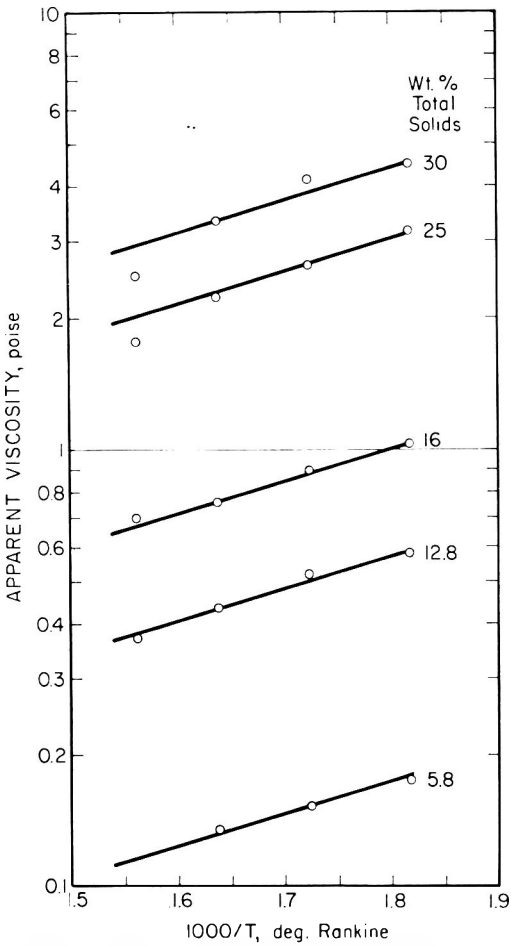

Fig. 2. Effect of temperature on apparent viscosity of tomato concentrates.

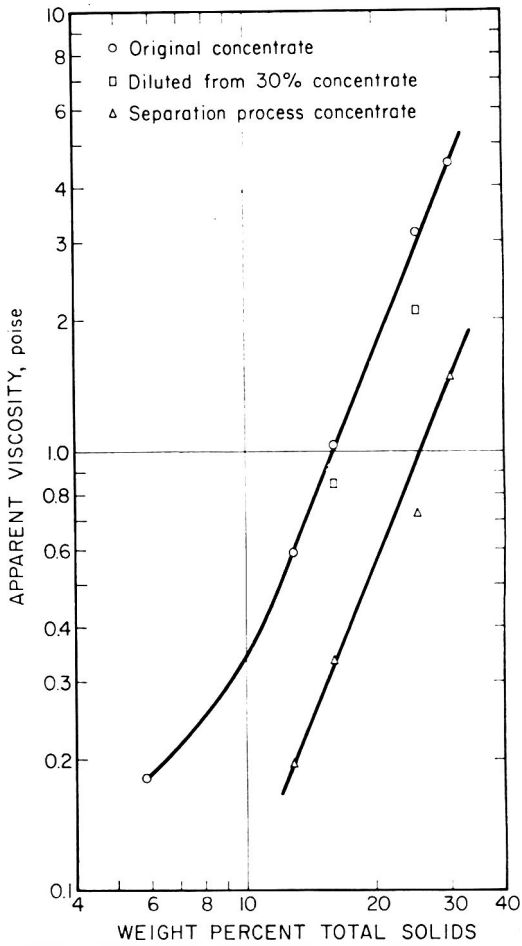

Fig. 3. Effect of concentration on apparent viscosity of tomato concentrates.

than from any actual differences in behavior. These lines can be represented by an equation of the form:

$$\mu_a = \mu_0 e^{1710/T}$$

where T is deg Rankine (deg F + 460). These materials show a considerably lower viscosity-temperature coefficient than water, for which the numerical constant in the above equation for this temperature range is 3070. Viscosities at 90°F, taken from the lines in Fig. 2, are plotted against concentration in Fig. 3. It can be seen that the four higher concentrations fall on a straight line on the logarithmic plot. It is to be expected that the slope of the curve will decrease at lower concentrations, since the viscosity approaches the constant value of water as the concentration goes to zero. The lower line in Fig. 3 represents the data for samples reconstituted from the centrifuged pulp and serum. The straight-line portion of the upper curve can be represented by the equation

$$\mu_a = 81.4C^{2.4}$$

where C is the weight fraction of total solids. The concentration and temperature effect can be combined into a single equation:

$$\mu_a = 3.62C^{2.4}e^{1710/T}$$

In this equation, the numerical coefficient will vary with the source of the raw material and the method of processing. The exponents that express the effects of concentration and temperature should be relatively independent of the material. As a comparison, the data of Harper and Lebermann (1962) for pear purées show the concentra-

tion exponent to be about 2.8 and the temperature exponent to range from 1600 to 3100, depending on concentration. The data of Charm and Merrill (1959) for applesauce and banana purée can be represented with a temperature exponent of 2100.

It is to be expected that viscosities will depend to a certain extent on the evaporation procedure. The magnitude of this effect can be observed from viscosity measurements on samples diluted from higher concentrations. Fig. 3 gives points representing the viscosities of 25 and 16% total solids concentrates prepared by dilution from 30% concentrate. The viscosity of the 25% sample appears to be inconsistent with the other data in the figure, and it is probable that some error was made. Only a small error in concentration will have a substantial effect on the viscosity correlation.

The serum obtained by centrifuging became increasingly non-Newtonian at higher concentrations, showing good agreement with the power law over the entire concentration range. Table 2 lists values of n, K, and apparent viscosity for both the serum and the reconstituted samples. The apparent viscosities of the reconstituted samples are only about one-third those of the corresponding original concentrates, so that this method of producing concentrates does not appear attractive. A similar result was observed by Mannheim and Kopelman (1964), who prepared a 22% tomato concentrate both by straight evaporation and by the centrifuge-separation procedure, each in both a scraped-surface vacuum evaporator and an atmospheric-pressure open-pan evaporator. The 22% concentrate was then reconstituted to the original juice concentration, and apparent viscosities were determined in a Brookfield viscometer. With vacuum evaporation, they found that the apparent viscosity of the centrifuged material had been reduced almost to half that of the material from the conventional process. With atmospheric evaporation, the conventionally processed material also had a low viscosity as a result of the prolonged heating at elevated temperatures.

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Table 2. Viscosity constants of tomato serum and reconstituted samples at 90°F.

Total solids (wt. %)	μ_a, poise ($\gamma = 500$ sec^{-1})	n	K, dyne sec^n cm^{-2} ($\gamma = 500 - 800$ sec^{-1})
Serum			
5.8 [a]	0.018	0.95	0.0247
12.8 [a]	0.044	0.91	0.0764
16.0 [a]	0.072	0.83	0.207
25.0 [a]	0.147	0.77	0.616
30.0 [a]	0.38	0.67	2.95
62.5	5.1	0.415	193.0
Reconstituted concentrate			
12.8	0.195	0.64	1.84
16.0	0.334	0.58	4.54
25.0	0.725	0.46	20.9
30.0	1.48	0.435	50.0

[a] Serum corresponding to stated concentration of total solids in reconstituted sample.

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A Comparison of Glycolysis and Associated Changes in Light and Dark Portions of the Porcine Semitendinosus

SUMMARY

Post-mortem glycolysis and related properties in light and dark portions of porcine semitendinosus were studied to determine differences in composition as well as rate and extent of change at two post-mortem holding temperatures. These post-mortem changes in the light and dark portions of the semitendinosus were studied in animals evidencing a death reaction, after insensibilization by a captive-bolt pistol, as well as in animals which were insensibilized by sodium pento-barbital which yielded no evident death reaction. Dark-muscle portions contained higher initial values for pH and glycogen and exhibited a shorter delay phase of rigor mortis. Lowering the post-mortem holding temperatures from 37 to 4°C slowed post-mortem glycolysis and increased the water-binding capacity in both muscle portions. Lactic acid concentrations (24 hr) in both portions were similar at both post-mortem holding temperatures. Insensibilization with sodium pento-barbital slowed post-mortem glycolysis but increased the accumulation of lactic acid in both portions. The delay phase of rigor mortis from animals injected with sodium pento-barbital was lengthened more in the light portions than in the dark portions. These studies indicate that only light muscle portions have an increased glycolytic rate which may be attributable to the struggle associated with the death reaction upon insensibilization with a captive-bolt pistol.

INTRODUCTION

Muscle fibers, although varying widely in size and composition, have been categorized on a physicochemical basis into red (dark) and white (light) groups. Red fibers are highly pigmented and contain a high oxidative enzyme activity, whereas white fibers have a high phosphorylase and/or glycolytic activity (Perry, 1956; Ogata, 1960; Beatty *et al.*, 1963). Consequently, there are major differences in metabolism in these two types of muscle fibers. Red skeletal muscle contracts slowly and tonically, while white skeletal muscle contracts rapidly and strongly (Szent-Györgyi, 1953). Intact muscles are considered mixtures of red and white fibers, the color of the muscle depending on the predominant type of fiber (Denny-Brown, 1929).

The porcine semitendinosus is a unique muscle in that it contains a portion of predominantly white (poorly pigmented) fibers and a portion of predominantly red (highly pigmented) fibers (Cassens *et al.*, 1963; Beecher *et al.*, 1963). Beecher *et al.* (1964) have noted, in a comparative histological study, that the light portion contains less than 20% dark or red fibers, whereas the dark portion of the porcine semitendinosus contains over 40% dark or red fibers. The porcine semitendinosus, therefore, although thought to function uniformly as a single muscle, possesses two portions with marked differences in physical and chemical properties. The study of post-mortem glycolysis in these two portions of the same muscle, which differ so markedly in red fiber content, offers a unique opportunity to closely estimate comparative differences in post-mortem glycolysis which are attributable to red and white fiber distribution. The effect of the death reaction (visible struggle and muscular contraction) immediately after insensibilization and exsanguination, and the effect of post-mortem holding temperature on post-mortem glycolysis in red and white portions can also be more specifically ascertained than has been done previously in studies involving distinctly different red and white muscles of the carcass.

Studies were made: 1) to investigate post-mortem glycolysis and related properties in light and dark portions of the porcine semitendinosus; 2) to compare the effect of post-mortem temperature on the rate and extent of glycolysis in each portion; and 3) to compare the rate and extent of post-mortem glycolysis in light and dark portions of the porcine semitendinosus in animals evidencing a death reaction, after insensibilization

by a captive-bolt pistol, with the rate and extent of post-mortem glycolysis in light and dark portions of the porcine semitendinosus in animals insensibilized by sodium pentobarbital which showed no evident death reaction.

EXPERIMENTAL

In the first phase of this study, 10 Chester White and 10 Poland China pigs were insensibilized with a captive-bolt pistol and exsanquinated.

Both semitendinosus muscles were excised immediately after exsanguination. Samples of both portions (light and dark) of the right semitendinosus were frozen and stored in a liquid-nitrogen refrigerator for subsequent analyses. Remaining muscle portions were placed in a 4°C constant atmosphere for 24 hr. The time course of rigor mortis was determined in each portion of the left semitendinosus muscles with a "rigorometer" apparatus as described by Briskey *et al.* (1962). Remaining portions of the left semitendinosus muscles were placed in a 37° constant-humidity chamber for 3 hr and then transferred to a 4°C chamber.

pH values were determined on all samples at 0, ½, 1, 2, 3, and 24 hr post-mortem by placing a combination glass-calomel electrode directly on the freshly cut cross-sectional surface of the muscle fibers. Objective color measurements were made at 0, ½, 1, 2, 3 and 24 hr post-mortem on the freshly cut surfaces of all muscle samples, with a Bausch and Lomb "Spectronic 20" colorimeter with reflectance attachment. The color values (485 mμ) are reported as percent reflectance based on solid magnesium carbonate as 100% reflectance. Glycogen (Dubois *et al.*, 1956) and lactic acid (Barker and Summerson, 1941) concentrations were determined on samples which were frozen at each post-mortem interval, except that samples for glycogen analyses were not taken at 24 hr post-mortem. Water-binding capacity (24 hr) was measured by the filter-paper absorption technique of Grau and Hamm (1953) as modified by Briskey *et al.* (1959). The results are expressed as the ratio of the water area to the meat film area. A larger ratio thus indicates an increase in the quantity of expressible juice or a decrease in the ability of the muscle to "bind" water.

In the second phase, similar experimental procedures were followed on a group of 3 Chester White and 3 Poland China pigs, insensibilized with intravenous injections of sodium pento-barbital (9.5 mg/kg) and exsanguinated 30 min post-injection. Results were analyzed by analysis of variance (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Since breed differences were not significant, results from animals of both breeds were combined and statistically analyzed according to muscle portion and holding temperature.

Phase I. *Properties of light and dark muscle portions.* pH values at the time of death, from animals insensibilized by a captive-bolt pistol, were slightly higher in dark portions of the semitendinosus muscle than in light portions (Table 1). As indicated by

Table 1. Properties of light and dark muscle portions.

Characteristic	Muscle portion			
	Light		Dark	
	$\bar{x}$[a]	$s_{\bar{x}}$[b]	$\bar{x}$[a]	$s_{\bar{x}}$[b]
Initial pH	6.11	.05	6.21	.04
Initial glycogen[c]	2.82	.31	4.14	.43
Initial lactic acid[d]	21.6	1.4	21.4	1.2
Rigor mortis (delay phase min)	81.9	10.4	39.6	6.4

[a] Mean value.
[b] Standard error of the mean.
[c] Mg/g tissue.
[d] Mg/g dry tissue.

Briskey (1963) the *in vivo* pH values of porcine muscles are not known; however, on the assumption that the *in vivo* pH values are similar, the slight initial difference of 0.1 pH unit between muscle portions may be due to differences in initial glycolytic rate (Bate-Smith, 1948). Another possible explanation is that the death reaction may be more severe in the light portions than in dark portions. If the death reaction were more severe in the light portion and resulted in greater contraction the pH would be lower (Bendall, 1951). Cori (1956) has also postulated that contraction of this type would markedly affect ATP breakdown and concomitant pH values. Another explanation for the initial pH differences might be found in the work of Infantee *et al.* (1964), who demonstrated a reduced rate of ATP breakdown in stretched fibers. It is conceivable that the vertical suspension of the carcass creates more tension on the dark than on the light portion of the semitendinosus; this would

result in greater stretch, reduced ATP breakdown, and higher initial pH values.

The initial glycogen levels were significantly higher ($P < .05$) in the dark portions than in the light portions, whereas there were no significant differences in lactic acid concentrations between the light and dark portions at death. These findings are in disagreement with the report of Beatty *et al.* (1963) that glycogen was higher in white than red muscle at death. However, in the latter study the death reaction was not taken into consideration. Higher glycogen levels in the dark portions of control animals were also noted by Beecher *et al.* (1963). Beecher *et al.* (1963) observed, however, that when the animals were subjected to ante-mortem temperature stress conditions immediately prior to death, the light portions exceeded the dark portions in glycogen concentration. These combined findings may imply that the glycogen in the dark portion is broken rapidly by ante-mortem stress under aerobic conditions, whereas the glycogen in the light portion may be more sensitive to the death reaction. If the light portion was more sensitive to the death reaction and actually experienced a greater glycogen breakdown before the initial samples were taken, the similarity between lactic acid levels in the two portions at this time period could be explained on the assumption that a greater quantity of glycolytic intermediates accumulated in the light portion.

The duration of the delay phase of rigor mortis in animals insensibilized by a captive-bolt pistol, was only one-half ($P < .01$) as long in the dark portions as in the light portions (Table 1). These results are similar to those reported by Briskey *et al.* (1962) for porcine semitendinosus. Lawrie (1952) also noted similar rigor mortis durations in separate light and dark muscles. It may, therefore, be inferred that the rigor mortis features of the dark portion of the semitendinosus are comparatively similar to other typically dark muscles.

Effect of post-mortem temperature on pH decline. Fig. 1 shows the rates of post-mortem pH decline in the light and dark portions (animals insensibilized by a captive-bolt pistol) at 4 and 37°C. The 4°C temperature retarded the rate of pH decline below that at 37°C, which is in agreement

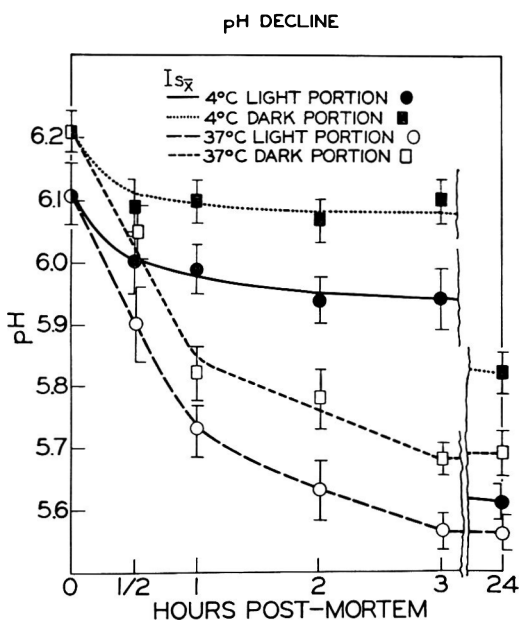

Fig. 1. pH changes in light and dark muscle portions held at 37°C and 4°C post-mortem (all animals insensibilized with a captive-bolt pistol).

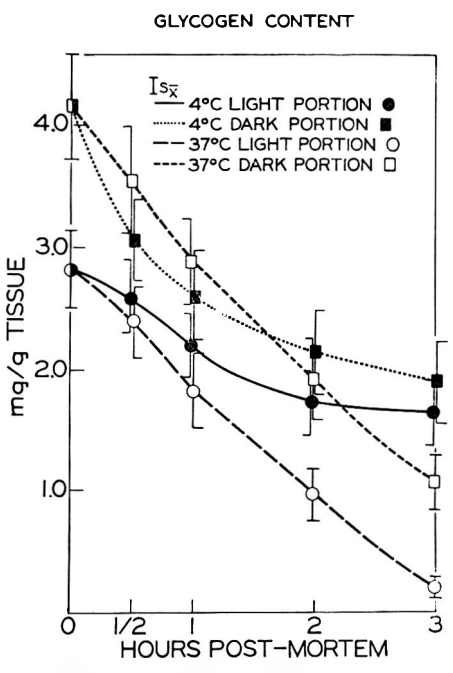

Fig. 2. Glycogen depletion in light and dark muscle portions held at 37°C and 4°C post-mortem (all animals insensibilized with a captive-bolt pistol).

with the findings of Wismer-Pedersen and Briskey (1961) using longissimus dorsi muscle of the Landrace pig. The present findings, however, are new in that they show the effect of temperature to be similar in retardation of pH decline in both light and dark muscle portions from pigs of two different breeds. By 3 hr post-mortem, pH values had declined to their ultimate (24 hr) levels in portions held at 37°C, but had decreased only .2 pH unit from initial pH levels in portions held at 4°C. pH values at 24 hr post-mortem in dark portions held at 37°C were significantly ($P < .05$) lower than pH values in dark portions held at 4°C. This is particularly interesting since no significant differences were observed between the 24-hr pH values of the light portions at 4° and 37°C. The lower 24-hr pH values in the dark portions held at 37°C, even though the lactic acid concentrations were similar (Table 2), may be explained by greater ATP breakdown at this high temperature as shown by Cassens *et al.* (1964). A greater accumulation of glycolytic intermediates in the dark portions at 37°C, which had faster glycolytic rates, would also contribute to this difference in pH level and partially explain why temperature did not have a significant effect on the 24-hr pH in the light portions, since in the latter case the glycolytic rate was relatively rapid regardless of temperature. Within each temperature treatment, ultimate pH values were significantly ($P < .05$) higher in the dark portions than in the light portions. This again may be due to differences between the muscle portions in buffer capacity, ATP breakdown and glycolytic intermediate accumulation.

Bate-Smith (1948) and Bendall (1951) pointed out that a normal rate of post-mortem anaerobic glycolysis usually permits marked reduction in muscle temperature before substantial acidity develops. Extreme variations, however, have been noted in rate of pH decrease in porcine muscle immediately post-mortem (Briskey and Wismer-Pedersen, 1961; Sayre *et al.*, 1963). These variations permit combinations of pH and temperature which may be detrimental to the gross morphology, color, and protein solubility of the tissue (Wismer-Pedersen and Briskey, 1961; Sayre and Briskey, 1963). Briskey and Wismer-Pedersen (1961) originally described four types of post-mortem pH patterns. When muscle temperatures were kept at 37°C for 3 hr, pH decreased relatively rapidly (Wismer-Pedersen and Briskey, 1961; Briskey, 1963). It is pertinent to point out that in the present study the dark portions started with a higher pH and therefore were not subjected to combinations of pH and temperature like those of the light portions. The pH values of dark portions kept at low temperatures (4°C) changed only slightly (from 6.21 at death to 5.82 at 24 hr post-mortem).

Glycogen depletion. The rate of glycogen disappearance was similar in light and dark portions kept at 37°C for the first 3 hr post-mortem (Fig. 2). Although glycogen concentrations at death and 3 hr post-mortem were significantly higher ($P < .05$ and $P < .01$, respectively) in the dark portions than in the light portions, the difference in glycogen levels between the two portions varied only slightly over the same time period. When the post-mortem temperatures were decreased to 4°C the rate of glycogen depletion was generally reduced in both portions. As a result, the 3-hr glycogen levels were significantly higher ($P < .05$) in both portions held at 4°C than in portions held at 37°C. However, dark portions held at 4°C had only slightly higher glycogen concentrations than light portions at 4°C. It is pertinent that dark portions held at 4°C had mean glycogen values indicating a trend toward a very rapid initial depletion of glycogen; after 1 hr post-mortem, however, the rates of glycogen reduction were similar in both portions held at 4°C. This indicates that high temperatures will accelerate glycolysis similarly in light muscle (adapted for anaerobic glycolysis) as in dark muscle (adapted for oxidative metabolism).

Lactic acid accumulation. Table 2 shows the lactic acid concentrations of the light and dark semitendinosus portions from animals insensibilized with a captive-bolt pistol. Muscle portions tended to accumulate lactic acid more rapidly at 37°C than at 4°C. Mean values indicate that the production of lactic acid was initially (½ hr) more rapid in

Table 2. Effect of post-mortem temperature on lactic acid accumulation in light and dark muscle portions.[a]

Time (hr) post-mortem	Muscle portion: Light, 4°C $\bar{x}$[b]	Light, 4°C $s_{\bar{x}}$[c]	Light, 37°C $\bar{x}$[b]	Light, 37°C $s_{\bar{x}}$[c]	Dark, 4°C $\bar{x}$[b]	Dark, 4°C $s_{\bar{x}}$[c]	Dark, 37°C $\bar{x}$[b]	Dark, 37°C $s_{\bar{x}}$[c]
0	21.6[d]	1.4	21.6	1.4	21.4	1.2	21.4	1.2
½	24.2	1.7	27.3	2.8	22.2	1.2	21.7	1.2
1	27.8	3.3	26.8	1.3	23.8	2.1	26.8	2.4
2	25.9	1.2	29.6	1.4	23.6	1.7	29.9	2.1
3	28.4	1.7	33.7	1.6	26.2	1.4	28.0	1.4
24	31.6	1.5	32.4	1.8	32.1	2.1	31.7	1.1

[a] All animals were insensibilized with a captive-bolt pistol.
[b] Mean value.
[c] Standard error of the mean.
[d] Mg lactic acid/g dry tissue.

Table 3. Effect of post-mortem temperature on color reflectance values in light and dark muscle portions.[a]

Time (hr) post-mortem	Muscle portion: Light, 4°C $\bar{x}$[b]	Light, 4°C $s_{\bar{x}}$[c]	Light, 37°C $\bar{x}$[b]	Light, 37°C $s_{\bar{x}}$[c]	Dark, 4°C $\bar{x}$[b]	Dark, 4°C $s_{\bar{x}}$[c]	Dark, 37°C $\bar{x}$[b]	Dark, 37°C $s_{\bar{x}}$[c]
0	33.4[d]	0.9	33.4	0.9	25.5	0.6	25.5	0.6
3	32.1	0.9	38.5	1.2	24.0	0.4	27.7	0.4
24	34.4	1.0	39.8	1.3	24.9	0.6	28.3	0.7

[a] All animals were insensibilized with a captive-bolt pistol.
[b] Mean value.
[c] Standard error of the mean.
[d] Percent reflectance.

light portions than in dark portions held at similar post-mortem temperatures, which substantiates the fact that light muscle fibers permit a more rapid anaerobic glycolysis than dark fibers. After the 30-min period, the differences were no longer evident between light and dark muscles in lactic acid production.

It is important to note that the significant ($P < .05$) glycogen decrease in dark muscle portions which took place within the first ½ hr at 4°C was not reflected in an increase in the post-mortem production of lactic acid during the same period. These results indicate that within the first 30-min period there was: 1) a build-up of glycolytic intermediates; or 2) a distribution of intermediates into pathways other than glycolysis. Ultimate (24 hr) lactic acid values were similar at both post-mortem temperatures and in both portions.

Muscle color. Over the 24-hr post-mortem period both muscle portions became significantly ($P < .01$) lighter when held at 37°C than at 4°C (Table 3). The change in color reflectance from the time of death to 24 hr post-mortem for those muscle portions held at 37°C was greater in the light portions than in the dark portions. Only small changes in color reflectance over the same time period were observed in both light and dark portions held at 4°C. These data indicate that temperatures and/or slow rates of anaerobic glycolysis contribute greatly to the ability of both muscle portions to retain normal color even though it was most evident in the light portion.

Expressible juice. The water-binding capacities (expressible juice) of muscle portions at 24 hr post-mortem from animals insensibilized with a captive-bolt pistol were greatly improved by decreasing post-mortem

Table 4. Expressible-juice ratios of light and dark muscle portions.

	Muscle portion							
	Light				Dark			
	4°C		37°C		4°C		37°C	
Animal group	$\bar{x}$ [a]	$s_{\bar{x}}$ [b]	$\bar{x}$ [a]	$s_{\bar{x}}$ [b]	$\bar{x}$ [a]	$s_{\bar{x}}$ [b]	$\bar{x}$ [a]	$s_{\bar{x}}$ [b]
A [c]	1.33 [e]	.08	1.63	.12	1.40	.07	1.66	.08
B [d]	1.43	.10	1.55	.17	1.28	.08	1.72	.16

[a] Mean value.
[b] Standard error of the mean.
[c] Animals were insensibilized with a captive-bolt pistol.
[d] Animals were insensibilized by the injection of sodium pento-barbital.
[e] Ratio of water area to meat area.

temperatures to 4°C (Table 4). This supports findings of Wismer-Pedersen and Briskey (1961) with longissimus dorsi. No differences in water-binding capacities were noted between light and dark portions at the same temperature. This observation indicates that the dark semitendinosus may differ quite markedly from other dark muscles in the carcass in water-binding capacity and implies that the dark portion may differ in various properties from other dark muscles. It seems pertinent to refer again to the difference in the duration of the delay phase of rigor mortis between these two portions (Table 1). Sink *et al.* (1964) have observed that certain rigor mortis properties are related to the contraction state of muscle, which may relate to its water-binding capacity. This may explain the similar water-binding properties of these two muscle portions.

Phase II. *Elimination of death reaction.* In the second phase of this study, three pigs of each breed were insensibilized with intravenous injections of sodium pento-barbital (9.5 mg/kg) and exsanguinated 30 min post-injection. These animals showed no death reaction upon exsanguination. All other procedures were identical to those previously outlined.

pH decline. The mean pH values in both muscle portions at death were higher in animals insensibilized by sodium pento-barbital injection than in animals insensibilized with a captive-bolt pistol (Table 5). Sodium pento-barbital injection significantly ($P < .05$) increased the initial pH values in the dark portions. The dark portions and light portions from animals insensibilized with sodium pento-barbital had initial pH values .21 and .11 unit higher, respectively, than dark and light portions from animals insensibilized with a captive-bolt pistol. pH decline in light muscle portions held at 37°C was slower ($P < .01$) for animals insensi-

Table 5. Post-mortem pH decline in light and dark muscle portions (37°C).

	Muscle portion							
	Light				Dark			
	A [a]		B [b]		A [a]		B [b]	
Time (hr) post-mortem	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]
0	6.11	.05	6.22	.08	6.21	.04	6.42	.10
½	5.90	.06	6.14	.09	6.05	.05	6.24	.11
1	5.73	.04	6.16	.17	5.82	.04	6.15	.16
2	5.63	.05	6.02	.14	5.78	.05	6.07	.10
3	5.56	.03	5.87	.10	5.68	.03	5.96	.09
24	5.56	.04	5.50	.08	5.69	.04	5.66	.07

[a] Animals were insensibilized with a captive-bolt pistol.
[b] Animals were insensibilized by the injection of sodium pento-barbital.
[c] Mean value.
[d] Standard error of the mean.

Table 6. Post-mortem glycogen depletion in light and dark muscle portions (37°C).

Time (hr) post-mortem	Muscle portion: Light, A [a] $\bar{x}$ [c]	Light, A [a] $s_{\bar{x}}$ [d]	Light, B [b] $\bar{x}$ [c]	Light, B [b] $s_{\bar{x}}$ [d]	Dark, A [a] $\bar{x}$ [c]	Dark, A [a] $s_{\bar{x}}$ [d]	Dark, B [b] $\bar{x}$ [c]	Dark, B [b] $s_{\bar{x}}$ [d]
0	2.82 [e]	0.31	4.40	1.14	4.14	0.43	3.99	1.28
½	2.41	0.33	3.23	0.97	3.55	0.46	3.34	1.27
1	1.82	0.33	2.99	1.33	2.86	0.42	3.74	1.60
2	0.98	0.21	2.97	1.09	1.92	0.33	3.32	1.31
3	0.19	0.08	1.98	0.87	1.07	0.22	2.35	1.24

[a] Animals were insensibilized with a captive-bolt pistol.
[b] Animals were insensibilized by the injection of sodium pento-barbital.
[c] Mean value.
[d] Standard error of the mean.
[e] Mg glycogen/g tissue.

bilized with sodium pento-barbital than for animals insensibilized with a captive-bolt pistol. Elimination of the death reaction, however, did not affect the rate of pH decline in the dark portions kept at 37°C. Ultimate pH values in muscle portions from animals insensibilized with sodium pento-barbital did not differ significantly from those in corresponding portions from animals insensibilized with a captive-bolt pistol.

Glycogen depletion. Sodium pento-barbital insensibilization tended to slow post-mortem glycogen depletion in both portions held at 37°C below that in portions from animals which were insensibilized by a captive-bolt pistol (Table 6). It is also pertinent that animals which exhibited no death reaction (sodium pento-barbital injected) had mean glycogen levels which were slightly higher in the light portions than in the dark portions. These findings are in agreement with those of Beatty *et al.* (1963), who reported higher glycogen concentrations in white than in red muscles of the rat. These data also imply that although red muscle reacts more extensively than white muscle to antemortem temperature treatments (Beecher *et al.*, 1963), white muscle reacts more extensively than red muscle to the struggle associated with the death reaction upon insensibilization with a captive-bolt pistol. Bate-Smith (1948) has shown that the pH after exsanguination may be partly due to lactic acid formed as a result of glycogen breakdown during the death struggle. Since the animals which were injected with sodium pento-barbital did not struggle upon exsanguination these data may be considered, to a large degree, to emphasize the difference between red and white muscle in their response to the death reaction. It must also be recognized, however, that part of the general difference between sodium pento-barbital and captive-bolt-pistol-insensibilized animals may be due to the fact that barbiturate anesthesia decreases the sympatho-adrenal response which must precede the release of epinephrine, from the adrenal gland, for the breakdown of glycogen.

Lactic acid accumulation. Sodium pento-barbital tended to decrease initial lactic acid concentrations and the rate of lactate formation during early post-mortem periods (1 hr) in both portions (Table 7). It seems pertinent that Bunker (1963) also noted that serum lactate levels were reduced by barbiturate anesthesia, which was thought to indicate either decreased glycolytic activity or increased oxidative activity. Since both dark and light muscle portions are involved, it would appear that the lower lactate levels probably result from reduced glycolytic activity from the time of insensibilization to the time of the initial sample excision. Mean values indicate ultimate lactic acid concentrations in both portions to be higher for sodium pento-barbital insensibilized animals than for animals insensibilized with a captive-bolt pistol. This may be due to the retention of higher initial glycogen levels or to a more

Table 7. Post-mortem lactic acid accumulation in light and dark muscle portions (37°C).

	Muscle portion							
	Light				Dark			
	A [a]		B [b]		A [a]		B [b]	
Time (hr) post-mortem	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]	$\bar{x}$ [c]	$s_{\bar{x}}$ [d]
0	21.6 [e]	1.4	18.5	2.1	21.4	1.2	17.4	2.4
½	27.3	2.8	18.3	2.5	21.7	1.2	16.8	1.8
1	26.8	1.3	20.0	2.1	26.8	2.4	16.1	2.0
2	29.6	1.4	23.0	2.7	29.9	2.1	22.8	3.4
3	33.7	1.6	32.0	2.1	28.0	1.4	24.8	1.5
24	32.4	1.8	37.2	4.2	31.7	1.1	34.9	3.2

[a] Animals were insensibilized with a captive-bolt pistol.
[b] Animals were insensibilized by the injection of sodium pento-barbital.
[c] Mean value.
[d] Standard error of the mean.
[e] Mg lactic acid/g dry tissue.

complete conversion of glycogen to lactic acid in the muscles from the animals which had no evident death reaction.

Rigor mortis and associated properties. The duration of the delay phase of rigor mortis in the light portion was nearly doubled by sodium pento-barbital insensibilization over insensibilization with a captive-bolt pistol, but was only slightly increased in the dark portion. These data further substantiate the relation between the rate of post-mortem glycolysis and time course of rigor mortis in light and dark muscles (Briskey *et al.*, 1962; Briskey, 1963; Lawrie, 1952; Buttkus, 1963).

Sodium pento-barbital insensibilization tended to decrease initial color reflectance values slightly in both portions from that for animals insensibilized with a captive-bolt pistol (Table 8). Mean values also indicate that sodium pento-barbital insensibilization decreased the changes in color values which were observed between 0 and 24 hr post-mortem in the light portions held at 37°C. These findings indicate that the death struggle or muscle contraction, which was eliminated in the case of sodium pento-barbital injected animals, contributes to the fast glycolytic rate and concomitant color change in the light portions of the semitendinosus. The water-binding capacity 24 hr post-mortem of both muscle portions was not greatly affected by ante-mortem treatment (Table 4).

