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MICROBIOLOGY

NUTRITION

SENSORY EVALUATION AND CONSUMER STUDIES

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The Ascorbic Acid Content of Edible Liver

SUMMARY

The ascorbic acid content of a total of 120 specimens of each species of edible liver was estimated by the N-bromosuccinimide method. Statistical analysis of the results is given. Differences were found occasionally between males and females in liver ascorbic acid content, but were not statistically significant. The effect of cooking on liver ascorbic acid content is shown.

It is now settled that, among the organs of horned cattle, liver has the highest content of ascorbic acid (Kégl, 1944; Watts, 1950). Liver ascorbic acid is higher in adult than in young animals (Bonati *et al.*, 1956). Relatively few estimations of edible liver have been reported from abroad. The results show a remarkable variation in the liver values (Phillips and Stare, 1934; Watts, 1950).

There appears to be no report on the ascorbic acid content of edible liver in Egypt. Hence it seemed desirable to evaluate edible liver from animals bred locally. This paper deals with the estimation of liver ascorbic acid from various species of animals including cattle, buffalo, sheep, goat, camel, and pig. Also studied was the effect of cooking on liver ascorbic acid content.

EXPERIMENTAL

Collection of samples. The animals were slaughtered on the morning after they arrived at the abattoir; they had no access to food or water for at least 18 hr before death. Liver specimens were freshly collected under veterinary supervision from the abattoir as soon as the animals were slaughtered and decided to be suitable for human consumption. In addition, records were made of the sex of the animal. Immediately after slaughter, the liver samples were taken to the laboratory and the ascorbic acid content was estimated by the N-bromosuccinimide method (Barakat *et al.*, 1955).

Method. The liver sample was minced. Then 5 g of the homogenized specimen were accurately weighed and stirred well with a glass rod in a graduated centrifuge tube for 5 min with 10 ml of 20% trichloroacetic acid. The mixture was diluted with the trichloroacetic acid up to 20 ml and centrifuged for 10 min.

To 10 ml of the clear supernatant solution (equivalent to 2.5 g of the liver tissue) in an Erlenmeyer flask of 50-ml capacity were added 5 ml of 4% potassium iodide solution and 10 drops of 1% starch solution. Then 0.1% N-bromosuccinimide solution was run in, drop by drop, from a 5-ml microburette graduated in hundredths of a milliliter with continuous shaking until a violet color just appeared (end-point). The volume of 0.1% N-bromosuccinimide solution added was recorded.

Calculation. Ascorbic acid content $= V \times I \times 176/178 \times 100/2.5$ mg per 100 g of liver tissue, where V is the volume of 0.1% N-bromosuccinimide solution added.

Application of the method. The ascorbic acid content in a total of 120 samples each of cattle, buffalo, sheep, goat, camel, and pig liver was determined during October to March. The moisture content of the various species of raw liver varied from 74.2 to 70.4 with an average of 72.8%. Moreover, the fat content ranged from 4.8 to 3.2, with a mean value of 3.8%.

In addition, the opportunity was taken to ascertain whether cooking influenced the ascorbic acid content of raw liver. Thus, ascorbic acid was estimated on 20 samples of raw edible liver and simultaneously on each liver sample after being roasted or fried. In case of roasting, the time of cooking lasted for 5 min and the internal temperature of the roasted liver was 70°C. But in the frying process, the liver was added after the butter fat had melted and continued for 2 min; the internal temperature of the liver tissue was 74°C.

RESULTS

The results were analyzed statistically. The whole series of estimations is summarized in Table 1, which shows the mean values, their standard errors, and the maximum and minimum values.

Fig. 1 shows the range of ascorbic acid content of edible liver obtained from various species of locally bred animals.

Table 2 shows statistical analysis of the ascorbic acid content of 60 samples of each male and female liver of the various species.

Table 3 gives the statistically analyzed results of ascorbic acid content of 20 samples of the same raw, roasted, and fried edible liver.

DISCUSSION

Evered (1960) recently reported that the N-bromosuccinimide method is not affected

Table 1. Ascorbic acid content of raw edible liver (mg%).

Liver	No. of samples	Max.	Min.	Mean	S.E.
Cattle	120	94.10	40.71	71.22	±1.07
Buffalo	120	92.12	50.60	72.37	±0.98
Sheep	120	129.70	48.62	77.58	±1.25
Goat	120	90.15	54.55	76.66	±0.76
Camel	120	74.33	38.73	58.06	±0.69
Pig	120	133.65	34.78	69.39	±1.18

Table 2. Ascorbic acid content of male and female liver (mg%)

Liver species	No. of samples	Sex	Max.	Min.	Mean	S.E.
Cattle	60	Male	94.10	50.60	71.14	±1.67
	60	Female	94.10	40.71	71.30	±1.35
Buffalo	60	Male	92.12	50.60	74.03	±1.59
	60	Female	84.21	50.60	70.71	±1.15
Sheep	60	Male	129.70	54.55	80.09	±1.87
	60	Female	103.99	48.62	75.08	±1.60
Goat	60	Male	88.17	60.48	75.54	±1.12
	60	Female	90.15	54.55	77.78	±0.99
Camel	60	Male	72.35	40.71	60.61	±1.03
	60	Female	74.33	38.73	55.52	±0.78
Pig	60	Male	90.15	48.62	67.80	±1.27
	60	Female	133.65	34.78	70.98	±1.97

Table 3. Ascorbic acid content of raw and cooked liver (mg%) (20 samples of each).

	Raw liver (72.8% moisture)	Roasted liver (57.2% moisture)		Fried liver (59.8% moisture)	
Ascorbic acid	Content	Found	Loss	Found	Loss
Max.	79.10	61.30	23.19	47.46	44.20
Min.	41.53	33.62	14.86	21.75	38.78
Mean	68.88	51.51	19.18	34.80	40.26
S.E.	±1.24	±1.52	±0.45	±1.20	±0.53

by reductones, reductic acid, or ferric salts.

It is obvious from Table 1 that the mean amount of vitamin C (or reducing substance) present in cattle, buffalo, sheep, and goat liver is respectively 71.2, 72.4, 77.6, and 76.7 mg per 100 g. There appears to be no wide variation in the average liver ascorbic acid content of these species of animals. This is not unexpected, since all of these animals are ruminants. Although the majority of the estimations were close to the mean value, there were occasional large variations and the table therefore includes the maximum and minimum values. At any rate, individual differences may account for much of this variation, for instance, the capacity of the ruminant to synthesize vitamin C (Wallis, 1940, 1942).

Although the camel is a ruminant, the liver results (a mean value of 58.06 mg per 100 g of liver tissue) are lower than those recorded for other ruminants, though the range is considerable. Thus camel liver shows a maximum of 74.33 and a minimum of 38.73 mg per 100 g of liver. It is possible that ration, breed, age, or climate, especially extremes in temperature, may account for at least part of this variation.

On the other hand, the pig shows a wide variation in the liver ascorbic acid content, with a maximum of 133.65, a minimum of 34.78, and a mean value of 69.39 mg per 100 g of liver tissue. This evidence is analogous to the wide variation in the blood glucose content of the pig. Thus a parallelism appears to exist between the liver ascorbic acid content and the blood glucose content of the pig.

From Table 2 it is evident that there are differences between males and females

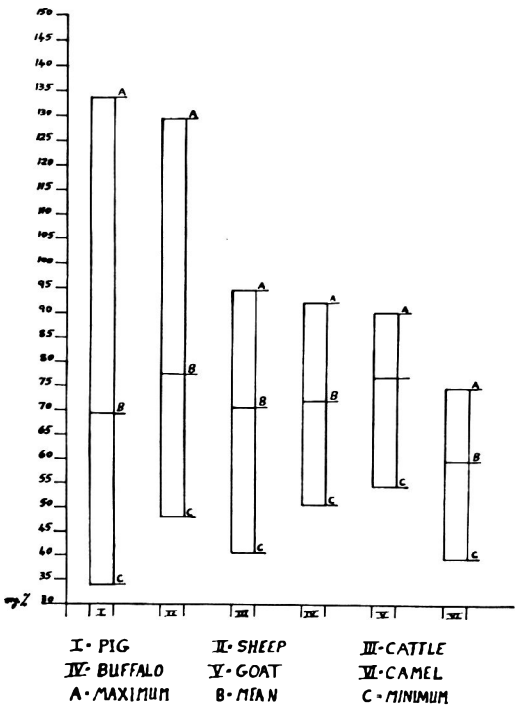

Fig. 1. Range of ascorbic acid content of edible livers.

in liver ascorbic acid content, though the results are not statistically significant. This differs notably from the liver of the rats, where the vitamin C content is about 50% greater in the males than in the females (Giovanni *et al.*, 1957–58). It is interesting to note that the rat liver, with a mean value of 80.7±4.1 mg% (Merezhinskii, 1959), is somewhat higher than the ruminant liver in ascorbic acid content.

On the whole, our results are higher than those previously reported abroad, e.g., the ascorbic acid content in the liver of cattle is recorded as 15 mg%, and calf and pig liver is 20 mg% (Lerche *et al.*, 1958). However, this difference would be expected because of variations in breed, age, ration, environment, and season. Furthermore, Radeff (1941) was working under the same general conditions in southeast Europe as Sinko (1936), whose values for the liver (mean, 49.3 mg per 100 g) are approximately twice those of other workers (cited after Watts, 1950).

The maximum, minimum, and mean loss of liver ascorbic acid on cooking is given in Table 3. The change in water content during cooking has been taken into account in determining loss of ascorbic acid. Edible liver is usually ingested either as roasted or fried liver. It is notable that the mean loss of liver ascorbic acid in roasting and frying is respectively 19.18 and 40.26 mg per 100 g of liver tissue. Thus, as regards ascorbic acid content of cooked liver, roasted liver surpasses fried liver.

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Water Activity of Flour at High Moisture Contents as Measured with a Pressure Membrane Cell

SUMMARY

A method was developed for the determination of water activities of flour at high moisture contents. In this method a flour sample was allowed to equilibrate with water contained in a porous membrane while under a certain constant mechanical pressure. The cell was fitted with a porous membrane which allowed the transfer of moisture from and to the sample. After equilibrium was achieved the sample was analyzed for moisture content. A thermodynamic relationship enabled calculation of the water activity corresponding to the pressure used in the cell. Results obtained with this method were in agreement with those obtained with the constant-relative-humidity desiccator technique in the over-lapping range of water activities. The two methods were also in agreement that the water activity of flour was not affected by the particle size distribution.

INTRODUCTION

Water activity is defined as the ratio between the vapor pressure above the material and the vapor pressure of water at the same temperature. Measurement of water activity by the pressure membrane cell is possible by utilizing the thermodynamic relationship between the free-energy change and the water activity, as follows: $\Delta F = -RT \ln a_w$ [1]

Where

ΔF = the free energy change of adsorbed water
T = the absolute temperature
R = the gas content
a_w = the water activity.

However, at constant temperature:

$$\left[\frac{dF}{dP}\right]_T = \overline{V} \qquad [2]$$

Where

P = pressure
$\overline{V}$ = molar volume of water.

If the molar volume is assumed to be independent of the pressure, Eq. 2 can be integrated and solved for ΔF, and the latter substituted in Eq. 1 to obtain the relation between pressure and water activity:

$$\Delta P = \frac{-RT}{\overline{V}} \ln a_w \qquad [3]$$

Thus, the molar volume of adsorbed water must be known in order to calculate equilibrium water activity by means of Eq. 3. Molar volume is equal to the molecular weight of water divided by the density of adsorbed water.

It was shown (Gur-Arieh, 1963; Gur Arieh *et al.*, 1964a) that the density of adsorbed water in flour-water systems was a function of moisture content in the flour. At the moisture level of interest in this paper, the average density of adsorbed water was shown to be above unity. An expression for this density as a function of moisture content can be derived by assuming that the volumes of flour and adsorbed water are additive.

$$W_t/d_t = W_f/d_f + W_w/d_w \qquad [4]$$

where d is density, W is weight, subscript t refers to the moist flour, subscript f refers to dry flour, and subscript w refers to the adsorbed water.

If we divide Eq. 4 through by W_t and let X be w_w/W_t, then $W_f/W_t = 1 - X$ and Eq. 4 becomes:

$$\frac{1}{d_t} = \frac{1 - X}{d_f} + \frac{X}{d_w} \qquad [5]$$

Gur-Arieh *et al.* (1964a) showed that d_f is 1.482 g/cc and that d_t, although it varies with moisture content of the flour, may be approximated by the expression $1/d_t = 0.40\ X + 0.631$ at moisture contents above 25%. Substituting these values into Eq. 5 and rearranging gives:

$$d_w = \frac{X}{1.075\ X - 0.044} \qquad [6]$$

If the moisture content is expressed on a dry basis so that $X_d = X_w/(1 - X_w)$, Eq. 6 becomes:

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$$d_w = \frac{X_d}{1.031\ X_d - 0.044} \qquad [7]$$

Eq. 7 was used with Eq. 3 to calculate water activity as shown by the following sample calculation:

In desorption studies a parent flour sample showed an equilibrium moisture fraction of 0.1952 (dry basis) under a pressure of 6550 psig and a temperature of 21°C:

$$d_w = \frac{0.1952}{1.031 \times 0.1952 - 0.044} = 1.241 \text{ g/cc}$$

$$-ln\ a_w = 2.3 \log \frac{1}{a_w} = \frac{\frac{6550 \text{ psi}}{14.7 \text{ psi /atm}} \times \frac{18 \text{ g/g mol}}{1.241 \text{ g/cc}}}{82.08 \text{ cc atm/g mol °K} \times 294 \text{ °K}}$$

$$a_w = 0.766$$

MATERIALS AND METHODS

The pressure membrane cell. The cell, a modification of the one proposed by Olsen (1962), consisted of three essential parts: the cylinder, the piston, and the base. Fig. 1 is a diagram of the cell.

The cylinder and piston were made of stainless steel, and the base of brass. The piston was fitted with an O ring to prevent leakage between the piston and the cylinder. The cylinder fitted into the base. The base had a concentric hollow section just below the piston to take a porous brass membrane. Underneath the membrane the base was grooved 3/16 in. wide by 3/16 in. deep, in a spiral pattern. A 3/16-in. hole was drilled through the base to each end of the spiral. The hole at the center was connected to a water reservoir fitted with a stopcock while the outer hole led to a microburette fitted with a 3-way stopcock. Copper tubing extending from these two holes was filled with water which flowed into the spiral groove, thus keeping the porous plate saturated with water. The cylinder was attached to the base by a brass cap bolted to the base by six metal screws (not shown).

Operation of pressure membrane cell. The cell was first disassembled. A cellulose acetate film 4 in. in diameter was spread over the cutout section of the base, on top of the porous brass membrane. In sorption studies, a sample of 3 to 4 g of flour was dispersed on the cellulose acetate film in the area of the porous brass membrane. The flour sample was then sandwiched with another cellulose acetate film. An asbestos gasket was placed on top of the film, and the cylinder was placed in the cut-out section of the base. Then the cap was placed on top of the cylinder and bolted to the base. Finally the piston was inserted into the cylinder and the entire assembly was placed in the Carver press. The copper tubes extending from the base of the cell were connected to a water reservoir and to the side arm of a 3-way microburette as indicated in Fig. 1. The reservoir was then filled with water while arranging both stopcocks so that the water flowed down through the cell and up into the burette. The burette stopcock was closed and a drop of Apiezon oil added to the burette to prevent evaporation. The desired pressure was then applied by forcing the piston down with the press. This forced the air that was above the sample down through the membrane and out through the reservoir. After air removal was completed by applying suction to the top of the reservoir, the reservoir stopcock was closed, and the burette stopcock opened to the cell.

In the initial few hours the pressure in the cell usually dropped. However, after adjusting the pressure at intervals of 2 hr for 6 hr, the pressure remained constant thereafter and required very little if any adjustment until equilibrium was achieved. Then the cell was disassembled, the sample was removed from between the films, and its moisture content was determined (American Association of Cereal Chemists, 1957).

The sample was equilibrated when the level of the water in the microburette remained constant. However, since the microburette was graduated in 0.01-ml graduations and since there existed the possibility that some air remained in the membrane, this was considered only a rough estimation. The confirmation of equilibrium was done in preliminary studies by allowing replicate flour samples to remain in the pressure membrane cell under a certain constant pressure for increasing periods, after which the moisture content of each sample was determined. For example, in sorption studies at 10,000 psi pressure and 2% initial moisture it was found that 36 hr were needed for equilibration. When lower pressures were used, the time required for equilibration increased.

In desorption studies, the only difference in procedure was in the preparation of the flour sample. A dough was formed by mixing 150 g of flour with 100 ml deionized water in a small Hobart mixer for 1 min at low speed and for an additional 2 min at medium speed. The dough was then

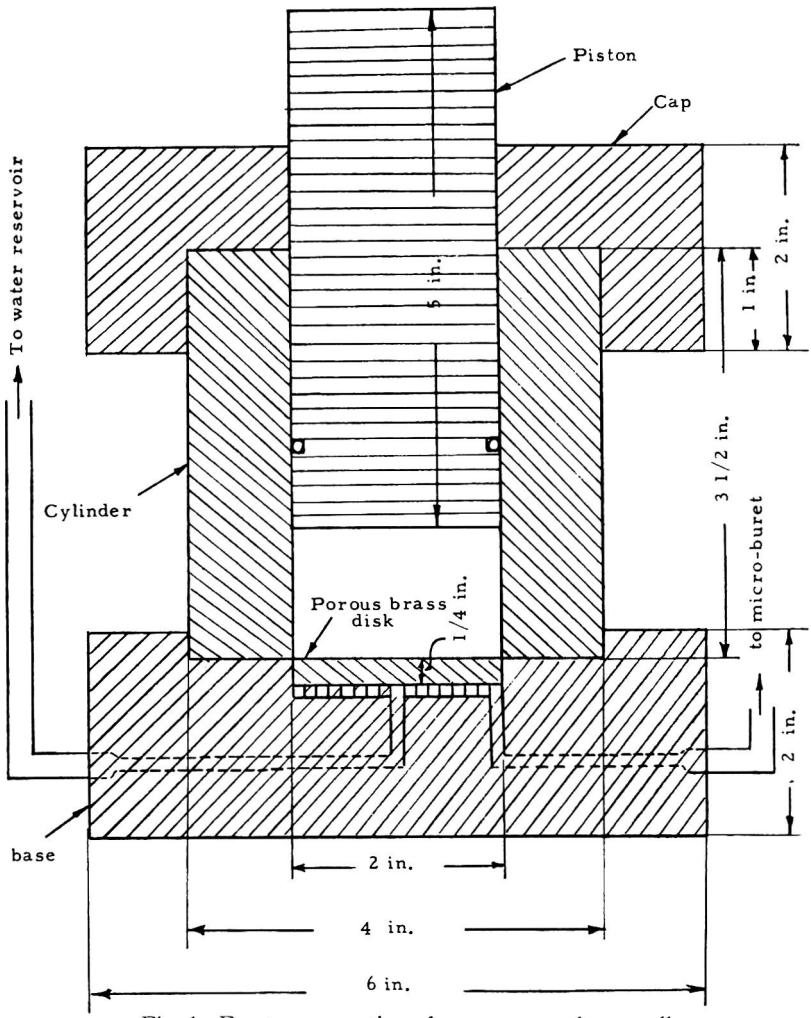

Fig. 1. Front cross-section of pressure membrane cell.

rolled in a sheet approximately ⅛-in. thick, and a 2-in.-diameter circle was cut out to serve as the sample. In desorption studies the time required for equilibration at pressures under 5000 psi was about 48 hr.

After equilibrium was achieved, the pressure membrane was immediately disassembled, the sample was removed from between the cellulose acetate films, and its moisture content was determined.

RESULTS AND DISCUSSION

The pressure membrane cell was used for the determination of adsorption isotherms at high moisture contents of six flour samples having different particle-size distributions. This apparatus enabled determination of the water activity in the range from 0.67 to 0.96. The pressures required ranged from approximately 10,000 psi to 800 psi. At pressures lower than 800 psi the pressure did not remain stable, so this became the lower pressure and upper water activity limit. If, in future work, water activities higher than 0.96 are desired, the source of pressure in the cell should be changed. Instead of applying force on the piston with the press, an external compressed air supply should be used for better control. In this case, special care must be taken to saturate the membrane with water prior to pressurizing the cell in order to prevent passage of gas through the membrane and consequent loss of pressure.

Fig. 2 presents the sorption-desorption isotherms obtained at 70°F. For comparison, the lower part of this graph also contains points determined with the constant relative humidity air circulation technique (Gur-Arieh *et al.*, 1964b). Fig. 2 shows that there is very good agreement between the results obtained by both methods in the overlapping range of 0.68 to 0.81 water activity. Furthermore, the data obtained with the pressure membrane cell show a smooth continuation at

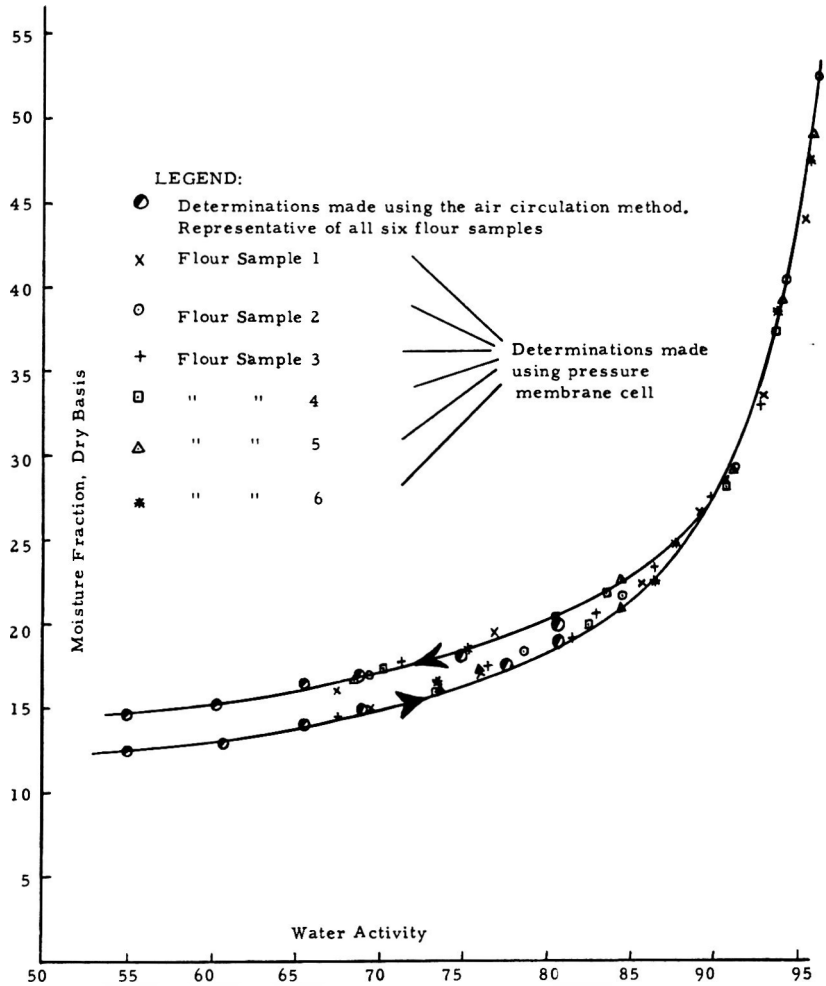

Fig. 2. Sorption-desorption isotherms at high water activities for six flour samples of different particle-size distributions at 70°F.

moisture contents above this overlapping range. It is interesting to note the coincidence of sorption and desorption isotherms at water activities above 0.9.

Fig. 2 shows that the water activity of flour is not affected by its particle-size distribution even at high water activities, just as shown at low water activities (Gur-Arieh *et al.*, 1964b).

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Volatile Components of Tomatoes

SUMMARY

Methods were developed for the preparation in reproducible yield (2–5 ppm) of odor concentrate from fresh ripe tomatoes. Sufficient concentrate was obtained to allow its separation by gas chromatography and collection of its components for direct investigation. Examination of the fractions resulted in rigorous identification of previously unknown tomato constituents.

Basic research on the nature of compounds responsible for flavor in natural foodstuffs is necessary to provide a basis for eventual understanding of the chemistry of flavor, and of flavor changes which occur during the ripening, storage, or processing of a food product. To this end, isolation, separation, and identification of the volatile compounds which contribute to the odor of fresh ripe tomatoes were undertaken.

Knowledge of the volatile constituents of ripe tomatoes is limited. Spencer and Stanley (1954) reported acetaldehyde, *i*-valeraldehyde, numerous other carbonyl compounds, alcohols, and unsaturated substances in tomatoes, and indicated the probable presence of citral and terpene-like compounds. The change in ethanol and acetaldehyde content during ripening was reported by Rakitin (1945). Chromatograms of a tomato concentrate showing numerous components have been published by Bidmead and Welti (1960). Matthews (1961) tentatively identified furfural, acetaldehyde, and acetone in ripe tomatoes. Hein and Fuller (1963) reported the presence of diacetyl, α-pinene, citronellal, limonene, and citral. A comprehensive review of the literature on the chemistry of the tomato and of tomato products is available (Beltran and Macklin, 1962).

EXPERIMENTAL PROCEDURES

Fresh vine-ripened tomatoes of high quality and known variety (No. 146) were obtained through the courtesy of Campbell Soup Co. in approximately 800-lb quantities during the tomato seasons of 1960 and 1961. The fruit was washed and sorted according to degree of ripeness. Fully ripe fruit was processed by removal of volatile components about 24 hr after picking. Less-ripe fruit was stored at 36–40°F. In no case did storage exceed 4 days, and only fruit of highest quality was investigated.

Preparation and investigation of tomato odor distillates. Fig. 1 shows the apparatus (a modified Turba-Film Processor, Rodney Hunt Machine Co., Orange, Mass.) used for continuous preparation of odor distillates from 35- to 130-lb batches of tomatoes. Known quantities of tomatoes were added, immediately after being cored and quartered, to a stainless-steel pulper (1) at a rate just sufficient to maintain an adequate supply in a 4-L glass reservoir (2). The resulting slurry was drawn through glass tubing, a flowmeter (3), and a flow-controlling stopcock (4) to the stainless-steel processor, where it was immediately distilled in a modified falling-film evaporator (7) at 20–30 mm pressure Hg and an average temperature of 28°C. A thin turbulent film of pulped tomato was maintained in contact with the heated wall of the evaporator by a high-speed motor-driven rotor. A separator section above the evaporator acted as a mechanical foam breaker and returned liquid entrained in the rising vapor to the evaporator. Tomatoes from which the volatile components had been removed were collected in a 20-L carboy cooled by ice water. The distillate was condensed in a vertical shell-and-tube-type heat exchanger (8) cooled by circulating (19) ice water, and collected in a series of receivers maintained at 0°C (12), −78°C (13, 14), and −196°C (15). Re-

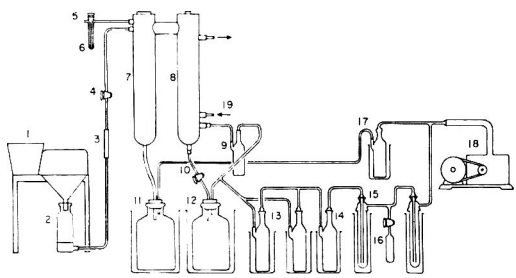

Fig. 1. Apparatus for preparation of odor distillates.

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duced pressure (20–30 mm Hg) was maintained in the system with a two-stage mechanical vacuum pump (18). Average vapor temperature at the top of the evaporator was 28°C. Average temperatures at the evaporator and condenser outlets were respectively about 30° and 28°C. All tubing was 12- or 25-mm ID Pyrex connected by minimum lengths of Tygon vacuum tubing to allow necessary movement of the apparatus. Polyethylene stoppers were fabricated for the large receivers. Connections on all other receivers were ground glass. A gas-sampling bottle (16) allowed collection of noncondensable gases for subsequent study. Throughput of the distillation system was approximately 50 lb per hr. Ultimate vacuum obtainable in the empty system was 0.2 mm of Hg measured with a McLeod gage. The major portion of odor distillate was collected in the 20-L carboy (0°C). The rest was distributed among the −78°C traps. The volumes of distillates collected during distillation of some typical batches of ripe tomatoes are given in Table 1. Also given is the quantity of oxidizable carbon present in these distillates as determined by the method of Gertner and Ivecovic (1954).

The distillates were saturated with sodium chloride and extracted with diethyl ether, and the ether extracts were dried over anhydrous sodium sulfate. The dry extracts were concentrated by careful distillation to the minimum practical volume (about 250 μl). Table 2 gives the amounts of odor concentrates thus obtained and estimated gas chromatographically on an ether-free basis.

Table 2. Yield of tomato odor concentrates.

Batch	Weight of concentrate (ether-free basis, mg)	Yield from tomato fruit (ppm)
1	92.0[a]	1.8[a]
2	222.2	5.2
3	171.0	4.4

[a] From 0°C condensate only.

The odor concentrates were stored at 0°C in the dark and in ether solution for periods up to a year without deterioration [as judged by gas chromatographic analysis under standard conditions (see below)].

Sensory evaluation of distillates and odor concentrates. Immediately after collection of the tomato distillates from batch 3, the odor of the combined condensates was compared in a triangle test with the odor of freshly pulped tomato. Each panel member was given 2 samples of fresh tomato and one sample of distillate in 60-ml glass-stoppered bottles covered with aluminum foil so the contents were not visible. The samples were at room temperature and were identified by two-digit numbers selected at random. Panelists were asked to indicate which samples were identical, which sample differed, and which had the most desirable tomato odor. Of the 18 persons who participated, 15 differentiated the samples correctly. An odor difference significant at the 0.1% level (Boggs and Hanson, 1949) was noted. Only 8 of the 15

Table 1. Oxidizable carbon content and volume of tomato odor distillates.

Batch	Fruit processed (lb)	Nonvolatile material (lb)	Distillate		
			Collected at (°C)	Volume (L)	Mg carbon per lb tomatoes
1	89	46	0°	13.2	23.2
			−78°	2.0	22.7
			Total	15.2	Total 45.9
2	94	44.4	0°	15.0	21.7
			−78°	0.6	5.4
			Total	15.6	Total 27.1
3	85	44.2	0°	14.4	22.2
			−78°	0.6	5.7
			Total	15.0	Total 27.9
4	87.8	41.0	0°	16.4	21.7
			−78°	0.7	.....[a]
			Total	17.1	Total 21.7
Water	66	31.2	0°	15.1	1.9
Blank			−78°	0.4	.03
			Total	15.5	1.93

[a] Value not determined.

judges who had matched the samples correctly preferred the odor of the fresh tomato to that of the combined distillate. This difference in preference was insignificant.

Informal odor evaluation of tomato odor concentrates isolated by ether extraction of the distillates confirmed that they exhibited characteristic fresh tomato odor. The odor concentrate obtained by ether extraction of the distillate from the water blank was odorless.

Gas chromatographic separation of odor concentrates and examination of resulting fractions. Headspace analyses of crushed fresh tomato, of pulped tomatoes, of the nonvolatile tomato residue, and of the contents of the various cold traps were determined with a home-made instrument fitted with an argon-diode ionization detector and associated amplifier-power supply (Jarrell-Ash Co., Newtonville, Mass.).

Analytical chromatograms of odor concentrates were obtained on a Research Specialties hydrogen flame ionization chromatograph, Model 60-1. Preparative chromatograms from which individual fractions were trapped for further investigation were carried out on an apparatus 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 155°C.

Analytical separations of from 100 μl to 1.5 ml of headspace vapor or of about 0.2 μl liquid samples were obtained on 6-ft × ⅛-in. columns containing 1% Ucon 50HB-2000 on 100/120–mesh Gas Chrom A at 34 or 50°C. Preparative separations of odor concentrates were carried out on the following columns:

1) 20% Ucon 50HB-5100 on 48/60-mesh firebrick (4 mm ID × 2 m) at 90°C. Flow rate, 50 ml/min helium. Back pressure 8.5 psig. Sample sizes, 3 μl to 30 μl.

2) 20% DEGS on 60/65-mesh acid-washed Chromosorb P (8 mm ID × 2 m) at 70°C, 148°C. Flow rate, 150 ml/min helium. Back pressure, 9.6 psig. Sample sizes, 6 μl to 55 μl.

3) 20% Quadrol on 60/65-mesh Chromosorb P (4 mm ID × 2 m) at 102°C. Flow rate, 75 ml/min helium. Back pressure, 12 psig. Sample size, 3 to 35 μl.

4) 20% Carbowax 4000 on 60/65-mesh Chromosorb P (4 mm ID × 2 m) at 126°C. Flow rate, 34 ml/min helium. Back pressure, 11 psig. Sample sizes, 6 μl to 35 μl.

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 0.05 mm nominal path length. 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 1% Ucon 50HB-2000 or 5% β,β'-oxydipropylnitrile (OPN) on 100/120-mesh Gas Chrom A. Both columns were 6-ft × ⅛-in. stainless steel.

RESULTS AND DISCUSSION

About 1200 lb of fresh vine-ripened tomatoes were processed by rapid continuous distillation under reduced pressure (20–30 mm) in the apparatus shown in Fig. 1. Volatile tomato components were condensed in traps maintained at 0, −78, and −196°C within 5 min after the fruit had been cored and quartered. Residence time within the evaporator itself was less than 1 min. Thus, changes in the composition of the volatile fraction due to possible enzymatic action were minimized. The volumes of aqueous condensates collected during the distillation of typical batches of fruit varied from 13.2 to 16.4 L in the 0°C carboy and from 0.4 to 2 L in the −78°C traps (Table 1). The −196°C traps contained only noncondensable vapors. The quantity of oxidizable carbon present in the combined condensate from each preparation was normally (Table 1) about 27 mg of carbon per lb of tomatoes processed. In one case it was as great as 45.9 mg of carbon per lb of fruit. Although this increased quantity of organic material may have resulted from inadvertent contamination by the distillation apparatus, it is not believed likely, since a condensate obtained by processing a water blank (66 lb) contained (Table 1) only 1.93 mg of carbon per lb of water distilled.

Sensory comparison in a triangle test of the odor of the combined aqueous conden-

sates with the odor of freshly pulped tomatoes showed that a difference existed which was significant at the 0.1% level (Boggs and Hansen, 1949). However, no significant preference for the odor of either sample was expressed by persons who had differentiated the samples correctly. It was thus concluded that the condensates contained tomato odor of good quality. The condensate obtained from distillation of the water blank had essentially no odor.

It is not surprising that the judges were able to differentiate the samples correctly, since any method of isolation of volatile components, no matter how mild, alters the delicate balance of volatile constituents. The vapor pressure and concentration of individual constituents in the fruit differ, and it is assumed that the relative concentration of components found in the condensates would be determined by these factors.

Tomato odor concentrates were obtained in yields of 2–5 ppm (see Table 2) by ether extraction of the aqueous condensates and concentration of the ether extract by distillation. The concentrates exhibited typical fresh tomato odor. A concentrate isolated from the water blank was odorless.

Yields in the range of 3–4 ppm were reported by Spencer and Stanley (1954) from tomato paste extracted with *iso*-pentane or benzene. A yield of 1.6 ppm was reported when ether extraction or vacuum distillates were used. Bidmead and Welti (1960) obtained 12.5 ppm when vacuum distillates of tomatoes were extracted with ethyl chloride.

The composition of headspace vapor over crushed tomato or freshly pulped tomato, of the nonvolatile stripped tomato residue, and of the various condensates was studied gas chromatographically. Fig. 2 shows that meaningful chromatograms were obtained only from the −78 and −196°C traps. The other chromatograms were almost identical. Their only significance was that the few components detected obviously did not contribute to tomato odor, since the stripped residue (curve *c*, Fig. 2) was odorless whereas the samples for curves *a*, *b*, and *d* exhibited typical tomato aroma.

Fig. 3. compares the composition of the vapor over a concentrate with the composition of a liquid sample of that concentrate. The components seen in the vapor (curve 1) are major constituents of the liquid concentrate. Separation of the liquid sample re-

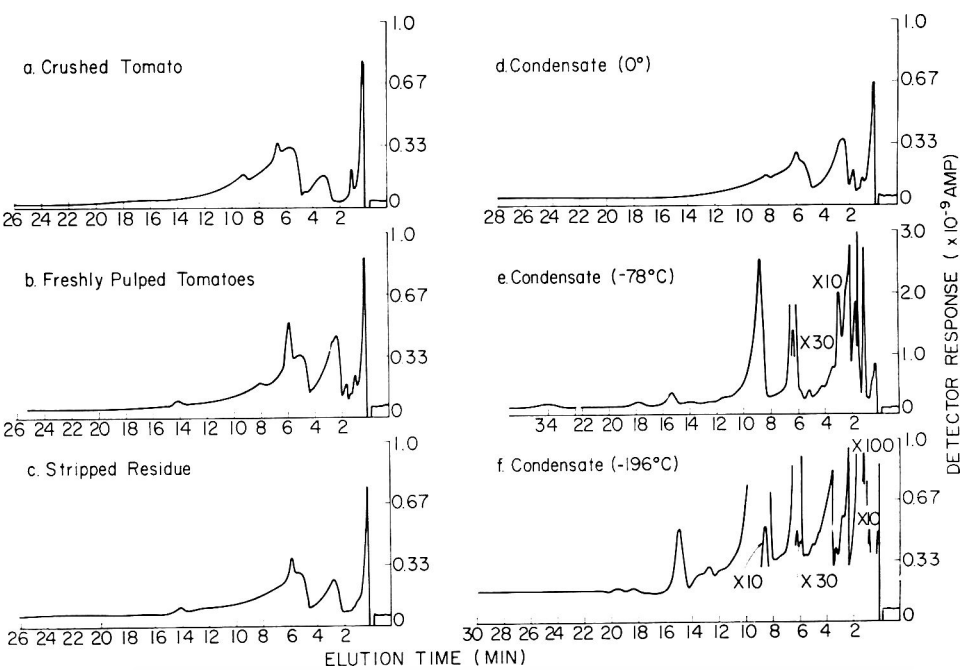

Fig. 2. Chromatograms of headspace vapor samples. Sample size, 1.5 ml; column, 1% Ucon 50HB-2000 on 100/120-mesh Gas-Chrom A at 34°C (⅛ in. ID × 6 ft).

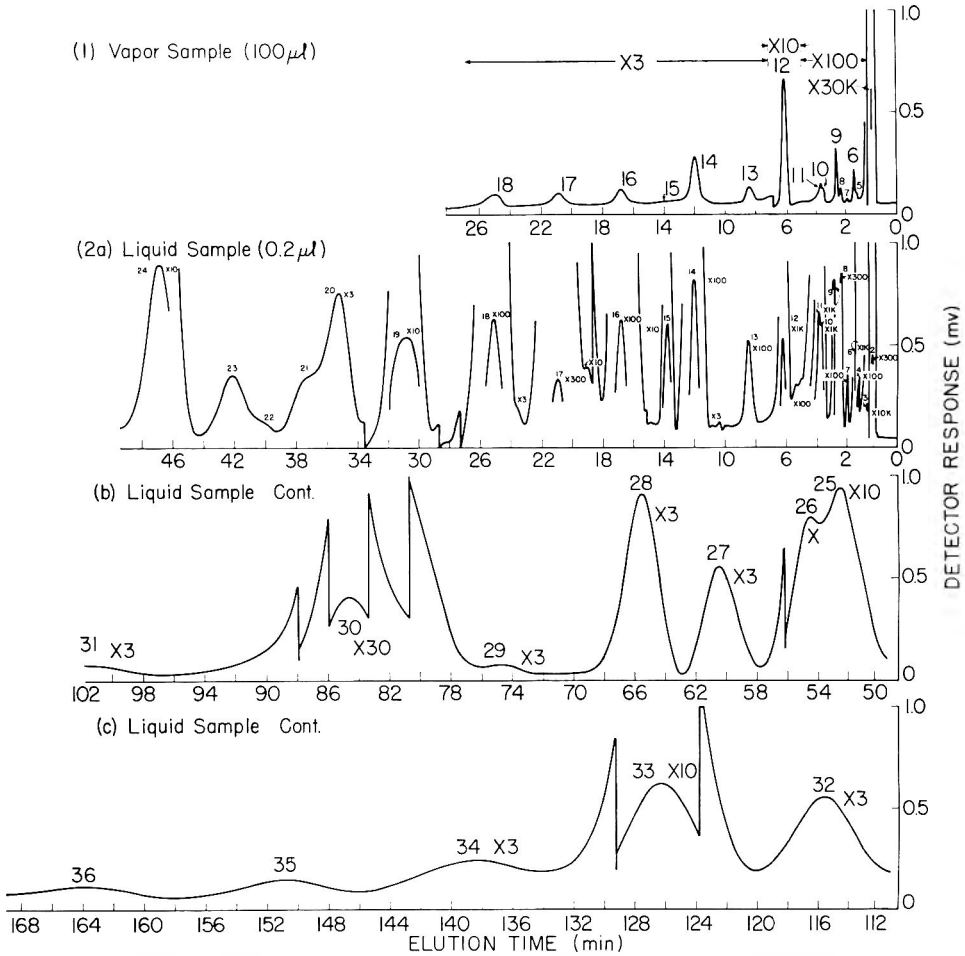

Fig. 3. Chromatograms of a vapor (100 μl) and liquid sample (0.2 μl) of tomato odor concentrate. Column, 1% Ucon 50HB-2000 on Gas-Chrom A at 50°C.

quired almost three hr. The number of detectable components doubled, the newly detected substances being present in small quantity. It is probable that man's nose can detect all these substances and more. If a capillary column had been used and its temperature had been programmed, many additional minor components would undoubtedly have been detected.

Separation of odor concentrates was carried out for isolation and identification of their individual components. The results are shown in Figs. 4, 5, 6, 7. Individual fractions were trapped. Comparison of infrared spectra and retention data with those of authentic reference compounds gave the identifications listed in Table 3. Also indicated is the identity of fractions detected in the vapor and liquid samples of Fig. 3. Review of these results shows that the relatively more volatile components were successfully isolated from the Ucon (90°C) and DEGS (70°C) columns. The Quadrol (126°C) and Carbowax (102°C) columns allowed isolation and identification of high-boiling constituents such as methyl salicylate and benzaldehyde but at the temperatures used were unsuitable for isolation of low-boiling components. A detailed study of Table 3 and Figs. 3–7 shows that numerous fractions from each column remain to be identified. The primary reason that they remain unknown is that they contain mixtures of components. For example, Fig. 8 shows the spectrum of fraction 9 from the Quadrol column (Fig. 6) and the chromato-

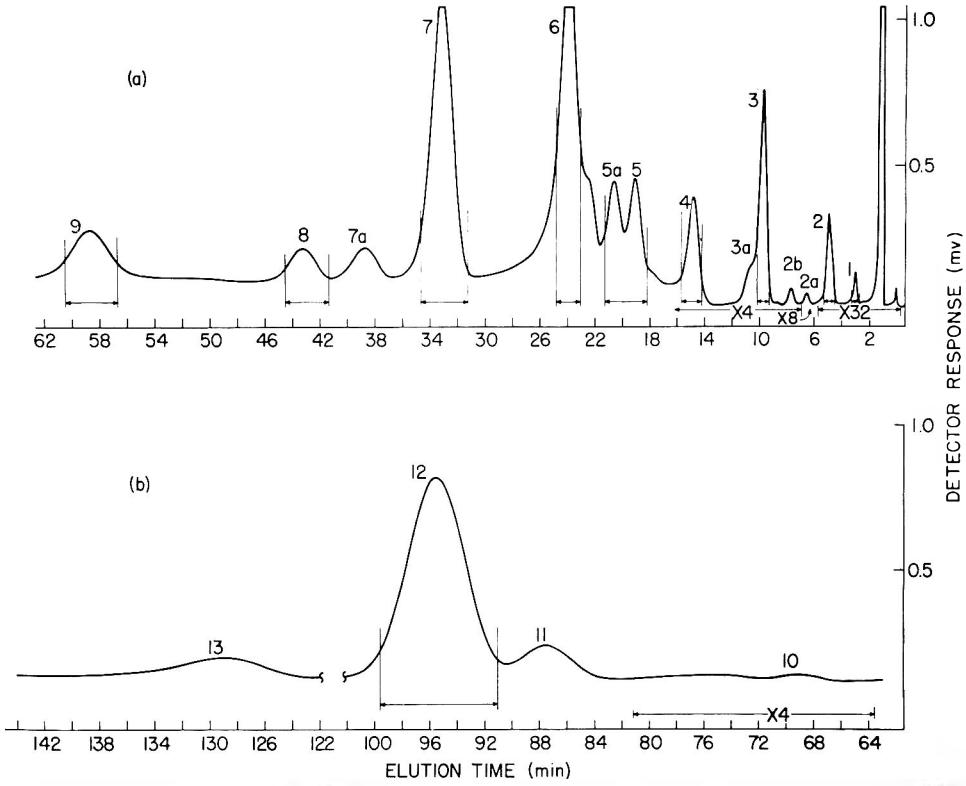

Fig. 4. Separation of 25 μl of tomato odor concentrate. Column, 20% Ucon 50HB-2000 at 90°C (8 mm ID × 2 m).

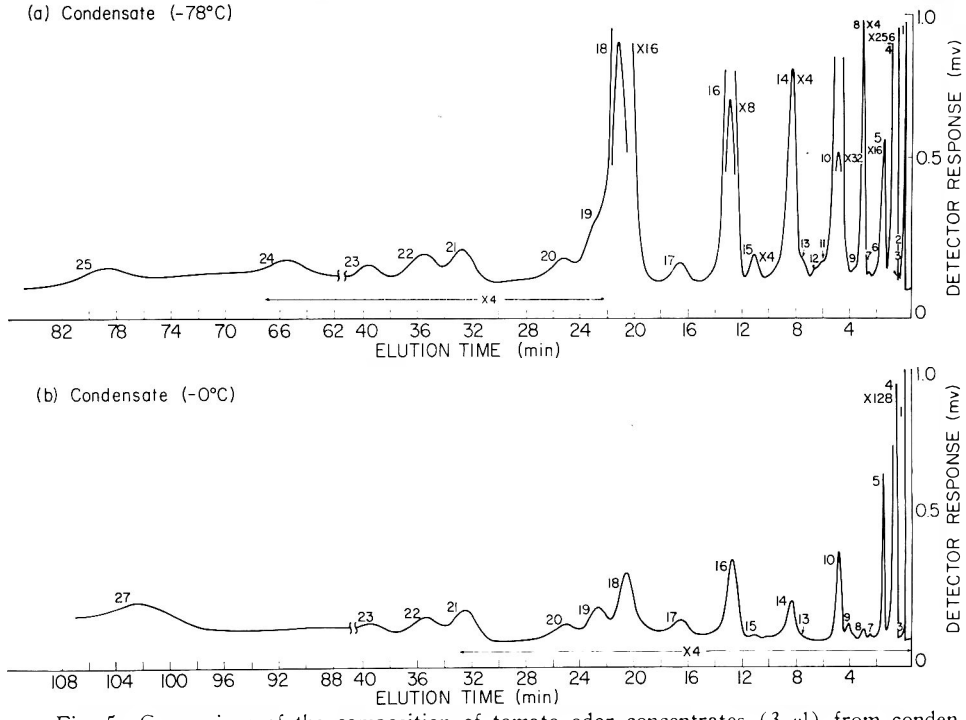

Fig. 5. Comparison of the composition of tomato odor concentrates (3 μl) from condensates collected at −78°C and 0°C. Column, 20% DEGS at 70°C (4 mm ID × 2 m).

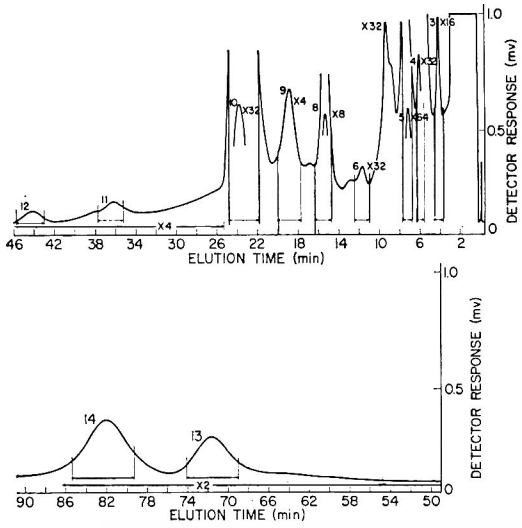

Fig. 6. Separation of 35 μl of tomato odor concentrate. Column, 20% Quadrol at 102°C (4 mm ID $\times$ 2 m).

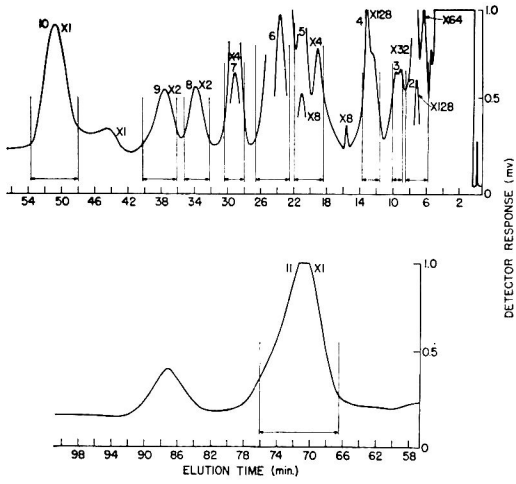

Fig. 7. Separation of 35 μl of tomato odor concentrate. Column, 20% Carbowax 4000 at 126°C (4 mm ID $\times$ 2 m).

gram which resulted from rechromatography of the infrared sample. It is obvious that the fraction contains at least two major components and that further separation is necessary to interpret the spectrum. Fig. 9 shows that fraction 7a from the Ucon column (Fig. 4) was essentially pure when

Table 3. Volatile tomato components.

Component	Fraction number in which component was isolated				
	20% Ucon (90°C)	20% DEGS (70°C)	20% Quadrol (126°C)	20% Carbowax (102°C)	1% Ucon (50°C)
Acetaldehyde [a]	....	1	....	....	....
Ethanol [a]	1	6	....	....	3
2-Propanol [a]	....	6	....	....	?
2-Butanone [a]	....	5	....	....	....
Ethyl acetate	2	4	....	....	4
3-Methyl butanal	2a	....	....	....	4
n-Propanol and an acetate	3	....	....	....	....
n-Hexanal	5	8	....	....	9
2-Methyl propanol-1	4	9	3	....	8
n-Butanol	5a	12	4	....	10
Two unsaturated carbonyl compounds, one an aldehyde	6	10	....	2	11
3-Methyl butanol-1	7	14	....	2	12
2-Methyl butanol-1	7	13	....	2	12
trans-2-Hexenal	7a	14	5	3	12
n-Pentanol	8	....	6	3	13
n-Hexanol	11	....	....	5	17
cis-3-Hexenol-1	12	16	10	6	17
Unsaturated alcohol	9	....	8	....	?
Unknown	10	15	....	5	?
Unknown	....	....	....	4–5	?
Unknown mixture	....	....	9	7	?
Benzaldehyde	....	....	....	10	?
Methyl salicylate	14	....	14	12	?

[a] Tentative identification based on retention data only.

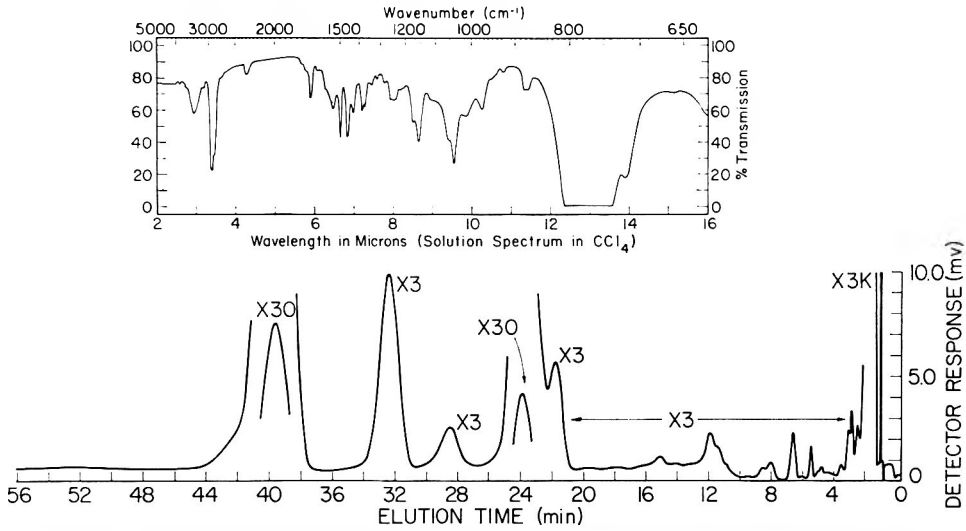

Fig. 8. Infrared spectrum and rechromatography of fraction 9 from 20% Quadrol column.

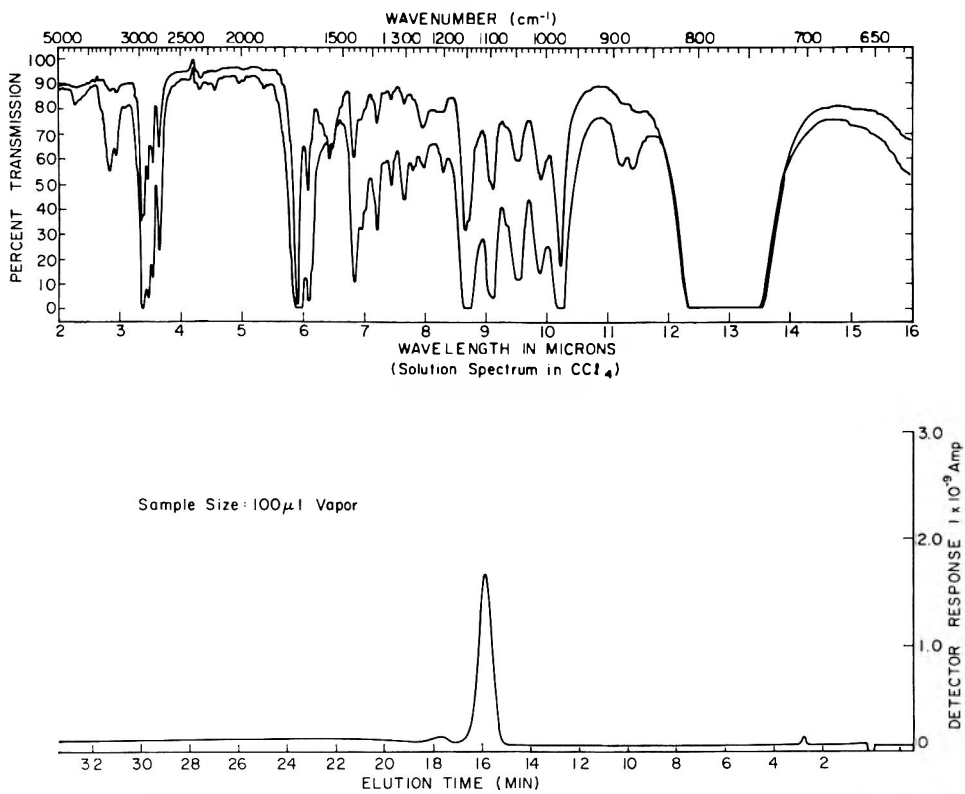

Fig. 9. Infrared spectrum and rechromatography of fraction 7a (*trans*-2-hexenal) from 20% Ucon 50HB-2000 column.

trapped. It was identified by its spectrum as *trans*-2-hexenal.

Fig. 5 compares the composition of odor concentrates from condensates collected at 0 and −78°C. As was expected, the concentration of the more volatile constituents was greater in the −78°C condensate than in the 0°C condensate.

General information concerning the contribution to tomato odor of the 27 fractions

shown in Fig. 5b was obtained by collecting them in a single trap and submitting them to informal sensory evaluation. The combined material exhibited "green tomato" aroma. When the effluent from the column was collected for an additional 80 min after fraction 27, a "ripe tomato" odor was observed. "Musty" and "spicy" odors were noted if collection was continued for 40 more min. Sensory evaluation of combined fractions from the Ucon column (Fig. 4) gave generally similar results. On the basis of this evidence it is clear that relatively high-boiling components which have not yet been isolated are major contributors to typical ripe tomato aroma. Components thus far identified appear to contribute primarily to "green tomato" aroma.

Although many volatile tomato components remain to be identified, thus far most of the known constituents (Table 3) are alcohols. Alcohols as tomato constituents had been reported by Spencer and Stanley (1954), who isolated 3,5-dinitrobenzoates from fractions which exhibited tomato odor. *cis*-3-Hexenol is a major component of the odor concentrates, and it is likely that its characteristic "green" note contributes significantly to tomato aroma. The amyl alcohols are substantial components of the odor concentrates since the *normal, iso,* and "active" isomers are present. The small quantity of ethanol and the apparent absence of methanol are of interest since they indicate that fermentation and/or demethylation of pectin had not occurred during distillation of the fruit.

The greatest frustration during the investigation was the inability to identify one of the largest fractions present (6 in Fig. 4, 10 in Fig. 5, and 2 in Fig. 7). Based on infrared data, two unsaturated carbonyl compounds were present, one of which was an aldehyde. Attempts failed to resolve the mixture. Retention data indicated a chain length of about 6 carbon atoms and suggested the possible presence of *cis*-3-hexenal. Since the fraction has a strong pleasant "green" odor, it may be a contributor to tomato aroma.

n-Hexanal, *trans*-2-hexenal, benzaldehyde, 2-butanone (tentative), 3-methylbutanal (tentative), and a very small amount of acetaldehyde (tentative) were the only carbonyl compounds identified. Spencer and Stanley (1954) had reported carbonyls as major components (35 ppm) of vacuum distillates of fresh tomatoes, with acetaldehyde accounting for 70% of the total. Since, in the present work, acetaldehyde cannot be expected to be isolated in other than very small amounts (due to the ether extraction and concentration procedure employed), the results are not necessarily in disagreement with those of Spencer and Stanley (1954). It is expected that additional carbonyl compounds such as citronellal and citral (Hein and Fuller, 1963) will be found during continued investigation of volatile tomato constituents.

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The Texture of Ice Cream

SUMMARY

Ice cream mix and frozen ice cream were studied. The aspects examined include the importance of the electrical charge on fat globules to the stability of mix against flocculation, the flow properties of mix at low rates of shear, the coagulation of mix at low temperature, the churning and coagulation of fat when mix is frozen, and the rheology of frozen ice cream. From the accumulated data a theory is proposed which indicates factors likely to influence the texture of frozen ice cream. A small initial fat globule size is desirable. This should be accompanied by rapid denaturation of milk protein during emulsification and homogenization. When mix is frozen, the solid fat particles produced by churning and coagulation should remain relatively small and be distributed throughout the lamellae between the air cells as small aggregates. The strength of the lamellae, on which texture largely depends, is also influenced by ice-crystal size and distribution, and the resistance of air cells to deformation. The fat particles in the aggregates are held together by van der Waals attraction forces.

INTRODUCTION

The general structure of ice cream has been established with a microscopic technique at low temperature (Arbuckle, 1960). This technique has been used as an alternative to subjective assessment to determine, in a general way, the influence of recipe ingredients and processing conditions on texture. More precise information can be obtained by rheological methods (John and Sherman, 1962). If the maximum benefit is to be derived from these data it is necessary to develop a better understanding of ice cream structure. Although the various components of ice cream structure have been identified, little is known about their interrelationships.

Studied in the present work is the structure of ice cream mix, in greater detail than hitherto, and the structural changes which ensue when mix is frozen. The accumulated data are used to develop a theory of ice cream structure.

EXPERIMENTAL

Ice cream mix recipe		% composition (w/w)
Oil phase	Vegetable fat	10.0
	Commercial-grade glyceryl monostearate	0.53
Water phase	Water	60.6
	Non-fat milk solids	13.1
	Sugar	15.6
	Vegetable gum	0.18

The mix was pasteurized 15 sec by the HTST process at 175°F, homogenized at 2,500 psi, and cooled to 4.5°C.

The electrical charge on fat globules in ice cream mix. The electrophoretic mobility (u) of the fat globules was determined in a modified Smith and Lisse (1936) cell as described elsewhere (Sherman, 1963). All tests were made at 4.5°C. Various field strengths within the range 2–10 volts/cm were used to ensure that u did not depend on the actual voltage applied. Sample dilutions for the tests were prepared by diluting 0.5 ml mix to 100 ml by the further addition of aqueous phase. A minimum number of 20 readings were taken for each mix dilution, at any one voltage, before reversing the direction of flow and taking a further 20 readings.

The viscosity of ice cream mix. Mix viscosity was determined at 4.5°C for a wide range of shear rates (0.071–571.1 sec^{-1}) with a Rotovisko coaxial-cylinder viscometer. The mean globule volume diameter (D_m) was determined for each sample.

The stability of ice cream mix. The coagulation behavior and the rate of slow globule coalescence (K) were deduced from the rate of decrease in the number of globules (N) per unit volume of mix with time (t) at 4.5°C (Sherman, 1962). The influence of milk protein concentration on K was studied by adjusting the milk powder content of the mix recipe between 50 and 150% of its normal value, the water content being altered accordingly.

Freezing of ice cream mix. To study the effect of high-speed whipping and low freezer temperatures, mix was frozen in a Sweden Soft Server. Samples were withdrawn for microscopic examination and churned-fat determinations (Keeney, 1958) after suitable time intervals ranging from

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15 sec to 5 min. Sampling after different freezing times is carried out more conveniently with a soft ice cream freezer than with the normal batch, or continuous, freezer for hard ice cream. The samples frozen in this way had overrun contents of about 70%, and the percentage churned fat was of the desired order.

Microscopy was used to determine the mean size of the fat particles, their size distribution, and to assess qualitatively the degree of fat-particle aggregation.

RESULTS AND DISCUSSION

Electrical charge on fat globules in ice cream mix and its influence on flocculation. The fat globules were negatively charged, and they showed an average mobility (u) of -0.63 μ/sec/volt/cm. When both monoglyceride and milk protein were omitted from the recipe the average u was $+3.2$ μ/sec/volt/cm. Mixes of the latter type coagulated more rapidly than standard-recipe mixes, in spite of their greater charge. This indicates that the stability of ice cream to coagulation cannot be attributed entirely to electrical repulsion forces. Other forces must be involved, e.g., the surface rheological and physical properties of the emulsifier-protein film around the fat globules.

The potential (ζ) of the innermost part of the diffuse portion of the electrical double layer can be calculated from the equation for the electrophoresis of nonconducting spheres (Davies and Rideal, 1961).

$$\zeta = \frac{6\pi\eta_0 u}{RF\epsilon} \qquad [1]$$

where η_0 is the viscosity of the aqueous phase, ϵ is its dielectric constant, and R is a correction factor which allows for the time-dependent relaxation of the ionic atmosphere. F is a correction factor which allows for interaction between the applied field and the electrical double layer, a very important effect for small particles, and also for possible interaction with the surface of the fat globules.

If the double layer thickness is small with respect to globule radius so that $\chi D_{m/2} > 300$ where χ is the reciprocal thickness of the diffuse double layer, $F = 3/2$, and $R = 1$ (Davies and Rideal, 1961). On this basis the value of ζ at 4.5°C is 8 mv. At this low ζ, and because of the relatively large thickness of the adsorbed emulsifier-protein layer with respect to fat-globule size (Sherman, 1961), it is unlikely that the potential (ψ) of the outermost portion of the diffuse double layer exceeds ζ by more than 10%. The ψ potential is taken as 8.8 mv.

The potential energy of repulsion (V_R) between fat globules is given by

$$V_R = \frac{\epsilon D_m \psi^2}{4}\left\{\ln\left[1 + \exp(-\chi H_0)\right]\right\} \qquad [2]$$

where H_0 is the distance separating fat globules (Verwey and Overbeek, 1948). A value of 10^{-7} cm was taken for $1/\chi$.

The potential energy of attraction (V_A) is effective to greater distances than V_R. For small values of H_0

$$V_A = AD_m/24H_0 \qquad [3]$$

where A is the van der Waals constant (Verwey and Overbeek, 1948). Calculated values of A must be corrected for time (retardation) effects.

The net potential energy of interaction (V) between fat globules is the sum of V_A and V_R. Its magnitude depends on the value adopted for A. For colloidal dispersions of solid particles in liquid media, A usually has a value of the order of 10^{-12} erg. For the present work it is necessary to adopt a much lower value for A, *viz*, 10^{-14} erg. This choice can be justified on the following grounds.

a) The coagulation pattern of mix (Sherman, 1962) indicates that V has a value of only a few kT, where k is the Boltzmann constant and T is the absolute temperature. Because ψ is only 8.8 mv, which is a very much lower value than for most O/W emulsions, V_R is low, and consequently V_A must also be low. If A fell anywhere within the range 10^{-11} to 10^{-13} erg, V would always be negative, i.e., attraction would predominate, and the fat globules would flocculate. In practice, flocculation is never pronounced.

b) For pure substances, A can be calculated in several ways. The physical data required to do this cannot be deprived very easily for such a complex

mixture as a vegetable fat. For a dispersion of fat-in-water

$$A = \left(\sqrt{A_{F-F}} - \sqrt{A_{W-W}} \right)^2 \quad [4]$$

where A_{F-F} and A_{W-W} are the interaction constants between fat molecules and water molecules, respectively (Vold, 1961). The value of A for stearic acid particles dispersed in water is 0.79×10^{-14} erg, or 3.68×10^{-14} erg (Ottewill and Wilkins, 1962) depending on the value chosen for A_{W-W}. The vegetable fat employed contains a fair proportion of stearic acid, so that ice cream mix may be expected to show a similar value of A when the emulsifying agent and milk protein are absent. When they are present, A may be reduced still further (Vold, 1961).

Using Eq. 2 and 3, the $V - H_0$ relationships have been calculated for two values of A and three values of D_m (Figs. 1, 2). A must have a value less than 1.25×10^{-14} erg; otherwise, V is always negative. The larger the value chosen for A, the smaller is the maximum height of the potential en-

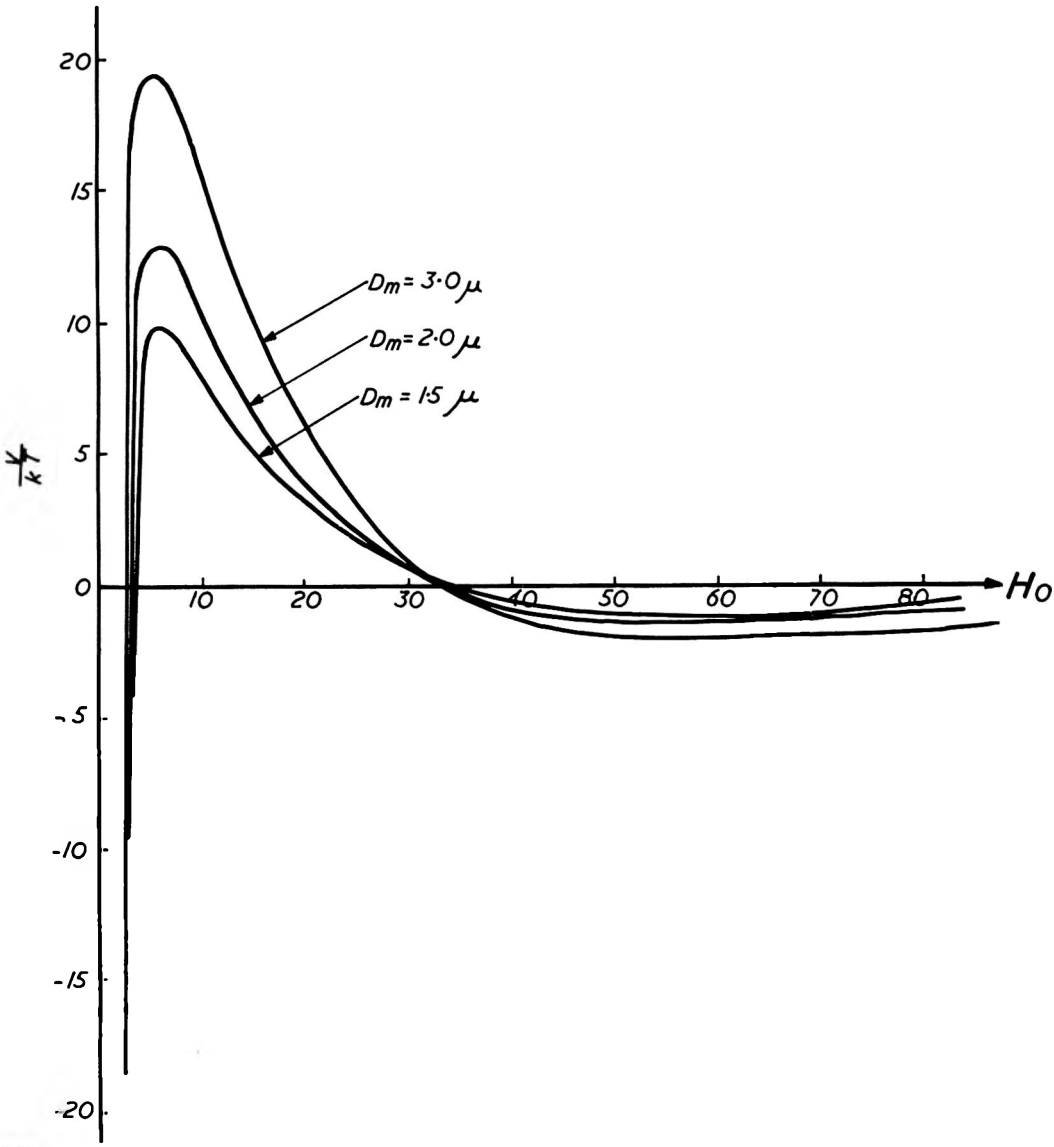

Fig. 1. Potential energy of interaction between fat globules in ice cream mix. $A = 0.75 \times 10^{-14}$ ergs.

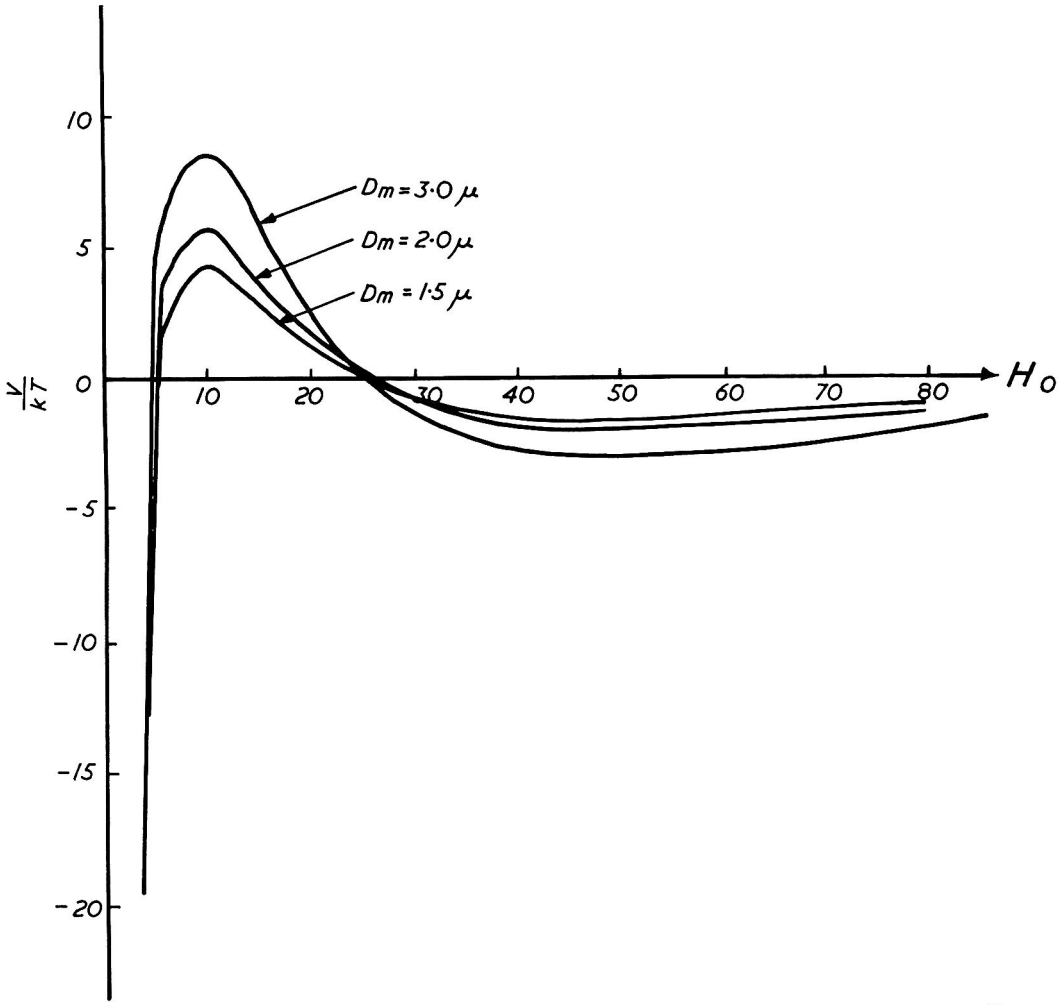

Fig. 2. Potential energy of interaction between fat globules in ice cream mix. $A = 1.0 \times 10^{-14}$ ergs.

ergy barrier (V_{max}) preventing the globules from coming close together. Both rheological and coagulation data, which will be discussed in later sections, suggest that A should have a value of 0.75×10^{-14} to 1.0×10^{-14} erg.

When H_0 exceeds 30Å, V_A predominates and a region of weak attraction—the "secondary minimum"—extends to H_0 values approaching 100Å. Since the globules are able to enter the secondary minimum more readily than they can surmount V_{max} they are still separated by about 50Å when they flocculate. The flocculated globules are held together by only weak forces of attraction, so that the preparation of a diluted sample for microscopic examination provides sufficient energy to cause deflocculation.

The use of D_m in Eq. 2 and 3 is valid provided the spread of globule sizes is narrow. If the size range is much broader, and it extends to larger sizes, it is more difficult to predict flocculation behavior. The probability of flocculation is reduced because V_{max} then has a larger value, but the rate of collision between the globules is increased.

Rheological properties of ice cream mix. Fig. 3 shows viscosity data for a mix with $D_m = 1.9\ \mu$. The rates of shear ranged from 0.071 to 1.90 sec^{-1}. At the lowest rate of shear, the viscosity (η) was 223.0 poise, and it decreased very rapidly with increasing rate of shear to 19.2 poise at 1.9 sec^{-1}. With further increase to 114.2 sec^{-1}, η fell to a steady value of about 1 poise (Fig. 4), and this value was maintained irrespective of any further increase in shear rate.

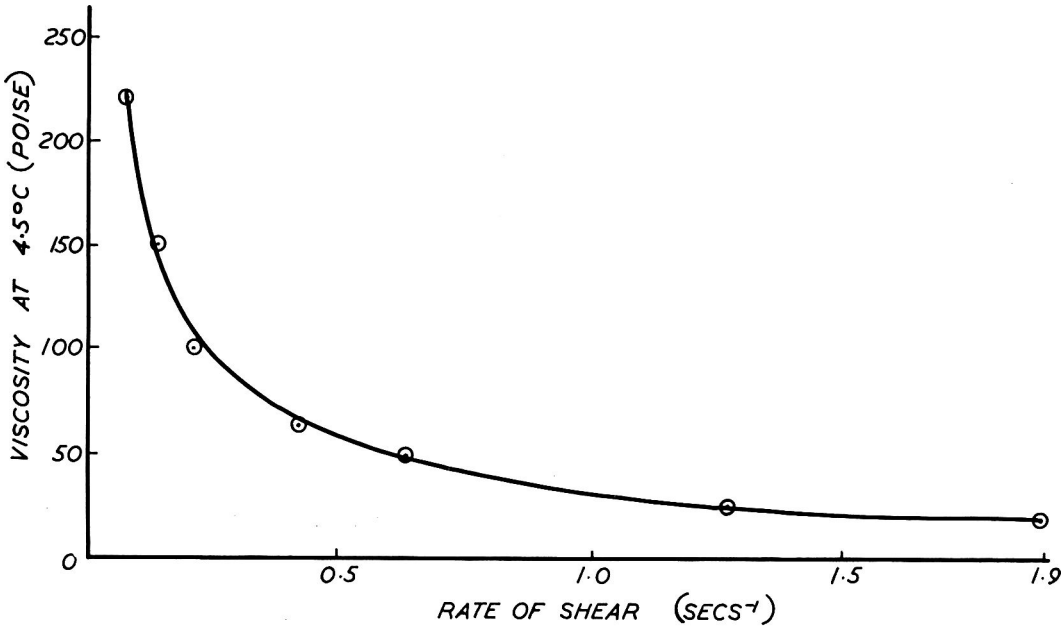

Fig. 3. Viscosity of ice cream mix at low rates of shear.

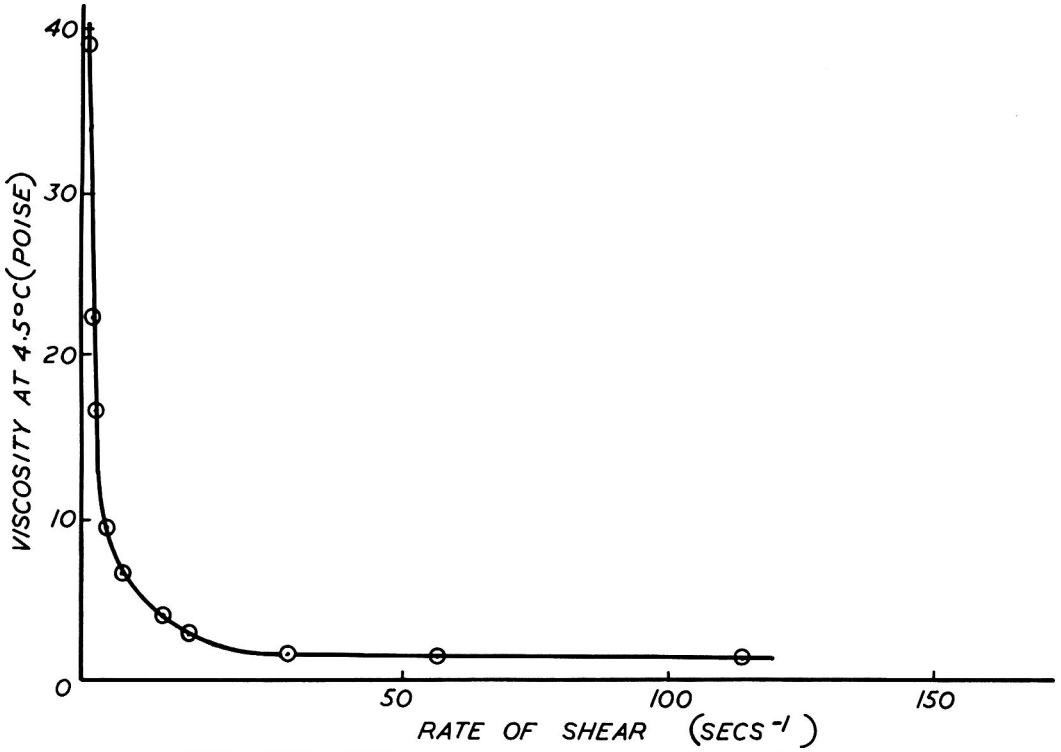

Fig. 4. Viscosity of ice cream mix over a wider range of shear rates.

At very low rates of shear the globules collect into aggregates. Each aggregate behaves as if it had a volume greater than the sum of the volumes of the individual globules from which it is constituted due to immobilisation of aqueous phase in the spaces between the globules. The aggregate rotates around its center of mass like a single globule. When the rate of shear is gradually increased the aggregates break down along

their weakest planes and η decreases. The final stage in this disruption may involve only pairs of globules. Flow, due to shear, not only makes these pairs rotate, but it also sets up a tension which promotes their separation. When the tension (v_{min}) exceeds the attraction force between the globules, they separate (Albers and Overbeek, 1960).

For nondeformable globules, v_{min} can be calculated from

$$v_{min} = \frac{A}{18\pi\eta_0 D_m H^2 \sin(2a)} \qquad [5]$$

where a ($\sim 30°$) is the critical angle between the line joining the centers of the two globules and the direction of shear at the time when the globules separate, and H is the distance between globules in the paired condition. It is unlikely that globules in ice cream deform when sheared. Even if they were not surrounded by a visco-elastic film of adsorbed protein and monoglyceride (Sherman, 1961), they are too small to deform significantly at high rates of shear (Taylor, 1934). Taking $A = 0.75 \times 10^{-14}$ or 1.0×10^{-14} erg, $D_m = 1.19 \times 10^{-4}$ cm, $\eta_0 = 0.015$ poise, since nearly all the protein is adsorbed at the fat/water interface, and $H_0 = 50 \times 10^{-8}$ cm, v_{min} is found to be 35 sec^{-1} or 47 sec^{-1}. These values agree very satisfactorily with the value derived from Fig. 4. Backward extrapolation of the linear part of the curve suggests that the minimum rate of shear for flow to become Newtonian is about 50 sec^{-1}. These observations provide indirect confirmation of the low value selected for A in calculations of the preceding section.

