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Lipid Antioxidants in Plant Tissue

SUMMARY

The antioxidant activities of several flavone glycosides and cinnamic acids were determined in lipid-aqueous systems. Flavone glycosides, except rutin, possessed approximately the same antioxidant activity as their respective aglycone. Rutin was not as effective as its corresponding aglycone, quercetin. Quercetin derivatives were the only glycosides isolated from green onions, green-pepper pods, green-pepper seeds, and potato peels. Green-onion tops also possessed a glycoside of myricetin. Caffeic acid was an effective antioxidant but chlorogenic acid had no antioxidant activity.

The water solubility of lipid antioxidants facilitates their distribution in low-fat aqueous foods, so that the antioxidants dissolved in the aqueous phase are in intimate contact with the lipid phase. The flavones occur in nature mainly as glycosides; usually the sugar moiety imparts water solubility to the compounds. The cinnamic acids are also sufficiently soluble in water for distribution in aqueous foods.

The antioxidant effectiveness of several hot-water vegetable extracts and of flavone aglycones isolated from the extracts has been discussed by Pratt and Watts (1964). Extracts in which flavone glycosides were unhydrolyzed possessed approximately the same antioxidant activity as extracts which contained the flavone aglycones. The present study was designed to determine the antioxidant activity of individual flavone glycosides and other closely related water-soluble phenols.

EXPERIMENTAL

Preparation of extracts. Hot-water vegetable extracts were prepared as described by Pratt and Watts (1964). In the present study, extracts of green onion tops were used to develop and perfect analytical methods, and the developed techniques were applied to the other extracts.

Chromatographic fractionation of extracts. Phenolic compounds of the extracts were separated by paper chromatography as previously described (Pratt and Watts, 1964). In the initial separation, descending chromatography was used with water as the developing solvent. The chromatograms were irrigated until the solvent front had migrated approximately 30 cm. In this system the flavonoid glycosides and other water-soluble components moved with the solvent.

The water-developed chromatograms were cut into three bands: 1) the origin, including approximately 5 cm of developed chromatograms (band A); 2) the next 15 cm of developed area (band B); and 3) the section adjacent to the solvent front including approximately 10 cm of the chromatogram (band C). The bands were eluted by three extractions in boiling water. Spectrophotometric comparisons of the eluates with fractions eluted three times in boiling methanol and uv examination of chromatographic papers following elution demonstrated that elution was essentially complete.

In order to separate the groups of phenolic compounds into individual compounds and remove contaminating material, the water-separated fractions were streaked and chromatographed using the upper layer of a 4:1:5 (v/v/v) two-phase mixture of 1-butanol–acetic acid–water (BAW). Eluted compounds to be used for antioxidant studies were diluted so that the concentration represented that which would be present in a 20% extract of the original vegetable.

Identification of extract components. Chromatographic and uv spectral analyses were used to identify phenolic components of the extracts. Because of the limited number of known glycosides available, isolated glycosides were hydrolyzed and the aglycone and sugar moieties identified. Sugars were identified by cochromatography with knowns. For those containing glucose the concentrations were determined by the Folin-Wu micromethod (AOAC, 1955). When known flavonoid glycosides were not available, the position of the sugar moiety was determined from reported R_f values (Harborne, 1959; Roux, 1957; Seikel, 1962) and reported uv spectral characteristics (Jurd, 1962). Color-developing sprays were also used in the chromatographic identification. The color-developing reagents are shown in Table 1.

Tests for antioxidant activity. The antioxidant effectiveness of the isolated compounds and known standards was ascertained by determining: 1) the TBA (2-thiobarbituric acid) number of beef slices covered with test solutions; and 2) the bleaching time of carotene-lard solutions in contact with the test solutions. The methods used for these determinations have been described (Pratt and Watts, 1964).

Table 1. Color-developing reagents for flavonoids.

Reagent	Reactive compounds	Reaction	References
Ammonia fumes	All flavonoids except anthocyanins	Forms colored phenolic anion	Bate-Smith (1956)
1% sodium carbonate	All flavonoids	Forms colored phenolic anion	Gage *et al.* (1951)
5% aluminum chloride in 95% ethanol	Flavones and flavanones	Forms complex with 4 carbonyl and 3 or 4 hydroxyl groups	Gage *et al.* (1951)
1% ferric chloride + 1% potassium ferricyanide	Phenols	Forms colored complex	Barton *et al.* (1952)
0.1*N* silver nitrate in 5*N* ammonium hydroxide + heat	(General reagent for many reducing compounds, in flavonoids spot intensity is proportional to degree of hydroxylation of B ring)	Reducing groups responsible	Bate-Smith (1950)
2% phosphomolybdic acid followed by ammonia fumes	Ortho-dihydroxy phenols	Reduction of phosphomolybdic acid	Riley (1950)
3% P-toluenesulfonic acid in ethanol + heat	Flavanones	Oxidizes flavanones to flavones	Roux (1957)

RESULTS AND DISCUSSION

Of the several extracts studied, green-onion-top extracts fractionated into the greatest number of phenolic compounds. All phenolic compounds identified from green-onion tops were flavones and flavone derivatives. Table 2 shows the R_f values of the separated fractions and their identified aglycones. Table 3 shows the R_f values of the sugars obtained from acid hydrolysis of the

Table 2. Chromatographic characteristics of green-onion-top flavonoids.

BAW fraction	R_f values			Aglycone
	BAW	80% phenol	2% acetic acid	
1–7	0.13			
8	0.26	0.22	0.24	Quercetin
9	0.42	0.52	0.00	Myricetin
10	0.44	0.33	0.04	Myricetin
11	0.51	0.49	0.01	....
16	0.57	0.35	0.15	Quercetin
13	0.64	0.33	0.03	Quercetin
14	0.70	0.44	0.19	Quercitin
15	0.95	0.67	0.00	....

Table 3. Characteristics of sugar moieties of green-onion-top flavonoids.

BAW fraction	R_f values of sugars[a]				Glucose concentration $\times 10^{-5}M$
	Aglycone	BAW	80% phenol	Butanol +1% NH_4OH	
8	Quercetin	0.17	0.40	0.68	6.72
9	Myricetin	....	....	....	0.00
10	Myricetin	0.18	0.38	0.08	0.36
12	Quercetin	0.18	0.39	0.08	0.71
13	Quercetin	....	....	....	0.00
14	Quercitin	0.37	0.60	0.22	0.00

[a] R_f values of glucose were 0.17, 0.39, and 0.09 in BAW, phenol, and butanol solvents, respectively; R_f values of rhamnose were 0.38, 0.61, and 0.21.

glycosides. The seven bands with the lowest R_f values in BAW (R_f values <0.13) had only slight activity, and the concentration was so low that identification was not practical. Two other phenolic compounds with R_f values of 0.51 and 0.95 in BAW were also not identified.

Of the 15 bands that were separated from green-onion-top extracts by paper chromatography using BAW as the developing solvent, six were identified. Two were the aglycones quercetin and myricetin: three were glycosides of quercetin; and one was a glycoside of myricetin. The R_f values in BAW, 80% phenol, 15% acetic acid, and water were nearly identical with values obtained with standard aglycones. The aglycone, obtained by hydrolysis of the glycosides, could not be separated from standards when cochromatographed in the above solvents.

One of the glycosides was identified as quercitrin (quercetin 3-rhamoside). This identification was comparatively easy since a standard was available. The maximum absorbance peaks for standard quercitrin solution (in absolute ethanol) were 259 and 351 mμ; isolated quercitrin had absorbance peaks of 259±1 and 353±1 mμ. Chromatographic comparisons with the standard also confirmed quercitrin.

The remaining glycosides possessed glucose as the sugar moiety; the sugars were identified by their chromatographic characteristics (Table 3). Quantitative glucose determinations were used to supplement chromatographic determinations in ascertaining the amount of glucose present in the glycoside. The glucose concentration ranged between 80 and 90% of the theoretical values for the identified glycoside (Table 3). No reason is readily apparent for the discrepancy.

The identities and the antioxidant activities of the isolated flavones are summarized in Table 4. The concentrations of the glycosides were determined by hydrolyzing the separated fractions and measuring the concentration of the aglycone spectrophotometrically. Table 5 shows the flavones that were isolated and identified from green onions, green-pepper pods, green-pepper seeds, and potato peels. The potato extract fails to show antioxidant activity, and no flavonoid was isolated from the extract.

Table 4. Antioxidant activity of green-onion-top flavonoids.

BAW fraction	Compound	Concentration[a] $\times 10^{-6}M$	TBA number (3 days at 3°C)
Control	....	....	6.6
8	Quercetin 3-triglucoside	2.5	1.6
9	Myricetin	0.4	3.8
10	Myricetin 3-monoglucoside	0.5	4.2
11	....	....	4.4
12	Quercetin 3-monoglucoside	0.8	4.5
13	Quercetin	0.4	4.7
14	Quercitrin	0.4	4.8
15	....	....	5.5
20% extract	....	....	1.1

[a] Based on aglycone concentration in 20% extract.

Commercial preparations of quercetin and rutin (quercetin 3-rhamnoglucoside) were available for antioxidant assays and comparison with isolated compounds. Quercitrin (antioxidant index = 3.6) possessed approximately the same activity as quercetin (antioxidant index = 3.5). Rutin (antioxidant index = 1.5), however, had only slight antioxidant activity. Kelley and Watts (1957) also found rutin somewhat inferior to quercetin and quercitrin, but the differences were not as great as those reported here. No reason is apparent for the variation in antioxidant activity between rutin and quercitrin. Chromatographic purification and the use of several available samples of rutin (to eliminate the effect of possible contamination) did not alter the finding.

Identification and antioxidant activities of substituted cinnamic acids. Two phenolic compounds other than flavonoids were isolated from extracts. Caffeic acid was separated chromatographically (in BAW) from eluate of the B bands of potato peel and pepper seed extracts. A compound identified as chlorogenic acid was isolated from potato and potato peel extracts. Recent work by Maier and Metzler (Anon. 1963), however, has shown that chromatographic properties of caffeoyl-

Table 5. Antioxidant activity of vegetable flavonoids.

Source	Compound	Concentration [a] $\times 10^{-5}M$	TBA number (3 days at 3°C)
Control	.....	.....	7.3
Green onions	Quercetin	0.5	5.2
	Quercitrin	0.8	4.7
	Quercetin 3-triglucoside	1.5	3.9
Green-pepper pods	Quercetin	0.9	5.1
	Quercitrin	1.9	3.9
Green-pepper seeds	Quercetin	0.8	5.6
	Quercitrin	1.2	4.2
	Quercetin 3-monoglucoside	3.5	2.8
	Quercetin 3-diglucoside	1.7	4.0
Potato peels	Quercetin	1.0	5.1
	Quercetin 3-diglucoside	1.3	4.4

[a] Based on aglycone concentration in 20% extract.

shikimic acid are very similar to chlorogenic acid, and they have almost identical uv spectra. Maier stated that identification based on chromatographic properties and uv spectra could not be considered conclusive. Since these methods are used for identification, the possibility cannot be overlooked that the compound may be caffeoylshikimic acid. Both chlorogenic and caffeoylshikimic acids have the orthodihydroxy configuration important in antioxidant activity. The antioxidant activities of these compounds are reported in Table 6. While caffeic acid was an effective antioxidant, chlorogenic acid had no activity. The reason is not readily apparent; in view of the findings that caffeic acid had a marked positive influence and quinic acid had a slight positive influence (antioxidant index = 1.7), chlorogenic acid would be expected to be a good antioxidant.

Table 6. Antioxidant activity of caffeic and chlorogenic acids.

Compound	Source	Concentration $\times 10^{-5}M$	TBA no. (3 days at 3°)
Control	.....	.....	7.5
Caffeic acid	(standard)	5.0	1.9
	Potato peels	4.4	2.1
	Pepper seeds	0.9	4.8
Chlorogenic acid	(standard)	5.0	7.1
	Potatoes	9.8	7.6
	Potato peels	2.3	7.3

[a] Concentration in 20% extract.

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Rehydration in Onion as a Function of Dehydration Regime

SUMMARY

Fresh, diced onions were dehydrated under different regimes (high-, intermediate-, and low-temperature, and lyophilization). Rehydration volumes were determined for the dice. Water vapor sorption properties and X-ray estimates of crystallinity were obtained for the cellulose extracted from the dice and for control samples of cellulose that had been extracted from fresh onions and then dehydrated. Rehydration occurs most rapidly in lyophilized tissues, in which at 24°C and at 91°C approximately the original fresh volume is attained in 15–30 min. The final rehydrated volume in other treatments is reached more slowly and is considerably smaller than the fresh volume. The highest crystallinity of cellulose is found in the lyophilized materials, probably as a freezing effect. Freezing also produces large, internal voids. Cellulose crystallinity is essentially unaffected by temperature differences during the dehydration process.

INTRODUCTION

Sterling and Shimazu (1961) previously found that the phloem of carrot root does not vary in its rehydration properties or in the extent of crystallinity of cellulose as a result of differences in the rate of dehydration. We also showed that the extent to which the tissue rehydrated is inversely related to the relative crystallinity of cellulose. Rehydration volume is smaller when crystallinity is high than when crystallinity is low. It appeared useful to extend these experiments to a vegetable with tissue structure different from that of the carrot, and to broaden the range of dehydration treatments. Chosen in the present instance was the onion bulb.

The onion bulb is a modified shoot, in which the internodes are unelongated and the sheathing leaf bases (bulb scales) are fleshy. Reserve food stored in these leaf bases serves to nourish the flowering shoot during subsequent internodal elongation. In contrast to the secondary phloem of the carrot root, the tissues of the onion bulb are all primary in origin.

MATERIALS AND METHODS

White Globe onion bulbs, 5–8 cm in diameter, were obtained from the Growers' Produce Co., of Nyssa, Oregon. After the removal of both ends and of dried surface scales, the bulbs were cut into dice of about 0.7 cm on an edge. The dice were either dehydrated directly or were first extracted of noncellulosic constituents and then dehydrated. The extraction procedure involved 8-hr treatment with ethanol in a Soxhlet extractor, washing with hot water, boiling with 2% HCl for 2 hr, washing with hot water, boiling with 2% NaOH for 2 hr, washing with hot water, and then refrigerating until use. The refrigerated cellulosic material was prepared for dehydration by filtration on a Buchner funnel and then casting into prisms, about 1 × 1 × 2 cm, on glass slides.