Conclusion. These studies show that when animals are permitted to undergo a death reaction upon insensibilization (captive-bolt)

Table 8. Effect of post-mortem temperatures on color reflectance values in light and dark muscle portions.[a]

	Muscle portion							
	Light				Dark			
	4°C		37°C		4°C		37°C	
Time (hr) post-mortem	$\bar{x}$ [b]	$s_{\bar{x}}$ [c]	$\bar{x}$ [b]	$s_{\bar{x}}$ [c]	$\bar{x}$ [b]	$s_{\bar{x}}$ [c]	$\bar{x}$ [b]	$s_{\bar{x}}$ [c]
0	32.2 [d]	1.0	32.2	1.0	23.2	0.7	23.2	0.7
3	29.4	1.0	32.1	1.1	23.2	0.5	24.8	0.8
24	29.9	1.8	36.1	1.8	24.0	0.8	27.5	1.2

[a] All animals were insensibilized by the injection of sodium pento-barbital.
[b] Mean value.
[c] Standard error of the mean.
[d] Percent reflectance.

and subsequent exsanguination, the light muscle portions have a faster glycolysis than the dark muscle portions as evidenced by a lower glycogen level in the initial muscle samples. When animals are injected with sodium pento-barbital, to prevent the death reaction, the light muscles have a higher initial glycogen level. These conclusions are further substantiated by the fact that the injection of sodium pento-barbital extended the duration of the delay phase of rigor mortis in light portions but had little effect on the duration of the delay phase in the dark portions. High temperature accelerated post-mortem glycolysis in both light and dark muscle portions; however, the dark portions retained higher pH values than the light portions at all post-mortem periods.

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Enzymatic Reduction of Metmyoglobin by Ground Beef

SUMMARY

A method is described for estimating the ability of ground meat to reduce metmyoglobin enzymatically. The method involves a complete oxidation of all pigments to metmyoglobin by the addition of ferricyanide, followed by measurement of metmyoglobin reduction by the meat in 1 hr. The work was confined to beef rib eye muscle. Metmyoglobin-reducing activity (MRA) showed great fluctuations in rib eyes from different animals. It was correlated with total pigment. In any one sample of meat, MRA increased with pH from pH 5.1 to 7.1 and with temperature from 3°C to 35°C. It declined only slightly in whole cuts of meat stored for several days in the refrigerator, but much more rapidly in stored ground meat. Chlortetracycline did not interfere with MRA, but 5% salt completely inhibited it. A possible reductive mechanism is discussed briefly.

INTRODUCTION

The maintenance of a desirable color in fresh meat is of interest to both the meat industry and the consumer. Bright-red oxymyoglobin and purplish-red reduced myoglobin are the normal ferrous pigments of fresh meat. The relative proportion of these two depends upon the oxygen tension at the pigment site. Where the pigments are in the ferrous form, exposed meat surfaces quickly assume the bright red of oxymyoglobin. On the other hand, oxidation of the meat pigments to metmyoglobin produces a brown color which is not acceptable to the consumer. Furthermore, there is substantial evidence in the literature that metmyoglobin (but not the ferrous heme pigments) can catalyze lipid oxidation, resulting in flavor changes.

There are numerous reports in the literature on the metmyoglobin discoloration of meats as affected by variations in oxygen tension, storage temperature, packaging materials, bacterial contamination, etc. The metmyoglobin actually formed during such storage studies is a result of two opposing factors, i.e., autoxidation of the ferrous pigments to metmyoglobin, on the one hand, and enzymatic reduction of the ferric metmyoglobin, on the other hand.

Walters and Taylor (1963) demonstrated a slow enzymatic reduction of pure metmyoglobin upon incubation under anaerobic conditions with pork muscle mince at 37°C, pH 6.0. While these appear to be the only published data directly demonstrating metmyoglobin reduction by meat, there is much indirect evidence in meat storage studies. For example, Dean and Ball (1960) showed the disappearance as well as the formation of metmyoglobin on the surfaces of beef cuts stored in various ways and stated that metmyoglobin is converted to oxymyoglobin by "natural processes."

Recent work of Cutaia and Ordal (1964) on ground beef demonstrates even more clearly the initial formation of metmyoglobin and its subsequent disappearance during a two-day storage period under anaerobic conditions. Those authors interpreted their results in terms of simultaneous autoxidation and enzymatic reduction of the meat pigments.

The observation (Stewart *et al.*, 1965) that reduction of metmyoglobin was substantial after ferricyanide treatment of ground beef suggested that this technique might be adapted to a study of the metmyoglobin-reducing activity of meats under various conditions. Thus, enzymatic reduction of preformed metmyoglobin could be separated from the situation of simultaneous oxidation and reduction, which complicates the study of spontaneous pigment changes in meat.

MATERIALS AND METHODS

Preparation of meat. Beef rib eye was trimmed and ground as described previously (Stewart *et al.*, 1965). Fifty-g portions were weighed out and placed in polyethylene freezer bags. If storage was required, samples were placed in a refrigerator at 3°C.

Temperature adjustment. Samples were removed from storage (or freshly prepared) and flattened with a hamburger press to a thickness of ¼ inch while in the bag. The packaged samples were then placed in water baths at various temperatures for 30 min. The standard conditions adopted were 30°C water bath for 30 min.

Ferricyanide addition. Varying amounts of $K_3Fe(CN)_6$ in 2 ml distilled water were added to the 50-g portion, followed by vigorous mixing for 3 min. Spectrophotometric measurements were made as described previously (Stewart *et al.*, 1965). Total elapsed time from the addition of ferricyanide to the start of the first curve was 4½ min. The initial curve was considered to be taken at 0 time, and subsequent curves were taken 30–60 min after this.

Chlortetracycline treatment. A stock solution was prepared of chlortetracycline HCl (American Cyanamide Co.) containing 1 mg per ml .01*N* HCl. One and one-half ml of this solution were added to 50 g meat, followed by thorough mixing. The meat was stored at 3°C and brought to 30° before ferricyanide addition.

pH adjustment. To ascertain the influence of pH on reducing activity, either 1*N* HCl or 5*N* NaOH was added to 50-g portions of meat immediately after grinding. After mixing, the samples were refrigerated for 1 hr and then brought to 30°C before addition of the ferricyanide. The addition of acid or base did not exceed 2 ml/50 g, and the volume of liquid added was kept constant.

RESULTS AND INTERPRETATION

Formation and reduction of metmyoglobin after addition of ferricyanide to meat. Fig. 1 is a photograph of a series of spectra obtained during a period of several hours after the addition of 0.2% potassium ferricyanide to a sample of ground rib eye muscle. The initial curve is that with highest absorption at 635 mμ and lowest at 573 mμ. Subsequent spectra, made at half-hour intervals except for the last two, which are 15 min apart, show a progressive decrease at 635 mμ and increase at 573 mμ. The meat was not removed from its position on the spectrophotometer during this 4¼-hr period. The spectra are recorded against a piece of gray cardboard, whose absorption against the magnesium carbonate reference standard is also shown as the uninflected line in the same figure.

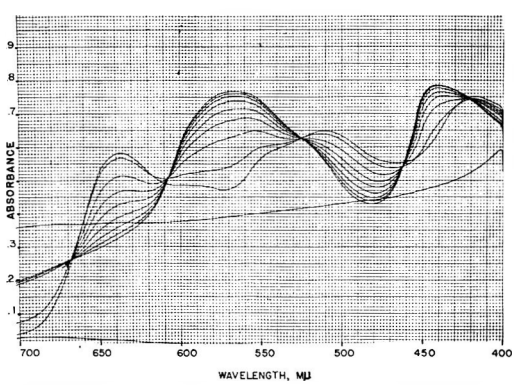

Fig. 1. Reduction of metmyoglobin in rib eye muscle during 4¼ hr.

The addition of ferricyanide resulted in oxidation of the muscle pigments to metmyoglobin. The first curve, recorded 4½ min after addition of the ferricyanide, indicates that a slight amount of oxymyoglobin is present with the metmyoglobin. Consequently, this curve is isobestic with the others only at 525 mμ. A number of isobestic points for reduced and metmyoglobin are clearly shown. The metmyoglobin is progressively reduced by the meat. The K/S ratio at 572 mμ/525 mμ for the final curve is 1.34, compared to an average value of 1.40 obtained with a number of samples of artificially reduced meat (Stewart *et al.*, 1965).

The amount of ferricyanide necessary to get complete oxidation of the pigments varied. With all samples, 0.2% ferricyanide was sufficient, but with meats of relatively low reducing activity 0.1% ferricyanide also oxidized the pigment and the subsequent reduction occurred more rapidly. Fig. 2 shows the reduction of metmyoglobin by two portions of the same sample of ground meat (of moderate reducing activity) to which had been added 0.1% and 0.2% potassium ferricyanide, respectively. With the smaller amount, the pigment was completely oxidized but reduction began immediately. With the larger amount, there was an initial lag in the reduction of metmyoglobin, presumably due to the excess oxidizing agent.

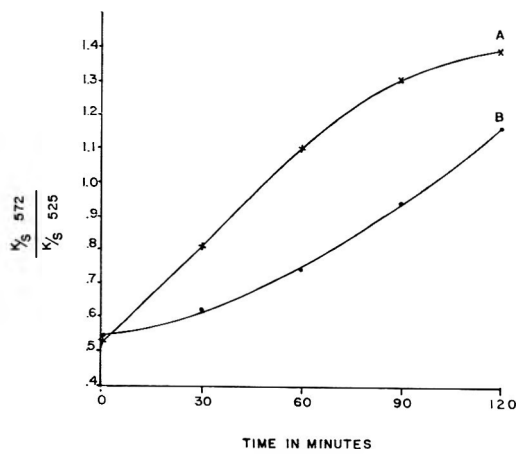

Fig. 2. Influence of ferricyanide concentration on reduction of metmyoglobin. A) 0.1% $K_3Fe(CN)_6$. B) 0.2% $K_3Fe(CN)_6$.

Table 1. Reducing activity of different rib eye samples.

Meat sample	% metmyoglobin [a]	
	0.1% ferricyanide	0.2% ferricyanide
A	100	100
B	48	79
C	43	76
D	28	62
E	12	91
F	2	28

[a] 60 min after $K_3Fe(CN)_6$ addition. Metmyoglobin concentrations obtained from Fig. 2 in preceding paper (Stewart *et al.*, 1965).

When a number of freshly ground samples of rib eye muscle are similarly treated with ferricyanide, the ability of the muscle tissue to reduce the metmyoglobin varies over wide limits. Table 1 summarizes the metmyoglobin remaining 1 hr after addition of ferricyanide to the rib eye muscles from six different animals. The extremes range from no measurable reduction to complete reduction within 1 hr.

A highly significant positive correlation was found between total pigment concentration and reducing activity of tissue. Table 2 lists 8 different rib eye samples in increasing order of hematin content, as estimated from K/S at 525 mμ. Application of the Spearman rank correlation coefficient (Siegel, 1956) gives an rs of + 0.83, significant at the 1% level. This correlation does not necessarily mean a true relation between the pigment and reducing activity. More highly pigmented muscles may also be richer in the enzyme systems responsible for the reaction. The interpretation will have to await further work.

Effect of refrigerator storage. Holding the unground rib eye muscle in the refrigerator for 6 days had only a slight effect on the reducing activity of samples taken from the muscle and ground just before reducing ability was measured. Refrigerator storage of preground samples resulted in much more rapid changes, although the rapidity of the loss varied in different samples of meat. A typical set of data is shown in Table 3. In this and in Table 4 the results are expressed as K/S ratios rather than percent metmyoglobin, because the ratios at zero time with these samples of meat were considerably higher than the average values (Stewart *et al.*, 1965) upon which metmyoglobin percentages were based.

Table 2. Relationship of total pigment concentration to the reducing activity of meat.

Sample	ppm hematin [a]	% metmyoglobin [b]
A	115	83
B	215	100
C	238	79
D	253	62
E	260	30
F	295	17
G	300	7
H	315	38

[a] Hematin concentrations obtained from K/S 525 mμ using Fig. 1 in preceding paper (Stewart *et al.*, 1965).

[b] 60 min after mixing with 0.2% $K_3Fe(CN)_6$. Metmyoglobin concentrations obtained from Fig. 2 in preceding paper (Stewart *et al.*, 1965).

Table 3. Reducing activity of refrigerator-stored ground beef.

Days after grinding of meat [a]	K/S 572 mμ/K/S 525 mμ Minutes after $K_3Fe(CN)_6$ addition		
	0	30	60
0	0.60	1.05	1.30
2	0.62	0.69	0.85
6	0.62	0.63	0.63

[a] pH 5.62; 0.2% $K_3Fe(CN)_6$.

Effect of antibiotics. The addition of 30 ppm chlortetracycline, a broad-spectrum antibiotic, to several samples of ground meats had no effect on the ability of the tissues to reduce metmyoglobin at least up to 3–4 days of storage. The antibiotic could be useful in prolonged storage studies where bacterial effects might obscure the results.

Effect of temperature on reducing activity. Fifty-gram samples of meat were held 30 min at temperatures ranging from 3 to 35°C prior to the addition of 0.2% $K_3Fe(CN)_6$ and during intervals between spectral analyses. The effect of these temperature variations is seen in Table 4.

Little reduction occurred at 15°C or lower, and the rate of reduction increased at least up to 35°C. In other experiments with meat having greater reducing activity than this sample, measurable reduction occurred at 15°C, but, again, the rate was much less than at room temperature. Similar results were reported by Cutaia and Ordal (1964).

As might be expected, cooking meat to 70°C completely destroyed all reducing activity.

Table 4. Effect of temperature on reducing activity.

Sample temperature (°C)	K/S 572 mμ/K/S 525 mμ Minutes after $K_3Fe(CN)_6$ addition		
	0	30	60
3	0.64	0.67	0.68
15	0.64	0.65	0.68
22	0.66	0.85	1.08
35	0.65	1.17	1.26

Effect of pH on reducing activity. Fig. 3 shows the results of an experiment in which the pH of a sample of meat (originally pH 5.8) was adjusted to various values from pH 5.1 to 7.1. The reducing activity becomes progressively higher as the pH increases. The initial K/S ratios vary greatly. The higher initial value for the sample adjusted to pH 5.1 than for those at pH 5.4 or 5.8 is believed to be caused by a difference in texture of the acidified meat. This difference, like that produced by cooking, increases the absorbance of pigment-free meat and so changes the numerical value of the ratios corresponding to the various pigment fractions. On the other hand, the higher initial values of the samples adjusted to pH 6.5 and 7.1 are ascribed to the greatly increased reducing activity of these samples during the time of mixing and placing on the spectrophotometer.

Cutaia and Ordal (1964) have also noted more rapid reduction of metmyoglobin at higher pH. The demonstrated dependence of enzymic reducing activity upon the pH of a given sample of meat does not necessarily mean that a correlation should be expected between the pH and reducing activity of different samples of meat. It is quite possible that a negative rather than a positive correlation could appear if a greater variety of muscles were tested. Berman (1961) found a highly significant inverse correlation between pH and content of the enzyme lactic dehydrogenase.

Effect of added salt. Table 5 demonstrates the inhibitory effect of sodium chloride on the reducing activity of a sample of ground beef. Salt in these concentrations is known to inhibit the activity of many enzymes. Taylor (1961) observed that 5% salt did not interfere with the ability of pork tissue to reduce nitrite to nitric oxide, but the capacity of the tissue to reduce the indigenous pigments to the ferrous nitric oxide myoglobin was appreciably reduced by the salt.

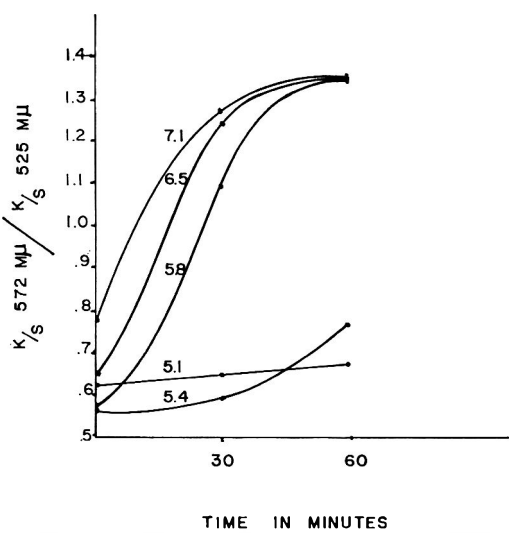

Fig. 3. Reducing activity of rib eye muscle as influenced by pH.

Table 5. Effect of added salt on K/S ratios at 60 minutes.

Sample	K/S 572 mμ/K/S 525 mμ [a]
Control, 0% NaCl	1.15
2% NaCl	0.82
5% NaCl	0.64

[a] 0.1% $K_3Fe(CN)_6$; initial ratio = 0.63.

DISCUSSION

It should be re-emphasized that the reducing activity of meat tissues, investigated here, is only one of several factors involved in the oxidative pigment changes occurring in stored meats. Although increasing the storage temperature, for example, accelerates the enzymatic reduction of metmyoglobin, it also accelerates the myoglobin autoxidation rate. Thus, the net result of a change in temperature (or other environmental factors) on metmyoglobin formation cannot be predicted easily and might not be the same in all meat samples. Great variations are evident in the metmyoglobin-reducing capacity of even a single muscle. Further work on meat of controlled postslaughter treatment from animals of known preslaughter history would be highly desirable.

There is no indication in any of the meat literature of the enzymatic pathways responsible for metmyoglobin reduction. Considering the conditions known to prevail in the postrigor meat, one possible reductive chain might be the following. Hydrogen may be transferred from lactate to diphosphopyridine nucleotide (DPN^+), by the enzyme lactic dehydrogenase (LDH). Meat is well supplied with both lactate (the end product of glycolysis) and LDH (Berman, 1961). The reduced pyridine coenzyme (DPNH) can, in turn, reduce metmyoglobin, but not directly. In work with model systems, Rossi-Fanelli *et al.* (1957) found a requirement for intermediate electron carriers, which may be other enzymes, quinones, methylene blue, etc. It may be postulated that the rate of the over-all reaction is limited either by the con-

centration of DPN^+ or by the availability of suitable electron mediators between the reduced coenzyme and metmyoglobin. DPN^+ is known to undergo enzymatic hydrolysis in tissue homogenates (Mann and Quastel, 1941; Gutmann, *et al.*, 1947). Obviously it will be necessary to approach the problem at a more fundamental experimental level in order to clarify the enzymatic pathways involved.

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Porcine Muscle Properties. B. Relation to Naturally Occurring and Artificially Induced Variation in Heart and Respiration Rates

SUMMARY

Studies were conducted to: 1) investigate the association between the physiological parameters heart and respiration rates, and the post-mortem properties of muscle; and 2) determine the physiological response of the pig to experimentally-imposed, abrupt changes in environmental temperature immediately prior to slaughter. In the first phase of these studies, observations were made on 55 untreated pigs representing 4 breeds. Heart and respiration rates were determined on restrained animals in standing position, just before slaughter. Abnormally high heart and respiration rates were observed under these conditions in comparison with resting rates. Nevertheless, very high heart and respiration rates, immediately prior to slaughter, were associated with rapid rates of post-mortem muscle pH decline, low post-mortem pH values and pale, soft, exudative musculature. In the second phase of these studies, a group of 42 Poland China pigs were subjected to 7 ante-mortem temperature treatments. Warm treatment resulted in marked increases in respiration rates and wide variations in heart rates. Cold treatments generally decreased both respiration and heart rates. In the combination treatments, a change from warm to cold environment tended to restore respiration rate to pretreatment levels. Both respiration and heart rate tended to increase as muscle temperature increased. Drastic increases in heart rates due to warm treatment were associated with the development of extremely pale, soft, exudative musculature.

INTRODUCTION

Limited information exists on the association of various physiological parameters of pigs to the post-mortem properties of the musculature. Since heart and respiration rates are associated with blood flow and oxygen supply to the muscle, the initial state of, as well as changes in, these physiological parameters immediately prior to exsanguination, may be associated with the post-mortem properties of the muscle. If naturally-occurring variations in heart and respiration rates are associated with the post-mortem properties of the muscle, the importance of environment immediately prior to slaughter may be more specifically explained. High-temperature exposure of short duration might be expected to increase heart and respiration rates (Robinson and Lee, 1941). Nevertheless the effects of specific ante-mortem environmental conditions on the animals' physiological parameters immediately prior to death has received limited attention. This is of utmost importance to the food industry since environmental conditions, immediately prior to slaughter, influence the ultimate post-mortem properties of porcine muscle. Ambient temperatures have been observed to markedly influence porcine muscle color (Ludvigsen, 1954; Wismer-Pedersen, 1959; Judge *et al.*, 1959; Briskey *et al.*, 1960; Forrest *et al.*, 1963). Experimentally-induced environmental changes, immediately prior to exsanguination, have also been shown to influence post-mortem muscle properties (Sayre *et al.*, 1961, 1963; Kastenschmidt *et al.*, 1963, 1965).

The present studies were made to: 1) investigate the association between heart and respiration rates and the post-mortem properties of muscle; and 2) determine the physiological response of the pig to experimentally-imposed, abrupt changes in environmental temperature immediately prior to slaughter.

EXPERIMENTAL

Phase I. *Naturally occurring variations.* In the first phase of this study, heart and respiration rates were obtained on 55 pigs (32 Poland China; 11 Yorkshire-Duroc; 6 Chester White; 6 Hampshire). Heart rate was obtained from the electrocardiogram recorded either with an E & M Physiograph or a Cardiopan EKG. A two-lead assembly was used with hypodermic-needle electrodes inserted directly under the skin. The dorsal electrode was inserted

to the left of the midline posterior to the scapula, while the ventral electrode was inserted left of and adjacent to the sternum. Respiration rate was simultaneously obtained from these two leads by channeling the signal through an impedance pneumograph. Heart and respiration rates were recorded while the animals were restrained in a standing position, after which they were immediately insensibilized with a captive-bolt pistol and exsanguinated.

Samples of the right longissimus dorsi muscles were immediately excised at the last thoracic vertebra. Muscle temperature and pH were determined, and samples were frozen in liquid nitrogen and held for determinations of lactic acid and glycogen concentration as described by Kastenschmidt *et al.* (1965). Muscle pH and temperature were also determined on the longissimus dorsi at ¼, ½, 1, 2, 3, and 24 hr post-mortem. The pH values were obtained by placing a combination electrode directly on the freshly cut cross-sectional surface of the muscle fibers. At 24 hr post-mortem the longissimus dorsi muscles were subjectively evaluated for gross morphology (color-structure) by two independent judges using the 5-point scale described by Forrest *et al.* (1963). A low score (0.5–2) indicated a pale, soft, exudative (PSE) muscle; a score of 2.5–3.5 was considered normal; and 4–5 represented a dark, firm, dry (DFD) muscle.

Phase II. *Artificially induced variations.* In the second phase of this study, the effect of experimentally-induced changes in preslaughter environmental temperature, which are known to alter post-mortem muscle properties, were studied in relation to their effect upon heart and respiration rate.

Forty-two Poland China pigs averaging 113 kg were randomly assigned to 7 ante-mortem temperature treatment groups as described by Kastenschmidt *et al.* (1965). Heart and respiration rates were determined before and after each warm or cold treatment regardless of whether the treatments were given singly or as combination treatments. The other procedures were identical to those described in phase I of this study.

RESULTS AND DISCUSSION

Phase I. *Naturally-occurring variations.* Heart rates were observed to be higher in animals which ultimately exhibited PSE longissimus dorsi muscle (morphology score

Table 1. Relation of heart and respiration rates to breed and post-mortem gross morphology score of the muscle.

Breed	Observations (no.)	Morphology score[a]	Heart rate[b]	Respiration rate[c]
Poland China				
PSE	20	1.3	163±22	51±12
Normal	12	2.8	142±30	52±19
Average	32	1.8	155±25	51±16
Yorkshire × Duroc				
PSE	4	1.4	135±18	30±5
Normal	7	2.9	121±12	39±18
Average	11	2.3	125±14	36±15
Chester White				
PSE	3	1.4	140±30	64±24
Normal	3	3.0	119±12	33±4
Average	6	2.2	129±22	49±23
Hampshire				
PSE	1	2.0	148	52+
Normal	5	2.9	120±14	33±12
Average	6	2.8	126±17	36±13
All breeds				
PSE	28	1.3	156±24	49±16
Normal	27	2.9	130±24	43±18

[a] Scored on a 5-point basis: 0.5–2.0 = PSE; 2.5–3.5 = normal; 4.0–5.0 = DFD.
[b] Beats per minute, determined on a restrained animal in standing position (± std. dev.).
[c] Inspirations per min (± std. dev.).

Table 2. Relation of heart and respiration rates to rate of post-mortem pH decline.

pH decline (rate)	Observations (no.)	Morphology score[d]	Heart rate[e]	Respiration rate[f]
Fast[a]	4	0.7	166±17	45±6
Intermediate[b]	8	1.8	145±33	38±12
Slow[c]	6	2.5	135±24	36±8

[a] pH 5.4 or lower within 30 min post-mortem.
[b] pH higher than 5.4 at 30 min but lower than 6.0 at 1 hr.
[c] pH 6.0 or higher at 1 hr post-mortem.
[d] Scored on a 5-point basis: 0.5–2.0 = PSE; 2.5–3.5 = normal; 4.0–5.0 = DFD.
[e] Beats per minute, determined on a restrained animal in standing position (± std. dev.).
[f] Inspirations per min (± std. dev.).

0.5-2.0) than in those with normal musculature (morphology score 2.5-3.5). When all animals were grouped into PSE or normal categories, the PSE animals had heart rates of 156±24, compared to 130±24 for normal animals (Table 1). Respiration rates were also observed to be slightly higher in PSE than normal animals.

The Poland China pigs in this study had the lowest morphology scores and were observed to have the highest heart and respiration rates. The other breeds (Yorkshire-Duroc; Chester White; Hampshire) in this study had higher mean gross morphology scores and lower heart and respiration rates.

Table 1 also shows that within each breed, heart rates tended to be higher in those animals which were classified as PSE than in those which were normal. Respiration rates were extremely variable among breeds and the association of respiration rate to the PSE condition appeared to be partially dependent upon the concomitant heart rates.

A group of animals from this study (8 Poland China and 10 Yorkshire-Duroc) which were slaughtered within a 2-week period, were classified according to rate of pH decline in the longissimus dorsi muscle from 0 to 1 hr post-mortem (Kastenschmidt *et al.*, 1965). The results are given in Table 2. Heart and respiration rates both tended to be higher in those animals with fast rates of pH decline than in animals with intermediate or slow rates of pH decline.

It is pertinent to point out that all animals in this study had been hauled a short distance (approximately .25 mile) within 1 hr prior to the estimation of heart and respiration rates. Many of the animals were visibly excited at the time the heart and respiration rates were determined. In order to evaluate the effect of handling animals prior to slaughter on heart and respiration rates, four animals (2 Poland China and 2 Chester White) were held in 9 × 10-ft pens in a constant-temperature room for at least one week prior to the estimation of heart and respiration rates. The recording electrodes were fixed so that the animals had complete freedom of the facility while respiration and heart rates were being recorded. Heart rates as low as 72 beats per min were observed in a Chester White in a resting state. It was observed that the mere change from a prone to standing position, without excitement, caused an increase in heart rate of as much as 30 beats/min. Standing unrestrained pigs were observed to have heart rates of 100-110 beats per min, which is considerably lower than that observed in animals immediately prior to slaughter. Resting respiration rates of 18-20 were observed although one animal had a very high respiration rate of 60 inspirations per min even while sleeping.

It was concluded from the Experiments in Phase I that the heart rate of the pig is extremely variable whether or not the pigs are excited. It was further concluded that the animals' ability to respond to handling, through appropriate adjustments in heart and respiration, may be an important factor influencing the post-mortem properties of muscle.

Phase II. *Artificially-induced variations.* Fig. 1 shows respiration rate before and after the various warm and cold treatments described by Kastenschmidt *et al.* (1965). In

the warm-treatment group, mean respiration rate increased from 40 to 78 inspirations per min, while in the cold-treatment groups respiration was essentially unaffected. Although not shown in Fig. 1 it seems pertinent to point out the specific effects of each portion of the combination treatments (warm + cold; warm + cold + spray; warm + cold + spray-short). In each of the combination treatments, respiration rates increased as a result of the warm portion of the treatment. A change from a warm to cold environment restored respiration rates to near pretreatment levels (Fig. 1). Although

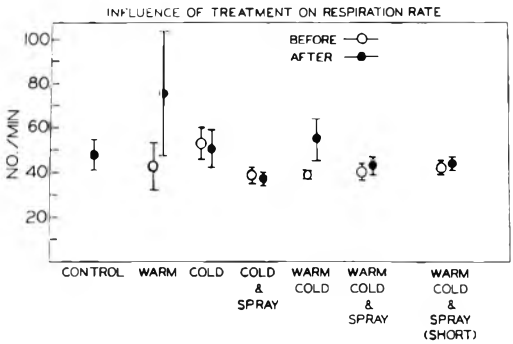

Fig. 1. The influence of treatment on respiration rate. I. Standard error of the mean. Treatments as described by Kastenschmidt *et al.* (1965).

different treatments were not compared, Robinson and Lee (1941) observed that spraying with water afforded an effective means of reducing the respiration rate of heated pigs.

While reasons for some of the specific treatment responses in respiration rate remain somewhat obscure, it may be assumed, as pointed out by Ingram *et al.* (1963), that high temperature increases the temperature of the hypothalamus and thereby increases respiration rate.

Fig. 2 shows the effect of treatment on heart rate. In the warm-treatment group the mean heart rate increased from 143 beats per min before treatment to 167 beats per min. It is important to point out that this increase in mean heart rate from the warm treatment was due to drastic increases in the heart rate of two animals while the other four animals actually exhibited slight decreases in heart rate. As was previously pointed out, respiration rates increased in

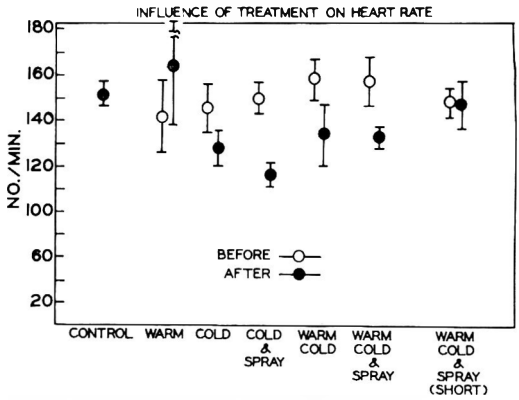

Fig. 2. The influence of treatment on heart rate. I. Standard error of the mean. Treatments as described by Kastenschmidt *et al.* (1965).

all animals as a result of heat treatment. However the greatest increase in respiration occurred in those animals which showed a decrease in heart rate. Additionally, those animals which decreased in heart rate during heat treatment were observed to produce darker muscles than those which increased in heart rates. Since one would normally expect an increase in heart rate with increasing temperature as reported by Robinson and Lee (1941), and Ingram (1964), it is postulated that the initial rate of 143 was considerably higher than the normal resting rate and that the animals which exhibited a decrease in rate were those which were able to adjust to the heat stress. These animals actually still had heart rates, after warm treatment, well above the expected normal resting level, but did not show evidence of the added factor of a hyperexcited state. This may also partially explain the observations of Heitman and Hughes (1949) that heart rate decreased with increasing environmental temperature. Nevertheless, the fact that two animals showed drastic increases in heart rate when exposed to warm conditions emphasizes the extreme variability in the animals sensitivity to its changing environment. The animals which responded in this way, however, were the ones which showed evidence of an extremely rapid post-mortem muscle pH decline and a very severe condition of PSE musculature.

Heart rate was markedly reduced by cold

treatment. Only two of 12 animals in these treatments (cold; cold + spray) showed small increases in heart rate, and from visual observations it would appear that these responses were related to the excited state of the animals.

Marked declines in heart rate were noted following the combination treatments; however, the major portions of these declines were due to the cold phase of the treatments. In the short treatment (warm to cold + spray-short), no significant ($P>0.05$) changes in heart rate were observed as a result of either the warm or cold treatment. Robinson and Lee (1941) observed that heart rate increased concomitantly with an increase in rectal temperature. In the present study, correlations between muscle temperature at death and respiration and heart rate immediately prior to death were .33 ($P<0.05$) and .35 ($P<0.05$), respectively, indicating that both respiration rate and heart rate tended to increase as muscle temperature increased. These results are in agreement with the recent findings of Ingram (1964). Continuous measurements of heart and respiration rates must be made during the treatment period in order to completely understand the specific relationship between these factors. Based on the present data it would appear that changes in heart rate were not solely the result of changes in body temperature. The animals' physiological responses *per se* to the environmental and handling conditions appeared to be of equal significance. Nevertheless, the stated correlations between heart rate and body temperature support previous reports on the ox (Ingram *et al.*, 1963), dog (Frankel *et al.*, 1963), and mouse (Richards, 1963).

Artificially-induced changes in heart rate were associated with post-mortem muscle pH changes and ultimate gross muscle morphology in much the same manner as naturally-occurring variations. Those animals which responded to treatment with increased heart rate tended to exhibit lower morphology scores. Respiration rates following the various treatments were not as highly related to muscle properties as the naturally-occurring variations. Respiration rates prior to treatment, however, were related to the post-mortem properties of the muscle. This would indicate that the treatment-induced changes of respiration rate of short duration were not as important as the naturally-occurring level of respiration in its relation to the ultimate properties of the muscle. It cannot be ascertained from the present data whether the respiration and heart rates are related directly or indirectly to post-mortem properties of muscle. However, because the relationships were lower after the various preslaughter treatments, even though muscle properties were markedly affected by treatment (Kastenschmidt *et al.*, 1965) it may be inferred that the relationship is indirect.