The pronounced decrease in η with increasing shear at low rates of shear suggests that A is more likely to be 1.0×10^{-14} erg than 0.75×10^{-14} erg. For the latter value the secondary minimum is too shallow, and the attraction forces decrease too slowly with increasing H_0 to explain the observed flow behavior. Even when the higher value of A is adopted, the secondary minimum potential is no greater than 1.2–2.0 kT when D_m is 1.2–2.0 μ. These values may be increased, however, as may V_{max}, if the size of the globules is significantly enlarged by the adsorbed hydrated protein-monoglyceride layer. If this layer has the structure proposed elsewhere (Sherman, 1961), viz., that the degree of protein denaturation varies with the distance from the interface, and consequently that retention of the ability to bind water increases with this distance, it is possible that the amount of water bound will also vary with the rate of shear. If binding involves weak electrostatic forces, and/or weak hydrogen bonding by the peptide groups of the polypeptide chains and by the side chains of the amino acids residues (Pauling, 1960), more water should be bound at low rates of shear. Hydration would then make a greater contribution to η at low rates of shear than at high rates of shear.

Coagulation of ice cream mix on aging. When globules diffuse toward one another and collide, and each collision results in the coalescence of two globules, N will decrease with t in accordance with (Smoluchowski, 1916, 1917):

$$-\frac{dN}{dt} = 4\pi \mathcal{D} \bar{\bar{R}} N^2 \qquad [6]$$

where $\mathcal{D}$ is the diffusion constant ($= kT/3\pi\eta_0 D_m$), and $\bar{\bar{R}}$ ($\sim D_m$) is the collision radius.

Integrating Eq. 6 yields

$$\frac{1}{N} - \frac{1}{N_0} = 4\pi \mathcal{D} \bar{\bar{R}} t \qquad [7]$$

where N_0 is the value of N when t is 0.

When $N = N_0/2$, $t = t_{1/2}$, and

$$t_{1/2} = \frac{1}{4\pi \mathcal{D} \bar{\bar{R}} N_0} \qquad [8]$$

For $D_m = 1.19$ μ, $N_0 = 1.2 \times 10^{11}$ per ml, so that $t_{1/2} = 58$ sec. The experimentally determined value of $t_{1/2}$ is far greater than 58 sec, so that only a fraction of the collisions between globules result in coalescence.

Eq. 7 should therefore read

$$\frac{1}{N} - \frac{1}{N_0} = 4\pi \mathcal{D} \bar{\bar{R}} t . \exp(-E/RT) \qquad [9]$$

where E is the energy barrier to coalescence. The value of E will depend on the electrical forces preventing flocculation, as discussed previously, on the rate of thinning of the thin film of fluid between the globules after they have flocculated, and on the physical

properties of the monoglyceride-protein film around the globules.

When milk protein and monoglyceride are omitted from the recipe, $t_{1/2}$ is still 96 min when N_0 is 6.94×10^8 per ml. In this instance the energy barrier primarily prevents flocculation rather than coalescence following flocculation, since, in the absence of the surface-active materials, the globules have a much larger u. From plots of $1/N$ against t, $\exp(E/RT)$ is found to be about 9. For satisfactory stability it should be at least 10^5.

The monoglyceride-protein film around the globules has polymolecular dimensions (Sherman, 1961). Further confirmation of this structure is provided by data on the influence of milk protein concentration on coagulation of ice cream mix. It was previously shown (Sherman, 1962) that coagulation proceeds in two stages provided the initial D_m is below a critical value. A phase of relatively rapid coagulation is followed by a phase of slow coagulation, the change from rapid to slow coagulation occurring at a certain value of the interfacial area. It was suggested that coagulation is governed by the structure of the adsorbed monoglyceride-protein film around the globules, and that this film becomes more condensed as the interfacial area decreases. Because of the polymolecular nature of the adsorbed film it should be possible to alter its thickness, and the tightness of molecular packing in the film, by adjusting the milk powder concentration. The value of K, and the value of the interfacial area ($\propto N^{1/3}$) at which rapid coalescence gives way to slow coalescence, should also be affected. These suppositions are confirmed by Fig. 5 and the data in Table 1. As the milk powder concentration increases so the interfacial area at which slow coalescence begins increases linearly (Fig. 6), while K decreases, so that E must increase with increasing concentration of milk protein.

Coagulation of fat in the freezer. Fat coagulation and solidification proceeds very rapidly within the first few seconds of freezing. Subsequently the main changes are slower growth of fat particles, and their continued aggregation, at rates dependent on

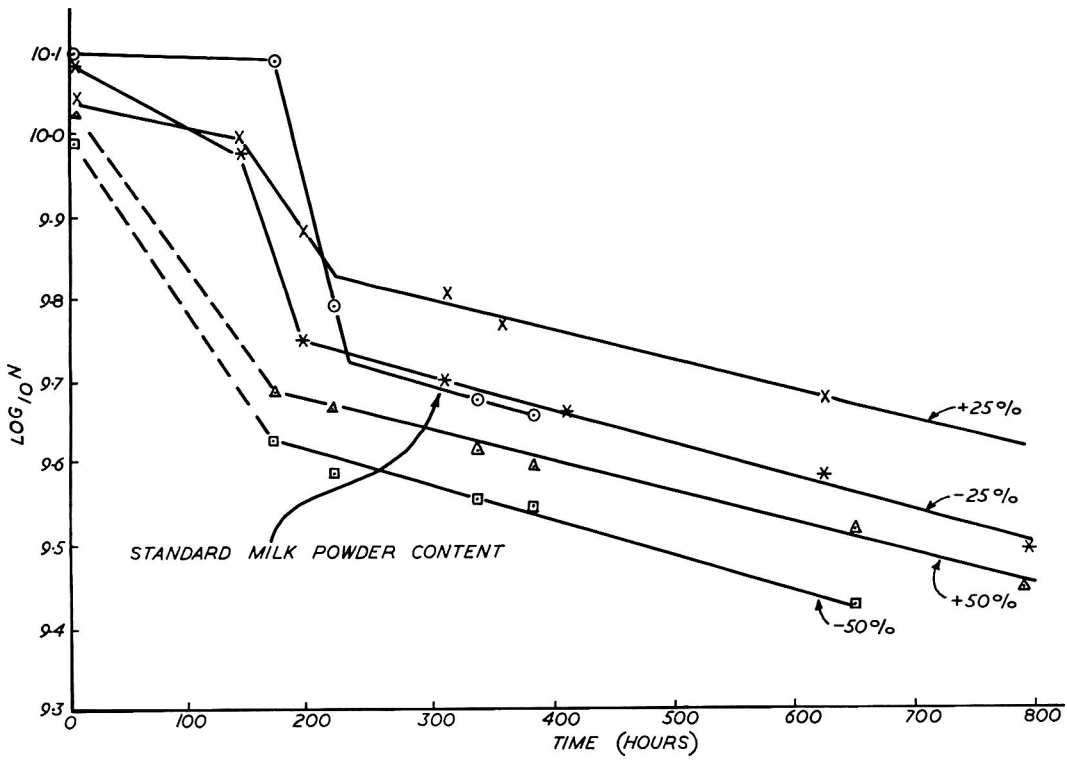

Fig. 5. Rates of coalescence of mixes containing varying concentrations of milk powder.

Table 1. Coagulation data for ice cream mixes at 4.5°C.

% milk powder concentration	N at onset of slow coagulation ($\times 10^{9}$)	$3\sqrt{N}$	Rate of slow coalescence, K ($sec^{-1} \times 10^{-5}$)
150	6.76	1,890	21.7
125	Insufficient data to assess accurately		22.4
100	5.5	1,760	Insufficient data to assess accurately
75	Insufficient data to assess accurately		26.9
50	4.17	1,610	27.8

freezer temperature (Fig. 7). The proportion of fat churned also depends on freezer temperature.

At 4.5°C the fat in the globules is still in the liquid state, because of supercooling. Rapid reduction of temperature in the freezer, plus the vigorous whipping action, weakens the monoglyceride-protein film around the globules and eventually ruptures it. The released fat then solidifies. The breakdown of emulsifier films on freezing has been attributed to dehydration by loss of water to the ice phase (Cole *et al.*, 1959). This process may be influenced by the nature of the fat, the constitution of the emulsifying agent, and the solubility of the emulsifier in the fat phase (Singleton *et al.*, 1960), presumably through their influence on the properties of the emulsifier layer.

The whipping action will increase both the frequency of collision between fat particles and their rate of separation. Smoluchowski (1916, 1917) calculated collision frequency resulting from a velocity gradient in simple shear flow, and from this the size distribution of particle aggregates as a function of time, but it is doubtful that the shear forces prevailing in a freezer are in any way comparable. Beyond a certain rate of shear in simple shear flow the collision frequency is independent of particle interaction, as discussed previously, and is proportional to the rate of shear and to the cube of the collision diameter (Vries, 1963). It is evi-

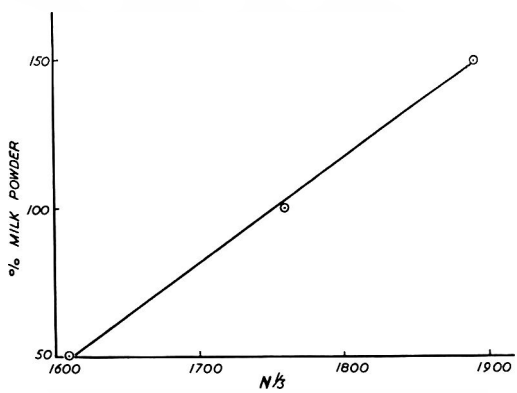

Fig. 6. The influence of milk powder concentration on the coagulation characteristics of ice cream mix.

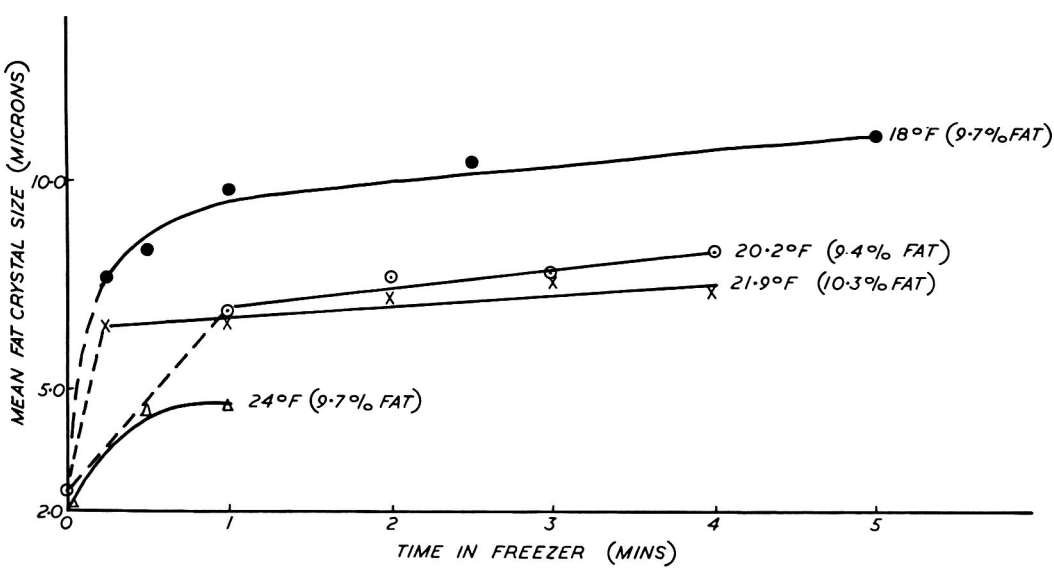

Fig. 7. The influence of freezing time and temperature on fat crystal size.

dent from Fig. 7 that the collision frequency must be greater than the rate of separation. Partial freezing of the aqueous phase brings the fat particles closer together. High-speed agitation provides sufficient energy for the particles to pass over the potential energy barrier to flocculation, even though V_{max} increases as particle size increases. As a result, fat particles will be separated eventually by only a few angstroms. At this small distance apart they are linked together by strong attraction forces. The magnitude of this attraction cannot be estimated accurately, since the electrical conditions in frozen ice cream will be different from those prevailing in ice cream mix, because of partial freezing of the aqueous phase, fat, etc. It is quite likely, however, to be several times $10kT$.

An interesting point emerges from this study regarding the significance of churned-fat data. Irrespective of the time for which any mix remained in the freezer the percent churned fat did not alter after the first minute or less. The mean fat-particle size, on the other hand, increased approximately linearly with freezing time (Table 2). Since fat-particle size, and the degree of particle aggregation, must influence the texture of frozen ice cream, churned-fat data should not be used on their own to draw conclusions about texture.

Table 2. Typical sets of churned-fat and fat-particle size data for ice cream mix frozen in Sweden soft server.

Time in freezer (min)	Temperature (°C)	% churned fat	Mean fat-particle size (μ)
0	4.5	0	2.48 (globules)
1	−6.7	48	6.88
2	−6.7	56	7.68
3	−6.7	58	7.76
4	−6.7	53	8.26
0	4.5	0	2.43 (globules)
1	−5.6		6.60
2	−5.6	37	7.17
3	−5.6	53	7.64
4	−5.6	46	

Rheology of frozen ice cream. The strong attraction forces between the fat particles will contribute to the textural properties of frozen ice cream. When stress is applied, the particles move apart, so that the attraction will decrease. Eventually, if sufficient stress is applied, the attraction forces between the particles become insignificant and the particles move independently of each other. The stress-strain behavior should show regions of instantaneous elasticity and viscous flow. Superimposed on these effects will be the rheological behavior of the larger air cells surrounded by a visco-elastic protein film and chains of fat particles, the rheological behavior of the ice crystals, etc.

A study was recently made of the rheological behavior of ice cream at low shearing stress (Shaw, 1963). The results are considered to support the theory (Shaw and Walker, 1964) that the structure of ice cream is due to ". . . a denatured protein film which envelops the fat particles and air bubbles to form a network throughout . . ." The stress-relaxation curve composed for frozen ice cream suggests a non-cross linked structure, however. This is in accord with the present theory.

CONCLUSIONS

Frozen ice cream may be regarded as an aerated emulsion, or foam. A foam is defined as an "agglomeration of gas bubbles separated from each other by thin liquid films" (Bikerman, 1953), or alternatively, as a "coarse dispersion of gas in a liquid, most of the phase volume being gas, with the liquid in thin sheets called lamellae, between the gas bubbles" (Davies and Rideal, 1961). Because of the high air content of detergent foams, the thickness of their lamellae can fall to as little as 100Å. In ice cream, as manufactured in Great Britain, air constitutes about 50% of the total volume, so that the lamellae are very much thicker. Microscopic examination of sections held at low temperature indicated lamellae thicknesses ranging from 30 to 300 μ. Nevertheless, the structure of ice cream resembles that of a solid foam because, in both cases, texture depends largely on the physical properties of the lamellae. In ice cream, this involves fat particle size, the size and distribution of fat particle aggregates, the size and distribution of air cells, the physical properties of the protein-monoglyceride film adsorbed

around fat particles, and the physical properties of the protein-enveloped air cells with their oriented fat particles.

Most structural changes occur during freezing. The attainment of satisfactory texture depends on several factors:

1) Small globule size (1–2 μ) and narrow size distribution in the ice cream mix. The globule size should be too small for the globules to form aggregates, i.e., the rate of coalescence should be greater than at the stage when globules form aggregates prior to coalescence. This implies the use of emulsifiers, etc., which produce a substantial reduction in interfacial tension, since homogenization on its own cannot produce the desired effect.

2) Denaturation of protein at the fat-water interface should proceed at a rate which quickly establishes an effective barrier to too rapid growth in globule size. When fat is emulsified in an aqueous medium, the fat not only disperses as small globules, usually of microscopic dimensions, but an opposing tendency is established whereby the globules diffuse toward one another and coalesce. The rate of the latter process should be reduced to a reasonably low level.

3) Resulting from the implementation of 1 and 2, small fat particles are promoted on freezing. These are distributed throughout the aqueous medium between the air cells. Small fat particles, and small particle aggregates, provide better air retention and whipping properties (Keeney and Josephson, 1962).

4) The solubility of the emulsifier in the oil phase. Unsaturated emulsifiers contain fatty acids which remain soluble to lower temperatures than saturated fatty acids (Stistrup and Andreasen, 1962). The protective monoglyceride-protein film around the globules may remain intact to lower temperatures with unsaturated emulsifiers than with the conventional saturated monoglycerides. This could influence the rate of film rupture during freezing and the rates of fat solidification and coagulation.

5) Processing conditions will exert an influence on each stage of manufacture. Ice cream is extruded from the freezer at a relatively low shear rate approximating to "plug flow," i.e., the inner part of the ribbon flows as a single unit, and only the surface layers are sheared.

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Proteolytic Action of Pepsin on Glutenin

SUMMARY

Pepsin rapidly decreases the viscosity of a glutenin dispersion having an acid reaction and rapidly increases the amount of water-soluble fragments. The molecular weight of the solubilized fragments, estimated by the gel-filtration method, indicates that most of the fragments are large and have molecular weights greater than 10,000. A comparison of data obtained by gel filtration and by determination of terminal amino acids in pepsin hydrolysates of glutenin indicates that cleavage of a few peptide bonds rapidly produces these large molecular polypeptides from glutenin. Further pepsin action causes a substantial increase in small peptide fragments.

INTRODUCTION

During investigations on the preparation of a milk-like product from wheat flour, pepsin treatment was found to be useful for extracting protein in high yield and for modifying the wheat protein. In an attempt to explain the action of pepsin, studies were conducted on the proteolytic action of the enzyme on purified glutenin, which is one of the main fractions and the least dispersible fraction of wheat protein.

MATERIALS AND METHODS

Pepsin. Pepsin used in this work was crystalline pepsin (Nutritional Biochemical Corporation).

Glutenin. Kesco (Keever Starch Co., Columbus, Ohio) vital wheat gluten containing 80.5% protein was used as the starting material for the preparation of glutenin. Lipid removal was accomplished by extracting five times with *n*-butanol. Purification was by fractional precipitation in acetic acid and sodium acetate according to the procedure of Jones *et al.* (1959). The solvent-free gluten was nearly completely dispersed by the initial acetic acid treatment. The final precipitate was dispersed in 0.1*N* acetic acid and dialyzed against 0.05*N* acetic acid. The dialyzed dispersion was used as the glutenin sample.

Hydrolysis of glutenin by pepsin. The pH of a glutenin dispersion was adjusted with hydrochloric acid, and pepsin hydrolysis was carried out mainly at 40°C.

TCA-soluble nitrogen. Nitrogen soluble in 3.33% trichloroacetic acid was determined by the micro-Kjeldahl method.

Soluble nitrogen. One part of sample was mixed with one part of McIlvaine buffer (mixture of 228 ml of 0.1*M* citric acid and 772 ml of 0.2*M* Na_2HPO_4) having a pH of 6.8. The resulting precipitate was removed by centrifugation. Final pH was 6.5. The nitrogen content in the supernatant was determined by the micro-Kjeldahl method and is defined as soluble nitrogen.

Dialyzable nitrogen. Five ml of pepsin hydrolysate was placed in a cellophane tube and neutralized to pH 6.5 by addition of 5 ml of the buffer solution. This material was dialyzed against 4 L of distilled water for 2 hr at room temperature under constant agitation. The same treatment was repeated once more, and the nitrogen content in the tubing was determined by the micro-Kjeldahl method. The difference in nitrogen content before and after the treatment was taken as dialyzable nitrogen.

Viscosity measurement. Viscosity of the pepsin hydrolysate was measured with an Ostwald viscometer. The flow rate of distilled water was 42.1 sec at 40°C when 10.5 ml of distilled water was employed. Ten ml of glutenin dispersion at pH 2.0 was mixed with 0.5 ml of pepsin solution, and the hydrolysis of glutenin was carried out in the viscometer. The flow rate of the reaction system was measured periodically, and the time at the middle of the measurement was taken as the treatment time.

Gel filtration. Sephadex G-100 was used for gel filtration analysis. The gel bed, consisting of a 40 × 2.5-cm column was equilibrated with pH 7.0 McIlvaine buffer (824 ml 0.2*M* Na_2HPO_4 plus 176 ml 0.1*M* citric acid).

The glutenin hydrolysate was prepared as follows: To 10 ml of glutenin dispersion (4.85 mg N/ml) were added 0.22 ml of 1.076*N* HCl, 0.18 ml NH_3-free distilled water, and 0.10 ml of 0.5% pepsin. The mixture had a pH of 2.0 and was incubated at 40°C. After incubation, 10.5 ml of McIlvaine buffer (pH 7.0) was added to stop the reaction. One ml of glutenin hydrolysate was added to the column and chromatographed with McIlvaine buffer (pH 7.0).

Used as a molecular weight reference in this experiment were gamma-globulin (Protein Foundation, Inc.), α-chymotrypsin (Nutritional Biochemicals Corporation), and cytochrome C (Sigma Company). The molecular weight of each fraction

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was estimated by assuming a linear relationship between the ratio of the elution volume V_e to the void volume V_o; and the logarithm of molecular weight according to the procedure of Whitaker (1963). The nitrogen content of the effluent was determined by the colorimetric method of Lowry *et al.* (1951). Optical absorbance was measured at 750 mμ.

N-Terminal amino acids. N-Terminal amino acids of pepsin hydrolysates of glutenin were determined by the DNP-method of Sanger (1945). A modification was made in that 3/10 part of dimethylformamide (DMFA) was added to 1 part of sample to attain sample solubility at the dinitrophenylation stage.

One ml of hydrolysate, from various stages of the pepsin hydrolysis, was mixed with 0.3 ml of DMFA and 0.1 g of sodium bicarbonate, and then 2 ml of 5% 1-fluoro-2,4-dinitrobenzene (FDNB) ethanolic solution was added to the mixture. The dinitrophenylation was carried out by constant shaking of the mixture for 2.5 hr at room temperature. Then the reaction mixture was diluted with 2 ml of distilled water and extracted five times with ethyl ether. The aqueous layer was acidified with hydrochloric acid and evaporated in a vacuum flash evaporator. The dried material was transferred into a test tube with 10 ml of 6*N* hydrochloric acid, and the tube was then sealed. Hydrochloric acid hydrolysis of the dinitrophenylated materials was carried out by heating the sealed tube for 24 hr in a boiling-water bath. After dilution, the hydrochloric acid hydrolysate obtained was filtered to remove humins, and then extracted three times with ether. The ether extract was washed twice with a small quantity of diluted hydrochloric acid and evaporated in vacuum. The water layer was also extracted three times with *n*-butanol, washed twice with diluted hydrochloric acid, and evaporated in vacuum. These two fractions were applied separately to the paper for chromatography.

The recoveries of these DNP-amino acids were estimated by comparing them with the recoveries obtained from a known amount of the corresponding standard DNP-amino acids treated under the same conditions of acid hydrolysis and chromatography as those for the dinitrophenylated pepsin hydrolysate of glutenin. Recoveries varied from 31 to 59%, for glycine and valine, respectively, in the N-terminal determination under the described experimental conditions.

C-Terminal amino acids. C-Terminal amino acids of the pepsin-treated glutenin were estimated by the hydrazine decomposition method of Akabori *et al.* (1952). One ml of solution from various stages of the pepsin hydrolysis was freeze-dried. After 0.5 ml of anhydrous hydrazine was added, the sample was sealed. Hydrazine decomposition of the sample was carried out by heating the sealed ampoule for 16 hr in boiling water. The content of the ampoule was dried for 3 days in a vacuum desiccator with phosphorus pentoxide, and dissolved in 2 ml of distilled water. The solution was shaken for 1 hr at room temperature with 5 ml of benzaldehyde, and the benzaldehyde layer was removed after centrifugation. The benzaldehyde treatment was repeated twice, and then free amino acids in the water layer were dinitrophenylated by addition of 0.2 g of sodium bicarbonate and 4 ml of 5% alcoholic solution of FDNB. The C-terminal DNP-amino acids thus obtained were separated by two-dimensional paper chromatography and determined spectrophotometrically by the method given here for N-terminal groups. It should be noted that this method is not suitable for C-terminal glutamine.

The recoveries of DNP-amino acids were estimated by comparing them with the recoveries obtained from known amounts of the corresponding standard amino acids treated under the same conditions of hydrazine decomposition and of chromatography as those for the freeze-dried samples. Minimum recovery was obtained with glycine (4.1%) and maximum recovery with leucine (40%).

The carboxypeptidase method was also employed. One ml of the sample was treated with 0.1 ml of 0.6 mg/ml carboxypeptidase *A* solution in the presence of 0.1 g of sodium bicarbonate at room temperature. After specific reaction times, the reaction mixture was dinitrophenylated and the DNP-amino acids produced were separated by two-dimensional paper chromatography.

Chromatographic separation and determination of DNP-amino acids. The DNP-amino acids thus obtained for determination of N- and C-terminal groups were separated and identified by two-dimensional paper chromatography on a Whatman No. 1 filter paper. The solvent systems used were *n*-butanol saturated with 0.8*M* ammonia solution in the first dimension, and 1.5*M* phosphate buffer, pH 6, in the second.

DNP-Glutamic and aspartic acids were separated by an additional single-dimensional chromatography. The mixture of the two kinds of DNP-amino acids was extracted from the spot on the two-dimensional chromatogram. The above solvent systems did not separate these amino acids and gave only one spot for them. The extract was rechromatographed on Whatman No. 1 paper buffered with sodium acetate, using the ethyl acetate-isoamyl alcohol-sodium acetate buffer, pH 4.8, solvent system of Portugal *et al.* (1963).

DNP-valine, leucine, and isoleucine, which were difficult to separate on the two-dimensional chro-

matogram, were separated and identified by additional single-dimensional chromatography. The mixture of the three DNP-amino acids was extracted and regenerated by heating 1 hr at 100°C with saturated barium hydroxide in a sealed tube according to the procedure of Mills (1950). The regenerated sample was treated with CO_2, heated, and extracted with ether after being acidified. The water layer was concentrated in vacuum and chromatographed on Whatman No. 1 filter paper with the solvent of tertiary amyl alcohol saturated with 0.04% 8-hydroxyquinoline.

The quantity of DNP-amino acids was determined spectrophotometrically at a wave length of 360 mμ after removal of the spot from the chromatogram by extraction with 1% sodium bicarbonate.

RESULTS

Optimum pH for pepsin action on glutenin. The amount of TCA-soluble nitrogen produced from glutenin by the action of pepsin was determined at various pH values.

As shown in Fig. 1, the amount of TCA-soluble

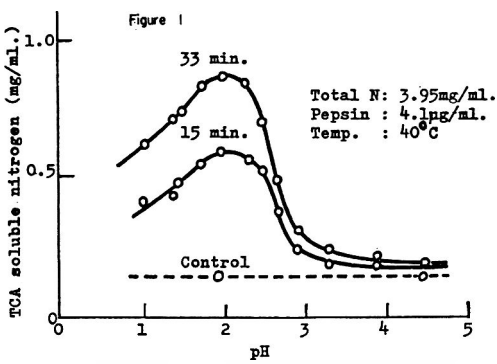

Fig. 1. Optimum pH for pepsin action on glutenin.

nitrogen produced in the reaction time employed was highest at pH 2, and decreased rapidly on either side of this value.

Increase of TCA-soluble nitrogen. The increase of TCA-soluble nitrogen was followed in the presence of various amounts of pepsin at the optimum pH value, and the time required to give a definite amount of TCA-soluble nitrogen (1 or 2 mg/ml) was determined. Table 1 shows that, under the experimental conditions employed, reaction velocity is directly proportional to enzyme concentration. The increase of TCA-soluble nitrogen is also shown in Fig. 2.

Increase of soluble nitrogen. The same glutenin-pepsin system was employed to determine the amount of nitrogen soluble in a MacIlvaine buffer at neutral pH.

Table 1. The effect of pepsin concentration on the hydrolysis of glutenin.

Concentration of pepsin (μg/ml)	Time required to give a definite amount of TCA-soluble N (min)	
	1 mg/ml	2 mg/ml
1.9	228	.....
9.5	44	184
45.6	10	35
236.0	2	7

Total N: 4.47 mg/ml. pH: 1.93. Temperature: 40°C.

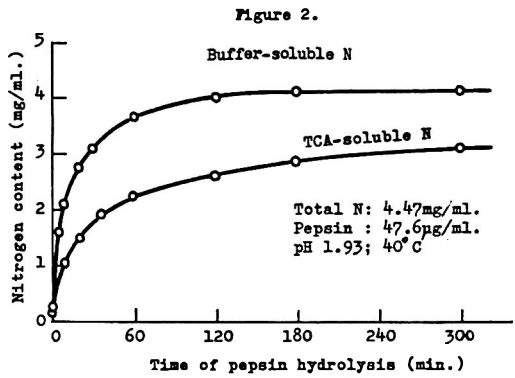

Fig. 2. Increase of buffer-soluble and TCA-soluble nitrogen during pepsin hydrolysis.

As shown in Fig. 2, the rate of formation of material soluble in buffer is considerably higher than that of material soluble in TCA.

The final constitution of the hydrolysate obtained after treatment for 48 hr is shown in Table 2. More than 90% of the nitrogen was soluble in the buffer with neutral pH, and 15% of the nitrogen was dialyzable.

Viscosity change in acid medium. The change in viscosity of a glutenin dispersion, produced by the action of pepsin, was followed at the optimum pH.

Fig. 3 shows that the viscosity of the glutenin dispersion decreased rapidly in the presence of the enzyme. The rate of decrease in viscosity was proportional to the concentration of the enzyme, as was the case of the TCA-soluble nitrogen.

Table 2. Nitrogen distribution in a 48-hr pepsin hydrolysate of purified glutenin.

Fraction	N-content (mg/ml)	%
Soluble-nondialyzable [a]	3.51	78.5
Soluble-dialyzable [a]	0.67	15.0
Insoluble [a]	0.29	6.5

[a] pH: 6.5.
Total N: 4.47 mg/ml. Pepsin: 47.6 μg/ml. Temperature: 40°C.

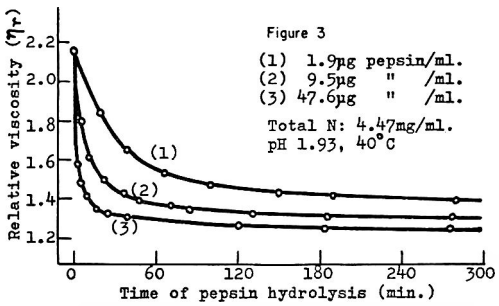

Fig. 3. Decrease of viscosity with time for glutenin solutions containing three different concentrations of pepsin.

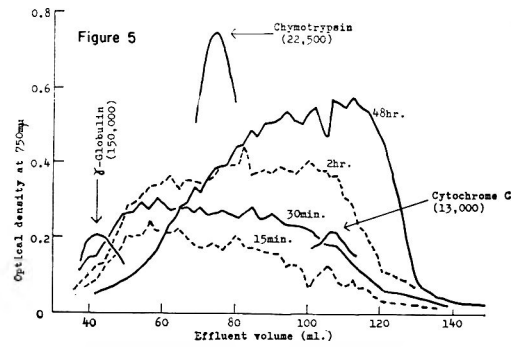

Fig. 5. Gel filtration of buffer-soluble fraction of pepsin hydrolysate of glutenin.

Fig. 4 shows the effect of temperature on the decrease of viscosity of glutenin. The rate of decrease was approximately doubled by an increase of 10°C in the reaction temperature, and the temperature coefficient, Q_{10}, was estimated to be about 2.

Molecular weight distribution of pepsin hydrolyzed glutenin. To observe the change in molecular weight distribution with respect to time of pepsin treatment in pepsin-hydrolyzed glutenin, the solubilized fraction was examined with a Sephadex G-100 column. Molecular weights were estimated by the method of Whitaker (1963).

Fig. 5 indicates that the molecular weight of most of the material in the fractions treated for different times is distributed from 10,000 to 30,000, and that increasing the time of hydrolysis shifts the molecular weight distribution toward the smaller value. After 48-hr treatment, the molecular weight still remains in the range of 10,000 to 20,000 even though the hydrolysis is almost complete.

Terminal amino acids determination. The increase in N- and C-terminal amino acids in a hydrolysate of glutenin was investigated.

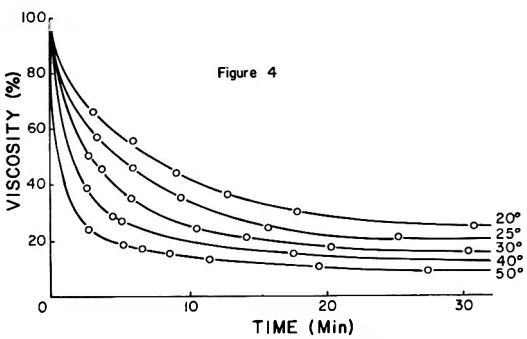

Fig. 4. Decrease of viscosity with time at 20, 25, 30, 40, and 50°C for glutenin solutions containing 4.47 mgN/ml and 47.6 μg/ml pepsin at pH 1.93 (zero-time viscosities are taken as 100% and viscosities after 24 hrs are taken as 0%).

As shown in Fig. 6, pepsin treatment of glutenin produces a large increase in N-terminal glutamic acid and valine. Phenylalanine, serine, alanine, threonine, aspartic acid, glycine, and lysine were detected as minor amino acids. Since the rechromatography of the DNP-valine fraction regenerated by barium hydroxide showed approximately 20% of isoleucine, isoleucine was also considered as a minor N-terminal amino acid. At 48 hr of hydrolysis, the total amount of N-terminal amino acids was computed to be 389 μmol/g of protein and that of free amino acids detected by the DNP-method was estimated to be 30 μmol/g of protein.

C-terminal determinations were considered to be semiquantitative because of low recoveries ranging from 4.1 to 40% for hydrazine-treated amino acids. Fig. 7 shows that leucine, phenylalanine, and alanine were the major C-terminal amino acids.

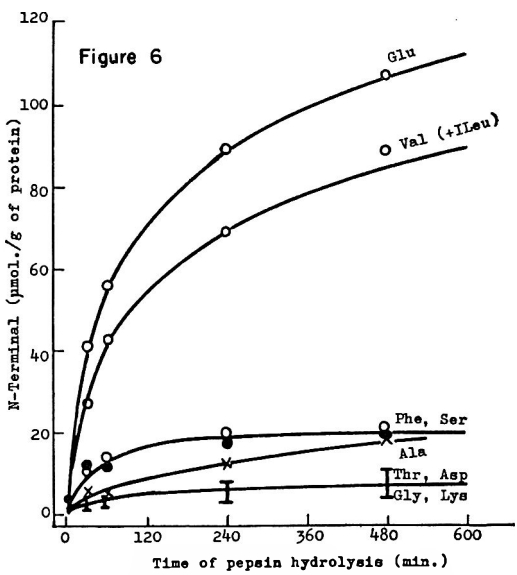

Fig. 6. N-Terminal amino acids appearing during treatment of glutenin with 47.6 μg/ml pepsin at pH 1.93 and 40°C.

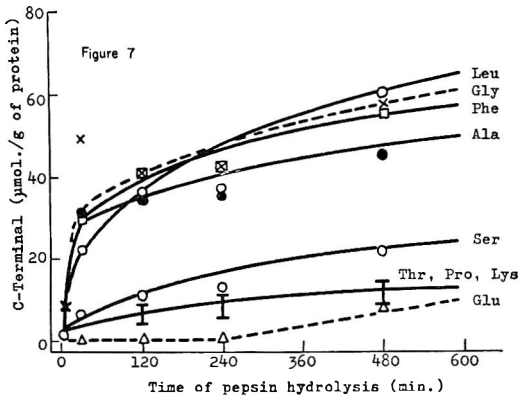

Fig. 7. C-Terminal amino acids appearing during treatment of glutenin with 47.6 μg/ml pepsin at pH 1.93 and 40°C.

In this case, the leucine fraction did not include valine and isoleucine. Glycine was also detected as a major acid, but its status is highly questionable because of only 4.1% recovery for hydrazine-treated glycine. Carboxy-peptidase treatment of a pepsin hydrolysate also gave a rapid increase in leucine and phenylalanine, but not of alanine and other amino acids, as shown in Table 3. Accordingly, this suggests that leucine and phenylalanine, and perhaps alanine, are the major C-terminal acids appearing during pepsin hydrolysis.

DISCUSSION

Major changes observed during pepsin hydrolysis are summarized in Fig. 8. Changes are expressed in terms of percentage of reaction obtained during hydrolysis for 48 hr. Considerable differences are indicated between rates of change in different properties of glutenin. As an indication of the degree of modification, the time required to give 50% of each of the changes ($T_{0.5}$) is given in the first column of Table 4. The values in the second column are the actual

Table 3. Detection of C-terminal amino acids in pepsin hydrolysate (24 hr) by the use of carboxypeptidase A.

Amino acid	Amount of amino acids increased (μmol/g of protein)		
	Blank (no enzyme)	15 min	120 min
Phenylalanine	3.2	32	32
Leucine	trace	16	61
Alanine	1.3	1.2	1.6
Glycine	trace	trace	trace

Concentration of carboxypeptidase A: 0.06 mg/ml. Sodium bicarbonate: 0.1 g/ml. Temperature of reaction: 27°C.

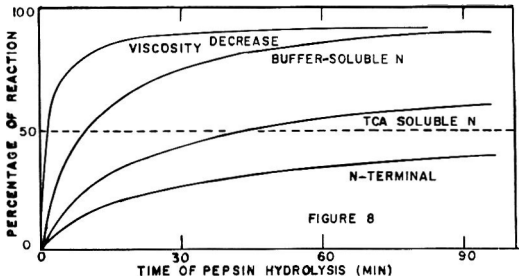

Fig. 8. Comparison of changes in viscosity, soluble nitrogen, TCA-soluble N and N-terminal groups during pepsin hydrolysis of glutenin.

amount of the terminal groups appearing at this stage. The values in the last column are the average molecular weights of the products in the hydrolysate as estimated from the amount of terminal groups. The high molecular weight value of 91,000 calculated from N-terminal groups after 1.3 minutes treatment, suggests that in fairly intact glutenin some end groups may be inaccessible to the dinitrophenylation reagent.

The estimations of molecular weights of the solubilized products in the hydrolysate by the gel filtration method indicate values from 10,000 to 30,000 for the early stage of hydrolysis, and 10,000 to 20,000 for the final stage. At the early stage of hydrolysis these values are in the same range as values estimated from terminal groups. After 48 hr of treatment, the values estimated from the terminal groups are much lower than those from the gel filtration method, as would be expected if many of the peptides produced contain disulfide bonds.

Nielsen *et al.* (1962) indicated that glutenin has a basic peptide unit with a molecular weight of about 20,000, and that various numbers of these units are linked via disulfide bonds to form the large protein molecule. The contribution of disulfide bonds was estimated to be 1.3 molecules per

Table 4. Comparison of rates of change of properties of glutenin upon pepsin treatment.

Reaction	$T_{0.5}$ (min)	Total N-terminal groups (μmol/g)	Av. MW
End group	180	184	5,300
TCA-soluble N	46	117	8,600
Soluble N	9.9	59	17,000
Viscosity	1.3	11	91,000

$T_{0.5}$ = Time for 50% of the change measured.

unit of polypeptide based on the cystine content of glutenin. With such a molecular constitution, two kinds of cleavage of peptide bonds can be considered with glutenin molecules. One consideration is that pepsin splits a peptide bond between two disulfide bonds on a polypeptide and divides the large molecule of glutenin into two high-molecular-weight fragments. Such cleavage can reduce the molecular weight of the protein and the viscosity very rapidly. Another possibility is that cleavage outside the disulfide bond produces only small peptides, and there is less effect on the size of the glutenin molecule.

It is to be expected that the rapid decrease in viscosity is due to the cleavage of the molecule between disulfide bonds. It appears likely that most of the solubilization is due to this kind of cleavage. However, the production of dialyzable nitrogen may be due to cleavage of the molecule outside of the disulfide bonds. In this case, the distribution of pepsin-sensitive peptide bonds might be greater near the ends of the polypeptides rather than at the center because further treatment results in only a small shift in the molecular-weight distribution of the main fragments of the hydrolysate.

It is not possible to designate the specific pepsin-sensitive bonds at this time, because the amino acid sequence in glutenin is unknown. However, glutamic acid and valine were the major N-terminal amino acids, and leucine, phenylalanine, and alanine were the major C-terminal amino acids produced during pepsin hydrolysis. This suggests that pepsin-sensitive peptide bonds in the glutenin molecule involve some combination of these specific amino acids.

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Papaya Pectinesterase Inhibition by Sucrose

SUMMARY

Pectinesterase (PE) was inhibited by sucrose at the same concentrations that delayed gelation of papaya purée. The inhibitory effect was linear with sucrose concentration throughout the range investigated (up to 50% sucrose). Conditions for optimum PE activity, pH 7.5 and 0.2*M* NaCl, were not affected by inhibitory concentrations of sucrose. Evidence was obtained against competitive inhibition, transferase activity, and an effect on the binding of PE as possible mechanisms for inhibition. Other sugars tested, including glucose, maltose, and corn syrups of varying degrees of hydrolysis, as well as glycerol were also inhibitory.

INTRODUCTION

Pectinesterase (PE) activity results in gelation of papaya purée. Heat inactivation of PE (Aung and Ross, 1964) and the use of polygalacturonic enzymes to prevent gelation have been reported previously (Seagrave-Smith and Sherman, 1954). Recent investigations showed that gelation of papaya purée was inhibited when the natural dissolved solids were increased by adding sucrose in 13% concentration immediately after preparation of the purée (Allen and Chan, 1964; Yamamoto and Inouye, 1963). Inhibition of gelation only when sucrose was added immediately, suggested that gelation inhibition was due to inhibition of PE activity. This paper concerns investigations on inhibition of papaya PE by sucrose and other sugars in model systems.

EXPERIMENTAL METHODS

Assay methods. Protein concentrations were estimated by absorbance at 260 and 280 mμ. Pectinesterase activity was determined at 30°C by constant pH titration of unbuffered solutions with 0.02*N* NaOH using a Coleman Model 32 automatic titrator. The reaction vessel, an 80-ml jacketed beaker, was kept at 30°C by circulating water through the jacket. The reaction mixture was stirred continuously during titration with a teflon coated magnetic bar and magnetic stirrer of the automatic titrator. Unless otherwise indicated, 0.2 ml enzyme solution, containing approximately 6×10^{-1} units PE, was added to 30 ml of test solution, previously adjusted to within 0.5 pH of the desired experimental condition. After addition of enzyme, the solution was adjusted to within ±0.1 pH of the desired value and the reaction was followed for 5 min. At pH's above 7.5, a boiled enzyme solution was used to correct for alkali consumption due to nonenzymatic de-esterification. The unit of PE activity is the μmole of acid released per minute in a solution of 0.5% pectin and 0.2*M* NaCl. Nutritional Biochemicals "Pectin NF" was used throughout this study.

Partial purification of papaya pectinesterase. Fully ripened whole papayas were chopped in a Fitz Mill, Model D, and then passed through a Langsenkamp pulper fitted with a 0.020-inch screen. The resulting purée was held at room temperature for 4 hr to promote gelation and then stored at 0°F until used.

After the purée was thawed overnight, Hyflo-Supercel (5% by weight) was added to it and the mixture pressed in a horizontal bag press. The first press juice, containing 8% of the total fresh activity, was discarded. The press cake was reconstituted with a volume of 2*M* NaCl equal to the amount of first press juice, and the mixture was re-pressed. The second press juice was collected and Hyflo-Supercel (approximately 1 g/unit PE) was added as carrier. The mixture was first dialyzed overnight against 0.05*M* $NaHSO_3$, and, finally, against several changes of demineralized water until free of bisulfite. The dialyzed preparation was centrifuged and then lyophilized. PE eluted from the dried preparation with 0.5*M* NaCl had a specific activity ranging from 27 to 37 units/g protein, approximately a 20× purification.

RESULTS AND DISCUSSION

Effect of pH and NaCl concentrations on sucrose inhibition of pectinesterase activity. Preliminary experiments demonstrated that sucrose concentrations inhibiting gelation of papaya purée also inhibited PE activity. The pH and salt dependency of PE is well known (Lineweaver and Ballou, 1945; MacDonnell *et al.*, 1945). The relative activity of partially purified papaya PE from pH 6.0 to 9.0 in 0.2*M* salt concentration, and the relative activity in varying salt concentrations at pH 7.5, were deter-

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mined. Fig. 1 clearly shows that PE activity was inhibited by 25% sucrose throughout the pH range investigated. The optimum pH of 7.5 was apparently the same in the presence and absence of sucrose, although in the presence of sucrose a plateau rather than a decrease in activity at alkaline pH's was observed. Because the inhibitory effect of sucrose was greatest at pH 7.5, further studies were done at this pH. Fig. 2 shows that 0.2*M* salt concentration gave optimum activity at pH 7.5 in the two systems. These results indicated that inhibition by sucrose

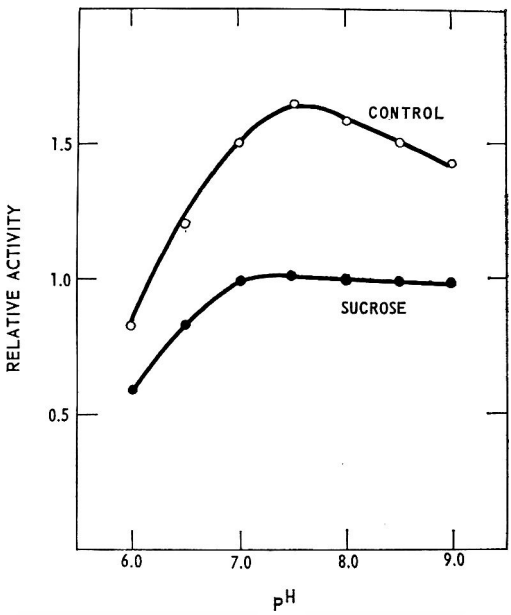

Fig. 1. Effect of pH on papaya pectinesterase activity.

was not due to alteration of the pH optimum or of the effective salt concentration.

The effect of varying sucrose concentration. Both 7 and 13% sucrose concentrations, which are respectively equivalent to final soluble solids concentrations of approximately 20 and 26° Brix, inhibited gelation of papaya purée (Yamamoto and Inouye, 1963). Several investigators (Bissett *et al.*, 1953; McColloch *et al.*, 1956; Rouse *et al.*, 1960; Wenzel *et al.*, 1951) have shown that the degree of concentration influences gelation and the cloud loss of frozen orange concentrates. Stability decreased with increased concentrations up to approximately 40° Brix; further increases in concentration promoted stability of the concentrate. Rouse *et al.* (1961) attributed stabilization by higher solids concentrations to PE inhibition. McColloch *et al.* (1956) showed that the effect of concentration on cloud stability was the result of changes in sugar and citrate concentrations. However, they did not attribute the phenomenon to an effect on PE activity.

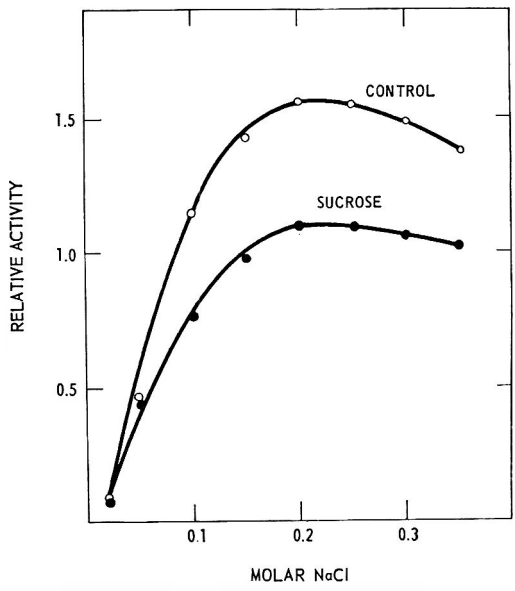

Fig. 2. Effect of salt on papaya pectinesterase activity.

It appeared that stabilization of orange juice concentrates at high Brix might be related to the gelation inhibition of papaya purée by sucrose. The inhibitory effect of sucrose in concentration up to 50% was therefore investigated. Fig. 3 shows that PE inhibition was linear throughout the range investigated. It is evident that PE inhibition by sucrose could be demonstrated at concentrations which inhibit gel development in papaya, as well as at concentrations which are effective in promoting cloud stability of orange juice.

The effect of other sugars on PE activity. Further investigations were performed to characterize the inhibition and to attempt to elucidate the mechanism. PE activity in the presence of 25% concentrations of sucrose, glucose, corn syrups of three different degrees of hydrolysis (Corn Products low-DE Type 1021, regular-DE Type 1132, and high-DE Type 1621), and glycerol were

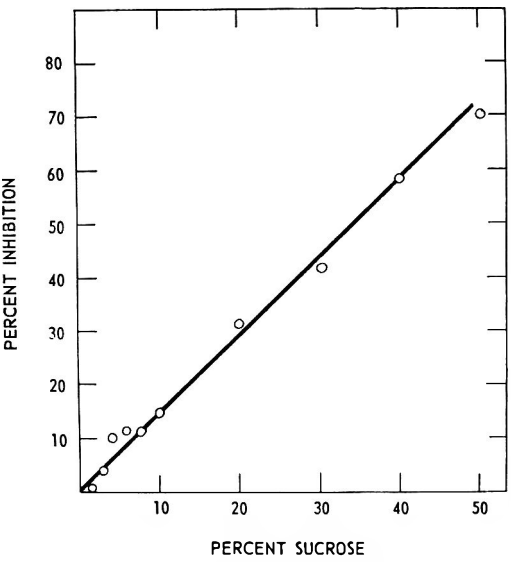

Fig. 3. Effect of sucrose concentration on papaya pectinesterase inhibition.

determined at different pectin concentrations to determine their inhibitory capacity as well as to characterize the inhibition. Fig. 4 shows the result of this study in the form of Lineweaver-Burke plots. Km for uninhibited papaya PE was 0.03 percent pectin. It is evident that all compounds tested were

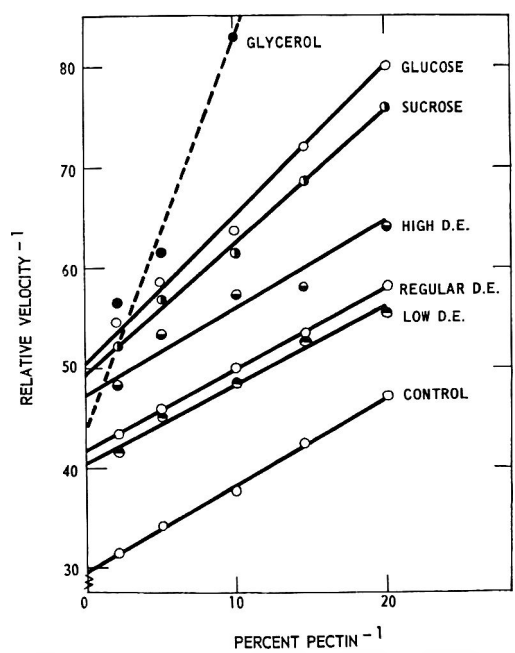

Fig. 4. Papaya pectinesterase inhibition by 25% concentration of various sugars and glycerol.

inhibitory to varying degrees of effectiveness. The relative order of increasing effectiveness, as indicated by the relative positions of the slopes, was: low DE, regular DE, high DE, sucrose, glucose, and glycerol. Since this order corresponds inversely to molecular weight, the observed differences may have resulted from differences in molar concentration of inhibitor. High sugar concentrations utilized in these studies in effect result in lowered water activity, which is less suitable for enzymatic action. The general inhibitory effect shown by all the sugars gives support to this hypothesis. The relative slopes indicate that glycerol, glucose, and sucrose were noncompetitive inhibitors, while the corn syrups were uncompetitive inhibitors. Although the inhibition may be classified in these terms it is doubtful whether the usual interpretations of such results could be applied in these cases. The high concentrations of inhibitor and possible interaction of inhibitor and substrate undoubtedly cause complex interactions. It is clear, however, that the sugars are not competitive inhibitors.

Effect of inhibitor on maximum degree of ester hydrolysis. The possibility that sucrose may affect not only the *rate* of hydrolysis but also the *maximum degree* of hydrolysis was investigated by comparing maximum hydrolysis obtained in the presence and absence of inhibitors. The results in Fig. 5 indicate that both sucrose and glycerol affected only the rate of hydrolysis and not the degree of hydrolysis.

Transferase activity. Hydrolytic enzymes often exhibit transferase activity. An inhibitory effect would be expected if sugar molecules were being transferred for methoxyl groups in pectin. Transferase activity as a possible mechanism was investigated by allowing PE to act on pectin in the presence of 25% glucose containing 6×10^5 counts per minute (cpm) in UL-C^{14} glucose. The reaction was carried out for 5 min in 10 ml of 0.1% pectin and 0.2*M* NaCl. A heated enzyme preparation served as control. The degree of inhibition was equivalent to 13.9 meq of methoxyl groups and was expected, theoretically, to give a total difference in incorporation of 392 cpm. After the reaction was stopped with hot ethanol, pectin was

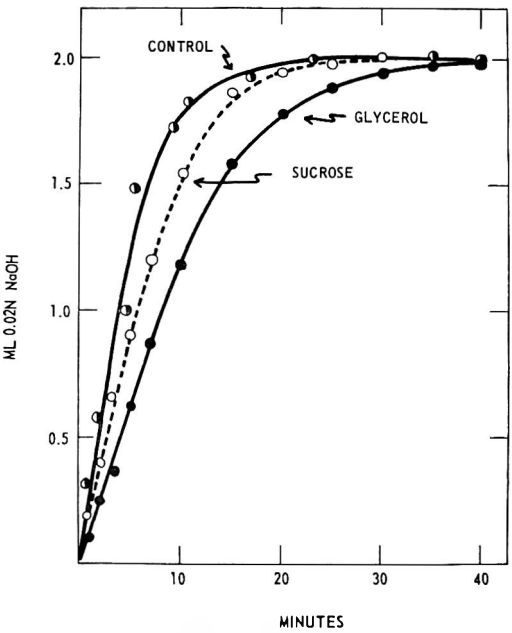

Fig. 5. The effect of inhibitors on the extent of pectin de-esterification.

reisolated by two precipitations with ethanol, followed by the addition of 1 g cold glucose and dialysis of the mixture for 48 hr against several changes of distilled water. The dialyzed pectin was reprecipitated with ethanol, washed with acetone, dried in a desiccator, and ground to a fine powder, and the radioactivity determined. Since no difference in activity was observed between the control (315 cpm) and inhibited sample (330 cpm), transferase activity was presumed to be absent.

Solubilization of pectinesterase from cell wall material. Jansen *et al.* (1960), investigating the binding of orange PE, showed that bound PE was inactive at pH 4–4.5 but that extracted PE showed activity in this range. Solubilization of bound orange PE at pH 3.8 required soluble pectin and salt. At pH 7.5, only salt was required for solubilizing of bound PE but de-esterification of cell-wall pectic material paralleled enzyme solubilization.

The possibility was investigated that sucrose inhibited gelation of papaya purée by affecting PE solubilization. The effect of sucrose on soluble PE concentration in papaya purée was observed by comparing PE activity in the supernatant of purée centrifuged after preparation and after 19 hr at 36°C. The development of gel was determined as previously described (Yamamoto and Inouye, 1963). The results (Table 1) show that the change in the soluble PE concentration initially and after 19 hr of incubation was nearly identical for samples with and without sucrose. On the other hand, gel strength increased appreciably in the sample without added sucrose and only slightly in the sample with sucrose. These results therefore indicate that gelation inhibition is probably not an effect of sucrose on the solubilization of enzyme.

The results do not indicate the mechanism of PE inhibition by sucrose. They do suggest, however, that the inhibition is probably the result of several factors, including an environment of lowered water activity, inhibitor-pectin interaction, and perhaps some degree of interference of the active enzyme site. The inhibition is not the result of altered pH optimum or salt requirement. The results clearly show that PE activity is inhibited by sugar concentrations normally found in juice and concentrates. The total factors involved in the effect of sucrose on papaya purée gelation are probably complex, involving several physiocochemical and biochemical effects. The present results show that a significant factor in the inhibition of gelation of papaya purée by sucrose is the inhibitory effect of sucrose on PE activity.

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Table 1. The effect of sucrose on solubilization of PE and gel strength in papaya purée.

Time	Gel strength (g)		PE units/ml × 10^{-1} in supernatant	
	No sucrose	13% sucrose	No sucrose	13% sucrose
Initial	5	5	3.4	3.0
19 hr at 36°F	17	6	2.7	2.5

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The Influence of Ante-Mortem Factors and Gamma Irradiation on the Degradation of 5′-Ribonucleotides in the Muscle of English Sole (Parophrys vetulus)

SUMMARY

The influence of feeding, activity and pasteurizing doses of gamma irradiation on the nucleotide composition and breakdown in English sole *(Parophrys vetulus)* was studied. Feeding did not cause any significant change in the total amount of nucleotides. Active fish had lower amounts of ATP and increased levels of IMP, but this difference was not significant after they had been stored 7 days in ice. In contrast to the results reported for other species of sole, inosine was present in English sole after 7 days in ice. Irradiation, 0.5 and 1.0 Mrad, did not affect the changes in the nucleoside and nucleotide composition.

INTRODUCTION

Irradiation pasteurization of fish results in quantitative and qualitative changes in the associated microbiological flora (Mac Lean and Welander, 1959; Shewan and Liston, 1958; Masurovsky *et al.*, 1963a,b). As a result, the common indices of quality for fish, TMA (trimethylamine), TVB (total volatile bases), VRS (volatile reducing substances), and TVA (total volatile acids), which are essentially measures of bacterial activity, are not entirely satisfactory when extrapolated from results obtained with unirradiated fish (Shewan and Liston, 1958).

Post-mortem formation of hypoxanthine in the muscle of fish is found to correlate well with freshness, even during the period preceding significant microbiological invasion of the tissues (Saito *et al.*, 1959a; Kassemsarn *et al.*, 1963; Jones and Murray, 1962; Spinelli *et al.*, 1964). The formation of this product in fish tissues can be assayed rapidly (Spinelli, personal communication) and has been proposed as an index of freshness for fish (Saito *et al.*, 1959a; Kassemsarn *et al.*, 1963; Shewan and Jones, 1957; Spinelli *et al.*, 1964). It is possible that this index of freshness could be applied to irradiated products should the enzymes involved in the degradation of nucleotides and nucleosides be unaffected by pasteurizing doses of gamma irradiation.

In addition to their potential value as indices of freshness, nucleotides and related compounds have been found to play an important contributory role in the flavor of fish and other food products (Saito, 1960; Jones, 1961; Kassemsarn *et al.*, 1963; Kuninaka *et al.*, 1964; Shimazono, 1964; Wagner *et al.*, 1963). Of particular importance is the fact that IMP has been reported to be "an effective suppressor of sulfide-like flavor notes" and some radiation off-flavors have been associated with sulfur compounds (Wagner *et al.*, 1963; Merrit *et al.*, 1959).

Before the hypoxanthine test of freshness can be applied readily to irradiated products, it is necessary to determine the effect of both ante-mortem and post-mortem changes upon the activity of endogenous enzymes responsible for the formation and degradation of nucleotides and nucleosides.

METHODS AND MATERIALS

English sole (*Parophrys vetulus*) were caught by the Research Vessel Commando with an otter trawl. They were transported alive to the salt-water aquarium of the College of Fisheries. Fish, 12–16 inches long and weighing 300–400 g, that survived the initial ten days in the aquarium were separated into two groups. One group was starved for three weeks prior to sampling. The second group was force-fed for the same period. Force feeding was necessary since the fish refused to eat voluntarily. Prior to feeding they were anesthetized with tricaine methane sulfonate (MS 222) (1:10,000 w/v) dissolved in salt water. The food (50% beef liver, 10% salmon meal, and 40% bottom-fish meal) was packed in gelatin capsules (No. 000, Eli Lilly and Co., Indianapolis) and was inserted into the stomach with long forceps.

The fish were killed by a blow on the head, either in the rested condition or immediately following violent exercise. Exercise consisted of turning the fish on their backs in a pan containing a few inches of water causing them to struggle to right themselves. This was repeated until the fish were unable to turn over. Ten-gram pieces from the anterior dorsal muscle of each side of two fish were sealed in polymylar bags. One half

of each group of samples were irradiated at 0.5 and the other at 1.0 megarads at 0–4°C in a cobalt-60 source. The samples were stored in melting ice (0–1°C) for seven days.

"Sterile" samples were prepared by washing the skin of the fish with tap water, rubbing with ethanol (95% w/v), and then flaming. The skin was peeled from the flesh, and portions were dissected and freed with sterile instruments. The dissections were carried out inside a cabinet that previously had been exposed to ultraviolet light.

Viable cell count. Duplicate 40-g samples for all treatments described above were homogenized with four parts of phosphate-buffered water (0.3 m*M* phosphate, pH 7.2) (Butterfield, 1932) in a Waring blender for 2 min at high speed. Appropriate serial decimal dilutions were made in buffered water, and aliquots were plated on tryptone glucose extract agar (Difco). Total counts were made after incubation at room temperature (22–25°C) for 7 days.

Nucleotide determination. After treatment, duplicate 40-g samples were extracted with 80 ml of cold 0.7*N* perchloric acid by blending in the cold for 2 min. The blended material was filtered through a Whatman No. 1 filter paper, and the material retained was washed with several 10-ml portions of chilled 0.7*N* perchloric acid. The filtrate was neutralized to a final pH of 6.8 with 10% KOH. After standing in the cold for 30 min the insoluble potassium perchlorate was precipitated on a refrigerated centrifuge. The samples were made up to a known volume with distilled water and then were kept frozen at −40°C until analyzed.

An aliquot of the extract equivalent to 10 or 20 g of flesh was analyzed for nucleotides on a 1.3 by 18-cm refrigerated (0°C) column of Dowex 1 × 8, 200 to 400 mesh (formate). The elution system used was essentially that of Jones and Murray (1960), but sodium formate was substituted for ammonium formate. The peaks were detected with a Vanguard Model 1056 automatic UV analyzer and collected in 10-ml fractions. The system was standardized against nucleotides obtained from Sigma Chemical Co., St. Louis, Mo. (purity confirmed by column chromatography). The various chromatographic fractions from four different fish flesh extracts were kept separate, and were analyzed for total phosphorus by the methods of LePage (1957) and Fiske and Subbarow (1925), and for labile phosphorus by the method of Jones and Murray (1960). In addition, the ultraviolet absorption spectra of these peaks were plotted (Beckman DK spectrophotometer) and compared with standard curves in Circular OR-10, Pabst Laboratories.

The total concentration of nucleotides was estimated by measuring the OD at 260 mμ on the combined samples corresponding to one peak after they had been adjusted to pH 2.0.

In general, the resolution of all peaks was good, but in some cases DPN and AMP eluted partially superimposed. In these cases they were estimated by simultaneous equations reading the OD at 325 and 260 mμ by the cyanide addition method of Ciotti and Kaplan (1957).

The samples were read against formate blanks and the concentrations were calculated on the basis of absorbance values in Circular OR-10, Pabst Laboratories.

Nucleoside determination. All the effluent absorbing at 260 mμ that was not retained by the formate column when eluted with distilled water was combined and is referred to as the nucleoside sample. These were kept frozen at −40°C until analyzed by the method of Jones (1960). The concentration of individual nucleosides was determined on the basis of OD at 251 mμ for inosine and 248 mμ for hypoxanthine (pH 2). The hypoxanthine concentration was confirmed by the xanthine oxidase enzyme method of Kalckar (1949).

RESULTS

After seven days in ice, less than 10^2 to 10^3 bacteria/g were present in the sterile unirradiated or irradiated samples. Thus bacterial enzymes could not cause any significant changes to the nucleotides or nucleosides in these experiments.

The ion-exchange system used for the analysis of nucleotides was found to be satisfactory. Sodium formate, because of its higher ionization, resolves the nucleotides more rapidly than ammonium formate. However, the sodium formate cannot be removed completely from the collected nucleotides and interferes with the spectrophotometric measurements at shorter wave lengths (220–235 mμ) as indicated by the red shift in the minimum wave length absorption for ATP at pH 2 and 11 (Table 1).

Total and labile phosphorus for ADP, IMP, and AMP varied greatly (Table 1). This variation was probably due to contamination of the pooled samples with sugar phosphates, which do not absorb at 260 mμ. The presence of sugars in these samples was confirmed by the anthrone method as described by Ashwell (1957). However, no separation of the nucleotide from the sugars was undertaken.

DISCUSSION

The total adenine nucleotides (excepting NAD) and IMP extracted from the muscle of rested English sole (4.95–5.71 μmoles/g) (Table 2) were lower than most of the

Table 1. Identification of nucleotides from English sole *(Parophrys vetulus)*.

Fraction no. (10-ml volume)	Elution similar to	Absorption peaks (λ in mμ) Max pH 2	Max pH 11	Min pH 2	Min pH 11	As ratios at pH 2 250/260	As ratios at pH 2 280/260	Phosphorus/purine[a] Labile	Phosphorus/purine[a] Total
230–290	ATP	257	259	237	234	0.86	0.22	1.8	2.9
110–130	ADP	257	259	230	231	0.91	0.24	[b]	[b]
90–110	IMP	249	254	222	224	1.65	0.23	[b]	[b]
42–52	AMP	257	NT[c]	229	NT	0.88	0.22	[b]	[b]
35–42	NAD	257	NT	235	NT	0.92	0.29	0	1.8

[a] μmoles phosphorus per μmole purine.
[b] Unable to determine.
[c] NT, not tested.

values reported for other species: codling 7.24 μmoles/g (Jones and Murray, 1960), carp 6.74 μmoles/g (Saito and Arai, 1958), trout 6.47–7.80 μmoles/g (Saito *et al.*, 1959b), sockeye salmon 1.45–7.90 μmoles/g (Tarr and Lerroux, 1962). They are similar to those reported for halibut 4.48 μmoles/g (Tarr and Lerroux, 1962), and higher than the 2.41 μmoles/g reported by Tarr and Lerroux (1962) for Atlantic cod.

Table 2. Nucleotides and nucleosides (μmoles/g) in English sole *(Parophrys vetulus)* muscle immediately after death.

	Rested Fed	Rested Not fed	Active Fed	Active Not fed
ATP	4.42	3.91	2.83	2.15
ADP	0.52	0.23	0.70	0.94
AMP	0.09	0.08	0.23	0.17
IMP	0.68	0.73	1.66	2.11
NAD	0.06	0.05	0.02	0.04
Total nucleotides	5.77	5.00	5.44	5.41
Total nucleotides minus NAD	5.71	4.95	5.42	5.37
Inosine	0.06	0.40	0.10	0.09
Hypoxanthine	0.01	0.13	trace	trace
Total nucleosides	0.07	0.53	0.10	0.09
Total nucleotides and nucleosides	5.84	5.53	5.54	5.50

There was no decrease in the total nucleotides (5.44–5.41 μmoles/g) in the fresh, unstored fish muscle due to activity (Table 2). The conversion of ATP to IMP in the muscle of exercised fish was considerably less than has been reported before (Jones and Murray, 1960). This difference may be real, or, more likely, the fish may not have been completely exhausted. It is difficult to duplicate physiological conditions prior to killing.

The levels of IMP (Table 3) in the muscle of English sole after seven days of ice

Table 3. Nucleotides (μmole/g) in English sole *(Parophrys vetulus)* muscle after 7 days' storage in ice.

Radiation dose	Rested 0	Rested 0.5 Mrad	Rested 1.0 Mrad	Active 0	Active 0.5 Mrad	Active 1.0 Mrad
Not fed						
ATP	0.16	0.11	0.09	0.07	0.13	0.13
ADP	0.11	0.04	0.17	0.05	0.14	0.14
AMP	0.05	0.02	0.11	0.06	0.08	0.10
IMP	0.07	0.00	0.10	0.14	0.07	0.07
NAD	0.02	0.00	0.01	0.01	0.02	0.01
Total	0.41	0.17	0.48	0.33	0.44	0.45
Fed						
ATP	0.10	0.11	0.11	0.06	0.10	0.08
ADP	0.10	0.14	0.10	trace	0.09	0.10
AMP	0.07	0.11	0.11	0.04	0.11	0.09
IMP	0.13	0.10	0.06	0.11	0.05	0.06
NAD	0.01	0.01	0.01	trace	0.01	trace
Total	0.41	0.46	0.39	0.21	0.36	0.33

storage (0.05–0.14 μmoles/g) are very low when compared to those reported for other species after similar storage: cod approx. 1 μmole/g, plaice approx. 0.8 μmole/g (Kassemsarn *et al.*, 1963), and Pacific salmon (5 days in ice) 1.08 μmole/g (Creelan and Tomlinson, 1960). Saito *et al.* (1959b) reported values of 5.42 and 0.62 μmoles/g of IMP for the dorsal and red lateral muscle of rainbow trout after 72 hr in ice. Tarr and Lerroux (1962) found 0.27 μmole/g of IMP in the muscle of ling cod held six days at 0°C.

Conversely, the levels of hypoxanthine (Table 4) found after 7 days of ice storage (1.80–5.20 μmoles/g) are higher than those reported for other species after similar storage: cod 1 μmole/g (Jones and Murray, 1962), haddock approx. 0.5 μmole/g, lemon sole 1 μmole/g, plaice approx. 1.5 μmole/g (Kassemsarn *et al.*, 1963). Hypoxanthine was not found in any significant amounts in Pacific salmon (Creelman and Tomlinson, 1960). Hypoxanthine values for the dorsal and red lateral muscle of rainbow trout after 72 hr in ice are respectively reported as 0.58 and 1.25 μmole/g (Saito *et al.*, 1959b).

The seven-day values for inosine (Table 4) were variable (0.00–0.69 μmole/g). In one case, 0.40 μmole of inosine was found in a fresh (unstored) rested fish. English sole varies in this respect from the pattern of nucleotide breakdown described for two other species of sole, lemon sole (Kassemsarn *et al.*, 1963) and petrole sole (Spinelli, personal communication), where no accumulation of inosine was evident.

The over-all post-mortem degradation of nucleotides seems to proceed much faster in English sole than in most other described species. This could be due to the greater activity of the 5'-nucleotidases in this species. It would be of interest to study the degradation of nucleotides in this species in more detail and establish the intermediate values of the different compounds for storage periods between 0 and 14 days in ice.

So far, as the results indicate, pasteurizing doses of gamma-ray irradiation have no significant effect on the action of nucleotidases and nucleosidases—at least no significant effect that manifests itself in different rates of accumulation of the intermediate and final products of nucleotide breakdown.

The average of the total nucleotide and nucleoside content of all fed-fish was 5.41 μmole/g, as opposed to 5.02 μmole/g for the unfed fish. However, this difference was not significant when tested statistically at the 95% confidence level. At the same level, there was no difference between the average of the total nucleotide and nucleoside content of active fish (5.15 μmole/g) and rested fish (5.28 μmole/g).

Table 4. Nucleosides and nucleotides (μmoles/g) in English sole *(Parophrys vetulus)* muscle after 7 days' storage in ice.

	Rested			Active		
Radiation dose	0	0.5 Mrad	1.0 Mrad	0	0.5 Mrad	1.0 Mrad
Not fed						
Inosine	0.00	0.15	0.24	0.30	0.69	0.40
Hypoxanthine	5.00	1.80	5.20	3.76	4.59	4.77
Total nucleosides	5.00	1.95	5.44	4.06	5.28	5.77
Total nucleotides and nucleosides	5.41	2.12	5.88	4.39	5.72	5.62
$\frac{\text{Total nucleotides}}{\text{Total nucleosides}} \times 100$	8.2	8.7	8.8	8.1	8.3	8.7
Fed						
Inosine	0.12	trace	0.12	trace	0.12	trace
Hypoxanthine	4.95	5.05	4.95	4.96	4.91	4.59
Total nucleosides	5.07	5.05	5.07	4.96	5.01	4.59
Total nucleotides and nucleosides	5.48	5.51	5.46	5.17	5.37	4.92
$\frac{\text{Total nucleotides}}{\text{Total nucleosides}} \times 100$	8.1	9.1	7.7	4.2	7.2	7.2

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Volatiles from a Commercial Pea Blancher. Mass Spectral Identification

SUMMARY

Volatile components from the steam above peas in a commercial blancher were isolated by adsorption on charcoal, freeze-drying of the charcoal, solvent extraction, and distillative concentration. The concentrate was further fractionated by distillation and gas chromatography. Ethanol and dimethyl sulfoxide were most abundant. The latter compound formed by the oxidation of dimethyl sulfide adsorbed on the charcoal. The less abundant components of the concentrate fractions were identified by capillary-column gas-chromatography fast-scan mass spectrometry. The most novel group of minor components thus identified was the diethyl acetals of C_1–C_6 aldehydes. It was not possible to determine from model experiments if the acetals were original components of the blancher steam or artifacts of the isolation. A control concentrate from unexposed charcoal and purified solvent differed quantitatively and qualitatively from the blancher steam concentrate.

The major effect in the initial stages of a program designed to improve canned-pea flavor was devoted to studying the composition of blanched, frozen peas. A large number of nonvolatile and volatile components of peas were identified in that study. However, it was apparent after the levels of the more abundant volatile compounds had been measured (Ralls, 1959, 1960), that many additional components were present at very low concentrations.

Isolating components present at parts-per-billion concentrations in amount sufficient for identification requires large quantities of foods. The isolation of volatile components in experiments using 2000 and 5000 lb of peas gave such small amounts of extract concentrate that artifacts of isolation confused the results. The most logical source of material from large quantities of peas is a commercial preservation operation.

This paper reports isolation of material from a steam blancher in a pea cannery during the 1962 season in California. The compounds isolated were identified primarily by capillary-column time-of-flight mass spectrometry (CAP GC-TOF MS) (McFadden and Teranishi, 1963).

EXPERIMENTAL

Collection of volatiles. The volatiles from the steam blancher were collected with the equipment shown in Fig. 1. This was installed on a roof adjacent to a commercial steam blancher, and the steam from above hot Perfection peas was pumped out of the blancher by a Nash pump. It was then led through a 3-in. pipe to a water-cooled condenser. Vapor from above this condensate was further pumped through 1-in.-diameter pipe containing activated charcoal (Columbia AC, 8–10-mesh). The collection equipment was operated continuously for 17 days (except when the blancher was washed out at the end of each shift period). A total volume of 200 gal. of condensate was collected. A total weight of 24 lb of charcoal was exposed to the condensate vapor in 12 separate 2-lb portions which were changed every 24 or 48 hr. The wet charcoal had a very characteristic odor of peas.

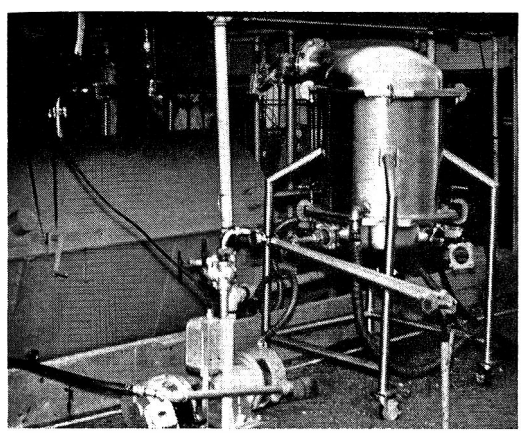

Fig. 1. Photograph of equipment used for isolation of volatile components from vapor in a commercial pea blancher.

[a] Collaborator employed by the National Canners Association with which this research was conducted cooperatively.

[b] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

The odor on the charcoal was largely retained after the removal of water by freeze-drying. Extraction of portions of the dried charcoal with four different solvents (ethyl ether, methanol, ethyl acetate, and dichloromethane) gave extracts which, after concentration, showed a number of components by gas-liquid partition chromatography (GLPC). Control runs carried out in an identically unexposed (fresh) charcoal gave much less complicated GLPC recordings. On the basis of the results of the trial solvent extractions of the exposed charcoal, the remainder of the dried charcoal (20 lb) was extracted (Soxhlet extractors) with 6 L of purified (distillation through a 25-plate Oldershaw fractionating column) dichloromethane, boiling point 40.3–40.5°.

The dichloromethane extract of the charcoal was concentrated by distillation through a fractionating column at atmospheric pressure. The residual liquid (*ca.* 150 ml) was distilled at reduced pressure. The 94.5 ml of distillate was redistilled to give 42.6 g of liquid, boiling point 70–78°/754 Torr., n_D^{25} 1.3636. This material was purified by preparative GLPC and identified as ethanol by infrared spectra and by preparation of two derivatives (ethyl 3,5-dinitrobenzoate, melting point 90.5–91.5°; and ethyl α-naphthyl urethane, melting point 77.5–78°.

After removal of the ethanol, the residue separated into 2 phases. The upper layer had a volume of 33 ml, and the lower phase a volume of 24 ml.

The major component of the lower phase of the concentrated extract was isolated by GLPC fractionation and identified as dimethyl sulfoxide (DMSO) by carbon-, hydrogen-, sulfur-analysis, infrared spectra, mass spectra, and oxidation to dimethyl sulfone, melting point 109.5–109.9°. This layer was separated by GLPC into 4 crude fractions on a 6-ft × ¼-in. 10% Carbowax 1540 column at 105° and a helium flow rate of 45 ml/min.

The upper layer (nonpolar phase) was also separated into 4 fractions on a 20-ft × ⅜-in. 25% Carbowax 20 M on Chromosorb P column operated at 108° with a helium flow rate of 170 ml/min (Fig. 2).

Mass spectrometric and gas chromatographic analysis. The individual fractions from the nonpolar and DMSO phases were qualitatively analyzed with the combined mass spectrometric-capillary GLC technique previously described (McFadden *et al.*, 1963). The nonpolar-phase fractions were analyzed on a 250-ft × 0.01-in. capillary column coated with Dow-Corning 710 silicone fluid. The polar-phase (DMSO) fractions were analyzed on a 200-ft by 0.01-in. capillary column coated with Carbowax 1540. In this technique, the effluent from a capillary column (exit at vacuum) is introduced directly into the mass spectrometer, and, as a component is eluted, the mass spectrum is recorded in about 1–4 sec.

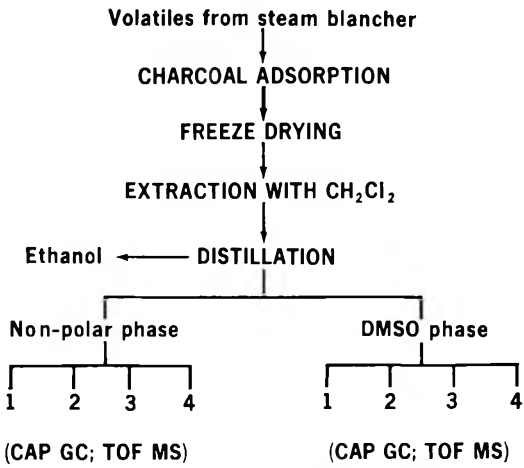

Fig. 2. Flow sheet of isolation and partial fractionation of pea steam-volatile components.

Effluent mass spectra were monitored by visual display of the pattern on an oscilloscope, and a simultaneous GLC trace was obtained by recording the ionization due to mass 43 ($C_3H_7^+$, CH_3CO^+). Because the percent ionization due to a particular mass varies from one material to another, chromatograms obtained by this method are completely nonquantitative but nevertheless provide a useful record for general reference.

As an example, Fig. 3 shows the chromatogram thus obtained during a temperature-programmed separation of the DMSO fraction 1. Fig. 4 shows representative mass spectra taken during the elution of peaks in the tempreature range 103–110°C.

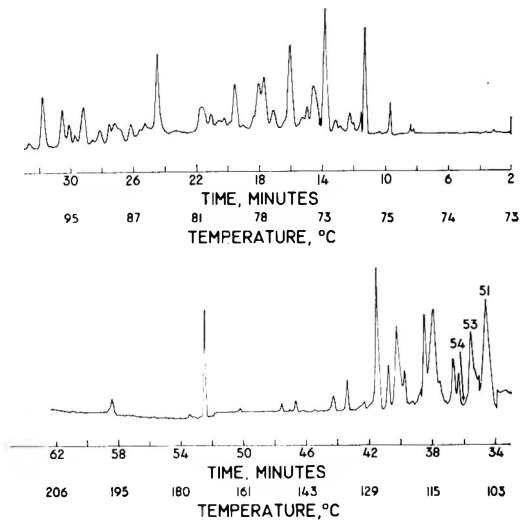

Fig. 3. Recording of mass 43 ionization from capillary-column separation of DMSO fraction 1.