Dehydration conditions. All dehydration methods were carried out to yield an approximately uniform terminal moisture content of 5%.

1). *High temperature* (HT). Drying was carried out in two stages: 82°C, with dewpoint at 22°C, for 1 hr; 49°C, with dewpoint at 12.5–14°C, for 20 hr.

2). *Intermediate temperature* (IT). The material was dehydrated at 44–49°C, with dewpoint at 4.4–15.5°C, for 24 hr.

3) *Low temperature* (LT). The dehydration was again a 2-stage process: 38°C, dewpoint at 11.7–14.5°C, for 94 hr; then in a desiccator at 24°C for 24 hr.

4) *Lyophilization* (LYO). The samples were frozen at −29°C in a moving air stream. Drying took place at 100 μ Hg pressure for 48 hr, the temperature on the warming plates being 54.5°C.

Analytical methods. Immediately upon completion of the dehydration process, the samples were analyzed:

1) *Moisture content.* The dice were weighed into tared aluminum moisture dishes and then dried at 70°C under 125 mm Hg pressure until a constant weight was reached. Results are expressed in percentage of the total sample weight.

2) *Rehydration.* Rehydration was measured in distilled water at 25 and 91°C. Five to 10 g of dehydrated onions and 50 g of fresh onion dice were used in the volumetric measurements. The volume of the samples was measured as the difference between the volume of a volumetric flask

(250 ml) and the volume of the water drained off after 2 min of flask inversion.

3) *Extraction of cellulose for sorption and X-ray measurements.* The dehydrated samples were soaked in distilled water for 2 hr and then washed in ethanol. The succeeding extraction process was identical to that described above for the preparation of cellulose for dehydration. The refrigerated cellulose was pressed to remove excess water, and then dried in air for 3 days at room temperature. The dried cellulose was ground to 60-mesh size for use in the following analyses:

4) *Water-vapor sorption.* Adsorption and desorption of water vapor were measured dynamically, with moisture-bearing N_2 gas passing through 3 sulfuric acid-water solutions and then through the samples at 50 ml/min. The solutions were made up to yield relative humidities of 20, 40, or 60% at 25±0.01°C. The samples and the two solutions immediately preceding it in the gas stream were held in a water bath at that temperature. After each equilibrium state was reached, the sample was weighed. When the two series of measurements were completed, the sample was dried at 70°C under 125 mm Hg pressure and weighed. The sorbed water vapor is expressed as a percentage of the dry weight of the sample.

5) *X-ray measurement.* The technique of this measurement and the calculation of crystallinity have been described (Sterling, 1960; Shimazu and Sterling, 1961).

6) *Histological examination.* Rehydrated tissues were embedded, sectioned, and stained for microscopy by the usual histological techniques.

RESULTS

Moisture content. The final moisture content in the dehydrated samples is given in Table 1. The values extend from 4.35 to 5.81%, for a maximum range of ±16% from the desired value.

Table 1. Moisture content of dehydrated onions.

Treatment	Percentage moisture
Fresh	89.9
HT [a]	
1st stage	60.9
2nd stage	4.67
IT [b]	5.81
LT [c]	
1st stage	5.34
2nd stage	4.79
LYO [d]	4.35

[a] High temperature dehydration. See text.
[b] Intermediate temperature dehydration. See text.
[c] Low temperature dehydration. See text.
[d] Lyophilization. See text.

Table 2. Rehydration volume (ml water/g dry matter) at 25°C for various dehydration regimes.

Time (hr)	Dehydration regime				
	Fresh	HT [a]	IT [a]	LT [a]	LYO [a]
0.5	12.8	4.88	5.09	5.45	11.5
1	12.8	5.80	6.03	6.36	11.6
2	12.8	6.71	6.92	7.14	11.7
4	12.9	7.25	7.50	7.62	11.8
6	12.8	7.53	7.70	7.96	11.8
8	12.8	7.75	7.96	8.13	11.8
24	12.8	8.01	8.06	8.28	11.8

[a] See footnotes, Table 1.

Table 3. Rehydration volume (ml water/g dry matter) at 91°C for various dehydration regimes.

Time (min)	Dehydration regime				
	Fresh	HT [a]	IT [a]	LT [a]	LYO [a]
15	11.9	7.13	7.12	7.43	12.4
30	12.1	7.72	7.79	8.03	13.5
60	12.1	8.09	8.02	8.39	12.5
120	12.1	8.17	8.18	8.69	12.3

[a] See footnotes, Table 1.

Rehydration volume. Volumes attained during rehydration are reported in Tables 2 (25°C) and 3 (91°C). It is immediately apparent that the lyophilized sample comes closest to attaining the volume of the fresh onion at 25°C, and at 91°C it even exceeds that volume slightly. The lyophilized onions appear to have a spongy texture upon rehydration, and they seem to be somewhat softer than the other onions—particularly at 91°C. There is little difference in rehydration volume among the samples with standard dehydration treatments. All these latter samples are significantly smaller than the lyophilized sample.

Water-vapor sorption. The moisture content in dehydrated onions during desorption is given in Table 4. The values for the water content during adsorption are similar, and therefore are not presented here. These values are more readily compared if related to the amount of water sorbed by cotton cellulose, as a standard. If the crystallinity of cotton cellulose be taken as 70%, the relative crystallinities in the other cellulose samples may be ascertained by computing a sorption ratio (Hermans, 1949) for each relative humidity. These ratios have been averaged for each sample, and the estimates of crystallinity are presented in Table 5. The lowest crystallinity is found in the extracted onion cellulose that is simply dried in air, and the highest occurs in extracted cellulose that is lyophilized. Among the samples subjected to standard dehydration treatments there appears to be a more or less uniform degree of crystallinity.

Table 4. Moisture content (%) during desorption after different dehydration regimes.

Dehydration regime	Relative humidity	Cellulose of dehydrated onion	Dehydrated (onion) cellulose	Cotton cellulose
HT [a]	60	10.4	10.3	
	40	7.49	7.47	
	20	4.93	4.87	
IT [a]	60	10.2	9.78	
	40	7.34	6.90	
	20	4.87	4.61	
LT [a]	60	10.2		
	40	7.34		
	20	4.85		
LYO [a]	60	10.1	9.51	
	40	7.39	6.82	
	20	4.86	4.44	
Air-dried	60		10.7	6.52
	40		7.83	4.71
	20		5.20	3.13

[a] See footnotes, Table 1.

Table 5. Estimates of crystallinity in cellulose from different dehydration treatments.

Sample	Treatment [a]	Crystallinity from sorption ratio: Adsorption	Desorption	Average	X-ray crystallinity
Cellulose extracted from dehdrated onion	HT	51	52	52	53
	IT	52	53	53	53
	LT	52	53	53	54
	LYO	52	53	53	55
Dehydrated extracted onion cellulose	HT	51	53	52	53
	IT	52	56	54	53
	LYO	56	57	56	55
	Air-dried	49	50	50	48
Cotton cellulose	Air-dried	70	70	70	70

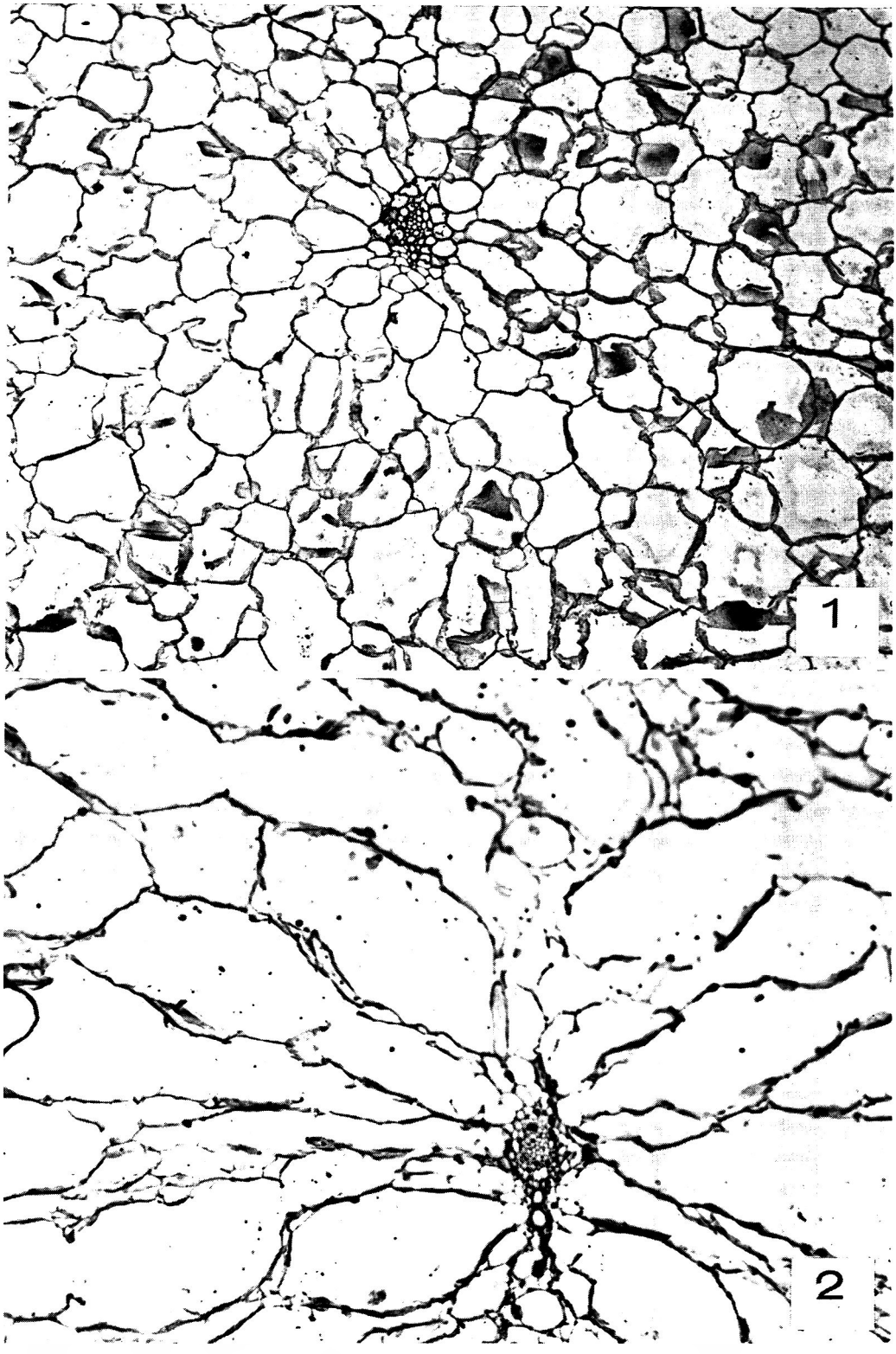
[a] See footnotes, Table 1.

X-ray diffraction measurements. Crystallinity determinations, based on areas of crystalline peaks and heights of amorphous backgrounds in X-ray diffraction photographs, are also given in Table 5. There is a general agreement with the sorption results, in both ranking and the magnitude of values. Note that the cellulose of lyophilized samples has the highest crystalline content.

Histological examination. Fig. 1 shows rehydrated onion bulb tissue after low temperature dehydration. The aspect is identical to that of tissue rehydrated after high temperature dehydration. The cells are intact, but their walls are somewhat wrinkled, in accord with the shrunken macroscopic appearance of the bulb scale segments. The lyophilized sample (Fig. 2) is strikingly different. There are few intact cells, and these are found mostly among the thick-walled elements of the vascular bundles. The remainder of the cells have been crushed and torn by the ice crystals so that a large, open meshwork has been formed. Capillary voids are now situated in a reticulum formed of the compressed cell walls.

DISCUSSION

The most striking result of the present experiments is the relatively complete reconstitution of lyophilized material during rehydration, despite the fact that lyophilization brings about the greatest extent of crystallization of cellulose. Obviously, the freezing environment is the agent responsible for the high crystallinity. It may be imagined that freezing occurred slowly, with the formation of large ice crystals (Meryman, 1956). The



Figs. 1, 2. Cross sections of rehydrated onion bulb scale segments. Fig. 1. After low temperature dehydration. Fig. 2. After lyophilization. Both figures ×65.

formation of such crystals could have withdrawn enough water from amorphous zones of the cellulose gel to permit molecule approach, alignment, and crystallization. Further drying accents the crystallization. The toughening of frozen apricot skins (Hohl, 1948) may be due to such cellulose crystallization. In other gels, similarly, it appears that slow freezing accelerates the crystallization process (Sterling, 1957).

The rapid rehydration of the lyophilized tissues, despite the higher crystallinity of its cellulose, can be referred to the presence of many open, irregular capillary spaces in the tissues, again caused by the expanding ice crystals. The effect of freezing in increasing the extent of cell separation has been shown by Maurer and Murray (1951), and it may be interpreted from the results of Cox and MacMasters (1942). It is apparent here that the cell walls are themselves ruptured (*cf.* Levitt, 1941; Tressler and Evers, 1947; Joslyn and Diehl, 1952; Meryman, 1956). (Parenthetically, it may be remarked that the lesser volume of the fresh onion at 91°C than at 25°C [Tables 2, 3] would seem to be due to the death of the cells and loss of turgor at the higher temperature.)

With conventional dehydration treatments, the tissue which is dehydrated at a high temperature shows a slightly lower crystallinity of cellulose than that dehydrated at a low temperature. There is probably no real significance in this slight percentage difference. These results agree with those reported for carrot (Sterling and Shimazu, 1961). In carrot there are no consistent differences in crystallinity or in rehydration volume as a result of the rate of dehydration. The present study shows small decreases in rehydration volume as the temperature of dehydration increases, but these differences are not significant.

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Changes in Individual Date Polyphenols and Their Relation to Browning

SUMMARY

The main simple polyphenols of immature dates (*Phoenix dactylifera* var. Deglet Noor) are flavans, flavonol glycosides, caffeoylshikimic acids, and a cinnamic acid derivative. Tannins are both water-soluble and -insoluble condensed tannins of the leucoanthocyanidin type. Of the simple polyphenols, the flavans and caffeoylshikimic acids undergo the greatest decrease during maturation and storage. Tests with date phenolase show these compounds to be the most susceptible to enzymic browning. Several new phenols form during storage. Treatment of the tannins with hot strong acid produces cyanidin chloride, suggesting the presence of leucocyanidin units in the tannins.