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Studies on the Composition of Lysosomes

SUMMARY

Studies were made on the components of lysosomes. Analyses were based upon the distribution of constituents between the insoluble fraction containing the membrane and bound proteins and the soluble fraction containing released enzymes. Results were compared with those obtained for mitochondria and microsomes. The composition of the lysosomal membrane was characteristic of a unit phospholipid-protein membrane. The sialic acid content of lysosomes was not much greater than that found in other subcellular fractions, suggesting that sialopolymers are not involved in a special role in lysosomes. The released enzyme fraction, which contained about 40% of the total protein, also contained free flavins and free amino acids in amounts very much larger than those observed for mitochondria. Fe, Zn, Cu, Mn, and Mo were the principal metals concentrated in lysosomes. The relationship between the chemical composition, structure, and function of lysosomes is discussed.

INTRODUCTION

The major hydrolytic enzymes of animal tissues are organized within subcellular organelles, the lysosomes (de Duve, 1963). Lysosomal enzymes include cathepsins, nucleases, and glycosidases, which can respectively catalyze the hydrolysis of proteins, nucleic acids, and mucopolysaccharides. Lysosomal enzymes are important in many hydrolytic processes in meats, including those of post-mortem autolysis, aging and tenderization, and hydrolytic production of flavor constituents. Lysosomes have been identified in many animal tissues (Shibko *et al.*, 1963). Among commercially important animals, lysosomes have been measured in livers and spleens of ox, hog, and sheep and in chicken pectoral muscle. Detailed investigations of isolated lysosomes are presently limited to laboratory animals, especially the rat.

Following the development of methods for separating the subcellular components of liver cells, a large amount of significant information has appeared concerning the chemical composition of mitochondria, microsomes, and nuclei (Getz and Bartley, 1961; Bienzenski and Spaet, 1961; Bienzenski *et al.*, 1963). Because of the difficulties involved in separating lysosomes from mitochondria and microsomes (Beaufay *et al.*, 1959b), only milligram amounts of purified preparations had been available, and consequently no studies had been made on their chemical composition. In our laboratory, however, methods have been developed for obtaining lysosomes (Sawant *et al.*, 1964; Tappel *et al.*, 1963) in quantities sufficient to allow some studies on their composition. Further, since lysosomes can be readily separated into a soluble fraction containing the characteristic hydrolytic enzymes and an insoluble fraction consisting mainly of membrane, analyses were made of constituents of these fractions. Additional analyses of mitochondria and microsomes made possible the comparisons of the compositions of these three organelles.

METHODS

Cell fractionation and evaluation of homogeneity of subcellular fractions. Mitochondrial, microsomal, and lysosomal fractions were separated from rat liver by centrifugal procedures previously described (Sawant *et al.*, 1964; Shibko and Tappel, 1963). Membrane and soluble fractions of the subcellular fractions were obtained by freezing and thawing suspensions of the preparations three times, diluting with deionized water, and centrifuging for 2 hr at 100,000 × G.

Measurements of enzymes characteristic of the subcellular fractions were used to establish the degree of homogeneity of the preparations. Mitochondria were characterized by assay of succinoxidase (Sawant *et al.*, 1964) and glutamic dehydrogenase (Hogeboom and Schneider, 1953), lysosomes

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Table 1. Distribution of protein, phosphorous, and lipid in subcellular fractions of rat liver.

	% total in subcellular fractions					
	Lysosomes		Mitochondria		Microsomes	
	Membrane	Soluble	Membrane	Soluble	Membrane	Soluble
Protein	50	50	50	50	90	10
Phosphorus	60	40	47	53	90	10
Lipid	100	0	86	14	53	46
Membrane phosphorus as phospholipid	24	—	42	—	27	—

by assay of acid phosphatase and other lysosomal enzymes (de Duve *et al.*, 1955), and microsomes by assay of glucose-6-phosphatase (de Duve *et al.*, 1955).

Lipid determination. Lipid was determined by extracting the fractions with chloroform-methanol mixtures as described by Bligh and Dyer (1959). Corrections were made for the extraction of non-lipid material by determining the amount of chloroform-insoluble material in the dried extract. Lipids were separated by thin-layer chromatography with chloroform, methanol, and water (65:25:4 v/v) used as the solvent system (Vogel *et al.*, 1962). The chromatograms were developed with iodine vapor, followed by ninhydrin, followed by 20% antimony trichloride (Neher and Wettstein, 1951).

For determination of phosphorus the subcellular fractions or lipids extracted from the fractions were digested with 72% perchloric acid in the presence of concentrated nitric acid, and the phosphorus determined by the method of Smith *et al.* (1959).

Sialic acid determination. For quantitative measurements of sialic acid in the sialoproteins, the subcellular fractions were hydrolyzed by treating with 0.1*N* sulfuric acid for 1 hr at 80°C. Sialic acid in the supernatant solution was separated by the method of Svennerholm (1957) on a small chromatographic column of Dowex 50 W (1 ml) superimposed on a column of Dowex 1 (1 ml). After elution of the neutral sugars with deionized water, the sialic acid was eluted with 1*M* acetate buffer, pH 4.6. With standard solutions of sialic acid it was shown that 70% of the sialic acid was recovered in this fraction and this factor was used to calculate the final concentration of sialic acid in the sample. Sialic acid in the eluate was estimated by the thiobarbituric acid method of Warren (1959). Corrections were made for the presence of desoxyribose derivatives by making dichromatic readings at 549 and 532 mμ. Subcellular fractions containing about 2 mg of protein were used for these determinations.

Flavin and amino acid determinations. Fluorescence spectra were measured with a Beckman DK-2 spectrophotometer fitted with a fluorescence attachment. The instrument was calibrated with a solution containing 0.1 mg of quinine sulfate per liter of 0.1*N* H_2SO_4.

For determination of free amino acids in the subcellular fractions, ethanol was added to suspensions of the fractions containing about 4 mg protein, to a final concentration of 80%. After boiling for 5 min, the precipitated protein was removed by centrifugation and the free amino acids in the supernatant solution were determined by the method of Troll and Cannon (1953). For identification of the free amino acids, the supernatant solution was first subjected to the ion-exchange procedures described by Carsten (1952) to remove the high concentration of sucrose present as a result of the preparative procedures. Amino acids were then separated by descending paper chromatography with phenol-H_2O-phosphate buffer used as solvent system (McFarren, 1951). Metals in the subcellular fractions were determined by spectrographic analysis by the method of Voth (1963).

RESULTS

Results may be divided into two main sections: 1) major constituents of the lysosome, which include lipids, lipoproteins and phospholipids of the membrane, and the proteins contained in the soluble fraction; and 2) minor constituents: sialic acid, pyridine nucleotides, flavins, amino acids, and metals.

Protein and lipid composition of subcellular fractions. Table 1 shows the distribution of protein between the membrane (insoluble fraction) and the soluble fraction of the subcellular fractions after freezing and thawing, a process which disrupts the mitochondrial and lysosomal membranes, releasing soluble constituents. Only small amounts of protein were released from microsomes by this procedure. Lipid analysis of the membrane and soluble fractions of lysosomes, mitochondria, and microsomes showed that almost all the lipid of lysosomes and mitochondria was associated with the membrane. However, considerable quantities of lipid were solubilized when microsomes were subjected to this treatment (Tables 1, 2). Phosphorus analysis of the lipid fractions (Table 1) indicated that some of the phosphorus must have been associated with fractions other than phospholipid. In the microsomal fraction, the high nonlipid phosphorus is probably associated with RNA, which can readily be identi-

Table 2. Phosphorous and lipid content of subcellular fractions.

	μg/mg protein		
	Lysosomes	Mitochondria	Microsomes
Phosphorus	25	23	50
Lipid	470	535	1100

fied by its UV absorption spectra. No RNA was found in lysosomes. Since neither the lysosomes nor the mitochondria contain RNA, the non-phospholipid phosphorus of the membrane is probably associated with phosphoproteins and bound phosphate compounds like pyridine nucleotides.

Chromatographic separation of the lipids showed that the major identifiable fractions appeared to be similar in the three subcellular fractions. The major constituents identified in lysosomes were sphingomyelin, lecithin, cephalin, neutral triglycerides, and cholesterol.

Sialic acid determination. Sialic acid was detected only in the membrane fractions of the subcellular preparations. Analyses showed that the lysosomal fraction had a higher sialic acid content than the other subcellular fractions (Table 3).

Table 3. Distribution of sialic acid in subcellular fractions.

	μg sialic acid/mg protein		
	Whole		Membrane
Lysosomes (6)[a]	9.2 ± 2.3	(4)	4.8 ± 0.9[b]
Mitochondria (1)	3.2		—
Microsomes (1)	6.5		—

[a] Number of samples analyzed.
[b] Based on protein of whole lysosome.

Although only part of the sialic acid was recovered in the lysosomal membrane fraction, analyses failed to indicate its presence in the soluble fraction.

Amino acid determinations. The nonprecipitable amino nitrogen in the mitochondrial and lysosomal fractions is shown in Table 4. It can be seen that the free amino nitrogen pool is five times as large in lysosomes as in mitochondria. Chromatography of the free amino acids of lysosomes and mitochondria gave similar patterns. The following amino acids were identified: aspartic, glutamic, serine, glycine, lysine, alanine, tyrosine, valine, and proline.

Metal analysis. Table 5 shows the metals detected by spectrographic analysis and their concentrations in the subcellular fractions. The most abundant metal was iron, probably derived from ferritin in the lysosomes (Beaufay *et al.*, 1959a), and cytochromes and protein-bound iron in the mitochondrial and microsomal fractions. In general, metals were present in highest concentrations in the lysosomal fraction.

Table 4. Free amino nitrogen in lysosomes and mitochondria.[a]

	μg amino N/mg protein
Lysosomes	4.1 ± 0.7
Mitochondria	0.8 ± 0.3

[a] Results of 5 analyses.

Fluorescence spectra. Fluorescence spectra of the subcellular fractions are shown in Fig. 1A. Interpretation of these spectra is complex because of the composite nature of the curves obtained. However, two principal peaks can be distinguished: one in the 510–530-mμ region and the other in the 476-mμ region. The respective peaks can be identified as flavin and pyridine nucleotide. Artificial mixtures of riboflavin and DPNH give similar curves. Lysosomal and mitochondrial preparations in the oxidized state showed distinct peaks in the 520-mμ region and only a small shoulder in the 469-mμ region, indicating that these fractions were rich in flavins but contained little reduced pyridine nucleotide. The microsomal fraction showed one peak in the 462-mμ region, corresponding to pyridine nucleotide; there was little fluorescence from flavins. It should be noted that no special precautions were observed in maintaining the structural integrity of the particles. The fluorescence spectra of freshly prepared mitochondria in the reduced state are normally dominated by pyridine nucleotides, and the flavin peak is hardly observed (Chance and Baltscheffsky, 1958). Further information on the distribution of flavins in the subcellular fractions was obtained by studying the fluorescence spectra of fractions that had been separated into soluble and insoluble, or membrane, fractions. The spectra shown are for equal amounts of protein in the respective fractions. Fig. 1C shows the spectra obtained with the soluble fractions. It can be seen that the soluble fraction of the lysosomes shows a very distinct flavin peak, whereas similar fractions derived from mitochondria and

Table 5. Distribution of metals in subcellular fractions.

	mg/g protein		
Metal	Lysosomes	Mitochondria	Microsomes
Fe	2	0.5	3
Cu	0.1	0.05	0.03
Mn	0.03	0.03	0.01
Zn	0.3	0.1	0.1
Mo	0.01	0.2	0.03
Ni	0.01	0 (<.0001)	0 (<.0001)
Co	0 (<.0002)	0 (<.0002)	0 (<.0002)
Cr	0 (<.0003)	0 (<.0003)	0 (<.0003)
V	0 (<.0003)	0 (<.0003)	0 (<.0003)

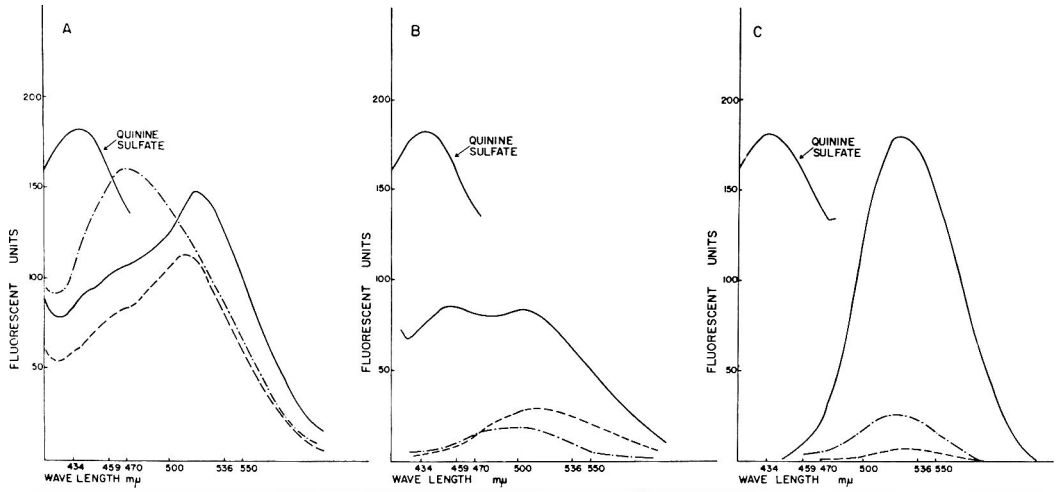

Fig. 1. Fluorescence spectra of subcellular particles. A. Whole subcellular particle. B. Insoluble, or membrane, fraction of particle. C. Soluble fraction of particle.
- - - - - mitochondria, ———— lysosomes, —•—•—• microsomes.

microsomes show very little flavin fluorescence. Spectra of the insoluble membrane (Fig. 1B) derived from mitochondria and lysosomes show the presence of flavin. Pyridine nucleotide appears to be present in the insoluble fraction derived from lysosomes and microsomes but is lacking in the mitochondrial fraction, presumably because of their redox state.

DISCUSSION

Studies of the composition of subcellular particles are important because: 1) they provide information about structure and function, and 2) they help in determining functional relationship; for example, the golgi apparatus is believed to be involved in part in the formation of lysosomes. Since the golgi apparatus may be derived from degenerate endoplasmic reticulum, there may be close similarities in the membrane composition of lysosomes and microsomes. Further, since mitochondria and microsomal fractions are digested in the lysosomes, their products could be expected to accumulate within the lysosomes.

The results presented here are comparative rather than a detailed analysis of the constituents of each fraction; for example, the lipid, phospholipid, and phosphorus content of each subcellular species has been estimated without detailed analysis of their constituents. The analyses show that total lipid and phosphorus content of lysosomes and mitochondria are similar; however, proportionally more of the mitochondrial phosphate is present as phospholipid. This can be related to differences in membrane structure, mitochondria possessing double membranes whereas lysosomes are known to have a single unit membrane (Novikoff, 1961). Microsomes, as has been previously demonstrated, contain far higher amounts of lipids and phosphorus.

One of the characteristic properties of lysosomes is that they are readily disrupted and separated into an insoluble fraction, containing the membrane, and a soluble fraction, which contains almost all of the hydrolases. Membrane stability is known to be associated with the latency of the lysosomal enzymes. It has recently been suggested that the latency of the lysosomal enzymes is due to their sequestration with sialopolymers and that the lysosomes do not have a membrane (Koenig, 1962). Sialic acid analyses show that it is present in lysosomes in amounts only slightly higher than those observed in the other subcellular entities, a result which suggests that sialopolymers are not involved in a special role in the lysosomes. Further, the amount of lipoprotein present in the lysosomal fraction is sufficient to account for the unit membrane postulated for these particles and seen in electron micrographs.

Interpretation of the fluorescence spectra, which must be semiquantitative, shows that

lysosomes contain a considerable amount of flavin in their soluble phase. Two possibilities may be suggested to explain its presence in lysosomes: 1) lysosomes are known to be involved in the intracellular catabolism of other subcellular particles, and the flavins represent an accumulation of products from this process; and 2) flavin is derived from flavoenzymes associated with function of the lysosome. Enzymes of this type are known to exist in two main categories: 1) those concerned with the direct oxidation of metabolites such as amino acid oxidase, aldehyde oxidase, and xanthine oxidase; and 2) dehydrogenases like DPNH-dehydrogenase. Amino acid oxidase is known to be associated with a group of particles quite distinct from the lysosome, the uricase particle (Baudhuin and Beaufay, 1963), and we have not found any evidence for aldehyde oxidase or xanthine oxidase in lysosomes. However, a specific DPNH-dehydrogenase has been identified in lysosomes (Ragab and Tappel, 1965) and part of the lysosomal flavin appears to be involved in this redox system.

The high concentrations of amino acids which accumulate in the lysosome as a result of intra-lysosomal digestion of proteins are probably transported to other parts of the cell and utilized in various metabolic processes. This has been observed in an analogous situation where labeled bacterial proteins were digested by macrophage granule enzymes, and were made available for utilization by the cell (Cohn, 1963). In our research on lysosomes we have found no evidence that the amino acids undergo further metabolism within the lysosome. Accumulation of iron in the lysosome is probably a reflection of the high ferritin content of these particles (Beaufay *et al.*, 1959a). Lysosomes may be involved in a regulatory role for the level of iron in the cell, although there is no evidence that they play a special role in erythrocytosis. Accumulation of iron and other metals also results from the general involvement of lysosomes in the digestion of mitochondria and microsomes and the removal of toxic material from the cell.

Compositional studies of lysosomes are complicated by the fact that the lysosomes appear to undergo a life cycle which is an expression of the degree of digestion of the autophagocytized material (de Duve, 1959). In addition, the lysosomal activity of the cell will also depend upon the physiological state of the animal and its diet. Thus, it is possible to present only a very general account of the composition of lysosomes. Further study of the lysosome will require more detailed material balances and assignment of components.

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Certain Physiological and Biochemical Changes in the Developing Tomato Fruit (Lycopersicon esculentum Mill.)

SUMMARY

The biosynthesis of nonvolatile compounds by tomato fruit parallels the growth rate of the fruit, regardless of varieties studied. Volatile reducing substances (VRS, mμ/100 g), reducing sugars (percent), water-soluble pectins (percent), and organic acids (mg/100 g) progressively increase in quantity with advancing maturity. Total titratable acidity (percent) and total pectic materials (percent) increased during the initial stages of maturation, but gradually decreased as the fruit ripened. Ascorbic acid (mg/100 g) increased with the maturity of fruit but declined slightly in the later (red and red-ripe) stages of maturation. Concentrations of compounds (chlorophylls, mg/100 g; carotene and lycopene, mg/100 g) contributing to the coloration changed significantly as the fruit passed through various degrees of maturation. The pattern of physiological and chemical changes during the development of tomato fruit was nearly identical in both varieties studied. However, V. R. Moscow fruit contained higher amounts of pigments, reducing sugars, pectins, organic acids, ascorbic acid, and VRS than the fruit of Fireball variety, regardless of maturities studied.

INTRODUCTION

The demand for and acceptance of fresh tomato fruit is based largely on its nutritional value, flavor, aroma, taste, and characteristics such as color and texture. These quality criteria are dependent primarily on the structure and chemical composition of the fruit. The extent and nature of physiological and chemical changes in developing fruits and vegetables other than tomatoes have been discussed by many investigators. Soule and Harding (1956) reported that minor differences were found in the various constituents in mango fruit of different maturities. Nonvolatile components such as starch, total sugars, total soluble solids, and sugar-solids ratio increased with the maturity of the fruit. Deshpande and Salunkhe (1964) studied the relation of weight and decay losses, firmness, acidity, pH, soluble solids-acidity, volatile reducing substances (VRS), sugars, total pectins, tannins, ascorbic acid, and carotenoids to the maturity of peaches and apricots. According to them, as maturity increased, firmness, acidity, total pectins, and tannins decreased, and pH, soluble solids, soluble solids-acidity, volatile reducing substances, ascorbic acid, and carotenoids increased. Results were similar with apricots. Lim and Romani (1964) recently indicated that volatiles, besides firmness, soluble solids, and carbon dioxide production, were markedly correlated with the maturity of peaches.

Information is very meager on physiological and biochemical changes in the developing tomato fruit. This article presents experimental data concerning changes in acidity, pigmentation, free reducing sugars, volatile reducing substances, and organic acids during various stages of maturation in tomato fruit.

EXPERIMENTAL

Tomatoes (var. V. R. Moscow and Fireball) were harvested at different stages of maturation. The fruits were sorted and classified into nine categories according to size, weight, and color (Table 1). Average weights for individual fruit were calculated by dividing the total weight of all fruit by the number of fruit in each category. Alcohol slurries were prepared by grinding 250 g of fruit in 250 ml of ethyl alcohol. The slurries

Table 1. Classification of tomatoes (variety: V. R. Moscow) according to size, color, and average weight of individual fruit.

Degree of maturation	Size (diameter, in inches) and/or color	Av. wt. of fruit (g)
1	½ or below, greenish	2.5
2	¾ to 1, greenish	8.3
3	1 to 1¼, greenish	10.3
4	1¼ to 1¾, greenish	35.7
5	2 to 3 or large green	50.0
6	Breakers	50.0
7	Pink	62.6
8	Red	83.3
9	Red-ripe	100.0

were preserved at 40°F and evaluated for the following subjective and chemical attributes.

Total titrable acidity and pH. Ten grams of fresh fruit from each category were homogenized with 100 ml distilled water and then titrated to pH 8.1 with 0.1*N* sodium hydroxide. Each sample was tested on a Beckman pH meter.

Color measurement. Fresh samples of tomatoes were obtained for determination of total chlorophylls and carotenoids.

Total chlorophylls and chlorophyll *a* and *b* were determined according to the procedures outlined by the AOAC (1960). Total carotenoids and beta-carotene were determined according to procedures outlined by the Assoc. Vitamin Chemists (1951).

Free reducing sugars. Free reducing sugars were determined by the Shaffer-Somogyi micromethod (AOAC, 1960).

Total pectic materials and soluble pectins. Total pectic materials and soluble pectins were respectively determined according to procedures outlined by McCready and McComb (1952) and Ruck (1956).

Volatile reducing substances (VRS). Fresh samples of tomatoes were obtained for VRS determinations. Fruit (250 g) was blended with a minimum quantity of distilled water and analyzed for VRS content according to the procedure outlined by Luh (1961).

Organic acids. Organic acids were separated by ion-exchange column chromatography and were identified by the method outlined by Marvel and Rands (1950).

Ascorbic acid (Vitamin C). Ascorbic acid was determined according to the procedure outlined in AOAC (1960).

RESULTS AND DISCUSSION

Total titratable acidity. Fig. 1 shows the percent total titratable acidity of tomatoes found in this experiment. Total titratable acidity increased as the fruit passed from green-mature stage to pink, but declined slightly as the fruit became red. Total titratable acidity was calculated as citric acid, which is the conventional method of expressing the acidity of tomato, although many other constituents contribute to total titratable acidity.

Color measurement. Fig. 2 shows total chlorophylls and the chlorophyll *a* and *b* contents of tomatoes at different stages of maturity. Total chlorophylls continued to increase as size and maturity of fruit increased, but decreased as the fruit started acquiring a pink color. Chlorophyll *b* and *a* existed in a 2:1 ratio. During the growing stage, the fruit contained green pigment but also had white patches of discoloration, which slowly disappeared with advancing maturity. The beta-carotene and lycopene content of tomatoes is shown in Fig. 3. The

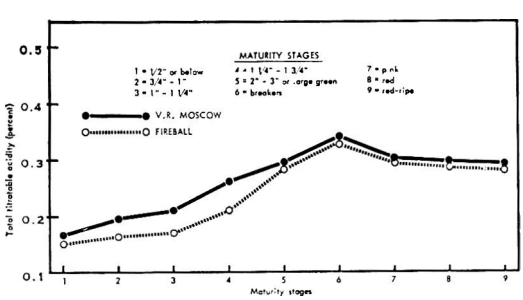

Fig. 1. Total titratable acidity of V. R. Moscow and Fireball tomatoes at various stages of maturation.

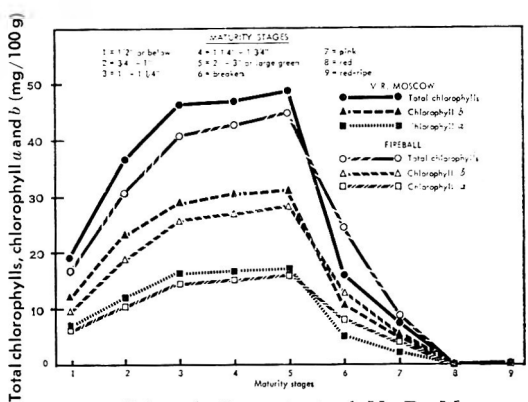

Fig. 2. Chlorophylls content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

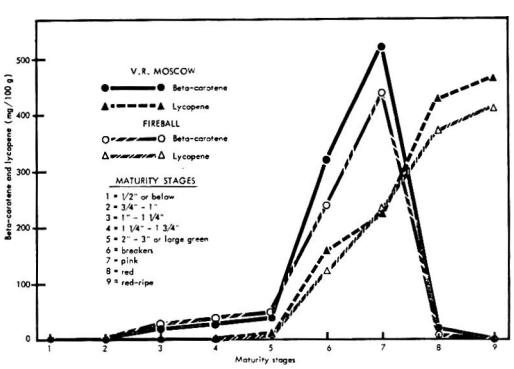

Fig. 3. Beta-carotene and lycopene content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

beta-carotene content was maximum at the pink stage. As the fruit became red, the beta-carotene changed, presumably to lycopene, since the ratios of these two substances varied markedly. The lycopene content of tomatoes was calculated by subtracting the beta-carotene content (mg/100 g) from the total carotenoids (mg/100 g). The exact nature of color development in tomatoes is not yet thoroughly understood. According to Wann and McFerran (1960), a complex system of pigmentation is responsible for tomato color. The major components of this pigment system are lycopene (red), beta-carotene (yellow), and other related carotenoid pigments.

Free reducing sugars. Reducing sugars constitute over a third of the soluble solids of tomato fruit. The sugar content increases uniformly from green mature to vine-ripe condition (Rosa, 1925). The free reducing sugar contents of the tested tomatoes are illustrated (Fig. 4). Reducing sugars such

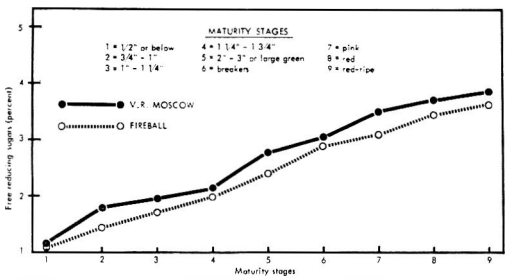

Fig. 4. Free reducing sugars content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

as glucose are found in tomatoes. Hydrolyzable dissaccharides such as sucrose were not found in this experiment.

Total pectic materials and soluble pectins. Kattan (1957) reported a significant difference in firmness between fruits harvested at the green-mature stage and fruits harvested at the pink or red stage. The softening process is thought to be due to conversion of the insoluble protopectin of middle lamella to water-soluble pectins. In this experiment protopectin content (responsible for firmness) increased up to the large green stage (described in Table 1) and decreased as the fruit attained full maturity (Fig. 5). Soluble pectins slowly increased

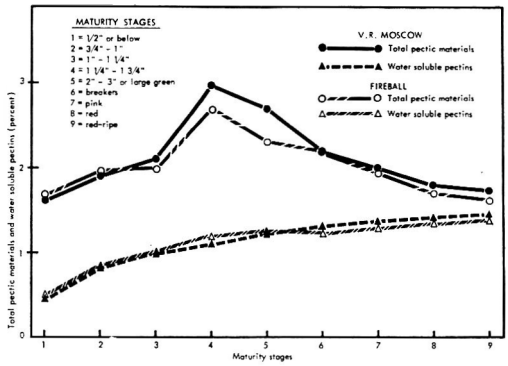

Fig. 5. Pectic values of V. R. Moscow and Fireball tomatoes at various stages of maturation.

during maturation, indicating progressive softening of fruit that accompanied the maturation process.

Volatile reducing substances. Flavor development is very closely related to the growth of the fruit. Temperature, relative lengths of day and night, humidity, and other environmental factors have considerable bearing upon plant growth and the flavor of the fruit. Tremendous differences in flavor can be developed both by genetic means and physiological controls. Increases in VRS (responsible for tomato flavor) were more or less correlated to the rate of maturity of the fruit in this experiment (Fig. 6). The VRS contents varied con-

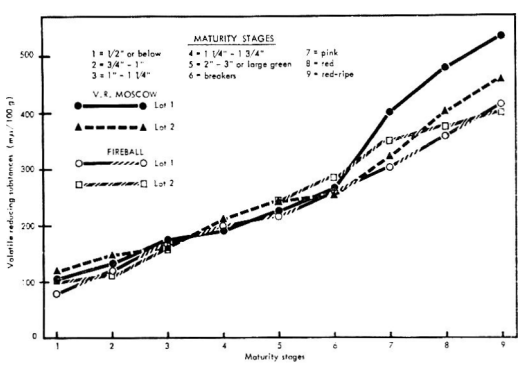

Fig. 6. Volatile reducing substances of V. R. Moscow and Fireball tomatoes at various stages of maturation.

siderably among the different samples of the single variety, indicating the highly sensitive character of the flavoring compounds.

Organic acids. Tomatoes can be classified as acid fruits because their soluble solids

are composed chiefly of organic acids and sugars. The acidity of tomatoes and tomato juice is due primarily to citric and malic acids (Hartmann and Hillig, 1934). Recalde and Blesa (1962) considered the metabolism of organic acids to be the function of enzymes. Fig. 7 is a typical chromatogram of malic and citric acids separated from red-ripe tomato fruits. The areas under the peaks, representing malic and citric acids, increased progressively with increasing maturity. Two-thirds of the total titratable acidity of the tomato fruit was attributable to the malic and citric acids. Many other acids belonging to Kreb's cycle, were eluted along with the malic and citric acids. No effort was made, however, to identify any of these organic acids, primarily because they were present in only extremely small concentrations. Concentrations of malic and citric acids were greater in the red-ripe tomatoes than in the other degrees of maturity tested (Fig. 8).

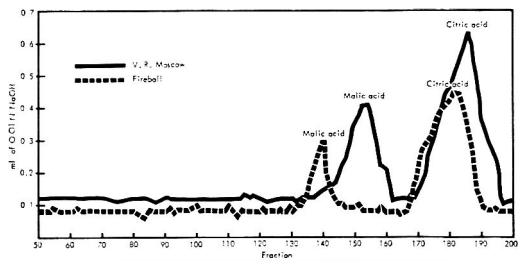

Fig. 7. Schematic representation of chromatographic separation of organic acids from red-ripe V. R. Moscow and Fireball tomatoes.

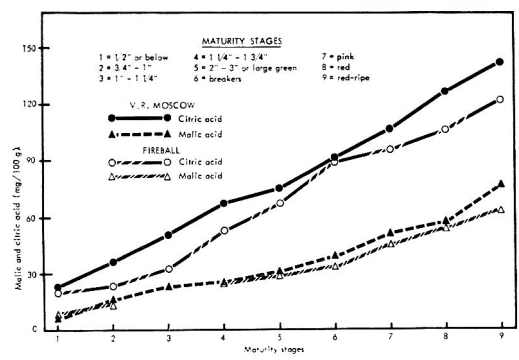

Fig. 8. Percent milligrams of malic and citric acids content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

Ascorbic acid (Vitamin C). Vitamin C content of fruits and vegetables varies considerably with variety, maturity, fertilizer, soils, and conditions of ripening (Brown and Moser, 1941). Fig. 9 shows the ascorbic acid content of tomatoes (mg/100 g). Ascorbic acid increased progressively up to pink stage and declined slightly as the fruit attained red-ripe stage. Utilization of ascorbic acid in the ripening process could be considered as a possible cause for this decrease of ascorbic acid content in the later stages of maturation.

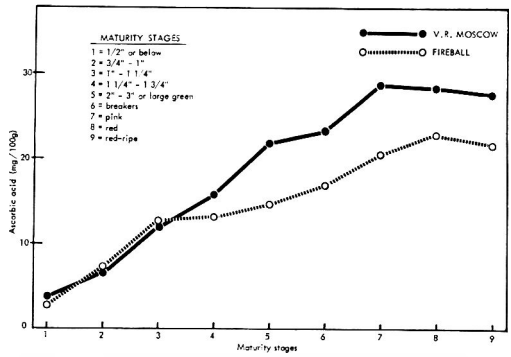

Fig. 9. Percent milligrams of ascorbic acid content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

Figures 1 through 9, in general, indicate rather similar patterns of physiological and biochemical changes in the developing tomato fruit in both varieties—V. R. Moscow and Fireball. However, V. R. Moscow variety contained higher amounts of pigments, organic acids, ascorbic acid, reducing sugars, pectins, and VRS than the Fireball variety, regardless of maturities studied.

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Chemical and Physiological Changes in Lime Fruits During and After Storage

SUMMARY

The rates of deterioration, weight loss, color change, and respiratory rate of lime fruits were determined during storage for 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C and after transfer to 20°C following the above storage treatments. The surface treatments compared were control, mineral oil, and wax. Changes in weight, juice content and soluble solids, acidity, and ascorbic acid in the juice were recorded during 4 and 8 weeks of storage at 5, 7.5, and 10°C and after one week at 20°C following the above storage treatments. The respiratory rate of limes at 20°C following storage was greater than limes placed directly at 20°C; the increase being greater, the lower the initial storage temperature, and the longer the duration of the initial storage period. Lime fruits to be stored and subsequently marketed at room temperatures should be waxed and stored at about 7.5°C for a period not to exceed 8 weeks. The amount of juice and the soluble solids, acid and ascorbic acid contents in the juice increased during storage based on the original fresh weight of the fruit. On a concentration basis, ascorbic acid decreased during storage. The relationship of changes after transfer to 20°C and chilling injury are discussed.

INTRODUCTION

Lime fruits, although not extensively grown in California, are an important crop in India, Mexico, Egypt, and Peru. Historically, limes played an important role in the prevention of scurvy among sailors, because of their ascorbic acid content. Today limes are used primarily in beverages, creating a constant demand throughout the year. However, production is seasonal; thus storage becomes a necessity, because in most countries where limes are produced no facilities are available for processing and storage of the processed material. Therefore, the period of availability is limited.