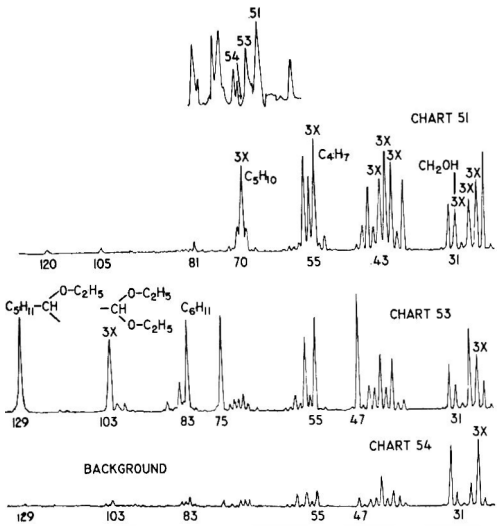

Fig. 4. Representative mass spectra of column eluates in separation of components of DMSO fraction 1 and the pertinent part of the chromatograph.

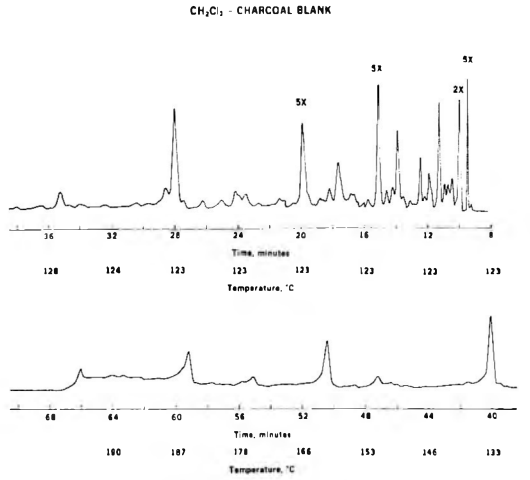

Fig. 5. Recording of mass 43 ionization from capillary-column separation of components in solvent-adsorbent blank.

The small section of the chromatograph shown in Fig. 4 indicates the places the mass spectra were run.

The various GLPC fractions were thus analyzed. Mass spectral identification was made with available reference spectra or by comparing the mass spectra from authentic compounds. Whenever possible, the retention times of the tentatively identified components were determined on the same columns with a hydrogen-flame ionization detector.

Control analyses and tests. A 300-g portion of unexposed charcoal was extracted for 8 hr with 500 ml of purified dichloromethane. The extract was concentrated to 0.5 ml by distillation. The material was still primarily dichloromethane as shown by GLPC. The extract was further concentrated to 0.072 g. This material was analyzed by the GLPC-TOF MS method, and the mass 43 recording is reproduced in Fig. 5.

Two liters of the original blancher condensate was extracted continuously with ethyl ether in a liquid-liquid extractor for 8 hr. The extract was dried and concentrated. Analysis by GLPC showed no peak corresponding to DMSO. Similar extractions of dilute aqueous solutions of DMSO with ether resulted in extract concentrates which, on GLPC analysis, gave a peak corresponding to DMSO.

The vapor above a dilute solution of dimethylsulfide in water was drawn for 8 hr through a U-tube filled with fresh charcoal. The charcoal was freeze-dried and extracted with dichloromethane. The extract was concentrated and analyzed by GLPC; a peak was found which had the same retention time as authentic DMSO.

An aqueous solution of 5 g of ethanol and 0.120 g of *iso*-valeraldehyde in 250 ml of water was placed in a 500-ml flask, and the vapor above the solution was pulled through charcoal-filled traps. The collection of the vapor continued over 16 hr, with two more additions of ethanol and *iso*-valeraldehyde to the flask. The 336 g of charcoal increased in weight to 363 g. The charcoal was freeze-dried and extracted with 500 ml of purified dichloromethane. The extract (400 ml) was dried with anhydrous sodium sulfate and concentrated by distillation to 10 ml. The concentrate was further concentrated in a rotary evaporator to 0.5 ml. Analysis of the concentrate was made on the Dow-Corning 710 column. There was a small peak corresponding in retention time to 1,1-diethoxy-3-methylbutane, but it was no larger in peak area than two adjacent peaks (unidentified). Analysis of the concentrate by GLPC-TOF-MS established the presence of 1,1-diethoxy-3-methylbutane and *iso*-valeraldehyde.

RESULTS AND DISCUSSION

The possibility of artifacts of isolation is always a serious problem in analysis of trace components. The material isolated from the impurities on the charcoal or in the solvent would have weighed a maximum of 25.92 g based on the weights of charcoal and of solvent used in the isolation. The compounds identified in the charcoal-dichloromethane blank by mass spectra are in Table 1.

Most of the major components identified in the blank run were not found as compo-

Table 1. Major components of the solvent-charcoal blank.

Halogenated	Aromatic hydrocarbons
1,2-dichloroethane	benzene
tetrachloroethene	toluene
bromobenzene	naphthalene
	6 indans and indenes
Aliphatic hydrocarbons	27 alkylated benzenes
16 $C_8 - C_{15}$ saturated and unsaturated	

nents of the exposed charcoal. Any components found in both the control and the exposed charcoal are not reported as components of the pea volatiles. With the question of artifacts from the charcoal and solvent now well defined, it is possible to state with some assurance which of the components identified in the various GLPC fractions of the charcoal extract concentrate came from the blancher steam.

The weight of the charcoal extract concentrate was 118 g. The major component of this mixture was ethanol. Ethanol has been identified in peas by a number of previous investigators (David and Joslyn, 1963; Wager, 1958).

Identification of the components in the various concentrate fractions is summarized in Table 2.

The nonpolar fractions were composed principally of saturated hydrocarbons in the range of C_{10} to C_{14}. The breadth of the eluted peaks and the mass spectral patterns showed that many isomers were present. A few unsaturated C_{10}–C_{14} hydrocarbons were observed. Since these hydrocarbons could have been from the steam used in the blanching process, they are not listed in Table 2.

As pointed out, the chromatograms were obtained by recording the ionization at mass 43 and cannot be used as a quantitative measure of the component. To a first approximation, designations in Table 2 are separated by a factor of 3–5 each so that very strong is approximately 300 times very weak.

It is well known that mass spectral evidence alone cannot always be relied upon to give positive identification, and even with supporting gas chromatographic evidence the

Table 2. Volatile compounds of green peas.

Alcohols	Abundance[a]	Aldehydes	Abundance
Methanol	m	Ethanal	s
Ethanol	excess	Butanal	m
1-Propanol	vs	2-Methylbutanal	s
2-Methyl-1-propanol	m	3-Methylbutanal	s
1-Butanol	w	Pentanal	vs
1-Pentanol	m	Hexanal	s
2-Methyl-1-butanol	s		
3-Methyl-1-butanol	m	Esters	
1-Hexanol	vs	Ethyl formate	s
3-Hexene-1-ol	vs	Ethyl acetate	vs
A heptanol	m	Ethyl propanoate	w
(primary, branched)		Ethyl-pentanoate	w
Acetals		Methyl octanoate	s
1,1-Diethoxy methane	m	(branched)	
1,1-Diethoxy ethane	m	Ethyl lactate	vs
1,1-Diethoxy butane	m	Hexenyl acetate	m
1,1-Diethoxy pentane	s	Hexanyl acetate	s
(presumably 2-me-butane		Sulfur compounds	
and 3-me-butane)		Dimethyl sulfide	s
1,1-Diethoxy n-pentane	s	Dimethyl disulfide	s
1,1-Diethoxy hexane	vs	Dimethyl trisulfide	m
1,1-Ethoxy pentoxy butane	w	Carbon disulfide	vw
1,1-Ethoxy pentoxy pentane	m	Methyl mercaptan	vw
1,1-Ethoxy propoxy hexane	vw		
1,1-Ethoxy pentoxy hexane	vw		

[a] vs = very strong, s = strong, m = moderate, w = weak, and vw = very weak on the basis of mass spectral ionization.

results should be regarded carefully. The entries in Table 2 are all fairly well established. Not listed are quite a few compounds (generally very weak to moderate) whose mass spectra only suggested structures. These include a few saturated and unsaturated esters, alcohols, acetals, a few additional aldehydes, and possibly some alkyl furanes and other heterocyclic structures. However, the mass spectra of furanes could be confused with those of conjugated dienals or some diunsaturated hydrocarbons so that further work will be required to establish identification.

The component eluted at 52 min from the fraction 1 (Fig. 3) is of considerable interest in that it does not seem to be in the general classes of other compounds found. The most abundant mass spectral peak was mass 43, so that the chromatographic trace is strong relative to the total mass spectral pattern. The molecular weight is apparently 132 (the largest mass peak observed). There is no indication of aromaticity or hydrocarbon chains longer than 3 or 4 carbons. Mass peaks observed at 47, 48, and 49 were weak but indicated that it might be a sulfur compound. However, the various propylbutyl sulfides (*t*-butyl not tested) did not match the observed spectrum. Isobutyl, sec-butyl, and *n*-butyl thio acetate were also synthesized, but did not conform. Further research will be necessary to identify this compound.

The question of artifacts produced from reactions of components originally present in the peas is of considerable concern. It was possible to get definite evidence for the catalytic autoxiadtion of dimethyl sulfide to the identified DMSO. There was no DMSO in the condensate collected directly from the blancher. A model experiment demonstrated that DMSO formed by oxidation of dimethyl sulfide adsorbed on the charcoal. Dimethyl sulfide was the second-most-abundant organic component of the pea blancher steam.

The identification of a number of aldehyde diethyl acetals in the charcoal extract concentrate is novel and exciting. An attempt was made to establish whether these acetals were originally present in the peas or were formed by reaction of the aldehydes and ethanol adsorbed on the charcoal. Some acetal formed under model conditions simulating isolation of the blancher steam components on charcoal. However, the ratio of the acetal to the parent aldehyde was much smaller than that found in the pea extract concentrate. It would be very difficult to duplicate the exact catalytic conditions existing on the charcoal used in the large-scale isolation. The total composition of the adsorbate could influence the position of equilibrium in the formation of acetals. Of special importance would be the contribution of acidic components to the total catalytic effect. Additional evidence will be required before it can be stated that acetals are true components of the vapor above heated peas.

ACKNOWLEDGMENT

The authors are indebted to I. I. Somers for suggesting a commercial pea blancher as a source of large quantities of volatile components; C. T. Townsend for arranging with William Lester, of the F. M. Wilson Company, to tap one of their pea blanchers at the Stockton, California plant; Edward Breitweiser and George Dolese for help in assembling and operating equipment in the isolation studies; and R. Teranishi for suggestions and for the use of capillary columns.

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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Inhibition of Pectinolytic and Cellulolytic Enzymes in Cucumber Fermentations by Sericea

SUMMARY

Pectinolytic and cellulolytic enzymes in cucumber flowers, when added to small-scale cucumber fermentations, were effectively reduced in activity by the use of a brine extract of sericea (*Lespedeza cuneata* Don) and by a freeze-dried substance isolated from this plant. Reduction of enzyme activity in the fermenting brines was directly related to the inhibitor concentration used. Higher levels of the inhibitor resulted in an increase in firmness of the cured salt-stock cucumbers. The judges rated the salt-stock from all inhibitor treatments *good* to *excellent* as to acceptability for commercial use. The addition of sericea, either as the brine extract or the isolated substance, appeared to exert no lasting inhibitory effect on the lactic acid bacteria responsible for acid fermentation in the experimental brines.

INTRODUCTION

The softening of cucumbers brined under commercial conditions was first shown by Bell *et al.* (1950) to be enzymatic in nature and the direct result of hydrolytic action by an enzyme system similar in behavior to polygalacturonase. Later, it was demonstrated that cellulolytic enzyme systems are also present in curing brines and may contribute to the total softening action (Bell *et al.*, 1955; Etchells *et al.*, 1955a). Continued studies on this perplexing and important problem implicated filamentous fungi as the actual cause of softening spoilage. Moreover, the hydrolytic enzymes pectinase and cellulase, of fungal origin, were shown to be introduced into the curing brines chiefly by way of fungus-laden flowers that remain attached to the green cucumbers, and to a lesser extent by the cucumbers themselves (Etchells *et al.*, 1958a; Raymond *et al.*, 1959). This work also demonstrated that the maximum concentration of softening enzymes diffused out of the flowers and into the brine within 24–48 hr after the vats were filled. This finding permitted development of a simple but effective softening-spoilage control measure consisting of draining off the original enzyme-laden cover brine 36–48 hr after filling, and replacing it with a new brine (Etchells *et al.*, 1955a,b). However, it has been our belief that a more satisfactory approach toward eliminating or significantly reducing the concentration of softening enzymes in commercial cucumber brines would be to use specific, nontoxic inhibitors of plant origin; recent investigations in our laboratory have been directed toward this end.

Bell and associates (Bell and Etchells, 1958; Bell *et al.*, 1960) first demonstrated the presence of a naturally occurring pectinase and cellulase inhibitor in leaves of the grape. This was followed by a more extensive study where in the water-soluble extracts of a wide variety of plants were screened for their ability to inhibit these two enzyme systems (Bell *et al.*, 1962). Of the 61 plant species in 32 families examined, 8 species were found to be good sources of the pectinase inhibitor: grape, persimmon, dogwood, blueberry, sericea, blackberry, raspberry and rose. The first five species also gave strong inhibition of cellulase activity. A recent paper (Bell *et al.*, 1964) reported that the leaves of muscadine grape, persimmon, and the leaves and stems of sericea also provided good yields of a purified inhibitor substance. Preparations from these three plants, isolated by the caffeine-complex method (Barnes, 1956; Porter and Schwartz, 1962), were rated about equal in their inhibitory properties against the pectinase and cellulase enzyme systems.

The present investigation was undertaken to test the effectiveness of sericea prepara-

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tions (brine extracts and the isolated substance) in small-scale cucumber fermentations for the inhibition of added softening enzymes, pectinase, and cellulase. It was also of interest to correlate, if possible, any reduction of softening activity with improved firmness of the cured salt-stock cucumbers. A further objective was to determine if the sericea inhibitor exerted any influence on the character of the lactic acid fermentation.

MATERIALS AND METHODS

The inhibitor source for the softening enzymes was the forage crop sericea. This material, harvested from four fields near Raleigh and from two in the vicinity of Laurinburg, North Carolina, was first refrigerated for several hours at 4°C and then chopped into 4-to-6-inch lengths, packed in freezer bags, and held at −10°C. Later, the frozen sericea was shipped by air with dry-ice to the pickle plant just prior to preparation and use as a brine extract in the cucumber brining studies and referred to herein as FSI. Frozen sericea from the same sources was used to prepare the freeze-dried inhibitor substance later used in the brining tests and referred to herein as SI. Methods for harvesting, handling, storage, and preparation of the inhibitor substance from sericea have recently been described in detail by Bell *et al.* (1964).

The effect of this plant material on small-scale cucumber fermentations in 45-to-50-gallon-capacity barrels or fiber drums was followed as to: 1) pectinolytic and cellulolytic enzyme activity of the brines; 2) brine acidity and pH; 3) optical density of the brines; and 4) firmness and general quality of the brine-stock cucumbers. The procedures for making the above-named series of tests were given in an earlier study on the use of grape leaves in cucumber fermentations to inhibit softening enzymes (Etchells *et al.*, 1958b). That work also gave information directly applicable to the present investigation on: source, variety, and size of cucumbers used; collection and handling of cucumber flowers; preparation of the brine extract from plant material; and brining and sampling procedures.

The two commercial pectinases, 41 and 41P-conc, were kindly supplied by Rohm and Haas Company, Philadelphia, Pennsylvania. The experimental work was done during the 1961 and 1962 brining seasons at a commercial pickling plant located in northeastern Texas. Brine temperatures during the active fermentation period were in the range of 28–32°C.

Table 1 gives details concerning the treatments employed during the study, together with information on the basic brining procedure used for the small-scale fermentations.

RESULTS AND DISCUSSION

Pectinolytic and cellulolytic activities of the brine samples presented in Table 2 were usually found to be at their maximum level 24 or 48 hr after the cucumbers were covered with brine. For this reason, all enzyme inhibition percentages were based on measurements made at these sampling intervals. Increasing levels of sericea inhibitor (FSI) caused decreasing activity for both enzyme systems originating from 2 lb of added cucumber flowers. The highest level of FSI used (2.0 lb) reduced the pectinolytic and cellulolytic activities 94 and 70%, respectively, and the salt-stock firmness was within 1 lb of the control. The two lowers levels of FSI (0.5 and 1.0 lb) did not improve salt-stock firmness over that of the softening control (treatment A).

The three levels of commercial pectinase-41 added to the curing brines resulted in pectinolytic enzyme activity of a very high order (535 to $>$ 10,000 units/ml). For each case, this activity was completely inactivated by the addition of sericea inhibitor. Cellulolytic activity, although present in less than 100 units/ml, was likewise reduced 100%. The salt-stock firmness readings for all treatments in this portion of the 1961 season's study were essentially the same. The three pectinase-added lots gave values between 14 and 15 lb firmness; the same was true for those receiving the sericea inhibitor in addition to pectinase.

The experimental work for the 1962 season followed the same general plan as for the previous year except that the substance isolated from sericea (SI) was used for enzyme inhibition, and the softening enzyme load was increased by the addition of an extra pound of cucumber flowers. Pectinase-41 was replaced with a more concentrated preparation.

The pattern of inhibition of the two enzyme systems from cucumber flowers by the sericea inhibitor (SI) shown in Table 3 was essentially the same as that described for the 1961 season (Table 2) where the brine extract of sericea (FSI) was employed. For example, pectinolytic enzyme inhibition for the highest levels added of both forms of the

inhibitor (Table 3, treatment *F*, and Table 2, treatment *E*) was within the narrow range 87–94%; for cellulolytic activity, both sericea preparations caused 70% inhibition. The same relationship was also true for within the 1962 season (Table 3); here the

Table 1. Treatments employed in the small-scale cucumber fermentations with added softening enzymes (cucumber flowers or commercial pectinases) and sericea inhibitor [brine extract of fresh-frozen material (FSI) or isolated substance (SI)].

	Softening enzyme added per barrel as:		Sericea inhibitor added per barrel as:	
Treatment [a]	Cucumber flowers (lb)	Pectinase-41 (ppm)	Brine extract (FSI)[b] (lb)	Isolated substance (SI) (ppm)
1961 season, Model var. cucumber				
A	0.0		0.0	
B	2.0		0.0	
C	2.0		0.5	
D	2.0		1.0	
E	2.0		2.0	
F	0.0		2.0	
G		25	2.0	
H		25	0.0	
J		5	2.0	
K		5	0.0	
L		1	2.0	
M		1	0.0	
N[c]	2.0		2.0	
		Pectinase-41-P conc. (ppm)		
1962 season, Model var. cucumber				
A	0.0			0
B	3.0			0
C	3.0			5
D	3.0			25
E	3.0			50
F	3.0			100
G	3.0		2.0	
H		10		100
J		10		0
MR-17 var. cucumber				
AA	0.0			0
BB	3.0			0
KM		10		0
L		10		100

[a] Each treatment consisted of 200–225-lb lots of No. 1B size Model or MR-17 variety pickling cucumbers brined in 45–50-gallon barrels or polyethylene-lined fiber drums of similar capacity. The basic brining treatment consisted of a 25° salometer (6.6% salt) cover brine plus sufficient salt added on the false head to maintain that concentration at equalization (24–36 hr). The initial brine strength was then raised 5° sal (1.3%) per week to a holding strength of 60° sal (15.8%). This procedure represented commercial practice at the plant where the work was done. In 1961, all treatments were in duplicate; in 1962, treatments A, B, H and J were duplicated.

[b] For all FSI-added lots except "N," the amounts indicated of fresh-frozen sericea were first blended in 1-gallon of 25° sal brine for 2 min, then the insoluble material was filtered out through cheesecloth and the brine extract was added to the cucumbers as part of the cover brine.

[c] For this lot, the sericea was not blended in brine to produce the extract but merely soaked 2 hr in 1 gallon of 25° sal brine and the total content (brine + sericea) added as part of the cover brine.

Table 2. Pectinolytic and cellulolytic enzyme activity of brines 24 hours after brining, and firmness of the cured, salt-stock pickles—1961 season.

	Treatment[a]	Pectinolytic enzyme		Cellulolytic enzyme		Firmness of cured salt-stock (lb)
		Activity (unit/ml)	Inhibition by sericea (%)	Activity (unit/ml)	Inhibition by sericea (%)	
A	Control, no flowers, no FSI	2	0	16	0	16
B	Flowers, no FSI	98	0	333	0	13
C	Flowers + 0.5 lb FSI	70	29	308	8	13
D	Flowers + 1.0 lb FSI	54	45	292	12	13
E	Flowers + 2.0 lb FSI	6	94	99	70	15
F	No flowers + 2.0 lb FSI	0	0	1	0	16
G	Pectinase, 25 ppm + 2.0 lb FSI	1	100	4	95	15
H	Pectinase, 25 ppm, no FSI	> 10,000	0	73	0	14
J	Pectinase, 5 ppm + 2.0 lb FSI	0	100	5	89	15
K	Pectinase, 5 ppm, no FSI	2,500	0	44	0	14
L	Pectinase, 1 ppm + 2.0 lb FSI	0	100	6	84	15
M	Pectinase, 1 ppm, no FSI	535	0	38	0	15
N	Flowers + 2.0 lb FSI	51	48	305	8	15

[a] Cucumber variety MR-17 used in all 1961 brining tests.

inhibitory action for SI and FSI (treatments *F* and *G*) was considered to be the same. Cucumber flowers alone (treatment *A*) reduced the salt-stock pickle firmness from 18 to 12 lb (33% loss). Five ppm of SI did not inhibit softening enzyme activity, and this was reflected by a 6-lb loss in firmness of the pickles, which was equivalent to that shown for the flower-added control. However, increasing levels of the inhibitor (25, 50, and 100 ppm) added to brines resulted in higher firmness values (14–16 lb) for the cured salt-stock pickles.

In considering the treatments with 10 ppm pectinase, this enzyme preparation was completely inactivated by 100 ppm SI, and the salt-stock pickles were adequately protected from softening action. This was true for fermentations of two cucumber varieties, Model and MR-17. In the absence of the inhibitor, the loss in firmness for both varieties amounted to 6 lb (33%). Although

Table 3. Pectinolytic and cellulolytic enzyme activity of brines 48 hours after brining, and firmness of the cured, salt-stock pickles—1962 season.

	Treatment	Pectinolytic enzyme		Cellulolytic enzyme		Firmness of cured salt-stock (lb)
		Activity (unit/ml)	Inhibition by sericea (%)	Activity (unit/ml)	Inhibition by sericea (%)	
Model var. cucumber:						
A	Control, no flowers, no SI	15	0	12	0	18
B	Control, flowers, no SI	93	0	300	0	12
C	Flowers + 5 ppm SI	90	3	255	15	12
D	Flowers + 25 ppm SI	80	24	260	14	14
E	Flowers + 50 ppm SI	34	64	215	28	14
F	Flowers + 100 ppm SI	12	87	90	70	16
G	Flowers + 2.0 lb FSI	10	90	86	71	17
H	Pectinase, 10 ppm + 100 ppm SI	1	100	1	98	18
J	Pectinase, 10 ppm, no SI	> 10,000	0	51	0	12
MR-17 var. cucumber:						
AA	Control, no flowers, no SI	64	0	29	0	17
BB	Control, flowers, no SI	116	0	250	0	14
KM	Pectinase, 10 ppm, no SI	> 10,000	0	60	0	11
L	Pectinase, 10 ppm + 100 ppm SI	2	100	1	98	16

both varieties responded in the same manner as to the degree of softening resulting from 10 ppm of the commercial pectinase, MR-17 var. may have offered some resistance to softening by the pectinolytic activity of cucumber flowers. Model var. was reduced in firmness by flowers by 6 lb (treatment *B*) more than the control (treatment *A*), but the reduction in firmness for MR-17 var. (treatment *BB*) as compared to its control (treatment *AA*) was only one-half that amount even though the enzyme activity of the brine was somewhat higher (116 units vs. 93). It will be recalled that MR-17 var. was used throughout in the 1961 brining studies, and that the reduction in brine stock firmness attributed to cucumber flowers (Table 2, treatment *B*) was the same (3 lb) as obtained with this variety for the 1962 season.

Fig. 1 shows the influence of both forms of sericea inhibitor (the brine extract and the isolated substance) on relative pectinolytic and cellulolytic enzyme activities at their maximum level in the flower-added brines. As the inhibitor (FSI or SI) concentration was increased, the activity of both enzyme systems decreased. Further, within a given enzyme system, both forms of the inhibitor produced similar inhibition curves. However, the cellulolytic enzyme system appeared to be less sensitive to the inhibitory properties of sericea than the pectinolytic.

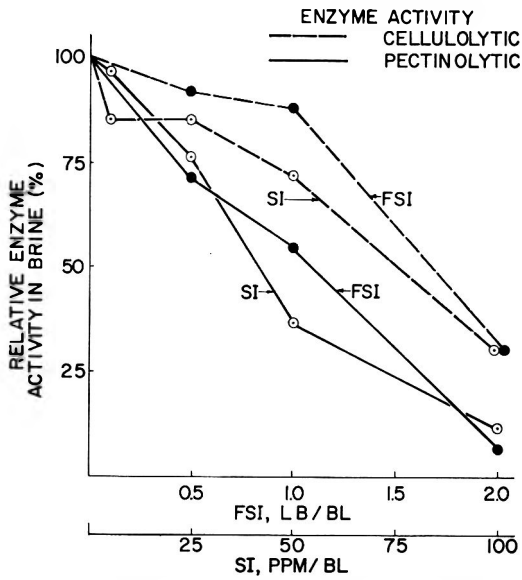

Fig. 1. Effect of sericea inhibitor on maximum pectinolytic and cellulolytic enzyme activity in cucumber brines. FSI = crude, brine-extract inhibitor solution prepared from fresh-frozen sericea and introduced in the cucumber cover brine in equivalent extracted amounts (lb/bl) of fresh material as indicated; SI = isolated inhibitor substance prepared from fresh-frozen sericea and introduced in the cucumber cover brine (ppm/bl) as indicated.

Table 4 shows total acidity and pH of brine samples from treatments typical of those examined during the two-season study. The values represent tests made on samples collected during the first 10 days after the

Table 4. The acidity and pH of brines from experimental treatments during the 10 days of fermentation.

Treatment	Fermentation in days: 1	2	3	5-6[a]	10	Fermentation in days: 1	2	3	5-6[a]	10
	Total acidity as lactic (%)					Brine pH				
1961 season:										
A Control, no flowers, no inhibitor	.08	.29	.43	.70	.77	5.45	4.12	3.87	3.50	3.50
B Control, flowers, no inhibitor	.07	.27	.52	.70	.80	5.77	4.47	3.72	3.50	3.50
C Flowers + 0.5 lb FSI	.07	.13	.37	.62	.75	5.85	5.70	3.90	3.50	3.50
D Flowers + 1.0 lb FSI	.08	.12	.22	.60	.66	5.90	5.65	4.15	3.50	3.55
E Flowers + 2.0 lb FSI	.08	.14	.31	.58	.72	5.90	5.30	3.90	3.50	3.55
1962 season:										
A Control, no flowers, no inhibitor	.02	.17	.31	.41	.39	6.25	4.47	3.73	3.57	3.45
B Control, flowers, no inhibitor	.02	.12	.38	.43	.55	6.75	4.80	3.80	3.75	3.40
C Flowers + 5 ppm SI	.02	.10	.35	.41	.52	6.75	4.90	3.85	3.75	3.40
D Flowers + 25 ppm SI	.02	.12	.36	.50	.46	6.80	4.75	3.90	3.65	3.50
E Flowers + 50 ppm SI	.03	.05	.19	.50	.56	5.70	5.90	4.35	3.65	3.40
F Flowers + 100 ppm SI	.02	.03	.17	.48	.48	6.10	5.90	4.50	3.65	3.55

[a] Brine samples taken on fifth day in 1962; sixth day in 1961.

cucumbers were brined; this time interval adequately covers the active acid fermentation period under the brining conditions employed. Within each season, the maximum amount of brine acid developed by the lactic fermentation was comparable for all treatments. The higher inhibitor levels (1961, *D* and *E*; 1962, *E* and *F*) appeared to retard the onset of acid production, but this initial delay was rapidly overcome and a vigorous lactic fermentation ensued. The resultant final acidities were essentially the same as in the control treatments, and typical of natural fermentations at 25° salometer. In each treatment, an increase in brine acidity was accompanied by a corresponding decrease in brine pH during the active fermentation period. By the tenth day, the pH values for all treatments had dropped to minimum levels in the range of 3.40–3.55.

The optical density (OD × 10 at 650 mμ) of the brine samples reached peaks for all treatments between the third and fifth days of fermentation. In general, the values obtained for the inhibitor-added treatments were lower (2–4 range) than for the controls (3–7 range). This finding might reflect a decrease in certain groups composing the total microbial population present in brines receiving the sericea inhibitor. However, final brine acidities and pH's would support the view that the addition of sericea, either as the brine extract (FSI) or the isolated substance (SI), appeared to exert no deleterious or lasting inhibiting effect on the lactic acid bacteria responsible for the acid fermentation in the experimental treatments.

A panel of experienced salt-stock judges, representing several Texas pickling companies, evaluated coded 50-lb lots of the material from the 1961 and 1962 experimental treatments listed in Tables 2 and 3. In brief, all inhibitor-treated lots were rated *good* to *excellent* as to acceptability for commercial use. This was the same rating given salt-stock from the control treatments.

Additional brining experiments with the isolated sericea substance (SI) were conducted during the 1962 season at two cooperating plants in northern production areas, Ohio and Minnesota. In all, twenty-six 50-gallon lots and four small commercial tanks were employed, together with inhibitor levels of zero, 5, 10, 25, 50, and 100 pm. A detailed account of this work will be given in a separate report; however, it is pertinent that the cured brine-stock from all inhibitor-added lots was again rated *good* to *excellent* as to acceptability for commercial use.

The two commercial pectinases used in the current brining tests gave extremely high values for pectinolytic enzyme activity, ranging from 535 to > 10,000 softening units per milliliter of brine. One might have expected such enzyme concentrations in the curing brines to have completely softened the brined cucumbers, particularly when our experience has shown that 150–200 softening units from cucumber flowers are usually sufficient to reduce firmness 50% or more. Earlier research on the behavior of pectinase in the presence of salt (Bell and Etchells, 1961) established that, as the salt content of cucumbers was increased, the softening action of three pectinases (AP-46, polygalacturonase, and filtrates from fungi) decreased according to a first-order reaction. This important relationship of the action of salt on softening also applies to the enzyme systems found in cucumber flowers; thus it does not provide an explanation for the discrepancy of the degree of softening action between flowers and pectinases experienced in the present study. Rather, a clearer understanding of the chemical composition and properties of pectinases is essential to determining the optimum conditions under which such complex fungal mixtures will readily soften plant tissue, particularly brined cucumbers undergoing fermentation. Recently, McClendon and Hess (1963) studied eight commercial pectinases with pH-gradient chromatography and found that all were different as to enzymic composition.

Although Etchells *et al.* (1958b) showed that grape leaves effectively inhibit pectinolytic and cellulolytic enzymes and thus protect brined cucumbers from their softening action in small-scale fermentations, their limited supply would exclude them as a source in quantity for the inhibitor substance. The same would be true for persimmon leaves, which were mentioned by Bell *et al.* (1964) as a good source of the inhibitor substance. The forage crop sericea, however, is another matter. This perennial is widely

grown as a forage crop for hay and pasture and lends itself to large-scale production and harvesting. In the southeastern states, sericea is grown on an estimated 1 million acres. Furthermore, it is grown on land of low fertility and low organic matter, and few plants can compete with it in yield per acre and drouth resistance (Cope, 1964). Thus it is believed that, based on present results demonstrating the effectiveness of sericea for inhibition of softening enzymes, plus its availability as source in quantity, this plant offers high potential for ultimately eliminating softening spoilage of commercially brined cucumbers.

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Influence of Post-Harvest Storage of Snap Beans on Chemical and Physical Changes During Canning

SUMMARY

Two varieties of snap beans that had been mechanically harvested were stored at 35, 55, and 85°F for 1, 3, and 5 days to determine the relationship of chemical changes to the quality of the resultant canned beans. Only approximately 50% of the weight loss during storage was regained by increase in drained weight in blanching and canning. There were losses in total sugars, starch, Calgon-soluble pectin, and hemicellulose, but gains in water-soluble pectin and cellulose. Higher storage temperature accelerated these changes. Variety and sieve-size influenced most of the chemical and physical changes. Storage increased sloughing in canned beans. Softening of canned beans occurred despite a loss of moisture during storage, and an increase in cellulose. There were significant changes in color, apparent in an increase in browning of bruised and broken beans, and a decrease in the Hunter $-a/b$ ratio.

INTRODUCTION

Snap beans have become a highly mechanized vegetable crop in recent years. Since harvesting and processing schedules can be readily controlled, delays in handling and transport should be at a minimum. However, the raw product is frequently transported farther and in greater quantities than most other vegetables for processing.

Chemical and physical changes take place during holding of fresh snap beans (Culpepper, 1936; Guyer and Kramer, 1952; McConnell, 1956; Parker and Stuart, 1935). Weight losses of 30% were recorded in 10 days when fresh beans were held at 70°F although there was no change in percent moisture (Guyer and Kramer, 1952). The loss was attributed to respiration losses.

Differences in percent fiber and percent seed were greatest among varieties (Gould, 1951). Fiber content did not increase during storage for 10 days at 35, 50, and 70°F when measured by the Food and Drug Method (Guyer and Kramer, 1952). Both organoleptic evaluation and blender determination of fiber showed differences due to storage.

Large differences in chemical constituents have been reported between varieties (Gould, 1951). Cellulose content was lower in frozen beans than in fresh or canned beans. Soluble solids, total solids, and easily hydrolyzable polysaccharides of fresh beans decreased significantly during canning.

The chemical composition of snap beans changes rapidly during maturation (Culpepper, 1936; Gould, 1951; Guyer and Kramer, 1952). Total nitrogen decreases in the pods during maturation, with a subsequent increase in the seeds (Culpepper, 1936). Hemicellulose is higher in seeds than in pods, and decreases during room-temperature storage for 58.5 hr (Parker and Stuart, 1935). The principal post-harvest changes in snap beans are those involving carbohydrates. Starch is hydrolyzed during storage, with an accumulation of total sugars. The rate is accelerated by cold temperature. The loss in crispness in storage of fresh beans is attributed to loss of water and an increase in soluble pectin. Soluble pectin increases in small beans at both 35 and 82°F, and in large beans, only at 35°F. Synthesis of protopectin continues during storage of fresh beans, especially at 82°F. Both soluble and protopectin values are smaller in seeds than in pods.

Sloughing of snap beans has been shown to be influenced by sieve size, maturity, blanching temperature, blanching time, and holding after blanching (McConnell, 1956; Moyer *et al.*, 1952; Sistrunk and Cain, 1960; Van Buren *et al.*, 1960). It has been shown that high blanching temperatures are detrimental to the quality of canned beans in terms of severe sloughing, splitting, and softening (Sistrunk and Cain, 1960; Van Buren *et al.*, 1960). Changes in the water-soluble and Calgon-soluble pectin during the blanching of beans for canning have been found to be correlated with sloughing.

Chemical changes during post-harvest storage have not been related to subsequent

changes during blanching and canning. Therefore, the purpose here was to report the effect of variety, sieve size, storage temperature, storage time, and blanching temperature on chemical and physical changes in canned beans.

Color, weight loss, and amount of sloughing were other quality measurements of primary interest.

EXPERIMENTAL

Raw material. Two varieties of bush beans (Earligreen and Gallatin 50) were obtained from commercial fields that had been mechanically harvested. The beans were brought to the Physiology and Processing Laboratory on the morning of harvest. Approximately 150 lb of beans were divided randomly into 10 lots, 1 being processed fresh and the other 9 lots stored for 1, 3, and 5 days at 35, 55, and 85°F prior to preparation for processing.

Preparation and processing. The beans were snipped and graded by commercial-size equipment. Sieve sizes 3 and 5 were cut to one-inch lengths and blanched for 1½ min at 165, 175, 185, and 200°F. One set of samples in each lot was filled into cans without blanching. The remainder of the processing procedures were as described previously (Sistrunk and Cain, 1960) except that 211 × 400 cans were filled to a constant weight of 160 g.

Analytical. No determinations were made on the fresh beans. After the canned beans were stored 3 months, drained weight, sloughing, viscosity, dry matter, total sugars, starch, pectin fractions, hemicellulose, and cellulose were determined on the drained canned beans. Dry matter was determined on the drained canned beans. Dry matter was determined on duplicate 20-g samples by weighing into tared aluminum cups and drying 24 hr at 60°C. Sloughing was determined by a method established by Van Buren *et al.* (1960).

All chemical analyses were made on ground beans prepared by blending 100 g of beans with an equal weight of distilled water for 2 min in an Osterizer. Three g of the blend was placed in 50-ml polypropylene centrifuge tubes. Starch was determined by adding 6 ml of distilled water and 1 ml of 0.4% solution of Taka-diastase to each

Table 1. Main effects of blanch temperature, storage time, storage temperature, and sieve size on drained weight and dry matter of two varieties of canned snap beans.

Main effect		Drained weight (g)		Dry matter (%)	
		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	168.9	168.1	5.36	6.52
	165	162.3	162.3	5.32	6.42
	175	161.0	162.3	5.19	6.38
	185	161.7	162.6	5.07	6.34
	200	160.9	161.8	4.81	6.29
F value		35.77**	40.73**	18.23**	4.46**
LSD @ 5% level		1.6	1.2	.21	.12
Time of storage (days)	0	160.7	161.7	5.30	6.16
	1	163.2	162.0	5.44	6.38
	3	163.5	164.4	5.28	6.65
	5	164.5	165.7	4.57	6.38
F value		10.44**	27.07**	71.30**	30.51**
LSD @ 5% level		1.5	1.1	.19	.11
Temperature of storage (°F)	35	161.8	162.6	5.36	6.48
	55	163.6	163.1	5.15	6.39
	85	163.4	164.6	4.94	6.31
F value		5.05*	10.77**	27.41**	7.48**
LSD @ 5% level		1.2	0.9	.16	.09
Sieve size	3	162.3	163.2	5.00	5.60
	5	163.6	163.7	5.31	7.18
F value		6.77*	1.93	46.16**	187.77**

*@ 5% level; **@ 1% level of statistical significance.

tube. The samples were digested for 1 hr in a water bath at 55°C with agitation. Duplicate tubes without enzyme received the same treatment for the purpose of determining total sugars. At the end of 1 hr, the tubes were filled to 40 ml with 95% ethanol. One-half teaspoon of Celite was added to each tube before stirring and heating on a water bath at 60°C. After heating for 10 min and centrifuging for 10 min, the supernatant liquid was decanted into volumetric flasks. After repeating the extraction by heating with 60% ethanol the enzyme-digested samples were extracted twice each with water, 0.5% Calgon, and 1*N* NaOH in the cold. Then 15 ml of 70% H_2SO_4 was added to each tube and stirred frequently during 1 hr before the contents of the tubes were made up to volume.

The ethanol, NaOH, and H_2SO_4 extracts were analyzed for sugar residues by the phenol method (Dubois *et al.*, 1956). These extracts were used to determine total sugars, hemicellulose and cellulose respectively. Water and Calgon fractions were determined as percent anhydrogalacturonic acid (Dietz and Rouse, 1953), with minor modifications described previously (Sistrunk and Cain, 1960).

Viscosity was determined on the 1:1 blend by recording the seconds necessary for 100 revolutions on the Stormer viscosimeter by using a 100-g weight.

The Hunter color and color-difference meter measurements were taken on the beans and liquor in a plastic container, using the green color standard: $Rd = 25.9$; $a = 27.5$; and $b = 7.0$.

RESULTS AND DISCUSSION

Shrinkage losses of fresh beans were as high as 13.4% in the Earligreen variety after 5 days at 85°F (Fig. 1). One of the objectives of this experiment was to determine the relation of moisture loss prior to processing to changes in the drained weight of canned beans.

There was less than 5% increase in drained weight during blanching and processing that was attributed to length of storage of fresh beans prior to processing (Table 1). The gain in drained weight was small when the beans were blanched at 200°F. This indicated that maximum imbibition was

Table 2. Main effects of blanch temperature, storage time, storage temperature and sieve size on total sugars and starch of two varieties of canned snap beans.

		Total sugars (%)		Starch (%)	
Main effect		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	1.146	1.442	.332	.865
	165	1.022	1.240	.378	.942
	175	1.020	1.171	.292	.899
	185	.965	1.201	.302	.885
	200	.978	1.212	.278	.865
F value		13.79**	24.81**	4.23**	0.73
LSD @ 5% level		.056	.064	.057	ns
Time of storage (days)	0	1.036	1.196	.416	1.105
	1	1.084	1.288	.343	.904
	3	1.049	1.303	.324	.821
	5	.935	1.227	.182	.737
F value		13.67**	6.68**	31.91**	22.91**
LSD @ 5% level		.050	.056	.050	.096
Temperature of storage (°F)	35	1.052	1.260	.342	1.049
	55	1.056	1.298	.326	.898
	85	.971	1.202	.280	.726
F value		10.40**	8.11**	4.62**	31.63**
LSD @ 5% level		.043	.048	.043	.082
Sieve size	3	1.110	1.311	.242	.632
	5	.943	1.195	.391	1.150
F value		93.37**	35.47**	73.58**	243.65**

* @ 5% level; ** @ 1% level of statistical significance.

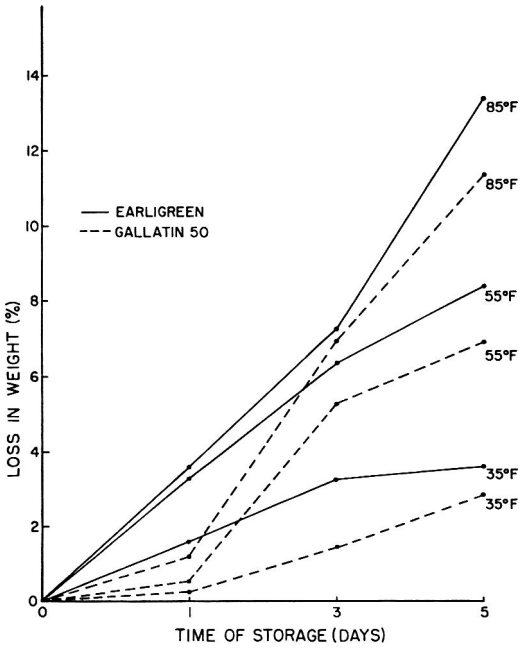

Fig. 1. Weight loss of fresh snap beans during storage.

reached during the 1½-min blanch and cooling, since the fill-in weight was 160 g. There was an increase in drained weight with an increase in storage temperature. The interaction between these factors indicated that drained-weight differences were greater at the higher storage temperatures, but only approximately 50% of the water loss was regained in any lot. Sieve 5 beans increased more in drained weight than sieve 3.

There was a decrease in dry matter of the beans with an increase in blanch and storage temperatures (Table 1). The loss of soluble constituents from beans during blanching and processing has been reported for canned beans (Gould, 1951). Sieve 5 beans were higher in dry matter. Dry matter increased slightly in Earligreen variety in the first day of storage at 35 and 55°F. In Gallatin 50, the dry matter increased at all temperatures for three days, but decreased at 55 and 85°F at 5 days.

As the blanching temperature was in-

Table 3. Main effects of blanch temperature, storage time, storage temperature and sieve size on water-soluble and Calgon-soluble pectin of two varieties of canned snap beans.

Main effect		Water-soluble (% AGA)		Calgon-soluble (% AGA)	
		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	.146	.196	.222	.145
	165	.109	.171	.329	.239
	175	.088	.185	.302	.260
	185	.128	.193	.241	.193
	200	.154	.222	.157	.128
F value		22.46**	25.31**	41.43**	41.91**
LSD @ 5% level		.016	.011	.031	.026
Time of storage (days)	0	.103	.186	.266	.216
	1	.137	.189	.260	.194
	3	.130	.194	.259	.193
	5	.129	.205	.216	.168
F value		8.67**	6.01**	5.85**	6.23**
LSD @ 5% level		.015	.010	.027	.023
Temperature of storage (°F)	35	.125	.187	.264	.197
	55	.130	.196	.243	.201
	85	.119	.198	.244	.181
F value		1.63	4.41*	2.08	2.39
LSD @ 5% level		ns	.008	ns	ns
Sieve size	3	.112	.183	.236	.190
	5	.137	.205	.265	.196
F value		23.90**	44.00**	9.68**	0.49

* @ 5% level; ** @ 1% level of statistical significance.

creased, the total sugars decreased except for minor variations (Table 2). Total sugars were lower in sieve 5 beans. Since there was a significant interaction between storage time and temperature, these data were not consistent. For example, the total sugars of Earligreen variety increased the first day at all temperatures, then decreased during the rest of storage. Gallatin 50 variety increased in total sugars for 3 days before showing a loss. Starch content was higher in beans that received "no blanch" and 165°F blanch (Table 2). There was a decrease in starch content during storage at all temperatures, although the decline was more rapid at 85°F. Sieve 5 beans contained a higher percent starch than the sieve 3 beans, especially Gallatin 50 variety.

Blanch temperature induced the widest range of differences in water and Calgon-soluble pectin (Table 3). Similar results have been reported (Sistrunk and Cain, 1960), with the highest Calgon-soluble pectin and lowest water-soluble pectin occurring when beans were blanched at 165–175°F. The varieties reacted differently in that water-soluble pectin was lowest at 165°F in Gallatin 50 variety, and at 175°F in Earligreen variety. In most instances, low water-soluble pectin and high Calgon-soluble pectin values were indicative of less sloughing of the beans and higher viscosity of the slurry. Softening of the medullary area of the pods was evident when more sloughing occurred. Sieve 5 beans contained a larger percentage of both fractions of pectin than sieve 3 beans except for Calgon-soluble pectin in Gallatin 50 beans. In general, the Calgon-soluble pectin of the beans decreased during storage, while the water-soluble pectin increased. The data for Earligreen variety, however, were not consistent even though differences were sizable.

Hemicellulose was a much larger fraction in Gallatin 50 beans. Again, as with Calgon-soluble pectin, these values for time and

Table 4. Main effects of blanch temperature, storage time, storage temperature, and sieve size on hemicellulose and cellulose of two varieties of canned snap beans.

Main effect		Hemicellulose (%)		Cellulose (%)	
		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	.170	.305	.946	1.169
	165	.193	.338	1.048	1.246
	175	.184	.317	.971	1.222
	185	.172	.316	.984	1.172
	200	.184	.298	.866	1.176
F value		1.61	.175	2.49	0.18
LSD @ 5% level		ns	ns	ns	ns
Time of storage (days)	0	.193	.367	1.019	1.071
	1	.183	.353	.923	1.107
	3	.176	.288	.993	1.173
	5	.158	.251	.918	1.437
F value		3.65*	11.70**	1.79	5.04**
LSD @ 5% level		.025	.051	ns	.213
Temperature of storage (°F)	35	.186	.321	.970	1.093
	55	.183	.323	.955	1.123
	85	.163	.301	.964	1.375
F value		3.59*	0.78	0.05	5.86**
LSD @ 5% level		.022	ns	ns	.183
Sieve size	3	.156	.243	.925	.989
	5	.198	.387	1.001	1.404
F value		29.42**	81.79**	4.17*	31.67**

* @ 5% level; ** @ 1% level of statistical significance.

temperature of storage decreased with an increase in storage duration and temperature, except there was no temperature effect in Gallatin 50 variety. Blanch temperature did not influence the percent hemicellulose although data were slightly higher at blanch temperatures of 165 and 175°F.

Cellulose was higher in sieve 5 beans of both varieties. The other main effects for Earligreen variety were not significant although interactions indicated that no consistent differences could be separated (Table 4). There was a marked decrease in cellulose, especially in beans stored 5 days at 85°F. Gallatin 50 beans reacted entirely different in that there was an increase in cellulose during storage at all temperatures. One explanation for this difference is that Earligreen beans were lower in dry matter and other constituents. Consequently, water loss was more rapid, shifting the soluble constituents either toward the insoluble or toward the soluble and loss by respiration.

Viscosity of a 1:1 blend of canned beans and water indicated that blanching altered or leached out some constituents that decreased viscosity (Table 5). Changes in viscosity were closely associated with chemical changes. Since there was a strong interaction between time and temperature of storage, the relation of viscosity with a single chemical constituent was meaningless. The interaction was due to a large increase in viscosity when beans were stored 3–5 days at 55 and 85°F. Concurrently, there were large increases in cellulose, decreases in starch, and high moisture losses. Viscosity was not affected by sieve size.

Sloughing, which has been shown to be correlated with firmness (Sistrunk and Cain, 1960; Van Buren *et al.*, 1960), was increased by improper blanching either with "no blanch" or a high-temperature blanch. The varieties differed in sloughing due to storage. Earligreen increased in sloughing the first day of storage at all temperatures, then went

Table 5. Main effects of blanch temperature, storage time, storage temperature, and sieve size on viscosity and sloughing of two varieties of canned snap beans.

Main effect		Viscosity (sec/100 rev)		Sloughing (ml)	
		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	19.8	27.2	8.4	7.3
	165	18.3	29.4	2.7	2.7
	175	17.7	27.9	2.9	2.8
	185	16.1	24.7	7.2	8.9
	200	16.7	25.7	17.7	13.1
F value		3.10*	3.52*	150.31**	107.33**
LSD @ 5% level		2.4	2.9	1.5	1.2
Time of storage (days)	0	19.6	32.1	7.8	3.3
	1	16.5	32.4	11.0	7.1
	3	15.0	23.7	4.8	7.7
	5	19.8	19.6	7.4	8.3
F value		10.44**	51.94**	33.05**	37.41**
LSD @ 5% level		2.1	2.6	1.3	1.1
Temperature of storage (°F)	35	16.6	30.5	7.5	6.0
	55	17.2	26.2	7.5	6.3
	85	19.4	24.2	8.3	7.6
F value		5.40**	17.95**	1.35	6.59**
LSD @ 5% level		1.8	2.2	ns	0.9
Sieve size	3	17.0	21.0	7.0	6.2
	5	18.4	32.9	8.5	7.1
F value		3.93	182.70**	21.15**	5.49*

* @ 5% level; ** @ 1% level of statistical significance.

Table 6. Main effects of blanch temperature, storage time, storage temperature, and sieve size on Hunter color values of two varieties of canned snap beans .

Main effect		Hunter *Rd* value		Hunter *-a/b* ratio	
		Earligreen	Gallatin 50	Earligreen	Gallatin 50
Temperature of blanch (°F)	check	12.72	10.96	.091	.083
	165	12.40	10.65	.109	.091
	175	12.73	10.51	.107	.083
	185	12.06	10.62	.100	.082
	200	12.84	10.50	.092	.071
F value		4.4177**	2.6291**	8.0080**	6.6696**
LSD @ 5% level		.44	.34	.008	.008
Time of storage (days)	0	12.80	10.19	.112	.102
	1	12.62	10.91	.104	.079
	3	12.23	10.67	.100	.077
	5	12.55	10.81	.084	.068
F value		3.0944**	9.5507**	21.2756**	33.6564**
LSD @ 5% level		.39	30	.007	.007
Temperature of storage (°F)	35	12.47	10.86	.105	.090
	55	12.66	10.69	.102	.080
	85	12.51	10.39	.092	.075
F value		0.7047	6.9328**	9.1644**	11.9637**
LSD @ 5% level		ns	.26	.006	.006
Sieve size	3	11.38	9.78	.102	.087
	5	13.72	11.52	.098	.076
F value		297.295**	281.665**	1.7341	20.9198**

* @ 5% level; ** @ 1% level of statistical significance.

through a period of decline in sloughing, followed by another increase by the fifth day of storage. These data substantiate results of McConnell (1956) in which there was a resistance to sloughing after 1 day of storage. However, Gallatin 50, a more fleshy variety, increased in sloughing during storage at all temperatures. Under all storage and blanching conditions, sieve 5 beans consistently sloughed more than sieve 3 beans.

Some of the color of beans was lost during blanching, as indicated by the lower *a/b* ratio when beans were blanched at 200°F (Table 6). The *a/b* value decreased during storage, especially at 55 and 85°F. Time of storage was important to the color of beans, as shown by the higher *Rd* values of Gallatin 50 after storage. The rapid development of browning during storage of Earligreen masked any consistent trends in *Rd* values, and accounted for the lower *Rd* value at 85°F storage in Gallatin 50 (Table 6). The increase in browning of the mechanically bruised and broken beans at 55 and 85°F probably masked increases in *Rd*; however, the decrease in $-a/b$ ratio during storage at all temperatures indicated a marked loss of green color.

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Isolation and Evaluation of the Saccharide Components of Starch Hydrolysates. I. Isolation

SUMMARY

A series of oligosaccharides of DP 3–10 was isolated from acid-converted 42-DE corn syrup with large-scale carbon chromatography. The samples thus isolated, obtained as amorphous powders, were shown to be chromatographically pure by paper chromatography.

INTRODUCTION

Corn syrups are an important part of a family of hydrolysate products which result from carefully controlled acid or acid-enzyme hydrolysis of corn starch. Starch hydrolysates may be classified on the basis of the "dextrose equivalent" (DE) of the product. "Dextrose equivalent" is a measure of the reducing-sugar content of a product calculated as dextrose and expressed as a percentage of the total dry substance.

The various saccharides present in starch hydroylsates are polymers of glucose. It has been assumed that the properties of a hydrolysate product result from the combination of the properties of the individual saccharide components making up the product. Several generalizations are known about the relationship between the number of glucose units or degree of polymerization (DP) in an oligosaccharide and its chemical and physical properties (i.e., viscosity increases with DP, reducing power decreases with DP), but little data are available regarding the actual values of properties of the oligosaccharides. Thus, it seemed desirable that basic chemical and physical property data be developed for the oligosaccharides found in starch hydrolysates.

Chromatography revolutionized the study of the structure of carbohydrate polymers. Paper chromatography has become the best way of identifying saccharides, and column chromatography the best way of isolating them.

French and Wild (1953) concluded that the papergram mobilities of homologous oligosaccharides form a straight-line characteristic when the logarithm of a partition function is plotted against molecular size. Those observations can be correlated into the generalization that increasing the size of a saccharide by one hexose unit will decrease papergram mobility by an amount which depends on the type of hexose unit added and on its mode of attachment.

Whistler and Hickson (1955) reported completion of the development of their quantitative technique for separating corn syrup into nine components. These components, in order of decreasing chromatographic mobility, were tentatively identified as: glucose, maltose, isomaltose, maltotriose, isomaltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. The components were quantitatively determined, after separation, by absorptiometric techniques.

In 1958 the Corn Industries Research Foundation, Inc., through efforts of the Analytical Procedures Subcommittee of its Technical Advisory Committee, adopted a standard method of quantitatively determining the oligosaccharide composition of starch hydrolysates.

Isolation of oligosaccharides by column chromatography developed during the same period, as did the application of paper chromatography to carbohydrate identification. Whistler and Durso (1950), in a significant advance, resolved a mixture of mono-, di-, and trisaccharides by chromatographic adsorption on charcoal and displacement with water, 5% ethanol, and 15% ethanol in succession.

The use of gradient elution of carbon columns with water containing increasing concentrations of ethanol was reported by Alm *et al.* (1952).

Whelan *et al.* (1953) used the basic carbon technique of Whistler and Durso to separate glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose from a product of the partial acid hydrolysis of potato amylose. The respective solvents used were water and 7.5,

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15, 20, 23, 25, and 28% ethanol. The progress of the elution was followed by measurement of optical rotation of each fraction.

Maltotriose, maltotetraose, maltopentaose, and maltohexaose were isolated in a chromatographically pure state by Barker *et al.* (1957) by fractionation with increasing concentrations of ethanol of the partial acid hydrolysis products of amylose on a charcoal column. The eluates were filtered, concentrated, freeze-dried, and then analyzed by paper chromatography. The purity of each fraction, measured by determination of the glucose formed after hydrolysis for 4 hr with 2*N* sulfuric acid at 100° was: maltotriose, 96%; maltotetraose, 95%; maltopentaose, 98%; maltohexaose, 95%.

The present research was done to isolate, in substantial quantity, the saccharide components of starch hydrolysates and determine various chemical and physical properties of these individual carbohydrates. Such information could lead to the development of new hydrolysate products which would be tailor-made to provide a particular combination of properties.

EXPERIMENTAL METHODS

Design and construction of the column system. Carbon column chromatography was selected as the means of isolating relatively large quantities of oligosaccharides from corn syrup. A column system was constructed as shown in Fig. 1.

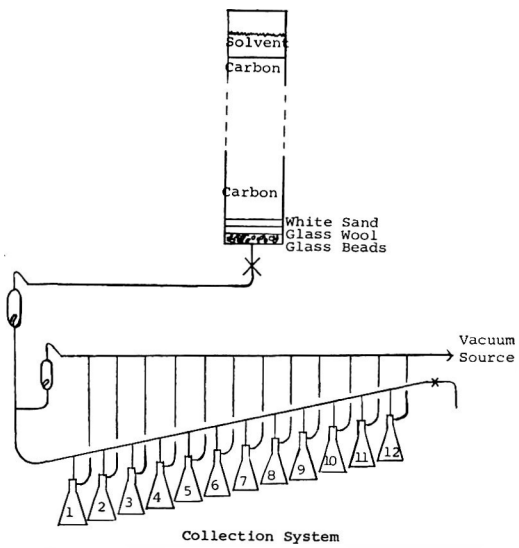

Fig. 1. Design and construction of the column system.

The column was constructed of two 4-ft sections of Plexiglass tubing of 6-inch diameter. The ends of the two sections were squared on a lathe, and the two sections were joined with epoxy resin. The 8-ft column was mounted in a frame formerly used for an ion-exchange column. This frame provided a flat base and top for the column, and leaks at these junctures were prevented with rubber gaskets.

It was necessary to design a collection system capable of collecting large fractions continuously while the entire system was operating under a vacuum. A vacuum of at least 20 inches Hg was necessary to increase the rate of elution of carbohydrates. Up to a certain maximum, the flowrate could be adjusted by controlling the amount of vacuum pulled on the system. The flowrate of solvent through the column greatly decreased as the alcohol content increased.

The collection system pictured in Fig. 1 proved completely satisfactory. It permitted continuous and automatic collection of 12 large fractions of eluate before it required attention or change of collection flasks. The fractions were collected in 350-ml vacuum flasks during the first primary separation, and in 1,000-ml vacuum flasks during the second run. Glass tees and tubing of 8 mm inside diameter were used to make the necessary piping and connections for the system. Rubber vacuum hose of the same size was also used. Kjeldahl connecting bulbs designed for Kjeldahl apparatus proved valuable for removing any entrapped air in the solvent entering the collection system. Air in the system at this point causes surging of the solvent up the inclined feed line to the collection flasks, which could result in simultaneous deposits of solvent in several flasks and thus cause a possible mixture of saccharides in the fractions.

A system similar to that of Fig. 1, but smaller, was constructed to separate mixed samples from the larger or primary separation column. This column was also 6 inches in diameter, but was only 4 ft high. The collection system was as described above, and 350-ml vacuum flasks were used to collect the fractions.

Packing the columns. Both columns were packed in the same way. A 1-inch layer of small glass beads was placed at the base of the column. This was covered with a ½-inch layer of glass wool, which, in turn, was covered with a ½-inch layer of fine white sand. The large column was packed to a height of 64 inches of carbon, and the small column to a height of 26 inches.

A 1:1 mixture of two carbons (supplied by the Atlas Chemical Industries Corporation) was used to pack the columns. Darco G-60, a powdered wood charcoal, and Darco S-51, a 20 × 40-mesh granular wood charcoal (<5% remains on a 12-

mesh screen, and <5% passed through a 40-mesh screen), were used. The carbons were mixed dry in a ribbon mixer, and just enough water was blended in to make the carbon "pack like a snowball" with moderate pressure in the hand. The mixture was then added to the column, three handfuls at a time. Between each addition of three handfuls, the carbon was firmly tamped. After the column was completely packed, water was added and pulled through the packed charcoal with a vacuum drawn on the bottom end of the column. Once packed, the carbon was always covered with water or solvent mixture.

The packed columns were treated to remove inorganic impurities by adding 1,000 ml of concentrated hydrochloric acid to the columns and continuously eluting with distilled water for several days until the pH returned to 3.5.

RESULTS

First column primary separation run. The column was charged with 470 g of carbohydrate solids from acid-converted 42-DE corn syrup, which was added to the column as a 10% solution in distilled water. The syrup was washed into the column with 41 L of distilled water before the gradient elution was started. Gradient elution was accomplished by joining in series two carboys containing distilled water and 30% ethyl alcohol. When vacuum was applied to the system, water was drawn from the first carboy, which resulted in vacuum syphoning of 30% ethyl alcohol from the second flask into the first. Solution from the first carboy was continually drawn through the carbon column and caught in the collection system. The contents of the first carboy were constantly stirred, and, as time passed, the ethyl alcohol concentration was increased toward the 30% concentration in the second flask.

Some of the early fractions collected had low pH values and tested qualitatively for the presence of chloride ions. These runs were put through an ion-exchange column. The fractions were first put through an Amberlite IRA-400 resin charged with $CO_3^=$ by washing with ammonium carbonate and then through an Amberlite IR-120 resin charged with H^+ ions from previous washing with sulfuric acid. It had previously been determined that using an OH^--charged resin caused a loss in optical rotation of a sample.

Fractions from the first primary separation run were evaluated for carbohydrate composition by paper chromatography, using the qualitative portion of the method of the Corn Industries Research Foundation, Inc., with 42-DE syrup used as reference material. Two of the ten combined fractions were found to contain single sugars, with the other fractions containing mixtures of two or three sugars. It was felt that the failure to obtain a better separation of the oligosaccharides was due primarily to gross undercapacity of the gradient elution system and mechanical difficulties in sealing the stirrer in the first carboy.

Second carbon column primary separation run. Used for the second run was a stepwise elution system rather than a gradient elution system. In this system, a solvent was run through the column until a peak appeared, and the concentration of solvent was then changed until a new peak appeared, etc. The solvents were water followed by 7.5, 15, 20, 23, 25, 28, and 30% by volume solutions of ethyl alcohol. The mixtures were prepared by blending appropriate volumes of water and 95% ethyl alcohol.

For convenience, the collection flasks were changed to 1,000-ml vacuum flasks, which held approximately 1,100 ml per fraction. After each collection flask was filled, hose clamps were fastened to the two rubber tubes attached to the flask. This ensured no mixing of the eluate into collection flasks which had previously been filled.

The column was charged with a 10% solution containing 468 g of carbohydrate solids from acid-converted 42-DE corn syrup solids.

Fig. 2 follows the course of the elution of carbohydrates from the column during the second primary separation run by plotting the optical rotation of the fractions collected.

Table 1 groups fractions from the second primary separation run found to be of the same carbohydrate composition by paper chromatography.

The second primary separation run was successful in that it did yield fractions containing only one of each of the desired oligosaccharides. It was felt that resolution could have been improved by using larger volumes of lower concentrations of alcohol to sepa-

rate some of the higher saccharides which came down in the same peak during this run. This theory was verified during the refining runs.

Refining runs. Fractions from the two primary runs containing similar mixtures of oligosaccharides were combined, concentrated to volumes containing 1–3% soluble solids, and run onto the smaller refining carbon column. The samples contained mix-

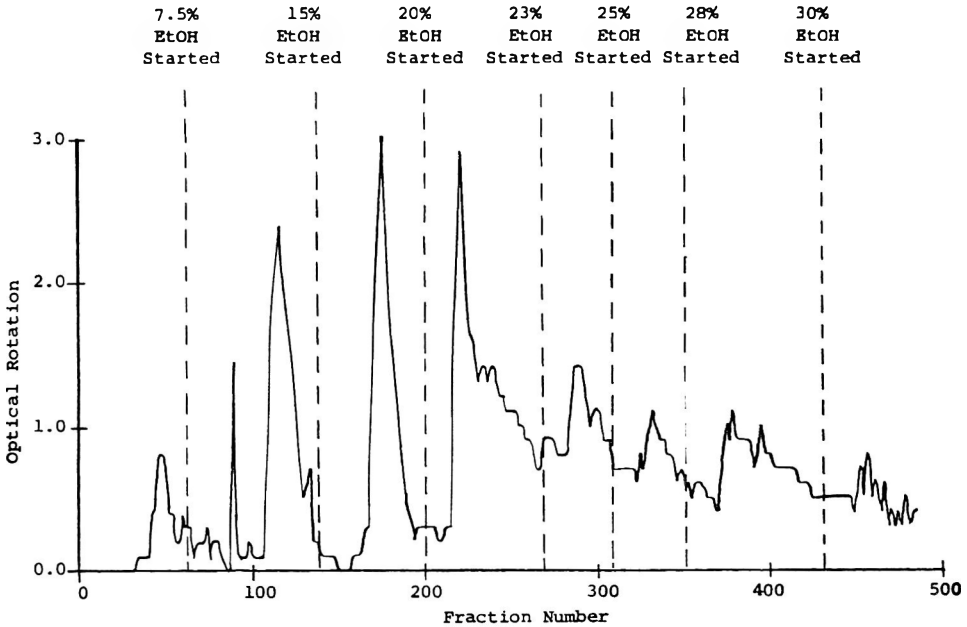

Fig. 2. Second primary separation.

Table 1. Carbohydrate composition of combined fractions from the second primary separation run.

Fractions	Composition	Fractions	Composition
164–191	maltotriose	342–344	maltoheptaose, maltooctaose
216–224	maltotetraose	345–347	(maltoheptaose), (maltooctaose)
225–242	maltotetraose, maltopentaose	348–353	maltooctaose (maltononaose)
243–253	maltopentaose	354–356	(maltooctaose), (maltononaose)
254–263	maltopentaose, (maltohexaose)[a]	357–359	blank
264–265	maltopentaose, maltohexaose	360–362	(maltononaose)
266–276	(maltopentaose), (maltohexaose)	363–365	maltooctaose, maltononaose
277–287	maltohexaose	366–368	maltooctaose
288–293	maltohexaose, (maltoheptaose)	369–371	maltononaose
294–299	maltohexaose maltoheptaose	372–380	maltooctaose, maltononaose
300–305	(maltohexaose), maltoheptaose	381–403	maltononaose
306–326	maltoheptaose	404–406	maltononaose, maltodecaose
327–341	maltoheptaose, (maltooctaose)	407–429	maltodecaose
		430–452	blank
		453–482	dextrins

[a] Parentheses indicate that the presence of a saccharide is questionable.

tures of oligosaccharides which were adjacent in the homologous series. They were separated by stepwise use of two solvents thought to be specific for the saccharides in the mixture. Again, the fractions were evaluated with paper chromatography. In this way, additional amounts of chromatographically pure maltotriose, maltotetraose, and maltopentaose were obtained.

During these refining runs it was found that concentrations of ethyl alcohol lower than those used in the second primary separation run would desorb the oligosaccharides if the volumes of solvent used were large enough. Specifically, it was found that large volumes of 15% ethyl alcohol would eventually desorb maltotetraose and that the use of 18% and then 20% ethyl alcohol would resolve mixtures of maltotetraose and maltopentaose.

Sample preparation. The fractions shown to contain one particular oligosaccharide were combined and concentrated in a vacuum pan (5-gallon capacity) operating at a vacuum of about 26 inches Hg or at a maximum temperature of 125°F. The concentrate was frozen in 2 × 4-ft stainless-steel trays at −40°F. The solution was then dried from the frozen state in a Stokes freeze-dryer operating at about 0.7 mm Hg. The plate temperature was controlled at a maximum of 110°F.

Some of the samples were light-brown when dried, and it was determined that these had a low pH and contained a substantial amount of chloride ions. These solids were redissolved, filtered, ion-exchanged, reconcentrated, frozen, and dried again. Even after this treatment the samples of maltotriose and maltotetraose were a light-cream color in the powdered form. By repeating the cycle just described, a white powder was finally obtained for maltotriose, but the maltotetraose was still off-white. All of the other oligosaccharides were white powders of very low bulk density in the dry form. All of the oligosaccharides thus prepared in the dry form gave only one spot when subjected to paper chromatography.

The yields of oligosaccharides were: maltotriose, 40 g; maltotetraose, 49 g; maltopentaose, 24 g; maltohexaose, 20 g; maltoheptaose, 10 g; maltooctaose, 5 g; maltononaose, 11 g; and maltodecaose, 8 g.

In this paper, for convenience, the oligosaccharides are referred to by specific names, i.e., maltotriose, maltotetraose, etc. This is done with the realization that there may be branched-chain polymers in each fraction; no attempt was made to separate polymers containing exclusively 1–4 glucosidic bonds from those which perhaps also contain 1–6 linkages.

Each fraction really represents, then, the family of saccharides which appear as one spot on a paper chromatogram of corn syrup. They have been assigned the names of the homologous series obtained from the hydrolyzed product of amylose. It is believed that, except for optical rotation, the properties of the saccharides evaluated will not be affected by the presence or absence of branching in saccharides having identical molecular weight.

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Isolation and Evaluation of the Saccharide Components of Starch Hydrolysates. II. Evaluation

SUMMARY

Oligosaccharides of DP 3–10 were evaluated for their properties of density, refractive index, viscosity, optical rotation, reducing power, and infrared absorption characteristics. Tables of data were developed giving the expected values of each property for each oligosaccharide.

As expected, density, refractive index, viscosity, optical rotation, and reducing power were related to the molecular weight of the sugars. The degree of the effect of molecular weight on each of these properties was established.

INTRODUCTION

The literature contains a wealth of knowledge and information on the properties of D-glucose and its solutions, and a significant amount of information on the properties of corn syrups. Most of this information has been compiled in Critical Data Tables published by the Corn Industries Research Foundation in 1957.

As yet, very little information has been published on the chemical or physical properties of the oligosaccharide components of corn syrup. Workers who developed or have applied chromatographic techniques to isolate these saccharides have done so to perfect analytical techniques to be used in research and quality control, to gain an insight into polysaccharide structures, or to aid in the understanding of enzyme action patterns.

French *et al.* (1949) determined the specific optical rotation of maltoheptaose to be $[a]_D$ + 176°.

Hickson and Whistler (1950) studied the preparation of crystalline maltotriose without success. To date none of the maltooligosaccharides of starch hydrolysis above DP 2 have been crystallized.

Thompson and Wolfrom (1952) confirmed the structure for maltotriose to be 4-*a*-maltopyranosyl-D-glucose.

Whelan *et al.* (1953) evaluated certain properties of glucose and the oligosaccharides of DP 2 through 7. They determined molecular weight by determination of the copper-reducing power of the saccharides using the colorimetric reagent of Somogyi (1952). Specific rotations of the saccharides were also determined.

Whistler and Hickson (1954) found the specific rotation of maltotetraose to be $[a]_D^{25}$ + 165.5°. Whistler (1954) reported the specific rotation of maltotetraose to be $[a]_D^{25}$ + 176.4°, and of maltopentaose to be $[a]_D^{25}$ + 179.4°. Whistler (1954) reported the specific optical rotation of maltohexaose to be $[a]_D^{25}$ + 182°.

Specific optical rotations for maltotriose and a hexasaccharide in water were respectively found to be $[a]_D$ + 158° ± 1 and $[a]_D$ + 177° ± 1 (Bird and Hopkins, 1954).

Whistler and Hickson (1955) observed the specific optical rotation of maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, and found them to follow a regular order as predicted by Freudenberg *et al.* (1932).

Isomaltotriose was isolated by Sato *et al.* (1962) and determined to have a specific optical rotation of $[a]_D^{21}$ + 136.1° in water.

The major drawback to the development of knowledge of the properties of the oligosaccharides has been a lack of sufficient quantities of the purified products necessary for study. In all of the foregoing work with paper or column chromatography, only minute quantities of the oligosaccharides were isolated. In the present work the isolated oligosaccharides and reagent-grade dextrose were studied to determine the following properties: density, refractive index, viscosity, optical rotation, reducing power, and infrared absorption characteristics.

EXPERIMENTAL METHODS

Dry substance. The vacuum-oven method was used to determine the percent dry substance.

Sample preparation for determining density, viscosity, refractive index, and optical rotation.

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Approximately 2.2 g of dry sample was weighed accurately into a 100-ml beaker. Approximately 8 ml of deionized distilled water was added with a graduated pipette. The sample dissolved in the added water was again accurately weighed. This solution was transferred to a test tube suspended in a water bath controlled at 20°C and was used for density, viscosity, and refractive index determinations. The solution remaining after measurement was returned to the original beaker, reweighed, and diluted with a predetermined aliquot of deionized distilled water, and the diluted solution was accurately weighed and subsequently measured. This procedure was repeated for four dilutions. The last dilutions (approximately 1% solutions), upon which optical rotation in addition to density, refractive index, and viscosity were measured, were prepared at a later time by weighing dry samples and dissolving in a weighed amount of water. Duplicate samples and dilutions were used for each oligosaccharide. All measurements were adjusted for the moisture content of the sample, and all calculations were based on dry weight.

Density. The apparent density of solutions of the malto-oligosaccharides was determined by weighing known volumes of their solutions in pycnometers in air. Previously calibrated 5-ml pycnometers were used, and the temperature of the solutions was controlled at 20°C.

Refractive index. The measurement was made on a Bausch and Lomb Abbé refractometer accurate to 0.0001 unit, which was checked for accuracy with deionized distilled water. Water from the 20°C water bath was pumped through the head of the refractometer to control the temperature of the sample and the prisms of the instrument. A drop or two of sample was introduced onto the test prism with a 1-ml pipette. A number of readings were made by approaching the target line from both directions, and the average of these readings was recorded. The measurements were precise to 0.0001 unit.

Viscosity. An Ostwald viscometer, type 100, was used. The viscometer was suspended vertically in the water bath. Exactly 5 ml of 20°C sample was added from a volumetric pipette. At least two, and sometimes three, observations were taken on each liquid sample.

Optical rotation. The samples were evaluated with a Franz, Schmidt, and Haensch polarimeter which was accurate to 0.001°. Measurements were made with the sodium D line, 598.3 mμ, and a two-decimeter tube length at 20°C.

Reducing power. The ferricyanide micromethod of Hagedorn and Jensen (1923) was selected, principally because it can be used with very small samples of carbohydrate. In fact, sample sizes must be selected that will contain reducing sugars equivalent to less than 0.385 mg of dextrose. Samples of the oligosaccharides were accurately weighed on watchglasses, quickly washed into 100-ml volumetric flasks, and diluted to 100 ml with deionized distilled water. The procedure followed is given by the National Bureau of Standards (1942; pp. 198–199).

Infrared absorption spectra. Three hundred milligrams of a mixture of 0.75 mg of sample and 375 mg of dry KBr powder were accurately weighed and placed in a KBr die. The die was evacuated under vacuum for 10 min. Twenty thousand pounds total force (equivalent to 80,000 pounds/square inch) was placed on the die and held for 3 min. The KBr pellet was then placed in a holder and scanned on a Beckman IR4 spectrophotometer. Operating conditions for the instrument were: 0–100% transmission; slit opening of 0.06 mm at 3.5 μ; scanning speed of 1 μ per min; and a gain of 6%.

RESULTS

Statistical evaluations. The method of least squares was used to develop equations expressing the relationship between the variables involved in the study of each property except infrared absorption. For all studies the following two equations were considered:

$$Y = a + bX \qquad [1]$$

$$Y = a_1 + b_1X + c_1X^2 \qquad [2]$$

The simple linear equation was used for each study except the reducing sugar property, where a straight-line relationship between reducing power and degree of polymerization obviously did not exist. In that evaluation, Eq. 2 was used. In addition, least-square lines using Eq. 2 were determined for density, refractive index, and viscosity, and a test was run to see if the addition of the second-degree component explained a significant amount of the variation over and above a simple linear relationship.

For those studies that used Eq. 1, the hypothesis was tested that there is no relationship between Y and X.

A statement on the goodness of fit of a particular regression model can be obtained by computing the fraction of the total variation of the dependent variable attributable to the model fit. This value ranges between 0 and 1, and the greater the value the better

the fit. The symbol for this value is R^2, and it is computed as follows:

$$R^2 = \frac{\text{explained variation}}{\text{total variation}}$$

An indication of the accuracy of the estimates of the true values is given by the estimate of the common standard deviations.

The deviation of the observed values from the expected values based on the selected model is due to variations, such as temperature changes, lack of precision in measuring instruments, and variations inherent in the experimental procedure.

For the properties of density and refractive index, a test was performed to determine if there was a significant difference between the slopes of the simple linear regression lines determined for the different maltooligosaccharides. The basis for these several statistical techniques is in Ostle (1954).

Density. The slopes of the simple linear least-square lines for the nine saccharides were tested, and no basis was found to reject the hypothesis that they are equal.

In testing the value of adding a second-order term, it was found that the second-degree equation explains a significantly greater amount of variation in density at the 1% level of significance.

The least-square equations for the saccharides were found to be:

Dextrose

$$Y = 0.99825 + 0.00338145\ X + 0.0000106085\ X^2$$

Maltotriose

$$Y = 0.99825 + 0.00396059\ X + 0.0000153398\ X^2$$

Maltotetraose

$$Y = 0.99795 + 0.00393281\ X + 0.0000136401\ X^2$$

Maltopentaose

$$Y = 0.99776 + 0.00400504\ X + 0.00000961340\ X^2$$

Maltohexaose

$$Y = 0.99788 + 0.00402459\ X + 0.00000799177\ X^2$$

Maltoheptaose

$$Y = 0.99812 + 0.00392586\ X + 0.0000158694\ X^2$$

Maltooctaose

$$Y = 0.99825 + 0.00380954\ X + 0.0000210111\ X^2$$

Maltononaose

$$Y = 0.99776 + 0.00398042\ X + 0.000013557\ X^2$$

Maltodecaose

$$Y = 0.99830 + 0.00391736\ X + 0.000017152\ X^2$$

Table 1. Expected density at 20°C (g/ml).

Concentration (%)	Dextrose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose	Maltoheptaose	Maltooctaose	Maltononaose	Maltodecaose
2	1.00606	1.00623	1.00587	1.00581	1.00596	1.00603	1.00595	1.00578	1.00621
4	1.01395	1.01434	1.01390	1.01393	1.01411	1.01408	1.01382	1.01390	1.01425
6	1.02192	1.02257	1.02204	1.02214	1.02231	1.02224	1.02186	1.02213	1.02243
8	1.02998	1.03092	1.03029	1.03042	1.03059	1.03054	1.03007	1.03047	1.03074
10	1.03813	1.03939	1.03864	1.03877	1.03892	1.03896	1.03844	1.03892	1.03865
12	1.04636	1.04799	1.04711	1.04721	1.04733	1.04751	1.04699	1.04748	1.04778
14	1.05467	1.05671	1.05568	1.05572	1.05579	1.05619	1.05570	1.05615	1.05651
16	1.06307	1.06555	1.06437	1.06430	1.06432	1.06499	1.06458	1.06492	1.06537
18	1.07156	1.07451	1.07316	1.07297	1.07291	1.07393	1.06682	1.07380	1.07437
20	1.08013	1.08360	1.08206	1.08171	1.08157	1.08298	1.08284	1.08279	1.08351

Using these equations, expected density values for solutions of each saccharide for concentrations of 1 to 20% were calculated, and those for ten concentrations are given in Table 1.

High R^2 values of 0.9995–0.9999 were obtained, indicating that the second-degree equation provides a reasonable model for explaining the variation of density with changes in concentration. The order of magnitude of the standard deviations (.00010–.00074) was reasonable considering the methods and instruments used.

Refractive index. Refractive-index values were observed. Both simple linear and second-order least-square lines, with refractive index as the dependent variable, were determined with these data.

The slopes of the simple linear least-square lines for the nine saccharides were tested, and no basis was found to reject the hypothesis that they are equal.

In testing the value of adding a second-order term, it was found that the second-degree equation explains a significantly greater amount of variation in refractive index at the 1% level of significance for dextrose, malotetraose, maltononaose, and maltodecaose; at the 5% level of significance for maltotriose, maltopentaose, and maltoheptaose; and not significantly different at the 5% level for maltohexaose and maltooctaose.

The least-square lines for the saccharides were found to be:

Dextrose

$$Y = 1.33305 + 0.0013687\ X + 0.0000074849\ X^2$$

Maltotriose

$$Y = 1.33326 + 0.0012796\ X + 0.00001377\ X^2$$

Maltotetraose

$$Y = 1.33298 + 0.0014322\ X + 0.0000073615\ X^2$$

Maltopentaose

$$Y = 1.33293 + 0.00145906\ X + 0.0000058228\ X^2$$

Maltohexaose

$$Y = 1.33300 + 0.00138994\ X + 0.0000095583\ X^2$$

Maltoheptaose

$$Y = 1.33304 + 0.00141273\ X + 0.00000963899\ X^2$$

Maltooctaose

$$Y = 1.33295 + 0.0014122\ X + 0.0000113764\ X^2$$

Maltononaose

$$Y = 1.33302 + 0.00144842\ X + 0.0000089287\ X^2$$

Maltodecaose

$$Y = 1.33307 + 0.00145636\ X + 0.0000066968\ X^2$$

Table 2. Expected refractive index values at 20°C.