The presence of polyphenols in dates has been widely known for many years (Bate-Smith, 1959; Ragab *et al.*, 1956; Rashid, 1950; Turrell *et al.*, 1940; Vinson, 1911). However, their relative abundance, identities, and properties have not been reported. We undertook to investigate the polyphenols because they are involved in the darkening of dates.

A recent paper reported the quantitative changes that take place in several classes of polyphenols during the growth, maturation, and storage of dates (Maier and Metzler, 1965). The results indicate that the polyphenols are involved in both enzymic and nonenzymic oxidative browning. In an earlier report (Maier *et al.*, 1964) the main enzymic browning substrates present in dates were identified as the isomeric monocaffeoylshikimic acids, dactylifric, isodactylifric, and neodactylifric acids. The present paper describes changes that take place in the major individual polyphenolic constituents of dates during growth, maturation, and storage. The results are interpreted in terms of browning pathways involved in the darkening of dates.

EXPERIMENTAL

Extraction and fractionation. Extracts of dates (*Phoenix dactylifera* var. Deglet Noor) were prepared for studies as reported previously (Maier and Metzler, 1965). Extracts were stored at 4°C, and all analyses were completed within one week of preparation. By washing the aqueous extract repeatedly with ethyl acetate the water-soluble polyphenols were separated into an ethyl-acetate-soluble, simple polyphenolic fraction and a water-soluble tannin fraction. Insoluble tannins that gave a leucoanthocyanidin test remained in the tissue marc after exhaustive extraction with aqueous methanol.

Paper chromatography. The simple polyphenolic and soluble tannin fractions were separated into a number of distinct spots by two-dimensional ascending paper chromatography. The 8.5-in.-square sheets of Whatman No. 1 filter paper, after spotting with an extract, were developed first with 10% aqueous acetic acid and then with butanol–acetic acid–water (BAW), 20:7:15. After drying, the chromatograms were observed in ultraviolet (UV) light before and after exposure to ammonia vapor. Replicate papers were sprayed with the following diagnostic reagents: bis-diazotized benzidine (Roux and Maihs, 1960), ammoniacal silver nitrate (Partridge, 1948), ferric chloride-ferricyanide, sodium borohydride (Horowitz, 1957), and vanillin (chromatogram sprayed with a solution containing 1% w/v vanillin in ethanol, dried, and fumed with hydrogen chloride gas). UV absorbance spectra of major compounds were determined with a recording spectrophotometer directly on the chromatographic paper. The spot was cut from the chromatogram and placed in the light path. Alkali shifts were determined by spraying the paper with dilute sodium ethoxide and drying before recording the spectrum. Browning substrates were detected by spraying dry paper chromatograms with an extract of peeled date tissue in 0.1*M* acetate buffer, pH 5.1, and holding the paper overnight in a humid atmosphere at 27°C (Siegelman, 1955). Control chromatograms were sprayed with the buffer solution and held under identical conditions.

Identification of dactylifric acids. The major spot on chromatograms of the simple polyphenolic fraction of green dates, spot No. 6, was isolated for further identification since it was the main enzymic browning substrate present. The ethyl acetate fraction from 196 g of green date tissue was streaked on 18.5 × 22.5-in. sheets of Whatman

No. 3 MM filter paper and developed with BAW. The blue-fluorescent band with R_f 0.76 was cut out and eluted with 70% aqueous methanol. The eluate was restreaked on chromatograms which were developed with 0.1*N* HCl solution. The blue-fluorescent band with R_f 0.40 was eluted and evaporated to dryness under vacuum. The UV spectra of the material in ethanol and with various diagnostic reagents were typical of a caffeoyl ester (Jurd, 1956, 1957): EtOH λ_{max} 332, ~ 303 mμ; EtOH + NaOAc λ_{max} 331, ~ 304 mμ; EtOH + NaOAc + H_3BO_3 λ_{max} 349, ~ 306 mμ. In addition, the eluted band gave a single spot when cochromatogramed with the authentic dactylifric acids (Maier *et al.*, 1964) in 0.1*N* HCl and BAW. Because dactylifric, isodactylifric, and neodactylifric acids do not separate on paper chromatograms, the relative amounts of each isomer in the eluted band could not be determined. Conclusive proof that the eluted band contained the dactylifric acids was obtained by identification of shikimic and caffeic acids following hydrolysis with 0.1*M* trisodium phosphate at 38°C in a nitrogen atmosphere. The hydrolysis products were separated into ether-soluble and water-soluble fractions. Paper chromatograms of the ether fraction gave a single blue-fluorescent spot which reduced ammoniacal silver nitrate and cochromatogramed with authentic caffeic acid in 10% acetic acid and in benzene–acetic acid–water (6:7:3, upper phase). An acetone extract of the dried aqueous fraction gave a single spot which cochromatogramed with authentic shikimic acid in BAW and gave immediate greenish-yellow color formation, as does shikimic acid with the periodate-permanganate spray of Lemieux and Bauer (1954).

Leucoanthocyanidin tannins. To identify the anthocyanidin produced from the insoluble leucoanthocyanidin tannin of ripe date tissue, 3.0 g of the exhaustively extracted marc was suspended in a stoppered round-bottom flask containing 100 ml of butanol and 5.0 ml of concentrated HCl. After heating for 20 min at 100°C an additional 2 ml of HCl was added and heating was continued for another 20 min. The intensely colored solution was filtered, the residue washed with methanol, and the filtrate evaporated to dryness under vacuum at 60°C to give a deep rose solid. The crude anthocyanidin was purified by column chromatography on silicic acid. A 19 × 4-cm (dia.) column was prepared in the usual way with a slurry of 200 ml of silicic acid (Mallinckrodt Analytical Reagent, 100-mesh powder) in 500 ml hexane. The crude anthocyanidin was dissolved in methanol, mixed with 3 g silicic acid, vacuum dried, suspended in hexane, and poured onto the column. Development with a linear gradient of butanol (containing 0.1% v/v concentrated HCl) in hexane separated one major and one minor anthocyanidin band. The eluate from the tubes containing the major anthocyanidin was pooled, excess hexane and a drop of concentrated HCl were added, and the solution was cooled. The deep-rose-colored solid which precipitated was washed thoroughly with ethyl ether and dried.

The purified anthocyanidin gave absorption maxima which were identical to literature values of cyanidin (Geissman and Jurd, 1955) in 95% aqueous ethanol (545 mμ), 1*N* hydrochloric acid (516 mμ), and amyl alcohol saturated with 1*N* hydrochloric acid (550 mμ). Also, the bathochromic shift caused by adding aluminum chloride to the anthocyanidin in acidified (HCl) 50% aqueous ethanol, 36 mμ, was in good agreement with the literature value for cyanidin, 33 mμ. On paper chromatograms the date anthocyanidin gave R_f values which were identical to authentic cyanidin: 0.51 with water–acetic acid–conc. HCl (10:30:3) and 0.24 with 90% formic acid–3*N* HCl (1:1). When the date anthocyanidin was treated with 12% sodium hydroxide by the method of Karrer *et al.* (1927), phloroglucinol and protocatechuic acid, the expected products of cyanidin hydrolysis, were obtained. They were identified by color tests and R_f values in three different solvent systems.

The major anthocyanidin produced by treating spot 14, the water-soluble tannin of the green and pink dates, with the butanol-HCl reagent was also identified as cyanidin by visible spectra and paper chromatography.

RESULTS AND DISCUSSION

Properties of the individual polyphenols. The principal soluble polyphenolic constituents of dates and their properties are listed in Table 1 in order of decreasing R_f values in 10% acetic acid. The general classes to which the compounds belong are given in the last column of the table. It should be understood that these are not all of the polyphenolic constituents of dates, but rather the principal ones.

Flavans. Spots 7, 9, 11, and 12 were invisible in UV light and gave the deep maroon color with bis-diazotized benzidine and the rose color with vanillin-HCl typical of flavans with a 5,7-dihydroxy substitution pattern in the A-ring. In addition, their reduction of ammoniacal silver nitrate indicates that the B-rings bear *ortho*-dihydroxy or *vicinal*-trihydroxy groups. Spot 11, the largest of the group, had an absorption maximum at 280 mμ which shifted to 290 mμ

Table 1. Properties and occurrence of the principal phenolic constituents of dates.

Spot No.	R_f values in:		Color with:								Spectral maxima[a] (mμ)		Present in:				Probable compound class
Simple phenols	A[b]	B[b]	UV	UV—NH_3	Bis-diazotized benzidine	Silver nitrate	Ferric chloride–ferricyanide	Borohydride	Vanillin-HCl	Phenolase	Untreated	+ alkali	Green	Pink	Ripe	Stored	
1	.77	.40	none	none	dull yel.	none	blue	not tested	none							X	oxidation product
2	.74	.96	none	none	none	none	blue	not tested	none							X	oxidation product
3	.67	.57	blue	blue	none	none	none	none	none	none				X	X	X	cinnamic acid derivative
4	.63	.95	none	none	red	brown	blue	not tested	none						X		hydrolysis product
5	.61	.65	blue	blue	none	purple	none	none	none	none			X	X	X	X	cinnamic acid derivative
6	.54	.76	blue	yel. gr.	brown	black[c]	blue	none	yellow	brown[c]	300,[d] 326	290,[d] 387	X	X	X	X	dactylifric acids
7	.52	.57	none	none	maroon	brown	blue	none	rose	pink-brown			X	X			flavan
8	.45	.91	blue	deep blue	purple	black	blue	not tested	none	none	286, 310[d]	308,[d] 330			X	X	cinnamic acid derivative
9	.44	.53	none	none	maroon	brown	blue	none	rose	pink-brown			X	X			flavan
10	.36	.73	shadow	yellow	pink	yellow	blue	yellow	yellow	none	272, 360	285, 400	X	X	X	X	flavonol glycoside
11	.36	.62	none	none	maroon	brown	blue	none	rose	yel. brown	280	290	X	X			flavan
12	.36	.57	none	none	maroon	brown	blue	none	rose	yel. brown			X				flavan
13	.35	.69	shadow	yellow	pink	brown	blue	yellow	yellow	none	272, 360	285, 400	X	X	X	X	flavonol glycoside
Soluble tannin																	
14	0—.2	.00	none	none	maroon	brown	blue	none	rose	none	278[e]	292[e]	X	X			leucocyanidin tannin

[a] Directly on paper. Blank space means spot was too weak to obtain a definite result.
[b] Solvent A is 10% aqueous acetic acid, and B is butanol–acetic acid–water (20:7:15).
[c] No reduction of silver nitrate and no darkening with phenolase in the stored stage.
[d] Inflection.
[e] In aqueous solution.

when treated with sodium ethoxide. These data suggest that spots 7, 9, 11, and 12 are flavans, probably either catechins (flavan-3-ols) or leucoanthocyanidins (flavan-3,4-diols) with 5,7,3′,4′-tetrahydroxy or 5,7,3′,4′,5′-pentahydroxy substitution patterns.

The yellow-brown observed when spots 11 and 12 were treated with crude date phenolase and the pink-brown color with spots 7 and 9 established that they are substrates for the enzyme.

Flavonol glycosides. Spots 10 and 13 have properties typical of flavonol glycosides. On paper chromatograms they appeared as shadows in UV light, but showed a yellow fluorescence when exposed to ammonia vapor. They gave a yellow color with sodium borohydride and pink with bis-diazotized benzidine; the pink suggests a quercetin hydroxylation pattern. The UV spectra of both spots together (they overlapped somewhat) is indicative of flavonol glycosides, λ_{max} 272 and 360 mμ, shifting to 285 and 400 mμ with alkali. Their mobility in 10% acetic acid also indicates that these flavonols are glycosides. Spot 13 reduced ammoniacal silver nitrate whereas spot 10 did not. These data indicate that spots 10 and 13 are flavonol glycosides with quercetin hydroxylation patterns in which the 3-OH is probably involved in the glycosidic bond. In spot 10, one or both of the *ortho*-dihydroxy hydroxyl groups is blocked (i.e. methyl ether, etc.). Thus, spot 13 could be a quercetin-3-glycoside and spot 10 an isorhamnetin-3-glycoside.

Neither flavonol darkened when treated with crude date phenolose.

Cinnamic acid derivatives. Spot 6, the major ethyl-acetate-soluble polyphenol in immature dates, was isolated and shown to be a mixture of dactylifric acids (isomers of monocaffeoylshikimic acid). The presence of these compounds in dates and their structures were recently reported by Maier *et al.* (1964). On chromatograms, spot 6 from green, pink, and ripe dates was darkened by date phenolase, whereas spot 6 from dark dates was not. Also, spot 6 from dark dates did not reduce ammoniacal silver nitrate but gave a very similar UV spectrum to that from green dates. It thus appears that spot 6 from dark dates is a different compound which lacks a free *ortho*-dihydroxy group. It may be a feruoyl- or isoferuoyl-derivative, possibly a feruoyl- or isoferuoyl-shikimic acid.

The small amount of spots 3 and 5 present limit the information obtained. Both were blue fluorescent, and negative ferric tests indicated the absence of free phenolic hydroxyls. With ammoniacal silver nitrate, spot 5 gave a purple color similar to that given by sinapic acid. These results suggest that spots 3 and 5 are cinnamic acid derivatives (esters, glycosides, or coumarins) that lack free phenolic hydroxyls.

Date phenolase darkened neither compound.

Spots that appeared during maturation and darkening. Spots 1, 2, 4, and 8 formed during maturation and postharvest darkening. Spots 4 and 8, which appeared first in the ripe fruit, seem to contain *ortho*-di- or tri-hydroxy groups since they reduced ammoniacal silver nitrate; however, the date enzyme did not darken spot 8. These compounds probably form by hydrolysis of preexisting esters or glycosides or by hydroxylation of other polyphenols. The blue fluorescence and the spectrum of spot 8 suggest that it is a cinnamic acid derivative. Spot 4 may be a phenolic acid.

Spots 1 and 2 appeared in small amounts only in the dark fruit. They were not fluorescent but gave positive ferric tests and may be oxidation products of other polyphenols, possibly the tannins.

The ethyl acetate extract of pink dates was violet, suggesting the presence of anthocyanidin pigments. The violet pigments were not present in sufficient quantities to locate them on the chromatograms.