General information on the storage of limes is available (Aref *et al.*, 1961; Mustard, 1950; Stahl and Mustard, 1948). Wright *et al.* (1954) recommended storage temperatures ranging from 5 to 10°C at a relative humidity of 85–90%. Various protective coatings on the fruit and film coverings for containers have been studied (Eaks, 1955; Hatton, 1958; Mustard, 1950; Stahl and Mustard, 1948). In general, waxing and latex on the fruit and the film wraps reduce weight loss. The green color of the rind has been prolonged during storage by 2,4,5-T (Hatton, 1958) and by mineral oil (Eaks, 1955).

The chemical changes in the lime fruit during and after storage have not received detailed attention. Aref *et al.* (1961) reported data on juice volume, acid and soluble solids during storage at 10°C and 70–80% relative humidity. Weight loss and changes in the amount of juice during storage at about 28 to 31°C and 70% relative humidity were reported by Lincoln (1949). Lynch (1942) presented weight loss, changes in juice content, acid, and degrees Brix for limes held up to 13 days at about 26°C. He reported changes in juice on the basis of initial fresh weight as well as on the final weight.

The present report concerning the physiological responses and chemical changes in lime fruits during cold storage and subsequent to cold storage at 20°C describes: a) the respiratory responses and physical deterioration; and b) the chemical changes in the juice (amount of juice, soluble solids, acid, and ascorbic acid).

MATERIALS AND METHODS

The lime fruits, *Citrus aurantifolia* (Christm.) Swing., used in this study were selected from freshly harvested fruit in commercial packing houses. Fruit from the Imperial Valley of California, graded for uniformity of size (average fruit weight about 75 g) but not washed or waxed, were used for the respiratory and deterioration rate studies. Random samples of 20 fruits each were selected, weighed, treated, placed in paper bags and put in storage. Fruit treatments were:

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a) control, no treatment; b) mineral oil, applied by rubbing each fruit with a piece of cotton toweling soaked with mineral oil; and c) wax, dipped in water emulsion citrus wax and dried with air from a fan at 25°C. The storage treatments consisted of 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C. Following the storage treatment the fruits were transferred to 20°C, where they were placed in respiratory chambers or placed on shelves in the paper bags. The respiratory rate was determined daily for a 14-day period by the colorimetric method of Claypool and Keefer (1942). The air stream, metered by capillaries over the limes in the respiratory chambers, was cleared of carbon dioxide by bubbling through cylindrical fritted gas-dispersion tubes into 1N NaOH containing Alizarine Yellow and then bubbled through water to trap any hydroxide carried over and to humidify the air stream. The dye indicated when the pH of the solution approached pH 10, at which time it was replaced. Amount of decay, rate of deterioration, and color change of all fruits were recorded when removed from storage and after two weeks at 20°C. All treatments were replicated four times.

Fruits for the chemical compositional study came from Escondido, California. These were harvested, held overnight at prevailing temperatures, and on the next day washed, graded, waxed, and brought to Riverside, and the experiment initiated. The bulk supply (average fruit weight 50 g each) was divided into random samples of 20 fruits each, weighed, placed in paper bags and put in storage, except for samples analyzed immediately. The storage treatments consisted of 4 and 8 weeks at 5, 7.5, and 10°C and one week at 20°C following 4 and 8 weeks at 5, 7.5, and 10°C. Each treatment was replicated five times. Characteristics investigated were: a) fresh-weight changes; b) amount of juice reamed from the fruits (Sunkist power reamer); c) soluble solids in the juice (Abbé Refractometer) corrected for acidity (Stevens and Baier, 1939); d) total titratable acid in the juice expressed as anhydrous citric acid; and e) ascorbic acid (AA) content of the juice (Loeffler and Ponting, 1942). The juice content is expressed as percentage based on fruit weight at the time of sampling and as grams of juice per 100 g of original fresh weight (OFW) of the fruits. Soluble solids and total acids are expressed as percentage in the juice and as grams per 100 g OFW of the fruits. The ascorbic acid content is expressed as mg per 100 g of juice and as mg per 100 g OFW of the fruits. Expressing the amounts of juice, soluble solids, acid, and ascorbic acid on the basis of 100 g OFW presents the actual quantitative changes in these components in the fruits.

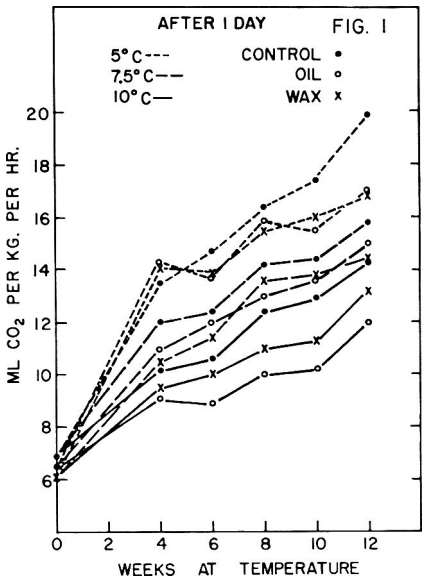
RESULTS AND DISCUSSION

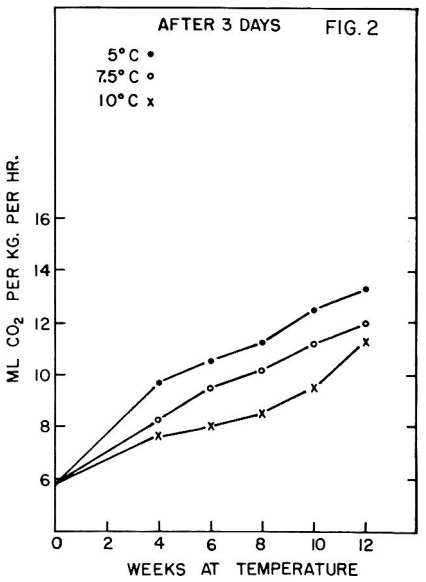
Figs. 1 and 2 show the average respiratory rate of lime fruits with no treatment and treated with mineral oil and wax after 1 and 3 days at 20°C following 0, 4, 6, 8, 10, and 12 weeks of storage at 5, 7.5, and 10°C. Although determinations were made daily at 20°C on all samples for 14 days after storage, presentation of the complete data would be cumbersome. The average value after 1 and 3 days illustrates the pertinent physiological responses observed. The initial respiratory rate of all fruits at 20°C prior to storage ranged from 6 to 7 ml CO_2/kg/hr (Fig. 1), with insignificant differences between the various surface treatments. Following the various storage periods, the respiratory rate 1 day after transfer to 20°C increased compared with fruit placed directly at 20°C and the increase was greater the lower the temperature during the previous storage period. Also, the degree of stimulation observed increased directly with the duration of storage. Fruits receiving no surface treatment had a higher rate than those that were treated, except for those fruits held 4 weeks at 5°C. Consistent differences between fruits treated with mineral oil and wax were not obtained for fruits held at 5 and 7.5°C, but for fruits held at 10°C, those treated with mineral oil showed less stimulation than those treated with wax.

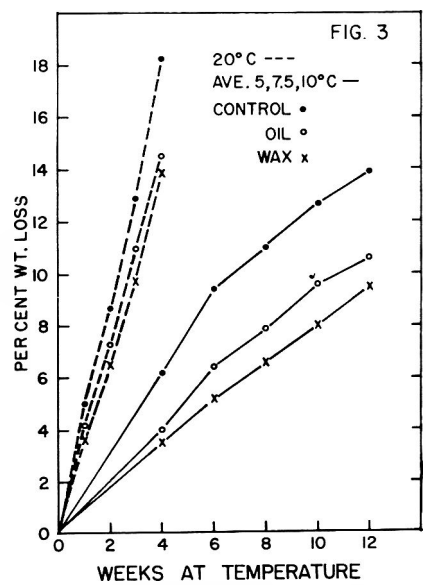
After 3 days at 20°C following storage the differences between surface treatments were small. Therefore, they were combined (Fig. 2). The respiratory rate of all fruits decreased from the first to the third days (Figs. 1, 2). However, after 3 days at 20°C the relationships between temperature and duration of storage remained the same as described above after 1 day at 20°C. From the third to the fourteenth days the respiratory rate of all samples declined except where decay was detected.

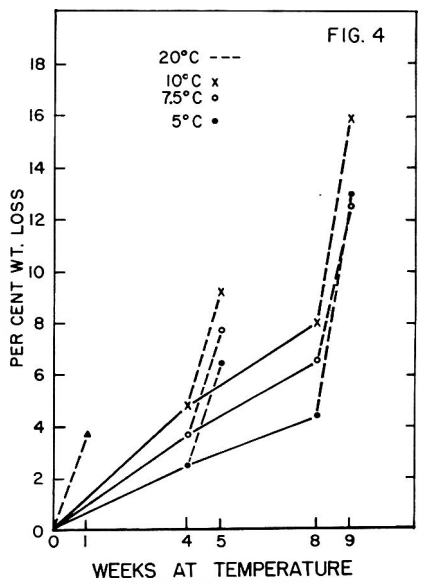
The stimulation in the metabolic activity at 20°C following low-temperature storage is indicative of physiological injury to the tissue. Responses similar to the ones observed here for limes have been reported for oranges, cucumber, and sweet potato (Eaks, 1960). The basic cause and source of the stimulated respiratory rate following

chilling exposures has not been delineated. However, it has been suggested that certain metabolic intermediate compounds may accumulate during the chilling exposure which are available for respiration. There is the possibility that these compounds may cause irreversible physiological damage to the plant cells (Eaks, 1960; Hulme *et al.*, 1964).









Figs. 1–4. 1) Respiratory rate (ml CO_2 per kilogram of fruit per hr) of control, mineral-oil-, and wax-treated limes after one day at 20°C following 0, 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C. 2) Respiratory rate (average for control, oil, and wax treatment) of limes after three days at 20°C following 0, 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C. 3) Weight loss of control, oil-, and wax-treated lime fruits after 1, 2, 3, and 4 weeks at 20°C and of control, oil-, and wax-treated lime fruits after 4, 6, 8, 10, and 12 weeks of storage (weights for fruits held at 5, 7.5, and 10°C were combined to get average percentage weight loss). 4) Weight loss of waxed limes used for the chemical analysis after 4 and 8 weeks at 5, 7.5, and 10°C and after 1 week at 20°C following 4 and 8 weeks at 5, 7.5, and 10°C.

Table 1. Percent acceptable surface-treated limes (control, mineral oil and wax after 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C (part 1) and after 2 weeks at 20°C in respiratory chambers (relative humidity 90–100%) and on the shelf in paper bags (relative humidity 50–60%) following 4, 6, 8, 10, and 12 weeks at 5, 7.5, and 10°C (part 2).

Storage temp (°C)	Relative humidity at 20°C	Percent acceptable Weeks at temperature											
		4			6			8			10			12		
		Cont.	Oil	Wax	Cont.	Oil	Wax	Cont.	Oil	Wax	Cont.	Oil	Wax	Cont.	Oil	Wax
Part 1. Day removed from temperature																
5		100	100	100	100	100	100	100	100	100	89	75	95	90	65	95
7.5		100	100	100	100	100	100	100	100	100	89	73	95	75	60	97
10		100	100	100	100	100	100	100	100	100	95	73	99	40	50	70
Part 2. After two weeks at 20°C																
5		100	97	100	40	60	98	0	0	80	0	0	60	0	0	30
7.5	50 to 60	89	88	98	30	5	95	5	0	90	0	0	8	0	0	10
10		80	90	92	30	60	85	2	0	60	0	0	2	0	0	0
5		97	95	94	98	92	98	55	23	80	58	0	71	15	1	37
7.5	90 to 100	95	88	100	95	70	97	93	41	94	53	41	86	40	22	65
10		85	90	98	90	88	100	80	65	90	40	30	63	5	10	15

Decay, deterioration, and color change data are summarized in Table 1. All causes contributing to unacceptability were combined. During the storage period at 5, 7.5, and 10°C, all fruits remained in good condition for 8 weeks, with some losses after 10 weeks and considerable loss after 12 weeks, especially at 10°C (part 1). After 2 weeks at 20°C following the various storage treatments, those fruits held in paper bags (relative humidity 50–60%) deteriorated primarily because of water loss, as evidenced by shriveling and hardening of the rinds. Lime frutis have little or no natural surface cuticle, and therefore are subject to excessive water loss (Lincoln, 1949). Waxing, as pointed out in several reports (Hatton, 1958; Lincoln, 1949; Mustard, 1950) and as indicated in Table 1 and Fig. 3, protects the lime fruit from water loss. In the respiratory chambers (relative humidity 90–100%) the lime fruits held up for a much longer time. Fruits treated with mineral oil showed surface discoloration as a cause of unacceptability. Symptoms of chilling injury, a surface breakdown similar to that previously described (Eaks, 1955), also appeared on many of the fruits after 6 weeks or longer at 5°C and after 8 weeks or longer at 7.5°C. The mineral oil tended to prevent yellowing, but its commercial value is questionable. Waxed fruit appeared better than control or mineral-oil-treated fruit in all cases. In general, the results suggest that lime fruits can be held in storage at 5, 7.5, and 10°C for about 10 weeks and that the storage period should not exceed about 8 weeks if they are to be held at room temperatures for any period after removal from storage.

The effect of the different surface treatments on the percentage weight loss of lime fruits is shown in Fig. 3. The values for different temperatures (5, 7.5, and 10°C) were averaged to illustrate the differences between surface treatments. Fruits placed directly at 20°C and 50–60% relative humidity lost weight rapidly. The control (untreated) lime fruits lost 18% of their weight in 4 weeks at 20°C, while the mineral-oil- and wax-treated fruit lost approximately 14% of their weight during the same period.

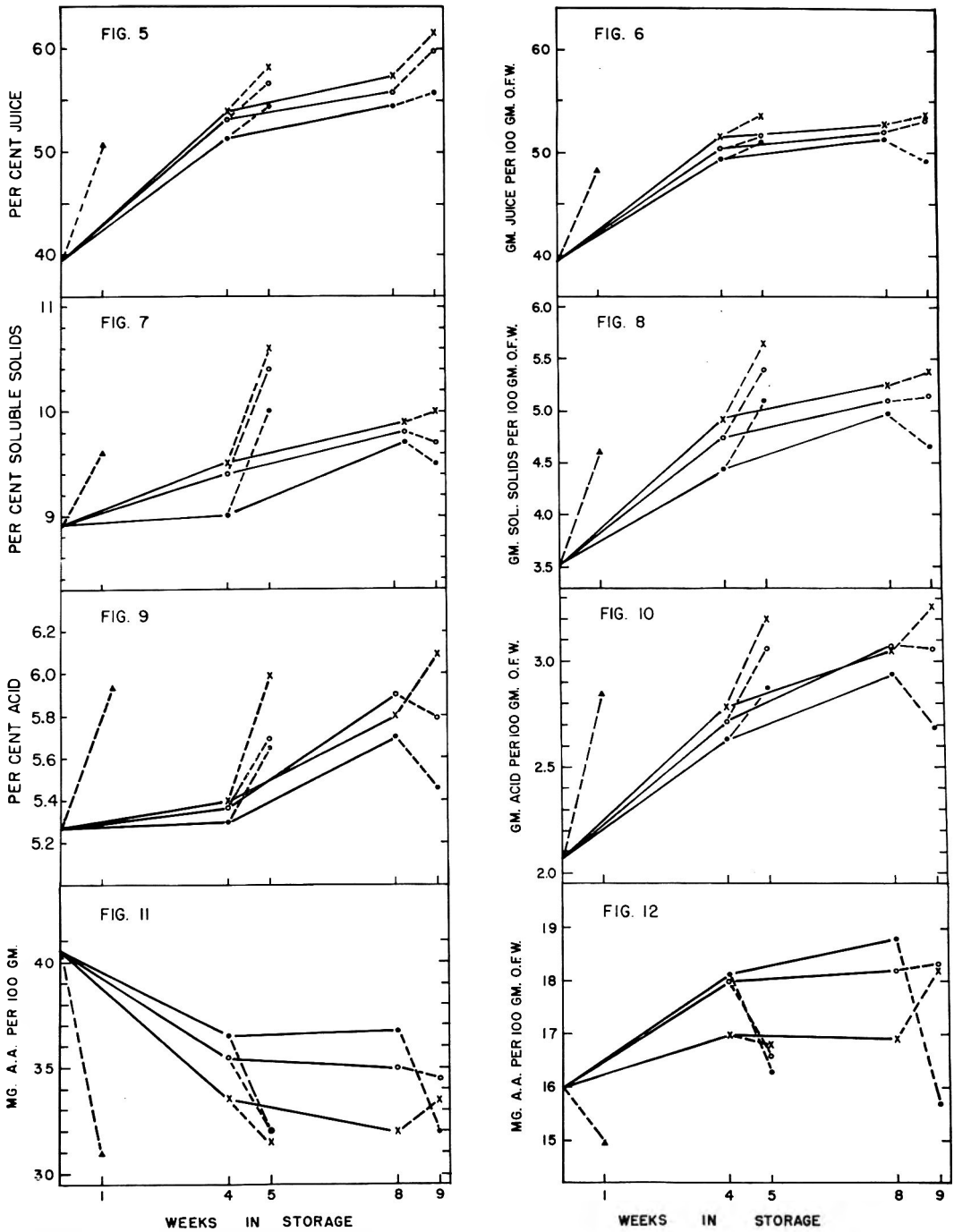
The averages for the fruits held at 5, 7.5, and 10°C clearly demonstrate the influence of the mineral oil and the wax on the prevention of weight loss; waxing was more effective than the mineral oil. The influence of wax on weight loss of lime fruits observed here is similar to previous reports (Hatton, 1958; Lincoln, 1949; Mustard, 1950).

The rate of weight loss of the commercially waxed fruits used for the chemical compositional study (Fig. 4) was similar to the fruits waxed in the laboratory (Fig. 3). An increase in the storage temperature increased the rate of weight loss (Fig. 4). After 4 weeks at 5, 7.5, and 10°C the fruit lost weight at essentially the same rate as fruits placed directly to 20°C. However, after 8 weeks in storage at 5, 7.5, and 10°C the rate of weight loss was faster at 20°C than for fruit held 4 weeks or placed directly to 20°C. This increase in rate of weight loss at 20°C after 8 weeks of storage, especially for fruits held at 5°C, may be associated with chilling injury.

The amount of juice reamed from the fruit increased during storage, based either on the weight at time of sampling or on the OFW (Figs. 5, 6). The higher the storage temperature the greater was the increase in juice. Fruit held one week at 20°C showed a marked increase in juice content. After 4 weeks in storage, the juice content increased considerably, with small additional increases after 8 weeks in storage. The fruits removed from storage and held one week at 20°C showed sharp increases in juice content, except for fruits held 8 weeks at 5°C when expressed on the basis of the OFW, which is probably the result of chilling injury. This decrease in grams of juice per 100 g of OFW from the fruit after 1 week at 20°C following 8 weeks at 5°C is reflected in the soluble solids, acid, and ascorbic acid. Presentation of the results as grams of juice per 100 g of OFW of the lime fruits gives an exact picture of actual changes in juice content. Lynch (1942) found an increase in juice content of limes held 13 days at about 26°C based on weight at time of sampling, but on the OFW basis essentially no change in juice content occurred. Aref *et al.* (1961), storing at 10°C, and Lincoln (1949), storing at 28–31°C, found an increase in the juice content of limes calculated as percent by weight or volume. Lemons held at 4, 13, and 24°C and sampled after 4, 8, and 12 weeks increased in juice content throughout the test period (Eaks, 1961). The limes used in the present study showed a slight increase in juice content on the OFW basis.

The soluble solids content of the juice increased during storage (Figs. 7, 8). The increase was greater the higher the storage temperature. After one week at 20°C following 4 weeks in storage, the soluble solids content of the juice showed a large increase similar to that observed for the fruit placed directly to 20°C. However, after 1 week at 20°C following 8 weeks in storage the grams of soluble solids per 100 g OFW in the juice from the fruit held at 7.5 and 10°C increased some, but decreased in the juice of the fruit held at 5°C. Increases have been observed in soluble solids in the juice of lime fruits held at 10°C (Aref *et al.*, 1961) and at 26°C (Lynch, 1942). Lemons held at 4°C showed very little increase in the soluble solids in the juice after 4, 8, and 12 weeks in storage (Eaks, 1961) in contrast to the sizable increase found in limes held at 5°C in the present study (Fig. 8). Lime fruits held at 7.5 and 10°C (Fig. 8) increased in soluble solids in a manner similar to lemons held at 13°C (Eaks, 1961).

Figs. 9 and 10 illustrate the changes in total titratable acid expressed as anhydrous citric acid. The changes in percentage acid during 4 weeks in storage were small. However, after 8 weeks the percentage acid increased in the juice of fruit held at all temperatures. Fruit held at 20°C produced juice that showed an increase in percentage acid after 1 week at 20°C without a previous storage period and following 4 weeks in storage. After 1 week at 20°C following 8 weeks at 10°C the percentage of acid increased, but decreased following 8 weeks at 5 and 7.5°C. The grams acid per 100 g OFW increased after 4 weeks in storage because of the increase in juice, and the increase after 8 weeks in storage is associated with the increase in percentage of acid. Small increases in the percentage of acid in the juice of lime fruits have been reported for fruit held at 10°C (Aref *et al.*, 1961) and 26°C (Lynch, 1942). The acid content



Figs. 5–12. 5) Percent juice based on fruit weight at sampling after 0, 4, and 8 weeks of storage at 5° (●), 7.5° (o), and 10° (x) Centigrade (solid line) and after 1 week at 20°C following 0, 4, and 8 weeks of storage at 5, 7.5, and 10°C (broken line). 6) Grams of juice per 100 g original fresh weight (OFW) of fruit after storage as described for Fig. 5. 7) Soluble solids in the juice (percent) after storage as described for Fig. 5. 8) Grams soluble solids in the juice per 100 g OFW of fruit after storage as described for Fig. 5. 9) Titratable acid as percent anhydrous citric acid in the juice after storage as described for Fig. 5. 10) Titratable acid as grams anhydrous citric acid in the juice per 100 g OFW of fruit after storage as described for Fig. 5. 11) Milligrams ascorbic acid (AA) per 100 g of juice after storage as described for Fig. 5. 12) Milligrams AA in the juice per 100 g OFW of fruit after storage as described for Fig. 5.

of the juice of lemons held at 4°C for 4, 8, and 12 weeks did not increase, but did increase in the juice of fruits held at 13 and 20°C (Eaks, 1961).

The ascorbic acid content of the juice illustrated in Figs. 11 and 12 appears to present contradictions. On a concentration basis (mg per 100 g) the ascorbic acid content of the juice decreased in all cases, except for the samples held 1 week at 20°C following 8 weeks at 10°C (Fig. 11). When the ascorbic acid is expressed as mg per 100 g OFW of the lime fruits, an increase was found following the 4- and 8-week storage periods (Fig. 12). This increase occurs because the increase in juice content was greater than the decrease in concentration of ascorbic acid. In all cases the ascorbic acid content of the juice decreased when the fruit was held at 20°C except in fruit held 1 week at 20°C following 8 weeks at 10°C (Fig. 12). The sharp drop in ascorbic acid during the 1 week at 20°C following 8 weeks at 5°C is similar to the reaction of lemons following 12 weeks at 4°C (Eaks, 1961). This response has been attributed to chilling injury in the lemon, and it appears that the same thing is true in lime fruits.

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The Exterior Structures of the Egg as Nutrients for Bacteria

SUMMARY

A study was made to see if the exterior structures of the egg (cuticle, shell, and shell membranes) can support the growth of bacteria. Two species of common egg-spoiling bacteria and a pathogenic bacterium were examined. The exterior structures supported extensive growth of all three organisms comparable to that found when the same bacteria were cultured in albumen. When these bacteria were seeded into sterile saline containing intact egg shells, they could be isolated, after some time, from the inside of the shell.

INTRODUCTION

The fact that egg-spoiling bacteria can utilize the material of the shell membranes for growth was established by several workers (Stokes and Osborne, 1956; Elliott and Brant, 1957; Garibaldi and Stokes, 1958). There are no reports on similar experiments regarding the cuticle and shell. The ability of bacteria to grow on the external egg structures is important and interesting both from the practical standpoint of handling eggs and for a better knowledge of the mechanism by which bacteria enter eggs.

MATERIALS AND METHODS

Used throughout the experiment were eggs from one strain of Single Comb White Leghorns from the Cornell University Poultry Farm. The eggs were used 1 day after they were laid.

Bacteria. Three representative species were selected:

1) *Pseudomonas fluorescens*, a very common egg spoiler, isolated from rotten eggs.

2) *Alcaligenes bookeri*, a common egg spoiler and a highly proteolytic bacterium (Garibaldi and Stokes, 1958).

3) *Salmonella paratyphi*, a pathogenic bacterium reported to be isolated from eggs and egg products (Hobbs, 1962).

Florian and Trusell (1957) reported that *Ps. fluorescens* and *A. bookeri* were responsible for 75% of the infected eggs that they had studied.

The cultures were maintained on glucose-yeast extract (GYE) agar slants. They were grown in GYE broth for 24 hr at 30°C. The cultures were then centrifuged twice and resuspended in phosphate buffer to avoid transfer of nutrients. This same buffer (American Public Health Association, 1958) was used for dilution.

Cuticle extract. The cuticle extract was prepared by rubbing nest-clean eggs with sandpaper. Distilled water was added to the powder, and the suspension agitated overnight. After filtration, a second aliquot of distilled water was added to the precipitate and it was further agitated to leach out soluble material. The filtrate from the second agitation was combined with that from the first and was sterilized by passing through the bacterial filter.

Shell suspension. After sanding off the cuticle and scraping out the membranes, the shell was placed for 1 hr in a sterile solution of 10% disodium ethylenediaminetetraacetate (pH 7.5) to facilitate complete removal of cuticle and outer shell membranes (Baker and Balch, 1962). The shell was washed repeatedly with sterile water, and then crushed in an Omni-mixer under sterile conditions.

Membrane homogenate. Eggs were sterilized externally in the manner used successfully in our laboratory (Lifshitz *et al.*, 1964). After the shell was cut and its contents discarded, the inside was washed well with sterile water. The membranes were then peeled off with a sterile spatula and put in a bottle of sterile water. After repeated washing to eliminate residual adhering albumen, the membranes were dispersed into very minute particles in an Omni-mixer. All work was performed in a sterile room under strict aseptic conditions.

Albumen. A 5% solution of albumen was prepared by mixing 10 g of sterile albumen in 190 ml of sterile water.

Inoculation of test media. Each of the 4 experimental media was incubated for 24 hr at 30°C and then tested for sterility. Three 50-ml portions of each test solution (cuticle, membrane, shell, albumen, and phosphate buffer blank) were transferred to separate sterile bottles. Each of the 3 species of bacteria was seeded into a different bottle of each test mixture, giving 15 inoculations in all. These bottles were kept at 30°C in a water bath, and samples for plate counts on GYE agar were taken

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at 0, 6, 12, 24, 40, 72, 120, and 144 hr. The plates were incubated for 48 hr at 30°C before counting.

Bacterial penetration of shell. Following the demonstration of the use of the shell and cuticle as nutrients for bacteria, experiments were conducted to discover if bacteria could pass a barrier of clean egg shell with no source of nutrients provided except those found naturally in the cuticle and shell. Shells were prepared by removing a small circular section from the pointed end of each egg. After the contents were discarded, the inside of the shell was scraped free of membranes and then washed repeatedly with sterile water. Six such egg shells were filled with sterile saline and placed in a desiccator containing sterile saline (Fig. 1). A suspension of bacteria (1×10^7 cells/ml) was added to the sterile saline surrounding the shell. Streak plates from the saline inside the shells were prepared daily. Results were considered positive when bacteria were recovered from 4 of the 6 models.

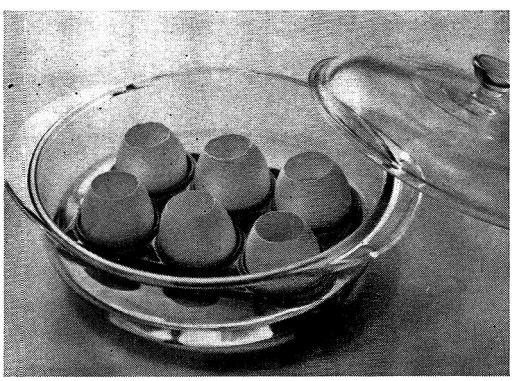

Fig. 1. Desiccator for studying bacterial penetration.

Effect of physiological state of bacterium on penetration. Two studies were made to distinguish whether penetration through the shell was active or passive. First, penetration by vegetative cells of lysozyme-resistant *Bacillus* species isolated in this laboratory was compared with penetration by spores of this same organism. The bacteria were grown in Roux bottles on a medium containing manganese ions to support sporulation. After 48 hr of growth, bacteria were harvested with sterile buffer and given a heat shock of 10 min at 85°C to kill vegetative forms. Malachite-green staining showed only spores under microscopic examination. The vegetative cells were grown under aeration in GYE broth. To eliminate residual nutrients the cells were centrifuged and resuspended in sterile buffer.

Six egg shells, containing phosphate buffer, were placed in each of 2 sterile desiccators containing phosphate buffer. After 48 hr of incubation to ensure sterility, the buffer surrounding the shells in 1 desiccator was inoculated with vegetative cells, and the buffer in the other desiccator was inoculated with the spore preparation. The interior of each shell was checked daily for passage of the organism through the shell by streaking a portion of the buffer on GYE agar and incubating for 48 hr at 30°C.

In a second part of this study, viable cells of *Ps. fluorescens*, inhibited by sulfathiazole, were compared with normal, reproducing cells of the same organism. The purpose was to find out if bacteria could penetrate through the shell when they were viable but not reproductive. Twelve sterile egg shells were prepared and filled with phosphate-buffered water. Six of these were placed in a desiccator containing buffer. The remaining 6 were placed in a second desiccator containing buffer supplemented with 2% glucose and 1% sodium sulfathiazole. A centrifuged, washed culture of *Ps. fluorescens* was added to the first desiccator to give a count of 1×10^7 organisms/ml. Enough cells were added to the second desiccator to elicit an initial count of 5×10^8 organisms/ml (50 times the inoculum in the first desiccator). Samples from inside the shells were streaked daily on GYE agar. In addition, bacterial counts of the medium containing the sulfathiazole were performed at regular intervals throughout the testing period.

RESULTS AND DISCUSSION

Results on the growth of bacteria on egg exterior structures are shown in Table 1. A short lag phase appeared in almost every case. It was somewhat more pronounced with *S. paratyphi* growing on shell homogenate.

There did not seem to be a great difference in the nutritive value of the exterior structures studied. Growth response was not the same with all organisms, but the general pattern was very similar. Albumen was found to be somewhat superior, though the bacteria grown in it had a longer lag phase because of the bacterial inhibitors it contains. Generally speaking, there was not a big difference among the three species studied. Results were essentially the same when some other species of *Salmonella*, including *S. derby*, were assayed.

A study on penetration showed that *Ps. fluorescens* was the first bacterium recovered from inside the models after 5 days of incubation. It took *A. bookeri* 17 days to penetrate 4 of the 6 models, and *S. paratyphi* required 23 days.

Table 1. Growth of bacteria on egg exterior structures.

	Time (hr)								
	0	6	12	24	48	72	96	120	144
	(log of counts: cells/ml)								
1) *Ps. fluorescens*									
Albumen	2.0	2.0	2.9	3.7	5.9	7.8	8.0	8.3	8.6
Cuticle	2.1	2.4	3.4	5.6	7.2	7.7	8.1	7.7	7.5
Membranes	2.1	2.0	4.0	6.2	7.6	7.9	8.0	8.3	8.0
Shell	2.0	2.1	4.0	6.1	7.7	8.0	8.1	7.8	7.6
Blank	1.9	1.9	1.7	1.8	1.8	1.7	1.7	1.8	1.9
2) *A. bookeri*									
Albumen	2.2	1.9	2.4	3.2	4.6	5.9	7.1	7.4	7.8
Cuticle	2.2	1.8	2.5	3.5	5.7	6.6	6.9	7.2	7.0
Membranes	2.3	1.8	2.9	4.3	6.2	7.0	7.3	7.1	7.1
Shell	2.1	1.9	2.8	4.0	6.0	7.0	7.8	7.9	7.9
Blank	2.3	2.0	2.2	2.3	2.0	1.9	1.9	1.7	1.9
3) *S. paratyphi*									
Albumen	2.8	2.8	3.2	4.5	6.3	7.3	8.2	8.0	7.9
Cuticle	2.2	2.2	2.6	3.6	5.8	7.2	7.2	7.1	7.0
Membranes	2.3	2.1	2.4	3.5	5.4	7.5	7.3	7.0	6.9
Shell	2.2	2.2	2.3	2.8	3.9	6.1	7.5	7.3	7.3
Blank	2.2	2.2	2.1	2.2	2.0	2.2	2.1	2.0	1.9

When vegetative cells were compared with spores for their penetrating power, results were conclusive. All 6 shells in the desiccator where vegetative cells had been added were penetrated in 4 days. In the desiccator inoculated with spores, not a single shell was penetrated even after 14 days. This experiment showed that only living bacteria can pass the barriers. Although spores were smaller, they did not penetrate. This indicated an active participation on the part of the bacterium, as opposed to a process of simple diffusion.

The study with sulfathiazole showed that merely a high challenge of bacteria is not enough to enable penetration through egg shells (Table 2). The bacteria remained viable but did not reproduce. As was the case with the study on spores, the penetration depended on the capacity of bacteria to reproduce. Sulfathiazole interferes with normal metabolism of egg shell nutrients. This would appear to support a theory that normal bacteria can utilize shell and cuticle, reproduce, and thus penetrate these structures.

It has been shown that several important egg-spoilage bacteria can utilize exterior egg structures as the sole source of nutrients. Further, clean egg shell was penetrated by physiologically active microorganisms in the

Table 2. Effect of sulfathiazole on penetration of *Ps. fluorescens* through egg shells.

Time of measurements (days)	Control		Sulfathiazole added	
	No. of shells penetrated out of 6 tested	Plate count	No. of shells penetrated out of 6 tested	Plate count
0	None	1.3×10^7	None	6.2×10^8
1	None		None	
2	2		None	5.8×10^8
3	3		None	
4	6		None	
8			None	5.0×10^8
12			None	4.8×10^8

absence of other nutrient materials. The failure of bacterial spores and of sulfathiazole-inhibited bacteria to penetrate the shell indicates that bacterial penetration involves active metabolism and reproduction by the invading microorganisms. These experiments explain, perhaps, why washing eggs without sanitizing compounds would be of little value. This treatment removes much of the dirt from the shell but does not eradicate the bacteria. Even in the absence of dirt, bacteria initially present on the shell can grow and multiply on the cuticle and shell in sufficient numbers to penetrate the egg.