Concentration (%)	Dextrose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose	Maltoheptaose	Maltooctaose	Maltononaose	Maltodecaose
2	1.33582	1.33587	1.33587	1.33588	1.33582	1.33590	1.33581	1.33595	1.33601
4	1.33864	1.33860	1.33882	1.33886	1.33871	1.33884	1.33878	1.33896	1.33900
6	1.34153	1.34143	1.34183	1.34190	1.34168	1.34186	1.34183	1.34203	1.34205
8	1.34448	1.34438	1.34914	1.34502	1.34473	1.34496	1.34497	1.34518	1.34515
10	1.34749	1.34743	1.34803	1.34811	1.34786	1.34813	1.34820	1.34840	1.34830
12	1.35055	1.35060	1.35122	1.35128	1.35106	1.35138	1.35153	1.35169	1.35151
14	1.35368	1.35387	1.35447	1.35450	1.35433	1.35471	1.35495	1.35505	1.35477
16	1.35687	1.35726	1.35777	1.35777	1.35769	1.35811	1.35845	1.35848	1.35808
18	1.36011	1.36075	1.36114	1.36108	1.36112	1.36159	1.36205	1.36198	1.36145
20	1.36342	1.36393	1.36456	1.36445	1.36462	1.36515	1.36574	1.36556	1.36487

Using these equations, expected refractive index values for solutions of each saccharide for concentrations of 1 to 20% were calculated, and those for ten concentrations are given in Table 2.

High R^2 values (0.9962–0.9999) indicated that the second-degree equation provides a reasonable model for explaining the variation of refractive index with changes in concentration. The order of magnitude (0.00014–0.00067) of the standard deviations was reasonable considering the methods and instruments used.

Viscosity. Measurement of the absolute viscosity of a liquid is difficult, but measurement of relative viscosity is simple and adequate for most purposes. Relative viscosity is the ratio of the viscosity of a liquid to that of a reference liquid, such as water. The absolute viscosity of the liquid may then be calculated from a knowledge of the absolute viscosity of the reference liquid.

When exactly the same volume of liquid is used in a given viscometer, the pressure driving the liquid through the capillary is proportional to the density of the liquid.

Letting η_1, ρ_1, t_1 be the viscosity, density, and time of efflux of the unknown liquid, and η_2, ρ_2, t_2, the viscosity, density, and time for water,

$$\frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2}$$

or

$$\eta_1 = \eta_2 \frac{\rho_1 t_1}{\rho_2 t_2}$$

The intrinsic viscosity, η, is defined as the limiting value of $\eta sp/c$ as c approaches zero. The concentration, c, is expressed in grams per 100 ml of solution. ηsp is the specific viscosity of a solution and is defined as

$$\frac{\eta_1}{\eta_2} - 1.$$

Flow times of the several concentrations of each of the saccharides under investigation were determined at 20°C. These data were used to calculate the viscosity, specific viscosity, and specific viscosity/concentration for each saccharide.

Least-square lines, both linear and using a second-order component, were calculated to determine the relationship between specific viscosity/concentration and concentration. The use of the second-order equation explains a significantly greater amount of variation in specific viscosity/concentration at the 1% level of significance.

Using Y for specific viscosity/concentration and X for concentration, the least-square lines for the saccharides were found to be:

Dextrose
$$Y = 0.021819 + 0.000870614\ X + 0.0000116092\ X^2$$

Maltotriose
$$Y = 0.029038 + 0.000505559\ X + 0.0000454960\ X^2$$

Maltotetraose
$$Y = 0.031739 + 0.000446196\ X + 0.0000568888\ X^2$$

Maltopentaose
$$Y = 0.031584 + 0.00047686\ X + 0.0000684242\ X^2$$

Maltohexaose
$$Y = 0.032420 + 0.000454536\ X + 0.0000794918\ X^2$$

Maltoheptaose
$$Y = 0.032119 + 0.000749166\ X + 0.0000743899\ X^2$$

Maltooctaose
$$Y = 0.036299 + 0.000759073\ X + 0.0000720590\ X^2$$

Maltononaose
$$Y = 0.034445 + 0.000917747\ X + 0.0000884443\ X^2$$

Maltodecaose
$$Y = 0.035966 + 0.00116064\ X + 0.0000621544\ X^2$$

Using these equations, expected values for viscosity, specific viscosity, and specific viscosity/concentration for solutions of each saccharide for concentrations of 1 to 20% were calculated. Table 3 gives the expected values for viscosity for ten concentrations, and Fig. 1 plots the expected values for specific viscosity/concentration vs. concentration.

The high R^2 values obtained (0.9944–0.9986) indicate that the second-degree equation provides a reasonable model for explaining the variation of specific viscosity/concentration with changes in concentration. The order of magnitude of the standard deviations (0.00049–0.00214) is reasonable considering the methods and instruments used.

In general, the data plotted in Fig. 1 form an orderly family of curves, but there are some unexplainable irregularities. The lines of maltotetraose, maltopentaose, maltohexaose, and maltoheptaose converge with decreasing concentration. At lower concentrations the slope of maltooctaose is less than its adjacent polymers and thus has a higher

Table 3. Expected viscosity in poises at 20°C.

Concentration (%)	Dextrose	Maltotriose	Maltotetraose	Maltopentaose	Maltohexaose	Maltoheptaose	Maltooctaose	Maltononaose	Maltodecaose
2	.010524	.010658	.010710	.010710	.010726	.010732	.010816	.010786	.010825
4	.011075	.011328	.011434	.011440	.011478	.011510	.011678	.011639	.011722
6	.011706	.012083	.012249	.012276	.012342	.012419	.012670	.012651	.012774
8	.012424	.012944	.013181	.013248	.013358	.013497	.013827	.013865	.014008
10	.013234	.013934	.014260	.014391	.014564	.014778	.015185	.015323	.015456
12	.014143	.015074	.015511	.015737	.015998	.016300	.016778	.017068	.017147
14	.015155	.016386	.016963	.017320	.017699	.018096	.018640	.019143	.019111
16	.016276	.017893	.018643	.019172	.019705	.020204	.020806	.021591	.021378
18	.017512	.019616	.020579	.021327	.022054	.022660	.023312	.024453	.023979
20	.018869	.021577	.022797	.023817	.024785	.025498	.026191	.027774	.026942

intrinsic viscosity than might be expected from its relation to the viscosity of the other oligosaccharides at higher concentration. The maltononaose had an unusually steep slope, resulting in an intrinsic viscosity lower than for maltooctaose or maltodecaose, but a higher viscosity than either of these at higher concentrations.

A description of the solutions of these saccharides seems warranted to aid understanding of the variations in viscosity values. Dextrose formed a clear water-white solution. Maltotriose and maltotetraose formed clear light-yellow solutions. Maltopentaose formed a clear water-white solution. Solutions of maltohexaose contained a small flocculent precipitate at higher concentrations which appeared to develop after standing in the 20°C water bath. As the samples were diluted, this precipitate disappeared. Maltoheptaose formed a turbid off-white solution at high concentration which cleared upon dilution, but solutions of maltoheptaose were clear but opalescent even at the lowest concentration. Maltooctaose formed a very slightly turbid yellow solution at high concentration which cleared when diluted. Maltononaose made a clear water-white solution. Maltodecaose solutions were slightly yellow with very slight turbidity at high concentration, but cleared quickly upon dilution.

The bulk density of all the oligosaccharides except maltononaose was very low. Maltononaose, which was much more dense, took some time to dissolve and acted like a crystalline compound. Because of this and the unusual shape of its viscosity curve, maltononaose powder and the other oligosaccharides were examined under a microscope. No crystals were observed in any of these materials. Upon redrying of a solution in the freeze-dryer, maltononaose powder had the same appearance and low bulk density as the other oligosaccharides. Drying conditions have a great influence on the shape and size of the dry particles formed when solutions of oligosaccharides are dried.

Since intrinsic viscosity is normally determined by extrapolating a plot of specific viscosity/concentration vs. concentration to zero concentration, simple linear least-square lines were calculated using the observed

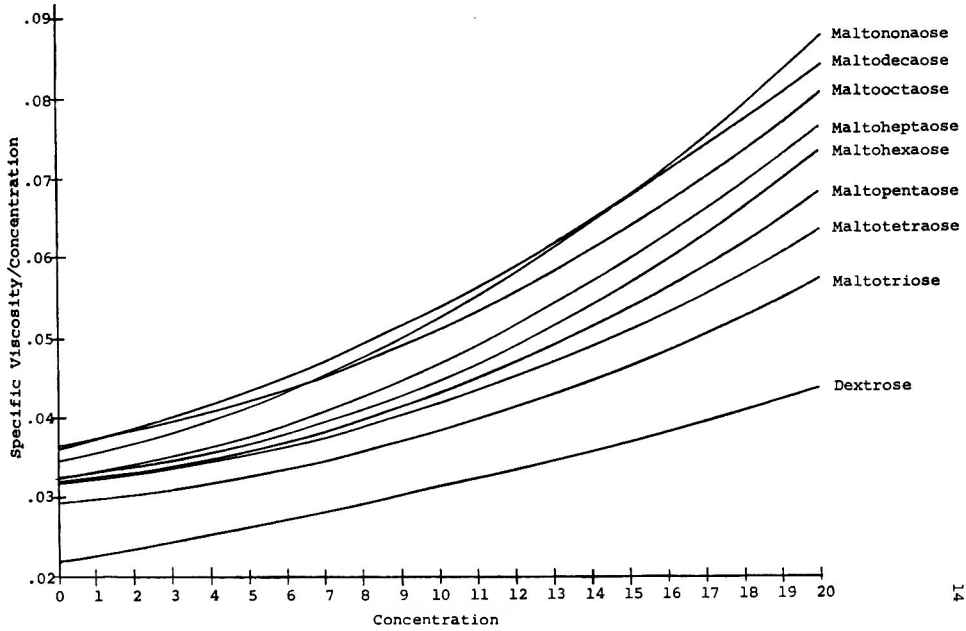

Fig. 1. Expected specific viscosity/concentration vs. concentration.

values for the lower three concentrations only. At concentrations under 10%, the linear equation adequately described the relationship. The intercept, a, for these lines should be the intrinsic viscosity.

A least-square line was calculated of the thus-determined intrinsic viscosities vs. DP. The expected intrinsic viscosities for the oligosaccharides based on this line are given in Table 4.

The linear relationship between log $[\eta]$ and log DP reported by Caesar *et al.* (1947) seems to be substantiated by this work.

Optical rotation. The optical rotations of duplicate samples of the oligosaccharides were measured. Average values were used to calculate the specific rotation $[\alpha]_D^{20}$, using the relationship:

Table 4. Expected intrinsic viscosities of oligosaccharides.

Saccharide	Expected intrinsic viscosity (poises)
Dextrose	.024766
Maltotriose	.027251
Maltotetraose	.028494
Maltopentaose	.029736
Maltohexaose	.030979
Maltoheptaose	.032222
Maltooctaose	.033464
Maltononaose	.034707
Maltodecaose	.035950

Table 5. Comparison of specific rotations.

Saccharide	Observed $[\alpha]_D^{20}$	Whistler $[\alpha]_D^{25}$	Whelan $[\alpha]_D^{15}$	Others $[\alpha]_D$
Dextrose	52.69	....	52.6	
Maltotriose	159.65	159	160	158±1, 160
Maltotetraose	163.03	165.5, 176.4	177	
Maltopentaose	178.31	179.4	180.3	
Maltohexaose	179.67	182	184.7	177±1
Maltoheptaose	182.90	179	186.4	176
Maltooctaose	182.23			
Maltononaose	186.09			
Maltodecaose	167.31			

$$[\alpha]_D^{20} = \frac{100\ \alpha}{2 \times \text{weight percent} \times \text{density}}$$

The resultant specific rotations are compared in Table 5 with those found by other workers.

The maltotetraose sample was known to contain a chloride ion contamination. This was not completely removed by repeated ion-exchange treatment, which may account for the observed specific rotation being lower than reported by other workers.

No explanation is offered for the unusually low value observed for maltodecaose. No unusual salt or acid contamination was found. This value was checked by determining the rotation of 6 and 4% solutions independently, and these were respectively found to be 169.62 and 170.74.

Reducing power. The reducing power of the oligosaccharides was measured, and the results were calculated in terms of dextrose equivalence. Commercial dextrose and maltose of reagent grade were used to complete the series of saccharides. Table 6 gives the DE values found for each reducing sugar.

Table 6. Reducing power.

Saccharide	DE	Meq of potassium ferricyanide consumed	
		Observed	Commerford
Dextrose	100.08	4.5	5.5
Maltose	71.95	6.0	8.3
Maltotriose	61.63	7.1	8.9
Maltotetraose	52.27	7.9	9.8
Maltopentaose	53.97	10.1	10.2
Maltohexaose	41.14	9.4	10.5
Maltoheptaose	38.04	10.2	10.0
Maltooctaose	33.84	10.3	9.9
Maltononaose	31.83	10.7	10.0
Maltodecaose	28.67	10.7	10.8

The value for maltopentaose was unusually high, and no explanation can be given. The determination for maltopentaose was repeated four times, and, each time, values close to the reported value were obtained. It must be emphasized that the precision of the test for DE was not good, and the results of repeated runs did not check well and had a range of 5 DE.

Commerford and Scallet (1964) reported the reducing power of the oligosaccharides in terms of the milliequivalents of potassium ferricyanide consumed in the reaction. Data using this basis of calculation of reducing power are also given in Table 6 and are compared with results of Commerford and Scallet.

Fig. 2 plots observed DE vs. DP for the saccharides evaluated. Because of the rather wide variation in observed values of different runs and the misleading effect that inclusion of the maltopentaose value had on a calculated least-square line, it was felt that a simple plotting of average values indicated a more meaningful relationship than a plotting of expected values based upon the least-square line.

Infrared absorption spectra. Infrared spectra have been used to determine the configurational and structural aspects of various carbohydrate molecules. The peak positions of the group frequencies of interest in carbohydrates have been established.

No unexpected peaks were found in any of the saccharides, indicating that there was little or no contamination in the samples with groups active in the infrared range. The spectra of all of the oligosaccharides greater than DP 2 were much more like that of unmodified starch than that of dextrose. No trends were noted in shifts of peaks or development of peaks with increase in molecular weight. The spectra of all of the saccha-

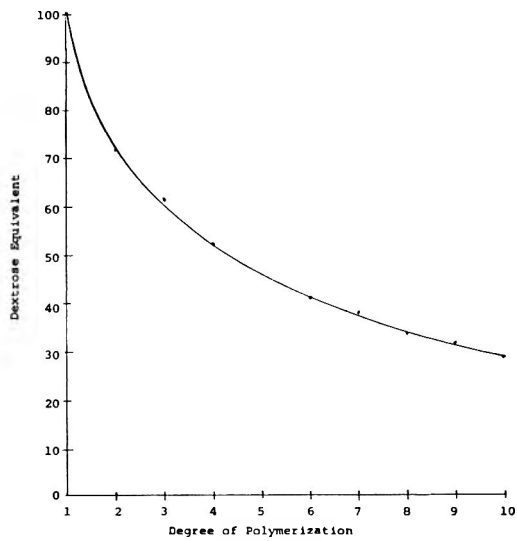

Fig. 2. Dextrose equivalent vs. degree of polymerization.

rides were so similar that they could not be used in identifying or distinguishing between the various malto-oligosaccharides.

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The Fatty Acid Composition of Free and Bound Lipids in Freeze-Dried Meats

SUMMARY

The fatty acid composition of free and bound lipids in freeze-dried pork, lamb, and beef was determined by gas-liquid chromatography (GLC). Both raw and cooked samples were studied to establish whether cooking affected the fatty acid content of either fraction. Sixteen acids were identified in pork, lamb, and beef. In the bound lipids fraction, traces of saturated C_{13}, C_{15}, and C_{17} were evident. Three peaks remained unidentified. These unknown peaks are probably unsaturated acids, although the possibility that they may be oxidation products cannot be ruled out entirely. The bound lipids fraction of the meat samples studied was found to have a greater quantity of polyunsaturated fatty acids than the free lipids fraction. The linoleic, behenic, and arachidonic acid content of the bound lipids exceeded that of the free lipids fraction. Cooking prior to freeze-drying appeared to have no significant influence on the fatty acid composition of either free or bound lipids. Lamb and beef are similar to each other in fatty acid composition, and quite different from pork. The myristic and myristoleic acid content is higher in lamb and beef than in pork, but the linoleic and arachidonic acid content of pork exceeds that of lamb and beef.

INTRODUCTION

The susceptibility of a natural fat to oxidative rancidity depends upon its degree of unsaturation and its antioxidant content. The fatty acid composition is then one of the inherent characteristics of the fat which has an effect on rancidity. Comparison of rancidity tests on extracted fat with tests on whole tissue shows that the lipid fraction primarily involved in the rapid oxidative reaction which takes place in cooked meats can be extracted with a mixture of chloroform and methanol but not with neutral fat solvents (Younathan and Watts, 1960). Tappel (1956) found that the oxidation of lipids that are unextractable by ether from freeze-dried beef appeared to account for half of oxygen adsorption; oxidation of ether-soluble lipids assumed a minor role, accounting, at most, for 10% of total oxidative reaction. This study was undertaken to determine the fatty acid composition of "free" and "bound" lipids of freeze-dried meats as an aid to understanding the changes that occur during oxidation. In this study, the lipid fraction extractable by solvents such as petroleum ether, diethyl ether, or chloroform is considered "free lipids." In mammalian non-adipose tissues, the major portion of the lipids is present as lipoproteins, and binding by water molecules plays an important part in this union. Dehydrating agents such as methanol and ethanol, which essentially rupture the lipid-protein linkage, may be included in the solvent used for extraction. The lipid fraction obtained only by use of a solvent mixture containing methanol and chloroform contains those lipids regarded as "bound lipids."

EXPERIMENTAL

Materials. The meat samples were taken as cross-sectional slices from commercial-grade pork (ham), lamb (leg), and beef (inside top round). Chemicals were methanol, absolute, analytical reagent; chloroform, analytical reagent; petroleum ether, certified reagent grade, bp 30–60°C; ethanol, 95%, refluxed and distilled over Mg ribbon; Amberlite IRA-400 or CG-400 (Rohm and Haas Co., Philadelphia, Pa.).

Preparation of meat. Each meat sample was cut into slices approximately one-half-inch thick and trimmed free of all visible fat. The meat was divided into two portions and treated in one of two ways prior to freezing: a) The meat was diced into pieces of about 1 × ¾ × ½ inch. b) The meat was cooked 30 min in boiling water on an electric range. It was then cut into pieces similar to those of the raw meat.

The diced-meat samples were frozen immediately following cooking, as single layers between aluminum foil on trays at −20°F and then freeze-dried in a Stokes freeze-dryer for 20 hr. During the final 16 hr of freeze-drying, the heating plate was set at 42°C. The internal vacuum was observed to fall to a minimum pressure of 70 microns.

After freeze-drying, the meat was ground with a mortar and pestle and the finely divided tissue

was subjected to lipid extraction immediately. Samples which could not be extracted the same day were stored overnight at −40°F.

Lipid extraction. *Free lipids.* A 10-g sample of the ground meat was extracted with petroleum ether in a Soxhlet type of glass apparatus, using a paper thimble with porosity permitting rapid passage of the solvent. The extraction period was about 8 hr at a condensation rate of 1–2 drops per second. Heating of the solvent was kept as low as possible. At completion of extraction, the solvent was removed by evaporation under vacuum on a Rinco rotating evaporator.

Bound lipids. The meat sample from which the free lipids had been extracted with petroleum ether was used for the bound lipids extraction. Extraction of bound lipids from muscle tissues was made by macerating in a Waring blender with chloroform, methanol, and water. The extraction procedure was essentially that of Bligh and Dyer (1959), modified to include washing of the extract according to Folch *et al.* (1957).

Fatty acid determinations. Dry lipid extracts were saponified for 6 hr with 25 ml of 0.5*N* methanolic potassium hydroxide per 200 mg of lipid material. The fatty acids were isolated by acidification of the diluted saponification reaction mixture with dropwise addition of concentrated hydrochloric acid and extraction with petroleum ether. The free fatty acids were adsorbed on an anion-exchange resin, and then, with anhydrous methanol-hydrochloric acid, converted directly on the resin to their methyl esters, following the procedure described by Hornstein *et al.* (1960).

Analysis of fatty acids by GLC. GLC was carried out on an F and M (Model 500) programmed high-temperature gas chromatographic unit fitted with a Disc Chart Integrator (Model 201 B). The partitioning medium was a polar ester (diethylene glycol succinate-LAC-3R-728), 20%, w/w, on Chromosorb W (F and M, 60–80-mesh). The Chromosorb W was treated with a 1% phosphoric acid solution for more effective resolution. The column was a 6-foot × ¼-inch OD coiled Cu tube. The helium flow rate was 75–85 ml per minute. The peak area was calculated from the pen trace made by the disc-chart integrator. The fatty acid composition of each lipid fraction was calculated as area percent of the total fatty acids.

Identification of fatty acids was made by comparing the sample components with a standard chromatogram of known high-purity fatty acid methyl esters, chromatographed under the same isothermal conditions of 210°C or by programming the operating temperature between 140 and 230°C at a constant rate of 4 or 7.9°C/min. Unknown peaks were tentatively identified by plotting log retention times of known esters against carbon numbers or degree of unsaturation. This applied to fatty acids 12:1 and 21:1 and three components remained unidentified.

RESULTS AND DISCUSSION

Fatty acid distribution. Saturated acids (8, 10, 12, 14, 16, 18, and 22 carbon atoms), monounsaturated acids (12, 14, 16, 18, 21, and 24 carbon atoms), and polyunsaturated acids (18:2, 18:3, and 20:4) were identified as present in raw and cooked pork, lamb, and beef. In the bound lipids fraction of all samples, traces of unsaturated C_{13}, C_{15}, and C_{17} were evident. Three peaks remained unidentified. It was not fully established whether these unknown peaks were those of oxidation products or acids. If they are acids it is probable that these peaks represent unsaturated rather than saturated acids. Table 1 shows the fatty acid composition of lipid fractions extracted from raw and cooked pork, lamb, and beef; the order in which the acids appear is the order of elution from the polar chromatographic column. The data are the values from three different animals of each species, and the average is the mean of these values.

Free lipids vs. bound lipids in raw meat. The bound lipids of the meat from the three species of animals studied contained a greater proportion of polyunsaturated fatty acids than the free lipids fraction. Unsaturated acids containing two or more double bonds make up 15.8, 7.0, and 6.3% of the free lipids fraction and about 41.1, 36.1, and 33.4% of the bound lipids fraction in raw pork, lamb, and beef, respectively.

Each class of lipid (free lipids, bound lipids) appears to have a characteristic fatty acid composition. The myristic, palmitoleic, and oleic acid contents of pork, lamb, and beef are much higher in the free lipids than in the bound lipids fraction. On the other hand, the linoleic, behenic, and arachidonic acid content of the bound lipids exceeds that of the free lipids. The major difference is the large amount of arachidonic acid found in the bound lipids fraction that has no counterpart in the free lipids fraction. Similarly, the linoleic acid content of the bound lipids fraction is greater than that of the free lipids fraction in all meat samples.

Table 1. Fatty acid composition of "free" and "bound" lipids of freeze-dried raw and cooked pork, lamb, and beef (% of total fatty acids).

Fatty acid[a]	Pork				Lamb				Beef			
	Raw		Cooked		Raw		Cooked		Raw		Cooked	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
8	Tr[b]	0.8	Tr	0.6	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
10	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
12	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
12:1[c]	Tr	1.0	Tr	0.9	Tr	Tr	Tr	0.9	Tr	0.6	Tr	0.7
14	2.3	1.2	3.1	1.2	4.7	0.9	4.8	1.0	4.8	1.0	4.2	1.2
14:1[d]	Tr	0.7	Tr	0.6	1.0	0.6	1.6	0.5	2.0	0.8	2.0	0.8
16	18.7	16.7	19.7	16.4	22.8	14.0	19.1	15.7	20.2	14.5	19.0	17.3
16:1[e]	8.5	2.1	9.3	2.1	5.0	2.9	8.4	3.1	9.4	4.2	9.5	5.5
18	13.2	13.1	15.0	12.2	16.9	10.6	19.6	12.2	19.3	10.3	20.6	10.6
18:1	35.1	15.9	34.3	15.4	38.7	23.3	33.8	25.1	34.0	23.3	33.0	30.0
18:2	12.1	29.5	12.8	29.0	5.1	24.6	6.3	25.1	4.4	21.1	5.2	17.5
18:3	2.6	1.1	2.1	1.0	1.9	2.7	2.6	2.7	1.9	3.3	2.3	3.3
21:1	0.9	0.9	Tr	0.7	Tr	1.0	1.0	0.9	Tr	0.6	Tr	Tr
22	Tr	1.8	Tr	1.7	Tr	0.8	Tr	0.8	Tr	2.2	Tr	1.7
20:4	1.1	11.5	0.7	12.3	Tr	8.8	0.7	7.0	Tr	9.0	0.7	6.1
x	2.0	0.9	0.7	1.5	1.0	3.6	Tr	1.9	1.0	4.0	1.0	2.1
24:1	Tr	1.3	Tr	1.5	Tr	1.1	Tr	Tr	Tr	0.6	Tr	Tr
y	0.7	0.5	Tr	1.3	0.5	2.8	0.8	1.6	0.8	3.0	0.8	1.9
z	1.2	Tr	0.8	0.7	0.8	1.8	0.8	0.7	1.0	1.1	0.8	Tr

[a] Number identifies the chain length and number after colon signifies the number of double bonds. x, y, z = unidentified components.
[b] Trace = <0.5%.
[c] 12:1 + 13.
[d] 14:1 + 15.
[e] 16:1 + 17.

These results are consistent with the observation of Kuchmak and Dugan (1964). A similar trend was noted in aged lean beef and pork (Hornstein, *et al.*, 1961).

Free lipids vs. bound lipids in cooked meat. There seems to be little difference in the fatty acid content of freeze-dried raw and cooked meat, either in the free lipids or bound lipids fraction. This does not indicate the liberation of any appreciable quantity of more tightly bound lipids on cooking. Only in beef is the oleic acid content of the cooked sample greater than that of the raw meat. However, this difference may be attributed to the variations in data rather than to the effect of cooking. In a study of the fatty acid content of meat and poultry before and after cooking, Chang and Watts (1952) attributed the differences between raw meat and cooked meat in fatty acid content of fat to a non-uniform distribution of triglycerides of raw meat rather than to a large difference in the extent of destruction of unsaturated fatty acids during cooking.

The fatty acid composition of meat from different animals. Prominent differences appear in the myristic, myristoleic, linoleic, and arachidonic acid content of the free lipids fraction. The amounts of myristic and myristoleic acid are higher in lamb and beef than in pork. However, the linoleic and arachidonic acid content of pork exceeds that of lamb and beef. Chang and Watts (1952) also found that the linoleic acid content of beef and lamb was less than that of pork, which in turn was less than that of chicken and turkey. The linoleic acid content of pork depot fat, too, exceeded that of lamb and beef (Ostrander and Dugan, 1962). In the bound lipids fraction, the same trend of a higher linoleic acid content in pork was observed. The greater proportion of linoleic

and arachidonic acid in pork tends to support and identify the greater susceptibility of pork to oxidation than that encountered in lamb and beef.

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Purification and Certain Properties of Avocado Polygalacturonase

SUMMARY

A procedure was developed for the extraction of polygalacturonase (PG) from avocado mesocarp. PG, which is absent from immature fruit, begins to develop at the blossom end and progresses toward the stem end. By ammonium sulfate fractionation, ion-exchange chromatography, and the use of calcium phosphate gel, a four-step procedure was developed resulting in a 36-fold purification in terms of specific activity. Avocado PG causes a random hydrolysis of polygalacturonic acid to D-galacturonic acid; it has an optimum pH of 5.5 in sodium acetate buffer. Partial inhibition was caused by ammonium, potassium, and phosphate ions, and inhibition was nearly complete by polymetaphosphate. Evidence is presented for the presence of a natural inhibitor of avocado PG in mesocarp tissue.

The polygalacturonase (PG) of higher plants, in contrast to their pectinesterases, have received considerably less attention than those produced by various microorganisms. This may be due to the usually much more abundant occurrence of pectinesterases in plant tissues than is the case with pectin glycosidases. Nevertheless, the presence of polygalacturonase in certain plant tissues is presently well established (Demain and Phaff, 1957; Patel and Phaff, 1960a,b; Hobson, 1962).

McCready and McComb (1954) and McCready *et al.* (1955) published the only accounts on avocado PG of which the present authors are aware. They blended the flesh of soft ripe fruit in 5% NaCl solution at pH 6.5 and filtered the insoluble residue. The enzyme was precipitated from the filtrate with $(NH_4)_2SO_4$ (0.9 saturated). This crude enzyme preparation was shown to randomly hydrolyze pectic acid at pH 5.0 to D-galacturonic acid as the final product of hydrolysis. The authors concluded that avocado PG and tomato PG were qualitatively similar. A brief report by Hobson (1962) indicates that, on a fresh-weight basis, tomatoes contain approximately 15 times as much PG activity as do avocados.

The present report describes methods for the purification of avocado PG and some factors which affect the activity of the enzyme.

EXPERIMENTAL PROCEDURE

Preparation of adsorbants. Calcium phosphate gel was prepared according to the procedure of Kunitz (1952). The final concentration was 25 mg per ml. Pectic acid gel was prepared by the procedure of Phaff and Demain (1956), and calcium pectate gel was prepared according to the method of Newbold and Joslyn (1952). Cellulose *N,N*-diethylaminoethyl ether (DEAE) was obtained from Eastman (No. 7392) and prepared as follows. One gram of DEAE cellulose was suspended in 100 ml of 0.1*M* phosphate buffer at pH 8.0. Entrapped air was removed under vacuum by slow stirring with a magnetic stirrer. The suspension was left to settle, and the buffer was decanted. The slurry was similarly treated with another 100-ml portion of the same buffer, followed by five successive 100-ml portions of distilled water. The final suspension was poured into a chromatography tube 1.1 cm in diameter and allowed to pack under flow induced by gravity. After washing with distilled water, the column was ready for use. Elution was done with a convexly increasing gradient in buffer strength. An apparatus similar to that described by Palmer (1955) was used. It was modified by the attachment of a hydrostatic pressure regulator for maintaining a constant head pressure of 80 cm of water.

Carboxymethylcellulose (CMC) columns were prepared from a commercial Whatman preparation (No. CM70). The general method of preparation was similar to that described for DEAE-cellulose except that the material was equilibrated against 0.1*M* sodium acetate buffer, pH 4.5, before washing it with distilled water.

Polygalacturonase assay. The substrate was a purified polygalacturonic acid (No. 491) purchased from the Exchange Lemon Products Co., Corona, California. It was used without further treatment. Enzymic reaction mixtures contained 0.5% polygalacturonic acid in 0.1*M* Na-acetate buffer at pH 5.5. This pH value was chosen since preliminary pH-activity curves with crude avocado PG indicated optimum activity at this pH in acetate buffers. This value was later confirmed

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with purified enzyme. Release of aldehyde groups during enzymic hydrolysis of polygalacturonic acid was measured by a modified hypoiodite procedure (Jansen and MacDonnell, 1945). One unit of polygalacturonase (PGu) is the amount of enzyme which releases 1 μmole of reducing groups per minute at pH 5.5 and 30°C.

A rapid assay for PG activity in fractions obtained during column chromatography was done by the cup-plate assay after Dingle *et al.* (1951). Polygalacturonic acid (1%) in distilled water was neutralized with NaOH to pH 5.5. Agar (2%) was added, and the mixture was melted and poured into a flat Pyrex dish. After solidification, holes, 6 mm wide, were made with a cork borer (using suction), and 0.05-ml quantities of enzyme solution were introduced into the holes. The plate was incubated 22 hr at 30°C and then flooded with 6*N* HCl. The holes containing active enzyme were surrounded by clear zones (representing degraded pectic acid), whereas the background appeared opaque from precipitated unhydrolyzed pectic acid. After 5 min the HCl was gently removed by washing with water, and the diameters of the clear zones were measured. This provided a reliable semiquantitative assay of the PG content of the various fractions.

For certain comparative experiments polygalacturonase was estimated by a viscometric assay procedure (Phaff and Demain, 1956). The substrate was sodium polypectate (obtained from Sunkist Growers Assn., Ontario, Calif.) purified by a single precipitation with hydrochloric acid. A 1% solution in water at pH 5.0 was used for the determinations in an Ostwald-Fenske capillary viscosimeter. The inverse of the time required to reduce the viscosity of the substrate by 50% was a measure of the enzymic activity. One viscometric unit $(PGu)^v$ is the amount of enzyme which will reduce the viscosity of the substrate 50% in 100 min. There exists a linear relationship between enzyme concentration and the reciprocal of the time required to reach 50% viscosity.

Determination of protein. Protein concentrations were ordinarily determined by the method of Lowry *et al.* (1951) with a commercially prepared Folin-Ciocalteu reagent. Crystallized bovine serum albumin was used to prepare standard curves. The protein content of the various fractions obtained during column chromatography was estimated by measuring changes in absorbance at 280 mμ with a Beckman DU spectrophotometer.

Chromatography. Paper chromatographic analysis of oligouronides was conducted at 30°C with ethyl acetate–acetic acid–water (2:1:2) on Whatman paper No. 4 (Demain and Phaff, 1954a). The spots were located by spraying with the benzidine reagent in trichloroacetic acid, followed by heating.

RESULTS

Extraction of polygalacturonase from avocado mesocarp. Hard-ripe avocados of the Fuerte variety were purchased periodically at a local market. At room temperature the fruit became soft-ripe in 3–10 days. A few lots of fruit of the Hass variety, picked from a single tree, were obtained through the courtesy of Dr. R. C. Bean, University of California, Riverside. After the skin and seed were removed, one part of mesocarp tissue was suspended in 2 parts (by weight) of a 6% (w/v) aqueous NaCl solution. The suspension was homogenized in a blender for 2 min and centrifuged for 30 min at 10,000 × G at room temperature. This operation produced a layer of solid matter, an aqueous phase, and an epiphase high in oil content. The aqueous middle layer was removed and dialyzed for 18 hr at room temperature against 0.1*M* Na-acetate buffer at pH 4.5 or 5.2. A certain amount of inert protein precipitated during dialysis, which was separated by centrifugation followed by filtration through fluted Whatman No. 12 filter paper. Activity determinations were possible with the clear, dark-yellow solution, whereas, prior to dialysis, inorganic salts and reducing materials interfered with activity assays. Extractions, as described above, were also tried with various additives in the NaCl solution. With 0.07% $NaHSO_3$, Na_2S, Versene, NaCN, or 0.1% Triton X-100 (Rohm and Haas Co.) added individually, the activities of the extracts were equal or in some cases inferior to those with salt used alone.

Degree of ripeness and enzymatic activity. During initial experiments with fruit of the Hass variety, picked at a certain day from a single tree, highly variable activity figures were obtained between individual fruits of comparable appearance and ripeness. A method was therefore developed to take samples from a single fruit. Because of the larger size, fruit of the Fuerte variety was used. An 11-mm-diameter cork borer was inserted into the mesocarp to the pit area. After the plugs were carefully removed, the holes were immediately filled with a sterilized 1:2 mixture of paraffin and Vaseline. The plugs were then extracted as described above. The results are given in Table 1. The blossom end of the fruit developed polygalacturonase activity much more rapidly than did the stem end, but when the fruit reached full maturity (after 4 days) the stem end had also developed considerable PG activity. The large increase in specific activity could indicate that active PG was formed from inactive protein during the 4-day ripening period, that inactive protein was broken

Table 1. Polygalacturonase activities found during the final stages of ripening of avocado fruit of the Fuerte variety. Plugs were removed from the stem end and from the blossom end in three individual fruits on four consecutive days and extracted with salt solution. Activities were measured by the viscometric method.

Time (days of ripening at 23–25°)	Region of the fruit where samples were taken	(PGu)^v per ml extract of the three fruits	Mean value	Specific activity (PGu)^v per mg protein Mean value
1	Blossom end	4.55; 4.58; 2.45	3.86	1.7
2		9.10; 9.10; 8.35	8.85	5.4
3		10.4; 12.5; 14.8	12.56	4.0
4		20.0; 18.7; 15.7	18.13	9.1
1	Stem end	1.95; 3.27; 1.57	2.26	2.4
2		4.76; 5.40; 2.56	4.24	2.5
3		5.50; 7.70; 3.10	5.43	3.1
4		13.8; 13.5; 10.6	12.63	8.8

down or transformed into an insoluble form, or that substances inhibitory to the activity of avocado PG were eliminated.

In another experiment, 5 samples were taken from a nearly ripe fruit at different locations ranging from the blossom end to the stem end. The following (PGu)^v per ml were found: 25.0 (blossom end); 28.5; 14.0; 12.5; 8.7 (stem end). With yet another fruit, the following (PGu)^v per ml were obtained: 16.6 (blossom end); 3.8; 1.8 (stem end). Thus, ripening or softening appears to commence at the blossom end and progress toward the stem end.

Purification of avocado polygalacturonase. The starting solution was a dialyzed salt extract of fully ripe fruit (see above).

Fractionation with ammonium sulfate. The crude extract was mixed with solid $(NH_4)_2SO_4$ to 0.5 saturation. The precipitated protein, which had negligible activity, was discarded after centrifugation. The resulting solution was brought to 0.9 saturation by addition of more solid $(NH_4)_2SO_4$. The active protein was collected by centrifugation at low temperature, and the pellet was dissolved in one-fifth of the original volume of distilled water in the cold, which, however, did not remove the color. The results of two individual experiments are given in Table 2.

Chromatography on a column of carboxymethyl cellulose (CMC). Preliminary experiments indicated that avocado PG was not adsorbed by CMC prepared as described under Experimental Procedures. In the hope that CMC might retain inert proteins, a column was prepared representing 12 g CMC. The enzyme solution prepared by $(NH_4)_2SO_4$ fractionation was passed over the column, followed by washing with distilled water. Further washing with 2% NaCl solution and with 0.5*M* Na-acetate solution at pH 4.5 eluted no further PG activity. The straight filtrate and the washings with distilled water were combined, giving a light-brown solution. Results of four individual experiments are presented in Table 3, indicating additional purification and partial removal of dark pigment.

Purification by chromatography over a DEAE-cellulose column. Preliminary tests showed that avocado PG which had been passed over CMC could be adsorbed on DEAE cellulose pretreated as described under Experimental Procedures. The enzyme was released from DEAE cellulose by suspending it in 0.4*M* Na-acetate buffer at pH 5.2.

Table 2. Fractionation of a crude extract of avocados with $(NH_4)_2SO_4$. The extract was prepared as described in the first section of "Results." Figures representing two individual experiments are given.

Step	Volume (ml)	PGu per ml	mg protein per ml	PGu per mg protein	Increase in specific activity	Recovery (%)
Crude extract	1480	0.24	4.18	0.06		
Fraction precipitated between 0.5 and 0.9 saturation with $(NH_4)_2SO_4$ (after dialysis)	300	1.45	5.74	0.25	4.2 ×	120
Crude extract	1600	0.37	5.30	0.07		
Fractionation as above	330	1.80	6.66	0.27	3.8 ×	100

Table 3. Results of passing a solution of avocado PG, previously fractionated by $(NH_4)_2SO_4$ and dialyzed against distilled water, over a column of carboxymethyl cellulose. The data represent four individual experiments.

	Step	Volume (ml)	PGu per ml	mg protein per ml	PGu per mg protein	Increase in specific activity	Recovery (%)
I	Initial solution	10	1.45	5.75	0.25		
II	Effluent from CMC column	25	0.43	0.77	0.56	2.2 ×	74
I		80	1.80	6.66	0.27		
II		125	1.45	2.11	0.69	2.6 ×	125
I		100	1.45	5.75	0.25		
II		140	1.20	2.44	0.49	2.0 ×	116
I		70	1.80	6.66	0.27		
II		105	1.95	2.56	0.76	2.8 ×	162

Next, a column was prepared containing 12 g DEAE cellulose. The enzyme solution (eluate from CMC column) was introduced at the top, and, after the column was washed with water, the enzyme was subjected to gradient elution with a constant volume of 250 ml in the mixing chamber. The two liquids were 0.005*M* phosphate buffer, pH 8.0, and 0.4*M* acetate buffer, pH 5.2. Fractions of 7.5 ml were collected at room temperature with a constant-volume fraction collector. The fractions which contained enzyme activity (as determined by the cup-plate assay) were pooled and assayed after dialysis for 16 hr against distilled water. The enzyme solution at this stage had a light-yellowish color. The results are shown in Table 4.

An attempt was made to chromatograph the enzyme precipitated between 0.5 and 0.9 saturated $(NH_4)_2SO_4$ directly on DEAE cellulose. In two separate experiments, specific activities of the eluates were 0.71 and 0.91 PGu per mg protein. Because these specific activities are not as good as those reported in Table 4, the CMC step prior to DEAE-cellulose was retained.

Adsorption and elution from various gels. Preliminary experiments with calcium pectate gel and with pectic acid gel indicated that there was little promise of further purification when the eluate from DEAE cellulose was adsorbed on these gels, followed by elution. The recoveries were very low and the specific activities did not increase. Results were much better with calcium phosphate gel. Virtually all of the PG activity could be adsorbed on calcium phosphate gel (10 ml of the gel were added to 100 ml of enzyme solution at room temperature) provided the eluate from DEAE-cellulose columns was thoroughly dialyzed against distilled water. After stirring for 20 min the gel was centrifuged, washed with distilled water, and then extracted with three portions of 1*M* Na-acetate buffer at pH 4.5. Dialysis of the eluate against distilled water gave a colorless enzyme solution. Besides a possible concentration of the enzyme, a significant additional increase in specific activity was obtained. The results are shown in Table 5.

One of the fractions eluted from calcium phosphate gel was rechromatographed on a DEAE cellulose column to establish if further purification might be obtained. After chromatography, a sample with a specific activity of 2.26 PGu per mg protein was recovered with a specific activity of

Table 4. Chromatography of effluents from carboxymethyl cellulose (*cf.* Table 3) over DEAE-cellulose. The active fractions in the eluate were located by the cup-plate assay and pooled. The data represent four individual experiments.

	Step	Volume (ml)	PGu per ml	mg protein per ml	PGu per mg protein	Increase in specific activity	Recovery (%)
I	Effluent from CMC	23	0.43	0.77	0.56		
II	Eluate from DEAE-cellulose	65	0.08	0.07	1.14	2.0 ×	52
I		100	1.45	2.11	0.69		
II		195	0.80	0.60	1.33	1.9 ×	107
I		100	1.20	2.44	0.49		
II		240	0.44	0.54	0.81	1.7 ×	88
I		100	1.95	2.56	0.76		
II		220	0.90	0.62	1.45	1.9 ×	101

Table 5. Purification of avocado PG with calcium phosphate gel. The eluate from DEAE-cellulose (*cf.* Table 4) was dialyzed against distilled water and adsorbed on calcium phosphate gel. After washing, the enzyme was eluted with 1*M* acetate buffer pH 4.5. The results represent four individual experiments.

Step	Volume (ml)	PGu per ml	mg protein per ml	PGu per mg protein	Increase in specific activity	Recovery (%)
I Eluate from DEAE-cellulose	10	0.80	0.60	1.33		
II Eluate from calcium-phosphate gel	9	0.43	0.16	2.68	2.0 ×	48
I	105	0.80	0.60	1.33		
II	30	1.17	0.44	2.66	2.0 ×	42
I	230	0.44	0.54	0.81		
II	70	0.73	0.32	2.28	2.8 ×	51
I	210	0.90	0.62	1.45		
II	90	0.64	0.38	1.68	1.2 ×	32

2.21 PGu per mg P and, thus, no further purification was obtained. For a better comparison of the results, Table 6 summarizes the purification obtained by the four steps discussed above.

Optimum pH of avocado PG and the effect of various ions. The optimum pH was studied in sodium acetate buffers, since many optimum pH values of polygalacturonases reported in the literature were obtained in the presence of this buffer. The results (Fig. 1) indicate that the optimum was at pH 5.5, one unit higher than that of tomato PG. A sodium ion concentration of 0.14*M* was selected because this level was found to be optimal. At higher sodium ion concentrations the activity gradually decreased, with inhibition being complete at a Na^+ concentration greater than 0.7*M*.

When the pH optimum of the enzyme was determined in 0.1*M* acetic acid adjusted to different pH values with 0.1*M* Na_3PO_4, an optimum pH of 5.2 was found, a shift of 0.3 pH unit to the lower regions. At the same time the maximum activity was significantly lower than that of the same enzyme preparation in sodium acetate buffer, indicating an inhibitory effect by phosphate. The influence of various ions was therefore determined on the same preparation of avocado PG. The results are shown in Table 7.

It is evident that NH_4^+ and K^+ inhibit the activity of avocado PG more than do sodium ions. Phosphate and polyphosphate (Calgon) ions were both strongly inhibitory. The inhibition by these two compounds may indicate that a divalent cation plays the role of an activator. However, none of the divalent cations tried (Table 7) exerted any stimulatory effect on the enzyme. It is possible, of course, that the substrate polygalacturonic acid, which was not subjected to purification, supplied a sufficient concentration of divalent ions to provide maximum activity.

Action pattern of purified avocado PG. The conclusion of McCready *et al.* (1955) that crude avocado PG causes a random hydrolysis of polygalacturonic acid to D-galacturonic acid, was rechecked with purified enzyme. Paper chromatographic analysis of the reaction products with time showed the initial appearance of intermediate oligogalacturonides, the higher ones disappearing with time. When 50% of the total number of glycosidic bonds in pectic acid were cleaved, mono-, di, and trigalacturonic acids were the products. With low

Table 6. Summary of the over-all purification of avocado PG by a four-step procedure.

Step of purification	Mean values: No. of experiments	PGu per mg protein	Increase in specific activity	Yield in % based on original extract
Crude dialyzed extract	2	0.065	1	100
Fraction precipitated between 0.5 and 0.9 saturated $(NH_4)_2SO_4$	2	0.26	4 ×	110
Eluate from carboxymethyl cellulose	4	0.63	9.7 ×	131
Eluate from DEAE-cellulose	4	1.18	18.2 ×	114
Eluate from calcium phosphate gel	4	2.33	35.8 ×	49

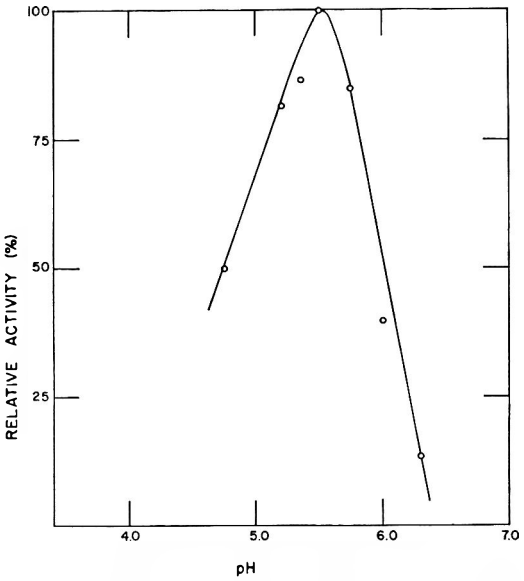

Fig. 1. Optimum pH of avocado PG. The purified enzyme solution was the eluate from calcium phosphate gel, specific activity 2.68 PGu/mg protein. The substrate polygalacturonate was 0.14*M* in sodium ions, adjusted to the required pH by means of acetic acid. The activities were obtained by the hypoiodite procedure.

enzyme concentrations, further degradation of these compounds was extremely slow at pH 5.5.

It is known that endo-polygalacturonase of yeast has a lower pH optimum for oligouronides than for pectic acid (Demain and Phaff, 1954b). In order to test if this would apply to avocado PG, a portion of the reaction mixture with avocado PG was removed and its pH was lowered to 4.5, but no noticeable increase in reaction rate was observed. However, pectic acid does appear to be degraded completely to D-galacturonic acid, because incubation of digalacturonic acid with a high concentration of avocado PG at pH 5.5, produced a slow but distinct hydrolysis to the monomer.

Evidence for the existence of a PG-inhibitor in avocados. The data in Tables 2, 3, and 4 show that the recovery figures of PG during purification are unusually high, often exceeding 100% by a significant margin. A possible explanation for this could be the presence in the crude avocado extract of a PG-inhibitor which was gradually removed during the purification steps.

Preliminary evidence of such an inhibitor was obtained in hard, immature fruit of the Fuerte variety. A salt extract was prepared in the usual manner and then dialyzed against 0.1*M* Na-acetate buffer at pH 5.5. This preparation was assayed and found to have negligible PG activity.

A highly purified avocado PG (eluate from phosphate gel) was used as the test enzyme. When 18% (v/v) of the immature avocado extract was added to a reaction mixture of the purified enzyme, 48% inhibition was obtained.

Next, the extract of immature fruit was fractionated with $(NH_4)_2SO_4$. The fractions precipitating between 0 and 0.5 saturation and 0.5–0.9 saturation were recovered and brought to the original volume. Neither of the two fractions had polygalacturonase activity. However, the first fraction (0–0.5 saturation) caused 54% inhibition of the purified enzyme when 18% (v/v) was added to a reaction mixture. The second fraction (0.5–0.9 saturation) caused no inhibition of the purified enzyme. The precipitation of inhibitor in 50% saturated ammonium sulfate very likely explains the high recovery percentages in Table 2. The lack of inhibitor in the fraction precipitating between 0.5 and 0.9 saturated $(NH_4)_2SO_4$ is probably due to the fact that hard, immature fruit was used, whereas extraction of enzyme was always done from soft ripe fruit in which the inhibitor may become more soluble in high concentrations of ammonium sulfate.

DISCUSSION

The work described above has shown that the salt-extractable polygalacturonase level in avocado mesocarp increases from virtual absence in hard, immature fruit to a concentration of 0.66 PGu per g of fresh tissue in soft, ripe fruit. This value is in good agreement with the value of 0.58 PGu reported earlier by McCready and McComb (1954) in their preliminary study of this enzyme.

Table 7. Influence of various ions on the activity of avocado PG at pH 5.2. The enzyme preparation was a fraction precipitated between 0.5 and 0.9 saturated $(NH_4)_2SO_4$ and contained 1.45 PGu per ml. The substrate was 0.5% sodium polygalacturonate at pH 5.2. The substrate contributed 0.03*M* sodium ions to each reaction mixture.

Substances added adjusted to pH 5.2 by corresponding acid of base	Molarity of cations added to medium	Activity (%)
Na-acetate	0.1	100
K-acetate	0.1	45
NH_4-acetate	0.12	65
NH_4-acetate	0.01	100[a]
NaH_2PO_4	0.1	55
$NH_4H_2PO_4$	0.1	26
Calgon sodium salt	0.001	2[a]
$CaCl_2$	0.0001	100[a]
Mg-acetate	0.0001	100[a]
$FeSO_4$	0.0001	100[a]
$MnSO_4$	0.0001	100[a]

[a] In these media of low ionic strength, 0.1*M* Na-acetate buffer at pH 5.2 was also added to the reaction mixture.

Whether the development of avocado PG with ripening is the result of *de novo* synthesis of the enzyme or due to removal of an inhibitor in the tissue, cannot be concluded from our work. Although hard, immature avocados were shown to contain a polygalacturonase inhibitor, this factor was precipitated by half-saturated $(NH_4)_2SO_4$, whereas the supernatant liquid contained neither PG nor inhibitor. However, both the physical state of the inhibitor and that of a possible PG-precursor may change upon maturation. In fact, the unusually high PG yields during the first three steps of the purification process point to the presence of inhibitor in the enzyme precipitated between 0.5 and 0.9 saturated $(NH_4)_2SO_4$. Presumably the inhibitor is gradually eliminated during subsequent purification steps.

The discovery of a polygalacturonase inhibitor in plants is not new. Weurman (1953) demonstrated a thermolabile "pectinase" inhibitor in pear sap, which was effective in specifically inhibiting pectic enzymes acting on pear or apple pectin. A thermostable pectinase inhibitor was isolated from grape leaves by Bell and Etchells (1958). Myrobalan tannins exert a strong inhibition of pectic acid breakdown by fungal polygalacturonases (Hathway and Seakins, 1958), and Cole (1958) found that enzymically oxidized leucoanthocyanins of apple tissue inhibited the polygalacturanose of *Sclerotinia fructigena*.

The polygalacturonase of avocados can be classified as a member of the endo-polygalacturonases (Demain and Phaff, 1957), a group of pectic enzymes which hydrolyze polygalacturonic acid by a random mechanism to either di- or monogalacturonic acid. It appears most nearly related to tomato polygalacturonase, but has a higher optimum pH (5.5 versus 4.5), which may be related to its origin in a nonacidic fruit. The highest specific activity of avocado PG obtained in this work (36-fold purification and 97% inactive protein removed) was 2.68 PGu per mg protein. This value compares closely with the value of 6 to 7 PGu per mg protein for tomato PG (Patel and Phaff, 1960b), but both values are much lower than the 163 PGu per mg protein for endopolygalacturonase of the yeast *Sacch. fragilis* (Phaff and Demain, 1956) or that of a partially purified preparation of fungal origin (Lineweaver *et al.*, 1949) which contained 272 units (converted from 1.7 of their polygalacturonase units per mg total nitrogen). In view of the rather considerable purification of the enzymes from tomatoes and avocados it appears that the specific activity of fungal polygalacturonases is much higher than that of plants. However, confirmatory evidence for this conjecture must await the preparation of fully purified plant polygalacturonases. The much higher specific activity of a salt extract of pressed tomato pulp (Patel and Phaff, 1960b) than that of avocado mesocarp (1.29 versus 0.065 PGu per mg protein) is undoubtedly due to the much greater protein content of avocados.

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Volatile Constituents of Black Pepper. III. The Monoterpene Hydrocarbon Fraction

SUMMARY

The monoterpene hydrocarbons of black pepper oil were isolated both by vacuum distillation and by thin-layer chromatography. The following compounds were separated by gas chromatography and identified by infrared and ultraviolet spectroscopy: α-pinene, β-pinene, sabinene, 3-carene, limonene, and *p*-cymene. There is evidence for the presence of α-thujene, α-phellandrene, myrcene, β-phellandrene, γ-terpinene, and terpinolene. The monoterpenes isolated by thin-layer chromatography also contained small quantities of α-terpinene and greater quantities of γ-terpinene, *p*-cymene, and terpinolene.

INTRODUCTION

Oil of black pepper is a complex mixture of terpenes, with a pungent aroma that has intrigued man for centuries. It differs from many essential oils in that it consists primarily of a complex mixture of hydrocarbons, most of which are monoterpenes. In this study our attention is centered on this more volatile fraction. The monoterpenes appear to possess the desirable attributes of pepper flavor and may account for the gourmet's insistence on freshly ground pepper.

As early as 1890, according to Guenther (1952), Schimmel and Co. reported the presence of α-phellandrene, caryophyllene, and dipentene in pepper oil. In 1901, Schreiner and Kremers confirmed this. Hasselstrom *et al.* (1957) isolated the monoterpenes α-pinene, β-pinene, l-α-phellandrene, and dl-limonene from pepper. Using gas chromatography and infrared spectroscopy, the authors (Jennings and Wrolstad, 1961) identified α-pinene, β-pinene, and limonene.

Ikeda *et al.* (1962) analyzed pepper oil in their study of a number of essential oils. The monoterpene hydrocarbons were isolated by a descending chromatostrip procedure (Stanley *et al.*, 1961). On the basis of retention times on two columns and the peak area of the individual monoterpene hydrocarbons, tentative identifications and relative concentrations were assigned as follows: α-pinene (9.4%), α-thujene (2.9%), camphene (0.1%), β-pinene (13.8%), sabinene (20.4%), 3-carene (15.1%), α-phellandrene (3.6%), α-terpinene (0.3%), myrcene (6.4%), d-limonene (21.6%), β-phellandrene (4.1%), γ-terpinene (1.0%), *p*-cymene + terpinolene (1.3%), and ocimene (0.1%).

METHODS AND PROCEDURES

Source. The oil examined was a steam distillate of Ceylon black peppercorns prepared by the research department of Wm. J. Stange Co., Chicago.

Knowns. Used for comparison with fractions isolated from black pepper oil were authentic samples of terpene hydrocarbons from sources previously listed (Wrolstad and Jennings, 1964). The mixture containing β-phellandrene, discussed in this previous paper, has now been resolved on Apiezon L (Klouwen and Ter Heide, 1962) and shown to be a mixture of β-phellandrene and 1,8-cineole. The infrared spectrum of β-phellandrene is shown in Fig. 1.

Isolation of monoterpene hydrocarbons. The monoterpene hydrocarbons were isolated by vacuum distillation on a semi-micro Podbielniak column with an efficiency of 75 theoretical plates. Also used to examine this class of compounds was the downward chromatostrip procedure of Stanley *et al.* (1961). Chromatostrips were prepared by the procedure of Kirchner *et al.* (1951) and utilized starch as a binder.

Gas chromatography. Preparative-scale gas chromatography utilized an Aerograph Autoprep with a 20-foot by ¼-inch aluminum column packed with 40-60 HMDS-treated Chromosorb P with 15% Carbowax 20M, and an Aerograph Model A-90, fitted with a 10-foot by ¼-inch stainless-steel column similarly packed.

The analytical instrument used to the greatest extent was an Aerograph Hy-Fi with a flame ionization detector, fitted with a 10-foot by ⅛-inch stainless-steel column packed as above.

A Beckman Thermotrac, with a Carle microcell thermistorized detector, was used for both pre-

A part of a thesis by Campbell Soup Fellow R. E. Wrolstad, Univ. of Calif., 1964. Investigation supported in part by Research Grant EF 92 from the Division of Environmental Engineering & Food Protection.

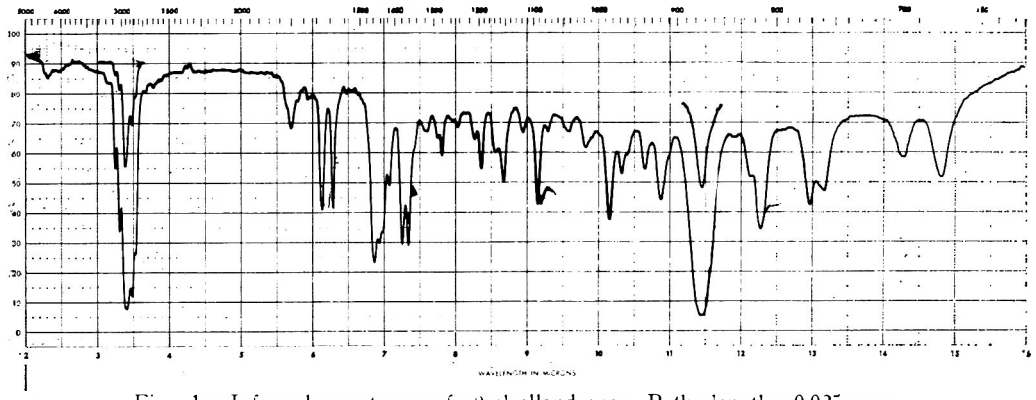

Fig. 1. Infrared spectrum of β-phellandrene. Path length, 0.025 mm.

parative and analytical work. This was equipped with a 23-foot by ⅛-inch stainless-steel column packed with 20% Apiezon L on Gas-Pack F.

The gas chromatographs were operated isothermally. The Autoprep and the A-90 were operated at 120°C and a flow rate of 60 ml He/minute. The Hy-Fi was maintained at 95° with N_2 and H_2 flow rates of 20 ml/minute. The Thermotrac was operated at 120° and a flow rate of 20 ml He/minute.

Relative retention times and percent composition of individual fractions were calculated from the chromatograms of the analytical instrument. Percent composition was estimated by calculating the percentage of the total peak area.

Fractions were collected from the A-90 and the Thermotrac by a method modified from that first suggested by Kratz (1963). One-foot lengths of thin-walled capillary tubing with a right-angle bend 2¼ inches from the end were inserted into the outlet part of either gas chromatograph. The 2¼-inch end was inserted into a 2-ml conical test tube the mouth of which contained a loose plug of glass wool. An inverted Styrofoam cup in which a hole had been cut supported the test tube by the lip. The cup, holding the test tubes, was placed over beakers of ice water.

Spectroscopy. Infrared spectra were determined on a Beckman Model IR5 spectrophotometer fitted with a Beckman beam condenser. Spectra were determined either in CIC Type D microcavity cells (path length, 0.05 mm) or the Beckman demountable ultramicro liquid cell (path length, 0.025 mm).

Ultraviolet spectra were determined on a Beckman DB spectrophotometer in spectrograde isooctane.

RESULTS AND DISCUSSION

The distillate collected from 10 ml of black pepper oil amounted to 8.5 ml. So that the distillate composition would be limited solely to monoterpene hydrocarbons, distillation was not carried to completion. Since some monoterpene hydrocarbons would remain in the pot residue, 85% is not an accurate estimate of the percentage of monoterpene hydrocarbons. A 1-μl injection on the analytical instrument was programmed from 100 to 175° in order to get relatively sharp peaks in the sesquiterpene fraction. The percentages of monoterpenes and sesquiterpenes were approximated from the chromatogram by the triangulation method. The oil used was estimated to be 96% monoterpene hydrocarbons and 4% sesquiterpene hydrocarbons.

Fig. 2 is a chromatogram of the distillate of Stange black pepper oil on Carbowax 20M. Table 1 lists the compounds making up this chromatogram, their percentage composition, and retention data. Ultraviolet

Table 1. The monoterpene hydrocarbons of black pepper.

Peak No.		Percent composition	Relative retention	
			Carbowax 20M	Apiezon L
1.	α-Pinene	26	0.40	0.48
1.	α-Thujene	3	0.40	0.41
3.	β-Pinene	13	0.64	0.67
4.	Sabinene	25	0.67	0.60
5.	3-Carene	12	0.77	0.83
5.	Myrcene	3	0.78	0.60
6.	α-Phellandrene	2	0.85	0.83
	α-Terpinene		0.91	0.92
7.	Limonene	13	1.00	1.00
8.	β-Phellandrene	2	1.06	0.96
9.	γ-Terpinene	> 1	1.26	1.22
10.	p-Cymene	1	1.44	0.86
11.	Terpinolene	> 1	1.57	1.47

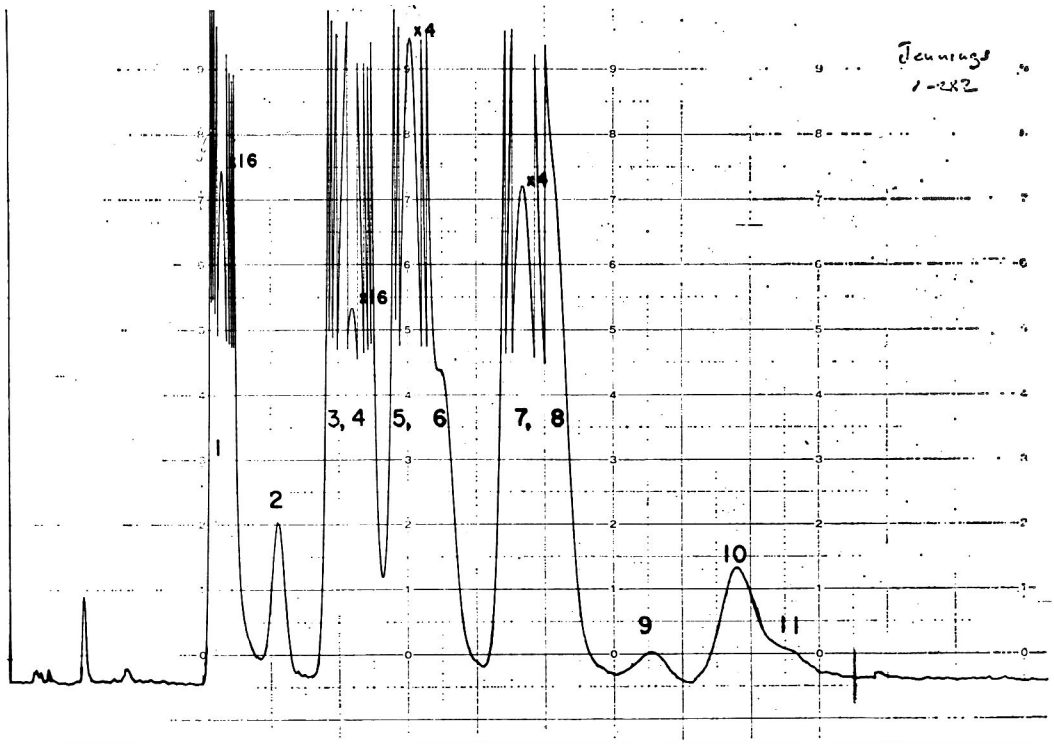

Fig. 2. Chromatogram of the monoterpene hydrocarbons of black pepper oil. Carbowax 20M on Chromosorb P at 95°. N_2 and H_2 flow rates 20 ml/min.

absorption and the infrared spectrum (Fig. 3) of peak 1 were identical to those of α-pinene except for weak absorption at 9.75 and 12.85μ. α-Thujene absorbs strongly at these wave lengths (Wrolstad and Jennings, in press) and has a retention equal to that of α-pinene on Carbowax 20M. Retention data reported by Klouwen and Ter Heide (1962) indicated that these two compounds could be separated on Apiezon L. When the symmetrical peak was collected from a Carbowax 20M column and rechromatographed on Apiezon L it was resolved into 2 components having retention times equivalent to those of α-thujene and α-pinene. The infrared spectrum of the major component, which was 88% of the two-component mixture, was then identical to that of α-pinene, the absorptions at 9.75 and 12.85 μ being eliminated.

Not enough of peak 2 was isolated to obtain infrared and ultraviolet spectra.

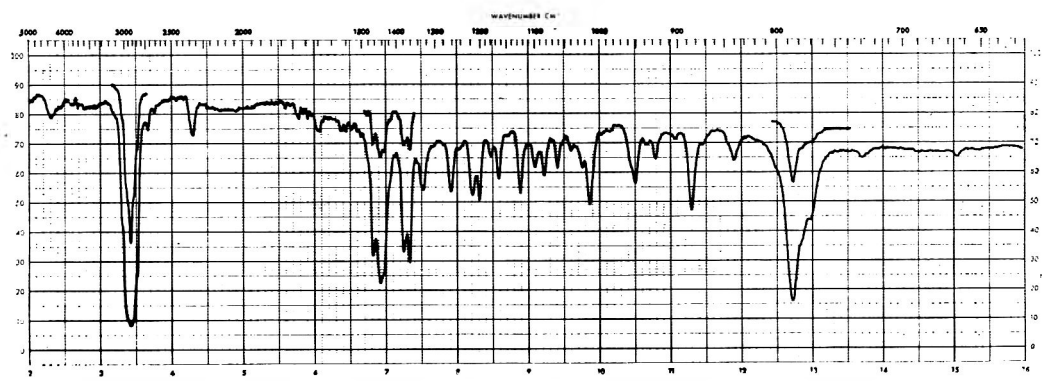

Fig. 3. Infrared spectrum of fraction 1 (α-pinene and α-thujene). Path length, 0.025 mm.

Camphene has retention times in agreement with those of this compound, but Zubyk and Connor (1960), along with Matsuura *et al.* (1961), gave evidence that the *p*-menthenes have retention times close to that of camphene. *p*-Menthenes could arise from disproportionation of p-menthadienes to *p*-cymene and *p*-menthenes. Hunter and Brogden (1963) reported the isomerization of limonene on silica gel at 100° and 150° to α-terpinene, γ-terpinene, terpinolene, and isoterpinolene, which subsequently disproportioned to 1-*p*-menthene, trans-2-*p*-menthene, 3-*p*-menthene, t-8(9)-*p*-menthene, and *p*-cymene. Since retention data were not obtained for the *p*-menthenes, it would be unjustified to identify peak 2 as camphene, even tentatively.

Peaks 3 and 4 were normally not resolved on Carbowax 20M. Peak 3 was identified as β-pinene, and ultraviolet absorption and the infrared spectrum (Fig. 4) of peak 4 were in agreement with those of sabinene. The fraction representing peak 5 and the shoulder, peak 6, were collected. The infrared spectrum of this cut (Fig. 5) was characteristic of 3-carene (Wrolstad and Jennings, in press) except for weak absorption at 6.3, 10.1, 11.25, 13.5, and 13.8 μ. The ultraviolet spectrum showed maxima at 225 and 260 mμ, respectively characteristic of an acyclic conjugated diene and a cisoid conjugated diene (O'Connor and Goldblatt, 1954). Rechromatography of this cut on Apiezon L resulted in two peaks, which were collected. The minor component absorbed at 225 mμ. Recovery of this compound was insufficient for infrared analysis, but examination of the larger fraction showed that the weak absorptions at 6.3, 10.1, and 11.25 μ (Fig. 6) had been eliminated. Myrcene is characterized by strong absorption in these regions (Wrolstad and Jennings, in press) and by absorption at 225 mμ. The infrared

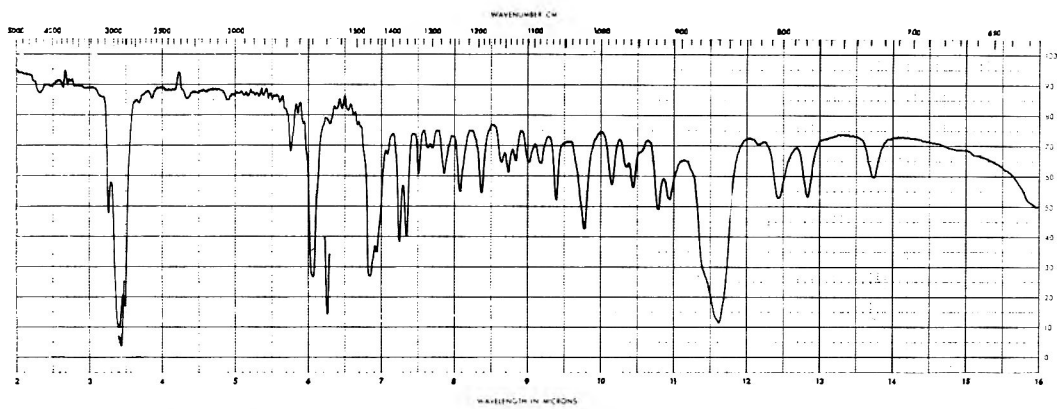

Fig. 4. Infrared spectrum of fraction 4 (sabinene). Path length, 0.025 mm.

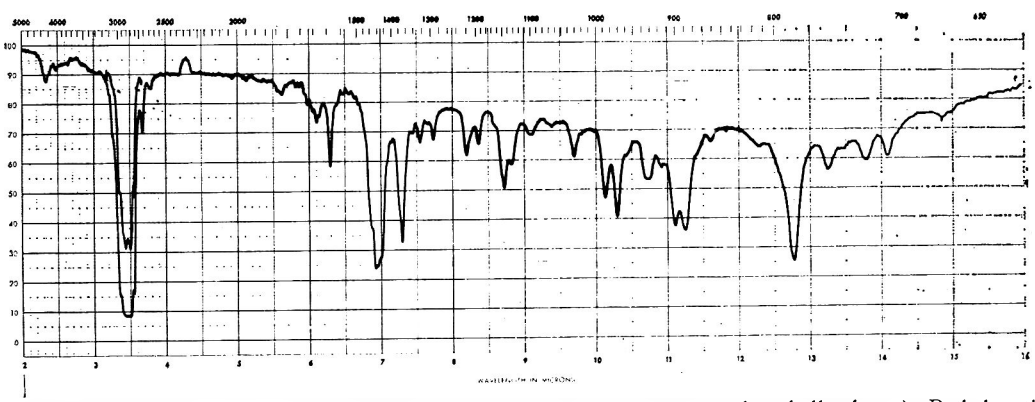

Fig. 5. Infrared spectrum of fractions 5 and 6 (3-carene, myrcene, and α-phellandrene). Path length, 0.025 mm.

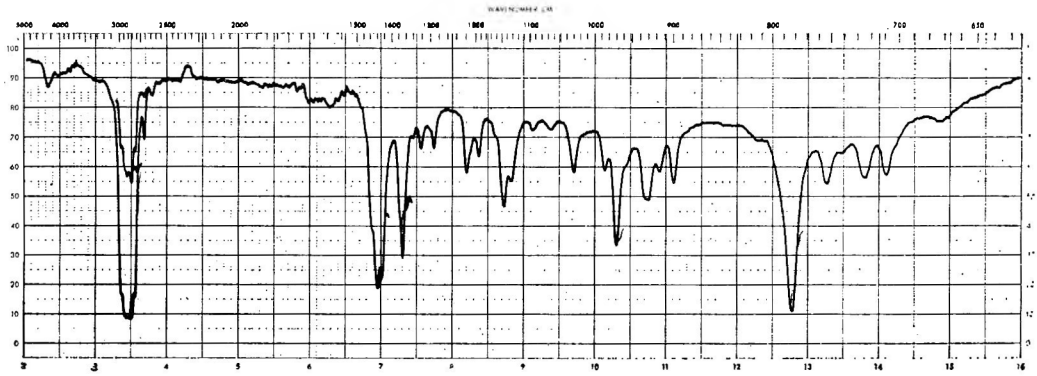

Fig. 6. Infrared spectrum of fractions 5 and 6 (3-carene and α-phellandrene). Path length, 0.025 mm.

spectrum of the major component collected from the Apiezon L column from this cut (Fig. 6) is identical to that of 3-carene except for weak absorption at 13.5 and 13.8 μ. The presence of α-phellandrene could account for this (Spectrum No. 12959, Sadtler Research Labs, 1959–65), and for the ultraviolet absorption at 260 mμ. Retention data as shown in Table 1 indicate that peak 5 could contain both myrcene and 3-carene, and peak 6 could be α-phellandrene.

The infrared spectrum of peak 7 matches that of limonene. The shoulder, peak 8, exhibits ultraviolet absorption at 232 mμ, as does β-phellandrene. Retention of this compound is also in agreement with that of β-phellandrene. Peak 8 was not sufficiently resolved from limonene for an infrared spectrum to be obtained of the pure material. Fig. 7 is an infrared spectrum of a collection of peak 8 shown by gas chromatography to be approximately 60% limonene and 40% peak 8. Absorption at 5.65, 6.1, 7.0, 7.3, 7.55, 8.4, 8.75, 9.55, 9.9, 10.5, 10.98, 11.3, 12.1, and 13.2 μ can be attributed to limonene (O'Connor and Goldblatt, 1954), while absorption at 5.8, 6.3, 6.9, 7.8, 8.55, 8.7, 9.15, 10.15, 10.65, 11.45, 12.25, 12.95, 14.3, and 14.8 μ can be attributed to β-phellandrene (Fig. 1).

The infrared spectrum of peak 10 is in agreement with that of the aromatic hydrocarbon *p*-cymene (Fig. 8) (O'Connor and Goldblatt, 1954); the ultraviolet spectra are also in agreement.

Peaks 9 and 11 have retention data respectively equivalent to γ-terpinene and terpinolene. Not enough material was collected for infrared spectra. The ultraviolet spectra of these two compounds show no absorption above 210 mμ.

The percentage composition of the various fractions in the distillate of Stange black pepper oil was calculated by the triangulation method from the chromatogram on Carbowax 20M. Fractions containing α-pinene

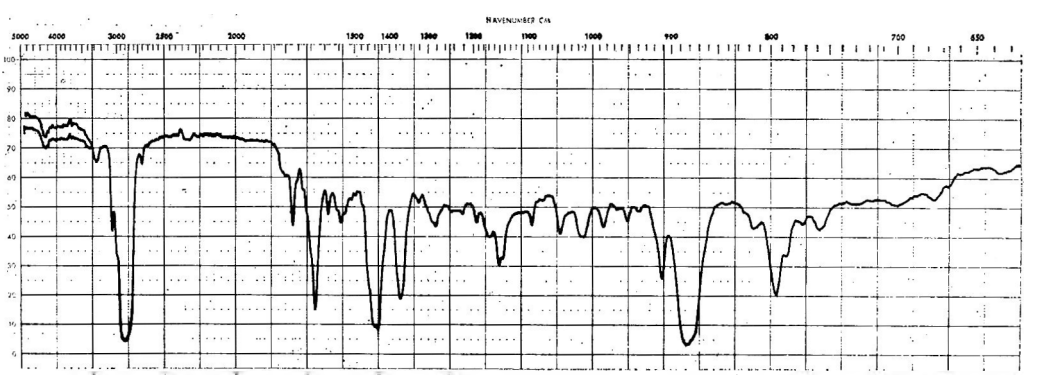

Fig. 7. Infrared spectrum of fractions 7 and 8 (60% limonene and 40% β-phellandrene). Path length, 0.025 mm.

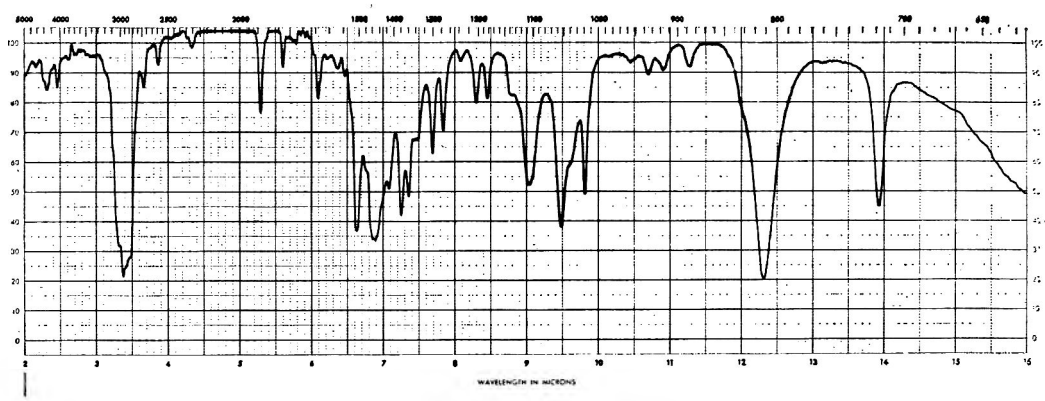

Fig. 8. Infrared spectrum of p-cymene. Path length, 0.05 mm.

and α-thujene, β-pinene and sabinene, and 3-carene and myrcene were collected and rechromatographed on Apiezon L, where they were resolved better. From the resulting chromatograms were estimated the relative amounts of these compounds. The results are shown in Table 1.

Monoterpene hydrocarbons isolated from pepper oil by the descending chromatostrip technique (Stanley *et al.*, 1961) exhibited α-terpinene (not previously present) and larger amounts of γ-terpinene, *p*-cymene, and terpinolene than a control dissolved in heptane and exposed to the same conditions of light and temperature. These results indicated that these 4 compounds arose from isomerization induced by the chromatostrip.

Later work (Wrolstad and Jennings, 1965) in which individual terpenes were subjected to the chromatostrip technique indicates that sabinene is the primary source of these isomerization products, and that the vast majority of the monoterpene hydrocarbons comprising pepper oil are stable to chromatostrip separations *per se*.

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Volatile Flavor and Aroma Components of Pineapple. I. Isolation and Tentative Identification of 2,5-Dimethyl-4-Hydroxy-3(2H)-Furanone

SUMMARY

An unstable compound with an intense odor of "burnt pineapple" was isolated as a major component of a pineapple flavor concentrate. This compound was tentatively identified as 2,5-dimethyl-4-hydroxy-3(2H)-furanone (I).

INTRODUCTION

The first study of pineapple flavor was carried out without benefit of modern techniques of isolation and identification (Haagen-Smit *et al.*, 1945). Nonetheless, eight aliphatic esters, one acid, one aldehyde, one ketone, and a unique sulfur-containing ester were reported. Several other esters, alcohols, aldehydes, and ketones were reported recently, and Haagen-Smit's results were confirmed (Gawler, 1962; Connell, 1964). In addition, Connell identified the ethyl ester homolog of Haagen-Smit's sulfur-containing compound, methyl β-methylthiopropionate.

Jennings (Jennings and Sevenants, 1964) divided flavor components into two classes: "character-impact compounds" and "contributory flavor compounds." The former compounds are unique to a particular flavor, and the latter contribute "fruitiness," "bouquet," or "fullness." Of the pineapple flavor components reported to date, it seems probable that only the two sulfur compounds are unique.