Leucocyanidin tannins. Spot 14, the water-soluble tannin, gave color tests typical of a flavan with a 5,7,3′,4′-tetrahydroxy substitution pattern. Its insolubility in ethyl acetate and its low mobility on paper chromatograms indicate that it is a polymer. It had an absorption maximum at 278 mμ in aqueous solution which shifted to 292 mμ when alkali was added, typical of catechins and leucoanthocyanidins. When treated

with hot concentrated hydrochloric acid in butanol the major anthocyanidin produced was cyanidin chloride (3,5,7,3′,4′-pentahydroxy flavylium chloride). These results indicate that the water-soluble tannin contains condensed leucocyanidin units (5,7,3′,4′-tetrahydroxy flavan-3,4-diol); however, they do not exclude the presence of other constituents.

Date phenolase does not darken the soluble leucocyanidin tannin even though it contains *ortho*-dihydroxy groups. Presumably the polymeric nature of the tannin prevents it from being a substrate for the enzyme. An irreversible reaction between these two large molecules (polyphenol polymer and protein) is another possible explanation. The astringency of the aqueous tannin solution supports this hypothesis.

The presence of insoluble leucocyanidin tannin in ripe date tissue is shown by the production of cyanidin when the marc is treated with hot, strong acid in butanol. Cyanidin was purified by column chromatography, and its identity was established by alkaline degradation, paper chromatography, and visible spectra in the presence of various solvents and reagents. Thus, the insoluble tannin is similar to the soluble tannin in containing condensed leucocyanidin units. The insolubility of tannin in ripe dates could be due to large molecular size or to interaction with other insoluble tissue fractions such as cellulose, pectin, hemicelluloses, or proteins (Swain and Goldstein, 1963). The insoluble tannins of date tissue are not darkened in the presence of date phenolase (Maier and Metzler, 1965).

Changes in the individual polyphenols. The occurrence of the principal soluble polyphenolic constituents in the different maturity stages is also shown in Table 1. Essentially the same compounds are present during the green and pink stages. The soluble tannins of the green and pink stages averaged 94.8% of the total soluble polyphenolic constituents. The green and pink stages differed qualitatively in the absence of spot 3 in the green stage and spot 12 in the pink stage.

The ripe stage lacked the three flavans of the pink stage and the soluble leucocyanidin tannins, but included two new spots, 4 and 8. In addition, with the exception of spots 4 and 8, the concentration of all polyphenols was lower.

The stored dates reflected the trend begun in the ripe stage, namely, a decrease in concentration of the spots present in the pink stage and an increase in the new spot (8) of the ripe stage. Two new spots, 1 and 2, appeared in stored dates, and spot 4, which had been present only in ripe dates, was absent. Spot 8 is the predominant ethyl-acetate-soluble polyphenol in the dark dates. Less of spot 6 (the dactylifric acids), the predominant simple polyphenol of immature dates, was present in the ripe and stored stages. In fact, spot 6 of stored dates appears to contain a different compound from that of the other stages.

The general decrease in the simple polyphenols and soluble tannin during growth, maturation, and storage as judged from the relative sizes and intensities of the spots on paper chromatograms agrees with the quantitative changes reported previously (Maier and Metzler, 1965).

Relation of the polyphenolic constituents to browning. Of the polyphenolic constituents in dates five have been directly shown to be enzymic browning substrates: the dactylifric acids (spot 6) and the flavans (spots 7, 9, 11, and 12). Of this group the only ones detectable in ripe dates are the dactylifric acids. The flavans disappeared during maturation, and their oxidation products probably contribute to the brownish color of ripe dates. Apparently the dactylifric acids, which disappear during postharvest darkening, are the primary substrates for postharvest enzymic browning of dates. This order of disappearance of enzymic browning substrates is in general agreement with the oxidation-reduction hypothesis of Roberts (1957). The formation of new enzymic browning substrates by tannin degradation during storage is possible; however, their rates of formation would probably be much slower than their rates of oxidation.

Direct tests have shown that date phenolase oxidizes neither the flavonol glycosides nor the cinnamic acid derivatives, spots 3

and 5. The presence of these compounds in dark stored dates confirms this fact. Spots 1, 2, 4, and 8 appear during maturation and postharvest darkening, and therefore may be oxidation or hydrolysis products of polyphenols (possibly tannins) present in immature fruit.

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The Mechanism of Sulfite Inhibition of Browning Caused by Polyphenol Oxidase

SUMMARY

The effect of sulfite on the reaction of mushroom polyphenol oxidase with o-diphenols was studied in model systems at pH 6.5. Spectrophotometric, manometric, chromographic, electrophoretic, and radiosotopic evidence is presented that sulfite prevented browning in the systems by combining with the enzymatically produced o-quinones and stopping their condensation to melanins. During preincubation of polyphenol oxidase with sulfite a gradual loss of the property of the enzyme to cause browning was observed.

INTRODUCTION

Sulfur dioxide is known to inhibit the browning caused by polyphenol oxidase (PPO) (Ponting, 1960; Diemair *et al.*, 1960). The mechanism of this inhibition is not completely understood. Ponting and Johnson (1945) thought that, in fruits to which SO_2 has been added, PPO catalyzes first the oxidation of SO_2 and then, when all the SO_2 is oxidized, the darkening of the fruit. On the other hand, Diemair *et al.* (1960) showed that, when potato tyrosinase is incubated with SO_2 prior to the addition of the substrate, considerable inactivation of the enzyme occurs.

The objective of this study has been to evaluate the inactivation of PPO by SO_2 in connection with the browning reaction and explore other mechanisms of the sulfite inhibition of this enzymatic darkening.

MATERIALS AND METHODS

The enzyme used in this work was mushroom tyrosinase (Sigma Chemical Co.). Its activity was found to be 800 units/mg (1 unit = 0.001 absorbancy increase per min at 280 mμ, in 0.16*M* phosphate buffer, pH 6.5, 25°C, containing $3 \times 10^{-4}M$ L-tyrosine). The substrates were pyrocatechol (Eastman) and caffeic and chlorogenic acids (Nutritional Biochem.). Sodium hydrogen sulfite (Merck reagent) was used in the unlabeled sulfur experiments. Radioactive SO_2 was prepared by boiling $H_2{}^{35}SO_4$ with copper, trapping the radioactive SO_2 in 1% NaOH soln., distilling the $^{35}SO_2$ after acidification with H_2SO_4 and receiving it in cold water. The reaction mixture used throughout this work consisted of 1 ml 0.5*M* phosphate buffer, pH 6.5, 1 ml substrate solution, *x* ml $NaHSO_3$ solution, 0.9 — x ml water, and 0.1 ml enzyme solution.

The spectrophotometric measurements at 290 and 420 mμ were made with a Beckman DU spectrophotometer connected to a Ledland log-converter and a Sargent SR recorder. Except in the preincubation experiments, the enzyme solution was the last component of the reaction mixture to be transferred into the 1-cm-path Beckman cuvette. Used for the transfer and mixing of the enzyme solution was a square Teflon plunger provided with a groove and three orifices and connected to a stainless-steel handle. In the preincubation experiments the substrate solution was added last.

A Warburg apparatus thermostated at 30°C was used for the manometric studies. In paper chromatography, No. 1 Whatman paper was employed, with *n*-butanol–acetic acid–H_2O (25:6:25) as solvent, in descending irrigation, at 25°C. Paper electrophoresis was performed with a Spinco cell, Model R, and accessories, and 450 V were applied for 2 hr. The electrolyte was 0.1*M* formic acid adjusted to pH 2.2 with NaOH. A 4-pi Vanguard 880 radiochromatogram scanner was used in tracing the radioactivity of chromatograms and electropherograms. A Spectronic 505 recording spectrophotometer was employed in spectral analysis.

RESULTS AND DISCUSSION

Preliminary spectrophotometric observations indicated that, in the presence of sulfite, there was a rapid increase in the absorbance at 290 mμ of the system PPO-pyrocatechol. [The term sulfite is used to describe the form of the sulfur at the beginning of the reaction, because at pH 6.5, about 95% of the salt added is dissociated to sulfite ions and the remainder to bisulfite ions (Vas and Ingram, 1949).] When levels of sulfite were used which were not sufficient to inhibit browning completely, the wave length of 420 mμ was found suitable for following the development of browning. Using these two wave lengths, the curves of Fig. 1 were

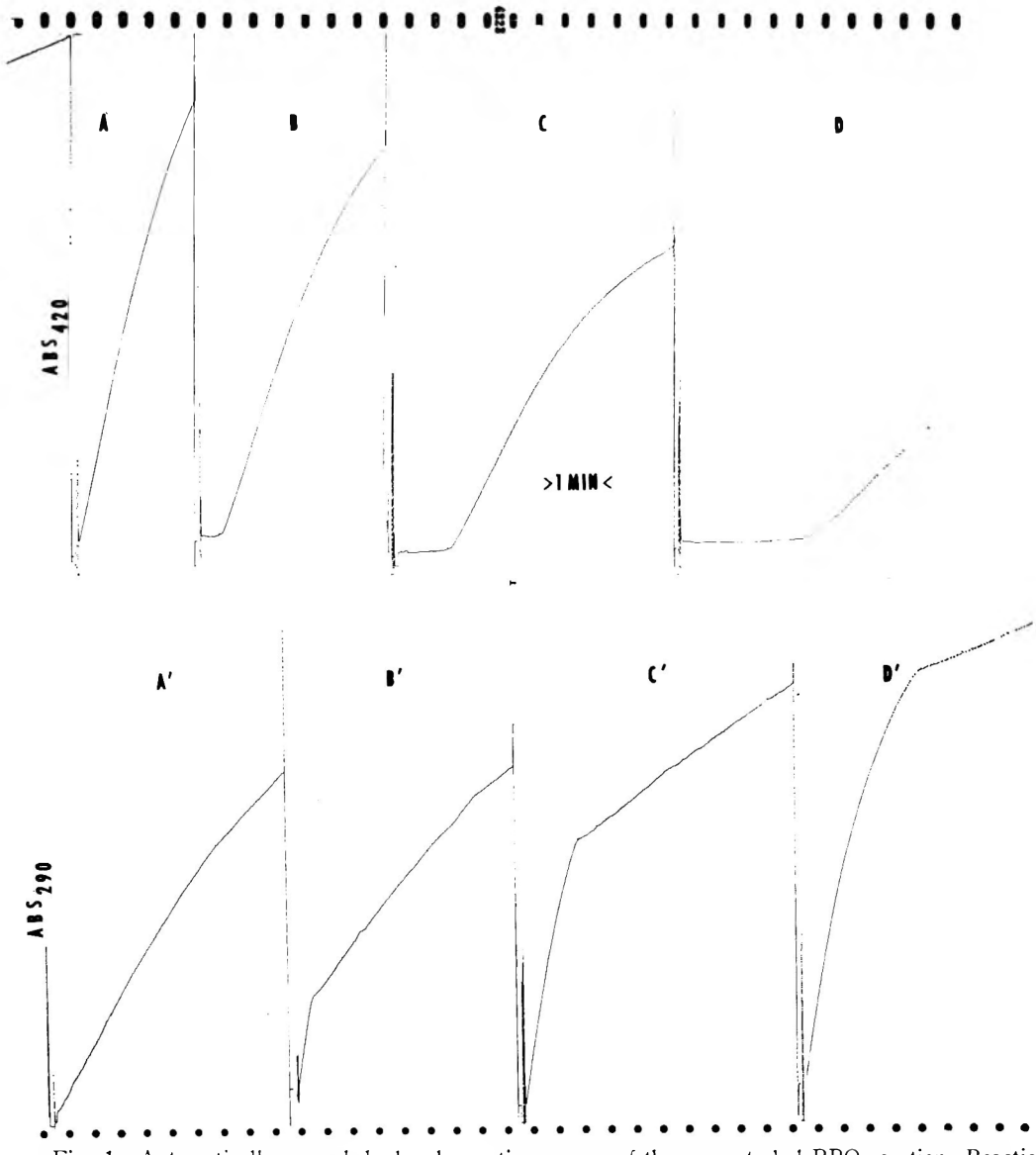

Fig. 1. Automatically recorded absorbance-time curves of the pyrocatechol-PPO reaction. Reaction mixture: pyrocatechol $1.3 \times 10^{-3}M$; PPO 1.3 unit/ml; $0.16M$ PO_4 buffer, pH 6.5; sulfite in A,A′ none, in B,B′ $10^{-4}M$, in C,C′ $2 \times 10^{-4}M$, in D,D′ $3 \times 10^{-4}M$.

obtained from the reaction PPO-pyrocatechol-sulfite. The presence of sulfite resulted in the appearance of a lag period in the development of browning. The length of this lag increased with sulfite concentration. If the slope of the absorbance curve at 420 mμ, after the lag, is taken as a measure of the rate of the browning reaction, it is obvious that the rate of this reaction decreased as the sulfite concentration increased. During the period corresponding to the lag at 420 mμ, there was a rise in absorbance at 290 mμ that was considerably steeper than in the absence of sulfite. After the initial rapid rise in absorbance at 290 mμ, a rather sharp decline in the rate of increase of this absorbance was observed; and the height of the breaking point appears to be proportional to the quantity of sulfite. Noteworthy also is the coincidence of the time at which the 420 mμ lag ends and the 290 mμ curve breaks, at all levels of sulfite.

To explain these data one may contemplate the various ways by which sulfite can interfere with the PPO-catalyzed browning. Joslyn and Braverman (1954) theorized that "SO_2 could act by reducing oxygen and making it unavailable for oxidation or by reacting with the quinones or other intermediates in polyphenol oxidation." The suggested reduction of the oxygen could be accomplished either directly by oxidation of SO_2 or indirectly through the quinones oxidizing the SO_2, in analogy with the oxidation of ascorbic acid; in both cases sulfate would be formed. To these possibilities two more can be added: the reaction of the sulfite with the substrate to render the latter unsuitable for the enzymatic reaction and the inactivation of the enzyme by the sulfite.

The hypothesis of a substrate-sulfite reaction was tested first. In the absence of enzyme, no spectral change was observed when sulfite was added to the buffered substrate solution. Apparently, the substrate was not modified.

The inactivation of the enzyme by sulfite was tested by incubating the enzyme with two concentrations of sulfite for several periods and subsequently measuring the rate of browning (420 mμ) development. As shown in Fig. 2, the enzyme loses activity when it is in contact with sulfite. Although this loss of activity could be a cause of the declining rates observed after both the lag in browning development and the initial increase in the 290 mμ absorbance, it cannot explain either the lag or the steep 290 mμ rise.

Furthermore, the oxygen uptake by the pyrocatechol-PPO system is the same whether sulfite is absent or present in concentrations approximating those of the spectrophotometric experiments (Table 1).