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Chemical and Microbial Activity Rates Under Square-Wave and Sinusoidal Temperature Fluctuations

SUMMARY

Temperature amplitudes to 160° and Q_{10} values from 1 to 30 were integrated to derive coefficients of fluctuation. Multiplication of the coefficient by the reaction rate at the mean temperature permits ready calculation of the average reaction rate when temperature fluctuations assume either a square-wave or sinusoidal form of cycling. The method is applicable to any temperature scale. Ascorbic acid solutions were less stable when the temperature was cycled from —6 to 30°F than when held steady at 12°F. *Aerobacter aerogenes* and *Staphylococcus aureus* cultures incubated under cyclic conditions from 40 to 80°F usually grew quicker than cultures incubated at a constant temperature of 60°F.

INTRODUCTION

Both fresh and processed foods are invariably exposed to fluctuating temperature from the time of production to the time of consumption. In fact, some foods such as those marketed through vending machines may be cycled 4-6 times at temperatures from approximately 32 to 140°F before the food is consumed or removed from the machine.

This paper presents a simple method of calculating the average reaction rate when temperature fluctuates, and data relative to the effect of fluctuating temperatures on the stability of ascorbic acid solutions and bacterial multiplication.

REVIEW OF LITERATURE

Hicks (1944) developed an equation to estimate the effective temperature and the influence of sinusoidal diurnal temperature fluctuations on reaction rates as applied to foods stored at ambient temperature. Schwimmer *et al.* (1955) extended Hicks' procedure by developing a more convenient method of obtaining effective temperature. Van Arsdel and Guadagni (1959) described a method for estimating accumulated quality change in frozen foods, and Van Arsdel (1961) developed an alignment chart for calculation of storage life.

At least 25 of the time-temperature-tolerance studies of the Western Regional Research Laboratory, USDA (Van Arsdel, 1957; Dietrich *et al.*, 1962; Guadagni *et al.*, 1963), have included the effects of fluctuating storage temperature on the quality of frozen foods. Gortner *et al.* (1948), Hustrulid *et al.* (1949), Winter *et al.* (1956) have conducted similar studies. Cecil and Woodroof (1962) extensively investigated the stability of canned and other processed foods stored at steady and fluctuating temperatures from —20 to 100°F. Ballantyne and Anglin (1955), Berryhill *et al.* (1955), Kemp and Ducker (1957), and Kemp *et al.* (1958) studied the stability of Canadian military rations under normal service storage conditions and under experimental steady and cyclic storage temperatures for two years.

Fluctuating temperatures affect biological entities as well as chemical reactions. Pickett *et al.* (1960) observed that bull semen transferred from —79 to —196°C, or in the reverse direction, decreased in motility as compared with storage at steady temperatures of —79 and —196°C. Michener *et al.* (1960) reported that daily fluctuation in temperature between 0 and 20°F led to as great destruction of microorganisms as storage at 20°F. Raj and Liston (1961) also observed that fluctuating temperatures affect bacterial survival. Weiser and Osterud (1945) found, on the other hand, that in the range —30 to —78°C repeated fluctuation of temperature resulted in lower mortality than storage at either temperature, but this effect was not evident at temperature ranges above —30°C or below —78°C. Gunderson (1961) observed that mold developed in frozen foods at temperatures below 32°F when the temperature fluctuated.

Above freezing, fluctuating temperatures have also been noted to affect microbial growth. Lark and Maaløe (1954) demonstrated that fluctuating temperatures between 25 and 73°C induced cellular and nuclear division of *Salmonella typhimurium*. Ng *et al.* (1962) found that bacterial cells assumed a higher growth rate without detectable lag when the temperature was suddenly increased provided the change occurred within a range where the Arrhenius plot of the growth rate was linear. When

the temperature was decreased, a similar effect, in reverse, was observed. Zahlan (1961) has calculated that sinusoidal variation in temperature affects the concentration of chemical constituents in microbial cells. Ingraham (1958) stressed the importance of holding temperature constant when determining growth rates.

EXPERIMENTAL

A high-speed computer was used to integrate the effects of temperature amplitudes of 2–160° and Q_{10} values of 1–30 when temperature cycling assumed either a square-wave or a sinusoidal form. The programs for the computer were prepared by Shackelford (1964).

To compare the theoretical calculations with actual trials, the stability of ascorbic acid solutions and multiplication of *Aerobacter aerogenes* and *Staphylococcus aureus* cultures were determined.

Ascorbic acid. Ascorbic acid was dissolved in water (20 mg/ml) and in apple juice (5 mg/ml). Three groups of sealed 9-mm-OD glass tubes containing 6 ml ascorbic acid solution were stored at steady temperatures of 30, 12.8, and —6°F. The respective temperatures were established by using eutectic solutions of Na_2SO_4, KCl, and NaCl (Venemann, 1948). To stabilize the temperature to the maximum, the vessels containing the eutectic solutions were in turn kept in refrigerated rooms controlled to the same temperature as each eutectic temperature. The fourth group of sealed tubes was alternated every 24 hr from —6 to 30°F to give an average temperature of 12°F and a square-wave form of fluctuation. The transfer did not bring about a detectable change in the temperature of the eutectic solution because the weight of the tubes and ascorbic acid combined never exceeded 1/20 of the weight of the eutectic solution. The contents of the tubes assumed the new temperature within 0.5 hr. The concentration of ascorbic acid was determined by the colorimetric method of Schmall *et al.* (1954).

Bacterial multiplication. Except for a few initial runs for which flasks containing 100 ml of medium were used, test tubes containing 10 ml of medium were inoculated with *A. aerogenes* or *S. aureus* and incubated at 40, 60, and 80°F in temperature-controlled water baths. Nutrient broth was used as the medium for *A. aerogenes*, and brain-heart-infusion broth for *S. aureus*. For the square-wave fluctuating trials, the test tubes were incubated 2 hr at 40°F, transferred to the water bath at 80°F for 2 hr, and alternated every 2 hr between these two temperatures for at least 60 hr. Because the water baths had good recovery rates and the weight of the medium and tubes was never more than 1/15 that of the 3 gallons of water in the bath, the temperature of the medium closely approached that of a square-wave function. For the sine-wave trials, the procedure was similar except the water and test tubes were placed in an 18-in. stock pot and the stock pot was transferred alternately from a refrigerated room at 35°F to an incubation room at 100°F so as to cycle the temperature of the water between 40 and 80°F. Because of the thermal inertia of the water in the stock pot, the temperature of the inoculated media was close to a sine wave in form. Viable-cell counts were made by the most-probable-number technique at zero time and at 3- to 6-hr intervals according to the temperature of incubation. To simulate practical food conditions, in which bacterial cells might be either fresh or dormant, separate trials were made with 18-hr actively-multiplying cultures as the inoculum and cultures that had been stored for at least a week at 85°F. Cultures for inoculation were grown at 85°F.

RESULTS

Theoretical calculations. The relation between the rates of reaction and temperature for nonsteady systems is given by the equation

$$\overline{R} = \frac{1}{Z} R_m Q^{\frac{1}{n}} (f(t) - T_m) \qquad [1]$$

where $\overline{R}$ = average reaction rate when temperature is a function of time, $f(t)$; Z = number of cycles per unit of time used for R_m; R_m = reaction rate at temperature T_m; Q = ratio of reaction rate at temperature $T_m + 10$ to that at temperature T_m; n = number of degrees of increase in temperature required to change the reaction rate Q times; T_m = any specified temperature on any scale for which the reaction rate equals R_m.

For $T_m = 0°$ and $n = 10°$, the reaction rate is:

$$\overline{R} = \frac{1}{Z} R_0 Q^{0.1T} \qquad [2]$$

where Q_{10} is the ratio of reaction at the temperature $T_0 + 10$ to that at T_0. If the Q_{10} value is not known but it is known for any other temperature difference, it may be obtained from:

$$Q_{10} = Q^{\frac{10}{n}} \qquad [3]$$

The sum of the reactions during time H according to Eqs. 1 and 2 is:

$$\Sigma RH = \frac{1}{Z} R_m \int_{t=0}^{t=H} Q_{10}^{0.1\, f(t)}\, dt \qquad [4]$$

when H = time in days and $f(t)$ = temperature as a function of time.

Sine-wave fluctuation. Temperature as a sinusoidal function of time may be expressed by the equation:

$$f(t) = T_m + A \sin 2\pi t \qquad [5]$$

where T_m = mean temperature and A = the amplitude of one-half cycle. Combining Eqs. 4 and 5, $\bar{R}$ during one cycle will be:

$$\bar{R} = \frac{1}{Z} R_m \int_{t=0}^{t=1} Q_{10}^{0.1(T_m + A \sin 2\pi t)}\, dt \qquad [6]$$

assuming $H = 1$ unit of time. The values Z, Q_{10}, R_m, and T_m are independent of time; therefore:

$$\bar{R} = \frac{1}{Z} R_m Q_{10}^{0.1T_m} \int_{t=0}^{t=1} Q_{10}^{(0.1A)(\sin 2\pi t)}\, dt \qquad [7]$$

R_m will often not be known, but from the term $Q_{10}^{0.1T_m}$ and any R which is known at any T, R_m can be calculated either mathematically or graphically, assuming, of course, that there is no change in Q_{10} value in the range which encompasses both R and R_m. The integrals for Eq. 7 are listed in Tables 1, 2, and 3. In practice, Eq. 7 becomes:

$$\bar{R} = R_m F_s \qquad [7a]$$

where F_s stands for the coefficient of fluctuation for a sine-wave curve.

The sine-wave form of fluctuation is probably the most common in the storage of foods because of thermal inertia of the stored materials.

Square-wave fluctuation. The square-wave mode of fluctuation causes the average reaction rate to increase more sharply than does the sine-wave mode. In this form of fluctuation, during one-half the cycle, the temperature is steady and equal to: $T_m + A$. The rate becomes:

$$\bar{R} = \frac{1}{Z} R_m Q_{10}^{0.1(T_m + A)} \qquad [8]$$

for one-half the cycle. During the other one-half cycle, the temperature is also steady but equal to $T_m - A$, and the rate is given by:

$$\bar{R} = \frac{1}{Z} R_m Q_{10}^{0.1(T_m - A)} \qquad [9]$$

For the whole cycle:

$$\bar{R} = \frac{1}{Z} R_m (Q_{10}^{0.1T_m})[0.5(Q_{10}^{0.1A} + Q_{10}^{-0.1A})] \qquad [10]$$

Here, as in Eq. 7, the reaction rate R_m may be calculated for the mean temperature from R at any temperature and Q_{10}. The coefficients of fluctuation within the bracket in Eq. 10 have been calculated and are listed in Tables 1, 2, and 3. For simplicity, Eq. 10 may be written:

$$\bar{R} = \frac{1}{Z} R_m F_{sq} \qquad [10a]$$

where F_{sq} stands for the coefficient of fluctuation for a square-wave cycle.

Examples. From Cecil and Woodroof (1962), the rate of deterioration of processed cheese at 100°F is 0.55% per 24 hr and $Q_{10} = 2.0$ in the range 32–100°F. Assume that this processed cheese is used in a prepared dish refrigerated in a vending machine with the vending machine set to cycle the food 6.7 times per day from 32 to 128°F and return to 32°F to coincide with meal times in an industrial plant. In the event a given dish is not purchased within 24 hr, it is picked up as a "return." How much will the food deteriorate in 24 hr? T_m and A may be calculated to be:

$$T_m = \frac{\max T + \min T}{2} = \frac{128 + 32}{2} = \frac{160}{2} = 80°F$$

$$A = \frac{\max T - \min T}{2} = \frac{128 - 32}{2} = \frac{96}{2} = 48°F$$

Since R = 0.55% per 24 hr at T_{80} and $Q_{10} = 2.0$, R_m (i.e., the rate at T_m or T_{80}) = 0.1375%. Assume that the cycling approaches a sine-wave in form. Having calculated R_m, Eq. 7a applies, and, accordingly, $\bar{R}$ is found to be:

$\bar{R} = R_m F_s = (0.1375)(6.3848) = 0.8879\%/24$ hr

by substituting for F_s from Table 2, the coefficient of fluctuation for a sine-wave function with a one-half amplitude of 48° when Q_{10} equals 2.0. From the average reaction rate of 0.887% per 24 hr, one may calculate that fluctuating the temperature from 32 to 128°F for 24 hr is equivalent to holding the temperature steady at 106°F for 24 hr. In other words, the effective temperature as defined by Hicks (1944) or Schwimmer *et al.* (1955) is 26°F above the mean temperature.

The striking thing about the example above is how misleading average temperature may be as an index of spoilage. The same situation might apply to microbial spoilage. Microbial multiplication could not occur over as extreme a temperature range as that specified above—at least not by the same organism—but within some temperature ranges the Q_{10} values for growth may be as high as 30 (Wolf and Wolf, 1947). On that account, the average activity rate may still be well above the activity rate at the average temperature although the range of temperature fluctuation is not great. Peterson (1961) and Gunderson (1961) both reported that the temperature of frozen food in retail sales cabinets may fluctuate nearly 40°F. Using Gunder-

Table 1. Coefficients of fluctuation for sine-wave and square-wave cycling when one-half amplitude ranges from 1 to 18°, and Q_{10} values from 2 to 9.

One-half amplitude	Q_{10} 2	3	4	5	6	7	8	9
1°	1.0012	1.0030	1.0048	1.0065	1.0080	1.0095	1.0108	1.0121
	1.0024	1.0060	1.0096	1.0130	1.0161	1.0190	1.0217	1.0242
2°	1.0048	1.0121	1.0193	1.0261	1.0324	1.0382	1.0437	1.0489
	1.0096	1.0242	1.0387	1.0523	1.0649	1.0767	1.0877	1.0981
3°	1.0108	1.0273	1.0437	1.0591	1.0735	1.0870	1.0997	1.1116
	1.0217	1.0548	1.0877	1.1188	1.1480	1.1753	1.2010	1.2252
4°	1.0193	1.0489	1.0784	1.1063	1.1326	1.1573	1.1806	1.2026
	1.0387	1.0981	1.1577	1.2145	1.2680	1.3185	1.3663	1.4117
5°	1.0303	1.0769	1.1238	1.1686	1.2109	1.2510	1.2891	1.3253
	1.0607	1.1547	1.2500	1.3416	1.4289	1.5119	1.5910	1.6667
6°	1.0437	1.1116	1.1806	1.2471	1.3105	1.3709	1.4287	1.4840
	1.0877	1.2252	1.3663	1.5036	1.6357	1.7626	1.8847	2.0024
7°	1.0597	1.1534	1.2496	1.3434	1.4337	1.5205	1.6041	1.6848
	1.1200	1.3106	1.5090	1.7047	1.8952	2.0803	2.2602	2.4352
8°	1.0784	1.2026	1.3319	1.4594	1.5835	1.7040	1.8211	1.9351
	1.1577	1.4117	1.6807	1.9499	2.2157	2.4771	2.7337	2.9860
9°	1.0997	1.2598	1.4287	1.5975	1.7637	1.9269	2.0870	2.2442
	1.2010	1.5300	1.8847	2.2458	2.6076	2.9679	3.3260	3.6815
10°	1.1238	1.3253	1.5413	1.7603	1.9787	2.1957	2.4107	2.6238
	1.2450	1.6667	2.1250	2.6000	3.0833	3.5714	4.0625	4.5556
12°	1.1805	1.4840	1.8211	2.1738	2.5358	2.9044	3.2781	3.6560
	1.3663	2.0024	2.7337	3.5218	4.3511	5.2136	6.1041	7.0191
14°	1.2496	1.6848	2.1880	2.7335	3.3112	3.9157	4.5435	5.1923
	1.5090	2.4352	3.5540	4.8177	6.1837	7.6555	9.2168	1.0860[a]
16°	1.3319	1.9351	2.6640	3.4854	4.3846	5.3531	6.3848	7.4754
	1.6807	2.9860	4.6492	6.6044	8.8189	1.1272[a]	1.3947[a]	1.6832[a]
18°	1.4287	2.2442	3.2781	4.4919	5.8677	7.3946	9.0646	1.0872[a]
	1.8847	3.6815	6.1041	9.0873	1.2599[a]	1.6617[a]	2.1124[a]	2.6108[a]

[a] Multiply by 10 the value listed. The upper row for each amplitude lists the sine-wave values, the lower row lists the square-wave values. Coefficients are not shown for $Q_{10} = 1$ because all values = 1.0000.

son's data, in which some packages fluctuated from —24.2 to —2.2°C during the daily automatic defrost, and assuming that a fungus grows in 8 hr at 10°C with a Q_{10} value of 30 per 10°C in the range —30 to 10°C, what effect might the fluctuating temperature have on the growth rate?

T_m may be calculated to be —13.2°C, and A to be 11°. If the organism grows in 8 hr at 10°C, then the growth rate per day is 3.0; using this rate and $Q_{10} = 30$, the rate of growth at —13.2°C is 0.0011. Judging from Gunderson's graph, in only ⅓ of the day is the temperature cycling, but the fluctuation is so drastic it approaches a square-wave form; accordingly:

$$\overline{R} = \frac{R_m F_{sq}}{3} = \frac{(0.0011)(21.09)}{3} = 0.0077$$

When $\overline{R} = 0.0077$, then the corresponding tempera-

Table 2. Coefficients of fluctuation for sine-wave and square-wave cycling when one-half amplitudes range from 20 to 80° and Q_{10} values from 2 to 9.

One-half amplitude	Q_{10} 2	3	4	5	6	7	8	9
20°	1.5413	2.6238	4.0679	5.8373	7.9166	1.0296[a]	1.2970[a]	1.5933[a]
	2.1250	4.5556	8.0312	1.2520[a]	1.8014[a]	2.4510[a]	3.2008[a]	4.0506[a]
24°	1.8211	3.6560	6.3848	1.0042[a]	1.4671[a]	2.0315[a]	2.7014[a]	3.4803[a]
	2.7337	7.0191	1.3947[a]	2.3806[a]	3.6865[a]	5.3363[a]	7.3520[a]	9.7536[a]
28°	2.1880	5.1923	1.0207[a]	1.7580[a]	2.7655[a]	4.0756[a]	5.7193[a]	7.7266[a]
	3.5540	1.0860[a]	2.4261[a]	4.5304[a]	7.5477[a]	1.1621[b]	1.6890[b]	2.3488[b]
32°	2.6640	7.4754	1.6527[a]	3.1160[a]	5.2759[a]	8.2731[a]	1.2250[b]	1.7352[b]
	4.6492	1.6832[a]	4.2230[a]	8.6236[a]	1.5455[b]	2.5310[b]	3.8802[b]	5.6565[b]
36°	3.2781	1.0872[a]	2.7014[a]	5.5732[a]	1.0155[b]	1.6942[b]	2.6469[b]	3.9306[b]
	6.1041	2.6108[a]	7.3520[a]	1.6416[b]	3.1646[b]	5.5122[b]	8.9144[b]	1.3622[c]
40°	4.0679	1.5933[a]	4.4475[a]	1.0038[b]	1.9681[b]	3.4931[b]	5.7577[b]	8.9638[b]
	8.0312	4.0506[a]	1.2800[b]	3.1250[b]	6.4800[b]	1.2005[c]	2.0480[c]	3.2805[c]
44°	5.0823	2.3491[a]	7.3642[a]	1.8181[b]	3.8354[b]	7.2414[b]	1.2592[c]	2.0552[c]
	1.0580[a]	6.2854[a]	2.2286[b]	5.9489[b]	1.3269[c]	2.6146[c]	4.7051[c]	7.9002[c]
48°	6.3848	3.4803[a]	1.2250[b]	3.3080[b]	7.5078[b]	1.5079[c]	2.7663[c]	4.7330[c]
	1.3947[a]	9.7536[a]	3.8802[b]	1.1325[c]	2.7170[c]	5.6943[c]	1.0809[d]	1.9025[d]
50°	7.1688	4.2428[a]	1.5824[b]	4.4686[b]	1.0519[c]	2.1790[c]	4.1059[c]	7.1927[c]
	1.6016[a]	1.2150[b]	5.1200[b]	1.5625[c]	3.8880[c]	8.4035[c]	1.6384[d]	2.9524[d]
55°	9.6176	6.9885[a]	3.0116[b]	9.5117[b]	2.4533[c]	5.4896[c]	1.1059[d]	2.0550[d]
	2.2638[a]	2.1045[b]	1.0240[c]	3.4939[c]	9.5236[c]	2.2234[d]	4.6341[d]	8.8573[d]
60°	1.2970[a]	1.1565[b]	5.7577[b]	2.0336[c]	5.7466[c]	1.3891[d]	2.9919[d]	5.8971[d]
	3.2008[a]	3.6450[b]	2.0480[c]	7.8125[c]	2.3328[d]	5.8824[d]	1.3197[e]	2.6572[e]
65°	1.7565[a]	1.9211[b]	1.1049[c]	4.3640[c]	1.3511[d]	3.5278[d]	8.1235[d]	1.6984[e]
	4.5260[a]	6.3133[b]	4.0960[c]	1.7469[d]	5.7142[d]	1.5564[e]	3.7073[e]	7.9716[e]
70°	2.3873[a]	3.2017[b]	2.1270[c]	9.3944[c]	3.1864[d]	8.9873[d]	2.2125[e]	4.9065[e]
	6.4004[a]	1.0935[c]	8.1920[c]	3.9062[d]	1.3997[e]	4.1177[e]	1.0486[f]	2.3915[f]
75°	3.2543[a]	5.3508[b]	4.1059[c]	2.0278[d]	7.5349[d]	2.2957[e]	6.0420[e]	1.4212[f]
	9.0512[a]	1.8940[c]	1.6384[d]	8.7346[d]	3.4285[e]	1.0894[f]	2.9658[f]	7.1745[f]
80°	4.4475[a]	8.9638[b]	7.9443[c]	4.3872[d]	1.7859[e]	5.8775[e]	1.6538[f]	4.1262[f]
	1.2800[b]	3.2805[c]	3.2768[d]	1.9531[e]	8.3981[e]	2.8824[f]	8.3886[f]	2.1523[g]

[a] Multiply by 10 the value listed.
[b] Multiply by 100 the value listed.
[c] Multiply by 10^3 the value listed.
[d] Multiply by 10^4 the value listed.
[e] Multiply by 10^5 the value listed.
[f] Multiply by 10^6 the value listed.
[g] Multiply by 10^7 the value listed.

The upper row for each amplitude lists the sine-wave values, the lower row lists the square-wave values. Values for $Q_{10} = 1.0$ are not listed, because all values equal 1.0000.

Table 3. Coefficients of fluctuation for sine-wave and square-wave cycling when one-half amplitude ranges from 1 to 80° and Q_{10} values from 10 to 30.

One-half amplitude	Q_{10}										
	10	12	14	16	18	20	22	24	26	28	30
1°	1.0133	1.0155	1.0175	1.0193	1.0210	1.0226	1.0240	1.0254	1.0267	1.0280	1.0291
	1.0266	1.0310	1.0350	1.0387	1.0421	1.0452	1.0482	1.0509	1.0535	1.0560	1.0584
4°	1.2236	1.2627	1.2986	1.3319	1.3631	1.3925	1.4203	1.4467	1.4719	1.4960	1.5191
	1.4550	1.5360	1.6109	1.6807	1.7462	1.8081	1.8668	1.9228	1.9764	2.0278	2.0773
7°	1.7629	1.9121	2.0533	2.1880	2.3170	2.4411	2.5609	2.6768	2.7894	2.8989	3.0056
	2.6057	2.9349	3.2503	3.5540	3.8476	4.1323	4.4092	4.6791	4.9427	5.2006	5.4532
10°	2.8350	3.2521	3.6628	4.0679	4.4681	4.8639	5.2559	5.6443	6.0296	6.4119	6.7916
	5.0500	6.0417	7.0357	8.0312	9.0278	1.0025[a]	1.1023[a]	1.2021[a]	1.3019[a]	1.4018[a]	1.5017[a]
15°	7.0978	8.9446	1.0905[a]	1.2970[a]	1.5131[a]	1.7383[a]	1.9720[a]	2.2137[a]	2.4631[a]	2.7199[a]	2.9837[a]
	1.5827[a]	2.0797[a]	2.6201[a]	3.2008[a]	3.8190[a]	4.4727[a]	5.1599[a]	5.8792[a]	6.6291[a]	7.4084[a]	8.2161[a]
20°	1.9180[a]	2.6515[a]	3.4953[a]	4.4475[a]	5.5066[a]	6.6713[a]	7.9405[a]	9.3133[a]	1.0789[b]	1.2366[b]	1.4045[b]
	5.0005[a]	7.2003[a]	9.8003[a]	1.2800[b]	1.6200[b]	2.0000[b]	2.4200[b]	2.8800[b]	3.3800[b]	3.9200[b]	4.5000[b]
25°	5.3864[a]	8.1629[a]	1.1628[b]	1.5824[b]	2.0786[b]	2.6551[b]	3.3151[b]	4.0618[b]	4.8981[b]	5.8269[b]	6.8508[b]
	1.5812[b]	2.4942[b]	3.6668[b]	5.1200[b]	6.8731[b]	8.9443[b]	1.1341[c]	1.4109[c]	1.7235[c]	2.0743[c]	2.4648[c]
30°	1.5480[b]	2.5710[b]	3.9571[b]	5.7577[b]	8.0235[b]	1.0805[c]	1.4151[c]	1.8111[c]	2.2733[c]	2.8067[c]	3.4159[c]
	5.0000[b]	8.6400[b]	1.3720[c]	2.0480[c]	2.9160[c]	4.0000[c]	5.3240[c]	6.9120[c]	8.7880[c]	1.0976[d]	1.3500[d]
35°	4.5185[b]	8.2226[b]	1.3672[c]	2.1270[c]	3.1442[c]	4.4636[c]	6.1317[c]	8.1971[c]	1.0710[d]	1.3723[d]	1.7288[d]
	1.5811[c]	2.9930[c]	5.1336[c]	8.1920[c]	1.2372[d]	1.7889[d]	2.4972[d]	3.3862[d]	4.4810[d]	5.8080[d]	7.3943[d]
40°	1.3336[c]	2.6590[c]	4.7762[c]	7.9443[c]	1.2457[d]	1.8642[d]	2.6860[d]	3.7506[d]	5.1007[d]	6.7824[d]	8.8450[d]
	5.0000[c]	1.0368[d]	1.9208[d]	3.2768[d]	5.2388[d]	8.0000[d]	1.1713[e]	2.6589[e]	2.2849[e]	3.0733[e]	4.0500[e]
50°	1.1892[d]	2.8460[d]	5.9652[d]	1.1341[e]	2.0008[e]	3.3272[e]	5.2738[e]	8.0340[e]	1.1837[e]	1.6952[f]	2.3687[f]
	5.0000[d]	1.2442[e]	2.6891[e]	5.2429[e]	9.4478[e]	1.6000[f]	2.5768[f]	3.9813[f]	5.9407[f]	8.6052[f]	1.2150[g]
60°	1.0835[e]	3.1120[e]	7.6107[e]	1.6538[f]	3.2826[f]	6.0656[f]	1.0576[g]	1.7577[g]	2.8057[g]	4.3272[g]	6.4785[g]
	5.0000[e]	1.4930[f]	3.7648[f]	8.3886[f]	1.7006[g]	3.2000[g]	5.6690[g]	9.5551[g]	1.5446[h]	2.4095[h]	3.6450[h]
70°	1.0017[f]	3.4530[f]	9.8527[f]	2.4470[g]	5.4644[g]	1.1219[h]	2.1520[h]	3.9017[h]	6.7473[h]	1.1207[i]	1.7977[i]
	5.0000[f]	1.7916[g]	5.2707[g]	1.3422[h]	3.0611[h]	6.4000[h]	1.2472[i]	2.2932[i]	4.0159[i]	6.7465[i]	1.0935[i]
80°	9.3603[f]	3.8722[g]	1.2891[h]	3.6592[h]	9.1933[h]	2.0973[i]	4.4252[i]	8.7529[i]	1.6398[j]	2.9332[j]	5.0414[j]
	5.0000[g]	2.1499[h]	7.3789[h]	2.1475[i]	5.5100[i]	1.2800[j]	2.7438[j]	5.5038[j]	1.0441[k]	1.8890[k]	3.2805[k]

[a] Multiply by 10 the value listed.
[b] Multiply by 100 the value listed.
[c] Multiply by 10^3 the value listed.
[d] Multiply by 10^4 the value listed.
[e] Multiply by 10^5 the value listed.
[f] Multiply by 10^6 the value listed.
[g] Multiply by 10^7 the value listed.
[h] Multiply by 10^8 the value listed.
[i] Multiply by 10^9 the value listed.
[j] Multiply by 10^{10} the value listed.
[k] Multiply by 10^{11} the value listed.

The upper row for each amplitude lists the sine-wave values, the lower row lists the square-wave values.

ture is —7.6°C (i.e., the effective temperature). From the growth rate of 0.0077, one may calculate that the organism would grow in 129 days. At the average temperature of —13.2°C, 909 days would be required for growth.

Actual trials. The illustrative problem above is based on assumptions. Trials with *A. aerogenes* and *S. aureus* supported some of the assumptions relative to microbial growth. Table 4 lists the average and maximum growth rates of *A. aerogenes*, actively-proliferating and dormant when the experiments were started. The average growth rates of the actively-reproducing cells were higher when the temperature fluctuated between 40 and 80°F than when it was constant at 60°F. This was true whether the form of fluctuation was a sine wave or a square-wave. The average growth rate of cells dormant at the time of inoculation was nearly the same (the sine-wave pattern resulted in a slightly higher average growth rate). The cells actively reproducing at the time of inoculation never attained as great a maximum growth rate under fluctuating temperature conditions as did cells incubated steadily at 60°F. The dormant cells did the reverse. Fig. 1 shows the patterns of growth for the dormant inocula of *A. aerogenes*.

Fresh cultures of *S. aureus* exhibited greater average and maximum growth rates under fluctuating temperature conditions than under steady conditions. The rates of growth are listed in Table 5. Figs. 2 and 3 show the growth curves.

Ascorbic acid. The stability of solutions of ascorbic acid was less when the temperature fluctuated in a square-wave form from —6 to 30°F (average temperature 12°F) than when the temperature remained constant at 12.8°F. For the water solutions there was a difference of 20.2% between the calculated rate of loss and the actual

Table 4. Average and maximum growth rates of *A. aerogenes* inoculated as actively-proliferating and dormant cells.

Temperature (°F)	Growth rate (hr)			
	Active inoculum		Dormant inoculum	
	Average	Maximum	Average	Maximum
80	0.917	1.438	0.543	1.149
60	0.232	0.613	0.217	0.250
40		0.044		0.024
40–80, sine, actual	0.400	0.488	0.246	0.625
calc'd	0.348	1.03	0.278	0.515
40–80, square, actual	0.415	0.459	0.216	0.500
calc'd	0.487	1.45	0.386	0.700
Q_{10} values, 40–60		3.76		3.78
60–80	1.99[a]	1.54	1.59[a]	2.17
40–80		2.15[a]		2.54[a]

[a] Q_{10} value used to calculate growth rates, using corresponding rate at 60°F as base.

Table 5. Average and maximum growth rates of *S. aureus* inoculated as actively-proliferating and dormant cells.

Temperature (°F)	Growth rate (hr)			
	Active inoculum		Dormant inoculum	
	Average	Maximum	Average	Maximum
80	0.414	0.704	0.251	0.463
60	0.098	0.166	0.102	0.200
40	0.049	0.105		
40–80, sine, actual	0.245	0.522	0.184	0.300
calc'd	0.137	0.216	0.130	0.250
40–80, square, actual	0.310	0.625	0.252	0.362
calc'd	0.172	0.299	0.156	0.298
Q_{10} values, 40–60	1.42	1.26		
60–80	2.07	2.10	1.55[a]	1.51[a]
40–80	1.76[a]	1.62[a]		

[a] Q_{10} value used to calculate growth rates, using corresponding rate at 60°F as base.

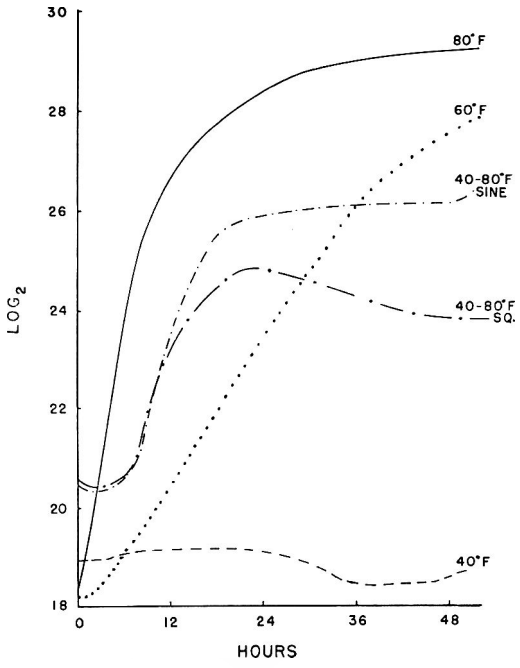

Fig. 1. Effect of steady and fluctuating temperatures on growth of *A. aerogenes*. The inoculation was one week old at time of inoculation. The curves labeled 40–80°F sine and 40–80°F sq show growth when temperature was fluctuated according to sine and square-wave patterns.