We initiated a chemical investigation and sensory evaluation study of volatile flavor and aroma components of pineapple in August, 1963, using methodology previously described (Bassler and Silverstein, 1963). This report is concerned with isolation and tentative identification of what appears to be a major "character-impact" component of pineapple flavor.

The preliminary investigation reported here was carried out on a flavor concentrate prepared under mild conditions from 250 freshly-picked pineapples which were pressed to yield 25 gallons of juice. The juice was saturated with sodium chloride and extracted with peroxide-free ethyl ether. The ether solution was concentrated, dried with sodium sulfate, and further concentrated to a thick syrup as described under Experimental. The syrup was subjected to rapid short-path distillation at a bath temperature of 25–120°C between atmospheric pressure and 0.1 mm Hg. Of the 18 g of syrup, 10 g remained undistilled. The fraction of immediate interest is that which was distilled at a bath temperature of 25–120°C at 0.1 mm Hg and collected in a receiver held at 25°C. This fraction, consisting of 0.95 g of the least volatile components, was labeled fraction 4. It was further fractionated by preparative gas chromatography on a silicone column into 5 fractions (A through E). The largest of these fractions was 4B, consisting of 300 mg of a light-yellow crystalline solid. Fraction 4B was fractionated on a Carbowax column. The major peak (Fraction 4B1) was collected as a colorless solid (100 mg, mp 70°C).

Fraction 4B1 had an intense odor, best described as "burnt pineapple." Although all of the distillate fractions and the first crude gas chromatograph fractions were recognizable as pineapple, the odor of Fraction 4B1 was by far the most intense; the term "character-impact compound" seems applicable. The possibility that 4B1 is an artifact formed either during distillation or during chromatography must be considered, though this seems unlikely.

Apart from its odor, an outstanding feature of this compound was its instability; much of the material was lost through polymerization on standing, and much through

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unsuccessful attempts to carry out chemical transformations. This instability may be a major factor in the loss of pineapple flavor during processing.

TENTATIVE IDENTIFICATION

Our tentative identification of this compound rests on the interpretation of four spectra: mass, infrared, nuclear magnetic resonance, and ultraviolet. These spectra are presented as Fig. 1. Synthesis of an authentic sample must be deferred until work-up of the flavor concentrate on hand has been completed.

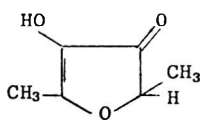
The first step in translating the four spectra of Fig. 1 into a molecular structure is to establish a molecular weight and an empirical formula from the mass spectrum. The parent peak (P) was found to be 128; thus, we have the exact molecular weight. Since this is an even number, we know that the molecule contains either no nitrogen or an even number of nitrogen atoms. Absence of nitrogen was confirmed by a micro-sodium fusion test. The P + 2 peak is too small to allow for the presence of sulfur or halogen atoms. From the calculated contributions of isotopes to the P + 1 and P + 2 peaks, we select $C_6H_8O_3$ as the best fit to our P + 1 peak of 7.18% and P + 2 peak of 1.18% (Silverstein and Bassler, 1963).

We turn now to the infrared spectrum and pick out the prominent features. We note a strong, broad hydroxyl band at 3.06 μ, a strong carbonyl band at 5.88 μ, and a very strong, broad band at 6.16 μ; the positions and relative intensities of the latter two bands suggest a conjugated carbonyl group, C = C − C = O. A 2, 4-DNP test was positive. The shape and breadth of the hydroxyl absorption indicate that the hydroxyl group is strongly hydrogen-bonded. The initial impression is that of a carboxylic hydroxyl group. However, that is quite firmly ruled out by the absence of absorption peaks below 1 τ in the NMR spectrum. Absence of aromatic peaks in the NMR spectrum rules out the possibility of phenolic hydroxyl groups. We are effectively limited to alcohol and enols.

Let us consider the NMR spectrum in some detail. From low field to high field we note: a broad absorption at 2.66 τ, a quartet at 5.62 τ, a singlet at 7.80 τ, and a doublet at 8.62 τ. The ratios are respectively 1:1:3:3. We make the following assignments in the same sequence: OH, CH, CH_3, CH_3. We note that the CH quartet at 5.62 τ and the CH_3 doublet at 8.62 τ are coupled; therefore these groups are vicinal. Furthermore, the strong downfield shift of the quartet suggests that the carbon atom to which the proton is attached may in turn be attached to an oxygen atom. We can justify the strong downfield shift of the OH peak (outside the usual range of about 4.5 τ to 9.3 τ for alcohols) by assigning it to an enolic proton. A strongly positive ferric chloride test (green) afforded confirmation. The CH_3 group represented by the peak at 7.80 τ is attached to a carbon atom that does not carry a proton. There are no olefinic protons. From the infrared and NMR data, we can write the following fragments:

There is one additional and, as it turns out, crucial bit of information. The UV absorption peak is at 289 mμ, ϵ 6700 (the molar absorptivity may be low because of decomposition of the solution on standing).

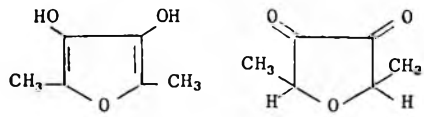
We were unable to write an acyclic structure which would satisfy all the spectral data. A number of unsaturated five-membered ring lactones were considered, and discarded because of discrepancies between predicted spectra and the evidence at hand; the requirement of absorption at 289 mμ, in particular, could not be satisfied. The only structure we are able to write that is consonant with the spectral data is:



2,5-dimethyl-4-hydroxy-3(2H)-furanone

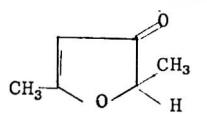
I

This compound, of course, can be written in either of two other tautomeric forms.



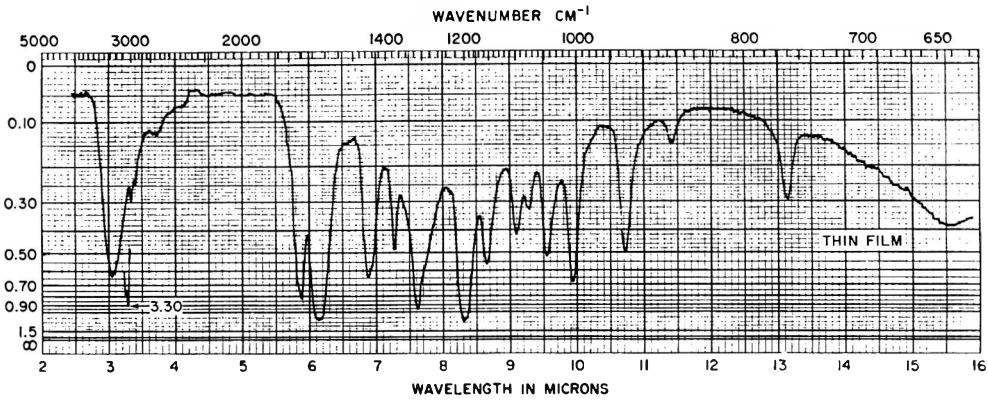
The lower homolog, tetrahydro-3,4-furandione, has been reported (Kendall and Hajos, 1960) to exist exclusively in the diketo form. However, the spectral evidence indicates that I exists exclusively as the monoenol. The 2,5-dimethyl compound I has not been reported in the literature.

Confirmatory evidence for the structure of I can be derived from the reported IR and UV values for a related compound, 2,5-dimethyl-3(2H)-furanone II (Eugster *et al.*, 1961).



II

INFRARED SPECTRUM



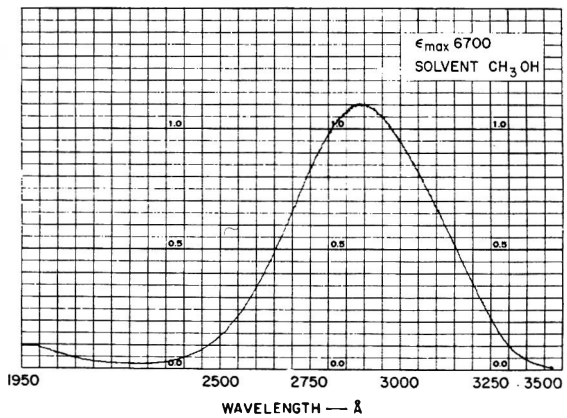
MASS SPECTRAL DATA (RELATIVE INTENSITIES)

m/e	% OF BASE PEAK	m/e	% OF BASE PEAK
14	5	44	8
15	16	45	11
17	3	53	4
18	11	55	18
26	4	56	10
27	28	57	61
28	16	58	3
29	35	72	4
31	7	85	21
39	5	128	95
41	4	129	6.82
42	5	130	1.12
43	100		

ISOTOPE ABUNDANCE

m/e	% OF P
128(P)	100
129(P+1)	7.18
130(P+2)	1.18

ULTRAVIOLET SPECTRUM



NMR SPECTRUM

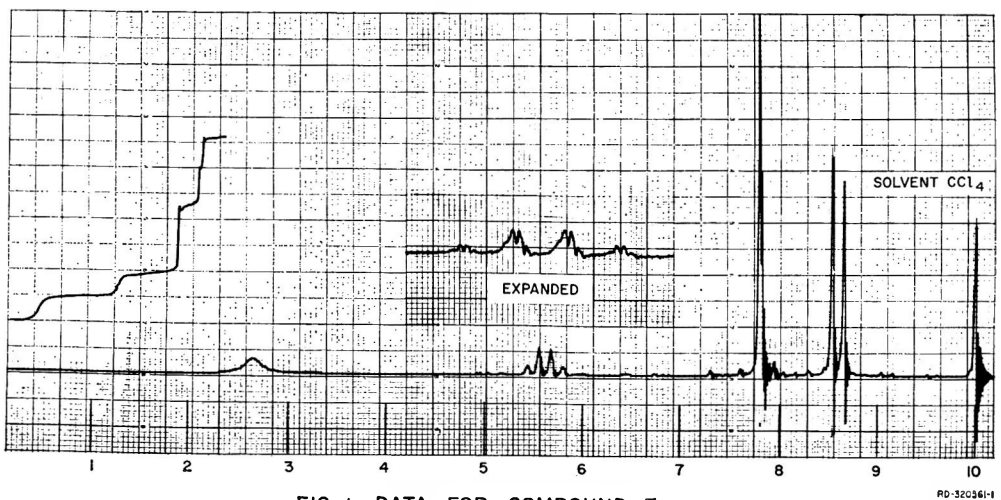
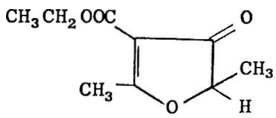


FIG. 1 DATA FOR COMPOUND I

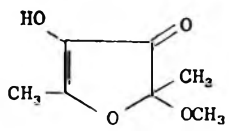
The carbonyl absorption in the IR spectrum was 5.84 mμ, and the UV absorption peak was 260 mμ ($\epsilon = 12{,}200$). The bathochromic shift proposed (Jaffé and Orchin, 1962) for an α-hydroxy substituent in an acyclic conjugated ketone is 35 mμ; the calculated value of 295 mμ is thus in fair agreement with our value of 289 mμ.

The shift position, in the NMR spectrum, of the methine proton of I (5.62 τ) is in good agreement with that reported (5.5 τ) for the methine proton of the related compound III (Rosenkranz, *et al.*, 1963):



III

Although attempts to carry out chemical transformations on I were unsuccessful, a satisfactory derivative was obtained inadvertently from a solution of I in a mixture of methanol and chloroform which had been stored for a week in the refrigerator. This derivative was identified from spectral data (Fig. 2) as 2,5-dimethyl-4-hydroxy-2-methoxy-2,3-furanone (IV):



IV

The mass spectral data show the molecular weight to be 158 (loss of one hydrogen, gain of methoxy group). The P + 1 peak allows for 8 carbon atoms; the P + 2 peak was too small to measure accurately. Loss of the methine proton in I is indicated by the absence of the quartet from the NMR spectrum of IV. The shift position of the new peak at 6.88 τ is reasonable for a methoxy group. The informative peaks of the IR spectrum are largely unchanged from those of the IR spectrum of I.

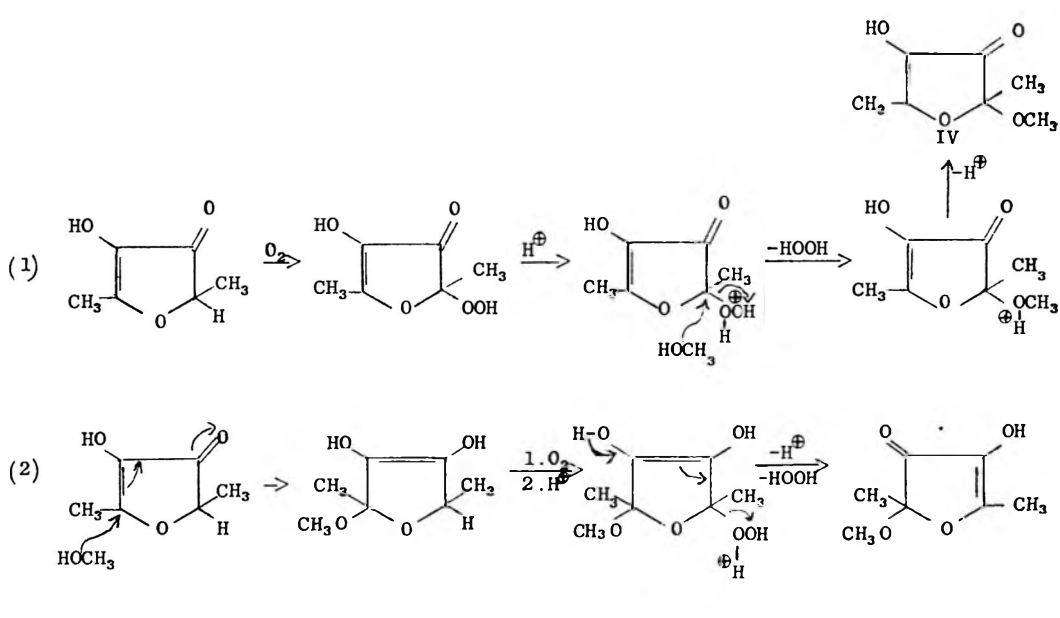
Two plausible mechanisms can be written to rationalize the transformation I → IV. These both invoke air oxidation to hydroperoxides (usually postulated in air oxidation of furans; Dunlop and Peters, 1953) and acid catalysis from degradation of chloroform.

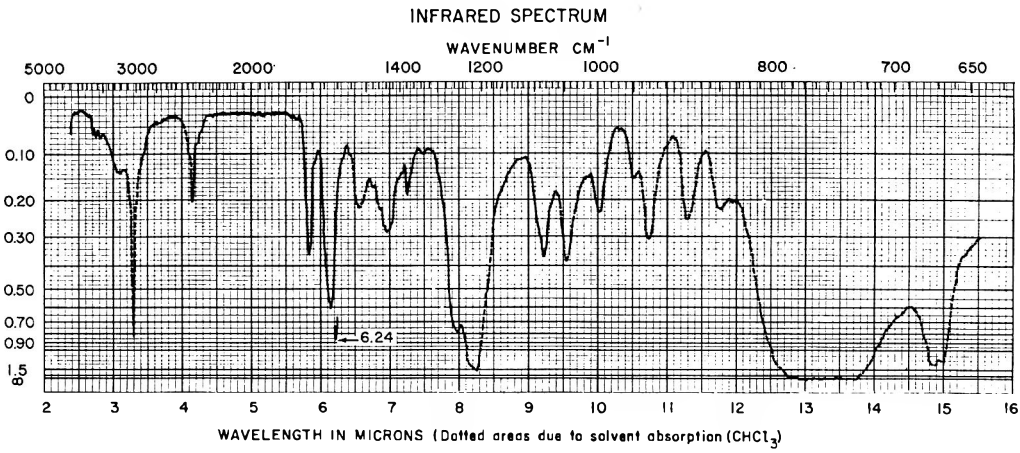
The first sequence depicts oxidation to a hemiketal type of hydroperoxide. Protonation of the hydroperoxide oxygen is followed by nucleophilic displacement of hydrogen peroxide by methanol, and loss of a proton. The second sequence assumes a nucleophilic attack by methanol at the β-carbon of a conjugated ketone. This is followed by air oxidation and protonation. Ketonization with loss of a proton displaces hydrogen peroxide.

Synthesis of IV has been reported (Steinbauer and Waldmann, 1958), but no spectral data were presented.

EXPERIMENTAL

Freshly picked pineapples [*Ananas comosus* (L.) Merr. var. Smooth Cayenne, winter harvest, field-grown at 700–800-ft ele-





MASS SPECTRAL DATA (RELATIVE INTENSITIES)

m/e	% OF BASE PEAK	m/e	% OF BASE PEAK
13	3	42	7
14	9	43	100
15	31	44	11
17	6	45	5
18	21	86	5
27	3	93	3
28	7	157	0.72
29	9	158(P)	0.24
31	7	159	0.020
32	3		

ISOTOPE ABUNDANCE

m/e	% OF P
158(P)	100
159(P+1)	8.2
160(P+2)	TOO SMALL TO MEASURE

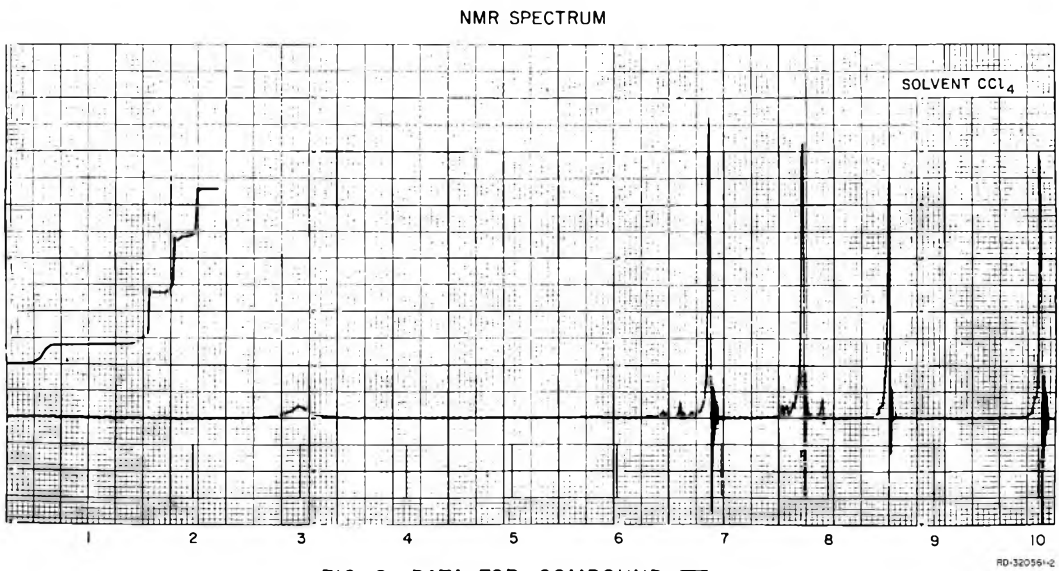


FIG. 2 DATA FOR COMPOUND IV

vation in the Waipio region, Oahu, Hawaii] were selected so that the fruit were half to fully yellow shell color, and the flesh was moderately to highly translucent. Five batches of 50 fruit each were converted to cored cylinders in a Ginaca machine. The cylinders were trimmed of portions of shell, all opaque flesh, and imperfections. They were dipped in water and passed twice through a screw-press juicer, obtaining 5–6 gallons of juice from 50 fruit. Sodium chloride was added to the juice (25 g/100 ml, 10.4 lb/5 gal), and the juice was extracted once, volume for volume, with peroxide-free ethyl ether. The ether layer was separated and centrifuged for 5 min at 1500 rpm.

The clear ether extract was concentrated to a small volume in a flash evaporator (Buchler Instrument, Inc., Fort Lee, N. J.) using a bath temperature of not over 40°C and a water aspirator at about 40–50 mm Hg. The final stage of the evaporation was carried out at room temperature to a volume of 100–200 ml. This concentrate was dried over anhydrous sodium sulfate for 1–2 hr, filtered, and returned to the flash evaporator for a final concentration at room temperature to a thick syrup. The syrup was transferred to a small vial, purged with nitrogen, and sealed. A total of 18 g of syrup was obtained from 250 fruit. The ampoules were stored in a freezer except for the time required for airmail between Hawaii and California.

Gas chromatography was performed on Wilkens Instrument Aerographs A350B, A90C, and A600B. Distillate fraction 4 was fractionated on a silicone column (G.E. SF96, 20% on firebrick, 6 ft × ¼ in, 140°C, 40 ml helium per min, each injection 50 μl of a 50% methanol solution, injector block 155°C, on-column injection, A350B). Fraction 4B was collected between 8 and 14 min (300 mg). Fraction 4B was fractionated on a Carbowax 20M column (15% on Chromosorb W, 3 ft × ¼ in, 145°C, 80 ml helium per min, each injection 20 μl of a 50% methanol solution, injector block 200°C, on-column injection, A90C). Fraction 4B1 (I) was collected between 11 and 15 min (100 mg). Compound IV was fractionated on the same Carbowax column under identical conditions, and was collected between 20 and 25 min. An analytical run of Fraction 4B1 (I) showed a sharp symmetrical peak at 6 min (15% Carbowax 20M on Chromosorb W, 6 ft × ⅛ in, 205°C, injection block 220°C, on-column injection, A600B; retention time for dibenzyl ether under the same conditions was 18 min).

Melting points were obtained on a micro hot-stage under polarized light. Infrared spectra were run on a Perkin-Elmer 221 and on a Beckman IR5, ultraviolet spectra on a Cary 14M, mass spectra on a CEC 21-103 C (peaks of less than 3% relative to the base peak were not reported), and the NMR spectra on a Varian HR60.

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Research Note

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Effect of Magnesium in Salting of Cod

Tressler observed in 1920 that impurities such as calcium, magnesium, and sulfate noticeably reduced the rate of chloride uptake by fish during dry-salting. This effect, though often quoted, has not been confirmed so far as the writers are aware. Boury (1934), in particular, did not detect substantial differences in the dry-salting of herring in the absence and presence of impurities. The present note describes experiments on brine-salting and dry-salting of cod (*Gadus callarias*) in the presence and absence of magnesium chloride.

An optical method was initially used to study brine-salting. A lamina (1–2 mm thick) of fish muscle, cut from frozen fish and thawed (as were all specimens used in later experiments) is sandwiched between two larger glass plates separated by spacers and held together by spring clips. The spacers are slightly thinner than the muscle, which is thus pressed into close contact with the glass surfaces. When saturated brine is run around the edge of the lamina it is observed that the muscle becomes more transparent as the salt diffuses inward. The boundary of the transparent region is quite sharp, and its movement is at right angles to the edge of the lamina. The rate of movement, which proves to be proportional to the square root of time, can be used to measure the diffusion coefficient by the equation

$$C/C_1 = 1 - erf\,(x/2\sqrt{Dt})$$

where C is the concentration in the aqueous phase of the fish tissue at the boundary (shown by other experiments to be $0.35M$),
C_1 is the external brine concentration,
x is the distance moved by the boundary in time t
and D is the diffusion coefficient.

Several measurements gave the following values of D:

$D = 1.10 \pm 0.13 \times 10^{-5}$ cm²/sec in NaCl brine, and

$D = 1.26 \pm 0.35 \times 10^{-5}$ cm²/sec in (NaCl + 1% $MgCl_2$) brine.

These mean values are not significantly different.

While this optical method is a useful rapid qualitative demonstration of the brining process, a quantitatively more accurate method is required. Regularly shaped blocks ($5 \times 5 \times 1.5$ cm³) of fish muscle are immersed in stirred saturated brine at 25 or 20°C for various periods; each piece is then analyzed for chloride (AOAC, 1960). In the early stages, as in most diffusion-controlled absorption processes, the salt uptake is proportional to the square root of time. The appropriate solution of the diffusion equation is

$$Q = 2C_2\sqrt{Dt/\pi}$$

where Q is the uptake per unit surface in time t
and C_2 is the surface concentration of NaCl in the fish tissue (taken as 58% of the external brine concentration).

Experiments give:

$D = 7.5 \pm 0.7 \times 10^{-6}$ cm²/sec in NaCl brine,

$D = 6.7 \pm 0.8 \times 10^{-6}$ cm²/sec in (NaCl + 1% $MgCl_2$) brine.

Again, the difference between the means is not significant, and Tressler's observation does not appear to be confirmed in brine salting. (The discrepancy between values of D by the two methods is due to approximations in both).

To study dry-salting, regularly shaped blocks of fish were embedded in a considerable excess of salt (at least 2 parts salt to one part fish) and removed for analysis after fixed times. The NaCl was sieved to between 30- and 60-mesh (British Standard), and the $MgCl_2$ was sieved to the same size before mixing. The results are given as uptakes rather than as diffusion

coefficients, since the process of dry salting may be controlled by factors in addition to diffusion of salt within the fish.

	1 hr	24 hr
NaCl	6.06%	18.99%
NaCl + 1.5% $MgCl_2$	6.08%	18.78%

Once more, the presence of magnesium has not affected the uptake of salt. (Data not quoted also show that changes in water content during brining and dry-salting are not altered by magnesium).

It is now clear that Tressler's observations, if valid, are due to some other factor (such as choice of species) as well as to the over-all composition of the salt used. He carried out his work on squeteague (*Cynoscion regalis*), and the present work and that of Boury show that his conclusions cannot be applied generally to all species.

This work was carried out as part of the program of the Department of Scientific and Industrial Research. The first author participated in the work as a Colombo Plan Fellow deputed by the Council of Scientific and Industrial Research, India.

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Identification of Volatile Constituents from Pure-Culture Fermentations of Brined Cucumbers

SUMMARY

A high-vacuum distillation method, with liquid-nitrogen trapping, was used to separate the volatile components present in pure-culture fermentations of cucumbers. The pure cultures used were *Lactobacillus plantarum*, *L. brevis*. *Pediococcus cerevisiae*, and *Leuconostoc mesenteroides*. The vapors were subjected to gas-liquid chromatography and the components identified by comparison of retention times with those of known compounds. Differences in the vapor chromatograms were obtained both between the different species of lactic acid bacteria and with strains of the same species. Comparison of the chromatograms with the organoleptic evaluation of the different pickle samples indicated that the flavor of pickles is due to a blend of volatile components rather than the presence or absence of a single component. Formaldehyde, acetaldehyde, acetone, ethyl alcohol, propionaldehyde, and butyraldehyde were isolated and identified.

A knowledge of the chemical composition of a flavor characteristic of a specific food is important because of the opportunities it offers to produce more uniform and flavorful products, to maintain flavor during processing and storage, and to produce new products.

The flavor of green cucumbers, unidentified as to variety, was attributed by Takei and Ono (1939) to nona-2,6-dienol and nona-2,6-dienal. The alcohol was present in larger amounts but the aldehyde was the primary flavor constituent. More recently, Forss *et al.* (1962) reported that nona-2: trans-6-cis-dienal, nona-2-enol, hex-2-enal, and three saturated aliphatic aldehydes were present in green cucumbers (Marketer, Super Market, Ashley) and several others unidentified as to varietal type. To our knowledge no other reports have appeared dealing with volatile constituents of green cucumbers and none on pure-culture fermentation of brined cucumbers.

The preservation of cucumbers by fermentation processes dates back to antiquity. The effects of fermentation are mediated through the metabolic activities of naturally occurring acid-forming microorganisms. The utilization of pure-culture fermentations appears to offer promise for the improvement of cucumber pickles (Etchells *et al.*, 1963). There is considerable precedent for this kind of work in other areas of food research. For example, Pederson (1960) has made an extensive study of the end products of fermentation of cabbage to sauerkraut by the lactic acid bacteria. Similarly, Vorbeck *et al.* (1961) reported on biochemical changes of minor constituents of cabbage by lactic acid bacteria. Wiseblatt (1960) and Hunter *et al.* (1961) characterized some of the flavor components in fermented dough, bread extracts, oven vapors, and pre-ferments for bread. Flavor and aroma compounds of several types are produced by pure cultures used in meat and dairy products. Single strains of bacteria usually do not give the desired development of flavor and aroma to dairy products, but, instead, a mixture of two or more species or strains of bacteria are needed (Foster *et al.*, 1957). Before pure-culture fermentation as a science can be applied to the production of pickles, more detailed information is needed as to the identity of flavor constituents introduced by organisms used in the fermentation. Consequently this study reports some of the flavor and aroma components produced by lactic acid bacteria normally isolated from natural pickle fermentations, and carried out under pure-culture conditions.

MATERIALS AND METHODS

Preparation of samples. The pure-culture cucumber fermentations were made at the M. A. Gedney Company Plant, Chaska, Minnesota, by means of a special process (Etchells *et al.*, 1963) consisting of the following steps:

1) SMR-15 variety of pickling cucumbers, 7/8–1 1/16 inches in diameter (size No. 1-B), were used for the study. The cucumbers were soaked in tap

water for a few minutes, washed by hand with a vegetable brush and drained.

2) The washed cucumbers, contained in deep-fry wire baskets fitted with hardware-cloth tops, were immersed for 5 min in a steam-jacketed kettle containing about 50 gal. of water maintained at 77°C. The average internal cucumber temperature, based on the 24 bulk heatings required for the experiment, was 63°C.

3) The heated cucumbers were immediately packed by hand, using aseptic precautions, in 48-oz-capacity glass jars which, together with their caps, had been previously rinsed with 70% alcohol and drained. The packed cucumbers were locked in position, to prevent floating, by wedging 2 or 3 larger cucumbers (No. 2) beneath the shoulder of the jar and parallel to the top.

4) Prior to brining, all jars scheduled for inoculation were acidified by the addition of 1.5 ml of 85% lactic acid.

5) The packed jars were then filled with a 30° salometer salt-brine solution (7.9% salt/wt) which had been previously boiled and cooled to approximately 4°C. This resulted in an equalized temperature for the brined material of 30–32°C.

6) The jars of acidified brined cucumbers were inoculated with 2.5 ml of 24–36-hr trypticase sugar broth cultures of the appropriate species of lactic acid bacteria. The jars were hand-sealed with 77-mm-diameter "Twist Off" closures (White Cap Co., Chicago, Illinois), incubated 72 hr at 32°C, and stored at room temperature (21–27°C) until examined.

7) Pasteurized uninoculated controls consisted of three jars of cucumbers prepared as described above except that 2.5 ml of lactic acid was added for acidification. These jars were pasteurized 30 min in a water bath at 77°C and promptly cooled. For unheated or natural controls, two jars were packed with washed cucumbers, acidified with 2.5 ml of 85% lactic acid, brined, and allowed to ferment naturally; two jars were handled in the same manner except that they were not acidified.

Cultures used. The lactic acid bacteria selected for study represented 34 cultures in the following four species: *Lactobacillus plantarum* (10 strains); *L. brevis* (10 strains); *Pediococcus cerevisiae* (11 strains); and *Leuconostoc mesenteroides* (3 strains). Most of the cultures originated from brined cucumber fermentations and were obtained from three sources: Dr. R. N. Costilow, Michigan State University (FBB-designation); Dr. William Haynes, Northern Regional Research Laboratory, Peoria, Illinois (B-designation); and Dr. A. F. Borg, Kansas State University (L-designation). All pure-culture fermentations were made in duplicate.

Distribution of samples. The pack totaled 75 (48-oz) jars. Except for the heated controls, they were divided into two equal lots. One lot was used for gas chromatographic studies, and the other was examined at the plant after 5 months of storage for certain chemical, physical, and organoleptic changes.

Preparation of extracts for analysis. Five hundred grams of pickles and 250 ml of the liquor were blended in a 1-gal-capacity Waring blender. The slurry, with rinsings (250 ml), was poured into a 5-L flask, and the flask was attached to a high-vacuum low-temperature distillation system (Fig. 1). The pressure was slowly lowered to

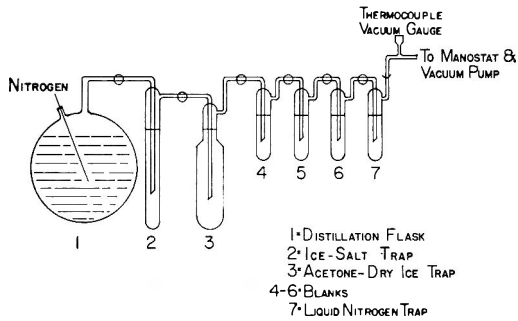

Fig. 1. High-vacuum low-temperature distillation apparatus.

750 μ of mercury, and nitrogen gas was then bubbled into the flask through a capillary tube. The slurry was distilled for 2 hr, during which time the temperature was maintained at 35°C.

Preliminary studies indicated that the flavor components were retained in the liquid nitrogen trap (Fig. 1). Consequently, this trap was removed from the system, fitted with a rubber septum, and allowed to warm to room temperature. A 5-ml vapor sample was removed for gas-liquid chromatographic (GLC) analysis. Immediately following the above step, 30 ml of 2,4-dinitrophenyl hydrazine (DNP) reagent (300 mg in 30 ml concentrated hydrochloric acid) was added to the trap and the reaction mixture allowed to stand overnight. The DNP hydrazones were collected by centrifugation, washed with *2N* hydrochloric acid and water, and dried under vacuum.

Fractionation of volatile components. A Barber-Colman model 10 chromatograph unit equipped with a flame ionization detector was used for the qualitative determination of the volatile components. The U-shaped columns were heavy-walled glass tubing, 5 mm ID and 6 ft long, packed with Carbowax 20M on 60–80-mesh GC-22 Firebrick (1:10 w/w). The columns were preconditioned by baking at 90°C prior to use. The operating conditions were: column temperature

70°C; detector 155°C; flash heater 190°C; helium pressure 14 psig; sensitivity 10^{-9} amps. Where applicable, the carbonyl derivatives were introduced into the column as described by Ralls (1960).

The resolved components were identified by comparing their retention times with compounds of known composition.

RESULTS

Representative chromatograms of the volatile components present in a natural nonacidified control and an uninoculated acidified pasteurized control are shown in Fig. 2. Differences existed

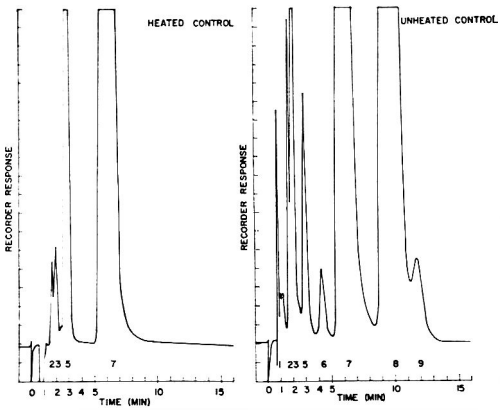

Fig. 2. Gas chromatograms of volatiles from a heated control (unfermented, pasteurized) and unheated control (natural fermentation, nonacidified). The peaks represent formaldehyde (2), acetaldehyde (3), acetone (5), butyraldehyde (6), ethyl alcohol (7), ethyl butyrate (8), isovaleraldehyde (9). Ordinate represents recorder response and abscissa represents time.

between the two control samples in that butyraldehyde, ethyl butyrate, and isovaleraldehyde (Peaks 6, 8, 9) were present in the nonacidified control whereas they were absent in the pasteurized control. This difference was due to the microorganisms present in the natural, unacidified fermented sample.

Representative chromatograms of the volatile components in pure-culture fermentations are presented in Fig. 3. The relative retention volumes of the volatile components in the distillates from the pure-culture fermentations and the controls are presented in Table 1. The data and chromatograms show marked differences between species. For example, formaldehyde and propionaldehyde (Peaks No. 2 and 4) were not detected in *L. brevis*; butyraldehyde (Peak No. 6) was absent in *P. cerevisiae*; all six compounds were present in *L. plantarum* and *Leuc. mesenteroides*. Similarly, differences existed for strains within

Table 1. Relative retention volumes of volatile compounds from cucumbers and pure-culture fermentations of cucumbers.[a,b]

Volatile compounds	Retention volume of known compound	Peak[c]	Natural control	Pasteurized control	Species and strains used for inocula							
					L. plantarum		*P. cerevisiae*		*L. brevis*		*Leuc. mesenteroides*	
					FBB-14	L-313	FBB-40	FBB-63	FBB-50	L-636	FBB-41	FBB-42
Formaldehyde	0.60	2	0.63	0.60	0.60	.....	0.60	.....	.....	.....	0.63	0.60
Acetaldehyde	0.68	3	0.70	0.68	0.70	0.63	0.68	0.68	0.68	0.68	0.70	0.68
Propionaldehyde	0.80	4	.....	.....	0.82	0.80	.....	0.80	.....	.....	.....	0.80
Acetone	1.00	5	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Butyraldehyde	1.40	6	1.48	.....	1.48	1.40	.....	.....	1.40	1.40	1.48	1.40
Ethyl alcohol[d]	2.20	7	2.22	2.20	2.22	2.20	2.20	2.20	2.20	2.20	2.22	2.20
Ethyl butyrate	3.70	8	3.66	.....	.....	.....	.....	.....	.....	.....	.....	.....
Isovaleraldehyde	4.67	9	4.66	.....	.....	.....	.....	.....	.....	.....	.....	.....

[a] Retention volumes relative to acetone.
[b] See Procedure for details.
[c] Peak No. 1 is the air peak.
[d] The jars and caps were rinsed in 70% alcohol and drained (see preparation of samples given under Materials and Methods); this may account in part for this component in the samples.

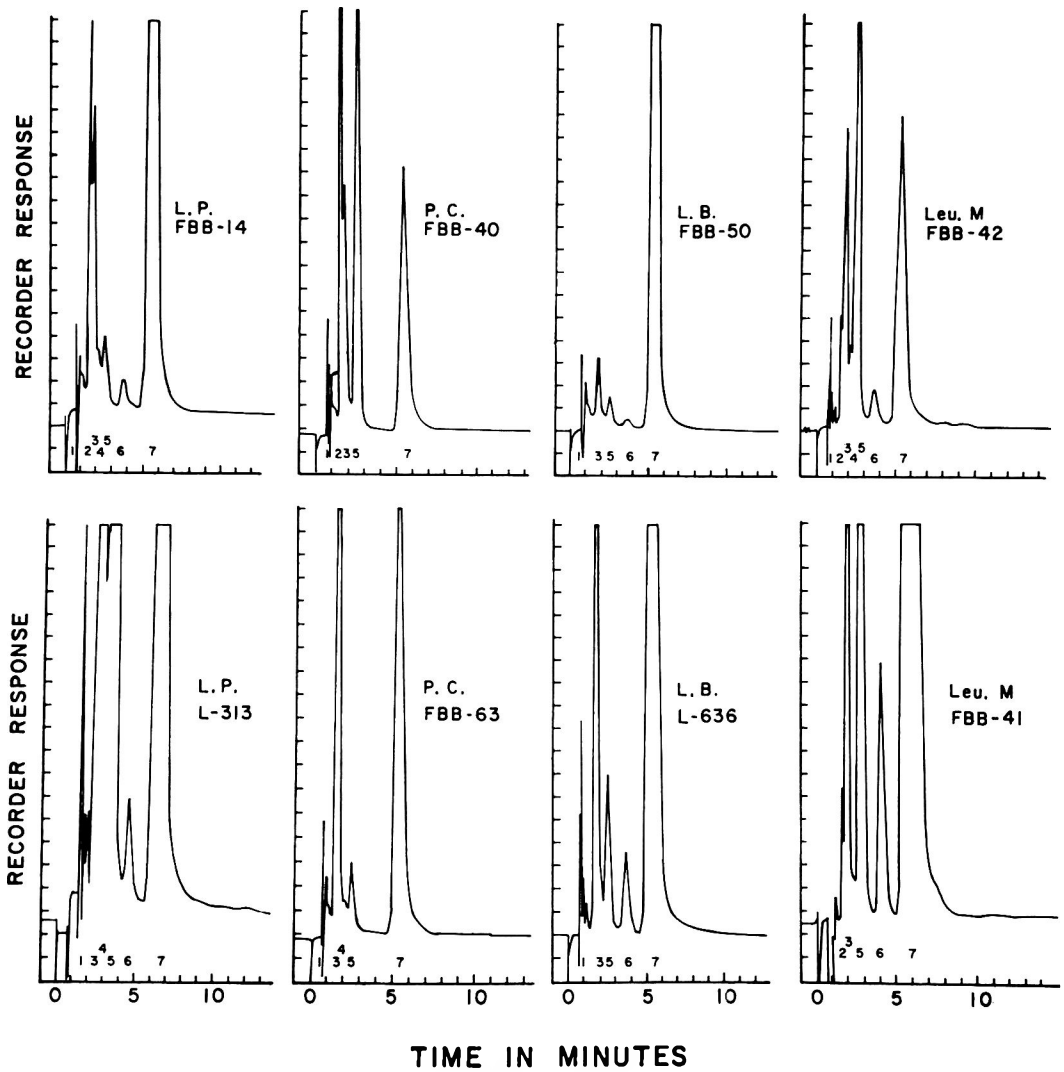

Fig. 3. Gas chromatograms of volatiles from pure-culture cucumber fermentations with lactic acid bacteria are shown with two strains each of four species from left to right; *Lactobacillus plantarum* (L.P.), *Pediococcus cerevisiae* (P.C.), *Lactobacillus brevis* (L.B.), and *Leuconostoc mesenteroides* (Leuc. M). The peaks represent air (1), formaldehyde (2), acetaldehyde (3), propionaldehyde (4), acetone (5), butyraldehyde (6), ethyl alcohol (7). Ordinate represents recorder response, and abscissa represents time.

species. Formaldehyde (Peak No. 2) was not detected in *L. plantarum* (L-313) and *P. cerevisiae* (FBB-63) whereas it was present in *L. plantarum* (FBB-14) and *P. cerevisiae* (FBB-40). Propionaldehyde (Peak No. 4) was absent in *Leuc. mesenteroides* (FBB-41) but present in *Leuc. mesenteroides* (FBB-42).

The chromatograms (Figs. 2, 3) show quantitative as well as qualitative differences for the natural control, pasteurized control, species, and strains within species. A comparison of the chromatograms for the two controls, or of *L. plantarum* and *L. brevis*, or of the two strains of *L. brevis* (FBB-50 and L-636) indicates the magnitude of their differences. Thus, the distinction between the various samples may be a matter of relative concentration of the volatile components rather than the presence or absence of a particular component.

The relative percent concentration of the volatile components from the distillates of the pure-culture fermentations is presented in Table 2. Flavor descriptions, as judged by an experienced taste panel, are presented in Table 3. Little, if any, relationship exists between the relative percent concentrations and flavor descriptions, as

Table 2. Relative percent concentration of volatile compounds present in the pure-culture fermentation samples.[a]

Species	Strain	Volatile compounds							
		Formaldehyde	Acetaldehyde	Propionaldehyde	Acetone	Butyraldehyde	Ethyl Alcohol	Ethyl Butyrate	Isovaleraldehyde
		(%[b])	(%)	(%)	(%)	(%)	(%)	(%)	(%)
L. plantarum	FBB-14	12	7	2	4	2	73	0	0
	L-313	0	32	3	13	2	50	0	0
	FBB-12	18	5	3	5	3	68	0	0
	L-346	10	15	3	3	3	65	0	0
P. cerevisiae	FBB-40	23	12	0	26	0	39	0	0
	FBB-63	0	20	1	4	0	75	0	0
	FBB-61	16	12	3	4	0	66	0	0
	L-358	19	7	1	12	0	60	0	0
	B-1325	12	7	0	25	1	56	0	0
L. brevis	FBB-50	0	12	0	12	10	66	0	0
	L-636	0	21	0	9	6	64	0	0
	L-106	0	6	0	6	3	85	0	0
	L-544	0	8	0	1	2	89	0	0
	B-1836	0	22	0	12	23	42	0	0
Leuc. mesenteroides	FBB-42	6	15	5	27	3	44	0	0
	FBB-41	3	9	0	14	8	66	0	0
Controls									
Natural (acidified)		0	27	1	12	6	54	0	0
Natural (nonacidified)		3	8	0	6	3	33	38	8
Pasteurized		3	3	0	24	0	70	0	0

[a] See procedure for treatment details.
[b] Percent to nearest whole number.

illustrated by comparing *L. plantarum* with the pasteurized control. The percent concentration of the several volatile components varied for the strains within *L. plantarum,* and propionaldehyde and butyraldehyde were absent in the pasteurized control. In contrast, the odor and flavor description of the pickles fermented with *L. plantarum* and the unfermented uninoculated pasteurized control were similar; the taste-panel reaction to these particular samples differed chiefly in response to the degree of acid present.

Since no definite correlation could be observed between the relative percent concentration of volatile components (Table 2) and flavor description (Table 3), the chromatograms of the various samples (Fig. 3) were compared with the flavor and odor descriptions of the same sample (Table 3). It appeared that the profiles of the volatile components were related to the odor description of the sample. A comparison of the chromatograms of *L. brevis* and *L. plantarum* indicates a greater amount of volatile components for the latter and a difference in the odor descriptions between the two. Similarly, the same relationship exists for strains within species as illustrated by comparing the chromatograms and odor descriptions of two strains of *L. brevis* (FBB-50 and L-636). The chromatogram of the latter culture (L-636) shows an increase in concentration of acetaldehyde, acetone, and butyraldehyde (Peaks No. 3, 5, 6, resp). The odor description was "aromatic" for this sample (L-636), and "slightly off" for the other strain (FBB-50).

DISCUSSION

McCarthy *et al.* (1963) reported consistent correlations between odor of ripe bananas and chromatographic patterns. Furthermore, it was suggested that identification of each volatile component in a chromatogram would give more precise flavor profiles. In view of those observations and those reported above, it seemed logical to determine, if possible, the relative importance of the sense of smell (odor) and taste in flavor profiles for pure-culture fermentations of brined cucumbers.

It will be recalled that flavor depends upon reactions to the physical senses (sight, taste, odor, touch, and hearing). Strictly

Table 3. Flavor and odor characteristics of the pure-culture fermentations of brined cucumbers.

Species	Strain	Odorant classification[a] Pungent (%)	Rancid (%)	Ethereal (%)	Flavor Description	Odor Description
L. plantarum	FBB-14	21	2	77	Clean, raw cucumber strong acid	Clean, raw cucumber
	L-313	35	2	63	Clean mild acid	Clean, raw cucumber
	FBB-12	26	3	73	Good, clean, acid cucumber	Clean, raw cucumber
	L-346	28	3	68	Very sharp acid	Clean, raw cucumber
P. cerevisiae	FBB-40	35	....	65	Mild acid, slightly bitter	Clean, mild acid cucumber
	FBB-63	21	....	79	Mild acid, bitter after taste	Clean, mild acid
	FBB-61	31	....	70	Mild, slightly aromatic, slightly undesirable after taste	Clean, raw cucumber slightly aromatic
	L-358	27	....	72	Clean, mild acid	Clean, mild fresh cucumber
	B-1325	19	1	80	Mild acid, salt slightly bitter, musty, hay-like	Clean, fresh cucumber
L. brevis	FBB-50	12	10	78	Mild acid, bitter after taste	Slightly off
	L-636	21	6	73	Medium acid, slightly bitter	Aromatic
	L-106	6	3	91	Bitter	Aromatic
	L-544	8	2	90	Raw cucumber taste	Aromatic, clean
	B-1836	22	23	54	Medium acid, slightly bitter	Mild aromatic
Leuc. mesenteroides	FBB-41	12	8	80	Medium acid, slightly fruity (apple), pleasant, clean, excellent	Slightly aromatic
	FBB-42	26	3	71	Slightly acid, salt	Aromatic
Controls						
Natural (acidified)		28	6	66	Predominantly acid secondary cucumber	Salt stock
Natural (nonacidified)		11	49	39	Acid and bitter, with undesirable after taste	Stale, unpleasant
Pasteurized		6	....	94	Mild, very slight acid, bland, raw cucumber	Raw cucumber

[a] Amoore, 1952

speaking, taste is the reaction resulting from stimulation of taste buds on the tongue which detect salt, sweet, sour, and bitter. Odor is determined by the reaction to stimuli on free nerve-ends in a relatively small area high in the nasal cavity. Flavor and odor are detected at the same place, with the distinction generally made that flavor is obtained through the mouth, whereas odor is detected through the nose. When a food is consumed, stimuli from the five physical senses are received simultaneously in the brain. Thus, it is difficult to separate entirely one sense reaction from the others, since they all influence each other. For purposes of discussion, the assumption was made that color (sight), touch, and hearing were common for all samples while odor and

taste were variables. In addition, flavor was defined as the combined effect of the sample on the taste and olfactory nerves.

The data (Fig. 3) indicated that a relationship existed between peak heights and organoleptic evaluation on the samples. The subjective measurements of the various samples (Table 3) were re-examined from the standpoint of comparing odor and flavor descriptions. The taste panel described the odor of the pasteurized unfermented control as "raw cucumber" and the flavor as "mild, very slight acid, bland, raw cucumber." The pasteurized control was slightly acidified with lactic acid to inhibit the growth of sporeformers, especially the anaerobes. Thus, it appears that good agreement exists between odor and flavor descriptions because, in the flavor and description, both taste (very slight acid) and odor (raw cucumber) were detectable. Similarly, the taste and odor description of cucumbers fermented with *L. plantarum* showed good agreement. The odor description was "clean, raw cucumber," and the flavor description was "clean, raw cucumber, strong acid, mild acid, etc." In contrast, the flavor and odor descriptions for the remaining pure-culture fermentations did not show good agreement. The taste of cucumbers fermented with *L. brevis* was described as "mild acid, salty, slightly bitter, etc." while the odor description was "aromatic, slightly off." It was noted above that the profiles of the volatile components appeared to be related to the odor description. Thus, it is apparent that flavor profiles *per se* cannot be used as a subjective measurement unless some other criteria are used in conjunction with the flavor profiles.

The stereochemical theory of olfaction proposed by Amoore (1952) appears to offer an explanation for the results obtained in this study. The theory has two prerequisites for an odorant: volatility and a molecular configuration complementary to the specialized receptors in the nasal cavity. After a comprehensive study of odorants (Amoore, 1952), seven primary odors were identified: camphoraceous, pungent, ethereal, floral, peppermint, musky, and putrid. Odorants like lemon, garlic, cedar, and rancid were classified as complex odors. Using this classification, the components identified in the chromatograms of the volatiles from the various pure-culture fermentations would be as follows: formaldehyde, pungent; acetaldehyde, pungent; propionaldehyde, pungent; acetone, ethereal; butyraldehyde, rancid; ethyl alcohol, ethereal; ethyl butyrate, rancid; isovaleraldehyde, rancid. The percent concentration of the volatile components was arranged according to the above classification (Table 3). A comparison between the odorants and odor description indicates there is a relationship between pungent and rancid components and odor. In the fermentation with *L. plantarum* the relative concentration of pungent odorants compared to rancid odorants was high, the odor description was "clean, raw cucumber," and flavor description was "clean, raw cucumber, mild to strong acid." There were no rancid odorants in the volatiles from *P. cerevisiae*. The odor was described as "clean, mild acid, raw cucumber," and flavor as "slightly bitter, mild acid, slightly aromatic, musty, hay-like, etc."

In contrast, pure-culture fermentations of cucumbers with *L. brevis* and *Leuc. mesenteroides* had greater concentrations of the rancid odorants and correspondingly lesser concentrations of pungent odorants. The odor descriptions by the taste panel were strikingly different when compared to the fermentations with *L. plantarum* and *P. cerevisiae*. The flavor descriptions for *L. brevis* were "mild acid, raw cucumber, bitter after taste, etc."; and for *Leuc. mesenteroides* "medium acid, slightly fruity, salt." The natural-fermentation control (nonacidified) has a higher concentration of rancid odorants than of pungent odors. The odor description was "stale, undesirable, unpleasant," and the flavor description was "acid and bitter with undesirable aftertaste." It was concluded that good agreement may be obtained between subjective and objective measurements if each volatile component in the chromatogram (objective measurement) is identified and classified according to the Amoore theory. It was also concluded: a) that the odor and flavor associated with an acceptable pure-culture fer-

mentation of brined cucumbers may be described in terms of the primary odorants, particularly the pungent odors; b) when the pungent odorants and rancid odorants (complex) are similar in concentration, the taste component of flavor is predominant; and c) when rancid odorants were in greater concentration than pungent odorants, the odor component is predominant for the flavor description, as illustrated for natural nonacidified control.

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The Long-Term Effect of Electrical Stimulation on the Post-Mortem Fall of pH in the Muscles of Landrace Pigs

SUMMARY

It has been shown previously, in a series of experiments on the post-mortem fall of pH in the muscles of Danish Landrace pigs, that the animals can be broadly divided into two groups, differing in rate of fall. One gives samples with low rates, called *A*, and the other with rates twice as high, called *B*.

The present experiments show that the slow fall of pH in group *A* muscles can be converted to a quick fall by subjecting excised samples to a short tetanus. This accelerating effect of electrical stimulation lasts during the whole course of the pH-time curves, and is easily distinguished from the well-known short-term effect, characteristic of this and many other types of muscle. Although the latter can be clearly demonstrated in rabbit muscle, for instance, the subsequent long-term acceleration is entirely absent. The latter must therefore be considered a new and hitherto undescribed phenomenon, so far reported only for the pig. It is due to an induced higher ATP-ase activity, although the mechanism is obscure.

These findings open up a new approach in the search for the cause of the rapid post-mortem fall of pH characteristic of pig muscles which subsequently yield pale and watery pork.

INTRODUCTION

In a previous paper (Bendall *et al.*, 1963), we described post-mortem chemical and physical changes in the muscles of Danish Landrace pigs. From experiments on samples of longissimus dorsi muscles held at 37°C under moist nitrogen, we found that the pigs could be divided into two broad groups, *A* and *B*, the former showing slow, and the latter fast, post-mortem changes. For example, the maximum rate of pH fall was 0.64 unit per hour in group *A*, and 1.04 units per hour in group *B*. There was a similar relative difference between the other changes. The quality of the meat on the carcasses from which the samples had been taken was related to these rates of change. Thus, on the day after slaughter, all carcasses of type *A* (slow) were of excellent quality, whereas those of type *B* (fast) were more or less pale and watery.

Further investigations along these lines have strongly supported the above findings. In 2 cases out of 25 we have, in fact, found rates of pH fall even greater than those mentioned for group *B*. The initial pH for these pigs was low, and the rate of pH fall from the start was of the order of 1.50 units per hour. The latter rates are similar to those found by Sayre and Briskey (1963) for their fast group *A* pigs, although initial pH values are not given for this particular group. The latter authors also describe slow groups *(D,E,F)*, with rates close to our slow group *A*.

Normally the rate of pH fall in the carcass itself, as indicated by the so-called pH_1 value, taken at 45 min after death, corresponds quite well to the rate in the excised samples held at 37°C, when corrected for the higher temperature (38–41°) of the carcass during the first 45 minutes after sticking. The two cases mentioned above, which gave an exceptionally high rate of fall in the excised samples, showed, however, a considerably lower rate in the carcass itself, indicating that some external influence had introduced changes in the excised muscle, leading to the faster fall. It was noted that these particular samples twitched very strongly when they were excised, and this suggested that electrical stimulation of excised muscles might induce a similar long-term acceleration. We found, in fact, that a short tetanus did have just this effect on excised muscles, changing the pattern from the slow type, *A*, to the fast type, *B*, that is, it nearly doubled the over-all rate of fall.

We must emphasize here that this induced acceleration of the chemical changes in pig muscles lasts throughout rigor mortis, that is, for 2 or more hours, and must not be confused with the well-known short-term

acceleration of lactic acid production associated with the process of recovery from a tetanus and lasting only a minute or less. The latter can be easily demonstrated in rabbit muscle, for example, but the long-term effect is completely absent.

METHODS

Sampling, preparation of experimental material, and measurement of pH were carried out as described by Bendall *et al.* (1963).

Stimulation. Two pieces of approx. 30 g were cut from the main sample of muscle. One was used for control, and the other was placed on a special Plexiglas plate in which were fixed six steel pins, connected three and three to the poles of a small inductor (35 V on primary, 200 cycles per sec). Two pieces were taken, immediately after cutting, from each end of the main sample for determination of initial pH. The piece on the plate was then stimulated for 30 sec. The stimulation normally took place about 10–15 min post-mortem. Samples were again taken from both pieces for pH determination—immediately after stimulation and then at intervals until pH fall had ceased.

RESULTS

The results showed that the pigs could be divided into two groups according to the response of their muscles to stimulation. One group of samples gave no visible response to stimulation. When the current was made, there were no signs of twitching, and the subsequent rate of pH fall was found to be the same for the control as for the stimulated samples. The rates indicated that the pigs in this group belonged to the "fast" type, *B*.

The other group of samples showed a strong tetanic contraction on stimulation, lasting 10 to 20 sec. The pH fall of the control piece was slow and corresponded to that found for type *A* pigs, whereas the stimulated piece showed a rapid fall of pH, most similar to type *B*.

Fig. 1 shows average pH-time curves for control and stimulated samples of type *A*. More detailed values for rates of pH fall are given in Table 1, with the average values for the original group shown for comparison. The Q_{10} values for rate of pH fall were also measured in samples from the same pigs and found to average 2.2 in the range 32–37°C, and 1.9 in the range 27–32°, irrespective of the absolute value of the rates in any particular case.

The experiments with rabbit muscle are not tabulated here, because in the ten cases investigated, five with longissimus dorsi muscles and five with psoas major muscles, no long-term effect of stimulation could be demonstrated, but only a small and immediate increase in rate of pH fall, which decreased to normal soon after stimulation had ceased—this in spite of the fact that the rabbit muscles responded more vigorously and longer than the pig muscles.

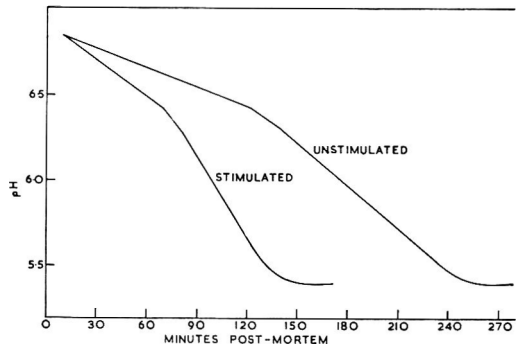

Fig. 1. Average pH-time curves for stimulated and unstimulated muscles of type A. The average values at various times were taken from the experiments in Table 1.

DISCUSSION

In attempting to assess the significance of the stimulation experiments we have described, it is essential to distinguish three factors which play a part in the phenomenon. First we must distinguish between the short- and long-term effects of stimulation; there is nothing new, for example, in the fact that stimulation under anaerobic conditions produces a rapid fall of pH, due to the production of lactic acid, but, as the classical experiments of Meyerhof, Hill, and the Coris have demonstrated (Cori, 1956), this is normally a short-term effect, lasting at most a minute or two, and representing the resynthesis, through the glycolytic cycle, of the ATP and creatine phosphate broken down during the tetanus. The rate of lactic acid production can rise during this process to about 200 times the resting rate, and would appear in our experiments as a sudden drop of pH at the very start of the pH-time curves, if it could be measured. The phenomenon we are discussing, however, is of quite a different order of magnitude from this: a twofold instead of a 200-fold increase in rate, and a duration, as we see from Fig. 1, of an hour or more instead of a minute or less. In terms of the potential rate of glycolysis of the muscle, it is therefore nugatory, but in terms of the acidification which occurs in the

Table 1. Collected results for effect of stimulation on rates of pH fall in pig muscle.

Ref. no.	Initial pH	Rate of pH fall (units per hour) Slow part of curve Control	Slow part of curve Stim.	Fast part of curve Control	Fast part of curve Stim.	Ultimate pH	pH_1 (on carcass)
1) Results from samples which did not respond to stimulation:							
8/3	6.50	....	....	1.25	....	5.45	5.80
12/3	6.70	0.55	....	1.02	....	5.38	6.10
30/4	6.75	0.43	....	1.10	....	5.35	6.30
29/5	6.50	0.42	....	0.78	....	5.45	....
Av.	6.62	0.47	....	1.04	....	5.41	6.07
Av. for Group B[a]	6.66	0.55	....	1.04	....	5.41	....
2) Results from samples which responded to stimulation:							
8/5	6.85	0.27	0.50	0.40	0.56	5.63	6.20
15/5	6.80	0.26	0.45	0.64	1.05	5.33	6.28
4/6	6.70	0.17	0.26	0.46	0.92	5.39	6.26
28/6	6.75	0.28	0.43	0.67	1.13	5.42	....
26/4	7.08	0.17	0.31	0.45	1.00	5.42	6.42
22/3	6.90	0.23	0.50	0.34	0.80	5.50	6.23
26/3	6.90	....	0.60	....	1.22	5.35	6.30
Av.	6.84	0.23	0.43	0.49	0.95	5.40	6.28
Av. for group A[a]	6.78	0.28	....	0.65	....	5.47	....

[a] Averages for groups A and B of Bendall *et al.* (1963).

muscles of a carcass, slowly cooling on the slaughter line, it is clearly of the utmost importance, and can make all the difference between acceptable meat, on the one hand, and pale and watery meat on the other.

The third factor which must be taken into account is initial pH itself, determined by the activity of the pig just before death and by the severity of the death struggle after stunning and sticking. It could be, for instance, that the lack of response of the rapid group (*B*) muscles to electrical stimulation post-mortem was due to their somewhat lower initial pH (see Table 1), which is probably quite critical in determining the strength of the response. Be that as it may, the very fact that these muscles had a lower initial pH shows that they had been more active during the death struggle than the muscles of type *A*, i.e., more highly stimulated while still in the carcass. And, like the artificially stimulated samples of the slow type (*A*), they therefore showed a higher sustained rate of pH fall. It is, indeed, very tempting to postulate that the high rates of pH fall almost invariably associated with the subsequent occurrence of watery meat are determined to a large extent by just this long-term after-effect of nervous stimuli which reached the muscles during the sticking process.

As we have said, Sayre and Briskey (1963) report a similar division of pigs into fast and slow groups, although they do not seem to have taken the possible effect of low initial pH into account in the faster groups. Where our results differ from theirs is that we have deliberately chosen muscles with high initial pH, in order to test the effect of stimulation over as wide a pH range as possible.

As a corollary to the effect of stimulation, we may note that in two pigs, successfully immobilized before death by injection of the motor-horn-cell paralyzant, myanesin, the rates of pH fall post-mortem were among the lowest yet recorded. In these pigs, almost no motor impulses could have reached the muscles during slaughter.

Although the actual ATP and creatine phosphate contents of the muscles were not measured in the present experiments, it follows from the general theory of rigor (*c.f.* Bendall, 1960), that the immediate cause of

the higher rates of pH fall in stimulated muscles and group *B* muscles is a higher ATP-ase activity, since it is this process, the splitting of ATP, which determines the rate of glycolysis. At present it is not easy to see how this comes about, although it could be due to partial depolarization of the muscle plasma membrane as an aftermath of stimulation under unnaturally acid conditions, and threfore not fully recoverable. If this were so, it would place the phenomenon in the same class as other depolarization contractures, such as those induced by caffeine or KCl. To this it can be objected that the phenomenon cannot be induced in rabbit muscles, but this also applies to the very similar phenomenon of "cold shock," which can be brought about in beef, sheep, and pork muscles merely by cooling them to 0°C (Locker and Hagyard, 1963). The latter is a typical contracture, and it is tempting to suppose that it, too, is brought about by upsetting the delicate sodium-potassium balance on which the resting membrane potential depends—in this case by cooling.

We must, however, be very cautious in applying conclusions drawn from far more vigorous and rapid contractures, such as those induced by caffeine, KCl, or prerigor freezing and thawing, to cases such as stimulation and cold shock effects, where the time scale is ten to a hundred times more drawn out, and which for that reason alone will require the most delicate methods of elucidation. Nevertheless, the stimulation effect, within the context of post-mortem changes, can clearly contribute significantly to the question of the variability of rates of pH fall, and together with the equally unexplained effects of environmental temperature, recently shown by Kastenschmidt *et al.* (1964), may be all that is required to explain how a pig carcass can so easily change from acceptable to watery, pale, and unattractive.

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The Anthocyanin Pigments of Boysenberries

SUMMARY

Anthocyanins in boysenberries were extracted and purified by ion-exchange and paper chromatographic methods. The pigments were identified by R_f values, sugar moiety, alkaline degradation products of the aglycone, partial acid hydrolysis, and absorption spectra. The major pigments were shown to be cyanidin 3-monoglucoside and cyanidin 3-diglucoside. Also present were smaller amounts of cyanidin 3-rhamnoglucoside and cyanidin 3-rhamnoglucosido-5-glucoside.

Boysenberries originated from a cross of blackberries with raspberries and loganberries. In California, most of the crop is packed as quick-frozen berries for pies. Berries are also used for canning, for syrups, jellies, preserves, wines, and other products (Rohrer and Luh, 1959). Under unfavorable processing or storage conditions, the attractive natural red color of boysenberry products tends to be replaced by an undesirable brown color. This presents a problem to the processors.

Anthocyanins are the compounds that constitute most of the red, purple, and blue pigmentation in plants. The subject has been reviewed by Bate-Smith (1950), Geissman (1955), Harborne (1958a,b), and Hayashi 1962). The anthocyanin pelargonin has been reported in strawberries by Sondheimer and Kertesz (1948). Lukton *et al.* (1955) found chrysanthemin in the same fruit. The anthocyanin pigments in cacao were reported by Forsyth and Quesnel (1957). Harborne (1960) studied anthocyanin production in the cultivated potato. Variations in the glycosidic pattern of anthocyanins were reported by Harborne and Sherratt (1957).

The recent development of methods for separating anthocyanins by paper chromatography has made possible rapid separation and identification of anthocyanins on a micro-scale (Harborne, 1958b; Chandler and Harper, 1962). The characterization of anthocyanins by spectral methods had been reported by Harborne (1958a). Abe and Hayashi (1956) used the partial hydrolysis method studying types of glycoside linkage in anthocyanin by paper chromatography. They demonstrated that a diglucoside was partially hydrolyzed to monoglucoside by mild acid hydrolysis, and then to the aglycone. The hydrolysis process can be traced distinctly on the paper chromatogram.

This paper presents the chemistry of anthocyanins in boysenberries.

MATERIALS AND METHODS

Extraction and purification of anthocyanins. Seven pounds of frozen boysenberries were thawed and mixed for 20 seconds in a blender with 1,000 ml of 0.05*N* HCl. The mixture was treated with Super-Cel, and filtered under suction through Whatman No. 2 paper in a Buchner funnel. The filtrate was mixed with 200 g of Dowex 50×4 cation-exchange resin in the hydrogen form and let stand 2 hr at 32°F. The supernatant was decanted off. The resin was washed five times with 5 L of distilled water, transferred to a 3.8 × 50-cm column, and eluted successively with a total of 8.5 L of 0.15*N* ethanolic HCl. The eluate was concentrated to 250 ml in a flash evaporator. The crude anthocyanin extract was separated by paper chromatography on Whatman No. 3MM paper in an ascending direction at 75°F for 15 hr, using a glacial acetic acid-H_2O (15:85 v/v) solvent mixture as the mobile phase. Four separate bands (A, B, C, D) were obtained. The bands were cut and eluted with 0.01*N* ethanolic HCl. Bands A and C were concentrated in a flash evaporator to a small volume, precipitated with diethyl ether, and then crystallized at 32°F from acidified 50% ethanol. The supernatant was decanted, and the crystals were washed three times, each time with 5 ml of diethyl ether. The crystals were dried and then kept in the dark in a vacuum desiccator.

Bands B and D are present in smaller amounts. Each fraction was concentrated in a flash evaporator to near dryness, dissolved in a small volume of methyl alcohol containing 0.01% anhydrous HCl, and then precipitated with diethyl ether. The anthocyanin was washed with ether, dried, and kept in the dark in a vacuum desiccator.

Paper chromatography of anthocyanins. Five

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μl of the purified anthocyanins in solution (5 mg/5 ml 0.01% methanolic HCl) were spotted on Whatman No. 1 filter paper and then chromatographed at 75°F in an asecnding direction with acetic acid–H_2O (15:85 v/v) solvent for 12 hr, and with the upper phase of *n*-butanol–acetic acid–water (4:1:5 v/v) for 48 hr. The spots were compared for R_f values under both visible and ultraviolet lights.

Sugar moiety in the anthocyanins. Five mg of the purified anthocyanin was refluxed for 1 hr with 5 ml of 1*N* HCl in a boiling-water bath. The mixture was cooled, treated with Dowex 50 × 4 cation exchanger and Dowex 1 × 8 anion exchanger (Dow Chemical Co.) to remove HCl and aglycone, and then filtered.

Thirty μl of the filtrate was chromatographed in a descending direction for 12 hr on Whatman No. 1 paper with the upper phase of ethyl acetate–pyridine–water (2:1:2 v/v), and with the upper phase of ethyl acetate–acetic acid–water (3:1:3 v/v) at 75°F (Jermym and Isherwood, 1949). The papers were dried, sprayed with a 2.5% aniline hydrogen phthalate in water-saturated butanol, and heated 5 min at 100°C (Partridge, 1949). Also used for this purpose was a spray of 0.5% 3,5-dinitrosalicylic acid in 4% NaOH solution. Reducing sugars form brown spots when heated 4–5 min at 100°C with either reagent.

Glucose in the anthocyanin hydrolysate was confirmed by the Glucostat (glucose oxidase and peroxidase, Worthington Biochem. Corp., Freehold, N. J.) method at pH 7.0. Glucose forms a yellow to brown color with Glucostat in the presence of oxygen and *o*-dianisidine (Kingsley and Getchell, 1960).

Diglucoside was indicated in pigment C by refluxing 6 mg of the pigment with 4 ml of 10% acetic acid (v/v) for 2 hr at 100°C (Chandler and Harper, 1961). The hydrolysate was treated with Dowex 50 and Dowex 1 ion exchangers to remove electrolytes and the aglycone. The sugars were compared by paper chromatography on Whatman No. 1 paper with ethyl acetate–acetic acid–water (3:1:3 v/v) and ethyl acetate–pyridine–water (2:1:2 v/v) solvents as described above. The R_f values of the sugars were compared with known samples of glucose, maltose, cellobiose, gentibiose, and laminaribiose.

Quantitative determination of sugars in the anthocyanins. The ceric sulfate titration method (Hassid, 1937) was used for quantitative determination of sugars in the anthocyanin pigments. Fifteen mg of the purified pigment was refluxed for 60 min with 10 ml of 1*N* HCl over a boiling-water bath. The mixture was cooled in ice water, and mixed with Dowex 50 × 4 cation exchanger in the hydrogen form to remove the aglycone. The resins were filtered and washed with water to recover the sugars. The filtrate and washings were combined, neutralized with 2*N* NaOH, and diluted to 25 ml. Five ml of the solution was heated for exactly 15 min with 5 ml of alkaline potassium ferricyanide (8.25 g $K_3Fe(CN)_6$ and 10.6 g Na_2CO_3/L) on a boiling-water bath. The samples were cooled, mixed with 5 ml of 5*N* H_2SO_4, and titrated immediately with 0.01*N* ceric sulfate to a golden-brown color, using 0.1% Setopaline C solution as an indicator. The ceric sulfate solution was standardized against c.p.-grade glucose.

Alkaline degradation of the aglycone. The structure of the aglycone in boysenberry anthocyanins was investigated by the alkaline degradation method described by Karrer and Widmar (1927). Fifteen mg of the anthocyanin was refluxed for 60 min with 10 ml of 1*N* HCl on a boiling-water bath. The product was treated with Dowex 50 × 4 cation exchanger in the hydrogen form to absorb the aglycone. The resin was washed with distilled water, and then eluted with CH_3OH containing 1% HCl (w/v) to recover the aglycone. The eluate was evaporated in a flash evaporator to remove the CH_3OH. The aglycone was transferred into a small flask, neutralized, and then refluxed for 30 min with 5 ml of 15% $Ba(OH)_2$ solution over a boiling-brine bath. A nitrogen atmosphere was maintained to avoid oxidation. The mixture was cooled, acidified with 2 ml of conc. HCl, and extracted with 2 ml of diethyl ether. The ether extract was chromatographed separately at 75°F on Whatman No. 1 paper in a descending direction with two solvent systems: 1) the lower phase of *m*-cresol–acetic acid–water (50:2:48 v/v, Bate-Smith, 1950) in a descending direction for 25 hr; and 2) the upper phase of benzene–acetic acid–water (2:2:1 v/v, Glick, 1954) in an ascending direction for 18 hr. The dried chromatograms were dipped into a freshly prepared diazonium salt solution made from 5 ml of 0.5% p-nitroaniline in 2*N* HCl, 0.5 ml of 5% sodium nitrite (w/v), and 15 ml of 20% sodium acetate (w/v, Swain, 1953). The R_f values of the alkali degradation products were compared wth those of authentic phenolic compounds.

Spectrophotometry. The absorption spectrum of the purified anthocyanin in 0.01% methanolic HCl was made in a Beckman DK-2 recording spectrophotometer.

RESULTS

Paper chromatography of anthocyanins. The anthocyanins in boysenberries were absorbed on a Dowex 50 × 4 cation-exchange column, eluted with 0.15*N* ethanolic HCl, and then chromatographed on paper. Table 1 shows the R_f values of the

Table 1. R_f values of anthocyanins isolated from boysenberries.

Pigment	Identification	R_f values: Acetic acid–water (15:85 v/v) ascending, 12 hr	R_f values: *n*-Butanol–acetic acid–water (4:1:5 v/v) ascending, 48 hr
A	Cyanidin 3-monoglucoside	0.43	0.42
B	Cyanidin 3-rhamnoglucoside	0.58	0.36
C	Cyanidin 3-diglucoside	0.71	0.33
D	Cyanidin 3-rhamnoglucosido-5-glucoside	0.79	0.25

purified anthocyanins with acetic acid–water (15:85 v/v) and *n*-butanol–acetic acid–water (4:1:5 v/v) as solvents. Four red pigments were observed on the chromatograms. The acetic acid–water (15:85 v/v) solvent separates the pigments into distinct spots in 12 hr. When *n*-butanol–acetic acid–water (4:1:5 v/v) solvent was used, a 48-hr period was needed for separation.

Pigments A and C were present in approximately equal amount, and constitute approximately 90% of the total anthocyanins. Pigments B and D were present in small amounts.

The aglycone. The structure of the aglycone in the boysenberry anthocyanins was investigated by the barium hydroxide degradation method and paper chromatography of the degradation products. Table 2 indicates that the aglycone of the four boysenberry anthocyanins is cyanidin, which was cleaved by barium hydroxide to yield phloroglucinol and protocatechuic acid.

The anthocyanins A, B, C, and D were hydrolyzed separately by refluxing with 10% HCl. The aglycones were purified on paper and cochromatographed with a known cyanidin sample. They have the same R_f values: 0.69 when irrigated with n-butanol–acetic acid–water (4:1:5 v/v), and 0.73 with *n*-butanol-2*N* HCl (1:1 v/v) solvents. This identification was further supported by the fact that the aglycone, when dissolved in CH_3OH containing 0.01% HCl, shows a maximum absorption peak at 535 mμ.

Sugar moiety. The sugars in each of the purified anthocyanins were determined by paper chromatography of the acid hydrolysis products. The aglycone and HCl were removed by ion exchangers as described earlier, and the resulting solution was tested for sugars. Table 3 indicates the R_f and R_g (glucose as reference) values of the sugars from acid hydrolysis of the anthocyanins. Pigments A and C both contained glucose and B and D contained both glucose and rhamnose. In both solvent systems, the glucose and rhamnose from the anthocyanins gave R_f and R_g values identical to those of authentic sugar samples. The R_f values were slightly different from those reported by Jermyn and Ishwood (1949). When the results were expressed as R_g values (distance traveled by glucose as reference), the sugar moiety

Table 2. Paper chromatography of phenolic compounds from alkali degradation of boysenberry anthocyanins.

Phenolic compounds	Identification	R_f: (1) Benzene–acetic acid–water (2:2:1 v/v, upper layer, ascending 18 hr)	R_f: (2) *m*-Cresol–acetic acid–water (50:2:48 v/v, lower layer, descending 25 hr)
Vanillic acid		.89	0.81
Syringic acid		.86	0.91
Protocatechuic acid		.15	0.30
Phloroglucinol		.00	0.15
Gallic acid		.01	0.07
From pigment A	Protocatechuic acid	.15	0.30
	Phloroglucinol	.00	0.15
From pigment B	Protocatechuic acid	.15	0.30
	Phloroglucinol	.00	0.15
From pigment C	Protocatechuic acid	.15	0.30
	Phloroglucinol	.00	0.15
From pigment D	Protocatechuic acid	.15	0.30
	Phloroglucinol	.00	0.15

Table 3. Paper chromatography of sugar moiety in boysenberry anthocyanins.

Sugar	Ethyl acetate–pyridine–water (2:1:2, v/v) upper layer descending, 12 hr		Ethyl acetate–acetic acid–water (3:1:3, v/v) upper layer descending, 12 hr	
	R_f	R_g	R_f	R_g
Pigment A	0.31 (Glucose)	1.00	0.21 (Glucose)	1.00
Pigment B	0.54 (Rhamnose)	1.70	0.42 (Rhamnose)	2.00
	0.31 (Glucose)	1.00	0.21 (Glucose)	1.00
Pigment C	0.31 (Glucose)	1.00	0.21 (Glucose)	1.00
Pigment D	0.54 (Rhamnose)	1.70	0.42 (Rhamnose)	2.00
	0.31 (Glucose)	1.00	0.21 (Glucose)	1.00
Rhamnose, authentic	0.54	1.70	0.42	2.00
Xylose, authentic	0.44	1.41	0.31	1.48
Arabinose, authentic	0.36	1.16	0.28	1.33
Glucose, authentic	0.31	1.00	0.21	1.00
Galactose, authentic	0.25	0.81	0.19	0.90

was found to be identical to glucose. Xylose, arabinose, and galactose were absent from boysenberry anthocyanins.

When anthocyanins with a diglycoside structure were heated 2 hr at 100°C with 10% acetic acid, the ether linkage between C_3 of the aglycone and the diglucoside was hydrolyzed in preference to the sugar-sugar linkage. This enabled us to determine the nature of the diglycoside by paper chromatographic method. When this method was applied to pigment B, rutinose was shown to be present, with an R_g value of 0.60 when *n*-butanol-acetic acid–water (4:1:5 v/v) was the solvent, and of 0.77 when ethyl acetate–pyridine–water (10:4:3 v/v) was the solvent. Thus it is evident that pigment B is cyanidin 3-rutinoside (cyanidin 3-rhamnoglucoside).

When the 10% acetic acid hydrolysis method was applied to pigment C, maltose was found in the hydrolysate. Table 4 shows the R_g values of various known diglucosides and the sugar in the hydrolysate: Luminaribose (1,3-β-diglucoside), cellobiose (1,4-β-diglucoside), and gentiobiose (1,6-β-diglucoside) were shown to be absent from pigment C. The test also excludes the possibility of cyanin (cyanidin 3,5-diglucoside), which could not yield a diglucoside on partial acid hydrolysis with 10% acetic acid. From the above results, it appears that pigment C is cyanidin 3-diglucoside.

When pigment D was hydrolyzed with 10% acetic acid, the presence of rutinose in the molecule was also indicated.

Quantitative determination of sugars. The reducing sugars in the purified anthocyanins were determined by the semimicro ceric sulfate titration method. The purified pigments were first hydrolyzed by refluxing for 1 hr with 1*N* HCl. Table 5 shows the approximate molar ratio of sugar to aglycone in each pigment. The number of intermediate anthocyanins derived from the pigments is also presented. These data indicate the identification of the pigments. Pigment A is cyanidin 3-monoglucoside, based on the evidence that: 1) its R_f value was same as that of a known cyanidin 3-monoglucoside; 2) it contained only one glucose per mole of cyanidin; and 3) it does not fluoresce under ultraviolet light.

Similar evidence indicates that pigment B is cyanidin 3-rhamnoglucoside, C is cyanidin 3-diglucoside, and D is cyanidin 3-rhamnoglucosido-5-glucoside.

Partial acid hydrolysis. Anthocyanins occur in nature in combination with sugars as glycosides, and occasionally in combination with organic acids as acylated glycosides. The sugar moiety is nor-

Table 4. Paper chromatography of sugar moiety in pigment C (cyanidin 3-diglucoside) after hydrolysis with 10% acetic acid for 2 hr at 100°C.

Sample	Structure	R_g values (glucose = 1.00) Ethyl acetate–acetic acid–water (3:1:3 v/v) upper phase, descending, 28 hr
Hydrolysate from pigment C	1,4-(α-D-glucosido)-D-glucose	0.55
Laminaribiose	1,3-(β-D-glucosido)-D-glucose	0.58
Maltose	1,4-(α-D-glucosido)-D-glucose	0.55
Cellobiose	1,4-(β-D-glucosido)-D-glucose	0.48
Gentiobiose	1,6-(β-D-glucosido)-D-glucose	0.44

Table 5. Characterization of anthocyanins in boysenberries.