Had the oxygen uptake been due to sulfite oxidation, sulfate should have been formed. To test the possibility of sulfate formation, tracer techniques were used. Chlorogenic acid was incubated with PPO and sulfite-^{35}S, and the reaction products were streaked on Spinco electrophoresis paper strips. Other strips were streaked with a similar reaction mixture but containing unlabeled sulfite and a trace of sulfate-^{35}S added to it. The two sets of strips were subjected to electrophoresis, as already described, and scanned for

Table 1. Oxygen uptake (μl) by the pyrocatechol-PPO-sulfite system. Pyrocatechol $6.8\times10^{-4}M$; PPO 1.3 unit/ml; 0.16M PO_4-buffer, pH 6.5.

Time (min)	Oxygen uptake (μl) with sulfite: 0	$3\times10^{-4}M$	$7.5\times10^{-4}M$
0	0	0	0
2	10.3	11.0	11.0
4	19.2	19.9	18.5
6	24.0	24.7	21.9
8	26.0	27.4	26.0
10	29.5	29.5	29.5
12	30.8	30.1	30.3
	Browning started immediately	Browning developed during expt.	No browning at the end of expt.

radioactivity. The scanning tracings appear in Fig. 3. No sulfate peak is present in chart A corresponding to the system which contained radiosulfite; all the radioactivity is associated with the fluorescent band (striated area). Chart B indicates the location of the sulfate and shows that the fluorescent compounds bind no sulfate.

While working with fluorescent substrates, such as chlorogenic or caffeic acids, it was observed that the products of the

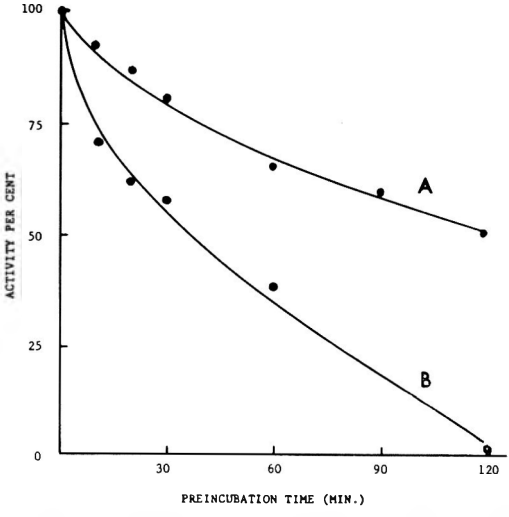

Fig. 2. Effect of preincubation of PPO with sulfite on the activity of the enzyme. Preincubation mixture: 1.0 ml of 0.5M PO_4 buffer, pH 6.5; 0.1 ml of enzyme solution (40 units/ml); A, 0.1 ml of $10^{-3}M$ $NaHSO_3$, 0.9 ml of H_2O; B, 0.2 ml of $10^{-3}M$ $NaHSO_3$, 0.8 ml of H_2O. Assay mixture: above plus 1.0 ml of $5\times10^{-3}M$ pyrocatechol. The activity was measured as increase in absorption at 420 mμ from tracings as in Fig. 1.

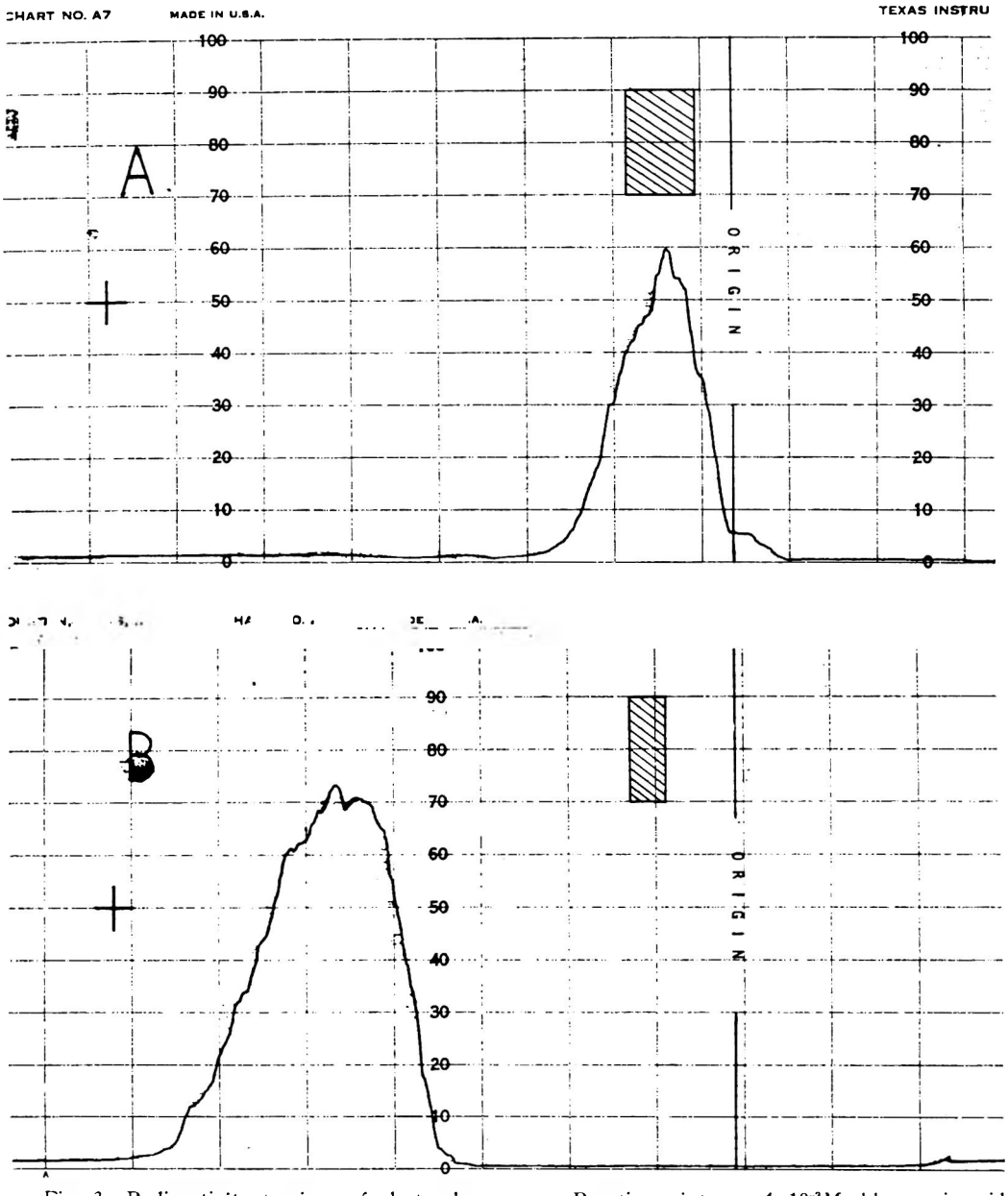

Fig. 3. Radioactivity tracings of electropherograms. Reaction mixture: *A*, $10^{-3}M$ chlorogenic acid, 1.3 units/ml PPO, $10^{-4}M$ sulfite-^{35}S, $0.16M$ PO_4 buffer, pH 6.5; about 0.01 ml was streaked on standard Spinco paper strip. *B*, same as *A* but containing unlabeled sulfite and trace of sulfate-^{35}S. Electrophoresis: 450 V, 2 hr, $0.1M$ formate, pH 2.2. Scanning range: 300 cpm. Striated areas indicate fluorescent loci.

reaction of PPO with these substrates, in the presence of sulfite, could be resolved by paper chromatography into a number of fluorescent fractions. When two of the brightest fluorescent bands of the paper chromatograms obtained from either the PPO-chlorogenic-sulfite or the PPO-caffeic-sulfite systems were eluted and subjected to the sodium formate fusion test (Feigl, 1960), positive results were obtained. This is a test for sulfonic acids, sulfinic acids, sulfonamides, and sulfones.

Later, the reaction between PPO and caffeic or chlorogenic acids was carried out in

the presence of sulfite-^{35}S. The products of the reaction were then chromatographed on paper and the chromatograms were scanned for radioactivity. Fig. 4 illustrates the results of the scanning. It can be seen that the fluorescent bands, and only these, are radioactive.

All these observations strongly suggest the formation of compounds between sulfite and the intermediary enzymatic products. It is known (Mason, 1959; Dawson and Tarpley, 1963) that quinones are formed during melanin formation, and that quinones form addition compounds with sulfite (LuValle, 1952; Schenck and Schmidt-Thomée, 1953). If such additional compounds between sulfite and the o-benzoquinone derived from the enzymatic oxidation of pyrocatechol should be formed, the condensation of the o-benzoquinone would be prevented and the appearance of browning would be delayed until all the sulfite is used up. This would explain the lag in absorbance at 420 mμ (Fig. 1). If the quinone-sulfite compounds absorb strongly at 290 mμ, as the quinone-sulfite derivatives studied by LuValle (1952) do, the initial rapid increase at 290 mμ (Fig. 1) would be understandable. The formation of the quinone-sulfites would also explain the manometric results, since the oxygen uptake by the enzymatic system would not be

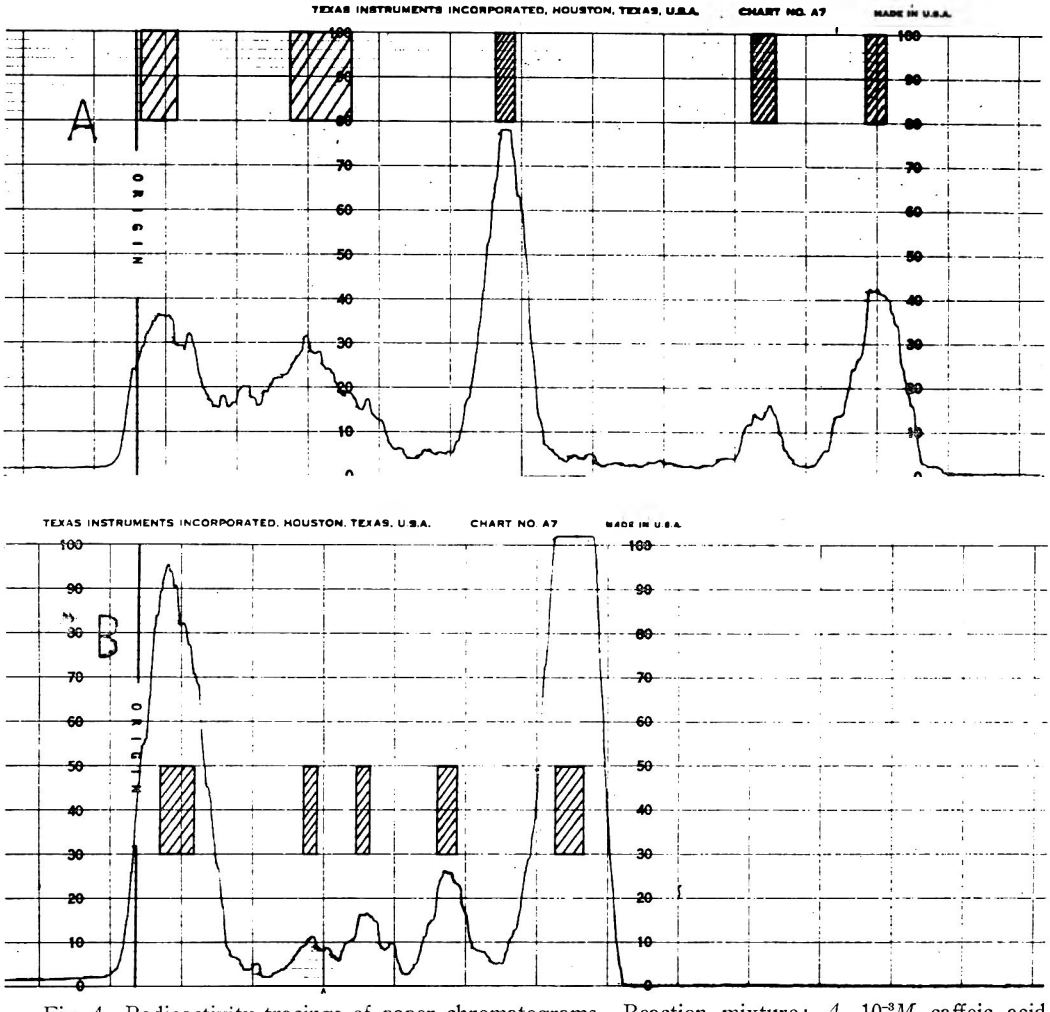

Fig. 4. Radioactivity tracings of paper chromatograms. Reaction mixture: *A*, $10^{-3}M$ caffeic acid, 1.3 units/ml PPO, $10^{-4}M$ sulfite-^{35}S, $0.16M$ PO_4 buffer, pH 6.5; *B*, $10^{-3}M$ chlorogenic acid, remainder as in *A*. Paper: Whatman No. 1. Solvent: *n*-butanol–acetic acid–H_2O (25:6:25), descending 24 hr. Striated areas indicate fluorescent loci.

affected significantly by the presence of sulfite during the initial phase of the reaction.

The failure of sulfate formation is obviously compatible with this theory, and the radioactivity of the fluorescent intermediates provides direct evidence of the presence of sulfur in these derivatives. The multiplicity of the fluorescent products is in agreement with the formation of many quinone-type intermediates in melanin production.

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Effect of Gamma Radiation Upon Cherries

SUMMARY

The effect of gamma radiation upon the quality of sweet cherries was studied. Softening was detected which progressed rapidly above a threshold dose of approximately 50 kilorads and was related to the degradation of pectic constituents of the fruit. Although respiratory O_2 consumption and CO_2 evolution were stimulated during irradiation, the response subsided slowly after irradiation ceased. Color bleaching occurred only at the high doses. Despite a marked initial reduction in microbial contamination, extended storage periods showed increased microbial spoilage. A reduction in the development of brown-rot during high-temperature short-time storage resulted from kilorad doses of radiation. There was a slightly increased rate of sulfur dioxide bleaching and increased yield of brined cherries, but this was accompanied by loss of texture of the finished product.