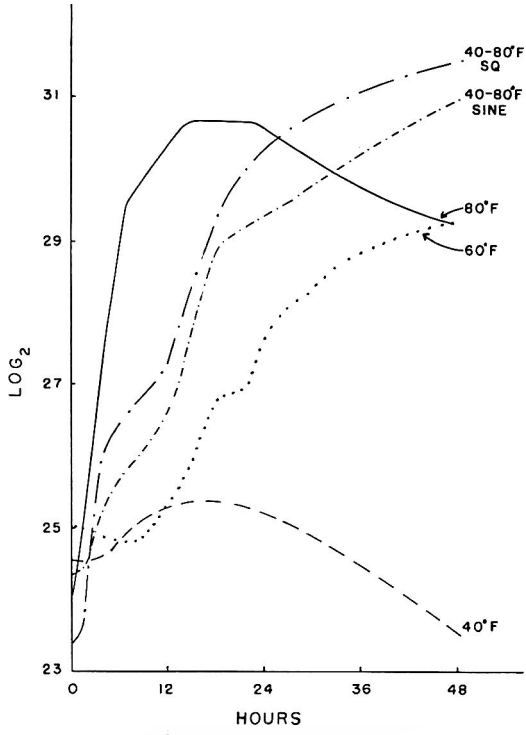

Fig. 2. Effect of steady and fluctuating temperature on growth of *S. aureus* inoculated as actively-proliferating culture. Curves labeled 40–80°F sine and 40–80°F sq show growth when temperature was fluctuated every 2 hr in sine or square-wave fashion.

rate when the temperature fluctuated. For the apple juice, the difference was 11.6%. The rates of loss are listed in Table 6.

DISCUSSION

The difference of 20.2 and 11.6% between the calculated rates of loss of ascorbic acid and the actual rates might seem to suggest that the calculated rate does not predict the actual rate. Very likely the reverse is true. To establish the rate of loss, three tubes of solution were analyzed separately every second day, then discarded, and another set of three tubes analyzed 2 days later. Determinations were made in duplicate on each tube. The duplicate determinations agreed closely, but among the three tubes there was usually 5-20% variation. The difference between calculated and actual rates was within this experimental variation; thus it was concluded that the two methods agree. The calculated rate includes, of course, any error involved in determining the actual rate at 12.8°F, the basis for the calculations.

Table 6. Rate of loss of ascorbic acid (mg/ml/day) in water and apple juice stored at constant temperatures of 30, 12, and —6°F and fluctuated between 30 and —6°F every 24 hr.

Temperature (°F)	Rate of loss (mg/ml/day)	
	Water	Apple juice
30	0.146	0.103
12.8	0.083	0.063
—6	0.035	0.048
30 to —6, actual	0.114	0.069
30 to —6 (calc'd[a])	0.091[b]	0.077[c]

[a] Calculated for square-wave fluctuation using actual rate determined at 12.8°F.
[b] $Q_{10} = 1.45$.
[c] $Q_{10} = 1.25$.

The calculated rates naturally indicated that fluctuating temperature would lead to greater microbial growth, and the actual rates within the particular temperature range selected usually did lead to greater growth. The data thus agree with observations of Ng

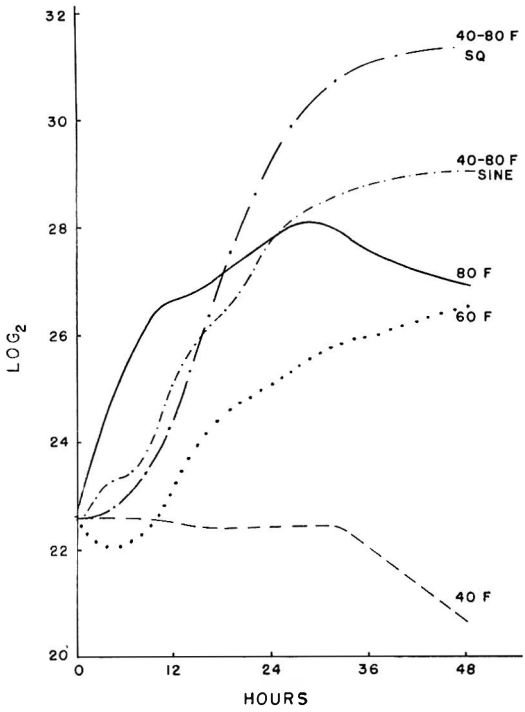

Fig. 3. Effect of steady and fluctuating temperature on growth of *S. aureus*. The inocula were one week old at time of inoculation. Curves labeled 40–80°F sine and 40–80°F sq show growth when temperature was fluctuated every 2 hr in sine or square-wave fashion.

et al. (1962) and Lark and Maalφe (1954). There are at least three reasons why the two methods did not always agree closely in magnitude. One, of course, is that microbes and enzymes have optimal temperature ranges of growth or activity, above and below which the rate falls off. Although not described here, bacterial trials were made in the range 60 to 100°F. Cultures that fluctuated from 60 to 100°F did not grow faster than those incubated steadily at 80°F, because 60 and 100°F were below and above the optimal growth range.

Secondly, above or below the range of optimal activity, there in turn may be variation in Q_{10} values—as with chemical reactions—according to the particular temperature range involved (Ingraham and Bailey, 1959; Jezeski and Olsen, 1961; Kiser, 1944; Sizer and Josephson, 1942). Even for chemical reactions, Q_{10} values sometimes hold true only for relatively narrow temperature ranges, and generally only for the same substrate. Guadagni *et al.* (1963) found that the Q_{10} values for the loss of flavor from apples for the three 10° intervals from 0 to 30°F were respectively 1.4, 4.0, and 4.5, whereas for peaches they were 1.7, 5.0, and 4.0. Ingraham (1958) observed, however, that the rates of growth of *Escherichia coli* and *Pseudomonas* strains were constant over a temperature range of approximately 20°C.

A third reason the actual and calculated rates did not agree exactly is that our average rates of growth include both the lag phase and the logarithmic phase. Each of these phases does not necessarily change to the same extent as temperature changes, i.e., their Q_{10} values may differ.

Although actual and calculated rates of growth under fluctuating temperature did not always agree, the trials were instructive as to practical food handling conditions. The fact that dormant or active cells held initially at 40°F but cycled between 40 and 80°F outgrew cells incubated at 60°F suggests that economic-spoilage or public-health problems could result in the storing or vending of foods which repeatedly attain a suitable temperature for growth although the time at the favorable temperature may be too short to seem important. Among such foods might be certain custard-type baked products, refrigerated dough, semipreserved fish or canned-refrigerated hams, and some types of sandwiches. Seemingly trivial, but repeated, excursions into favorable growth ranges could also affect microorganisms in foods in other temperature ranges or affect enzyme activity.

Gunderson's (1961) observation that frozen foods fluctuating in temperature become moldy bears this out. From the problem used to illustrate the method of calculation, the difference between an average temperature of —13.2°C and an effective temperature of —7.6°C may seem almost inconsequential for growth, but the rate at —7.6°C was 7 times as great as that at —13.2°C. Gunderson (1961) attributed germination to migration of moisture (Woodroof and Malcom, 1958) and continued growth in part to the establishment of a warmer microclimate (Rothbaum and Stone, 1961).

The establishment of a warmer climate could explain why *A. aerogenes* and *S. aureus* often outgrew the lots incubated steadily at 60°F although cells died off initially at 40°F. Perhaps the cells that were given a chance to initiate cell division at 80°F were never really at 40°F long enough thereafter, considering the rate of cycling and the momentum of the exothermic reaction, to bring cell division to a halt. From a practical point of view, the trials with microbes under conditions of fluctuating temperature have public-health and economic-spoilage implications, and the coefficients of fluctuation and the calculations show that for fundamental rate studies temperature must be held steady if valid reaction rates are to be derived.

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Although intermediate integrals for Tables 1, 2, and 3 may be obtained by graphing, the Department of Food Science can supply exact values to 8 decimal places for all whole-number amplitudes (1–80° half-amplitudes) and Q_{10} values 1–30.

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Factors Affecting the Nutritional Quality of Cottonseed Meals

SUMMARY

Eighteen samples of cottonseed oil meals from different countries in Central America were analyzed for proximate composition, free and total gossypol, epsilon-amino lysine groups and lysine, methionine, and threonine content. The meals were fed to weanling rats at a 10% protein level for 8 weeks, and protein efficiency ratios were determined at 4 and 8 weeks. The results showed a significant positive correlation between epsilon-amino groups of lysine content and PER and a negative correlation between weight gain and total gossypol content as well as between residual oil content and PER, or weight gain. L-lysine supplementation alone did not improve a poor-quality meal, while exhaustive extraction of the oil did increase the PER. The possibility of heat damage to the residual oil during the process of extraction was discussed.

Cottonseed oil meal is recognized as a potential source of good-quality protein for human consumption, especially in areas where the intake of both animal and vegetable protein does not meet the daily requirements of the population. INCAP Vegetable Mixture 9 (Bressani *et al.*, 1961) has been introduced successfully as a protein supplement into several populations in Latin America whose animal protein intake is low. This product has a biological value slightly lower than milk (Scrimshaw *et al.*, 1961), and its main source of protein is high-quality cottonseed oil meal. Cottonseed meals, however, vary greatly in composition and nutritional quality with the variety of cotton, the time of harvest, and, perhaps more important, the industrial processes to which the seed is subjected during the extraction of the oil (Altschul, 1958).

In the experiments reported here, variation in the chemical composition of 18 samples of cottonseed oil meals from different countries in Central America was determined by analyses of proximate composition as well as of free and total gossypol, epsilon-amino groups of lysine and total lysine, methionine, and threonine content to continue the studies on Central American cottonseed flours (Bressani *et al.*, 1964). Biological trials were also carried out with rats.

MATERIALS AND METHODS

The different meals were stored at 4°C until analyzed for moisture, ash, fat, crude fiber, and protein. The epsilon-amino groups of lysine were determined by the method of Conkerton and Frampton (1959), free and total gossypol by the method of the AOCS (1950), and lysine, methionine, and threonine by microbiological techniques (Bressani and Ríos, 1962). Urea fractionation of the residual fat was done according to the method of Crampton *et al.* (1953). Gas chromatography was also carried out on the methyl esters of the residual oils of some of the meals with a Perkin-Elmer fractometer, Model 154, an adipate polyester column, and helium as a carrier gas. Of the 18 samples analyzed, 7 (samples Nos. 4996, 5130, 5263, 5268, 5269, 5535, 5986) were produced by the prepressed solvent extraction method and 11 by the screw-press process.

For the biological trials, each meal was fed for 8 weeks at the protein level of 10% to 8 female and 8 male rats of the Wistar strain. The rats were housed in individual cages with raised screen bottoms in a constant-temperature room. Feed and water were supplied *ad libitum*, and the animals were weighed every week. Weekly records of feed consumption were kept, and protein efficiency ratios were calculated at the end of 4 and 8 weeks. The basal diet consisted of, in g per 100 g: 4.0 Hegsted mineral mixture (Hegsted *et al.*, 1941), 5.0 cottonseed oil, 1.0 cod liver oil, 38.0 dextrose, 52.0 cornstarch, and vitamin solution as recommended by Manna and Hauge (1953). Cottonseed meals replaced starch in sufficient amounts to provide 10% protein in the diet.

When L-lysine supplementation was studied, the amount of supplementary L-lysine was calculated taking into account only the epsilon-amino lysine group content of the meal. This was then increased to 3.6 g per 16 g of nitrogen in the meal with L-lysine so that all supplemented diets contained the same amount of available lysine, namely, 0.6% of the diet.

Residual fat in the meal was removed with hexane in a continuous extracting apparatus.

Table 1. Proximate composition (g%) of cottonseed oil meals.

Sample no.	Moisture	Ether extract	Crude fiber	Crude protein [a]	Ash
4996	6.2	1.7	5.0	51.9	7.3
5130	9.4	2.7	4.5	49.4	7.7
5259	10.6	6.8	4.5	45.7	6.9
5260	8.6	5.4	9.1	43.6	7.0
5261	9.0	8.1	4.9	46.1	7.4
5262	8.4	6.4	7.7	46.1	7.5
5263	10.5	0.7	4.2	46.6	7.7
5264	7.2	8.3	9.9	40.8	7.0
5265	9.8	9.7	4.4	42.1	6.5
5266	6.9	5.2	7.8	46.9	7.6
5267	10.3	6.2	3.2	53.0	6.2
5268	7.1	3.1	4.1	49.8	7.9
5269	6.8	3.7	4.4	51.1	7.8
5270	8.8	6.3	9.9	40.0	7.0
5535	5.8	2.2	4.7	50.0	8.2
5986	7.3	1.7	4.4	52.0	7.9
5987	7.6	10.4	8.7	40.8	6.2
6003	3.1	5.4	3.3	52.8	6.6
Mean	8.0±	5.2±	5.8±	47.2±	7.2±
standard deviation	1.9	2.8	2.4	4.4	0.6

[a] N content × 6.25.

RESULTS

Table 1 shows the results of the proximate analysis of the meals.

Table 2 gives the epsilon-amino groups of lysine, free and total gossypol and free lysine, and threonine and methionine content of the different meals.

Table 3 shows the weight gain and protein efficiency ratios in rats fed the different meals. The results are presented separately for males and females at 4 and 8 weeks of age. It is evident that the different meals differed greatly in these parameters.

Fig. 1 illustrates the correlation between epsilon-amino groups of lysine and PER, calculated at 4 weeks of age; there was a positive correlation between these two variables, with a correlation coefficient of 0.62, significant at the 1% level.

Fig. 2 demonstrates the negative correlation between total gossypol content and weight gain, calculated at 4 weeks of age. The correlation coefficient was −0.65, which was significant at the 1% level.

Fig. 3 shows the correlation between residual fat content in the meals and protein efficiency ratio at four weeks of age. Again, the correlation was negative, with a coefficient of −0.62, significant at the 1% level.

Fig. 4 shows the correlation between residual oil content in the meals and weight gain at four weeks of age. There was a negative correlation between these two variables, with a correlation coefficient of −0.77, significant at the 1% level. Sample No. 5265 was omitted from this calculation; when included in the analysis, the correlation coefficient was −0.52, which was significant at the 5% level. Results at 8 weeks showed the same trends as those obtained at 4 weeks.

A multiple-regression approach indicated that the cottonseed meal content of epsilon-amino lysine had a significant effect on both weight gain and PER, while a significant effect of total gossypol content could be shown only in the case of weight gain.

Table 4 shows the effect of lysine supplementation and of defatting of two cottonseed meals, one of which gave a low-PER (*KH*) and the other a high-PER (*B*). As can be seen from the table, lysine alone could not raise either the weight gain or the protein efficiency ratio of a low-PER meal to the level obtained with the high-PER meal supplemented with the same level of free lysine. Removal of residual fat from a cottonseed meal of good quality (high-PER) and a cottonseed meal of poor quality (low-PER) slightly increased the PER, but more so in the poor-quality meal than in the good-quality meal.

Table 5 shows the percentage of urea adducts in two samples of cottonseed oil, and the gas chromatography findings. As can be seen, the largest percentage of the oil did not react with urea; however, the products that did not react had a higher iodine

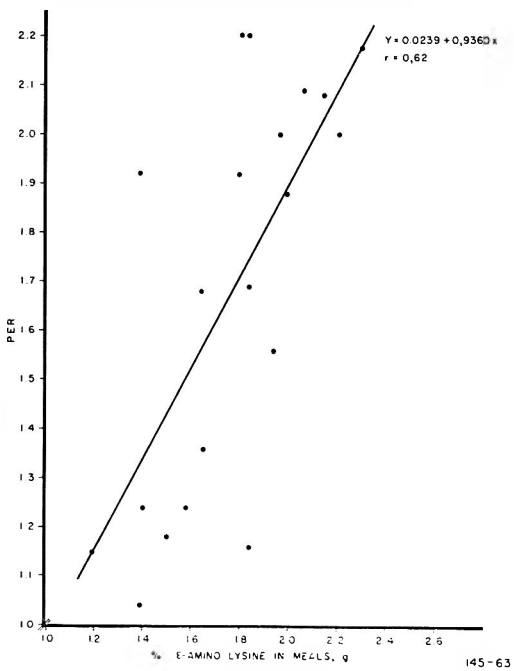

Fig. 1. Correlation between ε-amino lysine groups in cottonseed meals and PER in rats.

Table 2. Epsilon-amino lysine, free and total gossypol, and total threonine, lysine, and methionine content of cottonseed oil meals.

Sample no.	ε-Amino lysine (g%)	ε-Amino groups of lysine (g/16 g N)	Free gossypol (mg%)	Total gossypol (g%)	Threonine (g%)	Lysine (g%)	Methionine (g%)
4996	2.217	4.27	79.0	1.092	1.92	2.71	0.70
5130	2.161	4.38	80.9	1.058	1.77	2.08	0.74
5259	1.839	4.02	41.9	0.940	1.66	2.27	0.71
5260	1.652	3.48	51.4	1.223	1.66	2.26	0.66
5261	1.844	4.06	79.4	1.250	1.70	2.28	0.70
5262	1.390	3.01	84.1	1.224	1.64	2.19	0.71
5263	1.800	3.87	48.8	0.888	1.82	2.71	0.70
5264	1.500	3.67	56.2	1.264	1.70	2.15	0.63
5265	1.844	4.38	73.9	0.937	1.86	2.45	0.70
5266	1.585	3.38	50.5	1.184	1.65	2.10	0.65
5267	1.940	3.66	40.1	0.738	1.51	2.84	0.62
5268	1.992	4.00	95.6	1.155	1.88	2.47	0.67
5269	1.969	3.85	128.2	1.036	1.70	2.89	0.71
5270	1.398	3.49	62.2	1.172	1.51	2.16	0.68
5535	2.068	4.13	72.8	1.042	1.61	2.71	0.76
5986	1.806	3.47	67.4	0.880	1.76	2.90	0.56
5987	1.392	3.41	99.9	1.085	1.59	2.33	0.46
6003	1.640	3.10	61.0	1.071	1.69	2.66	0.48
Mean	1.780±	3.76±	70.7±	1.069±	1.70±	2.45±	0.66±
standard deviation	0.259	0.41	22.5	0.146	0.12	0.29	0.08

Table 3. Weight gain and PER of rats fed cottonseed oil meals.

Sample no.	Av. initial wt. (g)	Weight gain [a] (g)				PER [a]			
		4 weeks		8 weeks		4 weeks		8 weeks	
		Males	Females	Males	Females	Males	Females	Males	Females
4996	47	84	90	180	156	1.96	2.03	1.71	1.58
5130	48	83	86	171	162	2.02	2.13	1.77	1.79
5259	46	76	80	166	152	1.69	1.68	1.55	1.45
5260	50	51	55	94	117	1.36	1.35	1.16	1.27
5261	49	42	45	89	96	1.10	1.23	1.09	1.20
5262	46	34	34	62	66	1.11	0.96	0.94	0.88
5263	46	109	100	220	172	1.98	1.87	1.71	1.48
5264	47	45	48	92	97	1.21	1.14	1.08	1.05
5265	46	114	101	141	123	2.25	2.16	2.11	2.05
5266	55	44	56	96	112	1.14	1.34	1.11	1.20
5267	45	72	66	142	129	1.62	1.51	1.43	1.32
5268	47	92	90	194	153	1.92	1.84	1.64	1.43
5269	44	112	94	232	167	2.11	1.88	1.83	1.54
5270	44	54	54	112	110	1.23	1.24	1.10	1.09
5535	42	97	94	199	183	2.18	2.00	1.90	1.74
5986	48	96	92	200	178	2.25	2.15	1.96	1.84
5987	48	55	66	116	126	1.84	1.99	1.68	1.64
6003	44	63	62	150	142	1.69	1.67	1.64	1.53
Mean	47±	74±	73±	148±	136±	1.70±	1.68±	1.52±	1.45±
standard deviation	3	26	21	51	32	0.41	0.39	0.36	0.30

[a] 8 males and 8 females per group. PER: weight gain/protein consumed.

Table 4. Effect on the performance of rats of lysine supplementation and of defatting a high-PER (B) (CN) and a low-PER (KH) cottonseed oil meal.

Treatment of cottonseed meal	Av. initial wt. (g)	Weight gain [a] (g)	S.D.	PER [a]	S.D.	Total gossypol (mg%)	ε-amino lysine (g%)
B	50	94	11	1.90	0.13	1062	1.97
B + lysine	50	122	17	2.40	0.21		
KH	50	45	9	1.24	0.21	1186	1.22
KH + lysine	51	78	16	1.93	0.20		
CN	47	102	16	2.08	0.23	896	1.37
CN defatted	47	107	10	2.12	0.08	914	1.55
KH	48	52	10	1.34	0.15	1186	1.22
KH defatted	48	57	11	1.48	0.15	1211	1.27

[a] 16 rats per group (8 males and 8 females).

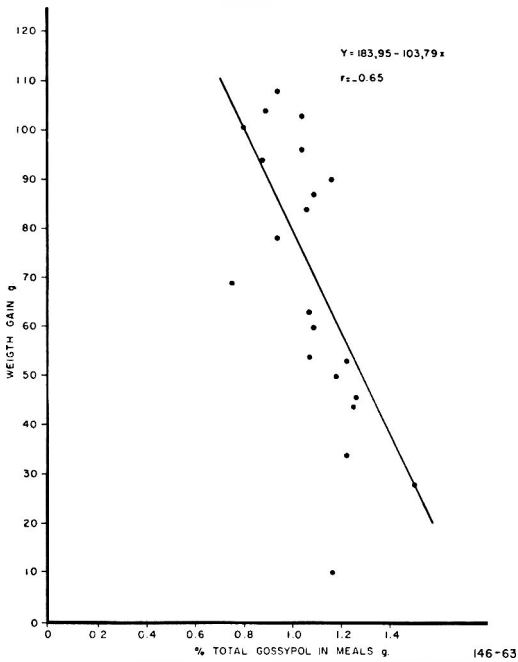

Fig. 2. Correlation between total gossypol in cottonseed meals and weight gain of rats.

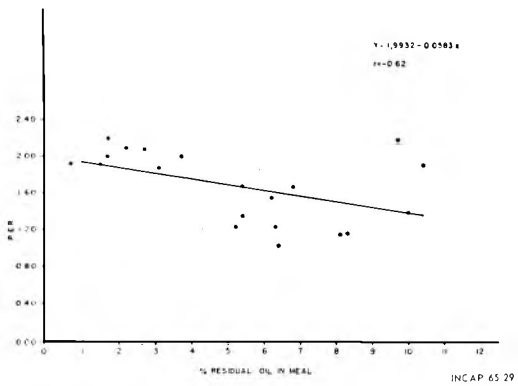

Fig. 3. Correlation between PER and fat content of cottonseed oil meals.

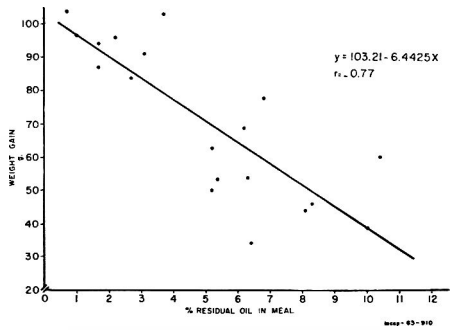

Fig. 4. Correlation between weight gain in rats and residual oil in cottonseed oil meals.

Table 5. Fatty acid gas chromatography and urea adducts of samples of residual cottonseed oil.

Fatty acid	% in cottonseed oil			
	Refined	KH	CN	B
C^{12}	0.21	0.11	0.08	0.07
C^{14}	0.98	0.99	0.91	0.83
C^{16}	25.82	26.94	26.23	27.90
$C^{16:1}$	0.81	1.25	1.57	1.50
C^{18}	2.59	2.65	2.98	2.90
$C^{18:1}$	16.97	16.59	15.87	16.50
$C^{18:2}$	52.60	50.90	51.54	49.50
Urea adducts	14.00	9.71	5.48	—
Urea non-adducts	61.34	74.00	75.98	—
IN [a] urea adducts	41.3	36.8	45.9	—
IN [a] urea non-adducts	101.6	82.2	92.2	—

[a] Iodine number.

number than those capable of forming urea adducts.

The gas chromatography findings of the oil from a good- and a poor-quality cottonseed meal and that of refined cottonseed oil, used as control, showed that the residual fats did not differ in fatty acid composition from those in refined cottonseed oil.

Table 6 shows the gossypol content expressed as bound gossypol percent and as percent of total gossypol. The meals were grouped according to the

Table 6. Free epsilon-amino groups, gossypol, and ether extract of cottonseed meals and their effect on rat growth and PER.

Meal no.	Free ε-amino groups of lysine (g%)	Free ε-amino groups of lysine (g/16 g N)	Gossypol Free (mg%)	Gossypol Total (g%)	Gossypol Bound (g%)	Gossypol Bound (% of total)	Ether extract (g%)	Rat (4 weeks) Wt. gain (g)	Rat (4 weeks) PER
4996 P	2.22	4.27	79.0	1.09	1.01	92.7	1.7	87	2.00
5130 P	2.16	4.38	80.9	1.06	0.98	92.4	2.7	84	2.08
5263 P	1.80	3.87	48.8	0.89	0.84	94.4	0.7	104	1.92
5268 P	1.99	4.00	95.6	1.16	1.06	91.4	3.1	91	1.88
5269 P	1.97	3.85	128.2	1.04	0.91	87.5	3.7	103	2.00
5535 P	2.07	4.13	72.8	1.04	0.97	93.3	2.2	96	2.09
5986 P	1.81	3.47	67.4	0.88	0.81	92.0	1.7	54	2.20
Av.	2.00 ± 0.16	4.00 ± 0.30	81.8 ± 24.9	1.02 ± 0.10	0.94 ± 0.09	92.0 ± 2.2	2.2 ± 1.0	88 ± 17	1.88 ± 0.11
5259 SP	1.84	4.02	41.9	0.94	0.90	95.7	6.8	78	1.68
5260 SP	1.65	3.48	51.4	1.22	1.17	95.9	5.4	53	1.36
5261 SP	1.84	4.06	79.4	1.25	1.17	93.6	8.1	44	1.16
5262 SP	1.39	3.01	84.1	1.22	1.14	93.4	6.4	34	1.04
5264 SP	1.50	3.67	56.2	1.26	1.20	95.2	8.3	46	1.18
5265 SP	1.84	4.38	73.9	0.94	0.87	92.6	9.7	108	2.20
5266 SP	1.58	3.38	50.5	1.18	1.13	95.8	5.2	50	1.24
5267 SP	1.94	3.66	40.1	0.74	0.70	94.6	6.2	69	1.56
5270 SP	1.40	3.49	62.2	1.17	1.11	94.9	6.3	54	1.24
5987 SP	1.39	3.41	99.9	1.08	0.98	90.7	10.4	60	1.92
6003 SP	1.64	3.10	61.0	1.07	1.01	94.4	5.4	62	1.68
Av.	1.64 ± 0.20 [a]	3.60 ± 0.41	63.7 ± 18.7	1.10 ± 0.16	1.03 ± 0.16	94.2 ± 1.6	7.1 ± 1.8	60 ± 20	1.48 ± 0.36

[a] Standard deviation. P = prepressed solvent extracted. SP = screwpress or expeller.

procedure used for extraction of the oil. As can be seen, the bound gossypol shows little correlation with the free epsilon-amino groups, which suggests that free epsilon-amino lysine groups are not the only active compounds which can bind gossypol.

DISCUSSION

From the data, it is evident that several factors affect the nutritive value of cottonseed oil meals. The main factors seem to be free epsilon-amino lysine groups, total gossypol, and residual fat content. The relation of free epsilon-amino groups of lysine to PER was expected, since it is known that cottonseed protein is limiting in lysine and that, during processing, free gossypol and the effect of heat bind certain groups like the epsilon-amino group of lysine, which renders them resistant to enzymatic hydrolysis. The correlation is, however, not as high as that reported by others (Frampton, 1960), suggesting that other amino acids are also possibly affected.

Total gossypol has also been known to affect weight gain in animals (Baliga and Lyman, 1957; Smith *et al.*, 1958), as it did in this study. Since total gossypol is the sum of bound and free gossypol and since free gossypol represents only a rather small quantity of the total, the negative relation between weight gain and total gossypol is really between weight gain and bound gossypol. This reasoning is logical since more bound gossypol would mean less available lysine, and less bound gossypol would mean more available lysine. This would in turn mean more or less gain in weight.

No significant relation was found between free gossypol and weight gain or PER. This is not surprising since the rat is not as susceptible to free gossypol toxicity as other animals, such as swine. Furthermore, the amounts present in the diet are relatively small. On the other hand, more free gossypol means more available lysine, which would possibly be more important to the young, growing rat and to PER determinations.

The effect of residual oil in the meal on PER is probably due to the fact that this oil has been overexposed to heat and oxidation during the industrial process of extraction. Sample No. 5265 was an exception, however. This sample had a high free lysine content with a low total gossypol, and a relatively higher amount of threonine than the other samples. It is evident that processing results in a significant decrease in lysine. It is also likely that lysine is not the only amino acid affected, since when a poor-quality meal was supplemented with free lysine to give 3.6 g of free lysine/16 g N, the improvement in weight gain and PER was far below that resulting from a good-quality meal with the same amount of free lysine.

Previous studies (Braham *et al.*, 1962) at this laboratory with swine fed rations adequate in all nutrients, but with different levels of cottonseed meal, showed that as the level of cottonseed meal in the ration was increased, certain symptoms of toxicity appeared in the experimental animals. These symptoms were characterized mainly by diarrhea, rough hair coats, and skin lesions, which had not previously been reported as symptoms of gossypol toxicity but rather as a result of a low fat intake, and especially as a deficiency of essential fatty acids (Hanson *et al.*, 1958). Since, in those studies, fat intake was adequate, and since, in the experiments reported here, all diets were supplemented with pure cottonseed oil, a deficiency either of fat or of essential fatty acids cannot be indicted as a cause of the gross symptoms observed in swine and, even less, of the negative correlation found between protein efficiency ratio and residual oil in rats.

Certain fractions of overheated edible oils were reported by Crampton *et al.* (1953) to be toxic for rats, probably due to the polymerization of fatty acids. Although the temperature used in their study can be easily attained in some of the industrial oil-extraction processes, in cottonseed meal the exposure time is much shorter. It is possible that the damage by heat and subsequent oxidation during storage would account, at least in part, for the negative correlation found between PER and residual oil content in rats. Further support for this speculation seems to be the lack of effect of lysine supplementation on the PER and the increase, though slight, of the PER when the residual oil was extracted from the meals.

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Proximate Composition of Chesapeake Bay Blue Crab (Callinectes sapidus)

SUMMARY

The proximate composition of the Chesapeake Bay blue crab *(Callinectes sapidus)* was determined on six bimonthly samples. The samples were taken with commercial crabbing methods as close to the same location and day of the month as possible. Data on a nitrogen-content basis proved to be more meaningful than data on a wet-weight basis. Proximate composition, together with the weight and carapace width, of the body meat, claw meat, and offal of the Chesapeake Bay blue crab were intercorrelated and interpreted at the 5% level of significance. The components of claw meat showed the greatest intercorrelation. Seasonal changes in the proximate composition coincide with the mating and spawning seasons of the Chesapeake Bay blue crab.

INTRODUCTION

The Chesapeake Bay blue crab fishery is currently valued at about 4.3 million dollars and is approximately 46% of the entire blue crab harvest taken from Atlantic and Gulf waters (Fishery Statistics, 1960). Very little data concerning the proximate composition of the Chesapeake Bay blue crab—and seasonal changes in composition with respect to any factors that might influence these changes—can be found in the literature.

Variations in the proximate composition of the blue crab are important to crab meat processors, to food technologists, and to nutritionists. The crab meat processor and food technologist can predict the type of meat and season best suited for the processing of crab meat, and the nutritionist can use data on proximate composition in the preparation of suitable diets.

Factors influencing variations in composition in fish may be attributed to: 1) changes in environment (Hart *et al.*, 1940); 2) changes in the reproductive cycle (Thurston and Newman, 1962); 3) changes in food and feeding habits (Stansby, 1953); and 4) difference in type of tissue (Stansby, 1962). The sampling procedure can be chosen to keep the geographical location constant and therefore minimize as much as possible the influence of environmental conditions and the food available.

This paper is intended to: 1) report the proximate composition of the blue crab, 2) determine whether interpretations become more meaningful when proximate composition data are evaluated on a nitrogen-content basis rather than on a wet-weight basis, 3) record and interpret seasonal variations in proximate composition of the body meat, claw meat, and offal of the blue crab, and 4) compare variations in the proximate composition of the various types of meat of the blue crab.

PROXIMATE COMPOSITION

Experimental. *Sampling method.* Blue crabs *(Callinectes sapidus)*, taken by commercial methods, were obtained on a bimonthly basis by members of the staff of the Bureau of Commercial Fisheries Technological Laboratory, College Park, Maryland, in Little Choptank River at Hudson Creek (middle Chesapeake Bay). The crabs were caught in as nearly the same geographical location and as close to the same day of the month as weather would permit. The whole crabs, less than 24 hr old, were frozen and shipped in dry ice to the Bureau of Commercial Fisheries Technological Laboratory, Pascagoula, Mississippi. The crabs, minus the claws, were divided into two groups—by sex when the ratio of males and females made it possible, or randomly when it did not. Weight to the nearest 0.1 g of the whole crab minus the claws, and carapace width of the nearest 0.1 cm, were determined for each crab. The crabs were cleaned and picked by experienced personnel. The edible body meat from each group was homogenized in a stoneware ball mill grinder. The claws from each monthly lot were combined into one sample, cleaned, picked, and ground similarly. The waste from the body and claws—excluding the back, which was discarded—was combined and ground in a Delta Cutter food grinder. Each ground sample was placed in a polyethylene bag. The bag was sealed,

Table 1. Standard deviation for proximate composition methods.

Type of tissue	Wet weight (%)			Protein content (mg/mg N)		
	Moisture	Oil	Ash	Moisture	Oil	Ash
Body	.575	.330	.073	.154	.013	.012
Claw	.210	.200	.066	.042	.010	.002
Offal	.440	.140	.170	.055	.006	.008

placed in a glass container, and held at 0°F until the contents were analyzed. A cube of ice was placed in each glass container to prevent dehydration of the sample during storage.

Chemical methods. The oil, ash, nitrogen, and moisture content of the blue crab were determined substantially according to the official methods of analysis of the Association of Official Agricultural Chemists, 9th edition (1960). The only deviation from the methods described was substitution of reagent-grade sand for asbestos in the moisture determination. Protein was calculated by multiplying the nitrogen content by 6.25. The data are reported as the average of four determinations (duplicate determinations of duplicate samples) in the case of body meat and as the average of duplicate analyses for the claw meat and the waste material. The standard deviation for each method on each type of tissue obtained by statistical analysis of all samples is shown in Table 1.