Pigments	Identification	Sugar moiety	Moles reducing sugar per mole aglycone	Number of intermediate anthocyanins on partial acid hydrolysis
A	Cyanidin 3-monoglucoside	Glucose	1	0
B	Cyanidin 3-rhamnoglucoside	Glucose + rhamnose	2	1
C	Cyanidin 3-diglucoside	Glucose	2	1
D	Cyanidin 3-rhamnoglucosido-5-glucoside	Glucose + rutinose	3	4

mally found at the C_3 or C_3 and C_5 positions. By determining the number of intermediates obtainable through partial acid hydrolysis and the nature and number of sugar molecules present in the molecule, the structure of anthocyanins can be quickly determined on a microscale by paper chromatographic methods.

The partial acid hydrolysis method was used to determine the structure of anthocyanins extracted from boysenberries. Pigment A gave no intermediate on partial acid hydrolysis, indicating a monoglycoside structure. Table 6 shows that, on partial acid hydrolysis of pigment B, rhamnose appeared earlier than glycose, indicating that glucose was attached to C_3, and rhamnose was attached to glucose to form rutinose in the molecule. Table 7 shows that pigment C was a diglycoside, forming one intermediate anthocyanin on partial acid hydrolysis. Table 8 indicates that pigment D forms four intermediate anthocyanins on partial acid hydrolysis. It appears to be a cyanidin-3-rhamnoglucosido-5-glucoside because of: 1) the formation of four intermediate anthocyanins through partial acid hydrolysis; 2) the presence of both glucose and rhamnose when hydrolyzed with 1*N* HCl; 3) the presence of rutinose in the 10% acetic acid hydrolysate; 4) the presence of 3 moles of sugar per mole of aglycone in the molecule, and 5) the R_f values of the intermediate anthocyanins formed during hydrolysis.

Table 6. Paper chromatography of sugars from hydrolysis of pigment B (cyanidin 3-rhamnoglucoside) isolated from boysenberries. Solvent: ethyl acetate–acetic acid–water (3:1:3 v/v), descending, 16 hr.

Hydrolysis with 1*N* HCL at 100°C (min)	Sugar moiety found	
	Glucose $R_g = 1.00$ [a]	Rhamnose $R_g = 2.00$ [a]
0	—	—
5	—	+
10	+	++
15	++	+++
60	++++	++++

[a] R_g represents the ratio of the distance traveled by the compound to that by glucose.

Spectrophotometry. The method described by Harborne (1958b) was used to study the chemical structure of boysenberry anthocyanin. Table 9 shows the absorption peaks of the pigments in 0.01% methanolic HCl. The absorption peak shifted from 523 to 567 or 568 mμ when aluminum chloride was added as a chelating agent. This indicates the presence of two ortho hydroxyl groups in the benezene ring of the anthocyanins. The extinction coefficient ratios (E_{440}/E_{max}) were either 22 or 23 for all the four anthocyanins, which agrees well with those in the literature (Harborne, 1958a).

From the above observations one may conclude that the anthocyanins in boysenberries are: 1) cyanidin 3-monoglucoside; 2) cyanidin 3-rhamnoglucoside; 3) cyanidin 3-diglucoside; and 4) cyanidin 3-rhamnoglucosido-5-glucoside. The pre-

Table 7. Paper chromatography of the products from partial acid hydrolysis of pigment C isolated from boysenberries. Solvent: acetic acid–water (15:85 v/v), 12 hr.

Hydrolysis with 1*N* HCl at 100°C (min)	Pigment C (cyanidin 3-diglucoside) $R_f = 0.71$	Intermediate (cyanidin 3-monoglucoside) $R_f = 0.43$	Aglycone (cyanidin) $R_f = 0.21$
0	+++++	—	—
5	++++	+	±
10	+++	++	+
15	+++	+++	++
20	++	+++	+++
60	±	++	++++

(+) represents relative color intensity of the spots.
(—) means absence of the anthocyanin.
(±) represents presence of trace amount of the anthocyanin.

Table 8. Paper chromatography of controlled acid-hydrolysis products of pigment D isolated from boysenberries. Solvent: acetic acid–water (15:85 v/v) for 12 hr in an ascending direction.

Hydrolysis with 1*N* HCl at 100°C (min)	Pigment D	Intermediates				Aglycone
	Cyanidin 3-rhamnoglucosido-5-glucoside $R_f = 0.79$	Cyanidin 3:5-diglucoside $R_f = 0.71$	Cyanidin 3-rhamnoglucoside $R_f = 0.58$	Cyanidin 3-monoglucoside $R_f = 0.43$	Cyanidin 5-monoglucoside $R_f = 0.41$	Cyanidin $R_f = 0.20$
0	++++++	−	−	−	−	−
5	+++++	++	+	−	+	+
10	+++	+++	++	++	++	++
15	++	++	++	+++	+++	+++
20	+	++	++	++++	+++	++++
30	−	+	+	+++	+++	+++++
40	−	+	−	++	+++	+++++
60	−	−	−	−	++	+++++
90	−	−	−	−	+	+++++
120	−	−	−	−	−	+++++

Table 9. Absorption characteristics of anthocyanin pigments isolated from boysenberries.

Pigments	Identification	Absorption characteristics in 0.01% methanolic HCl		
		Maximum absorption peak (mμ)	Maximum absorption peak after adding $AlCl_3$ (mμ)	E_{440}/E_{max} (%)
A	Cyanidin 3-glucoside	525	568	22
B	Cyanidin 3-rhamnoglucoside	525	567	22
C	Cyanidin 3-diglucoside	523	568	22
D	Cyanidin 3-rhamnoglucosido-5-glucoside	523	567	23

dominant pigments are cyanidin 3-monoglucoside and cyanidin 3-diglucoside, with the other two present only in small amounts.

ACKNOWLEDGMENTS

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Evaluation of Factors Affecting the Determination of Nitrogen in Soya Products by the Biuret and Orange-G Dye-Binding Methods

SUMMARY

Nitrogen content of soya protein was determined by a modification of the biuret method in which protein extraction and color development occur simultaneously in an alkaline copper tartarate solution and by the orange-G dye-binding method. Percentage of total nitrogen content extracted into the solution increased as total nitrogen content increased. Amount of extracted nitrogen was reduced in coarsely ground or severely heated samples. In finely pulverized and untoasted samples, correlation of Kjeldahl protein with the biuret method of protein estimation was 0.985, and with the dye binding method was 0.989.

INTRODUCTION

Nutritional value of soya flour has long been appreciated because of its superiority over other vegetable proteins and a cost lower than that of animal protein supplements. The most acceptable method of protein determination is the Kjeldahl method. Its main disadvantages are time, use of corrosive reagents, need for skillful manipulation, and expensive equipment. In the biuret method, proteins are peptized with potassium hydroxide and treated with a copper sulfate solution, and the intensity of the color formed is proportional to protein concentration. The biuret procedure is simple and rapid, and the equipment and apparatus used are relatively inexpensive and readily available. The biuret test involves reaction with the peptide linkage and measures protein-nitrogen only, whereas the Kjeldahl procedure measures total nitrogen and does not distinguish between protein and nonprotein nitrogen. The biuret method is widely used in biochemical laboratories to estimate small amounts of protein. It has been applied to the determination of protein in cereals (Jennings, 1961; Pinckney, 1961) and in meat (Torten and Whitaker, 1964).

Fraenkel-Conrat and Cooper (1944) showed that Orange G dye binds specifically under acidic conditions to free amino groups, the imidazole group of histidine, or the guanidyl group of arginine. Orange-G binding has been used to determine protein content of wheat flour (Udy 1956a), milk (Udy, 1956b), meat, fish, bean, and nut meals (Bunyan, 1959), and meat (Torten and Whitaker, 1964). Moran *et al.* (1963) found that toasting soybean meal lowered Orange-G binding as a result of decreased availability of the epsilon-amino group of lysine.

Work reported here evaluates the possibility of using the biuret and Orange-G methods to determine soya protein. A single-step protein extraction and color development procedure as proposed by Pinckney (1961) was employed to simplify analytical determination by the biuret method.

MATERIALS AND METHODS

Soya flour samples. Twenty-four samples of soya flour, obtained from four manufacturers, were chosen to represent variations in chemical composition, particle size, and heat treatment—variations believed to be typical of products marketed for use by food and feed industries. Composition and characteristics of the 24 soya samples are given in Table 1. Additionally, a sample of soya flour with protein dispersibility of 90% was autoclaved for various periods at 10 psi. Heated samples were reground in a laboratory micro-Wiley mill to pass a 60-mesh sieve.

Methods. Moisture and macro-Kjeldahl values were determined by the official AOAC (Horwitz, 1955) method. Percent nitrogen was converted to percent protein with the factor 6.25.

The modified biuret reagent was prepared as described by Pinckney (1961). To 930 ml of distilled water were added 10 ml of 10*N* potassium hydroxide solution and 20 ml of 25% sodium potassium tartrate solution followed by adding slowly, with vigorous stirring with a magnetic

Table 1. Chemical and physical properties of 24 soya samples from four manufacturers.

Soya sample no.	Heat treatment[a]	Particle[b] size	Moisture (%)	Protein[c] (%)	Fat (%)	Remarks
1	Toasted	14	8.4	51.0	1	
2	Toasted	18	8.7	51.2	1	
3	Toasted	25	8.8	51.5	1	
4	Toasted	50	9.2	51.0	1	
5	Toasted	100	5.5	52.9	1	
6	None	100	6.9	51.9	1	
7	Sl toasted	100	6.7	51.8	1	
8	Toasted	100	6.8	51.3	1	
9	Dbl toasted	100	7.0	51.0	1	
10	Sl toasted	100	7.0	51.4	2.0	1.75% lecithin
11	None	8	7.6	54.1	1	
12	Toasted	8	8.2	52.5	1	
13	Dbl toasted	8	8.2	52.1	1	
14	Toasted	28	7.1	52.9	1	
15	Toasted	64	7.2	54.1	1	
16	Toasted	100	6.8	52.6	1	
17	None	100	6.5	52.9	1	
18	None	100	5.5	42.2	19.0	
19	None	100	6.1	47.2	14.5	
20	None	100	6.8	52.5	4.0	
21	None	100	6.0	51.4	6.5	5% lecithin
22	None	100	8.3	63.9	1	
23	None	100	7.1	83.3	1	Isolated soya protein
24	None	100	7.4	82.3	1	Sodium proteinate

[a] Sl, slightly; Dbl, double.
[b] Products passing through given no. of mesh per inch.
[c] Nitrogen × 6.25.

stirrer, 40 ml of 4% $CuSO_4 \cdot 5H_2O$ solution. Unless stated otherwise, a soya sample of 0.1–0.2 g (depending on protein content) was weighed accurately and transferred to a 125-ml Erlenmeyer flask. The weighed portions were dispersed with 2 ml chloroform. Then 50 ml of the modified biuret reagent was added, and the stoppered flask was shaken for 15 min on a mechanical shaker and allowed to stand for 60 min. The contents were shaken, and portions of the extracts were centrifuged for 10 min at 4000 rpm in a Clay-Adams "Senior" centrifuge. The clarified extract was read in a Bausch and Lomb Spectronic colorimeter at 550 mμ 75 min after shaking began.

Orange-G binding was determined with the Udy Analyzer according to the manufacturer's instructions.

Color of laboratory-autoclaved soyaflour was determined by a reflectance method employing the Photoelectric Reflection Meter Model 610 (Photovolt Corporation, N. Y.). Samples were placed into depressions of a spot plate and flattened with a microscope slide. Three measurements were taken for each sample. In each case the green, blue, and amber tristimulus filters were used according to the manufacturer's instruction manual. The higher the reading, the less the color.

RESULTS AND DISCUSSION

Several important factors were investigated in establishing conditions for biuret protein assay procedure in soya products. Extraction flasks must be dry to avoid forming lumps that subsequently will not be wetted with chloroform. Samples must be completely wetted with chloroform to avoid turbidity from unextracted lipid material.

Increasing shaking time beyond 15 min or total extraction time beyond 75 min had no measurable effect on the optical density of clarified extracts prepared at room tempera-

ture (about 24°C). Extraction at higher temperatures (i.e., 40°C) with the modified biuret reagent should be avoided because it results in lowered optical density of the clarified extract and forms a brown-red precipitate, apparently as a result of reduction of Cu^{++} to Cu^{+} by soluble reducing substances in the soya flour.

Two series of samples of varied particle size were tested. In the first series, toasted soya products containing 51.0–52.9% protein were tested; in the second, protein contents of the untoasted soya products ranged from 52.5 to 54.1%. The consistently higher color intensity in finely ground samples shows a marked effect of particle size on results of protein determination by the biuret method (Table 2). Results of the Orange-G binding

Table 2. Protein determinations of soya products of indicated particle size.

Particle[a] size	Kjeldahl protein	Biuret optical density	Orange-G transmission
14	51.0	0.435	42.00
18	51.2	0.470	40.50
25	51.5	0.530	41.25
50	51.0	0.575	41.25
100	52.9	0.620	41.75
8	52.5	0.570	43.00
28	52.9	0.620	41.75
64	54.1	0.665	43.75
100	52.6	0.685	43.25

[a] Products passing through given no. of mesh per inch.

method were, however, affected only slightly, apparently as a result of the vigorous mixing in the reactor mixer of the Udy instrument.

To ascertain weight of a sample to be used in a biuret determination, four soya products were tested over the range of 50–300 mg. The products were finely powdered (passing a 100-mesh sieve) and included a 52.9%-protein toasted soya flour; a 51.4%-protein flour; a deflavored desugared 63.9%-protein product; and a 82.3%-protein soya flour sodium proteinate. Results are shown in Fig. 1. A linear plot of optical density compared with weight of soya products was found for up to 250 mg for the soya flours, 200 mg for the 63.9%-protein product, and 150 mg for the 82.3% sodium proteinate. This indicates that the amount of

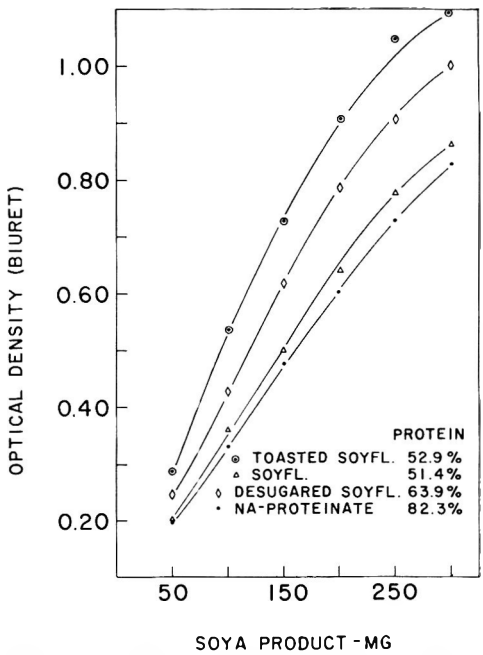

Fig. 1. Relation between sample weight and optical density of biuret extracts of soya products.

protein in a sample tested by the biuret method should be below 125 mg. $E_{550\ m\mu}^{1\ mg}$ for the 51.4, 63.9, and 82.3% soya products was respectively 0.056, 0.058, and 0.058 mg^{-1}.

Fig. 2 shows a plot of protein as determined by the Kjeldahl method and color of extracts of soya flours treated by the biuret test. Samples containing above 55% protein were determined at the 0.1-g level by the biuret method. Figs. 2 and 3 include only determinations of untoasted soya flours ground to pass a 100-mesh sieve. Excellent

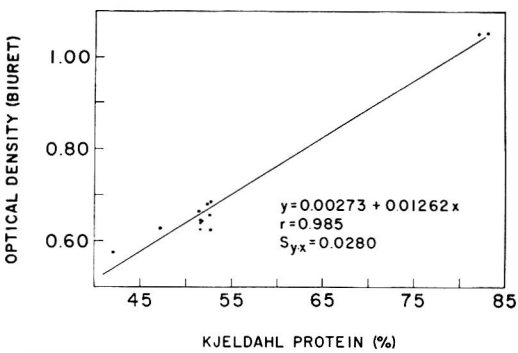

Fig. 2. Relation between percent protein of soya products as determined by the biuret and Kjeldahl methods. Y is optical density of biuret extracts, and X is percent Kjeldahl protein.

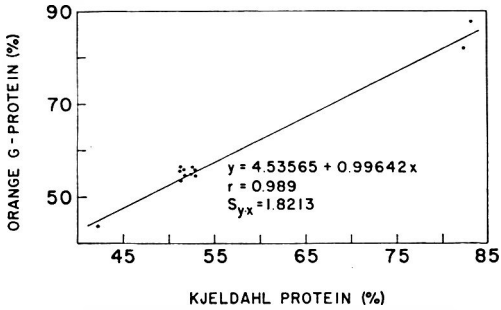

Fig. 3. Relation between percent protein of soya products as determined by the Orange-G and Kjeldahl methods. Y is Orange-G protein, X is percent Kjeldahl protein.

correlations between the Kjeldahl and biuret methods or dye-binding method show that the latter can be used to test finely ground soya flours.

Jankowski *et al.* (1962) found that heat-treating damp grain to dry it affected results obtained by the biuret method of determining the protein content of wheat. Soybeans processed for food or feed uses are generally heated to improve their nutritive value. Heating causes a series of changes, including decreases in protein solubility, loss of specific activity of the soya-flour-contained active enzymes and hormones, and changes in the availability of certain amino acids to enzymatic digestion. Additional properties such as visual color, fluorescence, and dye-binding capacity may be altered as a result of changes accompanying protein denaturation. Protein solubility is considered a valuable parameter in evaluating the effects of heat on the biological value of soya products. The solubility of protein (as percent of total protein) is around 20–40% in properly processed soya flours, below 20% in overheated samples, and around 75% in raw or untoasted soya flours. Reflectance decreases with severity of heat treatment. While it is realized that color indirectly measures changes that take place during heat treatment of soya products, determining reflectance is a rapid, simple, nondestructive, and fairly sensitive test.

Table 3 compares protein solubility and reflectance with biuret and Orange-G-binding estimates of protein of samples heated in the laboratory. Three samples of commercially toasted soya flours, containing 51.0–51.9% Kjeldahl protein, showed no significant differences in biuret or Orange-G-binding estimates of protein, despite their wide range of protein solubility (40–90%). Only in the sample that had a protein dispersibility index of 15% were biuret and dye-binding values lowered. The results show that both biuret and dye-binding protein results are lowered only in rather advanced stages of heating. Consequently, both methods seem feasible to use in detecting toasted or overheated samples.

Table 3. Protein solubility, reflectance, biuret protein and Orange-G binding of soya flour samples varying in extent of heat treatment.

Soluble protein (as % of total protein)	Total Kjeldahl protein (%)	Reflectance values employing filters:			Biuret optical density	Orange-G transmission
		Amber	Blue	Green		
A) Samples autoclaved in an autoclave at 10 psi						
	51.9	96	63	91	0.635	41.00
	51.9	97	62	93	0.640	40.00
	51.9	89	58	86	0.640	40.00
	51.9	98	57	89	0.630	39.75
	51.9	93	54	86	0.620	39.50
	51.9	84	43	76	0.550	39.00
	51.9	78	34	67	0.490	37.75
	51.9	43	5	35	0.140	33.50
	51.9	36	0	10	0.095	29.25
B) Commercial samples						
90	51.9	.....	.....	.....	0.630	42.00
75	51.8	.....	.....	.....	0.630	43.00
40	51.3	.....	.....	.....	0.635	42.75
15	51.0	.....	.....	.....	0.550	38.75

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Color Reversion in Processed Vegetables
I. Studies on Regreened Pea Purées

SUMMARY

Bright-green complexes were formed during the storage of canned green vegetable purées at room temperature or above. These pigments were isolated and purified, and their spectral characteristics analyzed. The occurrence of fluorescence and the phase test behavior were noted, and the hydrochloric acid numbers determined. Since copper and zinc were found in the green pigments, the ease of formation of these compounds on addition of metals in pea macerate was studied. The levels of metal content at which the phenomena of regreening can occur were determined, and the percentage conversion to the pigments formed was analyzed at these levels of metal. It was established that these pigments were copper complexes of mainly pheophytin *a* and, to some extent, pheophytin *b*.

INTRODUCTION

The authors reported in a previous publication (Schanderl *et al.*, 1962a) on the kinetics of the conversion of chlorophyll and several derivatives to pheophytin and pheophorbides.

Chlorophylls *a* and *b*, which are responsible for the green color in vegetables, are completely destroyed during normal heat processing, and partially destroyed during more modern methods of preservation such as aseptic canning. Even in aseptically canned vegetable products, the chlorophyll which may survive the brief heat processing is destroyed within 2–3 weeks of storage. The olive-green color of processed green vegetables is due to pheophytins *a* and *b*, a mixture of carotenoids, and some of their oxidation products. The conditions prevailing during heat processing are normally not severe enough to produce the phytol-free derivatives, pheophorbides *a* and *b*, although Jones *et al.* (1961) reported that these are formed during heat processing of such acidic products as pickled cucumbers and olives.

The present paper reports investigation of a phenomenon of regreening sometimes observed in the commercial vegetable packing industry. A preliminary review of this work was given by Schanderl *et al.* (1962b). Used in this investigation was green pea purée of approximately 85% moisture content, produced in the department during a study of aseptic canning techniques. The purée had been aseptically packed in 200 × 309 glass jars with lacquered closures and stored in the dark at temperatures ranging from 32°F (0°C) to 98°F (37°C). After about six months, the jars stored at 98°F exhibited a reversion of color to a green which closely resembled the color of unprocessed peas but was unusually stable. From these was isolated the pigment formed. The jars stored at 32°F were still olive-colored after two years, and samples of those were used in the subsequent experiments, trying to induce similar developments during refluxing.

METHODS AND MATERIALS

Isolation of the pigment. The content of a jar containing 77 g of pea purée showing regreening was extracted with successive amounts of acetone until a total volume of 200 ml was reached, and the cake was collected under suction on Whatman No. 1 paper. The collected extracts were refiltered and the pigment transferred to 50 ml petroleum ether, which was washed twice with water. The petroleum ether extract was dried over anhydrous sodium sulfate and kept under nitrogen at 32°F. Fractions of 2–3 ml of the petroleum ether extract were chromatographed on a column of powdered confectioners sugar (20 × 180 mm) and developed with increasing concentrations of acetone in petroleum ether, varying the acetone concentration of the eluant from 2 to 3%, etc., until reaching 8%. This procedure permitted an observation of all pigment constituents. It was observed that a large amount of carotenoids were eluted first, followed by wide gray bands of pheophytins. The next band was a thin yellow band of lutein. Above this band an unresolved green band followed, while some precipitated matter remained on top. The green band was cut out, extracted, and rechromato-

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graphed several times, until a homogeneous band was isolated which had a greenish-blue color.

Absorption spectra. The absorption spectra of 2 concentrations of the isolated pigment in a solution of petroleum ether, bp 30–60°C, with traces of acetone (less than 0.5%) were obtained. They were determined between 350 and 700 mμ with a Beckman DK_2 rapid-recording spectrophotometer (Fig. 1).

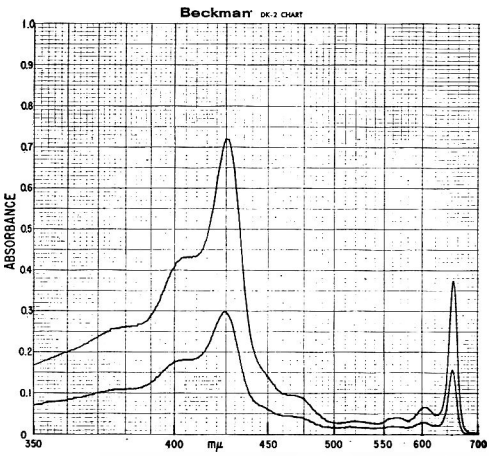

Fig. 1. Spectra of the pigment isolated from pea purée in petroleum ether (trace of acetone).

Phase test. Approximately 5 ml of an ether solution of the pigment were underlayered with an approximately equal volume of a 28–30% (w/v) methanolic potassium hydroxide solution. In a positive phase test a colored ring is formed at the interface of the two phases, which is yellow in the case of chlorophyll *a* and its derivatives and reddish in the case of chlorophyll *b*. After about 5 min, the ether layer becomes colorless if the chlorophyll is pure (Smith and Benitez, 1955).

Hydrochloric acid number. Ether solutions of the pigment were thoroughly shaken with an equal volume of a range of concentrations of hydrochloric acid. The concentration of pigment must not exceed 20 mg per 100 ml of ether. The hydrochloric acid number assigned to a pigment was the acid concentration in percent which extracted ⅔ of the dissolved substance during the shaking (Willstätter and Stoll, 1913).

Metal content. To determine the presence of metal, the pigment was examined by an emission-line spectrograph.

Formation of copper and zinc complexes in pea purée. A series of 25-g samples was prepared with aseptically canned pea purée. Ten to 100 ppm of zinc were added from a stock solution of zinc acetate (0.0908*N*) and sufficient distilled water to permit refluxing, about 25 ml. Each sample was refluxed for 1 hr. Another series of samples was prepared adding from 5 to 100 ppm of copper from a stock solution of cupric acetate (0.00394*N*). The concentrations for both metals were calculated on the basis of the original 25.0 g of pea purée. They were also refluxed for 1 hr, although it was observed in most cases that after 20 min no further increase in color intensity occurred.

Determination of the amount of complexes formed. Five ml of the refluxed sample were pipetted into a test tube, 28.3 ml acetone added to obtain a final acetone concentration of 85%, and this was thoroughly mixed and allowed to stand 3 min. It was filtered through Whatman no. 1 filter paper and the absorbance determined at 650 mμ, using acetone as the blank. The change at 650 mμ was plotted against zinc or copper concentration in ppm (Fig. 2).

Spectra of the complexes formed. The pigments formed during the refluxing of the pea purée with zinc and copper were extracted as in the above procedure and chromatographed on a powdered-sugar column. Their spectra are shown in Fig. 3.

Determination of the complexing agent in the purée. The pea purée stored at 32°F did not show any regreening. Therefore, 5-g samples were extracted with acetone until all pigment had been removed. The acetone extract, approximately 25 ml, was dried over anhydrous sodium sulfate, concentrated under vacuum to approximately 2 ml, and chromatographed on a powdered sugar column. Pigment separation was accomplished by increasing the amount of acetone from 2 to 6% in the

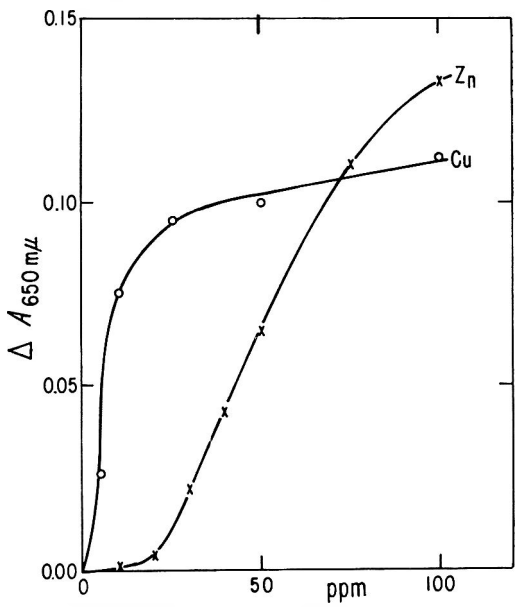

Fig. 2. The change in *A* at 650 mμ plotted vs. concentration of the ion added as a measure of the formation of copper and zinc complexes in the purée (solvent, acetone).

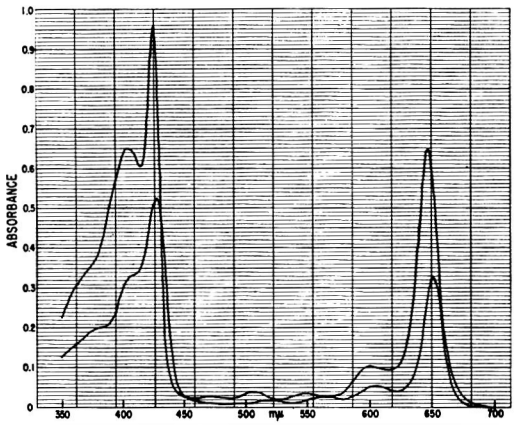

Fig. 3. Spectra of the prepared copper (upper curve) and zinc complexes (lower curve) at different concentrations in petroleum ether (trace of acetone).

petroleum ether used for development. It was possible to resolve all pigments into five zones. Ether solutions of all these were analyzed spectrophotometrically and identified in order of elution as: Zone 1, a carotenoid; 2, pheophytin *a*; 3, pheophytin *b*; 4, by spectrum and relative position on the column, a xanthophyll; 5, a second xanthophyll. There was no residual chlorophyll found.

Complex formation in pure solutions. Acetone and ether solutions of each of the above 5 compounds were refluxed with copper and zinc under neutral and acidic conditions. Acetic acid was used for acidification. The pheophytins *a* and *b* formed green colorations with the two metals, pheophytin *a* with ease in neutral solution, while pheophytin *b* required acidification. The reaction appeared to be somewhat faster with copper than with zinc.

Determining copper content of a regreened purée. Approximately 1-g samples of the purée were weighed in triplicate and wet-ashed, using 2 ml of concentrated sulfuric acid and 8 drops (approx. 0.4 ml) of 70% perchloric acid. A blank of sulfuric acid alone was carried through the procedure. After the digestion, 9 ml of deionized distilled water were added to the sample, and its copper content was determined with the colorimetric method devised by Marsh and described by Joslyn and Amerine (1941).

Determining percent conversion of pheophytin *a* to a metal complex. Pea purée (5.00 g) was thoroughly mixed with 28.3 ml of acetone. The mixture was filtered by suction through a 35-mm coarse-fritted glass filter and the cake rinsed twice with 2 ml of acetone. The filtrate was transferred to a 250-ml separatory funnel, and 10 ml of petroleum ether were added and mixed, followed by 80 ml of water. This mixture was agitated carefully to avoid emulsification and all the green pigment transferred to the petroleum ether layer, which was drawn off the aqueous acetone layer, dried over anhydrous sodium sulfate, and concentrated to about 2 ml. The pigment was chromatographed on an 18 × 130-mm sugar column, covered with a 3-mm layer of anhydrous sodium sulfate, and rinsed with petroleum ether. The chromatogram was developed with petroleum ether, slowly changing to 2% and finally 6% acetone in petroleum ether. The first eluates were carotenes and were discarded. These were followed by a red-fluorescing layer containing pheophytin *a*, which was saved. After this, pheophytin *b*, which fluoresced, and xanthophyll, which did not fluoresce, were eluted and discarded. Continued elution isolated the third fluorescent layer, representing the impure copper complex of pheophytin *a*. This and the pheophytin *a* were used in the following calculations.

Calculation. Using the molar absorptivity of pheophytin *a* of Zscheile and Comar (1941), its molar concentration can be calculated.

$$\frac{A_{663m\mu}}{50{,}880 \text{ liter mole}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}} \times \frac{0.5 \text{ liter}}{100 \text{ g}} = \frac{\text{moles of pheophytin } a}{\text{100-g sample}}$$

Likewise, using the molar absorptivity of the pheophytin *a*-copper complex from a table to be published in part II of this work, the molar concentration becomes:

$$\frac{A_{648m\mu}}{55{,}770 \text{ liter mole}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}} \times \frac{0.5 \text{ liter}}{100 \text{ g}} = \frac{\text{moles of pheophytin } a\text{–copper complex}}{\text{100-g sample}}$$

Percent conversion. The ratio of the two concentrations yields the percent conversion:

$$\frac{\text{Conc. of pheophytin } a\text{–copper complex}}{\text{Conc. of pheophytin } a\text{–copper complex} + \text{conc. of pheophytin } a} \times 100 = \text{percent complexing.}$$

RESULTS AND DISCUSSION

Spectra. The absorption spectrum of the isolated pigment is shown between 350 and 700 mμ in Fig. 1. It becomes obvious that the similarity of color of this pigment to that of chlorophyll *a* is due to the fact that it also displays the two typical peaks, one in the red region at 650 mμ and one in the blue region at 425–426 mμ. In addition to this, it has the similar shoulder to the left of the blue peak, in this case at approximately 405 mμ, and it has three minor peaks similar to those of chlorophyll *a*—one above 600 mμ, one between 560 and 570 mμ, and one lower peak at approximately 525 mμ.

The ratio of $red_{max}/blue_{max}$ serves as an indication of purity for the unknown compound, according to Zscheile and Comar (1941). It was found to be .52 in the best preparation.

In contrast to chlorophyll *a* the spectrum shows a shoulder at approximately 450 and a pronounced shoulder at 470 mμ. Since a dense band of xanthophyll had preceded the unknown pigment on the column, this may indicate a trace of xanthophyll remaining in the pigment. The trace of xanthophyll, then, contributing to the absorbance at 425 mμ would lower the ratio $red_{max}/blue_{max}$. The peaks in the region between 500 and 625 mμ could indicate traces of pheophytins, possibly the allomerized pheophytin *b* which had interfered so often in all phases of this work. The peak of pheophytin *b* at 653 mμ would, of course, be difficult to separate from the one of the unknown at 650 mμ.

The unknown pigment fluoresces brightly in solution, but on the column its fluorescence behavior is not always clear. During the development of the column, the green layer appears at times to be quenching the fluorescence.

The isolated pigment was phase-negative. It transferred into the aqueous phase without any transition color. This indicated that the cyclopentanone ring forming the isocyclic ring had been oxidized (allomerized) or the carbomethoxy group at C-10 had been removed. Colorless ether layers could not be obtained here but were achieved with the pigments isolated from refluxed purée.

The pigment did not transfer into 22% hydrochloric acid; the hydrochloric acid number determined was between 30 and 32. This test indicated that the phytol had remained on the complexing agent (the tetrapyrrole moiety of the complex).

These experiments showed, therefore, that the regreening of peas or pea purée at elevated temperatures was caused by the formation of a pigment which could be isolated on a powdered sugar column. The qualitative evaluation of the spectral curve showed the spectrum to be comparable to that of chlorophyll. The ratio of the absorption peaks did not permit a positive identification, and the spectrum gave the impression that the compound still contained impurities. The small amount of the pigment available did not permit further chromatography. Qualitative tests were done with the emission-line spectrograph and showed the presence of both copper and zinc in the isolated pigment, but no further information could be obtained, since the amount of pigment available was too small.

When copper or zinc in small amounts ranging from 0 to 100 ppm were added to a pea purée and the latter was refluxed to simulate the effects of storage at elevated temperatures, pigments containing copper or zinc were formed and isolated whose spectral characteristics were very similar.

Having found that the unknown compound contained either copper or zinc or both and after showing that the identical pigment can be formed by refluxing pea purée, it became necessary to show which constituent of the purée formed the complex. This was done in an experiment refluxing the five compounds separated by column chromatography from a pea purée which had not regreened since it had been stored at 32°F. Acetone and ether solutions of each of the above compounds were refluxed with excess copper or zinc acetate crystals under neutral and acidic conditions. Acetic acid was used for acidification. During refluxing the two compounds identified as pheophytins *a* and *b* formed green colorations with either of the metals. The copper complex seemed to form faster than the zinc complex, and pheophytin *b* required acidification. Pheophytin complexes of similar spectral characteristics have first been reported by Willstätter and Sjöberg (1924). The spectrum of the copper compound prepared by refluxing proved to be essentially identical to that of the unknown pigment. Rechromatography of the large amount of pigment obtained in treatment of the pea purée with copper produced pigments whose purity ratios were as high as 0.66.

The main difference between the copper and zinc complexes thus formed was that the copper complex did not fluoresce under uv radiation, and the zinc complex did. In this respect the unknown could be considered a zinc complex, and a study was therefore undertaken to observe the formation of copper and zinc complexes in purée. The results

are shown in Fig. 2. This figure shows that, upon the addition of copper, a complex forms at approximately 1–2 ppm and that 10–20 ppm are required to fully complex the pheophytin present in the purée in the time of refluxing.

Zinc complex formation did not start until approximately 25 ppm of zinc were present and was not completed until a concentration of 100 ppm was reached, as indicated by the leveling off of the absorbance values.

From this experiment it was concluded that the copper complex must form with greater ease at lower concentrations, and therefore is more likely to occur in vegetable purée. Natural content of copper could account for this phenomenon.

The zinc complex was investigated because of the fluorescence of the unknown compound. A noticeable decrease of fluorescence, however, can be observed in the purée itself during the process of regreening. This appeared to indicate that pheophytin was complexing with copper, in which case the fluorescence of the unknown pigment could be due either to traces of pheophytin remaining in it or a partial complexing with zinc. Phase test and hydrochloric acid numbers of the unknown were identical with corresponding values of the pigment isolated from refluxed pea purée.

To determine at what concentration of copper and zinc these pigments can form in storage, copper content was analyzed in a pea purée which had been stored 22 months at 98°F and had undergone regreening. The results of the analysis of three jars were 1.65, 1.76, and 2.2 ppm copper on the basis of the wet purée. The total solids of this wet purée were analyzed by AOAC procedures and found to be 15.0%. This copper content can be considered a low normal (Bridges, 1941) and shows that, given the other requirements such as elevated storage temperature and a pH which permits complexing, any normal vegetable purée may undergo regreening.

It was thought of interest to determine the percent conversion to this green complex in a case of a noticeably regreened sample. The above pea purée showed a conversion of 55.1 and 55.7% to the new complex in two separate determinations.

An additional interesting calculation shows that, for the concentrations of pheophytin *a* and copper present in the samples studied, a 100% conversion to the pheophytin *a* complex would be possible, time permitting.

From the above results it is concluded that, under favorable conditions, pheophytins and, in some foods, pheophorbides form green complexes with very small amounts of copper and zinc in stored vegetable products. These pigments are the cause of the so-called regreening and are remarkably stable (Willstätter and Stoll, 236, 1913). A more extensive study of the chlorophyll derivatives capable of forming complexes with copper was considered necessary.

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Ms. rec'd 7/3/63.

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Color Reversion in Processed Vegetables
II. Model System Studies

SUMMARY

Pure pheophytins *a* and *b* and pheophorbide *a* were isolated and the metal complexes of these compounds prepared in pure solutions. The metal content showed a 1:1 ratio of metal to pheophytin or pheophorbide. The spectral curves of the pure complexes were prepared and tables of absorptivities calculated. The molar absorptivities of the pheophytin *a* and pheophorbide *a* complexes proved identical.

INTRODUCTION

The formation of complexes of chlorophyll and its derivatives with metals other than magnesium was first reported by Willstätter and Sjöberg (1924).

The metal complexes of pheophytin *a* with three groups of metals were studied by Lamort (1955). The shifts in wave lengths of the red peaks were reported, but no quantitative data were given on the absorptivities.

Sweeney and Martin (1958) investigated the relative stability of chlorophylls and the effect of zinc chloride on the color of acetone extracts of treated plant tissues. They reported a change in color and described the spectra but did not attempt to separate the compounds into the different components.

The present authors reported earlier (in press) that canned vegetables can regreen during storage. They isolated and identified the pigments causing the so-called color reversion and showed that they were complexes of pheophytins *a* and *b* with copper and zinc. The metal concentrations normally found in vegetables seem to be sufficient to complex part or all of the pheophytin present, improving color greatly. As an example, analysis of a pea purée of 85% moisture content used in a previous study showed 2.9 μM of pheophytin *a* per 100 g. To complex this on a 1:1 basis, 0.184 mg of copper per 100 g is required. Since 0.21 mg can be found normally in this type of product (Bridges, 1941), all the pheophytin *a*, present in the largest amount, can be complexed, plus some of the pheophytin *b*. Since the color of the pheophytin *b* complex is light green, this will not produce much additional improvement in color.

This study was undertaken to obtain quantitative data on the absorptivities of pheophytins, pheophorbides, and metal complexes thereof to permit, among others, the calculations of the percent conversion of the parent compounds to the complexes in foods.

This part is a continuation of work begun by Lamort (1955) and has a parallel in some recent studies by Dilung and Butzko (1960) and Butzko and Dain (1961) using different metals.

Since it had been suggested by Aronoff (1953) that more than one metal ion can be complexed per molecule of pigment, the ratios of metal to pigment were studied.

The range of compounds used in these experiments has been limited to those normally occurring in fresh or heat-processed plant tissues. The products of severe treatments, e.g., chlorins and rhodins, were not studied at this time. Pheophorbide *a* was included, since its complexes could be responsible for the abnormal greenness sometimes found in Spanish-style olives and pickled products.

METHODS AND MATERIALS

Preparation of pure pigments. Pheophytin *a*: 15–20 g dry grape leaves were ground finely and extracted in a Soxhlet with 100 ml petroleum ether to remove most of the carotene content and then with anhydrous ether to obtain the chlorophyll compounds. The extract was dried over anhydrous sodium sulfate, evaporated in a stream of nitrogen, and taken up in acetone. The mixture of pigments was chromatographed on a dried powdered sugar column, 34 × 200 mm, and developed with a solution of 4% acetone in petroleum ether. When the movement of the pigments on the column became too slow, the concentration of acetone in petroleum

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ether was increased to 5 and finally 6%. The first fraction of gray-colored pheophytins, strongly red flourescent under UV radiation, was collected and rechromatographed four times.

The spectrum was clearly that of pheophytin *a*, and the hydrochloric acid number was 29. The compound gave a positive phase test, and the purity ratio was equivalent to that reported by Zscheile and Comar (1941).

Pheophytin *b*: Pheophytin *b* was prepared from the fraction which immediately followed pheophytin *a*. To obtain it in pure form it was rechromatographed with a solution of chloroform, isopropyl alcohol, petroleum ether (12, 0.4, 87.6 v/v). This eluate permitted the separation of the various compounds reported by Smith and Benitez (1955, p. 154) as pheophorbides of similar absorbance characteristics. The determination of the hydrochloric acid numbers permitted a clear separation of the pheophytins from the pheophorbides. The order of elutions was: pheophytin *a*, pheophytin *b*, small bands of phase negative pheophytins *a* and *b*, pheophorbide *a*, pheophorbide *b*. The hydrochloric acid numbers were 35 for pheophytin *b* and between 19 and 20 for pheophorbide *b*. The layers accompanying pheophytin *b*, which were difficult to separate from it in the acetone-petroleum ether mixture, were not pheophorbides. The phase test was negative for the pheophytin *a*-like compound which followed immediately after pheophytin *b*. The pheophytin *b* was identified by its spectral characteristics and its positive red phase test. The hydrochloric acid number was 35. The purity ratio was 1.90, compared to 1.88 (Zscheile and Comar, 1941) and 1.95 (Smith and Benitez, 1955). It was not possible to purify it any further.

Pheophorbide *a*: A purified solution of pheophytin *a* in ether was shaken with approximately 4.8 ml of 34% hydrochloric acid for every 3 ml of ether solution of the pigment. This mixture was allowed to stand for 40 min; then it was diluted with water to approximately 18% hydrochloric acid and extracted twice to remove traces of pheophorbide *b*. The aqueous phase was diluted to 11% hydrochloric acid and extracted twice with ether. This yielded the pure pheophorbide *a*. The ether solution was washed three times with deionized water and dried over anhydrous sodium sulfate. Its phase test was positive and the hydrochloric acid number 15.

Pheophorbide *b* could be present only in amounts up to 40% of pheophorbide *a* since the ratio of chlorophyll *a* to *b* is approximately 2.5 in higher plants. Since the color of its copper complex is yellow-green, it would not contribute much to the regreening of a product. For the above two reasons, pheophorbide *b* was not investigated here.

Preparation of metal complexes in model systems. The copper complexes of pheophytin *a*, pheophytin *b*, and pheophorbide *a* were formed by preparing a 50-ml solution of the pigment of known absorbance, calculating the concentration from the absorptivities given by Zscheile and Comar (1941) for pheophytin *a* and *b*. The solution was transferred quantitatively to a refluxing system, and 4 ml of an ether solution saturated with cupric chloride were added. This procedure provided an approximately 10-to-20-fold excess of cupric ion concentration over pigment concentration, depending on the concentration of the latter. In the case of pheophorbide *a* the concentration of copper had to be raised by adding a few crystals of cupric chloride to the refluxing mixture, thus providing an ether solution saturated in cupric chloride. The complex of pheophytin *a* formed readily in neutral solution; however, the formation of the complex of pheophytin *b* had to be accelerated by addition of 0.2 ml of glacial acetic acid per 50 ml of reaction mixture, and in the case of pheophorbide *a*, 2 ml of glacial acetic acid per 50 ml of reaction mixture. These mixtures were refluxed for 2 hr and then the ether solutions were washed 6 times with deionized distilled water, if acetic acid had been used, filtered over an 18 × 20-mm anhydrous sodium sulfate column to remove water, and each solution transferred back into a 50-ml volumetric flask and made to volume. The formation of each complex could be followed by observing the fluorescence. When completed, the original red fluorescence had disappeared. The absorbances of the final solutions were measured between 350 and 700 mμ in a Beckman DK2 recording spectrophotometer, and the molar absorptivities calculated for the metal complexes, using both newly formed peaks and isosbestic points. The concentration of pheophorbide *a* was calculated using the absorptivities of pheophytin *a*, since Holt and Jacobs (1954) showed that the side-chain on C-7 does not influence the absorptivity. Chlorophylls *a* and *b* did not form complexes in the neutral ether solutions. The solutions were not acidified, since in that case pheophytin would have formed and it would have complexed. It was observed, however, that in such concentrations of copper as can be obtained in acetone solutions, chlorophyll *b* formed a complex. Under the above conditions only a small shift in the spectrum was observed for chlorophyll *a*. The spectrum of the complex formed by chlorophyll *b* under high concentrations of copper is not identical with that of the complex formed by pheophytin *b*. The curve is reported qualitatively (Fig. 4, also compare Fig. 2).

Determination of the metal content of the complexes. The metal contents of complexes prepared as above or by chromatography from mixtures were determined. The ether solutions were

washed 4 times with deionized water, dried over anhydrous sodium sulfate, made to volume, and studied spectroscopically. The concentrations were calculated using the absorptivities of Tables 3, 5, and 7 expressed on a molar basis. Duplicate 10-ml samples of the pigment were then pipetted into a 25 × 150-mm Pyrex test tube and the ether was allowed to evaporate. The samples were submitted to wet-washing, using 2 ml of concentrated sulfuric acid and two drops of perchloric acid per sample; a blank was carried through the whole procedure. Nine ml of deionized water were added after cooling, and the samples were analyzed by the Marsh procedure (Joslyn and Amerine, 1941). The results are shown in Table 1.

Table 1. Metal content of pure complexes.

Pigment complexed	Concentrations (moles × 10^{-6})		Ratio, copper/pigment
	Pigment	Copper	
Pheophytin *a*	7.17	6.61	.923
	7.17	6.77	.945
	7.55	7.86	1.04
	7.55	7.55	1.00
Pheophytin *b*	6.18	5.89	.954
	6.18	5.89	.954
	6.18	5.98	.968
	6.18	5.98	.968
Pheophorbide *a*	9.62	10.2	1.06
	9.62	9.92	1.03
	9.62	9.76	1.02

RESULTS AND DISCUSSION

The spectra obtained by the complexing of copper with pheophytins *a* and *b* and with pheophorbide *a* and chlorophyll *b* are shown in Figs. 1–4. They show some of the characteristics of similar compounds quoted by Fischer and Orth (1940, p. 60), but the lack of some of the peaks they reported, which coincide with impurities, would indicate that these complexes are more pure. The similarity of the pheophytin *a*–copper complex to the unknown isolated from peas by Schanderl *et al.* (in press) becomes obvious on comparison of the spectral curves. The two main peaks are only about 2–3 mμ apart, and the pure pheophytin *a*–copper complex shows fewer peaks than the complex obtained from peas. Since the copper complex of pure pheophytin *a* is not fluorescent, the possibility of traces of pheophytin *b* may account for the observed fluorescence of the pigment isolated from pea purée. The other

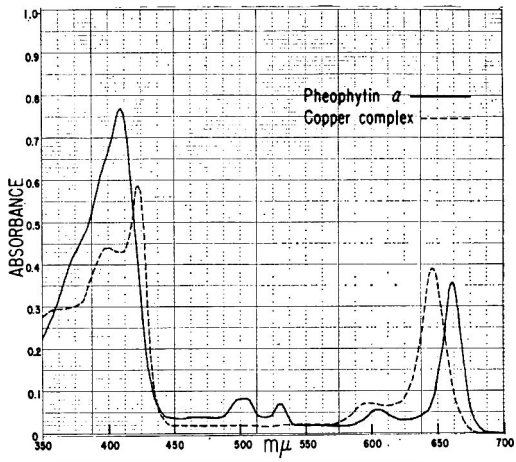

Fig. 1. Absorption spectra of pheophytin *a* and its 1:1 copper complex in ether.

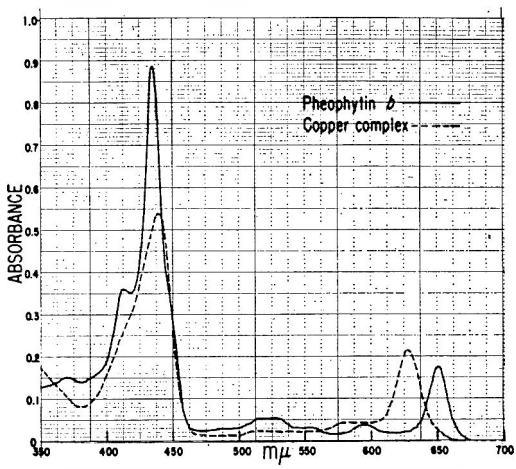

Fig. 2. Absorption spectra of pheophytin *b* and its 1:1 copper complex in ether.

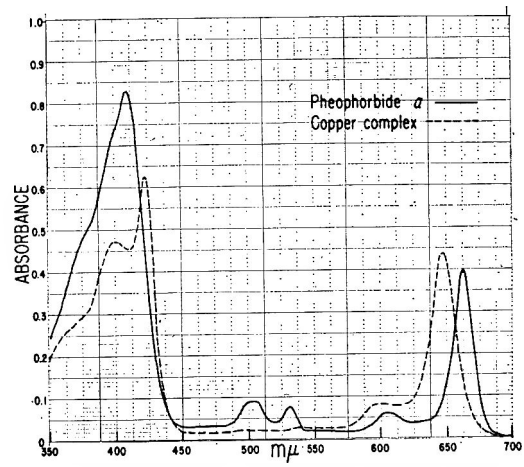

Fig. 3. Absorption spectra of pheophorbide *a* and its 1:1 copper complex in ether.

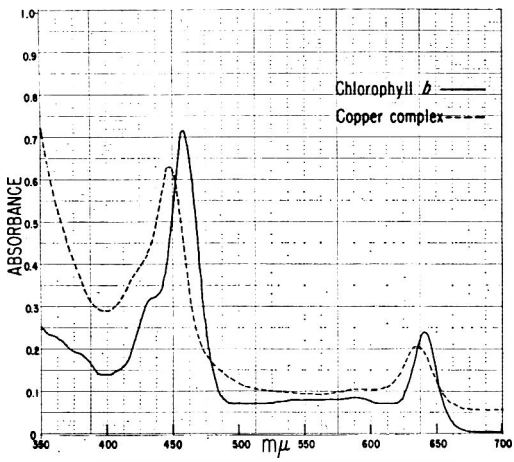

Fig. 4. Absorption spectra of chlorophyll *b* and an impure copper complex in acetone.

obvious difference between the isolated and prepared pheophytin *a*-copper complexes lies in the shoulders in the 450 mμ region of the isolated complex which are considered due to traces of remaining xanthophylls.

The obvious characteristics of the complex formation of pheophytin *a* (Fig. 1) are thus a shift of the red peak towards shorter wave length (hypsochromic shift), accompanied by a slight increase in absorbance (hyperchromic shift) and a shift of the blue peak towards longer wave length (bathochromic shift), with a decrease in absorbance (hypochromic shift). In addition to this, the minor peak at 608 mμ undergoes a hypsochromic shift, and the peaks at 532 and 505 mμ disappear. The spectrum of the pheophytin *b*-copper complex (Fig. 2) also shows a hypsochromic shift of the red peak with a slight hyperchromic shift and a small bathochromic shift of the blue peak, connected with a large hypochromic shift. In a manner similar to that of the *a* compound, a hypsochromic shift of the 599-mμ peak and a disappearance of the 558- and 523-mμ peaks can be observed. The shoulder of pheophytin *b* at 413 mμ is greatly minimized, while in the case of pheophytin *a* a strong shoulder had actually appeared at 399 mμ upon complex formation.

The complex formed by pheophorbide *a* (Fig. 3) is identical to that formed by pheophytin *a* (Fig. 1). The metal-free pigments were fully complexed, as indicated by the disappearance of the fluorescence. The newly formed compounds were made to volume and their spectra superimposed on the spectral curve of the original compounds, thus permitting the calculation of molar absorptivities for these compounds.

The spectral curve labeled copper complex in Fig. 4 is for a solution consisting of chlorophyll *b* plus an unknown amount of copper complex. Its purpose is to record that chlorophyll *b* does form a complex in neutral solvents if the concentration of copper is high enough, such as can be reached in an acetone solution of the pigment. It is further obvious that the spectral characteristics of the new complex are dissimilar to the pheophytin copper complex and therefore likely to constitute a different form of complex.

Since Aronoff suggested (1953) that there is more than one complexing site on the molecule, the complexes of pheophytins *a* and *b* and pheophorbide *a* were analyzed for the ratio of metal to metal-free pigment, as described below in the case of the copper complex of pheophytin *b*. A separate sample had been prepared as described, and the absorbances at 628 and 439 mμ were .250 and .647 respectively. Using the absorptivities from Table 5, the concentration can be calculated.

$$\frac{.25}{41{,}250 \text{ liter mole}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}}$$

$$= 6.06 \times 10^{-6} \text{ moles liter}^{-1}$$

and

$$\frac{.647}{102{,}750 \text{ liter mole}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}}$$

$$= 6.3 \times 10^{-6} \text{ moles liter}^{-1}$$

with an average of $6.18 \pm 0.1 \times 10^{-6}$ moles liter $^{-1}$

A sample of 10 ml carried through the procedure of metal determination gave a copper content of .375 mg per liter. From this the molar concentration can be calculated as

$$\frac{.375 \times 10^{-3} \text{ g liter}^{-1}}{63.54 \text{ g mole}^{-1}}$$

$$= 5.89 \times 10^{-6} \text{ moles liter}^{-1}$$

The ratio of these two concentrations is

$$\frac{5.89}{6.18} = .954 \text{ moles of copper per mole of pigment.}$$

Repeated analyses gave the results shown in Table 1.

Table 1 shows that the ratio of copper to metal-free pigment is 1:1 for the complexes of pheophytin *a* pheophytin *b*, and pheophorbide *a*.

Tables of absorptivities (3, 5, 7) were prepared for the complexes of pheophytin *a*, pheophytin *b*, and pheophorbide *a*. They are values taken from the graphs prepared specifically for this purpose. The concentrations of the starting material were calculated in the case of the pheophytins using the absorptivities given by Zscheile and Comar (1941). Only the major peaks were used for calculations. In the case of pheophorbide *a*, the molar absorptivities of pheophytin *a* were used to calculate the concentration of the starting material.

The atomic weights used in calculation of the molecular weights and the composition of the various pigments were: C, 12.01; H, 1.008; N, 14.008; Mg, 24.32; O, 16.00; Cu, 63.54.

A sample calculation is shown below:

where: A = absorbance
a = absorptivity
ϵ = molar absorptivity
c = concentration
b = sample path length (here always 1 cm)
$A = a\,c\,b$

Pheophytin *a*: $A_{410\ m\mu} = .768$

$$\frac{.768}{126 \text{ liter g}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}} = 6.095 \times 10^{-3} \text{ g liter}^{-1}$$

Five absorption maxima were used for calculation, and the average specific concentration was: 6.061×10^{-3} g liter $^{-1}$ (Table 2). From this, the average molar concentration was calculated:

$$\frac{\text{specific concentration}}{\text{molecular weight}} = \text{molar concentration}$$

$$\frac{6.061 \times 10^{-3} \text{ g liter}^{-1}}{871.18 \text{ g mole}^{-1}} = 6.957 \text{ moles liter}^{-1}$$

Using the average molar concentration, the molar absorptivity was calculated:

$$\frac{A}{\text{molar conc.} \times b} = \text{molar absorptivity } \epsilon$$

$$\frac{.768}{6.957 \times 10^{-6} \text{ moles liter}^{-1} \times 1 \text{ cm}} = 110.390 \text{ liter mole}^{-1} \text{ cm}^{-1}$$

The absorptivity *a* can be calculated for the isosbestic point at 656 mμ as follows:

$$A_{656m} = .232$$

$$\frac{A}{\text{conc.} \times b} = \text{absorptivity } a$$

$$\frac{.232}{6.061 \times 10^{-6} \text{ g liter}^{-1} \times 1 \text{ cm}} = 33.3 \times 10^{-6} \text{ liter g}^{-1} \text{ cm}^{-1}$$

Table 2. Absorptivities of pheophytin *a* (solvent: ether).

Zscheile and Comar		Schanderl			
Absorption maxima (mμ)	Absorptivity (liter g^{-1} cm^{-1})	Absorption maxima mμ	Absorbance	Concentration (mg liter^{-1})	Molar absorptivity (liter mole^{-1} cm^{-1})
410	126	410	.768	6.095	110,390
470	4.5	470	.038		
505	13.5	502	.081	6.000	11,640
532	11.3	532	.069	6.106	9,920
559	3.2	559	.019		
608	8.8	608	.053	6.022	7,620
	33.3	656[a]	.232		33,350
665	59.0	663	.354	6.084	50,880
			Average:	6.061	

[a] Isosbestic point.

The molar absorptivities for the pheophytin *a*-copper complex were calculated (Table 3), assuming 100% conversion and applying the molar concentration of the starting material.

Example: pheophytin *a*-copper complex

$$A_{893\ m\mu} = .440$$

$$\frac{.440}{6.957 \times 10^{-6} \text{ moles liter}^{-1} \times 1 \text{ cm}}$$

$$= 63{,}250 \text{ liter mole}^{-1} \text{ cm}^{-1}$$

From the knowledge that the complex contains one atom of copper per mole, the molecular weight was calculated as 932.64, which permits calculation of the specific concentration:

$$6.957 \times 10^{-6} \text{ moles liter}^{-1} \times 932.64 \text{ g mole}^{-1} = 6.4884 \times 10^{-6} \text{ g liter}^{-1}$$

The absorptivity, *a*, can be calculated:

$$\frac{.440}{6.488 \times 10^{-6} \text{ g liter}^{-1}}$$

$$= 67.8 \text{ liter g}^{-1} \text{ cm}^{-1}$$

Pheophytin *b*: The same calculations applied directly for pheophytin *b* (Table 4). The concentration used for the conversion experiment was 5.236×10^{-6} mole liter^{-1}. Its calculated molecular weight was 885.16, and the absorptivity calculated for the isosbestic point at 641 mμ was

$$17.26 \text{ liter g}^{-1} \text{ cm}^{-1}.$$

Table 5 shows the values of the pheophytin *b*-copper complex. The molecular weight for the complex was calculated as 946.69, and the concentration after 100% conversion was 4.957 mg liter^{-1}.

In the case of pheophorbide *a* (Table 6) the molecular absorptivity of pheophytin *a* (column 3) was used to calculate the average molar concentration to be 7.658×10^{-6}, and the absorptivity was calculated, based on the molecular weight of 592.676, giving a specific concentration of 4.539×10^{-6} g liter^{-1}. The absorptivity is obtained in the case of 410 mμ:

$$A_{410\ m\mu} = .827$$

$$\frac{.827}{4.539 \times 10^{-6} \text{ g liter}^{-1} \times 1 \text{ cm}}$$

$$= 182.2 \text{ liter g}^{-1} \times 1 \text{ cm}^{-1}$$

Table 3. Absorptivities of pheophytin *a*-copper complex (1:1) (solvent: ether).

Absorption maxima (mμ)	Absorbance	Absorptivity (liter g^{-1} cm^{-1})	Molar absorptivity (liter mole^{-1} cm^{-1})
399	.440	67.8	63,250
423	.586	90.3	84,230
540	.021	3.2	3,020
600	.070	10.8	10,060
648	.388	59.8	55,770
656[a]	.237	36.5	34,070

[a] Isosbestic point.

Table 4. Absorptivities of pheophytin *b* (solvent: ether).

Zscheile and Comar			Schanderl		
Absorption maxima (mμ)	Absorptivity (liter g^{-1} cm^{-1})	Absorption maxima (mμ)	Absorbance	Concentration (mg liter^{-1})	Molar absorptivity (liter mole^{-1} cm^{-1})
413	77	413	.361	4.688	68,950
433	197	434	.884	4.487	168,830
523	12.6	523	.054		
558	7.5	557	.031		
599	8.1	599	.037		
	17.26	641[a]	.080		15,280
653	37	652	.175	4.729	33,420
			Average:	4.635	

[a] Isosbestic point.

Table 5. Absorptivities of pheophytin *b*-copper complex (1:1) (solvent: ether).

Absorption maxima (mμ)	Absorbance	Absorptivity (liter g^{-1} cm^{-1})	Molar absorptivity (liter $mole^{-1}$ cm^{-1})
439	.538	108.5	102,750
523	.023	4.6	4,390
580	.043	8.7	8,210
628	.216	43.6	41,250
641[a]	.080	16.1	15,280

[a] Isosbestic point.

Table 6. Absorptivities of pheophorbide *a* (solvent: ether).

Absorption maxima (mμ)	Absorbance	Molar absorptivity (liter $mole^{-1}$ cm^{-1})	Molar concentration (moles × 10^{-6})	Absorptivity (liter g^{-1} cm^{-1})
410	.827	110,390	7.491	182.2
470	.032			
502	.090	11,640	7.731	19.8
532	.075	9,920	7.562	16.5
559	.020			
608	.059	7,620	7.744	13.0
656[a]	.269	33,350		
663	.395	50,880	7.762	87.0
			Average: 7.658	

[a] Isosbestic point.

Table 7. Absorptivities of pheophorbide *a*-copper complex (1:1) (solvent: ether).

Absorption maxima (mμ)	Absorbance	Molar concentration (moles × 10^{-6})	Molar absorptivity (liter $mole^{-1}$ cm^{-1})	Absorptivity (liter g^{-1} cm^{-1})
399	.470	7.431	61,380	93.9
423	.624	7.408	81,500	124.7
540	.026		3,400	
600	.080	7.950	10,450	16.0
648	.438	7.853	57,210	87.5
656[a]	.269	7.896	35,140	53.8
	Average:	7.647		
	Average including A_{600} mμ:	7.707		

[a] Isosbestic point.

Knowing the molar concentrations of 7.658×10^{-6} and assuming 100% conversion, the concentration of the pheophorbide *a*-copper complex was calculated (Table 7, column 3), using the molar absorptivities determined for the pheophytin *a*-copper complex. These values should be identical, if the assumptions are valid that the sidechain on C-7 is of no influence on absorbance. The result is 7.647×10^{-6} (vs. 7.658×10^{-6}) as an average of four readings, and 7.707×10^{-6} as an average of five readings.

The absorptivities and molar absorptivities for the pheophorbide *a*-copper complex were calculated (columns 4 and 5), based on the molar concentrations of pheophorbide *a* (7.658×10^{-6}) and a molecular weight of 654.206 for the complex.

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Viability of Escherichia Coli on English Walnut Meats (Juglans regia)

SUMMARY

The effect of walnut meats on *Escherichia coli* viability was evaluated. Water-washed and unwashed English walnut meats were immersed in *E. coli* suspension under controlled conditions. Meats were dried at room temperature, packed in plastic bags in 50-g quantities, and stored under various conditions at room temperature and in the cold. Periodic examination of meats by bag over eight months revealed the following. The *E. coli* population was reduced 99% during two days of drying on unwashed substrate, but not on washed substrate. On the one hand, both unwashed and washed nuts stored at room temperature failed to show any *E. coli* after three and four months storage respectively; on the other hand, there was no significant loss of *E. coli* viability on either unwashed or washed nuts stored in the cold up to 240 days.

The effect of walnut tannin extract on *E. coli* was determined. This effect was greater on cells suspended in saline than on cells suspended in 0.1% peptone water. In 0.9% saline, 1×10^7 cells were killed at 0.62% walnut tannin concentration and 1×10^6 cells at 0.16%, both within 1 hr, whereas 2.5% tannin concentration was required to kill 1×10^7 cells in 0.1% peptone water within 2 hr. The reliability of *E. coli* as the index organism to detect insanitation in English walnut meats is discussed.

INTRODUCTION

Members of the coliform group, particularly *Escherichia coli,* are commonly used as indicators of fecal pollution. Ostrolenk and Hunter (1939), Ostrolenk and Welch (1940), Ostrolenk (1942), and others have shown that nut meats with unbroken shells contain no *E. coli* and that these organisms in shelled nuts reflect insanitary practice.

Satisfactory correlation between plant insanitation and *E. coli* content was shown for a number of tree nut products; data on English walnuts, however, appeared to be inconsistent.

A review of data from 352 English walnut meat samples examined in this laboratory since 1961 showed that *E. coli* were present in 3.1% of the samples. Furthermore, *E. coli* were found in 2% of the samples consisting of halves and larger pieces and in 36% of the samples consisting of nugget-sized meats. The fact that many of the samples came from plants found to be operating under insanitary conditions suggested that this variance should be scrutinized.

It has been observed that fresh English walnut kernels, when embedded in nutrient agar seeded with a wild-type *E. coli,* displayed zones of what appeared to be inhibition of growth. Similar but smaller zones surrounded absorbent paper discs previously dipped in walnut wash water. These zones, however, did not appear when the kernel skin was removed prior to the test. Embedding the kernels in eosin methylene blue (EMB) agar produced banded zones of discoloration and questionable inhibition, but when gelatin was added, neither bands nor inhibition was noted. These observations pointed to the presence of some readily water-soluble gelatin-reacting substance in walnut skins that is capable of hindering the growth of *E. coli.*

Jurd (1956a,b, 1957) has shown that walnut skins consist of 10–15% by weight of tannin, a mixture of glucosides whose polyphenolic constituents were identified upon hydrolysis as ellagic acid, gallic acid, and methyl gallate, and the sugar portion as glucose. Smaller quantities of these polyphenols were also found in the uncombined state.

Martin and Fowler (1934), in determining the germicidal action of tannic acid (gallotannic acid), reported that although tannic acid strengths of 1 to 5% were not significant, application of the next higher, i.e., the 10% concentration, resulted in the complete kill of *E. coli* and several other organisms within 24 hr.

Concerned with survival of coliforms on commercial nuts, Ostrolenk and Hunter (1939) artificially contaminated 2-lb quan-

tities of six kinds of nut meats with *E. coli*, stored them in tin containers at room temperature, and tested portions of these samples at intervals over a period of 80 days. The authors were able to detect *E. coli* for 68 days, but gave no further details.

In view of this background information, it appeared that the water-soluble *E.-coli*-inhibiting substance in walnut skins might be comparable to tannins. Like tannic acid, walnut skins gave intense blue reaction with ferric chloride and precipitated gelatin. Because the tannins are readily soluble in water, washing the nut meats should produce a quick drop in tannin content. If the two substrates, the washed and the unwashed walnut meats, were artificially contaminated with *E. coli* cells, a significant population change on one substrate and not on the other would demonstrate the effect of the water-soluble substances in walnut skins on *E. coli* viability.

To limit the number of variables in this experiment, the rate of solution of tannins during washing of nuts, the quantity of water retained by walnut meats as a function of time, and the amount of water retained by walnut meats as a function of kernel size were studied.

The purpose of this work, then, was: 1) to evaluate the detrimental effect of walnut meats on *E. coli* under various conditions of storage; and 2) to study the effect of isolated walnut tannin extract on *E. coli*.

EXPERIMENTAL METHODS

Material. The walnuts used were commercial English walnut meats, composited from samples submitted to this laboratory for bacteriological examination, collected from the 1961 West Coast crop. All samples showed low coliform densities and aerobic plate counts (APC) of less than 30,000 per gram, and none contained *E. coli*. The nut meats were relatively unbruised, predominantly light-brown and normal in flavor, and consisted of halves and pieces retained by the U. S. Eq. No. 4-mesh sieve.

Method for examination of E. coli in nuts. The method employed for determination of *E. coli* was as follows: 50 g of nut meats were aseptically weighed into a one-pint wide-mouth sterile bottle and washed 5 min in 50 ml of 0.1% sterile peptone water on a rotary shaker (Adams Yankee rotator, 130 turns of 2-to-3-cm diameter per minute); this washing was preceded and followed by a short, vigorous shaking by hand. From this washing, consecutive decimal dilutions were prepared, using a 20-ml transfer to a 80-ml blank for the 10^{-1} dilution, and 10-ml transfers to 90-ml blanks for the 10^{-2} and greater dilutions. The blanks were pharmaceutical 6-oz screw-cap bottles containing sterile 0.1% peptone water. As each new dilution was prepared, the suspension was first shaken by hand and then placed on the shaker for 3 min.

"Most probable numbers" (MPN) of *E. coli* were determined at three levels incubated at 35°C in 3 lauryl sulfate tryptose lactose broth (LST) ferment tubes. Cultures showing gas within 48 hr were transferred to EC broth ferment tubes and incubated at 45.5°C in a constantly agitated water bath. Cultures showing gas within 48 hr were streaked on Levine EMB and the plates incubated at 35°C. After 24 hr, at least one characteristic *E. coli* colony per plate was fished out and tested by the indole-methyl red-Voges-Proskauer-citrate series (IMVIC). The *E. coli* MPN were computed from tables of APHA (1955).

Estimation of walnut tannin dissolution during washing. The dissolving of walnut tannin in water as a result of washing was estimated in the following manner:

Two 2-pint jars, each containing 100 g of walnut meats, were placed on the rotary shaker; another two were allowed to stand undisturbed. At zero time, 200 ml of distilled water was added to each jar. After 5, 10, 20, 40, and 60 min, 2-ml portions of washings were removed from each, and their serial dilutions (½, ¼, ⅛, etc.) in distilled water were tested to extinction with 0.1% aqueous ferric chloride solution. Tannins react with ferric chloride to form a blue-black precipitate; a visible amount of such precipitate constituted a positive test. In addition, three reference solutions, each 0.1% by weight, were prepared: a) tannic acid U.S.P. Powder, Merck .04541, FD Test No. 12455; b) crude walnut skin extract; and c) purified polyphenolic mixtures from walnut skins. These solutions were similarly tested to extinction.

Water retention of meats during washing. For properly controlled contamination of nut meats with *E. coli*, the amount of water retained by 100 g of nut meats during periods of 1, 3, 5, 10, and 15 min was determined. Ten 2-pint jars, each containing 69–72 kernels per 100 g, were immersed and gently swirled in 0.1% peptone water at about 20°C. After each period, two jars were drained and their kernels were transferred with forceps to a balance and weighed.

Water retention of meats as a result of kernel size. The relation between water retention and kernel size was determined similarly, except that in this experiment the time for immersion of nut

meats was kept constant at 3 min, but the number of kernels per 100 g was varied. In the 13 batches tested, the number of kernels varied from 33 to 122.

Washing of meats. The walnut meats were artificially contaminated with *E. coli* cells as follows: First, the pooled walnut meats were divided into two identical 15-lb batches. One was placed in a rubber-coated wire basket and washed by rotation for 5 min while immersed in approximately 15 L of distilled water at room temperature. The washed nut meats were drained and dried to their original weight in a single layer on absorbent paper at 35°C (taking approximately 30 hr). These nut meats were then referred to as "washed."

Preparation of E. coli suspension. A fresh-sewage *E. coli* isolate of typical cultural and biochemical characteristics, grown 18 hr at 35°C in lactose broth shake culture, was washed twice by centrifugation in sterile 0.1% peptone water. The resulting suspension was filtered through a sterile eight-layer gauze pad and diluted in 0.1% sterile peptone water to a concentration of 1×10^6 cells per ml, based on previously established spectrophotometric absorption data. The *E. coli* MPN and aerobic plate counts (APC) were then determined, using Levine EMB and milk protein hydrolysate (MPH) agars.

Seeding of meats with E. coli. Two rubber-coated wire baskets containing the two batches of nut meats, one washed and one unwashed, were immersed side by side in the *E. coli* suspension at about 20°C and gently swirled. After 3 min the baskets were removed and drained, and their contents were spread in a single layer over a nonabsorbent plastic sheet. At this point, three 50-g portions from each batch were picked out with forceps at random and examined immediately for the *E. coli* MPN and APC. After two days at room temperature, when dried to their original weight, the nut meats were packaged in 50-g portions in commercial "Nasco Whirl-Pak" plastic bags (23 × 11.5 cm), and three packages from each batch were examined for the *E. coli* MPN and APC.

Storage. Sets of matched bags were stored under the following conditions: Two sets were kept at room temperature, one in a cardboard box and the other in a desiccator evacuated to 15 inches Hg and filled with nitrogen to one atmosphere. Three sets were kept in cold storage at 9–14°C: one in a cardboard box, one in a sealed plastic container saturated with moisture, and the third in a similar sealed container hut over activated indicator silica gel. One set in a paper bag was kept in the freezer at 10°F. At intervals, matched bags were removed and examined for the *E. coli* MPN. A record was also kept of the number of kernels found in each bag.

Effect of walnut tannin extract on E. coli. Finally, the effect of walnut tannin extract on *E. coli* cells was evaluated. The extract represented the crude polyphenolic mixture from walnut meat skins isolated and identified by Jurd (1956a,b, 1957). First, a 7.2-g extract was dissolved in 18 ml of 0.9% aqueous saline and filtered through Whatman No. 12 filter paper. A series of twofold dilutions were performed as follows: Tube 1 contained 10 ml saline and 10 ml of the filtrate. Tube 2 was prepared by withdrawing 10 ml from Tube 1 and mixing with 10 ml saline; Tube 3 was prepared in the same manner by diluting 10 ml from Tube 2 with 10 ml saline; and so on for Tubes 4–10. Then, 2.5-ml quantities of each dilution were transferred to sterile screw-capped 15-ml tubes in triplicate. Six control tubes, each containing 2.5 ml sterile saline, were also prepared.

An 18-hr *E. coli* culture, identical to that used in the previous experiment, was divided into two portions, and each portion was washed twice by centrifugation. One portion was resuspended in sterile saline, and the other in sterile 0.2% peptone water. The two suspensions were diluted in their respective diluents to approximately 1×10^n cells per ml and filtered through sterile eightfold gauze pads.

At zero time, 2.5-ml portions of the two *E. coli* suspensions were added aseptically to tubes containing dilutions of extract in such a manner that one complete set of dilutions reacted with one *E. coli* suspension. Upon addition of cells, the tubes were mixed to uniformity and allowed to stand at room temperature.

At intervals, 2-ml portions were withdrawn aseptically from each tube for determination of *E. coli* numbers by the tube dilution method. One ml was inoculated into an LST ferment tube, representing the 10^1 dilution; 1 ml was transferred to a 9-ml sterile 0.1% peptone water blank for the 10^{-1} dilution; and so on for the remaining dilutions.

RESULTS

Dissolution rate of walnut tannins. Fig. 1 shows data for the rate walnut tannin dissolved in water. With continuous swirling, the rate was maximum during the first few minutes, but without swirling the tannin dissolved at a constant rate. The highest dilutions giving a positive ferric chloride test in 60 min were 1:16 and 1:8. The three reference solutions, 0.1% tannic acid, 0.1% crude extract, and purified walnut tannin extract, all gave the highest positive dilutions for this test at 1:8. Therefore, the three tannins can be detected on the basis of the ferric chloride reaction up to a concentration of almost 0.01%; also, if

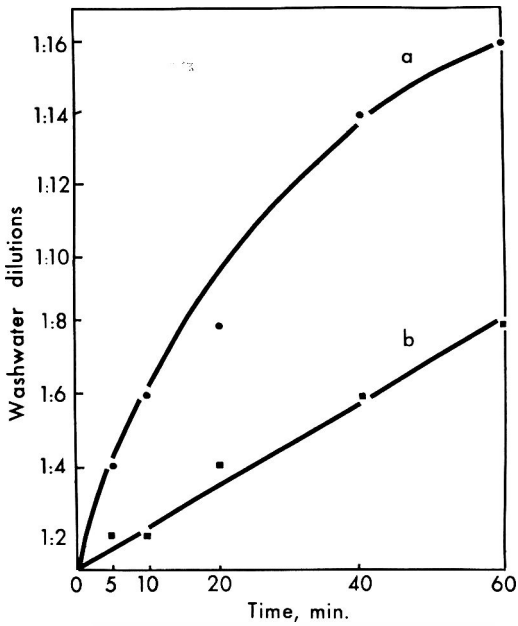

Fig. 1. Dissolution rate of walnut tannins in water as a result of washing procedure (a) by mechanical shaking (b) without shaking. Points show highest wash-water dilutions giving positive ferric chloride reaction in a given time interval.

the reaction is specific for the tannins, the washing giving a positive test at 1:16 should contain roughly 0.2% tannin by weight. Since this concentration was obtained by swirling 100 g of walnut meats in 200 ml of water, the soluble tannin content of walnut meats was shown to be about 0.4%. Considering that the walnut skin constitutes only a small fraction of walnut kernel by weight, the figure thus obtained appears out of proportion. No further attempts were made to determine the amount of tannin removed by washing.

Water retention of meats during washing. Fig. 2 shows water retained by walnut meats as a result of the extent of washing. Nut meats swirled 1 min in peptone water retained ½ as much water as was retained in 15 min. Walnut meats swirled for 3 min retained 6% of water by weight. Thus, in order to contaminate walnut meats by immersion with the desired one million *E. coli* cells per gram, a suspension of about 17 million cells per ml would be required.

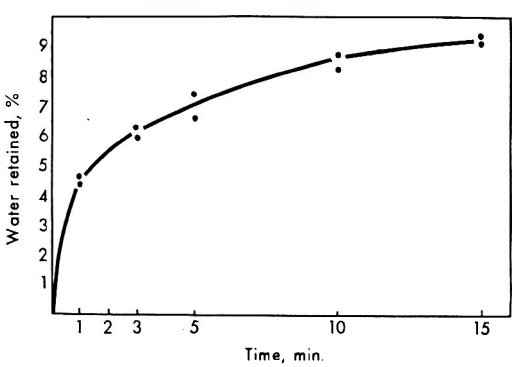

Fig. 2. Water retained by walnut meats as a result of the length of washing.

Water retention of meats as a result of kernel size. Fig. 3 shows the relation between the water retained by the nut meats as a result of washing and their surface area per given weight. More water tended to be retained as the number of nut meat pieces per 100 g increased, but the points on the curve were widely scattered. The following consideration may help explain this variation. In this work the variation in the number of walnut pieces was mostly due to broken kernels as a result of handling, rather than to variation in natural size of the walnut kernels. Breaking up a given amount of nut meats increases the surface area, but this newly gained surface consists of nut meat area devoid of skin. The imbibition of water by walnut skins, however, may be quite different from that of walnut meat. In general, the curve indicated that doubling the number of nut meat pieces per given weight raised the amount of water retained by roughly 3%. The walnut kernels used varied between 41 and 64 per g. This variable was considered negligible, and no corrections were made for this effect.

Effect of meats on E. coli during drying. Based on six determinations, the average *E. coli* MPN per g of the contaminated meats was 2.8 million for the washed and 3.6 million for the unwashed nut meats. After two days of drying, however, the average *E. coli* MPN per g, again

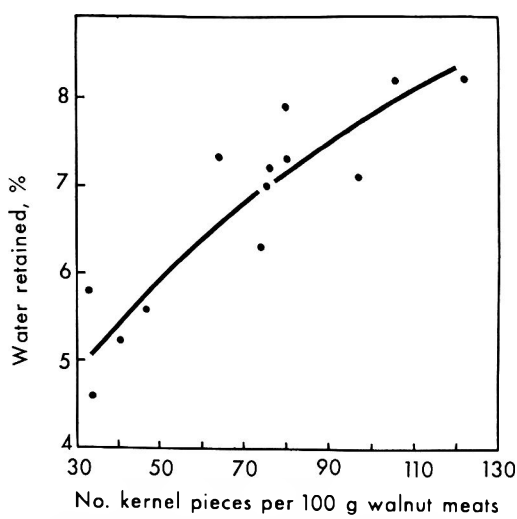

Fig. 3. The relation between the water retained and the number of kernel pieces per 100 g.

based on six determinations, was 5.4 million for the washed and 44,000 for the unwashed. Therefore, roughly 99% *E. coli* were killed within 48 hr on unwashed walnuts (Table 1). In view of the fact that contamination of meats by immersion had itself a washing effect, the effect of the intact walnut skin on *E. coli* should be considerably greater than that shown here. Furthermore, the diluent containing peptone, which reacts with tannins to form a precipitate, undoubtedly exerted some sparing effect on *E. coli.*

Effect of meats on E. coli during storage. Tables 2 and 3 show the *E. coli* MPN of dried nut meats placed in storage under various conditions and then examined at certain intervals for more than 200 days. Fig. 4 gives an over-all picture of *E. coli* MPN representing storage effect of contaminated nut meats at room temperature and under refrigeration.