Literature on the effect of gamma radiation upon sweet cherries is somewhat sketchy and often contradictory. One survey indicates that the shelf life of cherries can be extended by pasteurizing doses of ionizing radiations (Hannan, 1955). However, substantially accelerated rates of spoilage caused by radiation treatment have also been reported (Nehemias *et al.*, 1954) and verified (Cooper and Salunkhe, 1963). An increase in taste-panel acceptability of irradiated sweet cherries has been reported (Salunkhe, 1961), despite other reports of off-flavors and losses in texture (Nehemias *et al.*, 1954; Brownell *et al.*, 1954). Preliminary experiments have been reported (Romani *et al.*, 1961) showing a transient increase in respiratory activity of irradiated cherries followed by a subsequent return to near-normal.

This study endeavored to define the effect of gamma irradiation upon several quality factors of sweet cherries. Some of the extremes of radiation dose range, storage, and handling conditions utilized for this study are beyond the realistic range. They were included, however, to elaborate the particular response in question.

MATERIALS AND METHODS

Three varieties of sweet cherries were used (Schmidt, Windsor, and Napoleon), the last being used most extensively. All cherries were picked, sorted, and divided into samples at room temperature and irradiated within 18 hr. Stems were cut off even with the top of the fruit, when necessary. Except where noted, the cherries were packaged in 2-pound bags of 300-mil cellophane prior to treatment. The bags were closed by folding the top loosely and secured with one staple, so as to permit ready gas exchange but to minimize contamination with microorganisms. Preliminary experiments indicated that only a slight increase (less than 1%) of carbon dioxide could be detected in these bags during the experiments.

Irradiation. The irradiations were carried out with a cobalt-60 source at the Geneva Experiment Station. This source, heretofore undescribed in the literature, is a 4,000-curie cobalt-60 source placed in approximately the center of a specially constructed room 15 × 18 × 11 ft high. The source is of special design, as shown in Fig. 1. For operation, a hand winch located in an adjoining room (not shown) is turned to raise rod (*A*), which lifts the attached lead cover plug (*C*) and cobalt-60 capsule (*D*) assembly out of the lead storage pig (*E*) to a predetermined location in the guide tube (*B*). Objects (*G*) to be irradiated are placed in concentric arcs of predetermined dose-rate on a supporting table (*F*). Preliminary dosimetry performed using the ferrous-ferric system, with dose rates between 270 rads and 730 krads per hour obtainable, indicated a field uniformity at constant distance of better than ±5%.

Firmness and pectin evaluation. The Chatillon pressure tester was used for measuring cherry firmness. All readings were made at room temperature and, unless otherwise stated, within 1 hr of the radiation treatment.

Pectin extraction and evaluation was conducted in a manner described by Kertesz (1951). Briefly, immediately after firmness measurement, the alcohol-insoluble solids (AIS) of the cherries were

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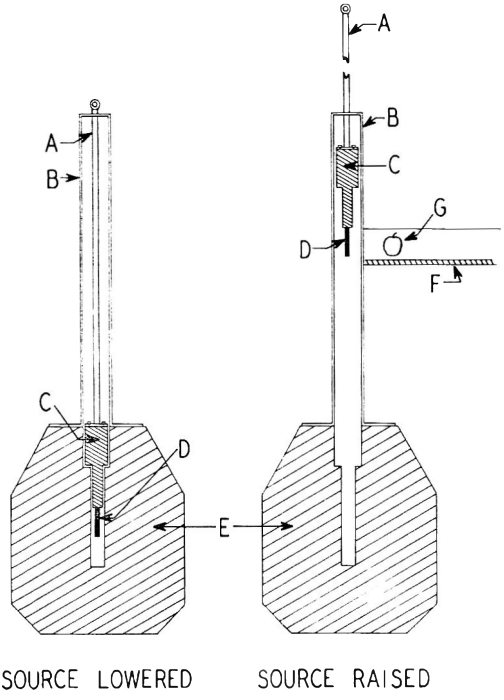

Fig. 1. Geneva Co^{60} source showing cobalt in lowered (exposed) and raised (shielded) positions. Lifting steel rod (A) up through the steel guide-tube (B) raises lead plub (C) and attached Co^{60} (D) out of the lead shield (E), permitting radiation of object (G) on supporting table (F).

prepared by boiling in 95% ethanol followed by filtration and dehydration in absolute ethanol, in air, and finally in vacuum over P_2O_5.

Extraction and characterization of pectic constituents of the AIS were performed (Glegg *et al.*, 1956). This procedure involved, first, a cold-water extraction to remove the water-soluble pectic constituents, a further extraction with dilute Calgon to remove the pectic acid and pectates, and, finally, extraction at 80°C with 0.05*N* HCl to extract the protopectin fraction. Each fraction was collected separately and made up to standard volume, NaCl concentration, and pH, and viscosity determination was made at 30°C in an Ostwald-Cannon-Fenske pipette. The "average specific viscosity" values were obtained by averaging the results of the water, Calgon, and acid extracts. Uronic acid determinations were conducted according to the method of McComb and McCready (1952).

Respiratory activity. The respiratory activity of the cherries was measured both during and after irradiation. The methods involved: 1) determining CO_2 production and O_2 consumption in a static atmosphere system for the during-irradiation phase; and 2) determining CO_2 production of the whole cherries in a Warburg respirometer for the post-irradiation phase. The during-irradiation techniques have been described (Massey *et al.*, 1961). The method was examined for complications arising from the possible accumulation during the relatively long exposure of gaseous respiratory influencing substances such as accumulated carbon dioxide, ozone, etc., known to be produced in air by ionizing radiation. Although it was possible to demonstrate aberrant respiration rates by the presence at high concentrations of some of these by-products, a careful comparison of respiratory stimulation in moving and in static atmospheric systems proved negative in experiments reported here.

Post-irradiation respiration rates were conducted on the same three fruit samples with standard Warburg techniques at 25°C. The fruit was carefully transferred to the Warburg flasks and permitted a suitable period for temperature equilibration, and respiratory measurements were taken every 15 min for 2 hr. The fruit was then stored for approximately 20 hr in the Warburg vessels at 25°C and maintained under gentle aeration (about 500 ml air per hr) to avoid anaerobiosis. Respiratory measurements were repeated. No evidence was noted of either abnormal physiological conditions due to bruising or microorganism growth.

Taste and color. Acceptability of the cherries was evaluated immediately following irradiation by taste panels of 18–20 persons by the multiple-comparison method. In each panel, the tasters ranked 4 samples of 15 fruit for color, texture, and flavor.

Additionally, reflected color of the cherry fruit was evaluated with a Hunter laboratory color-difference meter. For this test, Napoleon was used since any small change in red coloration would be more obvious with this pink variety than with the darker-colored ones. The cherries were packed, pinkest side exposed to the instrument, into a 4-inch-square sample holder. Reference Plate No. 25-303 was used as a comparative standard ($L = 85.5$, $a = -5.5$, $b = 22.4$).

Brined Napoleon cherries. After irradiation, cherries were placed in pH 3.0 brine containing 15,000 ppm of SO_2 and stored for 6 months. Then the cherries were pitted, their unit weight determined, and their color characteristics measured with the color-difference meter. Texture was obtained as the maximum resistance offered by the cherry to the passage of a blunt .125-inch-diameter pin traveling at 20 cm per min. After removal of the SO_2 by leaching in water the cherries were raised to 50% soluble solids in sucrose solutions. These raised, or "maraschino"-type, cherries were again examined for unit weight and texture.

Storage longevity. The influence of gamma irradiation on the storage longevity of cherries

was measured by both visual and microbiological assessment. For these experiments, the cherries were irradiated after packaging in 50-g lots in cellophane bags as previously described and stored for various periods at 21 and 2°C. Descriptions of appearance and texture were recorded both during and after storage. Microbiological assessment was done by homogenizing 50-g samples with an equal weight of water in a blender, followed by plating of appropriate dilutions. "Total" counts were obtained on standard plate count agar (Difco) incubated 2 days at 32°C. Yeast counts were estimated on potato-dextrose agar (Difco) acidified to pH 3.5 with tartaric acid and incubated 1 week at room temperature. Mold assay was restricted to visual observations.

RESULTS

Firmness and pectin content. Irradiation affected all 3 cherry varieties similarly. Fig. 2

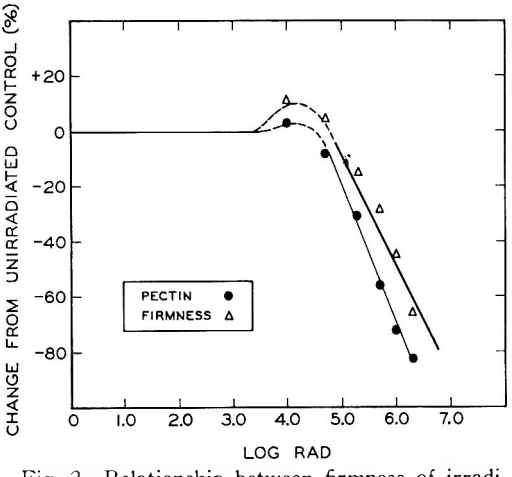

Fig. 2. Relationship between firmness of irradiated cherries as measured by changes in pectin viscosity corrected for constant anhydrogalacturonic acid content.

summarizes typical softening responses of the 3 varieties. Doses of less than 40 krad gave insignificant (less than 10%) softening. The radiation effect at about the point of inflection was irregular, a phenomenon observed before in our studies of other tissues (Glegg *et al.*, 1956; Boyle *et al.*, 1957). The highest dose used (2,000 krad) gave approximately 80% softening. Maturity exhibited only a slight effect upon the amount of softening due to radiation.

Comparison of the logarithm of radiation dose vs. percent change in firmness indicates a relationship similar to that observed before in the irradiation of other plant tissues (Boyle *et al.*, 1957; Massey *et al.*, 1961; Kertesz *et al.*, 1964). When the changes below 10% are disregarded, calculated regression lines indicate "threshold doses" (TD's) in the range of 50±20 krad.

Changes in the pectin content of Napoleon cherries from irradiation exhibited little consistent relationship to maturity of the cherry. The data in Table 1 are therefore an average of four harvest dates. These results indicate a progressive degradation of the pectin fractions as indicated by loss of viscosity of all individual fractions, both in specific viscosity and as corrected for constant uronide content. Changes in the soluble-insoluble pectin ratio appear to be an excellent index of response. These are similar to changes reported for some other tissues (Kertesz *et al.*, 1964; Massey *et al.*, 1964).

Fig. 2 shows that changes in pectin viscosity as corrected for constant anhydrogalacturonic acid content are correlated with changes in texture as measured by pressure-test readings. Disregarding measurements about the point of inflection, the TD for the degradation of pectin is nearly identical to the TD for softening. Although the deviation in slope of the two curves indicates that factors other than pectin changes *per se* may be involved, such as changes in turgor pressure, etc., it is clear that a positive relation does exist between tissue softening and pectin degradation in cherries similar to that reported for other tissues (Kertesz *et al.*, 1964). Of interest is the correlation between tissue firming and increase in viscosity of the extracted pectins at radiation doses near the point of inflection. Thus far, we have found no explanation for this phenomenon.

Respiratory activity. Results of the respiratory-activity measurements indicate that the response was independent of varietal differences, and that variations in response due to maturity were small and inconsistent. No real differences in either magnitude or type of response were found

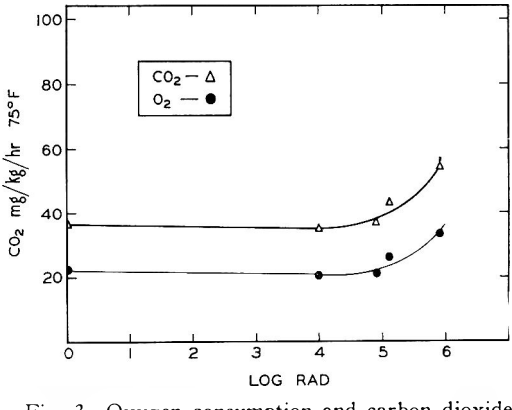

Fig. 3. Oxygen consumption and carbon dioxide evolution from cherry fruit during 18 hr of irradiation.

Table 1. Changes in pectin fractions of Napoleon cherries resulting from irradiation.[a]

Dose (krad)	Viscosity		Anhydrogalacturonic acid	
	Specific viscosity	$\frac{W}{C + H}$[b]	mg AGA per ml	$\frac{W}{C + H}$[b]
0	W 0.300	0.291	W 0.252	0.241
	C 0.538		C 0.332	
	H 0.492		H 0.730	
	Av. 0.443		Total 1.314	
10	W 0.328	0.323	W 0.238	0.244
	C 0.562		C 0.334	
	H 0.454		H 0.640	
	Av. 0.448		Total 1.212	
50	W 0.331	0.339	W 0.237	0.234
	C 0.504		C 0.337	
	H 0.471		H 0.667	
	Av. 0.435		Total 1.242	
200	W 0.354	0.457	W 0.349	0.359
	C 0.419		C 0.364	
	H 0.355		H 0.609	
	Av. 0.376		Total 1.322	
500	W 0364	0.701	W 0.429	0.530
	C 0.303		C 0.374	
	H 0.216		H 0.435	
	Av. 0.294		Total 1.238	
1000	W 0.312	0.869	W 0.543	0.797
	C 0.219		C 0.334	
	H 0.140		H 0.347	
	Av. 0.224		Total 1.224	
2000	W 0.192	0.857	W 0.560	0.854
	C 0.154		C 0.417	
	H 0.070		H 0.239	
	Av. 0.139		Total 1.216	

[a] Averages of data from four successive weekly harvests.
[b] Fraction W = soluble in cold water; C = soluble in 0.2% Calgon + 0.8% sodium chloride; and H = soluble in 0.05*N* HCl (80°C).

to exist between cherries varying in harvest date by as much as 4 weeks. Data in Fig. 3 indicate that little or no respiratory response could be demonstrated at doses below 100 krads. This lack of sensitivity is markedly lower than that previously reported for lettuce tissues measured under identical conditions (Massey *et al.*, 1961). The lack of respiratory response is further illustrated by the post-irradiation respiration data presented in Table 2. These data show that the during-irradiation response obtained by doses as high as 1,000 krad diminishes to near normal rates following treatment.

The during-irradiation measurement represents the sum total of respiratory activity during the 18-hr period of irradiation. Thus, even large fluctuations in respiratory activity could be occurring during this period without being evident. In some experiments, the CO_2 evolution rate during the first 9 hr of an 18-hr total exposure was com-

Table 2. Carbon dioxide evolution from cherry fruit during and following irradiation.