Results and discussion. The proximate composition of the body and leg meat and the offal, as well as the physical measurements of the whole crab, is listed in Table 2. There is considerable variation in the protein content throughout the period sampled. The protein content of the body meat varied from a high of 19.0% in January to a low of 13.8% in June. A similar situation prevailed in the leg meat, where a high occurred in March (19.3%) and a low in June (11.9%), and in the offal (January high of 14.8% and June low of 10.2%). Such variations are important not only from a nutritional viewpoint but also for determining the best processing and handling methods. In order that such changes and their effects could be evaluated, the data collected were subjected to further statistical analysis.

EVALUATION BASIS

There is the possibility that a greater statistical significance may be obtained by evaluating such data on a per milligram of nitrogen basis than is obtained by evaluating it on a wet-weight basis, as is the usual procedure. The former basis is seemingly more logical, since: 1) the nitrogen content is more closely related to the body functions than is the moisture content, and 2) the nitrogen content is much less susceptible to processing and storage variations (such as "drip" loss, freezer burn, and swelling), which are not related to the functional changes in tissue. Student t values (Table 3) calculated on a nitrogen-content basis show, in all but one case, a more meaningful relation between data pairs than values calculated on a wet-weight basis. The Student t and r^2 values increased, and in some instances became statistically significant, through the use of nitrogen-based data instead of wet-weight data. Accordingly, discussions in this paper are for the most part based on data calculated on a nitrogen-content basis; where moisture content is used as a basis, it is so specified.

SEASONAL VARIATIONS

The mating and the spawning habits of the Chesapeake Bay blue crab are well known (Cargo, 1955; Van Engel, 1958). Mating peaks during the months of August and September and is followed by migration of the female blue crab to the saltier water at the mouth of the Chesapeake Bay. Although eggs in the ovaries of each female develop almost to completion within the next 2 months, egg-laying usually does not occur until May and June —June being the peak month for spawning. Migration of the female crab in the Chesapeake Bay occurs during August and September. The lot of blue crabs caught in the middle of the Bay area during September contained no female crabs, as would be expected if migration takes place according to the schedule outlined above. Table 4 gives the proximate composition for male and female blue crabs caught in August. There were no significant differences between the means of the protein, oil, ash, moisture contents, carapace widths and weights of the male and female crabs during the mating season. If the reproductive cycles do affect composition to any great degree, there would be a significant difference. The period of mating and great activity is well defined and may last five days or longer. June is the peak month for spawning of the Chesapeake Bay blue crab. The large increases in the ash and moisture content and the decrease in the protein content observed from March to June could be related directly to spawning habits, since

Table 2. Weight, carapace length, and proximate composition of the Chesapeake Bay blue crab *(Callinectes sapidus)*.

Sample and Date	No. in sample	Weight (g)		Carapace width (cm)		Percent				mg/mg N		
		Range	Av.	Range	Av.	Av. prot. cont.	Av. moist. cont.	Av. oil cont.	Av. ash cont.	Av. moist. cont.	Av. oil cont.	Av. ash cont.
Body meat												
1/14/63	51	117.0-189.3	128.8	11.9-18.5	14.4	19.0	77.9	1.5	1.50	25.7	0.5	0.50
3/24/63	73	70.3-187.7	122.5	11.1-17.7	14.0	17.7	79.4	0.9	1.38	28.0	0.3	0.49
6/4/63	55	76.2-171.3	114.0	11.0-15.4	13.4	13.9	84.0	1.1	1.39	38.0	0.5	0.63
8/21/63	41	84.1-163.5	113.1	12.2-17.2	14.2	15.2	81.7	0.9	1.38	33.6	0.4	0.57
9/18/62	69	74.6-221.2	114.9	11.6-16.9	14.0	15.9	81.6	1.2	1.76	32.6	0.5	0.70
11/13/62	60	60.6-159.9	116.0	10.7-17.4	13.3	15.0	82.8	1.5	1.28	34.6	0.6	0.54
Claw meat												
1/14/63	97	117.0-189.3	128.8	11.9-18.5	14.4	19.0	77.4	0.8	1.38	25.4	0.2	0.45
3/24/63	133	70.3-187.7	122.5	11.1-17.7	14.0	19.2	78.3	0.8	1.59	25.4	0.2	0.52
6/4/63	93	76.2-171.3	114.0	11.0-15.4	13.4	11.9	86.7	1.2	1.81	45.4	0.6	0.95
8/21/63	78	84.1-163.5	113.1	12.2-17.2	14.2	15.9	82.1	0.7	1.67	32.2	0.3	0.65
9/18/62	114	74.6-221.2	114.9	11.6-16.9	14.0	15.1	82.7	1.3	1.69	34.6	0.5	0.71
11/13/62	127	60.6-159.9	116.0	10.7-17.4	13.3	15.0	82.6	0.8	1.50	34.4	0.4	0.62
Offal												
1/14/63	51	117.0-189.3	128.8	11.9-18.5	14.4	14.8	64.5	2.2	10.80	27.2	0.9	4.56
3/24/63	73	70.3-187.7	122.5	11.1-17.7	14.0	13.9	67.2	1.8	10.12	29.8	0.8	4.50
6/4/63	55	76.2-171.3	114.0	11.0-15.4	13.4	10.2	72.4	1.3	9.63	44.4	0.8	5.91
8/21/63	41	84.1-163.5	113.1	12.2-17.2	14.2	12.0	65.2	0.9	11.77	34.0	0.5	6.13
9/18/62	69	74.6-221.2	114.9	11.6-16.9	14.0	12.6	66.6	0.9	11.91	33.2	0.4	5.92
11/13/62	60	60.6-159.9	116.0	10.7-17.4	13.3	11.2	68.8	1.9	10.49	38.4	1.1	5.86

Table 3. Statistical measurements of the relationship between various data pairs.

Data pairs	Wet weight										Nitrogen content									
	Body meat			Claw meat			Offal				Body meat			Claw meat			Offal			
	S_{YX}	r^2	r	S_{YX}	r^2	r	P	S_{YX}	r^2	r	P	S_{YX}	r^2	r	P	S_{YX}	r^2	r	P	S_{YX}	r^2	r	P
Moisture-oil	.40	.0152	.1232	—	.17	.6122	.7824	.025	.48	.0714	.2672	—	.15	.0312	.1766	—	.074	.7825	.8845	.001	.22	.0027	.0519	—
Moisture-protein	.01	.0000	.0000	.000	.01	.0000	.0000	—	.92	.6491	.8056	.025	—	—	—	—	—	—	—	—	—	—	—	—
Moisture-ash	.16	.0000	.0000	.030	.08	.8571	.9257	.001	.59	.4893	.6994	.100	.075	.3149	.5611	.050	.031	.9608	.9802	.001	.46	.5648	.7515	.005
Moisture-weight	—	—	—	—	—	—	—	—	—	—	—	—	.200	.8333	.9128	.001	—	—	—	—	—	—	—	—
Moisture-width	.49	.3279	.5726	.050	.29	.5641	.7510	.050	.21	.7607	.8721	.005	4.4	.3105	.5572	.050	2.5	.6173	.7856	.001	2.9	.5168	.7188	.005
Protein-oil	.40	.0101	.1004	—	.17	.6122	.7824	.025	.43	.2532	.5031	.400	—	—	—	—	—	—	—	—	—	—	—	—
Protein-ash	.16	.0294	.1714	—	.10	.7857	.8863	.005	.80	.0689	.2624	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein-weight	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein-width	.53	.2093	.4574	.200	.27	.6239	.7898	.025	.25	.6667	.8165	.025	—	—	—	—	—	—	—	—	—	—	—	—
Ash-oil	.40	.0202	.1421	—	.18	.5918	.7692	.050	.40	.3506	.5921	.200	.15	.0170	.1303	—	.07	.7961	.8922	.001	.20	.1774	.4211	.200
Ash-weight	—	—	—	—	—	—	—	—	—	—	—	—	4.0	.5000	.7071	.005	—	—	—	—	—	—	—	—
Ash-width	.59	.0256	.1600	—	.25	.6667	.8165	.025	.35	.3675	.6062	.200	.03	.0000	.0000	—	.30	.4609	.6788	.010	.37	.2311	.4807	.100
Oil-weight	—	—	—	—	—	—	—	—	—	—	—	—	5.0	.3333	.5773	.025	—	—	—	—	—	—	—	—
Oil-width	.56	.1116	.3340	.400	.42	.0769	.2773	—	.43	.0171	.1307	—	.50	.1164	.3411	.400	.33	.3498	.5914	.025	.42	.0294	.1714	—
Weight-width	—	—	—	—	—	—	—	—	—	—	—	—	.73	.0000	.0000	—	—	—	—	—	—	—	—	—

this is a period of great activity. The oil content drops off after spawning and rises in November, 2 months after the peak month for mating. The eggs mature to almost completion within 1–2 months of mating. This may explain the rise in oil content during November for the body and offal tissues. A small increase in the oil content occurs during June, when the crab spawns, but is not large enough to be significant at the 5% level.

The blue crab does very little feeding or moving about during the winter months. The proximate composition of the blue crab for the month of January reflects this inactivity. The mating period —a period of great activity—produced a noticeable increase in protein content; however, the protein content increased, as mentioned above, during the period of restricted movement and feeding in the winter. Thus, there appears to be no relation between protein content and periods of activity—the relation appears more to be a function of egg-formation and/or a function of sperm-ripening prior to the mating season. The protein content of fish has been reported by Stansby (1962) as decreasing after mating or periods of inactivity; instead, however, the protein content of the blue crab increases from a yearly low in June to a high in January, covering the mating season and the winter inactivation period of the blue crab.

VARIOUS TYPES OF MEAT

Seasonal variations occur in the proximate composition of the body meat, leg meat, and offal of the blue crab. Figs. 1, 2, 3, and 4 show the seasonal changes in the proximate composition. The protein values in Fig. 2 are on a wet-weight basis whereas ash, oil, and moisture are reported as mg/mg N. The moisture content decreases in January and rises sharply in June for all types (Fig. 1); however, the inverse is true for protein (Fig.

Fig. 1. Seasonal variation in the moisture content of the Chesapeake Bay blue crab.

Table 4. Proximate composition of the body tissue of Chesapeake Bay blue crab *(Callinectes sapidus)* for the month of August.

Group	No. of males	No. of females	Av. protein %	Av. moisture mg/mg N	Av. oil mg/mg N	Av. ash mg/mg N
1	19	0	15.8	32.1	0.4	0.5
2	0	22	14.7	35.0	0.4	0.6

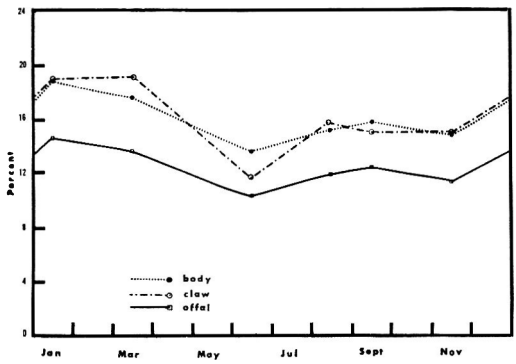

Fig. 2. Seasonal variation in the protein content of the Chesapeake Bay blue crab.

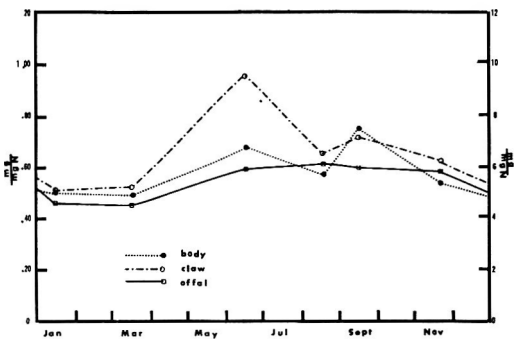

Fig. 3. Seasonal variation in the ash content of the Chesapeake Bay blue crab.

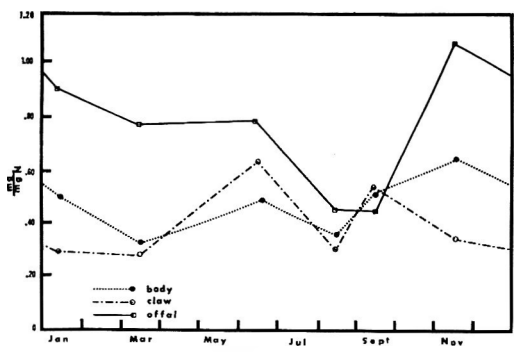

Fig. 4. Seasonal variation in the oil content of the Chesapeake Bay blue crab.

2). The same type of seasonal variation occurs in ash content (Fig. 3) as occurs in the moisture content, but only in the leg meat and offal and not in the body meat. The oil content rises in November and falls off in August for the body meat and offal. The sharp increase in the oil content of the claw meat occurs in September and June (Fig. 4).

Samples were tested for significant seasonal variations in composition. Because of the necessity of grouping individual crabs into a few larger samples, the usual statistical measure of significance of difference between the means was difficult to apply. Therefore, the samples were determined to be significantly different at the 95% level if the values exceeded the average of the two by more than two standard deviations. The ash content of the claw meat and offal changed significantly during January and June (Fig. 3). The protein content also changed during January and June, but oppositely to that of the ash content (Figs. 2, 3).

The offal material of the blue crab contains more moisture, oil, and ash than do the body or claw tissues. The protein content for the body and claw meat is slightly higher, however, than the protein content of the offal material. The nitrogen content of the offal material is such that any meal made from it should be comparable to other types of meals in gross protein nutritive value.

The greatest difference between the proximate composition of the body and claw meat occurs during June, at which time the ash and oil content of the claw increases and the ash and oil content of the body does not change. Claw meat should be a much more static tissue than either body meat or offal material since it is composed almost entirely of muscular tissue. Table 5 shows this effect in that more correlations occur in the claw

Table 5. Intercorrelation of the physical measurements and proximate composition of the Chesapeake Bay blue crab (*Callinectes sapidus*).

	Body meat					Claw meat					Offal				
	Weight	Width	Protein	Moisture	Oil	Ash	Weight	Width	Protein	Moisture	Oil	Ash	Weight	Width	Protein	Moisture	Oil	Ash
Wet weight to:																		
Weight									+	−		−			+			
Width				−				+			+	+				−		
Protein								−	+		+	+		+		−		
Moisture		−						+	−		+	+		−				+
Oil									+	+								
Ash								−	+		+							
Nitrogen content to:																		
Weight				−		−		−				−						
Width								−								−		
Protein						+						+						
Moisture		−						−						−				
Oil	−							−		+		+						
Ash	−			+				−		+	+							

meat than in the body meat or offal. The proximate composition of the claw meat may thus be predicted with greater accuracy than that of the body meat or offal.

Table 5 presents only the correlations that appear at the 5% level of significance. The table is divided into correlations calculated on a wet-weight and on a nitrogen-content basis (therefore, any correlation with protein would be found only on the wet-weight side of the table). On a wet-weight basis, protein content correlates positively with width, oil, and ash in the claw meat, and with width in the offal. Protein content correlates negatively with moisture in the offal, but does not correlate with any other component in the body meat. This is an indication that the nitrogenous content of the body meat is not completely related to the cell building processes as regards simple growth of the animal.

Protein content is, however, the best chemical measurement of growth, as indicated previously, and, therefore, the correlation pattern is best interpreted when calculated on a nitrogen-content basis. On this basis, width correlates negatively with moisture, oil, and ash in the claw meat. The positive correlations between oil and ash, oil and moisture, and ash and moisture in the claw meat show that, with the growth of the claw and the consequent increase in protein content of the claw, the oil, ash, and moisture contents all decrease. This relation would be expected in this type of tissue where only one function—that of muscular action—is predominant. This type of relation is obscured in the body meat—probably because of the numerous types of physiological functions taking place there.

With this type of analysis, there also appears to be a direct relation between the increase in protein content, growth of the crab in over-all size, and a decrease of the moisture content in the offal material.

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Balanced Protein Mixtures Based on Wheat and Peas

SUMMARY

Protein mixtures of wheat and peas alone, or with variable third constituents, were formulated to maximize the supplementary relationships among component amino acids. Biological quality was evaluated by rat assay methods. Data indicate that the quality of the plant protein mixtures is equal to that of egg when comparable levels of both essential amino acids and protein are provided.

INTRODUCTION

Numerous studies have demonstrated that the biological quality of wheat protein may be improved by supplementation with lysine or proteins high in content of this essential amino acid. Pea protein is one of these, and Murray (1948) has shown that mixtures of wheat and peas are higher in protein quality than is either alone. Combinations of wheat and peas rated somewhat below casein, however, when measured by rat growth.

This failure of the plant protein mixtures to match the growth-promoting quality of good animal proteins could be due to two factors:

1) An uncorrected deficit of the sulfur-containing amino acids. These are present only in low concentration in the legume protein although relatively abundant in wheat.

2) The lower percentage of total essential amino acids contained in plant proteins when compared with those of animal origin. Therefore, diets of equal protein content, customarily employed in assessment of protein quality, provide less essential amino acids in the former case.

Results of research reported here show that the biological quality of mixtures of plant proteins is equal to that of egg when fed at comparable levels of both essential amino acids and protein.

MATERIALS AND METHODS

Examination of published data on the amino acid content of food proteins (Orr and Watt, 1957) suggested that sesame seed, ragi (finger millet), corn gluten, Brazil nuts, and whole egg could be used effectively to increase the content of sulfur-containing amino acids in wheat-pea mixtures. Formulas containing varying proportions of the proteins were selected to match closely the essential amino acid patterns of egg protein. A description of the selected mixtures is given in Table 1, and Table 2 compares their amino acid content with that of eggs and with the FAO reference pattern (FAO, 1957).

Protein quality was determined from rat growth and, in a few groups, nitrogen balance data. For this purpose, diets containing 10% of protein were constructed, using the compositional data given in Table 1. To test the pattern of essential amino acids independently of quantity, a diet was included in which the essential amino acid contribution of egg protein was reduced to that of the plant protein diets by dilution with a nonessential amino acid. Approximate equivalence was obtained from 6.4 g of egg protein plus 3.6 g of l-glutamic acid.

An additional comparison was made by including a greater amount of protein in the diet, 16%, so that the wheat-pea-corn-gluten mixture would contribute the same amount of essential amino acids as the conventional 10% egg protein diet. The appropriate control in this case was a diet containing 10% of egg protein plus 6% of l-glutamic acid.

Diets were supplemented with corn oil to equate fat content to 10%; with USP Salts XIV plus zinc to yield 5% of minerals (two parts of $ZnCO_3$ added to 598 parts of USP Salts XIV); and with cellulose to provide 3% of total fiber. All diets included 10% of vitamin-B-fortified sucrose, 1% of glycerol, and cornstarch as needed to make up the total carbohydrate to 61% of the 10%-protein diets and 55% of the 16%-protein diets. The sucrose mixture provided, in mg per 100 g diet: thiamine HCl, 0.25; riboflavin, 0.50; pyridoxine HCl, 0.25; niacin, 2.0; Ca pantothenate, 2.0; inositol, 10.0; para-aminobenzoic acid, 5.0; biotin, 0.01; folic acid, 0.10; vitamin B_{12}, 0.005; and choline Cl, 100.0. Fat-soluble vitamins were given twice weekly by dropper, providing per rat per week: vitamin A, 600 I.U.; vitamin D_2, 10 I.U.; alpha-tocopherol, 3.0 mg; and 2-methyl-1,4-naphthoquinone, 0.3 mg.

Diets were prepared by cooking the specified amounts of cereal, legume, and starch components in distilled water of sufficient volume to yield a finished diet containing 60% moisture. After cool-

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Table 1. Composition of mixtures and ingredients.[a]

Substance	N/protein factor	%					
		Protein	Fat	Fiber	Ash	Moisture	Carbohydrate
Whole-wheat flour *(Triticum aestivum)*	5.83	14.4	2.30	2.42	1.62	8.9	70.4
Alaska pea flour [b] *(Pisum sativum)*	6.25	23.1	1.46	6.67	2.17	7.1	59.5
Ragi millet *(Eleusine coracana)*	5.83	6.0	1.69	3.36	3.66	9.8	75.5
Sesame seed *(Sesamum indicum)*	5.30	19.5	5.70	3.01	3.03	2.5	66.3
Corn gluten meal *(Zea mays)*	6.25	65.9	3.78	0.83	1.00	5.2	23.3
Brazil nuts *(Bertholletia excelsa)*	5.46	14.6	67.20	2.26	3.18	2.5	10.3
Dry whole egg	6.25	46.1	35.60	—	3.52	3.7	11.1
Wheat:pea:ragi N::50:35:15 [c]	5.98	13.5	1.9	3.6	2.4	8.8	69.8
Wheat:pea:sesame seed N::50:35:15	5.90	17.4	2.5	3.7	1.9	7.6	66.9
Wheat:pea:corn gluten N::50:30:20	6.04	19.9	2.2	3.5	1.7	8.2	64.5
Wheat:pea:Brazil nut N::60:25:15	5.88	16.0	12.1	3.2	2.0	7.6	59.1
Wheat:pea:egg N::50:30:20	6.04	19.4	5.0	3.3	1.9	8.0	62.4
Wheat:pea N::60:40	6.00	17.1	2.0	3.7	1.8	8.3	67.1

[a] Composition of diet ingredients was determined by analysis using AOAC methods, except carbohydrate content which was derived by difference.

[b] Pea flour was prepared by cooking whole peas for 20 min at a low boil, drying overnight at 46°C, and grinding finely. All other products were obtained from commercial sources.

[c] Mixtures were made from ingredients shown above, with the designated proportional nitrogen contribution from the specified components.

ing, the remaining diet ingredients were added with thorough mixing. One-day feeding allowances were packaged in polyethylene bags. Diets were frozen and thawed just prior to feeding.

Weanling male albino rats (Simonsen strain, cesarean-derived from Sprague-Dawley stock) were individually housed in metabolism cages in a temperature-controlled laboratory (78 ± 2°F). All rats were fed a low-protein (4%) egg diet, similar in all other respects to the test diets, during a one-week standardization period. Five rats were then assigned to each of the designated dietary treatments on a matched body-weight basis, and one group continued on the low-protein regime. For the next 3 weeks these diets were fed in amount equal to the average intake of the group showing the poorest consumption, the wheat-fed group. Distilled water was provided *ad libitum*. Food intake was recorded daily, and rejected food was saved for analysis of dry solids content (AOAC, 1960).

Animals were weighed 3 times weekly, and the amount of weight gained was used to compute protein efficiency ratios as follows:

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{g of weight gained}}{\text{g of protein eaten}}$$

The significance of differences between observed PER values was evaluated statistically with a multiple-comparisons test (Duncan, 1955).

During the second test week, urine and feces were collected from selected groups to determine protein digestibility and biological value. These samples and all diets were analyzed for nitrogen content (AOAC, 1960). Biological value was computed according to the method of Mitchell *et al.* (1945) using the following factors:

NI = nitrogen intake, mg

FN_m = fecal nitrogen of the 4% protein group (metabolic) = 1.136±0.100 mg per gram of dry food eaten

FN_t = fecal nitrogen of the test group, mg

UN_e = urinary nitrogen of the 4% protein group (endogenous) = 2.241±0.171 mg per gram$^{0.75}$ of body weight

UN_t = urinary nitrogen of the test group, mg

Absorbed nitrogen $(AN) = NI - (FN_t - FN_m)$

$$\text{Nitrogen digestibility } (ND),\ \% = \frac{AN}{NI} \times 100$$

$$\text{Biological value } (BV),\ \% = \frac{AN - (UN_t - UN_e)}{AN} \times 100$$

$$\text{Net nitrogen utilization } (NNU),\ \% = ND \times BV \div 100$$

To evaluate the correspondence between the precise measurement of net nitrogen utilization and the simpler measure of PER, these data were tested for correlation (Fisher, 1930).

At the end of the experiment, carcasses were examined for evidence of gross pathologic changes. Livers were weighed and analyzed individually for moisture; fat content of pooled dry samples was determined (AOAC, 1960).

RESULTS

When the diet provided 10% of total protein entirely from egg, the protein efficiency ratio was

Table 2. Calculated amino acid content (mg/g of nitrogen) of protein sources.[a]

Protein source	Histidine	Isoleucine	Leucine	Lysine	Methionine	Cystine	Total sulfur	Phenylalanine	Tyrosine	Total aromatic	Threonine	Tryptophan	Valine
Whole egg	150	415	550	400	196	146	342	361	269	630	311	103	464
Wheat	119	253	391	160	89	128	217	288	218	506	168	72	270
Pea	171	352	517	458	75	81	156	315	252	567	241	66	350
Wheat-pea	139	293	442	279	83	109	192	299	232	531	197	69	302
Wheat-pea-ragi	132	305	464	268	108	118	226	291	197+	488+	206	71	324
Wheat-pea-sesame seed	138	288	446	264	96	112	208	314	236	550	197	73	294
Wheat-pea-egg	141	315	461	297	105	117	222	310	239	549	218	77	333
Wheat-pea-corn gluten	136	287	546	239	101	106	207	308	258	566	199	63	304
Wheat-pea-Brazil nut	135	274	428	235	126	126	252	287	221	508	185	70	297
FAO Ref. pattern		270	306	270			270	180			180	90	270

[a] Data from Orr and Watt, 1957.

Table 3. Biological measurements of protein quality.

Protein source	Protein efficiency ratio (g gain/g protein intake)	Biological value (%)	True nitrogen digestibility (%)	Net N utilization (%)	Liver			
					Wet weight % of body weight (%)	Moisture content (%)	Fat content (%)	Moisture and fat-free weight, % of body weight (%)
4% of total protein								
Egg					6.04	54.6	27.1	1.10
10% of total protein								
Egg	3.59±0.20 [a]	96.4±0.6	99.2±1.2	95.7±1.6	5.38	69.1	9.4	1.16
Wheat-pea-egg	3.17±0.10				4.32	72.6	3.4	1.03
Wheat-pea-ragi	2.90±0.39				4.06	71.8	3.0	1.02
Egg + glutamic acid	2.88±0.18	78.0±1.4	99.4±0.6	77.5±1.2	5.24	64.4	14.0	1.13
Wheat-pea	2.75±0.23	71.6±1.9	90.6±1.5	64.8±1.9	3.90	72.2	2.9	0.97
Wheat-pea-sesame seed	2.63±0.21				4.24	71.9	2.3	1.09
Wheat-pea-Brazil nuts	2.62±0.46				4.38	71.5	3.7	1.09
Wheat-pea-corn gluten	2.36±0.06	64.1±2.2	93.0±1.2	59.8±2.9	4.34	72.0	3.7	1.05
Wheat	1.68±0.33	44.2±2.0	92.9±1.6	41.1±2.3	4.32	71.2	4.1	1.07
16% of total protein								
Egg + glutamic acid	2.56±0.12				4.70	69.8	5.7	1.15
Wheat-pea-corn gluten	2.44±0.30				4.02	71.9	2.0	1.05

[a] Mean and standard deviation. Means within a given bracket do not differ significantly from each other at the 5% confidence level, using Duncan's multiple comparisons test.

3.59, higher than that of any other diet (Table 3). However, in animals fed egg protein diluted with glutamic acid to equalize essential amino acid content with that of plant protein mixtures, growth response was not better than in the groups given wheat-pea mixtures (PER 2.88 vs. 2.62–2.90) with the single exception of the group receiving the corn gluten meal. Even the poorest mixture of the series, wheat-peas-corn gluten, represented a significant improvement over wheat protein alone (PER 2.36 vs. 1.68).

Fed at the 16% protein level, the mixture of wheat, peas, and corn gluten provided approximately the same total amount of essential amino acids as the diet containing 10% of egg protein plus 6% of glutamic acid. Growth response of these groups was similar, with PER's of 2.44 and 2.56, respectively.

From balance data, the biological value of egg was found to be 96% and digestibility, 99%, indicating net utilization of 95.7% of dietary nitrogen (Table 3). Dilution of egg with glutamic acid did not affect digestibility but reduced the retention of absorbed nitrogen (*BV*) to 78%.

The biological value of whole-wheat protein was 44%. This value was increased to 72% by supplementation with pea protein, and it was not improved by the further addition of corn gluten. Digestibility of the plant proteins was somewhat lower than that of egg, 91 to 93%, but within the normal range for foods of this type. Net nitrogen utilization was thus concomitantly reduced.

In the groups fed egg protein, liver made up a larger proportion of body weight, and livers were lower in moisture and higher in fat content than were those of all other groups (Table 3). The percentage of hepatic fat decreased as the dietary level of egg protein increased. Moisture and fat-free liver tissue approximated 1% of body weight of all groups, irrespective of source or level of dietary protein.

DISCUSSION

Objections can be raised to the use of protein efficiency ratio as a sufficient criterion of adequacy of protein nutritional status because gain in weight does not necessarily imply gain in body protein. Further evidence of deposition of lean body mass can be adduced from comparison of PER's with nitrogen balance data. Since animal growth reflects the combined effects of digestibility and retention of nitrogen, PER values should relate to net nitrogen utilization. However, for precision, growth must be referred to nitrogen rather than to protein intake because comparisons are to be made among proteins varying in nitrogen content. Appropriate conversion of data for the 5 groups in which both measurements were made yielded the relationship portrayed in Fig. 1,

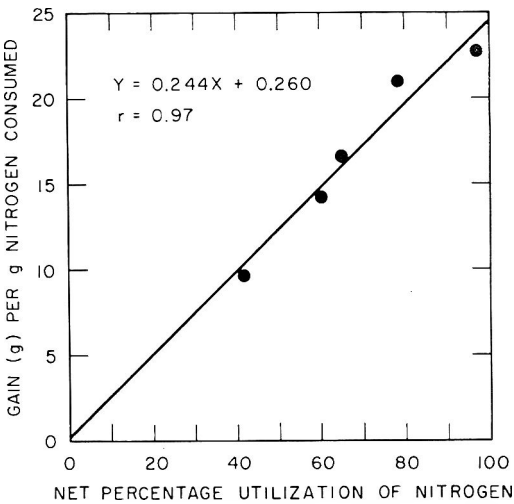

Fig. 1. Linear relationship of net nitrogen utilization and weight gain per gram of nitrogen intake.

with a product-moment correlation coefficient of 0.97. Regression of weight gain per gram of nitrogen consumed (Y) on net percentage nitrogen utilization (X) is described by the equation: $Y = 0.244X + 0.260$. This equation describes a relationship in which utilization of 1 g of nitrogen permits 24.66 g of body weight gain. The tissue gained must therefore contain 4% of nitrogen, or 25% of protein, a value fully consonant with the deposition of lean body mass.

Protein efficiency ratios recorded in this experiment indicate that mixtures of wheat and peas have a pattern of essential amino acids as good as that of high-quality animal protein. The differences in growth rate usually observed we ascribe to the higher proportion of nonessential amino acids in plant proteins than in animal proteins. This will limit growth if the test animal requires a proportion of essential to nonessential amino acids in excess of that of the dietary protein and if the diet is marginal with respect to total protein. These conditions exist in the case of growing rats fed diets containing 10% of total protein.

However, about 30 g of protein from these mixtures would supply sufficient amounts of the most limiting amino acids to meet the suggested minimum requirements of adult man (FAO, 1957). Thus, a diet based on these mixtures that met the total protein requirement would be more than adequate with respect to essential amino acids, so this limitation does not exist in the case of adult man.

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Palatability and Quantity of Pork as Influenced by Breed and Fatness

SUMMARY

The influence of breed, sex, and fatness on the quantity and eating quality of loins from 119 Duroc and 111 Yorkshire pigs was studied. Duroc pigs had significantly more intramuscular fat, smaller longissimus dorsi area, more tender, juicier loins, and less separable lean in the ham than Yorkshire pigs. Among Durocs, harrows had more desirable lean flavor than gilts. Among Yorkshires, barrows had more desirable fat flavor than gilts. In both breeds, an increase in backfat thickness was accompanied by a significant linear decrease in separable lean and increase in separable fat of the ham. Correlations among quantity indices and eating quality indicated that indices of quantity were not related to eating quality.

INTRODUCTION

Backfat thickness, because of its high negative relationship to percentage of lean meat in the carcass, has been an effective selection tool in development of the meat-type hog. The effect that changes in backfat thickness produce in both intramuscular fat and in eating quality is rather obscure.

Murphy and Carlin (1961) reported that the amount of backfat on the carcass did not have a significant effect on tenderness, juiciness, or flavor of braised pork chops. However, marbling of fat in the lean had a significant positive effect on both tenderness and juiciness. Henry *et al.* (1963) also reported a significant positive relationship between marbling score and tenderness.

Limited evidence is available to indicate that both fatness and breed influence eating quality and deposition of intramuscular fat in the lean of the longissimus dorsi (LD) muscle. Judge *et al.* (1960) reported that breed of hog significantly influenced loin marbling scores. Henry *et al.* (1963) indicated that at least two workers noted a positive effect between backfat thickness and marbling of the LD. These results suggested a more critical examination of effect of breed, backfat thickness, and intramuscular fat on pork eating quality in pigs with various levels of fatness.

The objective of this study was to evaluate eating quality, intramuscular fat in lean of the LD muscle, and quantity of pork in Duroc and Yorkshire pigs with various backfat thickness when slaughtered at approximately the same final feed-lot weight of 225 lb.

EXPERIMENTAL PROCEDURES

These data, collected at the USDA Agricultural Research Center, Beltsville, Maryland, originated from an experiment designed to study the inheritance of fat in swine and included 119 Duroc and 111 Yorkshire pigs. All pigs were fed a standardized fattening ration and slaughtered at approximately 210 lb. Barrows and gilts of each breed were sampled every year to obtain representative carcass and eating-quality information. Among the carcass information obtained was the physical separation of the right ham into lean, fat, bone and skin, and the area of LD muscle at the last rib. These measures were used to provide indices of muscle and fat deposition. A quantitative measure of intramuscular fat in the lean of the right loin was obtained by ether-extract fat analysis (AOAC, 1955). This sample was obtained from the same location as the sample for palatability evaluation. Loin samples, consisting of the last three thoracic and first three lumbar vertebra from the left side of the carcass, were cooked in an electric oven at 325°F to an internal temperature of 185°F, and evaluated for eating quality by a panel of five judges or more. Eating-quality factors included desirability of flavor of fat and lean, quantity of juice, and tenderness. Tenderness was also measured objectively by the Warner-Bratzler shear instrument.