The effect of storage conditions is somewhat obscured because all nut meats were dried at room temperature for two days after contamination, the period when the most drastic changes in population occurred. In storage at room temperature the survivors, shown by the two curves representing *E. coli* populations on washed and unwashed substrates in Fig. 4, declined steadily at similar rates for 3 and 4 months until no longer detected. The possibly beneficial inert gas atmosphere might have been lost, because of frequent exposures of nut meats to air during sample removals. On the other hand, *E. coli* on nut meats kept refrigerated or frozen showed little change in population numbers. In fact, most of these cells remained viable for the duration of the experiment. The last determination, made after 240 days in cold storage, indicated no significant decrease in viable cell numbers. Again, the type of storage had little or no effect.

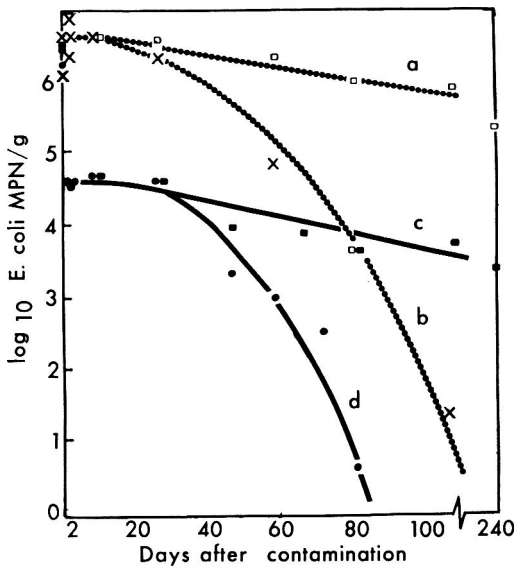

Fig. 4. Changes in *E. coli* populations on artificially contaminated washed (broken lines) and unwashed (solid lines) walnut meats at room temperature storage (*b, d*) and in the cold (*a, c*).

Effect of walnut tannin extract on E. coli. Fig. 5 shows the effect of walnut tannin extract on *E. coli* cells suspended in saline. Kill was complete within 1 hr at 0.62% extract concentration for 1×10^7 cells, and at 0.16% for 1×10^5 cells. Cells died off sharply within a relatively narrow zone. The lethal effect of the extract appeared rather sudden; the picture did not change appreciably within the following 24 hr. The ten million cells suspended in 0.1% peptone water (Fig. 6) showed a complete kill within 2 hr at 2.5% extract. This concentration was four times as great as that found fully lethal for the cells in saline suspension. The peptone-sparing effect was also observed as a

Table 1. *E. coli* populations on artificially contaminated washed and unwashed walnut meats during drying.

Days after contamination	Description	Washed				Unwashed			
		MPN/g	APC/g MPH	APC/g EMB	No. of kernels per 50 g	MPN/g	APC/g MPH	APC/g EMB	No. of kernels per 50 g
0	Drained nut meats	2,400,000	1,500,000	1,600,000	46	4,600,000	2,700,000	2,200,000	54
		1,500,000	1,800,000	1,700,000	56	4,200,000	2,400,000	2,000,000	50
		4,600,000	2,100,000	1,700,000	41	2,100,000	1,900,000	1,900,000	41
		Average 2,800,000	1,800,000	1,700,000	48	3,600,000	2,300,000	2,000,000	48
2	Nut meats dried at room temperature	9,100,000	5,300,000	4,000,000	59	46,000	24,000	16,000	45
		4,600,000	3,600,000	3,100,000	64	39,000	25,000	8,000	55
		2,400,000	1,100,000	8,300,000	58	46,000	59,000	61,000	49
		Average 5,400,000	3,300,000	2,600,000	60	44,000	36,000	28,000	50

Table 2. *E. coli* populations (MPN/g) on artificially contaminated washed and unwashed walnut meats during storage at room temperature.

Days after contamination	Nut meats in cardboard box		Nut meats under partial nitrogen	
	Washed	Unwashed	Washed	Unwashed
9	4,600,000	53,000		
26	2,400,000	45,000		
27			450,000	45,000
47		2,400		
39	74,000	1,000	45,000	450
72		350		74
81	4,500	4.3	1,000	5.3
107	24	0.0	0.9	0.0
115	0.0	0.0	0.0	0.0
240	0.0		0.0	

Table 3. *E. coli* populations (MPN/g) on artificially contaminated washed and unwashed walnut meats during storage in the cold.

Days after contamination	In refrigerator						In freezer	
	In cardboard box		In dry air		In damp air			
	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed
9	4,450,000	46,000						
26	4,200,000	44,000						
27			1,600,000	15,000	1,500,000	24,000		
47		9,300	4,300,000	24,000	4,500,000	25,000	2,400,000	15,000
59	2,400,000	44,000	2,500,000	11,000	2,100,000	14,000		
72		7,500						
81	1,100,000	4,400	1,100,000	4,400				
108	750,000	5,300			1,100,000	2,300		
115			2,400,000	5,300			1,100,000	12,000
240	240,000	2,400	460,000	4,300	910,000	2,400	740,000	4,300

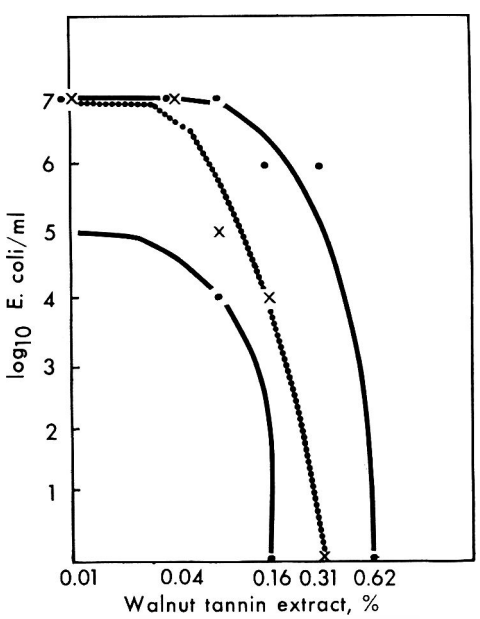

Fig. 5. The effect of walnut tannin extract on two *E. coli* suspensions in saline. Unbroken lines show the effect at the end of 1 hr, and the broken line the effect at the end of 24 hr.

gradual decrease in numbers, somewhat in step with increasing concentration, and spreading out the "complete kill and no-effect" zone from 0.08% to 2.5% extract. Although the cells in saline suspension did not increase or decrease within 24 hr, as indicated by the controls, the cells in peptone control tubes, while showing no change for the first 2 hr, increased to one billion per ml within the following 22 hr. This increase masked the effect of the extract exerted on the original population. Thus, the 24-hr curve apparently portrays the growth curve of survivors.

DISCUSSION

English walnut tannins show relatively high and fast-acting bactericidal properties. *E. coli* cells on fresh walnut meats, exposed to 10–15% tannin, have a small chance to survive and thus go undetected. In practice, however, additional factors enter the picture and tend to modify the tannin effect, so that at least some recovery of *E. coli* from walnut meats is possible. Contamination resulting from insanitary handling is not necessarily confined to naked, individual bacterial cells;

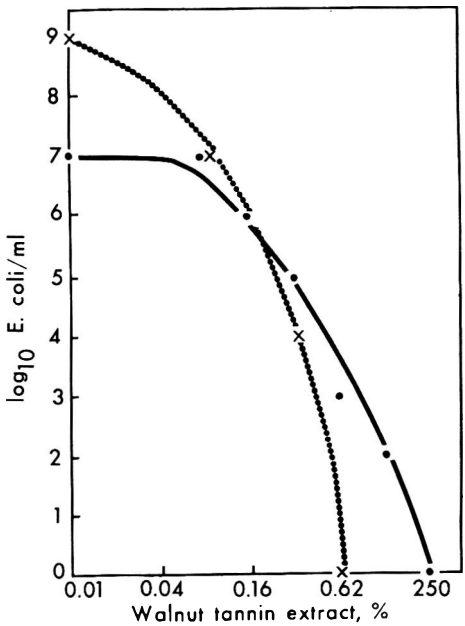

Fig. 6. The effect of walnut tannin extract on *E. coli* suspension in 0.1% peptone water. Unbroken line shows the effect at the end of 2 hr, and the broken line the effect at the end of 24 hr.

these may come in aggregates or embedded in dirt consisting of all sorts of matter. The filth may act as a physical barrier and may lessen tannin concentration by absorption or by reacting with it, and may even serve as a substrate for growth. These circumstances would favor survival of microbial life.

Thus, the *E. coli* indicator system appears weaker in English walnuts than in other food products. To many organisms, tannins are less detrimental. According to Martin and Fowler (1934), quoting Solis-Cohen and Githens, "One-half percent tannic acid will kill *B. coli* and *Staphylococcus aureus* in two hours, but 10% will not kill anthrax in twenty-four hours." Viruses are less susceptible to the effects of phenols and related preservatives, as shown by McCulloch (1945). Bacteriological examination, at best, can only detect survivors. Where insanitary practices are observed, the absence of *E. coli* in the product constitutes no proof that insanitation did not exist. Mere traces of *E. coli* in the product may well represent the remnants of a much larger, earlier contamination.

Jurd (1958) viewed walnut tannins as natural antioxidants in that the tannins were preferentially oxidized by oxygen of the air, thus temporarily sparing the kernel oil from oxidation. Oxidation of tannins, slower in unshelled nuts, is accompanied by browning of walnut skins; thus, the age of walnuts, particularly in the shelled state, further influences tannin content. This age effect, in turn, is modified by the conditions under which nut meats were stored. The moisture content of nuts, which varies directly with the relative humidity of the atmosphere of the storage room, favors oxidation, as does warm air. In general, the storage conditions that tend to keep nut meats in good quality, also favor the preservation of tannins.

These experiments suggest that walnut samples for *E. coli* examination should be placed in a refrigerator immediately after collection, kept in the cold, and examined promptly. The low temperature, by slowing chemical changes, not only prolongs the viable state of many microorganisms but may delay the deleterious tannin effect, which is rapid at room temperature. When refrigeration is not possible, it may be advantageous to collect samples in a predetermined amount of a diluent capable of neutralizing the tannin effect to the point of *E. coli* tolerance. Many conditions encountered in taking walnut samples cannot be changed, but if the sample is selected discriminately to minimize the detriments, detection of *E. coli* can be improved.

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Salmonella Derby Contamination of Eggs from Inoculated Hens

SUMMARY

Twelve hens were inoculated weekly for 11 weeks with *Salmonella derby* in peptone water. Another 12 were fed continuously on feed which had been contaminated with *Salmonella derby*. Another group of birds served as controls. Both the shell surface and interior of 2 eggs per bird each week were examined for salmonellae. Chicks hatched from 5 selected hens were also examined for salmonellae. Neither the shells nor the egg meats from the 622 eggs sampled yielded positive *S. derby* cultures during the trial. The feces of 2 birds receiving contaminated feed, and of 4 birds receiving the weekly inoculation contained the *S. derby*. *S. derby* was not recovered from any of the hens upon sacrifice or in any of the chicks from these hens.

INTRODUCTION

The frequent presence of salmonellae in feed and the implications of eggs as a source of salmonellae food poisoning of humans is of great concern to the poultry industry. A *Salmonella derby* outbreak in hospitals in 1963 was traced, on presumptive evidence, to cracked eggs being used in the hospitals and led the Surgeon General of the Public Health Service to issue recommendations against the use of cracked or unclean eggs (Anon, 1963).

Contaminated feed may be one source of *Salmonella* in the poultry flock. Morehouse and Wedman (1961), in a survey of laboratory analyses, reported the recovery of salmonellae from 718 of 5712 samples of feedstuffs. This leaves little doubt that there are salmonellae in feeds and feedstuffs. Morehouse and Wedman concluded, however, that definitive evidence is lacking that the animal by-products in rations are sources of causative organisms responsible for specific field occurrences of salmonellosis.

When Gibbons and Moore (1946) fed suspensions of *Salmonella bareilly* to pullorum-positive birds, the organism was eliminated by the birds for 2–38 days. They were able to isolate *S. bareilly* from the shells of 3 of the 37 eggs laid during the period the organism was being excreted. In a more recent study, Mundt and Tugwell (1958) reported on the role of the egg in transmission of 6 salmonellae (*S. oranienburg*, *S. derby*, *S. senftenburg*, *S. berdeney*, *S. paratyphi B*, and *S. montevideo*) from diseased hens. In that study, large doses of the *Salmonella* were given intraveneously. Each organism was given to 10 birds. This disease was considered severe and resulted in diarrhea and one death. Production dropped significantly for 2 groups (*S. derby*, 13.7%; and *S. senftenburg*, 20.7%). They recovered the inoculated organism from the feces in all groups on the second, third, and fourth days after inoculation, but not from every bird in each group. Of 489 egg meats cultured over a 14-day period after inoculation, none contained salmonellae.

Cantor and McFarlane (1948) in an investigation of market eggs reported finding 13 egg shells contaminated with salmonellae of 2132 eggs sampled. These proved to be strains of *S. montevideo* (5×) and *S. anatum* (8×). Of 2584 samples of egg meat, 30 were found to be *Salmonella*-positive, all of which were *S. pullorum*. *Salmonella* organisms were not isolated from shell scrapings and meat of the same egg, although samples of both were available for 2088 of the eggs sampled.

Thatcher and Montford (1962) reported isolating *Salmonella* organisms from commercial frozen egg products and cake mixes containing eggs. From 114 samples of frozen eggs, 27 positive samples were found (21%), and of the 119 samples of cake mixes containing eggs, 65 were positive (54%). The source of salmonellae in bulk egg products has generally been attributed to contamination from the shell during breaking. One would expect that samples containing high coliform counts would also have a greater chance for *Salmonella* contamination. They compared this relationship

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and found no direct correlation. They suggest that internal contamination is a likely alternative to contamination from the shell.

This study was initiated to attempt to recover *S. derby* from eggs laid by hens given a constant low inoculation as would normally be expected with contaminated feed. *S. derby* was chosen because of its recent high incidence of human isolations (Anon, 1964) and its linking with fresh eggs (Anon, 1963).

MATERIALS AND METHODS

White Leghorn hens, 12 months old, were placed in individual cages, with free access to feed and water. The wire egg tray under the cages was divided to keep the eggs from each hen separate. The hens were randomly assigned to the 3 tiers of 12 cages each. For 4 weeks prior to the test period, feed, feces and eggs were sampled from the 36 birds and no salmonellae were recovered. The hens in the top tier constituted a control and were not inoculated. The hens in the lowest tier were fed a ration prepared by mixing 1,500,000 cells of *S. derby* with 25 lb of normal breeder mash each week (feed consumption was estimated at 0.25 lb feed per bird per day). The 12 birds in the middle tier were inoculated by mouth weekly with a 0.1% peptone water dilution of *S. derby* calculated so that each bird received 100,000 organisms per week. These inoculations were made by inserting a 1 cc tuberculin syringe, without needle, into the firmly held extended esophagus beyond the tracheal opening before release of the inoculum. The heads were held long enough to ensure that the dose would not be rejected. These treatments continued for 11 weeks.

Two eggs per hen were sampled weekly for 12 weeks for salmonellae contamination. The eggs were picked up from the wire rack under each cage with sterile tongs and placed in individual pint jars containing approximately 100 ml of Selenite F (BBL) (to which had been added .01 g/L of cystine). The eggs were transported to the laboratory and allowed to remain in the solution for approximately 1 hr. The eggs were then removed with sterile tongs, dipped in alcohol, and flamed. The egg was then placed in a second jar containing Selenite-F-cystine, broken and mixed. Preliminary trials had shown that this alcohol flaming did sterilize the shell surface so that two samples could be considered. The egg meats sample would probably disclose any *Salmonella* in the membrane area of the shell and this would be considered to have entered the egg. Even though these second jars also contained shell, this was considered to be the egg meat sample. If the shell broke while the egg was in the first jar the sample was considered to be a whole egg sample. The inoculated jars were incubated 18 hr at 37°C. A loopful of the contents of each jar was streaked on brilliant-green agar (D) and incubated 24 hr at 37°C. Typical colonies were picked to nutrient agar slants for later identification.

Feces were sampled from 4 birds of each tier on each of 3 consecutive days each week. The samples were taken from fresh droppings under the cages with a sterile spatula. The sample was placed in a tube containing 10 ml of the Selenite-F-cystine broth, transported to the laboratory, and incubated, streaked, and treated in the same manner as the egg samples.

Feed was sampled on the same days as the feces by taking two samples of feed from each tier. Samples were taken by placing the feed in Selenite-F-cystine broth (10 ml) with the tip of a sterile spatula, shaking, transporting to the laboratory, incubating and treating like the feces samples.

On the ninth week of sample collection, three hens from each treatment group were artificially inseminated with pooled semen. Hatched chicks from the eggs from these hens were sacrificed and examined for salmonellae. Selected parts of the chick (spleen, genitals, and yolk sac) or the whole chick were ground in a Waring blender with 100 ml of Selenite-F-cystine broth. These samples were treated like all other samples.

Cloacal swabs were taken before the introduction of the *S. derby* and also just before sacrificing the hens. These swabs were incubated in 10 ml of Selenite-F-cystine broth and treated like all other samples.

At the end of 12 weeks of lay, all hens were sacrificed and the ovary, oviduct, duodenum and liver from each bird were ground separately in 100 ml of Selenite-F-cystine broth in a Waring blender. These samples were then treated like previous samples.

Suspect *Salmonella* cultures from brilliant green agar were screened by testing their biochemical reactions to: Krumwiede Triple Sugar Agar, Mannitol Broth, SIM Medium, Urea Broth, and polyvalent *Salmonella* antisera. Further serotyping was done at the National Animal Disease Laboratory, Ames, Iowa.

RESULTS

During the test period of 12 weeks, 622 eggs were sampled. With these 1209 samples (587 egg meat and 587 shell from each egg and 35 whole eggs), only 48 colonies were picked from the BGA plates as possible salmonellae (see Table 1). Of these, only 2 cultures appeared to be salmonellae after the screening tests. These 2 samples were

Table 1. *Salmonella derby* recovery from the shell surface and egg interior for the control and two treatment groups.

	No. of eggs	From brilliant green agar	Confirmed by biochemical tests	Sero-typed
Control	229			
Shell		7	0	0
Egg		4	0	0
Orally inoculated	193			
Shell		14	0	0
Egg		0	0	0
Fed contaminated feed	200			
Shell		15	2	0
Egg		8[a]	0	0
Total	622	48	2	0

[a] One of the cultures included here came from one of the sampled whole eggs.

from egg shells of hens being fed the inoculated ration. These organisms were reported by the Typing Center to be members of the "Aerobacter group." No cultures of the 13 picked from egg meat streaks passed the screening tests.

S. derby was recovered from the contaminated feed, but not with the regular consistency that had been expected. Positive samples were recovered for only 2 weeks, the third and fourth week of the trial. No other types of *Salmonella* were recovered from either the contaminated or uncontaminated feed.

S. derby was recovered from the feces of 6 birds during the trial. Four recoveries were made from birds receiving the mouth inoculation on the third, fifth, and tenth weeks of the trial. Two birds being fed the contaminated feed shed *S. derby* during the ninth week of the trial.

Salmonella-like organisms were found on one chick, but this proved to be a member of the "Aerobacter group" also.

No salmonellae were recovered from the cloacal swabs or when the birds were sacrificed.

Weekly egg production data are shown in Table 2 for periods before and during the inoculation.

DISCUSSION

It would seem from these results that *S. derby* does not infect the hen when given orally, at least when low doses are used. This is not to say that egg shells could not become contaminated from feed containing *S. derby*. It would be possible for *S. derby* in the feed to be transferred to the egg shell by the caretaker's hands or through the feces of the hen.

In this trial low doses were given over a long period to try to simulate a natural feed contamination. If higher doses had been given, infection may have been more evident, as in the trials of Mundt and Tugwell (1958), Gibbons and Moore (1946), and Ross *et al.* (1964). But in the light of these studies, it is doubtful if a higher dose would have resulted in *S. derby* transfer within the egg.

Table 2. Egg Production (no. of eggs) by groups for 5 weeks before and 12 weeks during the administration of *S. derby*.

		Group[a] 1	2	3
Before	Av.	50	52	44
	Range	49–58	50–63	40–53
	Total	300	313	265
During	Av.	48	42	43
	Range	46–60	40–52	41–54
	Total	578	502	519

[a] Group 1, uninoculated control; Group 2, inoculated weekly by mouth; Group 3, fed inoculated feed.

Since inoculations were calculated to give each bird approximately 15×10^3 cells daily, or 10^5 cells weekly, it was expected that, 1–2 weeks after beginning exposure, the hens would begin to shed *S. derby* in their feces regularly. This did not prove to be the case. With the methods employed in this study, only 6 feces samples proved to be positive of the 264 samples tested for *S. derby*. In no hens were *S. derby* cells found regularly. This may have been due to dying of the salmonellae in the bird or in the feed. Or it may have been due to lack of sensitivity of this method to isolate small numbers of salmonellae in the feces. But since this procedure was originally designed for feces (Leifson, 1936) it should have been most sensitive for these samples.

Feed and feces samples were taken the three days each week immediately following the mixing and feeding of freshly inoculated feed. This feed was expected to yield viable *S. derby* each week. In fact, it was expected that other salmonellae might be in the feed in addition to *S. derby*. Very few samples were positive for *S. derby* and no other salmonellae were found. This failure was probably due to the difficulty in recovering low levels of salmonellae or to the death of the cells in the dry feed as

noted previously by Leistner *et al.* (1961).

Galton *et al.* (1954) and Leistner *et al.* (1961) both reported a low incidence of salmonellae in hogs on farms but a very great increase as the hogs were transported to market. It is conceivable, despite the fact that statements often implicate contaminated feed when salmonella-contaminated eggs are found, that most of this contamination may come from handling rather than through the hen.

It has been suggested that to speed up the elimination of salmonellae from poultry flocks, the growers must be convinced that an economic loss is incurred through a low level *Salmonella* infection (Smith and Quist, 1964). Part of the data in Table 2 might seem to imply that this economic loss does exist. Group 2, the inoculated hens, suffered a drop in production far greater than that in either of the other groups. Group 3, however, which was fed the contaminated feed, did not suffer this drop in production. Any positive statement on this could not be made, however, because of the small numbers involved and the fact that the Group 2 birds were handled weekly whereas the others were not. No disease was noted in these birds except for some loose droppings from time to time. In the diseased birds of Mundt and Tugwell (1958), however, the production fell in five of the six groups and many eggs laid were soft shelled and malformed. This was not the case with the birds in this study.

The data in this paper agree with previous reports (Gibbons and Moore, 1946; Mundt and Tugwell, 1958; and Ross *et al.*, 1964) that most of the *Salmonella* species are not interior but egg shell contamination. It is thus necessary to develop methods to eliminate the salmonellae at the processing plant, besides elimination from feeds, flocks and farms in general.

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Miss Alice Moran verified and typed the cultures at the National Animal Disease Laboratory, Ames, Iowa.

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Prevention of Type E Clostridium Botulinum Toxin Formation in Smoked Whitefish Chubs with Tylosin Lactate

SUMMARY

The formation of type E *Clostridium botulinum* toxin was prevented in low-temperature laboratory-processed whitefish chubs by incorporating 100 ppm tylosin lactate in the brine. Although toxin formation occurred in brined fish processed 30 min at 130 and 150°F without tylosin, no toxin formation occurred in any of several commercial and laboratory batches of fish processed 30 min at 170 and 180°F. Studies of inoculated raw fish showed that 9 of 11 strains of *Cl. botulinum* type E were able to withstand 170°F for 30 min and that three of the strains tolerated 180°F. One sample of commercially packed fish having low brine and high moisture levels was capable of supporting toxin development by all 11 strains. Fish having a brine content of 5.5% were unable to support toxin formation whereas fish having 3% brine allowed production of toxin by the organism. The significant relationships between toxin formation, heat processing, brining, and the use of tylosin during processing are discussed.

INTRODUCTION

Recent incidences of Type E botulism arising from commercially processed smoked fish have stimulated research on many aspects of the growth and formation of toxin by cultures of *Clostridium botulinum* type E (Graikowski and Kempe, 1964; Segner *et al.*, 1964; Malin *et al.*, 1964). However, the many factors in the processing of smoked fish products which may be related to the formation of toxin by *Cl. botulinum* type E have not yet been investigated. Methods used by the industry for generations have come under close scrutiny by regulatory agencies. Some of these have been judged to be doubtful or dangerous practices. New processing conditions have been proposed and enforced upon the industry. These conditions have apparently caused the final products in many cases to be unacceptable to the consumer. According to one industry spokesman, a food product which has been consumed in this country for at least 100 years is now no longer available to the public, and a large part of the smoked-fish industry is now out of business.

Growth by various members of the genus *Clostridium* has been shown by Denny *et al.* (1961) and by Greenberg and Silliker (1962b) to be readily inhibited by low concentrations of tylosin lactate. The latter publication also suggests the possibility of a reduction in thermal processing in canned foods if tylosin levels can be maintained in the food for sufficiently long periods. Greenberg and Silliker (1962a) demonstrated that tylosin prevented toxin formation in minimally heat-processed pack of meat products inoculated with various strains of *Cl. botulinum* including type E.

This paper reports on experimental work which may help to define some of the relationships between smoked-fish processing and toxin production in the product by *Cl. botulinum* type E. Also discussed is the possibility of using tylosin lactate together with the control of some factors in the processing in order to ensure against toxin formation in the finished product.

EXPERIMENTAL PROCEDURES

All raw and processed fish used were obtained through the courtesy of Dornbos Fisheries, Grand Haven, Michigan. For the brining studies, frozen whitefish chubs, No. 2 size, were thawed out in cold water and brined in various concentrations of salt and tylosin for various periods at room temperature. In some experiments brine was adjusted with lactic acid to pH 6.0 from the normal pH of 6.8.

Tylosin residues in brines and in the fish were determined by a pen-cylinder modification of the assay method described by McGuire *et al.* (1961). For this purpose, extracts of the fish samples were prepared by macerating portions of the fish in a Waring blender with equal quantities by weight of distilled water. These mixtures were centrifuged to settle the particulate material, and the tylosin assays were run on the supernatant liquid.

Oven processing in most of the experiments was concluded with a cooking temperature of 180°F for 30 min. The time required to reach this temperature was about 3 hr for commercially prepared

batches and about 5 hr for the smaller-scale laboratory batches. For the latter, ordinary laboratory drying ovens were modified with hook hangers and aluminum foil to simulate the conditions of the commercial ovens. The laboratory preparations were not smoked. All commercial batches were prepared at the facilities of the Dornbos Fisheries.

The cultures used were strains of *Clostridium botulinum* type E isolated from the sources listed in Table 1. All cultures were grown for three days in the growth and sporulation medium used by Schmidt *et al.* (1962). The spores and vegetative cells were separated from the medium by centrifugation. They were then washed three times and resuspended in sterile saline so as to provide a concentration of about 10^7 viable organisms per ml. For the large commercial inoculation study, a mixed inoculum of cells and spores from two different strains, E40 and E105, was prepared. For the cell-free spore inoculum also used in the commercial inoculation study, three-day cultures were pasteurized for 10 min at 75°C. After washing, the spores from the same two strains, E40 and E105, were mixed together, giving a final concentration of 8×10^3 spores per ml of inoculum.

Table 1. Sources of strains of *Clostridium botulinum* type E.[a]

Designation	Source	Date of isolation
E13	Salmon eggs; Prince Rupert, B. C.	1957
E32	Home canned relish; Grand Forks, B. C.	1961
E40 (Pitt Lake No. 74)	Mud; Pitt Lake, B. C.	1962
E41	Jejunum of Eskimo; Labrador	1962
E105 (Russian E74)	Russian Sturgeon; Ukraine	1935
E110 (Iwanai)	Isushi; Japan	1951
1537/62	Human botulism; Sweden	1962
16/63	Human botulism; Sweden	1963
4318/63	Freshwater trout; Norway	1963
FDA Minn.	Smoked Cisco; Minneapolis, Minn.	1960
FDA No. 1	Smoked Whitefish Chubs; Nashville, Tenn.	1963

[a] Strains E13, E32, E40, E41, E105, and E110 were received from Dr. C. E. Dolman, University of British Columbia, Vancouver, British Columbia. Strains 1537/62 and 16/63 were isolated by Dr. A. Johannsen, Lund, Sweden, and forwarded by Dr. Anton Skulberg, Forshningsutvalget for Konserver, Oslo, Norway. Dr. Skulberg also sent his own isolate, strain 4318/63. Strains FDA No. 1 and FDA Minn. were supplied by Dr. D. A. Kautter, Food and Drug Administration, Washington, D. C.

In the large commercial experiment, 200-lb batches of fish were prepared with 100 ppm tylosin in the brine. Appropriate control batches were processed without the antibiotic. The fish were brined for about 18 hr at 60°F in a 7.7% salt brine. All batches of fish were finished at 180° and smoked in the oven. Portions of each batch were inoculated just before packaging in evacuated polyethylene bags. Three fish, with a total weight of about 200 g, were placed in each bag. Inoculation of the fish with the mixed cell-spore suspension consisted of spreading 0.1 ml of the inoculum over the surfaces of the skin and visceral cavity of each fish. In another inoculation study the washed spore inoculum was injected into the deep back muscle of the fish using 0.1 ml per fish. Appropriate uninoculated control fish were also packaged.

In experiments with ground raw and ground processed fish, the fish was passed through a meat grinder in a frozen state. Separate 25-g portions of ground fish were inoculated with 1 ml of saline suspensions of vegetative cells and spores of the cultures, prepared as indicated previously. The fish was mixed thoroughly and pressed firmly into screw-top 25-ml tubes. A set of tubes representing each of the eleven strains, plus one tube having no inoculum, was prepared for each of the five heat-treatment studies and for each of three different processed-fish studies. For the heat treatments, the twelve capped tubes of inoculated ground fish were immersed simultaneously into a water bath held at the desired temperature. After 30 min of exposure which included a 7-min come-up time, the tubes were placed in an ice bath. All tubes from the heat-treatment studies and the processed-fish studies were incubated 14 days before toxin assay.

Toxin assays were run on extracts of all fish samples. For the commercially prepared batches, 50 g of mixed macerated fish representing a single package of three fish was placed in a screw-top bottle. A 50-ml portion of gelatin-phosphate buffer (Duff *et al.*, 1956), pH 6.5, was thoroughly mixed with each sample. After 18–24 hr at 43°F, which allowed for toxin extraction from the sample, the mixture was centrifuged and the supernatant liquid was decanted off for toxin determination. The ground fish from the tubes was handled in a similar manner except that 25 ml of buffer was mixed with the contents of a single tube.

To prepare the fish extracts for toxin assay, 2-ml portions were trypsin-digested for 1 hr at 37°C. A 0.2 ml quantity of a 1% solution of

trypsin (Difco 1-250) in 0.2*M* phosphate buffer was added to each extract sample. According to Duff *et al.* (1956) and others, this step is necessary to assure detection of any toxin which may be present in the protoxin form. For each sample, a 0.5-ml portion of the trypsinized sample was injected intraperitoneally into 16–18-g Cox standard mice with and without type E antitoxin protection. The antitoxin was obtained from the National Institute of Health of Japan. For the protection dose the mice were injected intraperitoneally with 0.1 ml of antitoxin containing 1 International unit approximately 3 hr before the fish extract was administered. In the ground-fish studies, the samples were further assayed for toxin potency by serial dilution of the extract with gelatin phosphate buffer. The minimum amount of toxin detectable in the assay was 4.4 mouse lethal doses per gram of fish. Multiplication of this factor by the maximum lethal dilution of the fish extract indicated the approximate toxin potency in MLD per gram of fish.

Salt and moisture determinations in the processed fish were run according to methods indicated in the AOAC (1960).

RESULTS

Table 2 shows the absorption of tylosin lactate by whitefish chubs during brining. In general, as concentrations of tylosin in the brine were increased, the concentration in the finished fish increased. Approximately 5% of the brine tylosin could be recovered in the finished fish after a brining schedule of 18–24 hr. Increasing the ratio of fish to brine in the presence of higher concentrations of tylosin and salt does not appear to increase significantly the final concentrations in the finished fish. Also, it was found that adjustment of the brine to pH 6.0 with lactic acid or increasing brining time had little effect on the final concentration of tylosin in the finished fish.

Table 2. Tylosin absorption by whitefish chubs during brining at room temperature.

Tylosin in brine (ppm)	Brine salt concentration (%)	Brining time (hr)	Ratio fish to brine	Tylosin (ppm) in	
				Brined fish	Finished fish
75	8.5	24	3:2	8.6	3.6
100	8.5	24	3:2	10.5	4.1
150	8.5	24	3:2	13.5	6.8
100	7.0	24	1:1	17.7	5.1
60	7.0	24	1:1	13.0	2.0
75	7.0	18	1:1	7.1	2.5
100	7.0	48	1:1	22.5	5.5
100	7.0	69	1:1	19.6	6.6
100	7.0	18	1:1	14.7	3.2

The distribution of tylosin in the finished fish appears to be fairly uniform (Table 3). Although concentrations were somewhat higher in the exposed surfaces of the fish, especially in the skin, head, and tail, amounts were always significant in the thick muscle flesh of the fish.

During storage tests tylosin was found to be active in the tissues of the fish for considerable periods (Table 4). In most cases at least 75% of the tylosin in the finished fish was recovered after one month of storage at refrigeration temperatures. Table 5 shows the range of tylosin residues in 50 fish after 56 days of storage. Although the recoveries ranged from 10% to almost 90% of the amount of the original assay in the finished fish, 42% of the fish showed recoveries of 30–90% of the original tylosin, and 86% of the fish showed that more than 18% of the original tylosin was still active.

Table 3. Tylosin distribution in finished whitefish chubs.

Tylosin in brine (ppm)	Ratio fish to brine	Tylosin concentration (ppm) in:		
		Whole fish	Skin	Flesh
60	1:1	1.6	1.3	1.1
100	1:1	6.6	9.4	8.9
100	1:1	5.5	8.4	7.1
75	3:2	3.6	7.4	3.4
100	3:2	4.1	9.9	4.5
150	3:2	6.8	14.0	6.0
100	1:1	5.1	9.3	4.4
100	1:1	3.2	....	2.7
100	1:1	9.4	....	4.2
100	1:1	2.3	....	2.0

Table 4. Tylosin residues (ppm) in smoked whitefish chubs after storage.

Tylosin residues after processing	Tylosin residues after		
	14 days	28 days	56 days
5.5	5.0	4.2	....
6.8	....	6.7	....
4.1	....	4.0	....
2.0	....	1.8	0.7
6.6	4.8	3.6	....

Table 6 indicates the results of the inoculation and storage studies in the commercially prepared packs of fish processed for 30 min at 180°F. Regardless of the time and temperature of incubation or of the site or type of inoculation used (deep or surface; spores or mixed cells and spores), no toxin developed in any of the 560 packs, inoculated or uninoculated, control or tylosin-treated.

Table 5. Range of tylosin residues in 50 smoked whitefish chubs after 56 days at 43°F (Original assay, 2.26–3.23 ppm).

Tylosin assay (ppm)	% of fish
Less than 0.5	16
0.5 to 1.0	44
1.0 to 1.5	24
1.5 to 2.0	8
2.0 to 3.0	10

Table 7 shows the results of an experiment in which fish prepared at various baking temperatures were inoculated with a mixture of toxin-producing organisms and allowed to incubate. The ground fish processed at the lower temperatures, 30 min, 130 and 150°F, was able to support the formation of toxin by the organisms. However, the fish processed at the highest temperature, 170°F, did not develop toxin. Tylosin ranging in concentrations from 3 to 5 ppm in the fish processed at the lower temperatures prevented the development of toxin during the incubation period of 14 days at room temperature. In the entire fish-inoculation study the results were essentially the same with respect to the inhibitory effect of tylosin. In this case, however, both of the higher temperatures of processing resulted in fish which were unable to develop toxin.

Table 8 shows the results of analyses of various batches of fish produced under commercial conditions. Also, average toxin production is indicated, resulting from inoculation of the ground fish with the 11 strains of *Clostridium botulinum* type E. No evidence of toxin production was observed in fish from experimental packs produced in January 1964 or in the Michigan commercial pack No. 1, produced for sale in February, 1964. These packs were produced with the 180°F oven process for 30 min. They were quite similar in appearance and in analysis. Both were rather dark, dry, and wrinkled on the surface and had relatively low moistures and high brine concentrations. However, Michigan commercial pack No. 2, purchased on the open market in February, 1964, with the label of another manufacturer, was shown to be quite capable of supporting toxin formation. Appearance, brine, and moisture levels of this fish pack were considerably different from those of the other batches. These fish were brighter in color and smooth-surfaced, and had relatively high moisture levels and low brine concentrations. For comparison, analytical results are also shown for the old Michigan commercial pack No. 1, processed before October 1, 1963, in the old manner. The brine and moisture levels of this fish were similar to those of the Michigan commercial pack No. 2. Also, the appearances of these two packs were similar.

Table 6. Toxin production in inoculated new-process experimental smoked whitefish packs.

No. days incubation at:					Toxin produced (MLD/g)
43°F	86°F	77°F	65°F	59°F	
28	10	....	....	....	0
56	....	....	....	....	0
56	10	....	....	....	0
56	....	14	....	....	0
56	....	....	21	....	0
56	....	....	....	28	0

Table 9 shows toxin production in ground raw fish inoculated with the various strains of organisms and subjected to various heat treatments. All 11 strains produced high levels of toxin in raw fish without heat treatment. In fact, the uninoculated tube of raw fish showed a considerable toxin production, indicating the presence of a natural inoculum. The organism was subsequently isolated and identified as a strain of *Clostridium botulinum* type E. Following a heat treatment of 170°F for 30 min, 9 of the 11 strains were able to demonstrate toxin production. A 180°F treatment allowed 3 of the strains to survive, but none of the strains were able to tolerate the 190°F treatment.

Table 7. Toxin produced in inoculated baked brined fish.

Baking temperature (°F)	Tylosin in brine (ppm)	Toxin produced (MLD/g)
Ground fish		
170	0	0
	100	0
150	0	220
	100	0
130	0	44
	100	0
Whole fish		
170	0	0
	100	0
150	0	0
	100	0
130	0	220
	100	0

DISCUSSION

The large-scale experimental inoculation study showed that 180°F for 30 min given fish brined for 18 hr in 7% brine results in a finished product incapable of supporting the development of toxin by any of 11 strains of

Table 8. Moisture, brine concentration, pH, and toxin production in commercially processed, ground, smoked whitefish chubs (incubation: 14 days at room temperature).

Identity of pack	Production date	Brine (%)	Moisture (%)	pH	Toxin production (MLD/g)
Experimental pack	Jan. 1964	5.5	61.7	6.5	0
Michigan commercial No. 1, new process	Feb. 1964	7.8	57.3	6.4	0
Michigan commercial No. 2	Feb. 1964	3.0	67.2	7.0	440
Michigan commercial No. 1, old process	Sept. 1963	3.4	66.4	6.8	.....

Cl. botulinum type E tested. This result was the same for ground fish and for deeply or superficially inoculated whole fish. Results were similar in another study of commercially prepared smoked fish, similarly processed, marketed in February, 1964. All of the fish from this type of heat processing were similar in appearance and in brine and moisture levels.

In contrast to the fish described above, the product of another manufacturer, also marketed in February, 1964, was considerably different. Although actual conditions of processing of this pack are unknown, the moisture levels, brine concentration, appearance, and especially the demonstrated ability of this pack to support toxin production, did not in any way resemble the product of known 180°F processing conditions. This pack, however, did closely resemble a sample of fish produced before October 1, 1963. The latter was undoubtedly processed at temperatures much lower than 180°F and probably was quite similar to the fish implicated in the botulism incident of October, 1963, in Tennessee.

Table 9. Toxin production (MLD/g) in ground, inoculated raw fish subjected to various heat treatments.

Culture strain no.	Heat treatment			
	No heat	170°F, 30 min	180°F, 30 min	190°F, 30 min
No inoculation	220,000	0	0	0
E13	55,000	5,500	2,200	0
E32	88,000	22,000	2,200	0
E40	220,000	44,000	0	0
E41	220,000	22,000	0	0
E105	44,000	0	0	0
E110	44,000	0	0	0
16/63	220,000	44,000	0	0
1537/62	44,000	550	0	0
4318/63	220,000	2,200	0	0
FDA-No. 1	220,000	44,000	2,200	0
FDA-Minn.	44,000	660	0	0
Total no. strains producing toxin	11	9	3	0

The relationship between ability to support toxin and the oven processing of the fish was demonstrated in an experiment showing that higher processing temperatures (170°F) resulted in a product unable to support toxin production after inoculation, while fish processed at lower temperatures (130 and 150°F) did support toxin production. Thus, the evidence indicates that processing at high temperatures alters the brined fish sufficiently to make it incapable of supporting the formation of toxin by the organism.

Toxin production in ground unsalted raw fish was evident even when the fish was not inoculated. It is quite probable that many raw fish samples contain potential toxigenic organisms which, if not inhibited or destroyed during processing, would be capable of forming toxin in the finished product. The strain of organism indigenous to this particular sample of raw fish was apparently not very heat-resistant since it could not withstand 170°F for 30 min. However, at least 9 of the 11 strains used for inoculation were capable of resisting this temperature in ground raw fish. Although it had been shown previously that oven processing at 180°F for 30 min was able to prevent toxin formation in ground processed fish by 11 strains of toxigenic organisms, 3 of the strains were able to produce toxin in ground raw fish heat-processed in tubes at the same time and temperature. Thus, if heat processing alone was used to control the organism, even 180°F for 30 min would not be

effective. The highest heat process, 190°F for 30 min, was able to control all strains tested.

It is apparent that oven processing of the fish must be considered in relation to other factors such as brine content and moisture levels in order to define a process which will assure against toxin development in the finished product. Although brine and moisture contents required for toxin control in the finished fish have not been adequately established, it is probable that the oven process may exert its effect through control of those factors. The present evidence indicates that toxin can be produced in processed fish having 3% brine content whereas fish having 5.5% brine cannot support toxin production.

In past commercial practice, during normal smoked-fish processing, oven temperatures have probably varied considerably from batch to batch, and even from fish to fish in the same oven load. Also, it has been observed during this work that the brine content of fish, even from the same brine tank, may vary considerably both before and after the heat process. Thus, fish produced in the past may have ranged from being completely unable to support toxin production to a condition of brine, moisture, and heat processing which would allow toxin to be formed.

In a situation which allows a great deal of variation in processing, such as may be indicated in the commercial fish process, the antibiotic tylosin offers insurance against the production of toxin in fish which may have been in the lower range of the heat and brine process. The use of tylosin during the brining of fish in a normal commercial or slightly modified procedure will result in finished fish having predictable amounts of tylosin distributed throughout the fish. Moreover, significant amounts of tylosin, ranging to 5 or 6 ppm, can be maintained during storage of the fish for at least one month after processing whereas concentrations may be smaller after two months of storage. The effectiveness of tylosin in preventing toxin formation in processing fish has been demonstrated by the limited amount of work present in this paper. It is significant that inoculated low-heat-processed fish were protected by quantities of tylosin introduced during a normal brining operation.

Greenberg and Silliker (1962a) similarly indicated that toxin formation was prevented in deliberately underprocessed inoculated packs of canned corned-beef hash and of chili by respective initial concentrations of 5 and 10 ppm tylosin. The inoculum used in their study was 250,000 spores of *Cl. botulinum* type E per gram of product. The present study has shown that 3–5 ppm tylosin in the ground finished product is able to prevent toxin formation by a mixed inoculum of 400,000 vegetative cells and spores per gram of product. The use of tylosin lactate, together with a defined minimum heat and brine process, as an additional means of ensuring against toxin development in smoked whitefish chubs during the normal storage life of the product, is a sound proposal.

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Post-irradiation Survival of *Staphylococcus aureus* in Sea Foods

SUMMARY

***Staphylococcus aureus* is less sensitive to gamma radiation when suspended in fish homogenate or crab meat than in phosphate buffer. Comparative tests of various media confirmed that Staphylococcus No. 110 (Difco) plus egg yolk incubated at 45°C gave good recoveries of *S. aureus* from irradiated samples.**

The ability of *S. aureus* to survive and grow out in brain-heart-infusion broth, heat-sterilized crab meat, and unsterilized crab meat before and after irradiation was tested at various temperatures. The organism died out when stored at 1 and 8°C, regardless of treatment. At 12°C, *S. aureus* grew well in sterilized crab meat, reaching a count of 10^5 cells per gram after 14 days, but was greatly inhibited in nonsterile crab meat, whether irradiated or not. At 22°C (room temperature), *S. aureus* grew well and rapidly in sterilized crab meat, reaching a count of 10^9 cells per gram within three days, but was inhibited or grew poorly in untreated (nonsterile) crab meat. Apparently the inhibition of growth was due to competition by saprophytic bacteria naturally present in crab meat. The effect of such competition was largely eliminated when crab meat was irradiated at 100 and 200 Krad and *S. aureus* grew out well in such material when incubated at 22°C. There was some evidence that the ratio of staphylococci to saprophytic bacteria influenced outgrowth, but the major effect seemed to be due to the creation of inhibiting conditions in crab meat by the competing bacteria since staphylococci were completely inhibited in spoiling crab meat samples even after irradiation.

INTRODUCTION

A great variety of foods implicated in staphylococcal food poisoning have been found to contain a very high cell concentration of *Staphylococcus aureus* (Dack, 1956). The prerequisite of a food-poisoning outbreak is the presence of an enterotoxin-producing strain and its ability to grow and multiply.

The outgrowth of staphylococci is greatly influenced by chemical and physical factors. The effect of temperature and pH, as well as the presence and concentration of starch, sugars, and salt, has been studied by Peterson *et al.* (1962a,b, 1964a,b). Below 10°C the cells of *S. aureus* were unable to multiply, but pH fluctuation between 6 and 8 did not significantly influence cell multiplication. Starch, sugars, or salt favored the outgrowth of staphylococci in mixed population by inhibiting the saprophytes.

The overriding importance of the competitive growth of saprophytic bacteria in controlling the outgrowth of staphylococci has been shown by findings of Peterson *et al.* (1962b). In their studies staphylococci were held in check and eventually destroyed in the presence of an actively growing commensal microflora even at room temperature. Troller and Frazier (1963a) found that the ratio of staphylococcus to competing saprophytes might have some significance and that the efficiency of various bacteria as competitive repressors of staphylococcal growth is different. They suggested that competition for amino acids or the production of inhibitory compounds by competing microorganisms might be a significant cause of inhibition of staphylococci.

Since the natural or spoliage microflora apparently plays such a large part in controlling the outgrowth of staphylococci, treatments which alter this natural population may at the same time affect the suitability of a food as a vehicle for staphylococcal food poisoning. Substerilizing (pasteurizing) radiation treatment of sea foods is specifically designed to reduce spoilage microflora. We have therefore been concerned with the possibility of outgrowth of staphylococci in radiation-pasteurized foods.

MATERIALS AND METHODS

Organisms. *Staphylococcus aureus* strain S10A isolated from commercially processed Dungeness crab was used in all experiments. Strain No. 101, isolated from a food poisoning case in Yakima, 1958; and S6, obtained from the University of

Chicago, were also used in experiments on survival of *S. aureus* in crab meat stored at 1°C.

Inoculum. The inoculum was prepared by harvesting tryptone phytone yeast-extract (TPY) agar plates (Miyauchi *et al.*, 1963) which were surface-inoculated with a 24-hr brain-heart infusion (BHI) broth (Difco) culture and incubated 24 hr at 37°C. These cells were suspended in screw-capped test tubes containing 9 ml of phosphate buffer (Butterfield, 1932) and 5 g of sand. The cell suspension was agitated for 5 min in order to break up cell clumps, and appropriate decimal dilutions for inoculation were prepared.

Radiation sensitivity. Radiation survival of *S. aureus* S10A in phosphate buffer, fish extract, fish homogenate, and BHI broth was obtained by adding 1 ml of appropriate cell suspension to 9 ml of the suspending medium in a screw-capped tube and exposing for an appropriate length of time to gamma rays from Co^{60} Mark II food irradiator.

Suspending media. The fish extract was prepared by heating minced fish in distilled water (1:2) for 30 min at 100°C, filtering, and autoclaving. The fish tissue homogenate was prepared by blending 80 g of fresh fish tissue with 120 ml of phosphate buffer. Crab meat was obtained from a commercial plant to which the crab was delivered as live crabs or frozen in shell. Since the body meat was more suitable for our experiment than the leg meat, the former was used.

Storage study. Storage experiments were performed on Dungeness crab meat. The meat was distributed in 40-g quantities in polymylar bags, inoculated with 0.4 ml of *S. aureus* cell suspension, sealed, and irradiated. Where sterile meat was desired, the pouches were autoclaved 10 min at 10 lb pressure and cooled before inoculation and irradiation. The inoculum was chosen to yield an appropriate viable count of staphylococci after irradiation. Sampling was performed by homogenizing the entire contents of the bag with 160 ml of phosphate buffer for 2 min at high speed in a Waring blender.

Enumeration. Total viable counts of the mixed flora were obtained by the pour-plate method using TPY agar and the incubation temperature of 22°C. *S. aureus* counts in pure cultures were obtained by surface inoculation with 0.1- or 0.01-ml volumes of TPY agar, modified Staphyloccocus No. 110 (Difco) medium (S110-EY) (Carter, 1960), BHI agar, and/or blood agar (Difco) plates. *S. aureus* counts in a mixed culture were obtained by surface inoculation of S110-EY agar plates with 0.1-ml volumes. In all cases, the plates were incubated 24 hr at 37°C except the S110-EY plates, which were incubated for 48 hr at 45°C (Raj and Liston, 1961).

RESULTS

Identical *S. aureus* counts were obtained from pure cultures on TPY, BHI agar, S110-EY, and blood agar plates, regardless of suspending medium, irradiation dose, or storage time. Therefore, S110-EY was used as the standard count medium for staphylococci in all experiments.

As expected, survival of *S. aureus* was found to be proportional to radiation dose (Fig. 1).

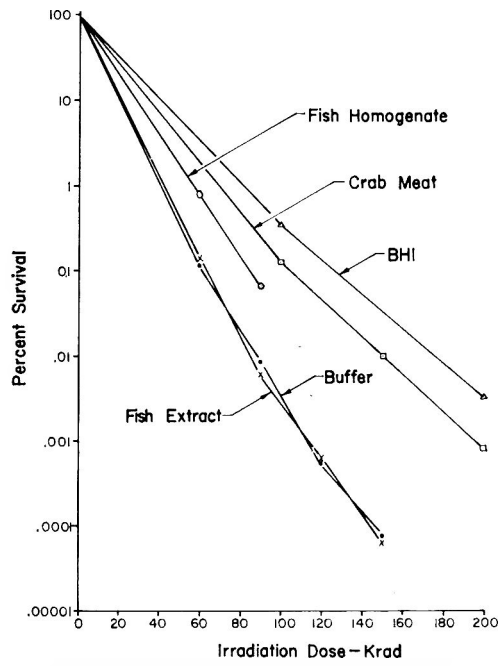

Fig. 1. Survival of *Staphylococcus aureus* S10A irradiated in different suspending media.

However, the suspending medium also affected recovery. In this regard, protection was highest with BHI broth and least with phosphate buffer and fish extract, with fish homogenate and crab meat giving intermediate levels of protection.

Storage experiments at 1 and 8°C (Figs. 2, 3, 4, 5) with BHI broth, fresh crab meat, and heat-sterilized meat show that all three strains of staphylococci tested die off slowly, whether or not a natural microflora is present. The death rate appears to increase as the initial viable cell concentration is decreased. The saprophytes, on the other hand, grew at a constant rate, reaching a maximum of about 10^8 cells per gram in 14 days at 1°C and 7 days at 8°C.

Heat-sterilized crab meat (Fig. 6) permitted good outgrowth of *S. aureus* (S10A) when stored at 12°C. A maximum cell concentration of about 10^8 cells per gram was reached in 14 days. In nonsterile crab meat (Fig. 7), staphylococci were

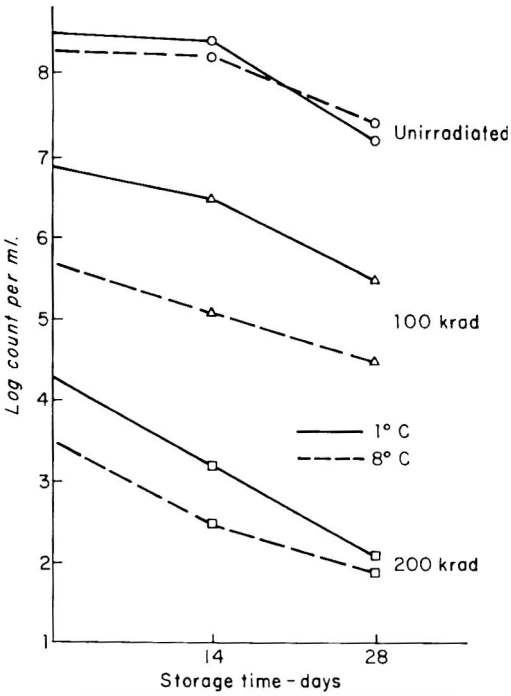

Fig. 2. Survival of *Staphylococcus aureus* S10A irradiated in brain-heart-infusion broth and stored at 1 and 8°C.

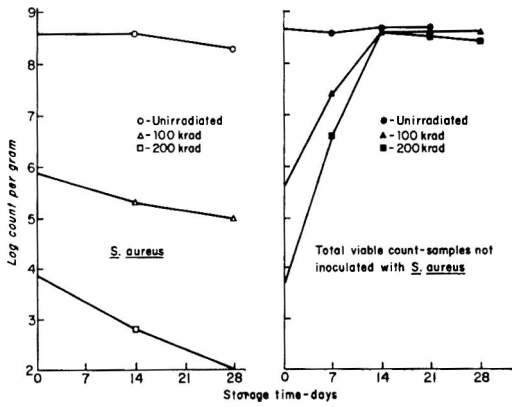

Fig. 4. Survival and outgrowth of saprophytes and *Staphylococcus aureus* S10A irradiated in fresh crab meat and stored at 1°C.

greatly inhibited by the spoilage organisms, which grew rapidly, reaching a maximum cell concentration of 10^8 cells per gram in 7 days.

Staphylococci grew abundantly in sterilized crab meat stored at room temperature (22°C), reaching a maximum of 10^9 cells per gram within three days. Inhibition of *S. aureus* (S10A) was observed in unirradiated samples. This occurred

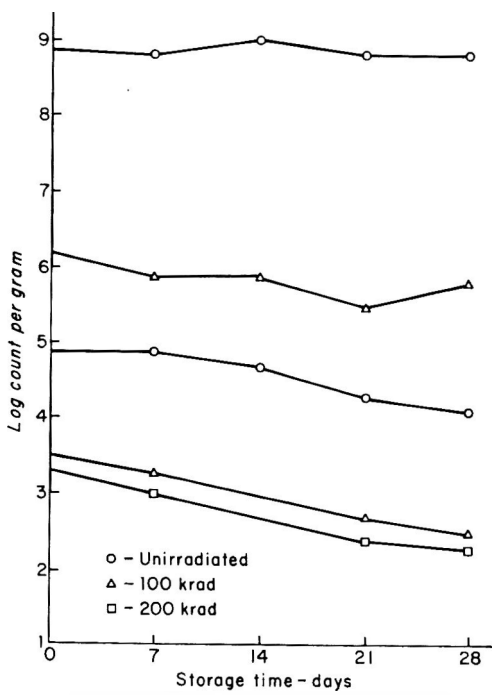

Fig. 3. Survival of *Staphylococcus aureus* S10A at different cell concentrations irradiated in heat-sterilized crab meat and stored at 1°C.

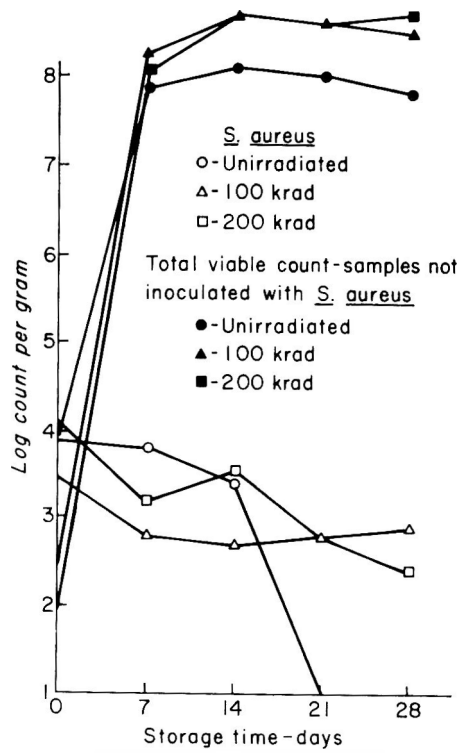

Fig. 5. Survival and outgrowth of saprophytes and *Staphylococcus aureus* S10A irradiated in fresh crab meat and stored at 8°C.

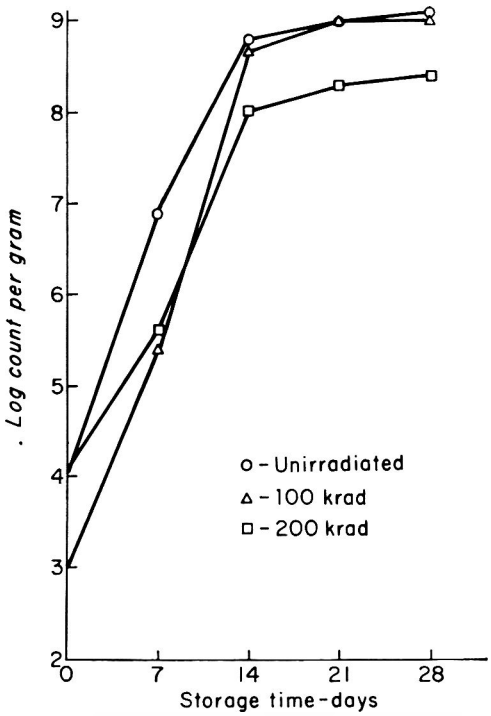

Fig. 6. Outgrowth of *Staphylococcus aureus* S10A irradiated in heat-sterilized crab meat and stored at 12°C.

when the ratio of staphylococci to spoilage organisms varied from 25:1 to 1:44 (Figs. 8, 9, 10). There is some evidence also that the actual initial level of saprophytes itself may be important (*cf.* Figs. 8, 9). In the irradiated samples the staphylococci were not inhibited and reached a maximum cell concentration of about 10^8 cells per gram within three days (Figs. 8, 9). Here the ratio of *S. aureus* to saprophytes varied from 290:1 to

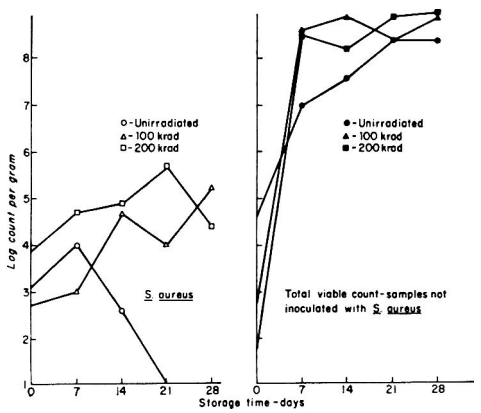

Fig. 7. Outgrowth of *Staphylococcus aureus* S10A and saprophytes irradiated in fresh crab meat and stored at 12°C.

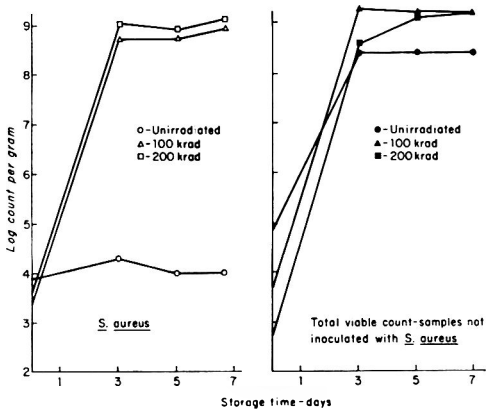

Fig. 8. Outgrowth of *Staphylococcus aureus* S10A and saprophytes irradiated in fresh crab meat and stored at 22°C.

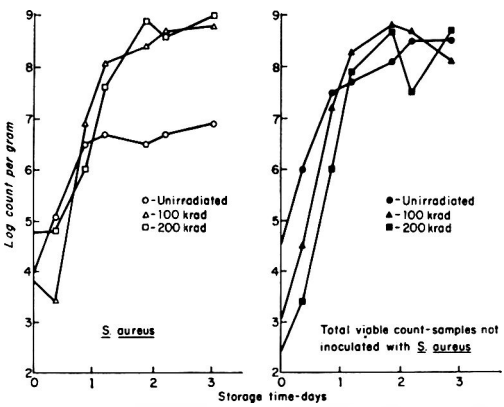

Fig. 9. Outgrowth of *Staphylococcus aureus* S10A and saprophytes irradiated in fresh crab meat and stored at 22°C.

1:2. Similar results are shown in Fig. 11 except that the samples having an estimated *Staphylococcus* to spoilage organism ratio of 1:1 up to 10:1 did not grow out as well as in the previous experiments.

S. aureus (S10A) did not grow out in crab meat in an advanced stage of spoilage even when the saprophytes had been reduced in numbers by irradiation (Fig. 12), yielding the relative concentration of staphylococci to saprophytes of 440:1. Here the freshly processed crab meat was stored at 22°C for 24 hr before inoculation with *S. aureus* and irradiation. The total viable count of spoilage organisms was 1×10^7 per gram before irradiation and 1.9×10^4 per gram after irradiation.

DISCUSSION

As expected, the sensitivity of *S. aureus* to radiation was to some extent dependent on the suspending medium. Food materials

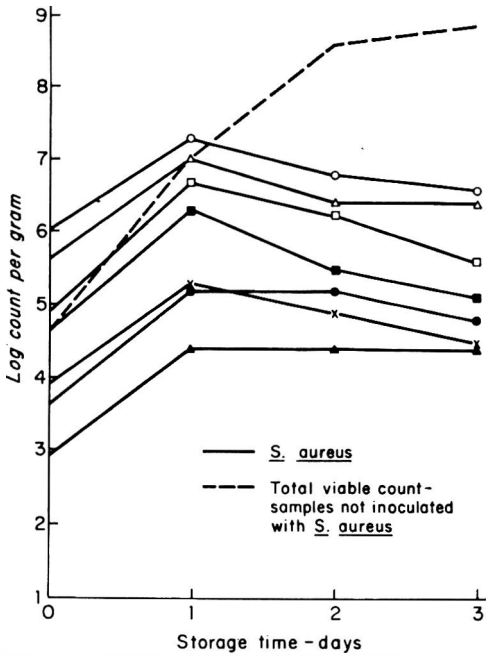

Fig. 10. Outgrowth of *Staphylococcus aureus* S10A inoculated at different cell concentrations into fresh crab meat and stored at 22°C.

(crab meat and fish) offered some degree of protection as compared to phosphate buffer, but did not permit as much recovery

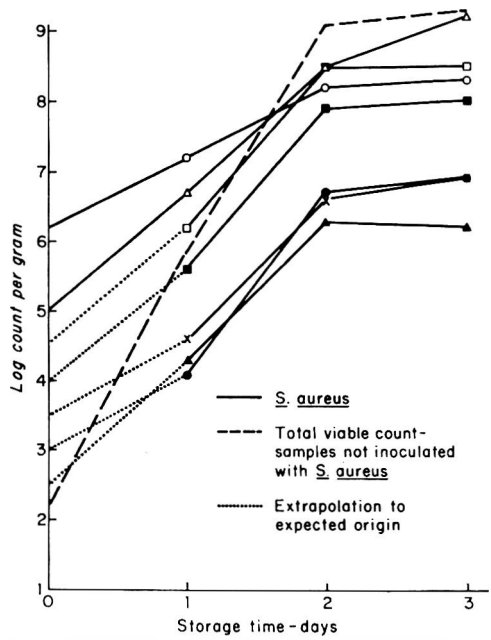

Fig. 11. Outgrowth of *Staphylococcus aureus* S10A inoculated at different cell concentrations into fresh crab meat, irradiated (200 Krad), and stored at 22°C.

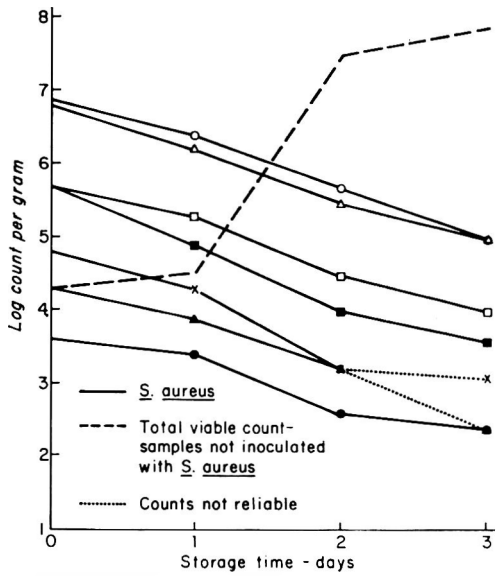

Fig. 12. Survival of *Staphylococcus aureus* S10A inoculated at different cell concentrations into spoiling crab meat, irradiated (100 Krad), and stored at 22°C.

as did BHI broth. Levels of recovery, maximum of 0.5% and minimum of 0.005% at 100 Krad, were lower than those reported by Ingram and Thornley (1959) with chicken meat (8%) and by Erdman *et al.* (1961) for broth (7.16%) and ground beef (13.1%). This difference may be due to variation in strain sensitivity or to an intrinsic difference in the protective capabilities of the suspending media used.

In our experiments, staphylococci failed to grow at either 1 or 8°C under any conditions tested. This is in accordance with findings of Peterson *et al.* (1962a,b, 1964a,b) and probably represents an intrinsic temperature limitation on growth (Angelotti *et al.*, 1961). Good growth was observed with the test strain in sterilized crab meat at 12 and 22°C in the absence of a competing microflora of spoilage bacteria. When added to untreated crab meat and incubated at 12°C, however, staphylococci rapidly disappeared, and even at 22°C under these conditions staphylococci inoculated at different levels showed only slight and generally rather transient growths (Figs. 8, 9, 10). These findings also correspond quite closely to observations of Peterson *et al.* (1962b). Apparently, therefore, at tempera-

ture ranges which will permit growth of staphylococci, the microflora present on crab meat competes so effectively that it suppresses staphylococcal growth.

The efficiency of competitive repression of staphylococcal outgrowth is apparently affected by radiation treatment since strain S10A grew out in samples irradiated at 100 and 200 Krad and stored at 12 or at 22°C (Figs. 7, 8, 9, 11) even though there was significant survival and rapid parallel outgrowth of the natural saprophytic bacteria. However, outgrowth of staphylococci at 12°C was restricted as compared with growth in a sterile sample (Fig. 6), indicating some residual competitive ability at this storage temperature, though this was not apparent in comparable tests at 22°C. Since work of Troller and Frazier (1963a) has suggested that the ratio of staphylococci to competitive bacteria may be important, tests were run at 22°C to investigate this point. There was some indication of such an effect in terms of the final level of staphylococci attained (Fig. 11). But an increase in the *Staphylococcus*-saprophyte ratio is not sufficient by itself to account for the outgrowth of staphylococci after irradiation and it is necessary to consider the possibility of qualitative changes in the competing microflora.

This effect is also suggested by Troller and Frazier's findings (1963a,b) concerning the relative competitive efficiency of particular strains of bacteria. Radiation at pasteurizing dose-levels certainly brings about significant changes in the composition of the bacterial populations on foods (Shewan and Liston, 1958; Masurovsky *et al.*, 1963). This is indicated in the growth patterns observed in counts on unirradiated crab meat (Figs. 7, 8, 9). Qualitative studies in our laboratories of bacteria on crab meat before and after irradiation and during storage have confirmed that there is a change in the composition of the microflora.

The actual selection of the saprophytic microflora involved in competitive repression has not been identified. However, the experiment in which spoiled crab meat was used as test medium (Fig. 12) provides some evidence of its nature and perhaps of its mode of action. Apparently the organism or organisms increase during normal chill storage of crab meat and can be assumed to be part of the spoilage microflora. These organisms, as a result of their growth, create conditions in the crab meat which are inhibitory toward staphylococci. Spoiling crab meat which was irradiated after the addition of staphylococci at various levels did not support growth of these organisms, and indeed showed a decline in staphylococcal count even before significant growth of the surviving saprophytes had occurred. It seems probable that some substance or substances produced before irradiation exert a toxic or at least inhibitory effect on the staphylococci, though, less likely, it is also possible that repression is due to exhaustion of some substance essential for the growth of staphylococci by the saprophyte population.

In conclusion, therefore, it appears that competitive growth by saprophytic bacteria naturally present on crab meat will repress growth of staphylococci. This growth repression is greatly reduced by irradiation at pasteurizing dose levels. Radiation appears to selectively eliminate organisms involved in competitive repression of staphylococci. This is to be expected since such organisms appear to be part of the normal spoilage flora. Some evidence has been obtained to indicate that repression is probably due to the production of a substance inhibitory toward staphylococci.

From the practical point of view it is important to note that radiation-pasteurized crab meat, properly handled, represents no greater hazard than untreated crab meat. Dose levels of 200 Krad are sufficient to eliminate all expected levels of staphylococcal contamination. Moreover, as shown in this report, staphylococci are quite unable to grow out in mixed populations at refrigeration temperatures.

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The Growth and Persistence of Staphylococcus aureus in Milk and Broth Substrates

SUMMARY

The growth of two strains of coagulase-positive *Staphylococcus aureus* was measured in whole and skimmilk and in broth at 5, 10, 22, 30 and 37°C. There was a continuous decrease in population in all media at 5°C. The medium had little effect on the duration of the logarithmic growth phase which varied from 12 hr at 37°C to 20 days at 10°C. Typical generation times in whole milk varied from 1642 min at 10°C to 45 min at 37°C. At 10 and 22°C there was no statistically significant difference in the generation times among media or between strains. At 30 and 37°C there was no significant difference between the generation times in skimmilk and whole milk, but the generation time was significantly shorter (at the 5% level) in the broth than in the milks. The cultures maintained a longer stationary growth phase and were more persistent in broth and skimmilk than in whole milk. More rapid decreases in population in the whole milk were attributed to the toxicity of fatty acids released by the lipase of *S. aureus*. The decline in pH of the substrate was approximately proportional to the increase in population.

INTRODUCTION

The growth or lack of growth of microorganisms is one of the most important factors involved in food processing and storage. The most numerous and persistent of the many types of disease outbreaks traced to food is poisoning caused by a toxin elaborated by enterotoxigenic coagulase-positive strains of *Staphylococcus aureus*. According to Dauer (1961), during the period of 1952 to 1960 there were 733 outbreaks of staphylococcal food poisoning reported, involving 28,332 persons. Informed medical authorities estimate that the actual number of outbreaks may have been 10–50 times as great.

In recent years there has been a greater incidence of staphylococci in milk, which is attributed to the ability of these organisms to tolerate the antibiotics used in mastitic therapy and to occasionally become the predominant organisms in the interior of the udder. The increasing interest in sterilized milk, the importance of milk and other dairy products in the formulation of a variety of foods, and the fact that dairy products have been involved in food poisoning make it imperative that we know the rate, amount and persistence of growth of coagulase-positive staphylococci in milk substrates.

LITERATURE REVIEW

Several investigators have studied the growth of staphylococci in various dairy products. George *et al.* (1959) determined that the growth of *S. aureus* strains 196-E and 1363 at 99°F was essentially the same in condensed skimmilk that had been subjected to precondensing heat treatments of 150, 165, or 185°F. Heinemann (1957) reported that raw milk was a poor growth substrate for *M. pyogenes* var. *aureus (S. aureus)* but that milk, either pasteurized or preheated 20 min at 205°F and condensed to 42% solids, supported rapid growth over a temperature range of 80 to 115°F. Jones *et al.* (1957) found that milk containing a natural staphylococcal infection, and milk aseptically drawn from healthy cows and inoculated with *S. aureus* supported good growth of the organisms at 30 and 37°C, but slow growth below 25°C. Takahashi and Johns (1959) reported that milk with standard plate counts of 33,000 to 260,000 per ml supported limited growth of *S. aureus* at 22°C and active growth at 32°C, but milk with a count of 5,100,000 per ml supported only limited growth of the organisms at 32°C. Clark and Nelson (1961) found no increase in the population of staphylococci in milk incubated 7 days at 4°C when the samples had an original staphylococcus count of 25–3,300 per ml, but at 10°C increases of up to 1000-fold in 7 days were noted.

METHODS AND MATERIALS

Several strains of coagulase-positive *S. aureus* were used to determine the effect of temperature and growth substrate on the population reached, generation time, length of logarithmic and stationary growth phases, and persistence of the organisms in the substrates. The data on two strains

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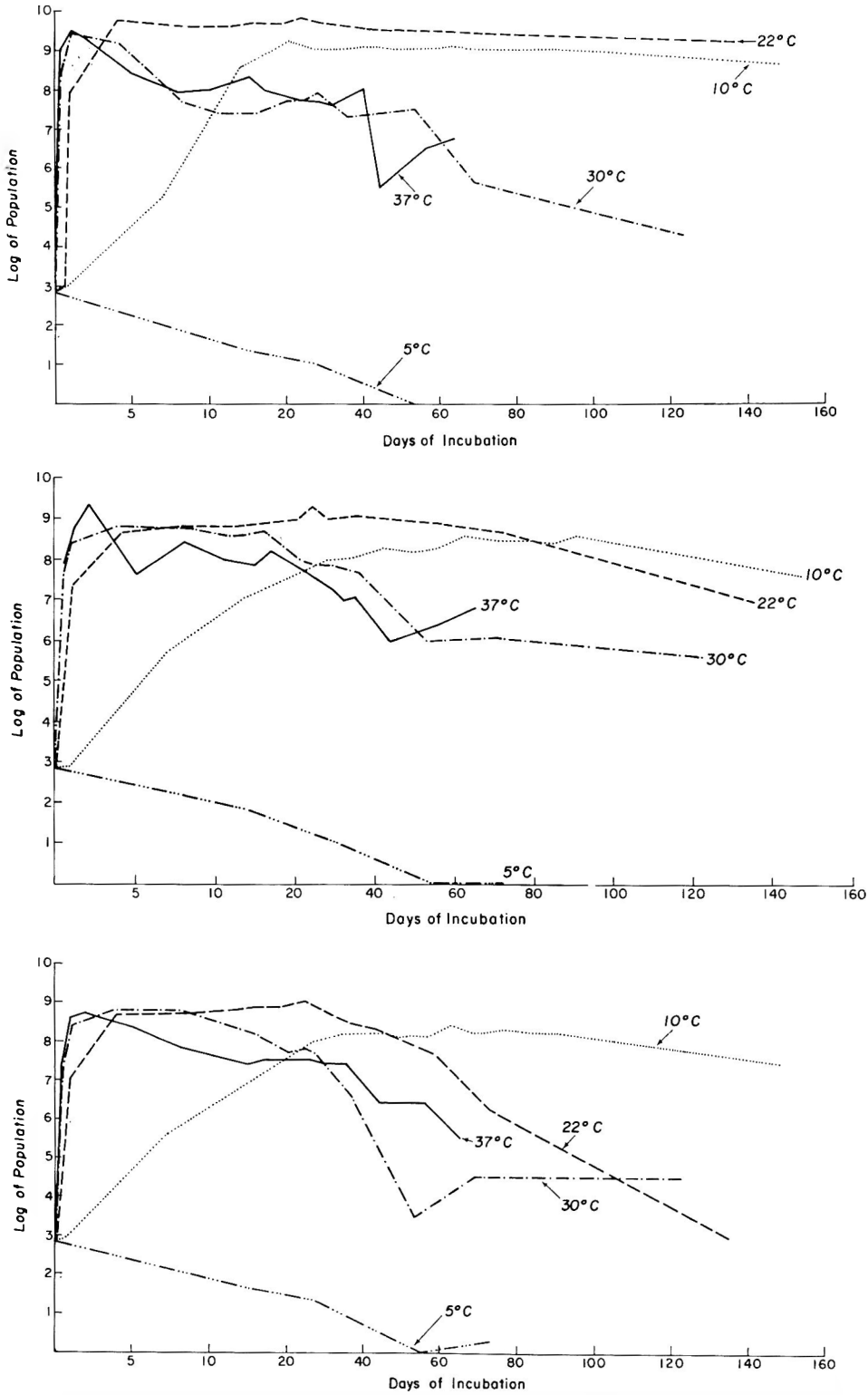


Fig. 1. Changes in the number of *Staphylococcus aureus* strain 161-C during incubation at various temperatures in the medium indicated (top, broth; middle, skimmilk; bottom, whole milk).

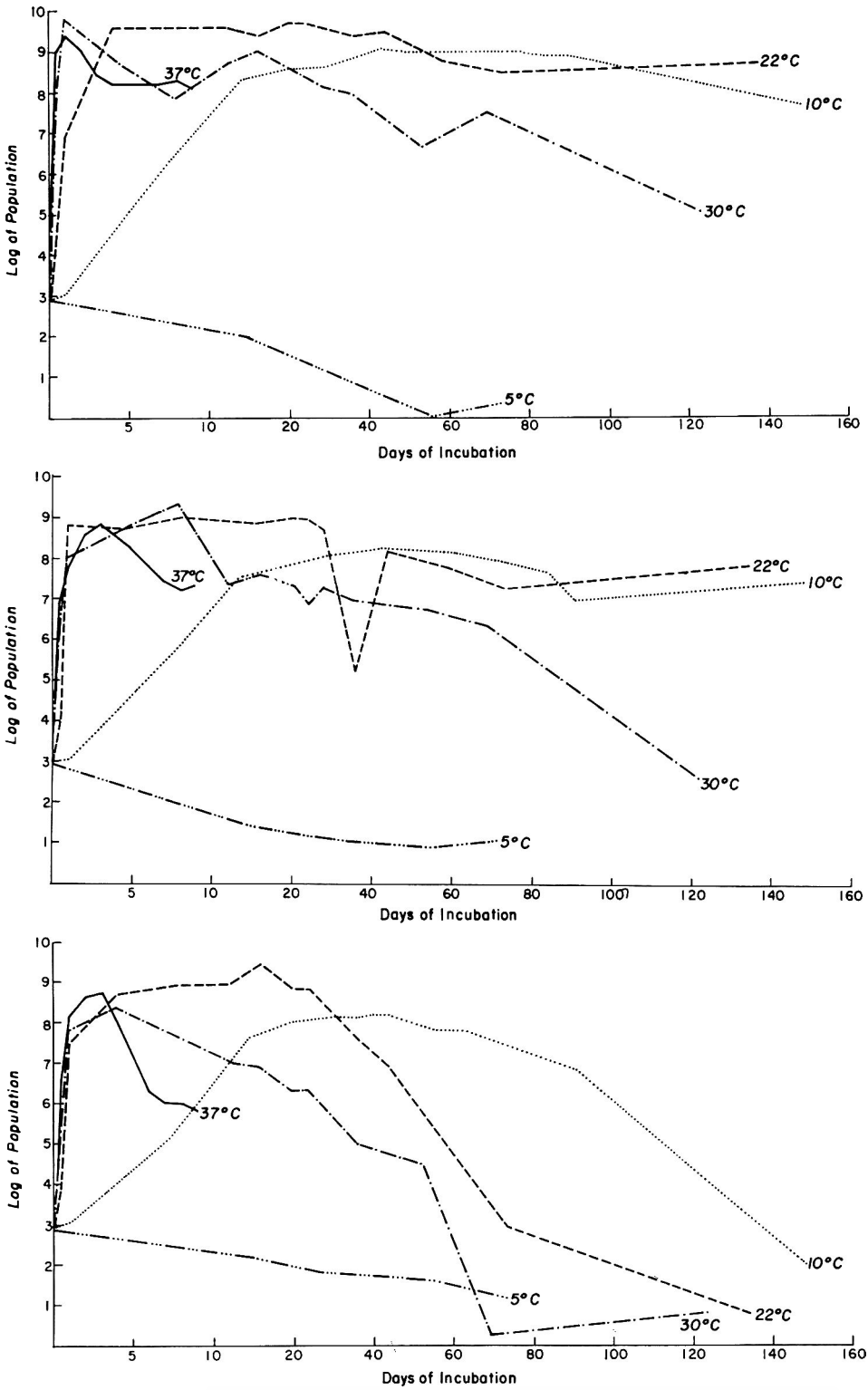


Fig. 2. Changes in the number of *Staphylococcus aureus* strain S-1 during incubation at various temperatures in the medium indicated (top, broth; middle, skimmilk; bottom, whole milk).

were selected as representative of the information obtained. Strain 161-C had been identified with a food poisoning outbreak and was obtained from the R. A. Taft Sanitary Engineering Center, 4676 Columbia Parkway, Cincinnati 26, Ohio. The other strain, designated S-1, was isolated from the milk from a cow with subclinical mastitis. Both strains conformed to the biochemical and morphological characteristics of the staphylococci listed by Breed *et al.* (1957).