Dose (krad)	Carbon dioxide production[a]		
	During irradiation	After irradiation	
		First 2 hr	After 24 hr
0	27	15	12
10	24	19	15
50	25	19	15
200	30	18	15
500	40	30	20
1,000	70	43	30

[a] As mg per kg fresh weight per hr.

Table 3. Effect of gamma irradiation on color of mature Napoleon cherries.

Dose (krad)	Hunter color-difference-meter reading[a]					Visual grade[b]
	L	a_L	b_L	a_L/b_L	$\sqrt{a_L^2 + b_L^2}$	
Control	41.3	18.0	18.9	0.95	26.1	48.3
50	38.8	20.1	17.8	1.12	26.8	55.2
200	41.5	18.1	19.2	0.94	26.4	45.6
500	38.8	18.9	17.9	1.06	26.1	54.5
1000	39.9	16.2	18.8	0.86	24.8	32.0

[a] Plate No. 25-303, packed in 4 × 4-inch square cell, pinkest side toward instrument.
[b] An estimate of percentage of total surface of cherry showing pink coloration.

pared with the rate during the last 9 hr. The results indicate a moderately consistent respiratory activity throughout the whole exposure.

Taste and color. The color evaluations of mature fruit are presented in Table 3. Only at the highest dose was there a real difference in color. A fading of the red coloration was visible, as well as recordable by the Hunter laboratory color and color-difference meter, as a reduction in the red-to-yellow ratio (a_L/b_L) and a reduction in color saturation ($\sqrt{a_L^2 + b_L^2}$). In addition to being less red, the cherries were described as "dull" or somewhat "translucent." Although color was not measured in the 2,000-krad samples, the destruction of red coloration was quite evident to the eye and included a pronounced browning of the tissues. At doses below 1,000 krad, no color differences could be detected.

Results of the taste-panel evaluation indicated no significant difference in flavor between samples irradiated at doses up to 1,000 krad. Softening of mature cherries, however, was detected at doses above 200 krad. Cherries irradiated at 1,000 krad in a mature condition were considered unacceptable for taste panel evaluation because of excessive browning and softening.

Brined Napoleon cherries. Irradiation of fresh cherries caused changes in the characteristics of the brined and maraschino cherries (Table 4). The weight of both brined and 50% soluble solids cherries increased as the irradiation dose increased. On the other hand, the textures of both products became less firm as the dose increased. There apparently was no threshold dose for the effect of radiation on texture or unit weight. No effect of radiation was found on the color of cherries held 6 months in brine, although it was observed that cherries receiving the higher dose lost their pink color more rapidly during the first several days in the brine.

Storage longevity. The shelf life of cherries contaminated with brown rot was extended by doses in the range of 100–400 krads. In one trial, for example, 60% of the unirradiated fruit were visibly infected after 5 days at 21°C, whereas the highest incidence in the irradiated fruit was 8%, in the 100-krad samples. After 11 days at 21°C, the unirradiated samples and the fruit given 400 krads showed a higher percentage of brown rot infection than the 100- and 250-krad samples.

Storage life of brown-rot-free cherries was not lengthened even though microbial counts were drastically reduced by doses above 50 krads (Table 5). The higher doses apparently made the fruit more susceptible to attack from molds, other than brown rot, that had survived the treatment.

DISCUSSION

These results show several distinct characteristics of irradiated cherries. As in most

Table 4. The influence of gamma irradiation of fresh cherries upon the characteristics of brined and 50%-soluble-solids cherries.[a]

Dose (krad)	Unit weight[b] (g)	Texture[c] (kg)	Hunter Color[d]			50%-soluble-solids cherries	
			L	a	b	Unit weight[b] (g)	Texture[c] (kg)
0	4.4	1.15	53	— .2	31	5.4	1.51
10	4.4	1.11	54	— .2	31	5.6	1.46
50	4.6	1.05	54	—1.0	31	5.6	1.40
200	4.9	.98	53	— .6	30	6.1	1.22
500	5.1	.84	53	— .6	30	6.3	1.02
1000	4.9	.67	53	— .3	30	6.1	.81

[a] Averages for 4 harvests of Napoleon cherries.
[b] After removing pits.
[c] Maximum resistance to the passage of a blunt pin.
[d] Plate No. 25-303, packed in 4 × 4-inch square cell. Random orientation.

Table 5. The influence of gamma irradiation upon the microbiological counts of Napoleon cherries immediately following irradiation and after storage.

Storage		Dose (krad)	Microbiological counts/g		Observations
Temp. (°C)	Period (days)		Total	Yeasts	
....	0	0	14×10^4	98×10^3	Normal appearing
		10	32×10^4	26×10^4	Normal appearing
		50	57×10^2	300	Normal appearing
		200	40	<10	Normal appearing
		500	<10	<10	Soft fruit
		1000	<10	<10	Soft fruit
21	7	0	91×10^3	21×10^3	Normal appearing
		10	6×10^2	44×10^3	Normal appearing
		50	48×10^3	7×10^3	Normal appearing
		200	<100	$<1 \times 10^3$	Some moldy
		500	<100	<100	Some moldy
		1000	....	....	All moldy, not plated
2	51	0	<100	1200	Normal appearing
		10	14×10^3	900	Normal appearing
		50	100	100	Normal appearing
		200	<100	<100	Some brown, firm
		500	100	<100	All brown, soft
		1000	<100	<100	All brown, soft

[a] Plated on standard plate count agar. Incubated 2 days at 32°C.
[b] Plated on potato-dextrose agar, acidified to pH 3.5. Plates incubated 1 week at room temperature.

fruit and vegetables, softening of the tissues is the principal detrimental factor limiting the process. In comparison with other plant tissues, cherries appear to have an intermediate susceptibility to softening. The calculated "threshold dose" (TD) of 50 krad is similar to the value of approximately 35 krad found as the average softening TD value for a large number of apple samples. However, this value is somewhat lower than that found for the softening of carrots (166 krad), beets (316 krad), and lettuce (800 krad).

Softening appears to be related directly to the degradation of pectic substances in the tissues in a manner similar to that known with several other tissues. The softness in brined cherries as well as in the fresh material indicates that the effect of irradiation on texture is largely an alteration of structural materials in the fruit. In many instances, a slight but significant increase in tissue firmness has been noted at radiation levels slightly above the TD. At present, the implication of this observation is unknown.

Fading of the red color or browning of the cherries did not occur at less than 1,000 krad of radiation. This did become most objectionable with the high doses, however, particularly following the holding of cherries at room temperature for a few hours. These color problems were accentuated in the light-colored variety but were also present in the dark-fleshed varieties. Off-flavors were not detected in cherries treated with doses of radiation as high as 2,000 krad.

It was found that cherries were relatively insensitive to radiation-induced respiratory activity stimulation. The large during-irradiation stimulation occurring at doses of about 1,000 krad is taken as an indication of excessive physiological damage. The uniform acceleration of both oxygen uptake and carbon dioxide evolution around 1,000 krads indicates a relatively general type of tissue injury rather than the selective effect at a specific location in intermediate metabolic pathway. The rather rapid return of the accelerated rates at the lower doses indicates that this effect is reversible unless pressed to the point of actual tissue disorganization.

The kinds of microorganisms contaminating cherries appeared to determine whether or not irradiation increased storage longevity.

Thus, while the shelf life of fruit contaminated with brown rot was extended, no benefits were observed with cherries free of this microorganism. The fact that higher irradiation doses shortened the storage life of fruit not contaminated with brown rot undoubtedly resulted in changes which made the fruit more susceptible to invasion by other spoilage organisms.

No significant differences in the qualities measured in this study were attributable to either variety or maturity.

In view of the degradative response to radiation, especially in texture, it is difficult to assess any really striking benefit of irradiation to cherries in the applied sense. One possible exception is the observation that brown rot development may be significantly retarded at elevated temperature storage. The full realization of the beneficial potential of gamma radiation upon cherries must await further developments in the control of objectionable changes, principally the loss of tissue firmness.

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Post-Mortem Changes in Muscle. I. Chemical Changes in Beef

SUMMARY

Chemical changes in the longissimus dorsi muscle of 5 beef carcasses were followed from less than 10 min after death through 20 days post-mortem. The average initial pH value of 6.99 declined to 5.46 at 48 hr and was 5.57 at 480 hr. Initial values for creatine phosphate, total acid-soluble phosphate, ortho-phosphate, lactic acid, total reducing sugars, and glycogen were respectively 9.1, 54.9, 22.1, 13.1, 7.9 and 56.7 μM/g of tissue. Creatine phosphate declined rapidly to only 16% of the initial value by 12 hr post-mortem, and was not detectable by 24 hr. Ortho-phosphate, lactic acid and total reducing sugars increased approximately 1.5, 6.5, and 2.25 times from their initial levels during 480 hr post-mortem. Glycogen appeared to be stoichiometrically degraded to lactic acid and reducing sugars, since the sum of these constituents was approximately constant at all times post-mortem, if expressed in terms of glucose equivalents. Results suggest that the onset of rigor occurred at 12–15 hr post-mortem. Development of rigor mortis was virtually complete 24 hr after death. The pattern of chemical changes observed with intact beef carcasses subjected to commercial cooling practices is in essential agreement with earlier results with isolated beef muscle strips.

An enzymic method for determining ATP levels was compared with the more commonly used method of acid hydrolysis. ATP values at 24 hr post-mortem were negligible when determined by the enzymic method, but amounted to about 27% of the original level when determined by acid hydrolysis. Reasons for the discrepancy in the two methods are discussed.

INTRODUCTION

Studies on beef muscle by Marsh (1954) and Howard and Lawrie (1956, 1957) established that chemical and extensibility changes were similar to those previously observed in rabbit muscle (Bate-Smith, 1939; Bate-Smith and Bendall, 1947, 1949; Bendall, 1951). Those studies on beef primarily involved observations on isolated muscle strips held at 37°C post-mortem. The current investigation was carried out to follow certain chemical and histochemical changes in longissimus dorsi muscle under post-mortem conditions similar to the usual commercial procedures. Results of the histochemical observations will be included in a subsequent paper (Bodwell *et al.*, 1965).

EXPERIMENTAL

Experimental animals and sampling procedures. Five Hereford heifers weighing approximately 850 lb were fasted 12 hr prior to slaughtering at ½-hr intervals. Immediately after death, the animals were partially hoisted and an incision was made through the hide in the vicinity of the last rib. Samples were removed from the longissimus dorsi muscle of each carcass with a 1-in. core borer. All initial samples were removed within 10 min of death. Each carcass was then dressed in the conventional manner and moved into a 3–4°C cooler. Further samples were removed at 6, 12, 24, 48, 126, 288, and 480 hr for pH and chemical observations. Additional samples were removed for pH measurements at 72, 96, 102, 173, and 198 hr. The holes resulting from the removal of a sample were packed with adsorbent cotton. Consecutive sampling locations were separated by approximately 2.3–5.1 cm.

Immediately after removal from the carcass, samples for glucose and glycogen analyses were frozen in liquid nitrogen, wrapped in Dow Handi-Wrap and stored at −29°C until analyzed. Samples (4–6 g) for lactic acid, CP (creatine phosphate), ATP (adenosine triphosphate), TSP (total acid-soluble phosphate), and ortho-phosphate analyses were weighed on an analytical balance and homogenized for 1 min in a Waring blender with 50 ml of cold (4°C) 7% TCA (trichloroacetic acid) solution. The homogenate was filtered through Whatman No. 1 paper in the cold (4°C). The homogenizing cup was rinsed with an additional 25 ml of 7% TCA, which was added to the original homogenate before filtering. The filtrate was adjusted to pH 7.1–7.3 with NaOH, made up to 100 ml with cold (4°C) distilled water, and stored at −29°C until analyzed. Prior to analysis, 0.5 ml of chloroform was added to each previously thawed sample.

Temperature measurements. A thermocouple, attached to a Minneapolis-Honeywell temperature recorder, was inserted into the center of the longissimus dorsi muscle of each carcass at the approximate level of the 4th lumbar vertebra immediately after the carcass had been placed in the cooler.

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Temperature recordings were taken continuously throughout the first 72 hr post-mortem.

Measurement of pH. A sample weighing approximately 2 g was homogenized for 0.5 min in 25 ml of 0.005*M* sodium iodoacetate in a Waring blender. Duplicate pH measurements were made with a Beckman pH meter, Model G.

Chemical analyses. Creatine phosphate was determined according to the procedure of Ennor and Rosenburg (1952), and lactic acid according to the method of Barker and Summerson (1941). The method of Allen (1940) as modified by Bendall (1951) was used to estimate ortho-phosphate and ATP, while the same method as modified by Howard and Lawrie (1956) was used to estimate TSP. ATP was also determined by the bioluminescent enzymic method of Strehler and Totter (1952) and Strehler (1953), using a Turner fluorometer. In order to avoid confusion, ATP values are all expressed as micromoles of ATP. The slightly depressing effect of neutralized TCA on both sample extracts and standard solutions did not interfere with the linearity of fluorescence to ATP concentration, i.e., the accuracy of the method.

For glucose or glycogen determination, 0.5–1 g of tissue was chipped from a frozen tissue block in the cold (−10°C). The tissue was introduced into a previously weighed digestion solution as outlined by Folin and Wu (1920) or an extraction solution as given by Hansen *et al.* (1952) for glucose and glycogen, respectively. After warming to room temperature, each solution was reweighed to obtain the sample weight. The analyses were then carried out according to the original methods.

Statistical analyses. Means and standard deviations were calculated for the chemical values for each sampling period according to Snedecor (1959). An estimate of measuring error was calculated for glucose, glycogen, and lactic acid determinations according to Magee (1964).

RESULTS AND DISCUSSION

Table 1 lists means and standard deviations of the values for chemical constituents and pH at various periods post-mortem.

Levels of pH. The average initial pH value was 6.99 (range 6.90–7.07). At 48 hr post-mortem, the mean pH was 5.46, and at 480 hr it was 5.57. The initial pH values are in agreement with those of Marsh (1954), who calculated the approximate pH at death for beef muscle and found the values varied from about 6.65 to 7.40. Similarly, the initial pH values are also in agreement with those of Howard and Lawrie (1956), who obtained an average pH value of 6.74 at 1 hr post-mortem. The values for 48 and 480 hr post-mortem are in agreement with the final pH value of 5.50 determined under similar conditions by Howard and Lawrie (1956).