Carcasses were cut according to the procedure described by Hiner (1949) at the Reciprocal Meat Conference. All weights were recorded to the nearest 1/10 lb. In the least-squares portion of the analysis, pigs were allocated to one of five discrete 10-mm backfat-thickness groups in each breed. All unadjusted and adjusted correlations were computed with backfat thickness used as a continuous variable.

The generalized fixed model

$Y^T = AX$ [1]

explained by Smith *et al.* (1962) was used to describe these data.

Y^T = an $(n \times p)$ matrix of observations.

A = an $(n \times m)$ design matrix.

X = an $(m \times p)$ matrix of unknown fixed effects.

Each of the p vectors in matrix X may be represented by the least-squares model,

$$Y_{ijkm} = u + t_i + f_j + s_k + e_{ijkm} \quad [2]$$

where Y_{ijkm} refers to the m^{th} observation (eating quality or quantity factor) of the k^{th} sex, j^{th} backfat thickness group, and i^{th} year. The e_{ijkm} are random errors, and u is the fixed population mean. Models 1 and 2 were computed separately for each breed. Multivariate procedures described by Anderson (1960) were used to study correlations among quantity indices and eating quality adjusted for fatness within each breed.

Table 1. Desirability of average yearly eating-quality factors and quantity indices.[a]

	Units	Year of slaughter				
		1958	1960	1961	1962	1963
Duroc breed						
Number of pigs		8	24	31	31	17
Fat flavor	Grade	5.71	5.46	4.99	4.08	3.91
Lean flavor	Grade	6.12	5.86	5.87	5.55	5.55
Panel tenderness	Grade	6.08	5.52	5.70	5.70	5.11
Juice quantity	Grade	5.06	4.92	4.89	4.94	5.04
Warner-Bratzler shear	lb	10.05	11.46	11.58	14.52	14.99
Percent inbreeding[b]	%	1.47	7.75	9.22	11.81	14.91
Intramuscular fat of LD	%	6.30	7.28	7.83	9.14	6.66
LD area	Sq. in.	3.42	3.50	3.65	3.47	3.20
Lean of right ham	lb	8.53	8.22	7.75	8.79	8.37
Fat of right ham	lb	5.70	4.64	4.41	5.04	4.71
Yorkshire breed						
Number of pigs		10	30	32	28	19
Fat flavor	Grade	5.34	5.40	5.10	3.81	4.00
Lean flavor	Grade	6.01	5.67	5.88	5.29	5.30
Panel tenderness	Grade	5.71	5.24	5.12	4.90	4.58
Juice quantity	Grade	4.36	4.33	4.03	4.06	4.62
Warner-Bratzler shear	lb	15.40	15.98	15.83	19.15	17.54
Percent inbreeding[b]	%	3.86	8.18	9.65	11.86	12.83
Intramuscular fat of LD	%	2.90	5.34	4.56	4.01	4.17
LD area	Sq. in.	4.16	3.98	4.17	3.75	3.69
Lean of right ham	lb	9.32	9.00	8.54	9.14	9.20
Fat of right ham	lb	4.93	4.36	3.97	4.66	4.18

[a] Least-square means.
[b] Unadjusted over-all means.

RESULTS AND DISCUSSION

Year effects. Table 1 shows the least-squares means for year effects separately for each breed. There were distinct linear trends in several of the eating-quality factors. Fat flavor, lean flavor, and tenderness showed a significant decline in eating quality from 1958 to 1963 in the Duroc breed. During this time loins were distinctly less tender as measured by the Warner-Bratzler shear instrument. These same eating-quality factors—flavor of fat, flavor of lean, and tenderness—followed the same trend in the Yorkshire breed. The unadjusted means for percentage inbreeding are also shown in Table 1, along with the year effects.

Sex effects. The least-squares means for sex effects are shown in Table 2. Barrows had significantly more intramuscular fat in the lean of the LD muscle than gilts in both breeds. In the Duroc breed this was accompanied by significantly more pronounced flavor of lean and tenderer, juicier loins. In the Yorkshire breed the increase in intra-

muscular fat in the LD muscle was accompanied by significantly more pronounced flavor of fat and juicier loins.

Backfat thickness groups. The least-squares means for backfat thickness groups are shown in Table 2. An increase in backfat thickness was accompanied by a significant linear decrease in separable lean and a significant linear decrease in separable fat of the ham in both breeds. Although backfat thickness had no significant influence on eating quality, the data indicated, in the Duroc breed, that tenderness increased with increase in backfat thickness.

Breed effects. The over-all means for breed effects are shown in Table 2. Significant breed effects indicated that pigs of the Duroc breed had more intramuscular fat in the LD muscle, smaller LD area, tenderer juicier loins, more separable ham fat, and less separable ham lean than pigs of the Yorkshire breed. Breed effects were meas-

Table 2. Desirability of eating-quality factors and quantity indices for breed, sex, and backfat groups.[a]

Effect	No. of Pigs	LD area (sq. in.)	Lean of rt. ham (lb)	Fat of rt. ham (lb)	Intramuscular fat of LD (%)	Fat flavor (grade)	Lean flavor (grade)	Panel tenderness (grade)	Juice quantity (grade)	Warner-Bratzler shear (lb)
Duroc breed	119	3.45	8.30	4.90	7.44	4.83	5.77	5.62	4.97	12.52
Males	60	3.27	7.98	4.93	8.34	4.88	5.86	5.90	5.16	11.01
Females	59	3.63	8.62	4.87	6.54	4.78	5.70	5.34	4.78	14.03
Backfat groups										
Below 31.0 mm	3	4.09	9.02	5.03	8.76	4.97	6.06	5.14	5.09	11.35
31.1 - 41.0 mm	26	3.89	9.50	4.14	6.37	4.73	5.62	5.60	4.66	12.99
41.1 - 51.1 mm	37	3.45	8.76	4.53	7.56	4.85	5.72	5.61	5.00	13.32
51.2 - 61.2 mm	42	3.29	7.65	5.14	6.49	4.79	5.68	5.67	4.97	12.87
Over 61.3 mm	11	2.52	6.57	5.66	8.02	4.81	5.82	6.09	5.13	12.97
Yorkshire breed	111	3.95	9.04	4.42	4.32	4.73	5.63	5.11	4.28	16.78
Males	59	3.71	8.73	4.43	5.06	4.94	5.67	5.22	4.53	16.18
Females	52	4.19	9.35	4.41	3.57	4.52	5.58	5.00	4.03	17.38
Backfat groups										
Below 31.0 mm	6	4.44	10.75	3.55	3.95	5.07	5.75	4.66	4.21	19.91
31.1 - 41.0 mm	50	4.19	9.76	4.06	4.17	4.68	5.48	4.82	4.16	18.27
41.1 - 51.1 mm	45	4.01	9.02	4.36	4.48	4.98	5.56	4.09	4.19	16.68
51.2 - 61.2 mm	8	3.97	8.84	4.18	3.04	4.60	5.67	4.95	4.38	17.17
Over 61.3 mm	2	3.14	6.83	5.95	5.96	5.14	5.68	6.03	4.51	11.87

[a] Least-squares means.

ured by adjusting each breed for significant effects shown in Table 3 and computing mean differences between breeds in analysis of variance. The least-squares constants were used to adjust for significant effects.

The mean squares for model 2 are shown in Table 3. A comparison of the mean squares for the same effects in each breed indicated distinct differences between the two breeds. The most apparent differences were in the eating quality of loins from barrows and gilts. Barrows had more pronounced flavor of lean and were significantly more tender than gilts in the Duroc breed. This difference was not observed in the Yorkshire breed. However, in the Yorkshire breed, barrows had more pronounced flavor of fat than gilts. This distinction in fat and lean flavor both between breed and between sexes within a breed indicates different reactions between components of fat and lean flavor in barrows and gilts of these two breeds. The correlation, unadjusted for breed effects, between intramuscular fat of the LD muscle

Table 3. Mean squares for year, sex, and backfat groups on quantity indices and eating-quality factors.

Source	d.f.	LD area	Age	Intra-muscular LD	Fat flavor desirability	Lean flavor desirability	Panel tenderness	Juice quantity	Warner-Bratzler shear	Lean of right ham	Fat of right ham
A) Duroc breed											
Years	4	.57	419.80	23.67	11.73**	1.01**	1.87**	.10	80.15**	3.87**	3.56**
Sex	1	3.66**	1151.29*	86.14**	.26	.68*	8.08**	3.93**	241.28**	10.64**	.13
Backfat thickness groups	4	3.63**	99.08	12.98	.08	.18	.63	.62	5.16	21.90**	6.16**
Residual	109	.27	244.39	10.52	.26	.11	.39	.31	11.70	.97	.40
B) Yorkshire breed											
Years	4	.92*	865.33	11.27	12.45**	1.86**	2.06**	1.12*	55.22*	1.97	2.52**
Sex	1	5.51**	1319.21**	54.28**	4.12*	.16	1.10	5.92**	35.01	9.28**	.01
Backfat thickness groups	4	.75	428.04	5.06	.74	.15	1.06	.09	36.20	8.88**	2.42**
Residual	101	.30	377.99	4.79	.66	.16	.51	.35	18.50	.84	.29

* P < .05.
** P < .01.

and panel tenderness was +0.48. Part of this extreme difference in tenderness between Duroc and Yorkshire pigs may be accounted for by differences in intramuscular fat; the ether-extract fat of the LD muscle from the Duroc breed was significantly higher than that from the Yorkshire pigs. Intramuscular fat of the LD was significantly related to panel tenderness in the Duroc breed but not in the Yorkshire breed, as shown in Table 4. These breed effects indicated that a certain amount of intramuscular fat is required to produce tender pork with acceptable satiety. Apparently the quantity of intramuscular fat deposited in the LD can be influenced by the genetic composition of the pig. This needs further study.

The correlations among all quantity indices and eating quality factors are shown in Table 4. Backfat thickness was significantly related to quantity of juice and tenderness in both breeds. Flavor of fat and lean and quantity of juice were significantly related to tenderness in both breeds.

The correlations among quantity indices and eating-quality factors adjusted for fatness (backfat thickness and intramuscular fat of LD) are shown in Table 5. These correlations indicate the influence that fatness has on both quantity indices and eating quality in each breed. It is obvious that if fatness is known or measured in both breeds, quantity indices furnish little additional information about eating quality. Perhaps the most obvious difference between the breeds is the relationship between Warner-Bratzler shear values and eating quality. When fatness is known, Warner-Bratzler shear values were significantly more descriptive of flavor of fat and tenderness in the Duroc breed than in the Yorkshire breed. This result suggests a study of the effect of more objective measures of fatness on eating quality in different breeds. Also suggested for further study is more extreme ranges of fatness, especially animals with more than 2½ inches of backfat as well as those with less than 1¼ inches of backfat.

Additional examination of these data as shown in Table 1 suggests that study should be made of inbreeding as it affects quality of pork, especially tenderness.

Table 4. Relationships among quantity indices and eating-quality factors.[a]

Source	Backfat thickness	Intramuscular fat of LD	LD area	Lean of ham	Fat of ham	Warner-Bratzler shear	Fat flavor	Lean flavor	Juice quantity	Tenderness
A) Duroc breed										
Backfat thickness	1.00	.07	— .61	— .72	.52	— .20	.13	.16	.26	.28
Intramuscular fat of LD		1.00	— .34	— .27	.11	— .29	— .13	.10	.46	.45
LD area			1.00	.77	— .43	.21	.01	— .04	— .47	— .32
Lean of ham				1.00	— .48	.38	— .11	— .15	— .41	— .37
Fat of ham					1.00	— .16	.00	.09	.18	.26
Warner-Bratzler shear						1.00	— .30	— .36	— .35	— .74
Fat flavor							1.00	.55	.07	.21
Lean flavor								1.00	.31	.41
Juice quantity									1.00	.53
Tenderness										1.00
B) Yorkshire breed										
Backfat thickness	1.00	.19	— .37	— .56	.35	— .26	— .00	.13	.39	.31
Intramuscular fat of LD		1.00	— .32	— .33	.14	— .26	.25	.16	.38	.22
LD area			1.00	.57	— .32	— .01	.10	.04	— .46	— .07
Lean of ham				1.00	— .38	.32	— .08	— .22	— .36	— .31
Fat of ham					1.00	— .20	— .07	.00	.17	.24
Warner-Bratzler shear						1.00	— .21	— .33	— .18	— .66
Fat flavor							1.00	.50	.20	.25
Lean flavor								1.00	.31	.52
Juice quantity									1.00	.39
Tenderness										1.00

[a] All correlations .25 or greater significant $P < .01$.

Table 5. Correlations among quantity indices and eating-quality factors (adjusted for fatness).[a]

Source	LD area	Lean of ham	Fat of ham	Warner-Bratzler shear	Fat flavor	Lean flavor	Juice quantity	Tenderness
A) Duroc breed								
LD area	1.00	.56	— .14	.01	.06	.11	— .04	— .28
Lean of ham		1.00	— .15	.28	— .07	— .03	— .14	— .22
Fat of ham			1.00	— .05	— .17	— .10	— .17	.20
Warner-Bratzler shear				1.00	— .34	— .33	— .70	— .22
Fat flavor					1.00	— .58	.28	.11
Lean flavor						1.00	.39	.27
Juice quantity							1.00	.36
Tenderness								1.00
B) Yorkshire breed								
LD area	1.00	.42	— .20	— .20	.19	.15	.10	— .30
Lean of ham		1.00	— .24	.16	— .03	— .15	— .13	— .10
Fat of ham			1.00	— .11	— .10	— .05	.14	.02
Warner-Bratzler shear				1.00	— .17	— .29	— .62	— .01
Fat flavor					1.00	.49	.23	.14
Lean flavor						1.00	.50	.26
Juice quantity							1.00	.17
Tenderness								1.00

[a] All correlations .25 or greater significant $P < .01$.

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Food Crushing Sounds: Comparisons of Objective and Subjective Data

SUMMARY

The sounds produced when certain foodstuffs were chewed were tape recorded, and the amplitude of frequency-filtered or total sound was measured from the recordings. On the same occasions, the subjects' opinions about toughness/tenderness or about loudness were noted. Some correlations between objective and subjective data obtained in this way are presented and discussed.

A previous article (Drake, 1963) describes a method used for objective analysis of food crushing sounds. Reported here are further results obtained with the same method in the USA and in Sweden.

METHODS

Apparatus. In the first part of the work reported here (series 1), the apparatus used was the same as described earlier (Drake, 1963). In the second part (series 2), a similar set-up was used, but with the following modifications.

The sound sensing device, consisting of the same hearing-aid earphone as that used earlier, was placed in a special holder to facilitate manipulation by the subjects (Fig. 1). The sounds were recorded on a Revox magnetic tape recorder (W. Studer, Zürich, Switzerland, Model E36) for ¼-inch two-track magnetic tape at a speed of 7½ inches per second. The recorded sounds were analyzed by recording their amplitudes on a 5-mv strip chart recorder with 1 second full-scale pen travel (Leeds & Northrup, Model H).

Foodstuffs. In series 1, tough and tender meat samples were used. The two lots of reconstituted freeze-dried meat were the same as those used earlier (*cf.* Tuomy *et al.*, 1963). In series 2, six different foodstuffs were compared. Five of these were of commercial quality and purchased in Göteborg: Swedish-type crisp brown bread, rusks, wafers, crackers, and raw carrots. Shortcakes (Swedish "småbröd," type "mörkakor") were specially baked for the purpose.

Subjects. People participating as chewers and/or judges were all healthy males and females 20–50 years old. Their teeth were said to be in good order.

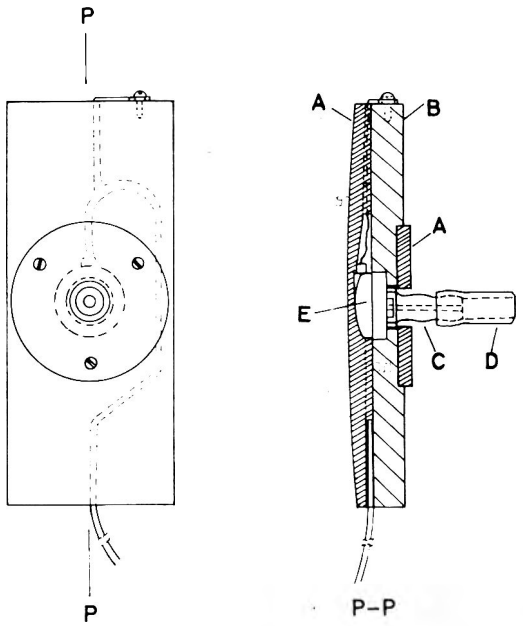

Fig. 1. Holder for hearing-aid earphone used as a microphone in recording of mastication sounds. A = polyvinyl chloride; B = brass; C = perspex; D = plastic tubing; E = earphone; P—P = cross section (half natural size).

Techniques. For a general description, reference is made to the introductory article (Drake, 1963).

In all work presented here, only the nonloop technique was used. The peak heights on the strip chart were measured and, when necessary, corrected for various amplification factors. Data were then compared after suitable averaging.

MEASUREMENTS AND RESULTS

Series No. 1. This series was performed in Chicago in 1962.

In the comparison of the two types of meat, 12 subjects each chewed 4 samples of tough and 4 samples of tender meat, giving a total of 96 chewings. Every chewing consisted of 15 consecutive bitings. Of the 12 persons, 4 were unaware of the purpose of the experiment and were not experienced in meat rating, whereas the other 8 knew the purpose and were somewhat versed in meat rating.

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No substantial difference was found between results obtained in these two groups.

During chewing, the plastic tubing of the earphone was held inserted into the ear canal on the side where the sample was being chewed. The recording was made at a tape speed of 15 inches per second. All samples were about 6 × 12 × 25 mm.

The samples were served in one of the orders ABBABAAB, BAABABBA, ABAABABB, and BABBABAA, each order being served to three persons. In this way, the two types of meat were arranged to occur in the same number of cases for each serving-order position. The following written instructions were given to the subjects:

> Insert the short plastic tubing of the earphone into one ear canal, so that the orifice of the latter is completely closed. Hold the iron block firmly, avoiding scratching, etc.
>
> Chew each sample 15 times at a regular rate, while the sound is being recorded. Start when a signal is given. Try to avoid "teeth chattering."
>
> After chewing each sample, indicate your judgment of tenderness/toughness on the appropriate scale below.

Peaks representing sound amplitude were recorded at a play-back speed of 7½ inches per second for sound filtered through frequency-analyzer channels centered on 630 and 800 c/s. This corresponds to the two original frequencies, 1250 and 1600 c/s.

Average sound amplitude for each chewing was determined as the median of the heights on the strip chart of the peaks obtained for the first 12 bitings. The arithmetic means of such values obtained for 12 persons and 4 samples were: tough meat 155, and tender meat 100. The quotient between these two values, 1.55, corresponds to a decibel difference of 3.8.

Concurrently with the sound recording sessions, ordinary sensory evaluation of tenderness was performed at the Food Acceptance Branch of the Armed Forces Food & Container Institute. There, 32 subjects judged the tenderness of two samples of tough and two samples of tender meat at one session. The 9-point unstructured scale of Raffensperger *et al.* (1956) was used (from 1, extremely tough, to 9, extremely tender). This special investigation (Eindhoven and Peryam, 1962) gave tenderness ratings of 3.41 (tough) and 6.31 (tender). These values should be compared with the corresponding values 3.17 and 6.04, obtained simultaneously with the sound recording.

Two coefficients of correlation between tenderness and sound amplitude were calculated, one at 1250 c/s and one at 1600 c/s. The respective coefficients found were $r = -0.26$ and -0.28. In both cases, the level of significance was 0.01.

Tenderness data and sound amplitude data, together with r values and the results of analyses of variance (Tables 1, 2), suggested the following conclusions.

Table 1. Results of analyses of variance for tenderness and sound amplitude data obtained in mastication sound sessions.

Source of variation	*df*	Mean squares and *F* for:		
		Tenderness	Sound amplitudes at	
			1250 c/s	1600 c/s
Between treatments *T*	1	198.3750	217.2017	169.3359
Between judges *J*	11	1.9280	269.2770	139.0371
Interaction *T* × *J*	11	2.7159	19.9592	12.4950
Error (samples within *T* × *J*)	72	1.2968	12.0596	15.3488
Total	95			
F (for T)		49.41	6.78	6.08
Signif.		0.001	0.025	0.025

Table 2. Results of analysis of variance for tenderness data obtained in an ordinary sensory evaluation session.

Source of variation	*df*	Mean squares	*F*	Signif.
Between treatments *T*	1	135.1406	40.53	0.001
Between judges *J*	31	3.9753		
Error (*T* × *J*)	31	3.3342		
Total	63			

1) The judgment of toughness/tenderness in the somewhat unnatural situation of making simultaneous sound measurements gives the same difference between tough and tender meat as an ordinary sensory evaluation under appropriate conditions. The level of significance is also the same in both cases.

2) The measurement of sound amplitude gives a difference between two meat lots with the "official" tenderness ratings 3.4 and 6.3. This difference is significant at a level of 0.025.

3) There is a slight but significant negative correlation between tenderness rating and mastication sound amplitude.

4) The discriminating power of measurements of sound amplitude is not so high as that of estimation of tenderness.

Series No. 2. This series was performed in Sweden in 1963.

Six subjects chewed six foodstuffs at each of six sessions, giving a total of 216 chewings. The samples were chewed in randomized orders, different for the six persons and for the six sessions. During each chewing, more than 10 bitings were recorded. Both recording and playback were performed at a tape speed of 7½ inches per second.

After each sample was chewed with the earphone tubing inserted into one ear canal, the subjects were asked to rate the loudness of their own mastication sounds, using any scale they found useful (*cf.* Stevens, 1962).

For each chewing, the peak heights for the first 5 bitings were averaged by calculating the arithmetic mean, *M*. Further processing was started by calculating the logarithm, *L*, of the geometric mean of *M* values, taken over six sessions, for each one of the subjects. This gave 36 *L* values for 6 subjects × 6 foodstuffs. In order to correct for possible differences between individual subjective scales, these 36 values were then adjusted for each subject by subtracting the corresponding arithmetic mean of personal *L* values. Except for the initial averaging of 5 values, the same procedure was applied to the figures for loudness. Data obtained in this way are plotted in Fig. 2. This figure leaves the impression that the points for the five types of bread fall within 3 subgroups along a slightly arcuate line, whereas the points for raw carrot seem to form a separate group.

The correlation coefficient for all data shown in Fig. 2 was found to be +0.80, whereas the corresponding value for only the five types of bread was +0.935. Individual *r* values for the six subjects, determined for the above mentioned *L* values, ranged from +0.74 to +0.97.

The results obtained in series 2 suggested the following conclusion: there is, for one type of foodstuffs, a high degree of correlation between objectively determined sound amplitudes and the simultaneously perceived loudness.

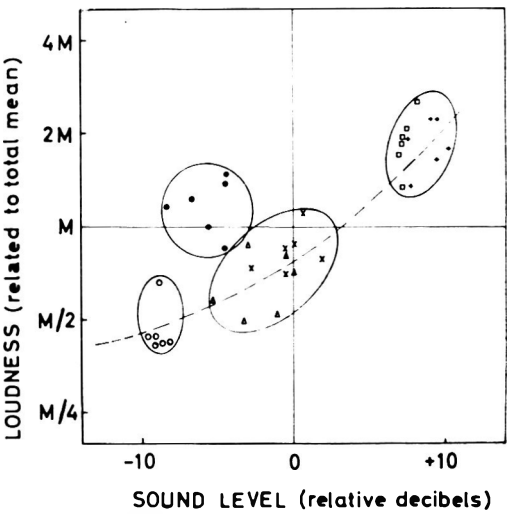

Fig. 2. Correlation between sound level and loudness of mastication sounds obtained with 6 foodstuffs.

○ = "småbröd" □ = crisp brown bread
△ = biscuits + = rusks
X = wafers • = raw carrot

DISCUSSION

Data presented in this article confirm previous findings (Drake, 1963) of a significant correlation between physically measured amplitudes of mastication sounds and the psycho-rheological property toughness/tenderness. Also, the positive correlation between mastication sound amplitudes and the subjectively perceived loudness of the same sounds confirms the idea that the physical method measures what is actually heard.

Taken together, the results indicate that an extended study of mastication sounds could be of value in food technology, especially after further improvements of the technique. Such work should be focused on qualitative differences, which could be expected to supplement the present results of a more quantitative nature. Such differences would probably be more closely related to frequencies and/or durations of the sounds than to their amplitudes.

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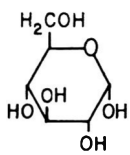
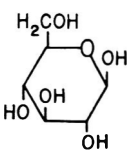
Configuration, Conformation, and Sweetness of Hexose Anomers

There are two general problem areas concerning the sweetness, configuration, and conformation of hexose anomers which are important in the field of carbohydrate chemistry. First, an uncertainty exists as to whether α- or β-D-glucose is sweeter. Second, the original work of Böeseken (1949) has been criticized. Kubler and Zepp (1963) and Sickels and Schultz (1964) have pointed out that the studies of Böeseken do not conclusively prove that α- and β-D-glucose have the presently accepted configurations as shown in the Haworth formulas:



α-D-GLUCOSE



β-D-GLUCOSE

Furthermore, Blom and Christiansen (1962) and Christiansen (1962, 1963) reported that mutarotation and other data can be calculated to fit a configuration for α- and β-D-glucose and mannose where the presently accepted assignment needs to be transposed.

As reported in his 1949 review article, Böeseken showed that compounds with *cis* vicinal OH groups complex with boric acid to cause an increase in the conductivity of the solutions. For mutarotating hexoses, the conductance of the solutions decreases if the anomer forming has *trans* OH groups on the anomeric and the adjacent carbon atom. On the other hand, the conductance will increase with time if the anomer forming has *cis* OH groups at these positions. Unfortunately, Böeseken was using the premise, accepted at the time (in 1911), that the monosaccharides possessed the furanose structure, where adjacent *cis* OH groups were *eclipsed*, and presumably quite able to complex with boric acid. Equally unfortunate was the inability to obtain satisfactory results with β-D-glucose to substantiate the theory, although α-D-glucose behaved ideally. Kubler and Zepp (1963) have again recently directed attention to this dormant inconsistency, although MacPherson and Percival (1937) were able to get β-D-glucose to behave as needed.

When it developed that monosaccharides have a six-membered pyranose ring where adjacent *cis* or *trans* OH groups are *gauche* and at equal distance from each other in the preferred chair conformations (Reeves, 1951), Böeseken's conclusions seemed completely untenable.

The observations of Tsuzuki and Mori (1954) have particular interest to food chemists. Those researchers pointed out that the sweeter sugar anomers have the *cis* configuration between the OH groups on the anomeric and the adjacent carbon atoms, although β-D-mannose and β-D-lactose are obvious exceptions. On the basis of this generalization, however, it might be expected that α-D-glucose would be sweeter than the β-anomer. Studies of sugars in solution (Pangborn and Gee, 1961; Schutz and Pilgrim, 1957; Cameron, 1947) show that freshly prepared solutions of α-D-glucose are sweeter than mutarotated solutions. Moreover, mutarotated glucose solutions are sweeter than freshly prepared β-D-glucose solutions (Pangborn and Gee, 1961). However, Shallenberger (1963) reported that crystalline β-D-glucose appears to taste sweeter than crystalline α-D-glucose. Thus, in spite of strong evidence to the contrary, sweetness data on crystalline compounds indicate, by analogy with the Tsuzuki generalization, that the configuration at the anomeric center of the glucoses may indeed be the reverse of that presently accepted.

In an attempt to obtain additional information on these problems, we have secured or prepared the anomers of the common hexoses in question. The studies of Böeseken were repeated with conventional laboratory

conductance equipment. Taste tests were conducted on the crystalline sugars. The basis of this note is results indicating the sweetest sugar anomers, probable reasons for varying literature reports on sugar sweetness, and an interpretation of results obtained in terms of the configuration and conformation of the sugars.

Table 1. Average sweetness score and physical constants for common hexose anomers.

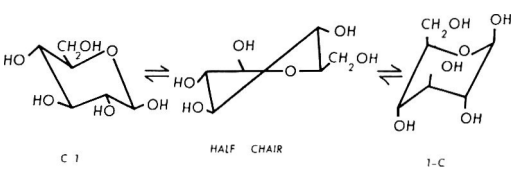
Sugar	Melting point (°C)	$[\alpha]_D^{23}$ Initial [a]	$[\alpha]_D^{23}$ Final	Sweetness score
α-D-Glucose	150–153	+ 112°	+ 52°	3.8
β-D-Glucose	146–152	+ 20°	+ 52°	4.9
α-D-Galactose	165–167	+ 138°	+ 76°	6.2*
β-D-Galactose	138–140	+ 52°	+ 77°	4.1
α-D-Mannose	121–122	+ 29°	+ 17°	5.5**
β-D-Mannose	132–133	— 16°	+ 15°	1.2[b]

[a] by extrapolation.
[b] Bitter.
* and ** denote significance at the 5% and 1% probability levels for anomeric pairs of sugars.

Table 1 shows average sweetness scores of crystalline sugar anomers along with sugar physical constants. The latter agree well with available literature values. The sweetness scores were obtained with the paired-comparison technique.

Twelve experienced tasters placed a few milligrams of sugar on the tongue, and scored the sweetness on a ten-point scale. Higher values generally indicate greater sweetness, but only anomeric pairs may be compared. The greater sweetness of α-D-galactose and mannose anomers is statistically significant as indicated by the *t*-test. β-D-Mannose was found to be bitter, as noted by others (Steinhardt *et al.*, 1964). The slightly higher, but statistically nonsignificant, score for β-D-glucose presents an interesting aspect concerning the sweetness of glucose anomers. Immediate judgments yielded rather higher scores for β-D-glucose. Delayed decisions gave high scores for α-D-glucose. No attempt was made to educate panel members on this facet of glucose behavior, and results are reported as the mean of all scores.

Both glucose anomers exist in the C1 conformation in the crystalline lattice structure. When β-D-glucose is dissolved in water, a conformational change apparently alters its sweetness. (To crystallize β-D-glucose effectively a temperature of 100°C needs to be employed. Upon dissolution, therefore, the occurrence of a conformational change does not appear too surprising.) Lentz and Heeschen (1961) found that although α-D-glucose still possessed the C1 conformation in water solution, the β-B-anomer appeared to have a half-chair conformation. Presumably, an unknown amount of the 1C conformation is also present:



Since conformational change vastly alters the arrangement of the OH groups on the pyranose ring, the taste properties of the sugar would also be altered since the disposition of OH groups affects sugar sweetness (Shallenberger, 1963). On the other hand, when crystalline compounds are tasted, sweet taste appears to be perceived prior to any appreciable conformational change.

Sugar anomers with OH groups positioned to complex most strongly with boric acid would also be capable of forming stronger intramolecular hydrogen bonds between these groups, and hence be less sweet. We have been able to confirm the conductivity studies of MacPherson and Percival (1937) to the effect that β-D-glucose, when dissolved in boric acid solution, does not initially increase the conductivity of the solution if corrections are made for the sugar and the boric acid alone. As mutarotation proceeds, however, conductivity increases. The changing conductance not only parallels the formation of the α-anomer (changing optical rotation) but correlates quite well. α-D-Glucose increases the conductance of boric acid solutions immediately upon dissolution, but with the formation of the β-anomer, conductance gradually decreases. The correlation with changing optical rotation is again good. These data are shown in Fig. 1.

The conductance behavior of galactose

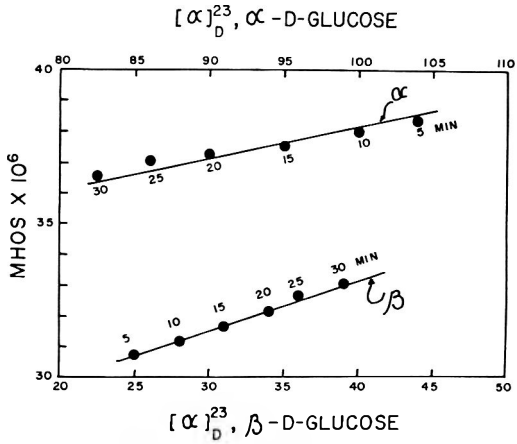
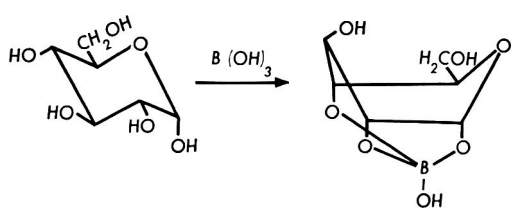


Fig. 1. Correlation between changing conductivity and optical rotation, with time, for 0.25 molar solutions of glucose anomers in 0.25 molar boric acid solution. Temperature of conductance studies, 23°C.

and mannose anomers in boric acid solution was also studied. The β-anomers reacted most strongly, but the change in galactose conductance during mutarotation is not easily related to α-β pyranose anomer interconversion. This is not surprising, since, at equilibrium, more than two glactose isomers are known to be present in significant amounts. Results with mannose were of the order of those shown for glucose, but the ability of these latter anomers to complex boric acid is much greater than for glucose.

Since neither *cis* nor *trans* vicinal sugar OH groups alone in the C1 conformation appear to be able to form coordination compounds with boric acid, as measured by conductance methods, we have deduced, by reference to Barton conformational analysis models (Barton, 1956) and the studies of Angyal and McHugh (1957), that the sugar conformation actually forming the complex with boric acid may be a boat form. The complex needed would be of tridentate structure. For α-D-glucose, this would be the B3 conformation of Reeves (1951):



The appropriate conformation for β-D-galactose and mannose may be the 2B and B3 conformations, respectively. The tridentate complex does not appear possible in other conformations, nor is it possible in the other anomers in any of the chair or boat forms. In essence, then, the sweeter sugar anomers in crystalline form, with fixed conformations, are those that react less strongly with boric acid. Those which react more strongly with boric acid are less sweet. The OH groups involved in this latter case are possibly better disposed to be hydrogen-bonded. If deductions made from conductivity and conformational studies are correct, there is no need to consider altering the presently accepted configurational assignment for α-D- and β-D-glucose and mannose.

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