Active cultures were maintained by frequent transfer in broth composed of 3.7% brain heart infusion, 2% mannitol, and 1% yeast extract. Two percent agar was added to prepare a solid medium for plate counts.

Prior to starting a growth trial an aliquot of a broth culture incubated for 12 hr at 37°C and containing in excess of 10^8 cells per ml was diluted with sterile buffered distilled water to obtain an inoculum ranging from 510 to 980 organisms per ml of growth substrate. One-hundred-ml portions of the inoculated growth media (sterile skimmilk, whole milk, and broth) were incubated at 5, 10, 22, 30 and 37°C. At appropriate intervals the inoculated substrates were removed from incubation, agitated vigorously for 5 min on a mechanical shaker, sampled for enumeration of organisms and pH of substrate, and returned to incubation. The total time out of incubation was approximately 7 min at each sampling interval. Populations were determined on agar plates containing the medium described above and using the quantitative surface inoculation technique of Punch and Olson (1961). The plates were incubated 48 hr at 37°C.

RESULTS AND DISCUSSION

Figs. 1 and 2 respectively show the trend in the growth of cultures 161-C and S-1 in broth, skimmilk and whole milk at the temperatures indicated. There was a continuous decrease in population in all media at 5°C. The medium had little effect on the duration of the logarithmic growth phase, which varied from 12 hr at 37°C for both strains, to 13 days at 10°C for strain 161-C and 20 days at 10°C for strain S-1.

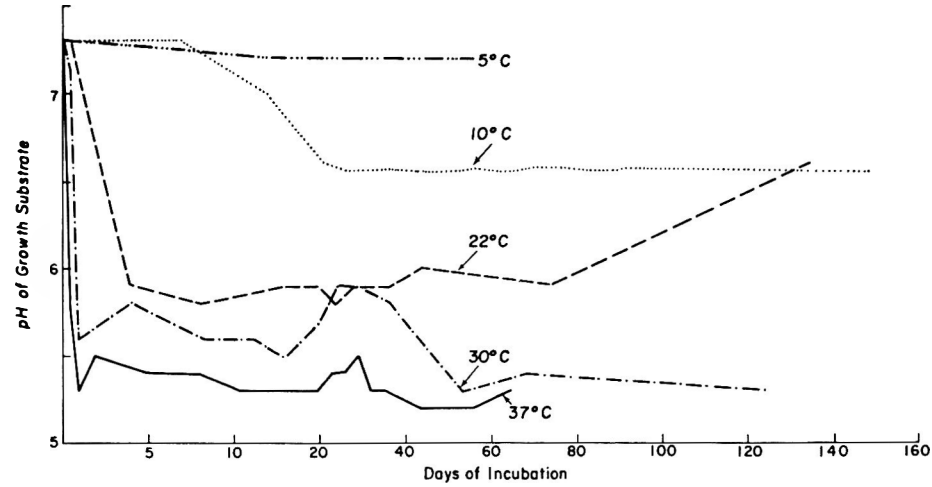
The generation times calculated from the data obtained during the logarithmic growth phase are summarized in Table 1. The influence of temperature on the generation time of the organisms would be anticipated. For example, in whole milk the generation time of strain 161-C was 15.2 times as long at 10 as at 22°C, and 2.1 times as long at 22 as at 30°C. With strain S-1 the generation time was 12.6 times as long at 10 as at 22°C and 1.5 times as long at 22 as at 30°C. The generation times of both strains in all three media were only 10–20% longer at 30 than at 37°C. These times are longer than those reported by Yotis and Teodoro (1957) who found generation times for *S. aureus* of 30.4 min at 30°C and 23.2 min at 37°C when milk was inoculated with 1–6 cells per ml; however, those workers selected the 3-hr interval within the logarithmic phase during which the generation times were minimum.

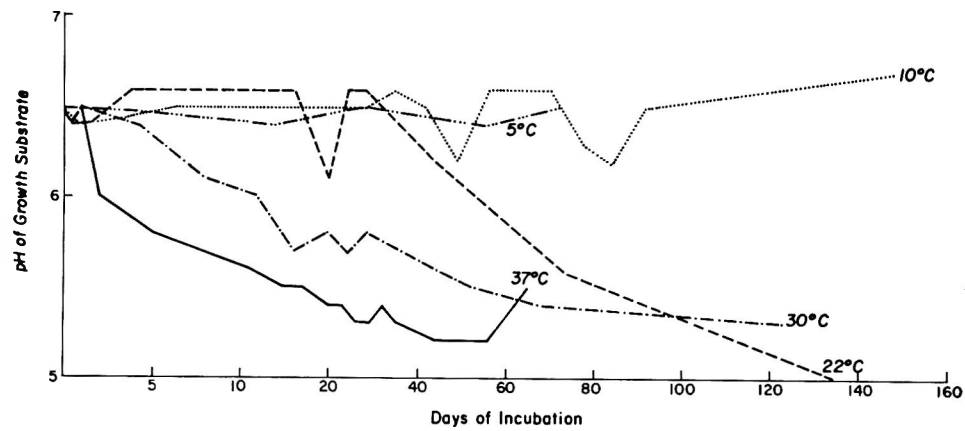
Table 1. Generation times (min) of *S. aureus* 161-C and S-1 at selected temperatures in broth, skimmilk and whole milk.

Growth medium	Temperature (°C)			
	10	22	30	37
Strain 161-C				
Broth	1051	108	38	34
Skimmilk	1066	102	46	42
Whole milk	1642	102	49	45
Strain S-1				
Broth	1152	126	44	35
Skimmilk	1130	125	66	58
Whole milk	1454	114	75	64

An analysis of variance of the data in Table 1 showed that at 10 and 22°C there was no statistically significant difference in the generation times among media or between strains. At both 30 and 37°C there was no significant difference between the generation times in skimmilk and whole milk, but the generation time was significantly shorter (at the 5% level) in the broth than in the milks. Also, the maximum populations attained were slightly higher in the broth than in the milks. There was no consistent difference between the maximum populations reached in the two milks. The cultures were routinely carried in broth and may have been better adapted to broth than to milk.

Allison (1949) and Wilson *et al.* (1959) postulated that populations of 500,000 coagulase-positive *S. aureus* per ml or g may produce sufficient enterotoxin to cause food poisoning. Unfortunately the population of coagulase-positive *S. aureus* present in a substrate at a given time is not a valid index of the potential hazard because the toxin may accumulate and remain in the substrate after the cells are destroyed. According to Dack (1956) the toxin is sufficiently heat stable to





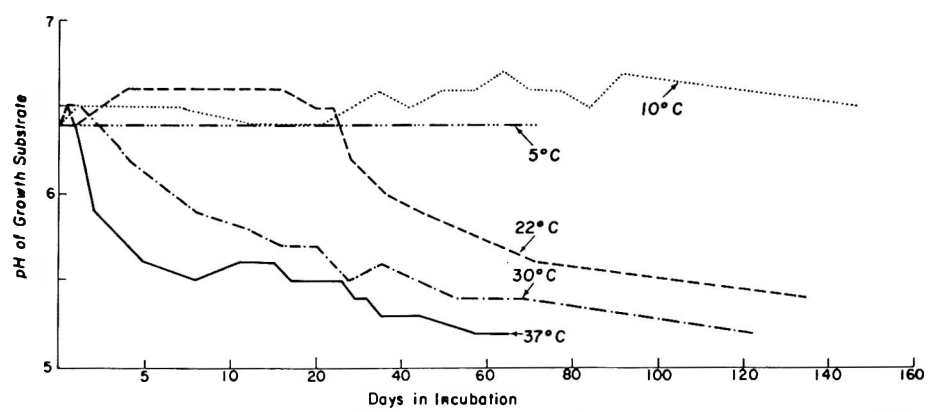


Fig. 3. Changes in the pH of substrates inoculated with *Staphylococcus aureus* strain 161-C and incubated at various temperatures (top, broth; middle, skimmilk; bottom, whole milk).

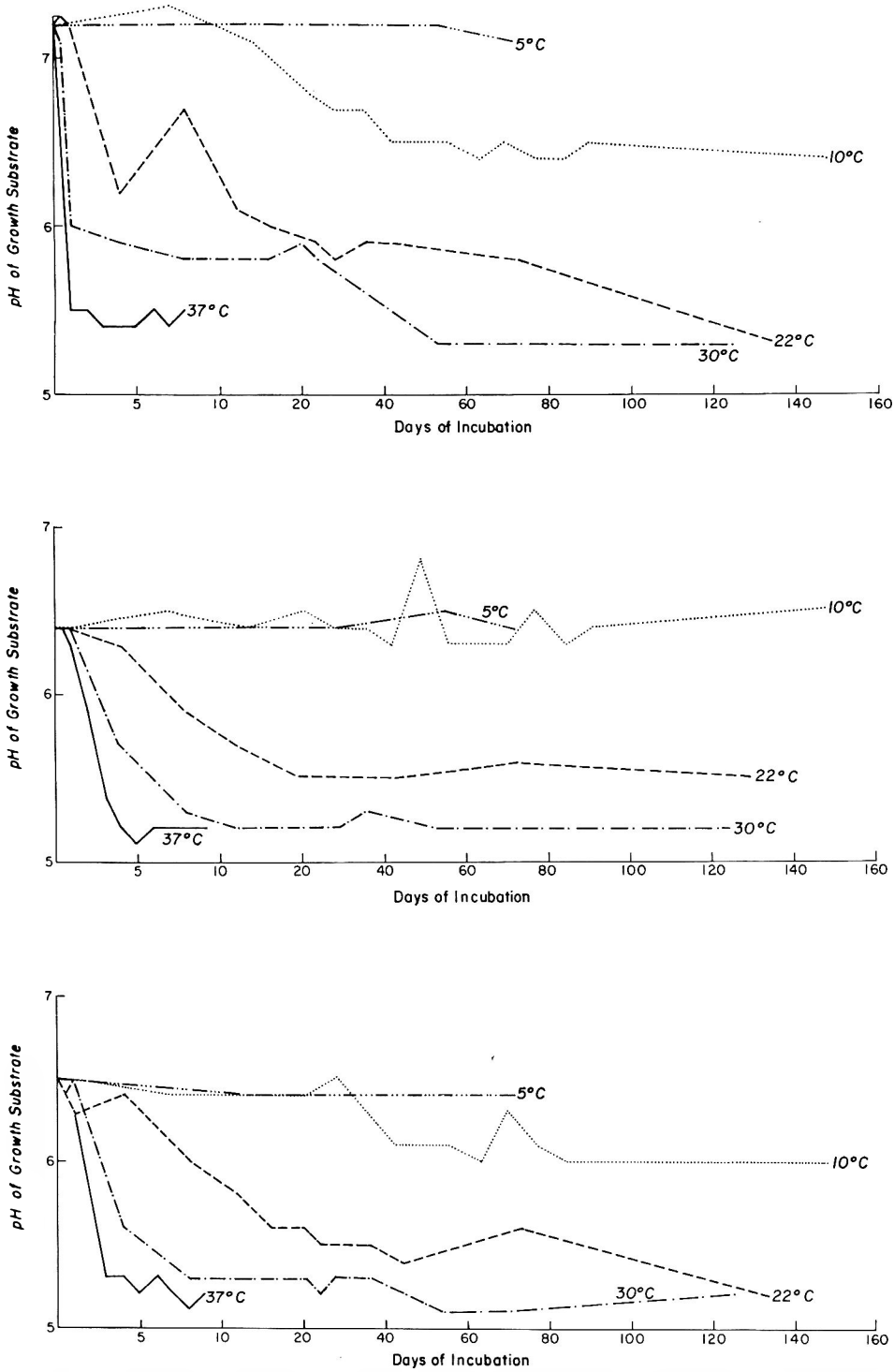


Fig. 4. Changes in the pH of substrates inoculated with *Staphylococcus aureus* strain S-1 and incubated at various temperatures (top, broth; middle, skimmilk; bottom, whole milk).

withstand autoclaving for 20 min at 250°F, and Jordan *et al.* (1931) reported that it was not destroyed by exposure to 0.09% chlorine solution for 3 min. The ability of staphylococci to cause food poisoning depends on environmental factors such as susceptibility of the individual person, chemical composition of the food, temperature, pH, air supply and the number and type of competitive organisms.

The high populations resulting in the inoculated sterile substrate used in this work do not necessarily imply that similar results would occur in normal pasteurized or raw milk containing mixed microbial populations of several hundred or thousand per ml. In such milk one would expect sufficient activity from miscellaneous competitive organisms to cause unpalatability before dangerous levels of toxin could be formed by the staphylococci. However, the data in Figs. 1 and 2 show the potential hazard of *S. aureus* contamination in poorly refrigerated sterile milk, or perhaps even in low-count milk which is subsequently held at time-temperature combinations commonly used in the manufacture of cheese or condensed milk.

The stationary and death phases of *S. aureus* in the three media were not well defined but the cultures exhibited a longer stationary phase and more persistence in broth and skimmilk than in whole milk. The more abrupt decrease in population in whole milk at all temperatures permitting growth is attributed to the toxicity of fatty acids released by the lipase of *S. aureus*. These samples had a distinctly rancid, butyric acid aroma after incubation for 2 or 3 days at 30 and 37°C. The elaboration of lipase from the staphylococci has also been noted by Trussel and Weed (1937) and Clark *et al.* (1961). Several reports in the literature including Kodicek and Worden (1945), Humfeld (1947), and Neiman (1954), attest to the toxicity of the fatty acids for the staphylococci.

As anticipated, the decline in pH is approximately proportional to the increase in population of the organisms (Figs. 3 and 4). Strain 161-C ferments lactose very slowly, a characteristic determined by appropriate tests performed during characterization of the organism. This slow fermentation of lactose, which is apparent from observation of the data in Figs. 3 and 4, probably accounts for the longer time required by strain 161-C to attain peak populations in milk and the greater persistence of this strain during prolonged incubation in milk.

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Control of Protein Level of Algae, Chlorella

SUMMARY

***Chlorella pyrenoidosa* No. 71105, grown under limited nitrate conditions (1m*M* NO_3^-) had protein levels of 21–64% of the total dry weight when grown in continuous culture, and only 5–20% protein when grown as stationary cultures. Light limited both the protein fixation and the total production of dry algal tissue. Protein per liter increased with light, but at a slower rate than total dry weight, resulting in yields of greater protein per liter but cells of lower percentage of protein. Up to limiting concentrations of nitrate the algal yield (dry matter) was directly proportional to light received. The conversion of light energy to net chemical energy was between 3 and 13%.**

INTRODUCTION

Spoehr and Milner (1949) demonstrated that the composition of *Chlorella pyrenoidosa* (Emerson's strain) responded to culture conditions, and particularly to fixed nitrogen in the form of NH_4^+ or NO_3^-. Cells containing 8.7–58.0% protein could be grown on media containing 2.5 and 25.0m*M* nitrate during periods of 15–75 days. The lipid content, which increased with decreasing protein levels, was analyzed by Milner (1948) who suggested that mass *Chlorella* culture was a possible means of lipid manufacture.

Continuous cultures of controlled quality were desired to supply a daily source of fresh cells for nutritional and ecological studies. A thermophilic strain, *Chlorella pyrenoidosa* No. 71105 Sorokin and Myers (1953), was chosen because of its fast growth and because of the extensive study of its composition and culture as a component in space air-food regeneration systems (e.g., Lubitz, 1961).

This study was made to devise a continuous method to supply a daily yield of algae cells of different protein levels. It was necessary to determine the relationship between retention times, illumination, and nutrient, which produced algae of predictable composition.

METHODS

Apparatus. The continuous-culture assembly (Fig. 1) consisted of: a) 18-L Pyrex carboys in which the media were prepared, autoclaved, and stored; b) Sigma finger pump (TM 11) to provide continuous delivery of culture medium; c) 2-L Erlenmeyer flasks, each containing 1 L of culture.

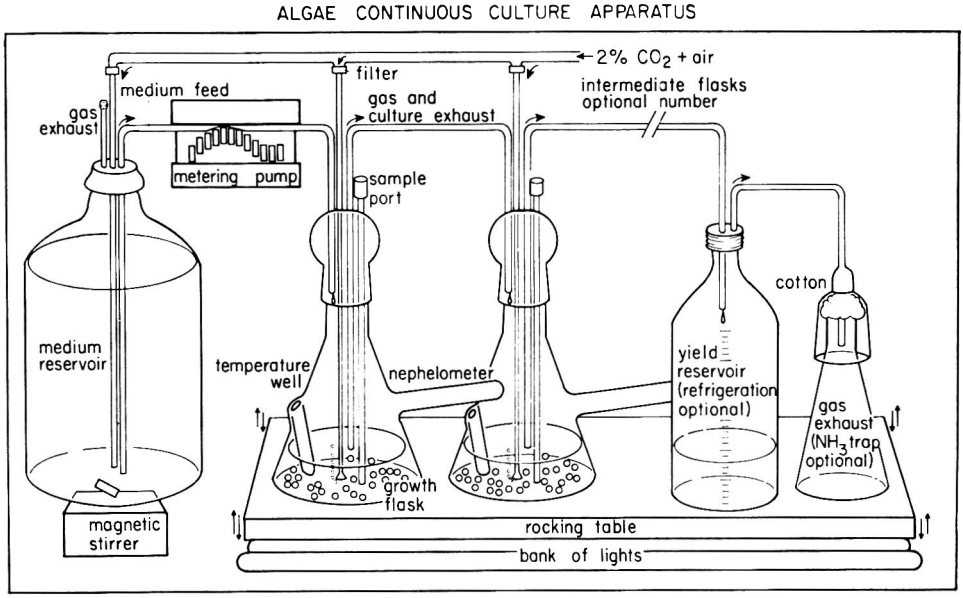

Fig. 1. Continuous-culture apparatus.

connected in parallel or in series; and d) 1-L receiving flasks stored either in the growth chamber in the light or at 5°C in the dark. Tubing was either Tygon or surgical rubber. Incubation was controlled at 38±2°C. Light was supplied to the flasks resting on a plexiglass sheet at 200 or 400 ft-c (color-corrected meter, G.E. 213) by two or four "Deluxe Cool White" General Electric fluorescent bulbs (40 w) located 11.5 cm below the flasks. Filter-sterilized air, enriched with 2% CO_2, was supplied from pressurized cylinders at a flow rate of about 200 ml/min/flask. Flow rate was determined by displacement in a gas burette. The cultures were agitated by rocking in a 15° arc at 17 cycles/min. The illuminated area (flask bottom) was self-cleaned by glass beads which rolled over the bottom as the cultures were rocked.

Periodically, aliquots of cultures were examined microscopically after gram staining, or were inoculated into nutrient broth, brain-heart infusion, and Sabouraud's medium. No microbial contamination was found.

Experimental cultures. *Series I.* The culture medium was supplied at a steady flow rate of about 1 L per day to each of two parallel systems: 1-A, which had three culture flasks in series; and 1-B, which had a single culture flask. In the 1-A system, the medium was introduced into the first culture flask; its overflow drained into the second growth flask; its overflow drained into the third growth flask; its overflow drained into a refrigerated receiving flask (4–7°C, dark). In system 1-B, the overflow from the single growth flask was stored in a separate refrigerated receiving flask. The algae cultured in system 1-A had a 3-day retention time and had therefore received 3-fold light energy. The cultures were illuminated for 24 days at 200 ft-c and then the illumination was changed to 400 ft-c for an additional 6 days. Cell counts (hemacytometer) and pH measurements were made daily. After the cultures became stable, the suspensions were stored several weeks at 4°C in the dark until analyzed.

Series II. Six culture flasks connected in series similar to 1-A, the last of which served as a removable yield reservoir, illuminated at 200 ft-c, were supplied with 1 L of culture medium per day. Five-hundred-ml samples were taken for analysis from each of the flasks after 13 days of culture, by which time cell concentrations were stable. The cultures were continued for 4 additional days, then the medium was discontinued. The cultures were permitted to grow as stationary cultures for 5 additional days and at this time were refrigerated for later analysis.

Culture media. The medium met the growth requirements of *Chlorella* and was nontoxic to *Daphnia* (Taub and Dollar, 1964). In Series I the medium consisted of $NaNO_3$ 1.0m*M*; $MgSO_4$ 0.2m*M*; KH_2PO_4 0.2m*M* with NaOH 0.07m*M*; $CaCl_2$ 0.1m*M*; NaCl 3.0m*M*; $FeSO_4$ 0.01m*M*, with EDTA 0.01 m*M* and NaOH 0.03 m*M*; H_3BO_3 0.03m*M*; $ZnSO_4$ 0.001m*M*, $MnCl_2$ 0.01m*M*; Na_2MoO_4 0.001m*M*; $CuSO_4$ 0.0002m*M*; and $Co(NO_3)_2$ 0.0001m*M*. In Series II, the $CaCl_2$ content was increased to 1.0m*M*. The pH is *ca.* 6.5. These media differ from most algal media by the predominance of Na^+ rather than K^+ and the relatively low H_3BO_3 content. To prevent precipitation during autoclaving, the medium, exclusive of the $CaCl_2$ and of the Fe solution, was autoclaved 1 hr at 250°C. The $CaCl_2$ solution was autoclaved separately and the iron solution was sterilized by filtration, and these were added shortly before use.

Methods of analysis. Most of the cells were collected by centrifuging at 4°C at 15,000 rpm, with a flow rate of approximately 1 L per hr, using a Szent-Györgyi and Blum continuous-flow attachment on a Servall RC-2 centrifuge. Cells remaining in the supernatant were harvested by filtration on H.A. .45 μ Millipore filters. Dry weight was determined by vacuum drying at 60°C. Ash weights were determined for Series II only.

Total nitrogen in the cells was determined with the semimicro Kjeldahl method. Complete conversion of the 1m*M* $NaNO_3$ to protein would yield 87.5 mg of protein/liter (1 meq. N/liter × 14 mg/meq. × 6.25 mg protein/mg N).

Nitrate and nitrite analyses were made on the cell-free spent media by the Strickland colorimetric method (1960).

Light energy. Light intensity was measured at the bottom of the culture flasks as either 200 or 400 ft-c (measured by G.E. 213 meter, color- and cosine-corrected). The illuminated area of each flask was 200 cm^2.

The light energy accumulated has been expressed in arbitrary units: One light-unit = 200 ft-c received over 200 cm^2 per day. While such arbitrary units permit an evaluation of the relative utilization of light in these systems, they cannot be used to estimate the percentage of energy conversion, nor can they be compared readily with other growth systems. To convert the light into energy units, several approximations and assumptions must be made. The wavelength distribution of the "Deluxe Cool Light" tubes is moderate over the ranges of 3,000 to 4,500 Å and 7,000 to 7,500 Å, and is high over the range of 4,500 through 6,500 Å. The color-corrected light meter is relatively insensitive to wavelengths less than 5,000 Å or greater than 7,000 Å. A correction factor was determined by integrating the published values for both the lights and the light meter, and it was found that the color-corrected light meter underestimates the true light intensity by a factor of

about 3. If 5,560 Å is taken to be the average wavelength, then 1 lumen = 0.000385 g calories/second (Hodgman, 1951), or 33.264 g calories/day. Two hundred ft-c received by one flask, 0.02 square meters, is equivalent to 129 lumen. Therefore, the total energy received during one day would be 4.3 kilocalories.

Yield. Yields have been calculated to represent 1 L of culture, which is approximately the daily yield. The retention time (ratio of the volume of medium introduced to culture volume) is 1 day for the first flask, 2 days for the second flask, etc.

RESULTS AND DISCUSSION

Complete data are shown in Table 1.

Series I. 200 and 400 ft-c; 1.2–4.8 days retention. The yield of dry cellular material for light units to 3.8 was directly related to the light received, irrespective of whether this was accomplished by doubling the light intensity or by increasing the retention time; *ca.* 29 mg, dry weight of cells, were produced per light unit (Fig. 2). This linear relationship is in agreement with Pipes and Koutsoyannis (1962), who defined the growth of *Chlorella pyrenoidosa* (20° strain) under light-limited conditions as $N = Ktr/V$, where $N \cong$ cell number; K is a function of light, temperature, and area; tr = average retention time; and V = volume. After 3.8 days' retention at 400 ft-c, both NO_3^- and light become limiting conditions and the relationship of yield of dry matter to light is no longer linear. The dry-weight yield is slightly lower—23 mg per unit of light.

The protein yield was also related to the light received; the theoretical limit of 87.5 mg/L (limited by the nitrate in the culture medium) was approached in an asymptotic manner (Fig. 2). Since the yield of total dry substance was more rapid than the yield of protein, there was a concomitant decrease in the percent protein.

As the percent protein decreased, an appreciable number of cells could not be harvested by centrifugation. The percent protein was less and the dry weight greater in these "floaters." In the order of increasing light received, 6.5, 13.4, 12.9, and 7.7%

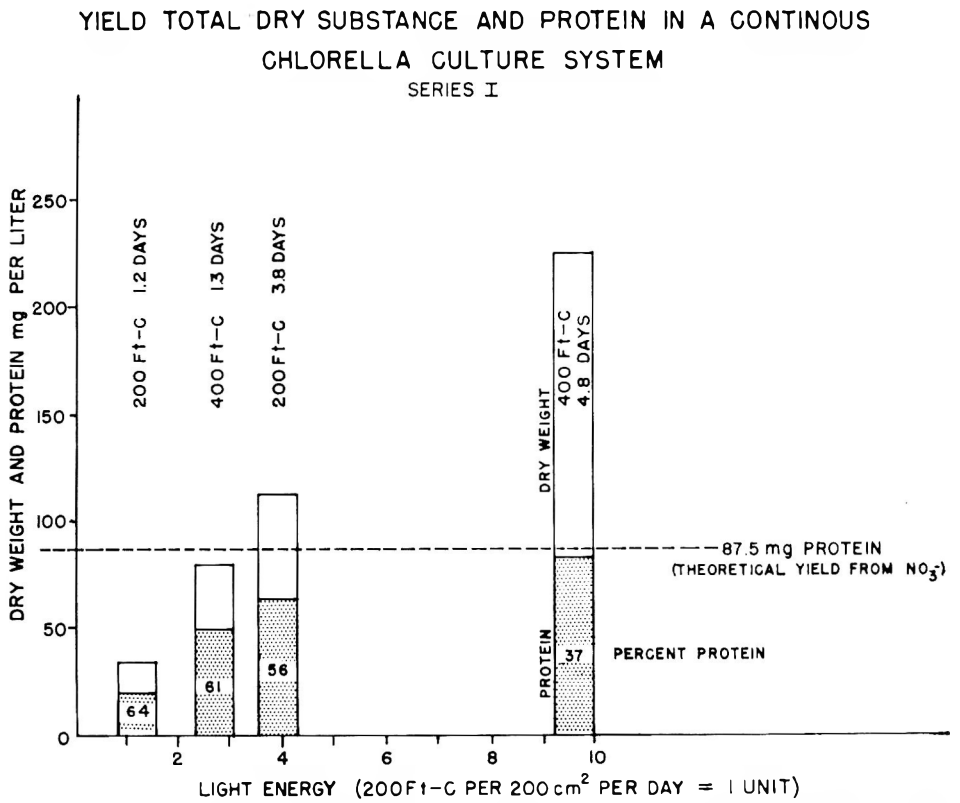

Fig. 2. Total yield of dry substance and protein in a continuous *Chlorella* culture system, series I.

Table 1. Effects of culture conditions on *Chlorella* composition.

Flask no.	Controlled variables: Light intens. (ft-c)	Controlled variables: Retention (days)	Controlled variables: Light units	Cellular products: Cell count ($\times 10^6$/L)	Cellular products: Dry wt. (mg/L)	Cellular products: Ash (% dry wt.)	Cellular products: Protein (mg/L)	Cellular products: Protein (% dry wt.)	Spent medium NO_3^- m*M*	N recov. (%)	Light util. (mg dry wt./unit)
Series I—continuous culture											
1 flask	200	1.2	1.2	6.9	33.8	....	20.2	63.8[a]	.613	84.6	28.2
	400	1.3	2.6	15.8	80.4	....	48.9	60.8	neg[b]	57.0	30.9
3 flasks	200	3.8	3.8	21.5	112.4	....	63.4	56.4	neg	72.7	29.6
	400	4.8	9.6	31.2	224.4	....	83.0	37.0	neg	95.0	23.4
Series II—continuous cultures											
Flask 1	200	1	1	17.5	109.9	6.2	66.2	60.2	.137	89.3	109.9
2		2	2	41.3	199.2	5.2	63.2	31.7	neg	72.2	99.6
3		3	3	33.8	235.8	5.3	58.2	24.7	neg	66.5	78.6
4		4	4	29.8	306.0	5.3	59.9	19.6	neg	68.4	76.5
5		5	5	40.0	278.6	6.1	49.4	17.7	neg	56.4	55.7
6		6	6	33.2	256.8	6.0	52.6	20.5	neg	60.2	42.8
Series II—stationary cultures											
Flask 1	200	6	6	46.7	501.5	4.0	97.3	19.4	neg	111.2	83.6
2		7	7	39.3	298.6	3.5	49.7	16.6	neg	56.8	42.7
3		8	8	24.4	276.1	12.3	56.8	20.6	neg	65.2	34.5
4		9	9	40.0	374.3	4.1	78.1	20.9	.003	89.6	41.6
5		10	10	31.2	312.8	5.4	15.6	5.0	neg	17.8	31.3

[a] Calculated on cellular material harvested by centrifugation only, 20.2 mg protein + 31.68 mg dry wt. An additional 2.16 mg dry wt. harvested by filtration was too small to be analyzed for protein.

[b] neg = less than .001m*M*.

of the cells had to be removed by filtration from the spent medium. The dry weights per cell (as compared to the cells harvested by centrifugation) were 6.0 (4.9), 8.8 (4.8), 6.9 (5.1), and 14.6 (6.9) $\times 10^{-12}$ grams. The percentage of protein (as compared to cells harvested by centrifugation) was –,* (63.8), 45.9 (63.1), 52.9 (57.0), and 33.7 (37.3). The values in Table 1 and Fig. 3 represent the sum of all cells harvested by the two methods.

Series II. 200 ft-c; 1–10 days retention. *Continuous cultures;* retention time 1–6 days. When the retention time was 4 days or less, the dry weight increased in direct proportion to the light received, while the total protein produced was limited by nitrogen lack (Table 1). Thus, while more dry cellular material was produced, the percent protein decreased from 60 to 20%. The increase in numbers of cells was limited to the first and second flasks, although the weight of dry cellular material continued to increase through the fourth flask.

The growth rate was almost twice that of Series I. The only change in the composition of the culture medium was the level of $CaCl_2$, from 0.1 to 1.0mM. Because of this rapid growth the yield of protein reached a maximum in the first flask; no increase was found in succeeding flasks because the level of nitrogen, as nitrate, was already limiting. The result is confirmed by the low nitrate in the medium, 0.137mM, and the lower percent protein in the cells. Although the cell count did not increase beyond the second flask, the cells increased in weight from 6.7 to 10.3 $\times 10^{-12}$ grams/cell. The protein/cell decreased from 3.8 to 2.0 $\times 10^{-12}$ grams.

Retention periods of 5 and 6 days resulted in a decrease of both dry weight and protein in one liter of culture medium. The percentage of protein was constant at approximately 20%.

Stationary cultures; retention times 6–10 days. After 5 days of additional retention time without receiving new medium, the first flask showed a 5-fold increase in dry weight, while subsequent flasks showed smaller increases. The protein level dropped from approximately 20% to only 5% in the fifth flask (10 days' retention time). The ash weight in the third flask increased to 12.3% from the 3–6% found under other conditions.

General trends. *Cell weight and protein.* There is a general tendency for cell weight to increase as the protein level drops. The range of weights was from 4.8 to 11.3 $\times 10^{-12}$ g per cell. The protein per cell decreased from 3.78 to 1.9 $\times 10^{-12}$ g as the retention time was prolonged from 1 to 9 days and declined to 0.5 $\times 10^{-12}$ g when the retention time was extended to 10 days.

Although Series I and Series II cultures were supplied with the same medium, cell concentration was less in Series I and NO_3^- level was not reduced as rapidly as in Series II (Table 1). It is evident that cultures became stabilized under limiting NO_3^- concentrations to yield the cells containing *ca* 20% protein (Table 1, Fig. 3). High protein (*ca* 60%) cells can be obtained when NO_3^- remains available in the medium.

Nitrogen utilization. Nitrate was utilized rapidly and free nitrate in excess of .001mM was limited to the first flasks in the continuous cultures. In succeeding flasks, nitrate was negligible (<.001mM) with the exception of 0.003mM in the flask having a 9-day retention period. Nitrite never exceeded 0.001mM in the spent medium. NH_3 was not measured in these cultures, but none was found in the gas exhausted from similar cultures during 1-day retention periods at 400 ft-c. The nitrogen recovered as protein, plus that recovered as nitrate or nitrite in the medium, accounted for 45–90% of the

* Insufficient sample for analysis.

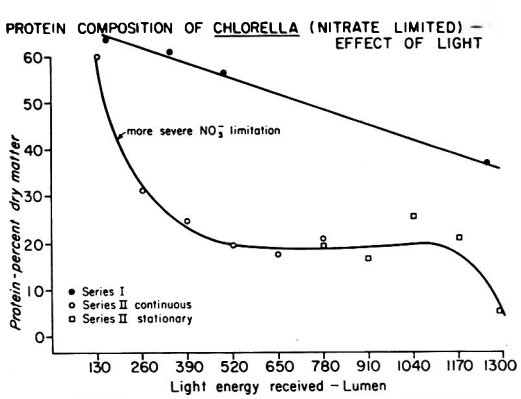

Fig. 3. The effects of light upon percent of protein in NO_3^- limited *Chlorella* culture.

nitrate-nitrogen originally present in the medium. The nitrogen not accounted for may have been nitrate or nitrite present in the cells and thus not included in the analysis.

Interestingly enough, the first flask of the stationary cultures shows greater than 100% recovery of the medium nitrogen (111.2%). Since the gas supplied to the first flask is not saturated with water vapor, it is quite likely that this anomaly is due to evaporation and subsequent concentration of the medium.

The other exception occurred in the final flask of the stationary cultures, where only 17.8% of the medium nitrogen was recovered. Here it is possible that NH_3 may have resulted from cellular deterioration, and this would have been lost since the spent culture medium was at pH 7.0.

Energy yield and light conversion. The cellular production could be expressed in energy units if it could be assumed that the calorie value of these cells was similar to values for *Chlorella* stated in the literature: 5.16 kcal/gram (dry weight) for *Chlorella* of 55.5% protein (bomb calorimetry) (Lubitz, 1961), and 4.67 kcal/gram of 20% protein (Vanderveen *et al.*, 1962). However, Spoehr and Milner's (1949) calculation of R values on *Chlorella* of various protein levels strongly indicates that the calorie content of the high-protein (low-lipid) cells should be considerably lower than that of the low-protein (high-lipid) cells. Although they do not convert their R value directly to calories, an approximation can be made by the data given in their paper. The calorie content of these cells should vary from 5.6 to 7.4 kcal/g for the high- and low-protein cells, respectively, assuming that the carbohydrate and fat content are similar for cells of similar protein levels.

Using 5 kcal/g for a first approximation, the energy yield varied from .1690 to 2.5 kcal/L of culture.

Light utilization may be calculated on a calorie basis by: (mg dry wt/unit of light) = [(5.0 kcal/g)/(1000 mg/g)] (1/4.3 kcal) since each arbitrary unit of light was approximately 4.3 kcal, and assuming each gram of dry weight to contain approximately 5.0 kcal/g, the light utilization then ranges from 3 to 13%.

Light utilization became poorer as the retention period was prolonged and nitrogen limitation became more severe. This meant that more light was required to produce low-protein cells. If these low-protein cells do have a higher calorie content, due to increased lipid as interpreted from Spoehr and Milner's data, then some of this increased light energy was not wasted but was incorporated into the cells.

The results confirm that it is possible to produce algae cells either high (approximately 60%) or low (approximately 20%) in protein content under continuous yield conditions.

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A Food Action Rating Scale for Measuring Food Acceptance

SUMMARY

A 9-point successive-category food action (FACT) rating scale for measuring food acceptance was constructed by psychological scaling techniques. Reliability tests using the scale in the laboratory with peaches and hominy (two groups of 24 people each) showed that the scale yields highly reliable results both with a single group and with different groups of people. When used as a survey questionnaire (54 foods, 100 people), the FACT scale means were always lower, but highly correlated with, hedonic scale means ($r = 0.97$). The distribution of ratings over nine categories was less skewed for the FACT scale than for the hedonic scale. Variance analyses of questionnaire ratings as well as examination of differences in means revealed that the FACT scale was more sensitive to food differences than the hedonic scale. Differences in mean ratings for foods between the hedonic and FACT scales were found to be correlated with carbohydrates in a normal serving ($r = 0.29$) and physical form ($r = -0.27$). The FACT scale has been used successfully in our Research and Development laboratory for many food products as well as in a market-research product test. The FACT scale is recommended as an additional tool for use by the food technologist in estimating over-all food acceptance.

INTRODUCTION

Measurement of food preferences as a predictor of consumer acceptance is a task which food technologists are faced with at one time or another during most research projects involving the development of new processes or new foods. Three common methods employed are the paired-preference, ranking, and rating-scale techniques. It is not the purpose of the present paper to evaluate the relative validity or efficiency of the three methods. However, it can be said that the rating-scale technique has certainly been quite popular and has the definite advantage of simplicity in handling situations where a large number of foods must be judged.

Within the rating-scale technique three types of successive-category scales can be distinguished. These are listed in Table 1, along with an example of each type.

The first type of scale, quality judgment, has been used primarily in the laboratory, with small panels whose members usually have some specific experience or knowledge about the products under investigation. Such subjects usually can provide reliable estimates of food quality with the scale, especially if the judges are highly experienced and have been selected for their ability to give discriminating and reliable ratings. Whether these judgments reflect food acceptance, however, is another question. It seems quite likely that such judgments could

Table 1. Three types of successive category-rating scales used for measurement of food attitudes.

Quality judgment	Like-dislike affect	Action
		I would like to eat this food:
Extremely poor	Dislike extremely	Rarely or never
Very poor	Dislike very much	Once a month
Poor	Dislike moderately	Once every two weeks
Below fair—above poor	Dislike slightly	Once a week
Fair	Neither like nor dislike	Twice a week
Below good—above fair	Like slightly	Several times a week
Good	Like moderately	Once a day
Very good	Like very much	Twice a day
Excellent	Like extremely	

not be easily translated into the attitudes and behavior of consumers. When inexperienced judges are used with such a scale, evidence exists from studies at the Quartermaster Food & Container Institute (Peryam, 1964) that they rate according to what they *like* rather than for inherent quality differences.

The second type of successive-category scale shown in Table 1 is the like-dislike scale, commonly known as the hedonic scale (Peryam and Girardot, 1952). This scale has been used extensively both as a laboratory and market research tool, with subjects who evaluate food products under taste-test conditions, as well as for questionnaire-type surveys of food attitudes in which only the food name is used as a stimulus. This scale measures how much a person likes or dislikes particular products. Average preference ratings on this scale have been correlated, for army personnel, with measures such as acceptance of food on a serving line, and amount eaten (Peryam *et al.*, 1960). The correlations obtained indicate a prediction of about 50% of the variation in actual food acceptance. The relationship, however, varies with food types. It is much stronger for foods such as desserts, snacks, and vegetables than it is over all foods. For example, a meat which receives a low rating is much more likely to be eaten than a dessert which receives a similar rating. The fact that there is still 50% of the variance to account for is not surprising, since many factors other than food attitudes determine the actual acceptance or consumption of a food at any particular moment (Pilgrim, 1957). Recent evidence indicates that ¾ of the variation in acceptance or rejection of food at an army serving line can be predicted if, in addition to food preference ratings, factors of satiety and amount of fat and protein are taken into account (Pilgrim and Kamen, 1963).

The third type of scale, which I will call an action scale, is illustrated in Table 1 in terms of frequency of eating measurement. This scale has also been used at the Quartermaster Food & Container Institute as a survey technique, but has not received extensive use. It requires the individual to be very specific about what actions he would take in terms of the number of times he would be interested in eating a food product in a given period.

It was my opinion that a successive-category scale which involved action as well as

Table 2. Scale values for eighteen statements describing food actions.

Statement [a]	Scale value
I would eat this rather than any other food of its type	2.84
I would eat this every opportunity I had	2.58
This would be one of my top favorites	2.53
I would go out of my way to eat this food	2.43
This is among the foods I like best	2.02
I would eat this very often	2.01
I would eat this more often than other foods of its type	1.79
I would frequently eat this	1.72
I like this and would eat it now and then	1.47
I would eat this if available but would not go out of my way	1.29
I neither like this nor do I dislike it and would eat it if it were easily available	1.25
I don't like it but would eat it on an occasion	1.06
I would hardly ever eat this	0.84
I would rarely eat this	0.78
I would eat this only if there were no other food choices	0.59
I would eat this if I were forced to	0.11
I would rather go without food than eat this	0.07
I would eat this only if I were starving	0.00

[a] Underlined statements were selected for final 9-point scale.

FOOD ATTITUDE RATING FORM

NAME	DEPT.	BOOTH #	DATE

CODES: _____ _____ _____ _____

I WOULD EAT THIS EVERY OPPORTUNITY I HAD

I WOULD EAT THIS VERY OFTEN

I WOULD FREQUENTLY EAT THIS

I LIKE THIS AND WOULD EAT IT NOW AND THEN

I WOULD EAT THIS IF AVAILABLE BUT WOULD NOT GO OUT OF MY WAY

I DON'T LIKE IT BUT WOULD EAT IT ON AN OCCASION

I WOULD HARDLY EVER EAT THIS

I WOULD EAT THIS ONLY IF THERE WERE NO OTHER FOOD CHOICES

I WOULD EAT THIS IF I WERE FORCED TO

COMMENTS:

CODE_____

CODE_____

CODE_____

CODE_____

Fig. 1. FACT scale for laboratory use.

affective-type statements would be one which could prove useful both in the laboratory and for questionnaire surveys. The development of such a scale and the testing of its reliability are described in this paper.

EXPERIMENTAL PROCEDURES

Construction of the scale. Eighteen statements which reflect an affective action toward food were constructed and are shown in Table 2. Twenty subjects (Hunt Research and Development per-

sonnel), unfamiliar with the purpose of the study, rank-ordered the 18 statements from 1, the most positive food attitude, to 18, the most negative food attitude. The 18 statements were then scaled with Guilford's composite standard technique (Guilford, 1954), and the resulting scale values are shown in Table 2, listed in order from the most positive to the least positive. Selected from this list of 18 were nine statements which represented approximately equal scale intervals. These nine are indicated by the underlines in Table 2. The property of approximate equality lends more confidence to statistical analysis of the ratings.

Nine categories were selected rather than some other number because of two conditions: Studies conducted for the Quartermaster Food and Container Institute on food rating scales of the hedonic-scale type indicated that the information obtained from a 9-point scale was about as high as from several other alternatives (Peryam *et al.*, 1960). Keeping the number of categories the same would permit direct comparison of the new scale with a hedonic scale to test its usefulness as a tool in a laboratory and in a survey questionnaire. The first letter of the word food and the first three letters of action were combined to yield the easily remembered scale name of FACT.

Laboratory studies. The first set of tests was designed to give information as to the reliability of the scale both for the same people and for different people. Two groups of 24 subjects judged two products, peaches and hominy, with two replications. The subjects were selected at random from 450 employees who make up the Hunt sensory-testing population. The scale as used in laboratory testing is shown in Fig. 1.

The products tested were selected to represent a highly liked food and one which was distinctly less liked, based on previous experience with the hedonic scale. The products for both tests were selected from the same pack so as to ensure comparability.

Serving amount for the peaches was ½ peach with one tablespoon of syrup, and one tablespoon of hominy. Peaches were served at room temperature, and hominy at 150°F. The hominy was prepared with a fixed amount of butter and salt.

Samples were presented one at a time, with a 30-second interval between samples. Order of presentation was position-balanced for both tests.

	Cream of Mushroom Soup	Oil Dressings	Baked Ham	French Fried Potatoes	Buttered Lima Beans	Strawberry Shortcake	French Fried Shrimp	Fried Pork Chops	Peanut Butter
I WOULD EAT THIS EVERY OPPORTUNITY I HAD									
I WOULD EAT THIS VERY OFTEN									
I WOULD FREQUENTLY EAT THIS									
I LIKE THIS AND WOULD EAT IT NOW AND THEN									
I WOULD EAT THIS IF AVAILABLE BUT WOULD NOT GO OUT OF MY WAY									
I DON'T LIKE IT BUT WOULD EAT IT ON AN OCCASION									
I WOULD HARDLY EVER EAT THIS									
I WOULD EAT THIS ONLY IF THERE WERE NO OTHER FOOD CHOICES									
I WOULD EAT THIS IF I WERE FORCED TO									
NEVER TRIED									

Fig. 2. FACT scale for questionnaire use.

Three-digit code numbers were assigned to the products so that each replication had a different number.

The subjects were instructed that they were to receive two samples of peaches and two of hominy, but were not told that the peaches and hominy were of equal quality.

Testing was conducted in individual booths lighted by 100-watt incandescent bulbs in bullet-type reflectors. The temperature of the booths is maintained at 72±5°F. Four groups of subjects were required for each test. All testing was conducted between 9:30 and 11:00 A.M.

Questionnaire study. In recruiting and selecting personnel for the sensory testing population, individuals filled out a questionnaire of the hedonic-scale type for 54 foods representing a wide variety of products. Of the 450 individuals completing the questionnaire, 150 were selected at random and sent a second questionnaire on the same 54 foods, but using the FACT scale form. Fig. 2 is an example of this form. A "not tried" category was included for use when an individual had never experienced a particular food. It had been at least two months, and on the average six months, since the individual had previously filled out the hedonic scale, so the problem of remembering previous ratings was considered minimal. The FACT scale questionnaire included an introduction sheet stating that we were interested in finding out more about their food attitudes. Of the 150 questionnaires sent out, 140 were returned. From these, 100 were selected at random, and all analyses were conducted on this sample of 100 individuals. There were 40 males and 60 females in the sample, with an average age of 33, and a range from 20 to 55 years. The subjects had originated from many areas of the United States.

RESULTS AND DISCUSSION

Laboratory studies. The ratings were treated as numerical values by equating the least-positive category as 1, and so on up to 9 for the most positive category, in the same manner as the hedonic scale is usually scored.

Table 3 gives the mean values for peaches and hominy in Tests I and II (representing the first and second group of 24 subjects). The means reveal no significant difference between the two groups of 24 people for either the peaches or the hominy mean ratings. The standard deviations for the comparable products also are not significantly different. Table 3 also shows the mean ratings within each group for first and second replication. Here also, no significant difference exists within each group of 24 for either peaches or hominy. Table 4 gives the means for each serving position over both products for Test I and Test II. It can be seen that there is very little evidence of a position effect. From this one set of tests we cannot conclude that a serving-position effect does not occur with the FACT scale. It would be much safer to balance serving position for any type of subjective food judgment.

Table 3. Results of reliability studies using FACT scale for laboratory panels.

Test	Product	N [a]	Mean rating [b]	Standard deviation [b]
I	Peaches	48	6.98	1.22
II	Peaches	48	7.21	1.18
I	Hominy	48	4.94	1.97
II	Hominy	48	5.17	1.85

Test	Product	N	Replication	Mean rating [b]
I	Peaches	24	1	7.25
I	Peaches	24	2	6.71
II	Peaches	24	1	7.46
II	Peaches	24	2	6.96
I	Hominy	24	1	5.28
I	Hominy	24	2	4.62
II	Hominy	24	1	5.38
II	Hominy	24	2	4.96

[a] 24 subjects judging twice.
[b] Values within brackets not significantly different at .05 level.

Table 5 summarizes an analysis of variance conducted for both test sessions. It reveals the high similarity between Test I and Test II, and also the fact that within each test the food by replication source was not a significant source of variation.

The FACT scale has been frequently used

Table 4. Means by position in reliability tests over both peaches and hominy (N = 24).

	Test I	Test II
First position	6.13	6.33
Second position	5.63	6.33
Third position	6.08	6.17
Fourth position	6.00	5.92

Table 5. Summary of analyses of variance for reliability tests.

Source	df	Mean square		F	
		Test I	Test II	Test I	Test II
Foods	1	100.04	100.04	25.01[a]	25.01[a]
Subjects	23	3.88	4.68	0.56	0.67
Replication	1	8.17	5.04	1.63	1.01
Foods by subjects	23	5.15	3.22	0.74	0.46
Subjects by replication	23	1.36	0.56	0.19	0.08
Foods by replication	1	0.04	0.04	0.01	0.01
Remainder	23	0.24	1.17	.....	.....
Total	95				

[a] Significant <.001 level.

in our research and development activities as a laboratory tool and has demonstrated that it discriminates among many varieties of food products. It has also been used in a large ($N = 1600$) home-placement product test for a product used in the preparation of foods rather than consumed as purchased. For this test the word "use" was substituted for "eat" in each rating category. The mean differences among test variables were higher for the FACT scale than for the hedonic scale with comparable variability of ratings (hedonic standard deviation 2.13, FACT standard deviation 2.18).

Questionnaire study. The means for the 54 foods over the 100 people on the FACT and hedonic scales are shown in Table 6 grouped by food types. Note that, with no exceptions, the FACT means are lower than the hedonic means.

An analysis of variance was conducted for each scale in order to obtain an estimate of relative discriminability on the two scales. These analyses of variance (summarized in Table 7) indicate that a greater proportion of the variance is accounted for by the foods source relative to the error remainder for the FACT scale than it is for the hedonic scale. This difference can be illustrated in terms of differences among food means as illustrated in Table 8, which shows the differences between the highest-rating food in each food category and the other foods in that category on both hedonic and FACT scales. As can be seen in all cases but one, there was a greater difference in means for the FACT scale than for the hedonic scale. The average mean difference was computed for both scales, and for the hedonic it was 0.96, and for the FACT 1.32. As another check on the larger differences between FACT means than between hedonic means, 40 pairs of foods were chosen at random from the set of 54, and a sign test (Siegel, 1956) was conducted on the differences between means for both scales. At the 0.05 level there were significantly more instances where the differences between means were larger for the FACT than for the hedonic scale.

Fig. 3 shows frequency of use of rating categories for both the hedonic and FACT scales over all 54 foods. The FACT scale has a more even distribution of ratings over the nine categories than the hedonic scale. The standard deviation was calculated for the frequency of ratings over the nine categories. For the hedonic scale it was 614.6, and for the FACT scale 416.2. This 67.8% smaller standard deviation for the FACT scale is significantly different at the 0.05 level.

A statistic commonly used for the hedonic scale is the relationship between mean preference rating and the percent dislike falling below the midpoint of the scale. The distribution relating these two values was of the same general shape for the FACT scale as for the hedonic scale.

One of the characteristics of the hedonic scale and any successive category-type scale, is that means are highly correlated with standard deviations. Table 9 shows that the correlation between the hedonic means and standard deviations is 0.78, and between the FACT means and standard deviations is 0.60. Although both correlations are highly significant, there is significantly less

Table 6. Mean ratings of 100 persons on FACT and hedonic scales for 54 foods, grouped by food type.

	Hedonic means	FACT means
Meats:		
Roast beef	8.39	8.14
Southern-fried chicken	8.16	7.58
Frankfurters	6.78	5.94
Breaded veal cutlet	7.17	6.45
Broiled hamburger steak	7.72	7.38
Baked ham	8.16	7.33
Fried pork chops	7.74	7.16
$\bar{X}$	7.73	7.14
Fish:		
Canned salmon	6.34	5.10
Tuna fish with noodles	6.75	6.00
French-fried shrimp	7.73	7.03
$\bar{X}$	6.94	6.04
Soups:		
Vegetable soup	7.19	6.50
Cream of mushroom soup	6.43	5.69
Clam chowder	5.91	5.49
$\bar{X}$	6.50	5.90
Snacks:		
Peanut butter	7.09	6.30
Potato chips	7.50	6.78
Pizza pie	7.44	6.70
$\bar{X}$	7.34	6.60
Fruits and desserts:		
Canned pears	7.40	6.79
Canned peaches	7.66	7.00
Fruit cocktail	7.46	6.62
Canned apricots	7.16	6.40
Pineapple sherbet	7.09	6.10
Apple pie	7.99	7.33
Strawberry shortcake	8.22	7.74
$\bar{X}$	7.57	6.85
Beverages:		
Milk	7.75	7.50
Tea	7.16	6.61
Instant coffee	5.78	5.20
Orange juice	8.08	8.05
Tomato juice	7.07	6.50
Buttermilk	3.56	3.08
$\bar{X}$	6.57	6.16
Condiments and dressings:		
Tomato catsup	6.92	6.45
Barbecue sauce	6.90	6.03
Margarine	6.13	5.61
Fruit preserves	7.31	6.39
Mayonnaise	7.01	6.67
Salad dressing	7.07	7.04
Oil dressings	7.00	6.59
$\bar{X}$	6.90	6.40
Vegetables:		
Corn-on-the-cob	7.69	7.09
Spiced beets	6.19	5.42
Broccoli with cheese sauce	6.69	6.12
Buttered asparagus	6.65	6.22
Pork and beans	6.91	6.20
Fried mushrooms	6.33	5.93
Chili beans	6.61	5.85
Glazed sweet potatoes	7.02	6.17
Canned tomatoes	6.62	5.90
Buttered carrots	6.57	6.22
Hominy	4.87	4.05
Stewed tomatoes	6.39	5.58
Canned potatoes	5.92	4.97
Buttered chopped spinach	6.26	5.59
French-fried potatoes	7.69	6.97
Buttered lima beans	6.71	5.98
Lettuce and tomato salad	7.65	7.47
Steamed rice	7.23	6.44
$\bar{X}$	6.67	6.00

Table 7. Summary of analyses of variance of ratings on 54 foods by 100 people on hedonic and FACT questionnaires.

	df[a]		Mean square		*F*[b]		% of variance[c]	
Source	Hedonic	FACT	Hedonic	FACT	Hedonic	FACT	Hedonic	FACT
Foods	53	53	69.39	81.41	24.52	28.12	17.40	18.53
People	99	99	20.65	32.94	7.30	11.38	8.62	13.12
Remainder	5155	5169	2.83	2.90	.....	.....	73.96	68.34
Total	5307	5321						

[a] *df*'s differ between scales because of differences in number of foods for which ratings were available.

[b] All *F*'s are significant at <.001 level.

[c] Used as estimate of variance contributed by each source (Anderson and Bancroft, 1952).

Table 8. Differences in means between highest rating food in each food type and other foods in type on hedonic and FACT scales.

	Δ Hedonic	Δ FACT
Meats:		
Roast beef	.....	.....
Southern-fried chicken	0.23	0.56
Frankfurters	1.61	2.20
Breaded veal cutlet	1.22	1.69
Broiled hamburger steak	0.67	0.76
Baked ham	0.23	0.81
Fried pork chops	0.65	0.98
$\bar{X}$	0.77	1.17
Fish:		
French-fried shrimp	.....	.....
Canned salmon	1.39	1.93
Tuna with noodles	0.98	1.03
$\bar{X}$	1.18	1.48
Soups:		
Vegetable soup	.....	.....
Cream of mushroom soup	0.76	0.81
Clam chowder	1.28	1.01
$\bar{X}$	1.02	0.91
Snacks:		
Potato chips	.....	.....
Peanut butter	0.41	0.48
Pizza pie	0.06	0.08
$\bar{X}$	0.24	0.28
Fruits and desserts:		
Strawberry shortcake	.....	.....
Canned pears	0.82	0.95
Canned peaches	0.56	0.74
Fruit cocktail	0.76	1.12
Canned apricots	1.06	1.34
Pineapple sherbet	1.13	1.64
Apple pie	0.23	0.41
$\bar{X}$	0.76	1.03
Beverages:		
Orange juice	.....	.....
Milk	0.33	0.55
Tea	0.92	1.44
Instant coffee	2.30	2.85
Tomato juice	1.01	1.55
Buttermilk	4.52	4.97
$\bar{X}$	1.82	2.27
Condiments and dressings:		
Tomato catsup	0.39	0.59
Barbecue sauce	0.41	1.01
Margarine	1.18	1.43
Fruit preserves	.....	0.65
Mayonnaise	0.30	0.37
Salad dressing	0.24	.....
Oil dressing	0.31	0.45
$\bar{X}$	0.48	0.75
Vegetables:		
Corn-on-the-cob	.....	0.38
Spiced beets	1.50	2.05
Broccoli with cheese sauce	1.00	1.35
Buttered asparagus	1.04	1.25
Pork and beans	0.78	1.27
Fried mushrooms	1.36	1.54
Chili beans	1.08	1.62
Glazed sweet potatoes	0.67	1.30
Canned tomatoes	1.07	1.57
Buttered carrots	1.12	1.25
Hominy	2.82	3.42
Stewed tomatoes	1.30	1.89
Canned potatoes	1.77	2.50
Buttered chopped spinach	1.43	1.88
French-fried potatoes	0.00	0.50
Buttered lima beans	0.98	1.49
Lettuce and tomato salad	0.04	.....
Steamed rice	0.46	1.03
$\bar{X}$	1.08	1.55

Note: Highest foods in each type are shown by .....

($p < 0.05$) relationship between the FACT means and standard deviations than there is between the hedonic means and standard deviations. Thus, the assumption of mean and variance independence made in analysis of variance is more nearly met for the FACT scale than for the hedonic scale.

The correlation between the two scales (Table 9) is 0.97. This is true even though, as pointed out earlier, the FACT means all are lower than their hedonic counterparts. The differences between ratings for foods on the two scales ranged from 1.24 down to 0.03. The question presented itself as to why the differences should vary for different foods. To help explain this fact, correlations were computed between these differences and various characteristics of the food products in the questionnaire. The characteristics examined were: grams

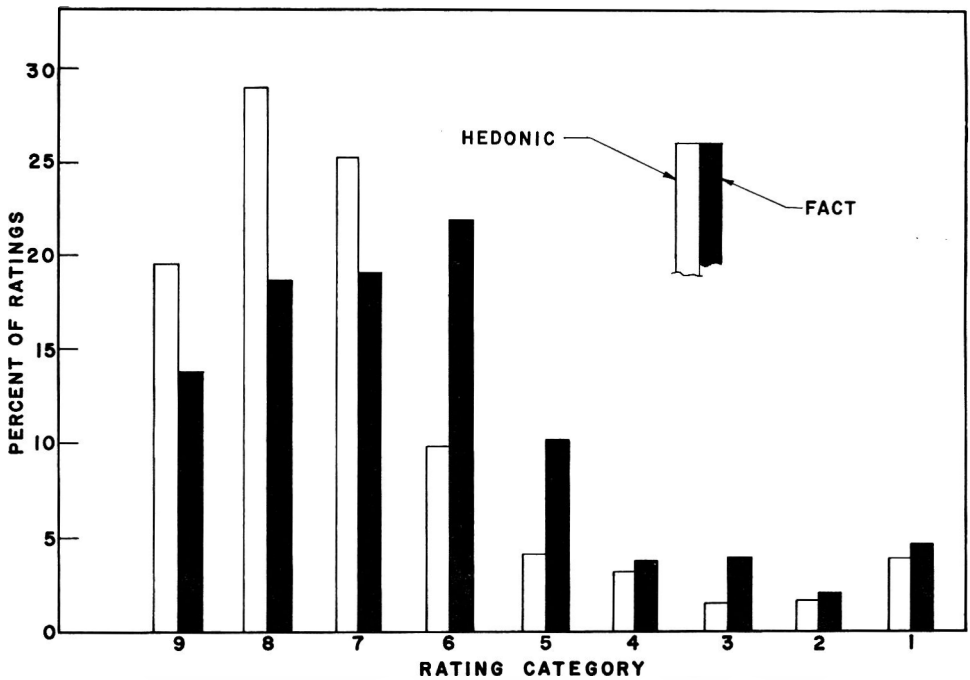

Fig. 3. Percent of ratings in nine categories for hedonic and FACT scales for 54 foods.

of fat, carbohydrate, and protein in a normal serving: grams of a normal serving; calories in a normal serving (preceding values were obtained from Watt and Merrill, 1950); and physical form, which was defined in terms of three levels: solid, semisolid, and liquid. Of these correlations, the two shown in Table 9 were statistically significant. They were, between the hedonic-FACT mean differences and grams of carbohydrate in a normal serving, and the hedonic-FACT mean differences and physical form. When the foods were examined which were high in carbohydrate, it was noted that these were of the dessert and snack type. It was hypothesized that when individuals rated these foods on the hedonic scale, there was a tendency to rate them high because of their general reward aspects, whereas on the FACT scale a more action-oriented attitude was taken. The physical-form relationship apparently is related to the idea that one would give relatively high hedonic ratings to solid foods, but would not eat them as often, as reflected in the FACT scale, as one would eat semisolid or liquid foods. These types of differences would be expected between a scale which measures mostly affect versus one which requires a statement of action.

Table 9. Correlation coefficients between various parameters of hedonic- and FACT-scale questionnaires.

Parameters	N	r[a]	p
Hedonic means FACT means	54	0.97	<.001
Hedonic means Hedonic standard deviations	54	0.78	<.001
FACT means FACT standard deviations	54	0.60	<.001
Δ Hedonic-FACT means Grams CHO in normal serving	49[b]	0.29	.05
Δ Hedonic-FACT means Physical form of foods[c]	54	−0.27	.05

[a] Pearson's product moment coefficient.
[b] CHO data were not available for five foods.
[c] 1 = solid, 2 = semisolid, 3 = liquid.

IMPLICATIONS

From the results of both the laboratory and questionnaire data the FACT scale appears to be a reliable and sensitive method of estimating food acceptance. This reliability holds within as well as between groups of people. The FACT scale appears to have

a higher discriminative sensitivity among foods than the hedonic scale, and this superiority could be due in part to the more realistic attitude that one has when one makes an action statement rather than simply an affective one concerning food products.

The FACT scale is by no means meant as a replacement for the hedonic scale or other methods of the affective type which can be used with effectiveness in applying to the specific characteristics of food such as appearance, texture, etc. The FACT scale can only be used as an over-all measure of food acceptance. It would appear that the FACT scale could be a useful addition to the tools already available to the food technologist for estimating food acceptance.

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The Relationships of Total, Bound, and Free Water and Fat Content to Subjective Scores for Eating Quality in Two Beef Muscles

SUMMARY

Subjective scores for eating quality of two beef muscles, longissimus dorsi (LD) and biceps femoris (BF), cooked to two final internal temperatures were related to fat content and to the amount of bound and free water. Steaks from muscle were cooked to final internal temperatures of 68 and 85°C. As the temperature increased the meat was drier, harder, and more mealy, and contained less connective tissue, but the fragmentation of muscle fibers was not changed. Softness scores were associated with higher amounts of fat and decreased amounts of total water in both muscles cooked to 68°C. As the amount of fat increased in raw and cooked beef, the amount of total water decreased. The amount of bound water, expressed as a percentage of total water, remained relatively constant in raw and cooked steaks. Differences between the muscles and the effects of heat were discussed.

INTRODUCTION

Neither the roles of fat and water in the juiciness and tenderness of beef nor the relationship between these two constituents in meat have been defined clearly. The concept that fatness results in the desirable qualities of juiciness and tenderness was expressed in early work by Armsby (1908). Maw (1935) gave the opinion that the degree of internal fat deposited in poultry determined the moistness of the meat. Proving these associations in the final product, after meat has been subjected to heat, has been very difficult.

Several investigators have reported juiciness and tenderness to be somewhat better in fatter animals (Black *et al.*, 1931; Hankins and Ellis 1939; Wanderstock and Miller, 1948; Husaini *et al.*, 1950b; Gaddis *et al.*, 1950). Others have found no significant differences between animals differing in fatness (Black *et al.*, 1940; Husaini *et al.*, 1950a). Recent work has indicated that fatness is neither a reliable indicator of (Cover *et al.*, 1956, 1958; Cover and Hostetler, 1960) nor consistently associated with (Lowe and Kastelic, 1961; Tuma *et al.*, 1962) tenderness or juiciness.

In earlier work the degree of fatness or marbling and the amount of juiciness as measured by press-fluid or water content have been related to either an objective measure of tenderness or to subjective scores for juiciness and tenderness. It has been recognized that tenderness encompasses several characteristics of meat and that a composite score will not adequately assess the eating quality of meat. The eating quality of beef has been divided into juiciness and six components of tenderness, and the relationships of these characteristics to each other have been studied by Cover *et al.* (1962b,c,d). The relation of bound and free water to juiciness and to softness scores were studied by Ritchey and Hostetler (1964b). Studies on the interrelationships of intramuscular fat, water, and panel scores for juiciness, softness, and tenderness of muscle fibers may lead to a better understanding of total tenderness or desirable qualities of beef. Such a study is the basis for this report.

EXPERIMENTAL

Meat from 13 different animals was purchased from local markets, and no control of the carcass up to the time of taking samples was possible. Steaks 1 inch thick were taken from the longissimus dorsi (LD) muscle opposite the last thoracic vertebra and from the dorsal end of the biceps femoris (BF) muscle in the round. Steaks were obtained from both muscles in the same carcass. The steaks were frozen until used.

The steaks were allowed to thaw in a refrigerator prior to cooking. Steaks were cooked on wire racks in electric ovens at 177°C to final internal temperatures of 68 and 85°C. Internal temperatures of the steaks, as well as the temperatures of the ovens, were followed with a recording potentiometer. Each steak had two thermocouples placed in the center; the temperature was recorded every 30 seconds. On reaching the desired temperature the steaks were removed from the oven and allowed to cool at room temperatures. Samples

Table 1. Scoring sheet used by panel.

Juiciness	Tenderness									
	Softness				Muscle fibers			Connective tissue		
	To tongue & cheek		To tooth pressure		Fragmentation		Mealiness	Softness		Amount
V. juicy	9	V. soft	9	V. soft	V. easy	9	V. mealy	V. soft	9	None
	8		8			8			8	
Juicy	7	Soft	7	Soft	Easy	7	Mealy	Soft	7	Tiny
	6		6			6			6	
Sl. juicy	5	Firm	5	Firm	Mod. easy	5	Sl. mealy	Firm	5	Small
	4		4			4			4	
Dry	3	Hard	3	Hard	Difficult	3	V. sl. mealy	Hard	3	Large
	2		2			2			2	
V. dry	1	V. hard	1	V. hard	V. difficult	1	None	V. hard	1	V. large

for the panel of four judges were removed from the center of the steak as described by Hostetler and Cover (1961). The order of scoring samples was randomly assigned and altered every day or judging session. The mean of the panel scores was used as the score for the steak. The score card (Table 1) is a modification of the one given by Cover *et al.* (1962b) and further altered by Ritchey and Hostetler (1964a). Definitions of juiciness, softness to tongue and cheeks, softness to tooth pressure, and mealiness are as given by Cover *et al.* (1962b,c,d). The scores for connective tissue—softness and amount—were defined by Ritchey and Hostetler (1964a). Scores for ease of fragmentation across the grain of muscle fibers and for apparent adhesion between muscle fibers (Cover *et al.*, 1962d) have been combined into a single score—fragmentation of muscle fibers, defined as the breaking of fibers into smaller fragments. This score involves both the reduction of fiber bundles to smaller groups of fibers and to individual fibers and the breaking of individual fibers into smaller parts as pressure is applied during mastication.

The reasons for combining two scores into fragmentation of muscle fibers were: 1) scoring procedure was simplified by reducing the number of judgments; and 2) there seemed to be a good possibility either that judges were not able to differentiate fragmentation from adhesion as previously defined or that the two scores were measurements of the same characteristic of meat. Scores for ease of fragmentation and apparent adhesion were essentially the same in most lots of animals reported by Cover *et al.* (1962d) and in animals studied by Ritchey and Hostetler (1964a). In scores obtained on 180 animals (Cover *et al.*, 1962d), correlation coefficients between ease of fragmentation and apparent adhesion ranged from 0.75 to 0.94 in steaks from two muscles cooked to three temperatures. In these same animals, correlations between mealiness and fragmentation and adhesion were almost the same at two temperatures and within two muscles. A similar situation existed in the correlation coefficients for the two scores and shear-force values (Cover *et al.*, 1962a). These observations do not prove that ease of fragmentation and apparent adhesion are indeed the same characteristics of meat, but would seem to indicate that the two are so closely related that subjective scoring will not differentiate between ease of fragmentation of muscle fibers and apparent adhesion between muscle fibers.

The steaks were trimmed of outside fat, cut into convenient pieces, and finely ground in an electric grinder. Ether extract was determined by the AOAC (1960) method. The fat represents that usually referred to as marbling, and that within the muscle that would be present in the samples judged by the panel.

Total moisture, bound water, and free water were determined on 10 animals as reported by Ritchey and Hostetler (1964b). Total moisture was determined on ground samples; bound water was determined on samples the size of samples given to the judges, as reported previously (Ritchey and Hostetler, 1964b), but pressed with a Carver laboratory press at 5000 lb pressure rather than at 12,500 lb, as in the above-cited study. Free water was the difference between total and bound water.

RESULTS AND DISCUSSION

Panel scores. Table 2 summarizes the average panel scores for juiciness, softness, tenderness of muscle fibers, and tenderness of connective tissue. These scores, being similar to panel scores reported for other groups of animals (Cover *et al.*, 1962b,c,d; Ritchey and Hostetler, 1964a), are not discussed at length in this report.

Table 2. Average panel scores for juiciness and tenderness for steaks from two muscles cooked to two internal temperatures.

	Scores [a,b]			
	L. dorsi		B. femoris	
	68°	85°	68°	85°
Juiciness	6.8 ± 0.6	2.9 ± 0.6	6.4 ± 1.0	2.3 ± 1.7
Softness to:				
Tongue and cheek	6.5 ± 0.7	4.8 ± 0.9	6.7 ± 0.6	4.2 ± 1.0
Tooth pressure	6.7 ± 0.9	5.1 ± 0.8	6.2 ± 1.0	4.4 ± 1.0
Muscle fibers				
Fragmentation	6.4 ± 1.0	5.6 ± 1.1	6.1 ± 0.9	5.9 ± 0.7
Mealiness	2.5 ± 0.5	4.5 ± 1.3	2.7 ± 0.7	5.4 ± 1.2
Connective tissue				
Softness	7.0 ± 0.8	6.9 ± 0.7	4.6 ± 0.7	5.2 ± 1.0
Amount	7.4 ± 1.2	7.1 ± 1.3	4.6 ± 1.1	5.6 ± 1.4

[a] Mean and standard deviation; 13 animals.
[b] Panel scores range from 1 to 9 with higher scores indicative of more desirable characteristics; mealiness may be exception.

Trends of scores for juiciness and softness were toward drier and harder meat at the higher internal temperature. Scores for juiciness decreased quite markedly from 68 to 85°C. The decrease in softness to both tongue and cheek and to tooth pressure was not as pronounced as that in the juiciness score.

The score for fragmentation of muscle fibers tended to be lower at the higher temperature, but the change was not significant in either LD or BF. Cover *et al.* (1962d) found that, when scoring for both ease of fragmentation and apparent adhesion, the trend was toward toughening in LD and toward tendering in BF. The greatest change usually occurred when steaks were heated to an internal temperature of 100°C and held there for 25 min. Seldom were differences in scores significantly different between intermediate temperatures, but the trends in the two muscles were different. In the present group of animals the scores indicated a slight toughening effect in both muscles as the temperature increased from 68 to 85°C. The major effect of temperature on tenderness of muscle fibers may occur beyond 85°C and under more severe conditions of heating.

The scores for mealiness of muscle fibers were similar in the two muscles as the temperature increased. There was no difference between the muscles at 68°C, but mealiness was somewhat more pronounced in BF at 85°C. Mealiness scores were much higher in BF than in LD in other groups of animals (Cover *et al.*, 1962d; Ritchey and Hostetler, 1964a) at 80 and 100°C. Whether differences in animals or in treatment of carcasses result in variations of mealiness scores can only be speculated on at this point.

The connective tissue scores were toward tendering in BF as temperature increased, but the scores were similar at the two temperatures in LD. The effect of heat on tenderness of connective tissue was demonstrated by Cover *et al.* (1962b) and by Ritchey *et al.* (1963). These previous studies, as well as the scores presented here, point out the difficulty in subjective scoring of connective tissue in muscles such as LD, in which the connective tissue is neither particularly abundant nor present in heavy strands.

Relation of fat to bound and free water. The relative amounts of fat and water within a particular sample of meat influence the eating quality. The total water within meat can be divided into bound and free water; the amount of each depends in part upon the method of determination and the definition assigned. The relationships of fat to total, bound, and free water were examined in raw and cooked steaks taken from two muscles of 10 different animals (Table 3). The amount of fat within the meat, as measured by ether extract, was calculated on both

Table 3. Percent bound and free water and percent fat in raw and cooked steaks from two beef muscles.

Muscle and treatment	Water (%)[a]		Fat (%)[b]	
	Bound	Free	Wet basis	Dry basis
L. dorsi				
Raw	60.1 ± 2.3	13.0 ± 3.4	4.5 ± 3.4	16.4 ± 9.8
68°	56.4 ± 4.8	10.1 ± 3.9	5.5 ± 2.8	16.9 ± 8.5
85°	46.2 ± 4.9	11.5 ± 4.5	7.2 ± 4.1	17.5 ± 7.8
B. femoris				
Raw	60.1 ± 4.6	13.6 ± 4.4	3.9 ± 2.0	15.2 ± 6.4
68°	57.0 ± 6.4	11.4 ± 5.2	4.7 ± 2.3	15.1 ± 6.9
85°	49.0 ± 3.6	7.2 ± 2.6	6.6 ± 3.1	15.9 ± 7.1

[a] Mean and standard deviation; 10 animals.
[b] Mean and standard deviation; 13 animals.

a dry-matter basis and as served to the panel (wet basis). The influence of cooking was to increase the percentage of fat in the steak because of the loss of moisture; when calculated on a dry-matter basis, the amount of fat was relatively constant.

Carcasses used in this study followed the generalization that, as total water increased, the amount of fat in the meat decreased. Correlation coefficients between total water and the amount of fat were negative and highly significant in raw steaks from both LD and BF (Table 4). In the steaks heated to 68°C, this inverse relation was statistically significant in both muscles. With heating to 85°C the correlation was significant in the LD but approached zero in BF. When steaks are subjected to heating, the loss of weight is mostly water. The loss of solids was calculated to be less than 2% in an earlier study (Ritchey and Hostetler, 1964b). As the amount of total (bound plus free) water decreased during cooking, the amount of fat on a dry basis remained relatively constant (Table 3). Prolonged cooking times associated with BF (at 85°C average cooking times were 52 minutes for LD and 75 for BF) destroyed the inverse relationship of fat and total water present in the raw steaks.

In this group of animals the relation between fat and free water was differen for the two muscles. In the raw LD the amount of fat and the amount of free water were highly significantly related (Table 4) indicating that as fat increased, the amount of free water decreased. In the raw BF the correlation between fat and free water was low. Correlation coefficients between fat and bound water were not significant in either raw muscle and were very low in the BF. Investigations on many additional animals and muscles within the carcass are required to completely elucidate these relations between fat and bound and free water.

Heating resulted in a decreased amount of both bound and free water (Table 3). The data showed that although total, bound, and free water decreased during cooking, the amount of bound water, expressed as a percentage of total water, remained fairly constant (ranged from 80.1 to 84.8%) except in BF cooked at 85°C (88.6%). In other groups of animals, Ritchey and Hostetler (1964b) found only small losses in bound water, calculated as percentage of the meat, in steaks from LD and BF cooked to internal temperatures ranging from 61 to 80°C. During heating, bound water is released and becomes free water; as the meat is subjected to higher temperatures, the rate of loss of free water exceeds the release of bound to free water and there is an increased loss of total water. When the loss of free water exceeds the release of bound to free, as in BF cooked to 85°C, a larger percentage of the total water within the meat exists as bound water.

None of the correlation coefficients between fat and bound or free water were significant (Table 4) in the cooked meat. While the coefficients were somewhat higher in LD, the trends in the two muscles were similar. The correlations between fat and bound water in both muscles were around −0.5 at 68°C and near zero at 85°C. The

Table 4. Coefficients of correlation between the amount of fat and total, bound and free water.[a]

Treatment	Correlation coefficients [b]					
	L. dorsi			B. femoris		
	Total	Bound	Free	Total	Bound	Free
Raw	— 0.93	0.54	— 0.83	— 0.86	0.02	— 0.27
68°	— 0.80	— 0.56	0.14	— 0.70	— 0.44	0.22
85°	— 0.72	— 0.11	— 0.48	— 0.09	0.03	— 0.15

[a] 10 animals.
[b] Coefficients above 0.63 significant at 5% level; above 0.77 significant at 1% level.

amount of fat deposited within muscle determines in a sense the amount of total water present in both raw and cooked meat, if high temperatures are avoided. But the relationship of fat to the type of water, bound or free, in cooked meat is not clear.

Relation of fat and water to panel scores. The eating quality of beef is a reflection of the constituents of the meat and their relations to the particular characteristic of meat. The relations between fat and water and the subjective measures of eating quality were examined in steaks from the two muscles cooked to 68 and 85°C. Correlation coefficients were calculated for all possible relationships between subjective scores (Table 2) and for the amount of fat and total, bound, and free water (Table 3). The correlations are presented only for fat and total water (Table 5) since no correlations between scores and either bound or free water were significant.

As steaks from both muscles were heated to 68 and 85°C there was a loss of water but an increase in the percent of fat in the samples given to the panel of judges. As the temperature changed, the meat became drier, harder, and more mealy, and fragmented with about the same degree of ease. The relationships between the changes in fat and water and the subjective scores were not very clear from the correlation coefficients.

The causes of juiciness within meat were studied and remain obscure. It would seem that water would be one of the major contributors to this sensation, and scores do follow similar trends as total, bound, and free water, but the correlations were low in this and in previous work (Ritchey and Hostetler, 1964b).

As the percent fat increases, total water decreases and softness scores increase. The positive correlations between fat and softness scores and the negative correlations between water and softness in LD cooked to 68°C were indicative of this relationship. The general trends of amount of fat and water and the softness scores were similar in BF cooked to 68°C, but the correlations were very low. It would appear that the

Table 5. Coefficients of correlation between amount of fat and panel scores and between total water and panel scores.

	Correlation coefficients			
	L. dorsi		B. femoris	
% fat vs.[a]	68°	85°	68°	85°
Juiciness	— 0.18	0.31	— 0.14	0.47
Softness to T/c[b]	0.63	0.25	— 0.06	0.36
Softness to TP[b]	0.45	0.23	0.02	0.36
Mealiness	0.47	0.08	0.72	— 0.28
% total water vs.[c]				
Juiciness	— 0.33	0.30	— 0.05	0.53
Softness to T/c[b]	— 0.71	0.45	0.03	0.48
Softness to TP[b]	— 0.59	0.26	— 0.23	0.39
Mealiness	— 0.17	— 0.11	— 0.23	0.15

[a] Correlation above 0.55 significant at 5% level; above 0.68 at 1% level.
[b] T/c, tongue and cheek; TP, tooth pressure.
[c] Correlation above 0.63 significant at 5% level; above 0.76 at 1% level.

causes of softness in the two muscles may be somewhat different. A likely influence on softness in BF is the large amount of connective tissue present at the lower temperatures compared to LD (Ritchey *et al.*, 1963). As the internal temperature to which meat is cooked increases, the major protein of the connective tissue, collagen, is converted to gelatin and the influence on the softness components of tenderness is decreased.

Mealiness has been defined as a special kind of fragmentation in which the fragments were tiny, dry, and hard. The higher scores were found in the meat cooked to 85°C which contained less water and more fat (wet basis) than samples heated to 68°C. The sensations of dryness and hardness would seem to be a reflection of the changes in water and fat, but the correlation coefficients (Table 5) were not consistent.

The effect of prolonged heating, as evidenced by the steaks from both muscles cooked to 85°C, is to alter the relations between panel scores and the constituents of meat. Changes in both amount and composition of connective tissue, fat, water (bound and free), and muscle proteins result in alteration of the eating quality. The ability of judges to detect these changes and score them in a significant manner seems to be the key at this time. Additional investigations on the subjective scoring of beef are needed to clarify the components of tenderness in different muscles cooked to various temperatures and relate these components to the chemical constituents and properties of meat.

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