Creatine phosphate. Creatine phosphate decreased to about 22% and 16% of the initial level (9.1 μM/g of muscle) at 6 and 12 hr post-mortem, respectively (Table 1). The initial CP value is in general agreement with Howard and Lawrie (1956), who reported an average value equivalent to about 7.3 μM/g in the longissimus dorsi muscles of 13 steers at 1 hr post-mortem.

ATP. The initial ATP_1 (acid hydrolysis) and ATP_2 (enzymic assay) values were 6.4 and 10.9 μM/g, respectively (Table 1). The ATP_2 value had decreased to 0.6 μM/g within 24 hr post-mortem, while the ATP_1 value was still 1.7. The ATP_1 value is in good agreement with that of Howard and Lawrie (1956), who reported an ATP value equivalent to approximately 5.3 μM/g of tissue at 1 hr post-mortem, and with that of 5.6 μM/g at 60–80 min post-mortem as reported by Marsh (1954).

The ATP_2 values were considerably higher than ATP_1 values during the first 12 hr post-mortem (Table 1). The acid-molybdate reagent of Allen (1940) used for color development in the ATP_1 analysis has been shown to hydrolyze the two acid-labile phosphates of ATP (especially the terminal phosphate) in varying degrees. The amount of hydrolysis is primarily dependent on temperature and time of exposure to the acid-molybdate reagent (Weil-Malherbe and Green, 1951). Since Allen's (1940) method allows the time of exposure to acid-molybdate reagent to vary from 5 to 25 min, values for ortho-phosphate estimated by this method would be expected to include some ATP phosphate. Consequently, the initial ATP_1 values in the current study as well as similar values of Howard and Lawrie (1956) and Marsh (1954) would be expected to be less than the true ATP value.

When using the acid-molybdate procedure, the combined residual ATP and ADP comprised less than 2 μM/g (ATP_1; Table 1). Bendall (1951) suggested that the residual ATP and/or ADP obtained by acid hydrolysis was due to ATP and/or ADP,

m	pH values [b]	Glycogen [c]	Glycose [d]	Lactic acid	ATP_1 [e]	ATP_2 [f]	CP	TSP	Ortho P
	6.99 ± 0.07	56.7 ± 5.9	7.9 ± 1.0	13.1 ± 6.0	6.4 ± 1.2	10.9 ± 1.4	9.1 ± 3.2	54.9 ± 4.2	22.1 ± 3
	6.57 ± 0.12	41.6 ± 10.4	6.3 ± 2.2	44.8 ± 15.8	5.0 ± 0.8	10.0 ± 2.3	2.0 ± 1.5	55.2 ± 3.6	23.2 ± 4
	5.96 ± 0.13	30.4 ± 8.5	12.2 ± 4.3	58.0 ± 18.6	3.9 ± 1.4	5.3 ± 3.9	1.5 ± 1.3	54.9 ± 3.9	25.9 ± 2
	5.74 ± 0.19	10.1 ± 3.8	18.1 ± 3.6	71.2 ± 11.2	1.7 ± 0.6	0.0 ± 0.2	.....	53.6 ± 5.2	21.6 ± 1
	5.57 ± 0.11	10.0 ± 0.9	15.9 ± 3.5	82.4 ± 7.4	1.1 ± 2.3	.....	.....	54.2 ± 3.6	27.5 ± 1
	5.46 ± 0.08	.....	.....	.....	.....	.....	.....	.....	.....
	5.36 ± 0.03	.....	.....	.....	.....	.....	.....	.....	.....
	5.42 ± 0.10	12.7 ± 4.2	12.1 ± 2.8	80.9 ± 5.0	.....	.....	.....	53.6 ± 3.9	30.3 ± 5
	5.50 ± 0.05	.....	.....	.....	.....	.....	.....	.....	.....
	5.53 ± 0.08	.....	.....	.....	.....	.....	.....	.....	.....
	5.54 ± 0.09	.....	.....	.....	.....	.....	.....	.....	.....
	5.63 ± 0.07	.....	.....	.....	.....	.....	.....	.....	.....
	5.59 ± 0.06	9.9 ± 5.0	16.8 ± 5.8	82.7 ± 3.3	.....	.....	.....	54.9 ± 3.6	33.6 ± 3
	5.46 ± 0.05	1.4 ± 0.6	17.9 ± 3.3	84.6 ± 2.9	.....	.....	.....	54.2 ± 4.2	35.5 ± 2

values are average observations on all 5 carcasses and are expressed in micromoles (microatoms for TSP and Ortho-P) per gram of fresh
ans ± standard deviations.
cogen is expressed in glucose equivalents.

which is tightly bound to the muscle protein. However, Bendall and Davey (1957) concluded that the acid-labile P content was a reliable guide to true ATP and/or ADP content only in the early stages of rigor, when CP and glycolytic resynthesis maintained ATP at a fairly constant level. They further concluded that when the rate of resynthesis begins to decrease, side reactions invalidate any simple equation for labile P and ATP. Furthermore, in support of the absence of detectable ATP_2 at 48 hr post-mortem in the current study (Table 1), it can be calculated from data presented by Davies (1963) that less than 0.2 μM of ATP and/or ADP/g of tissue would be bound to the muscle protein in full rigor for rabbit muscle.

Fredholm (1963) reported that 50 and 23% of the initial ATP was present in beef "gracilus-muscle" after storage at cooler temperatures for 3 and 5 days, respectively. This is not in agreement with results of the current study, where ATP_2 was not detectable at 24 hr post-mortem.

TSP and ortho-phosphate. The over-all mean for TSP was 54.4 microatoms/g of muscle (Table 1). Marsh (1954) and Lawrie (1960) reported respective values equivalent to 55.5 and 57.1 microatoms/g for beef muscle.

The initial level of ortho-phosphate was 22.1 microatoms/g of tissue. This level increased to 35.5 microatoms/g at 480 hr post-mortem. Comparable values are not available for beef. However, Bate-Smith and Bendall (1947) reported an initial level equivalent to 22.9 microatoms/g and a 444 min level (at 37°C) equivalent to 31 microatoms/g in rabbit muscle. Hence, the concentration of ortho-phosphate would appear to increase 1½ times after death in muscle from both beef and rabbit.

Glycogen. The initial glycogen level was 56.7 $\mu M/g$. This value decreased to approximately 54% at 12 hr and to less than 20% at 24 hr post-mortem. At 288 hr, the glycogen was still 17.5% of the initial value, but decreased thereafter to less than 3% at 480 hr (Table 1). It seems likely that the breakdown of residual glycogen occurred as a result of the combined activity of alpha-amylase, amylo-1:6-glycosidase, and maltase, which has been suggested by Sharp (1962). Although comparable values for all the various post-mortem time periods are not available, the values found in the present study are in good agreement with reports of previous workers at specified times post-mortem. Initial levels of glycogen calculated from the data of Howard and Lawrie (1956, 1957) are 51.9 and 51.7 μM glucose equivalents/g of longissimus dorsi muscle from 13 and 5 steers, respectively. Similarly, Swift *et al.* (1960) reported glycogen levels equivalent to 40.3 μM glucose equivalents/g at 2–3 hr post-mortem.

In the current study, the 24-hr value for glycogen was 10.1 μM glucose equivalents/g. This is in agreement with results of Howard and Lawrie (1956), who reported residual glycogen levels equivalent to as much as 8.3 μM glucose eqivalents/g in beef muscle frozen at 24 hr post-mortem and stored 20 weeks at −10°C.

Glucose. The average glucose (total reducing sugar) level increased from 7.9 to 17.9 $\mu M/g$ between the initial and 480-hr post-mortem sampling periods. The marked variation observed in glucose levels at intervening periods (Table 1) suggests that the amount of glucose may vary considerably from location to location, and/or it may be converted to a form other than a reducing sugar at certain times post-mortem.

The values for total reducing sugars (Table 1) are in good agreement with those reported by Sharp and Rolfe (1958). They reported an accumulation of free glucose equivalent to 5.6 $\mu M/g$ in beef muscle held 24 hr post-mortem at 18–20°C. The only other reducing substance detected in sizable concentration was glucose-6-phosphate, which increased during post-mortem glycolysis to a constant level of about 14 μM (glucose equivalents)/g of tissue. Hence, the combined value of free glucose and glucose-6-phosphate approached 20 $\mu M/g$. In the current study (Table 1), the comparable value for total reducing sugars at 480 hr post-mortem was about 18 $\mu M/g$.

Lactic acid. Lactic acid, which was present at an initial concentration of 13.1 $\mu M/g$, had increased by almost 3.5 times at 6 hr post-mortem (Table 1). A further, slower, but steady increase was observed up to 48

hr, to approximately 6.5 times the initial level. Thereafter, little or no increase was evident. Results support Marsh's (1954) suggestion that active glycolysis is complete in beef longissimus dorsi muscle within 36 hr post-mortem.

Temperature. The average temperatures of the longissimus dorsi muscle of the five beef carcasses are given in Table 2. Apparently, the cooling process was completed for this muscle prior to 20 hr post-mortem.

Table 2. Average post-mortem temperatures of the longissimus dorsi muscle of five beef carcasses.

Time post-mortem (hr)	Temperature (°C)
Initial	38.9
3	27.2
6	20.0
10	13.3
12	10.0
16	6.7
20	2.8
24	2.8
48	2.8

Interrelationship of ATP, CP, and pH. CP and ATP, as a percent of their initial levels, and their relation to pH are shown in Table 3. CP decreased to approximately 20% of its initial value within 6 hr post-mortem. During the same period, ATP_2 had decreased by less than 10%. At 12 hr post-mortem, ATP_2 values had decreased to about 50% of the initial value, with a concurrent average pH level of 5.96. The rapid depletion of CP prior to any significant decrease in ATP level is in agreement with observations of Marsh (1954).

Howard and Lawrie (1956) noted that the onset of rigor in beef muscle held at 37°C occurred when ½–¾ of the initial ATP had disappeared, with a corresponding pH level of 6.02. Bendall and Davey (1957) reported that the onset of rigor in rabbit muscle occurred when ½ of the initial ATP was depleted in muscle held at 37°C. If held at room temperature, however, onset of rigor occurred when ¾ of the initial level was depleted.

As previously discussed, the mean temperature of the longissimus dorsi muscle was 20.0, 13.3, and 10.0°C at 6, 10, and 12 hr post-mortem, respectively (Table 2). In the current study, it is suggested that the average time for the onset of rigor in the longissimus dorsi muscle was initiated at about 12–15 hr post-mortem, and that the development of rigor was virtually complete at 24 hr. This observation is supported by the data on lactic acid (Table 1).

Interrelationship of lactic acid and pH. The general relationship between post-mortem levels of lactic acid and pH is shown in Fig. 1. During the initial 24-hr post-mortem period, 46.5 μM of lactic acid/g of muscle were produced for every unit decrease in pH. During the same period, pH decreased from 6.99 to 5.74. This value is in good agreement with a similar increase of 44 μM/g of tissue per unit pH decrease as calculated from the data of Howard and Lawrie (1956) in beef longissimus dorsi muscle. However, an equivalent value for approximately the same pH interval of 63.3 μM of lactic acid per g of tissue for each unit pH decrease in rabbit psoas muscle was reported by Bate-Smith and Bendall (1949). Both the observations of Howard and Lawrie (1956) and those in the current study would suggest that the buffering capacity

Table 3. Levels of ATP, CP, and pH during initial 48 hr post-mortem.

Time post-mortem (hr)	pH	ATP as a % of the initial level		CP as a % of the initial level
		ATP_1[a]	ATP_2[b]	
Initial	6.99	100.0	100.0	100.0
6	6.57	78.1	91.7	22.0
12	5.96	60.9	48.6	16.5
24	5.74	26.6	0.0	.....
48	5.57	17.2	0.0	.....

[a] Based on acid hydrolysis values.
[b] Based on enzymic assay values.

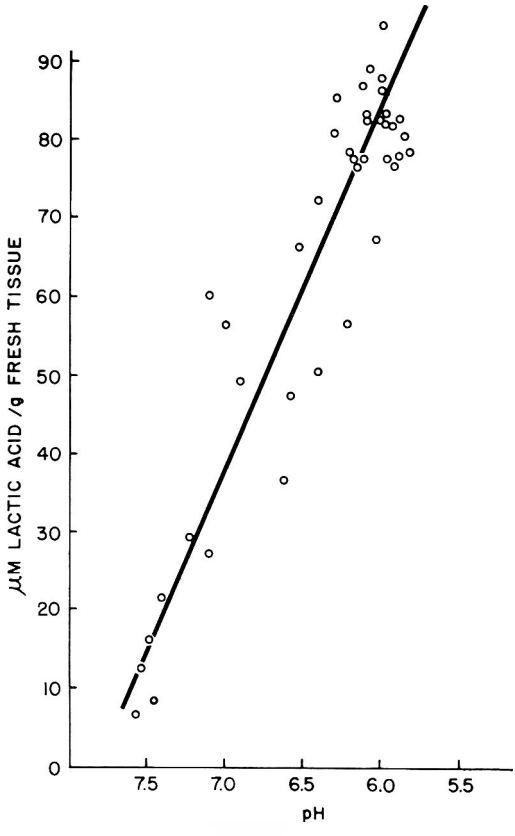

Fig. 1. The relationship between pH and lactic acid in beef longissimus dorsi muscle.

in beef longissimus dorsi muscle is considerably lower than that found in rabbit psoas muscle within the same pH range.

Interrelationship between glycogen, lactic acid, and glucose. The relation between post-mortem levels of glycogen, glucose (total reducing sugars), and lactic acid is shown in Fig. 2. The sum of glucose, glycogen, and lactic acid (expressed as micromoles of glucose equivalents) is approximately constant at all times post-mortem. Deviations from a completely constant relationship are within the limits of the errors of measurement, which were ± 2.2, ± 1.2, and ± 6.6 $\mu M/g$ for glycogen, glucose, and lactic acid, respectively.

It is concluded that glycogen in beef muscle is degraded to glucose and lactic acid during post-mortem glycolysis in an approximately stoichiometric manner. This is in agreement with work of Sharp and Rolfe (1958) on the accumulation of free glucose and glucose-6-phosphate. Furthermore, this conclusion supports observations of Howard and Lawrie (1956) that, in beef muscle, ultimate pH level could not be fully explained by assuming a stoichiometric degradation of glycogen to lactic acid.

In general, the data obtained in the present study confirm values for various post-mortem chemical changes in beef muscle reported by previous workers. However, the current investigation has resulted in hitherto unreported values for various chemical changes in relatively intact muscle from carcasses subjected to so-called "normal cooling and aging" conditions.

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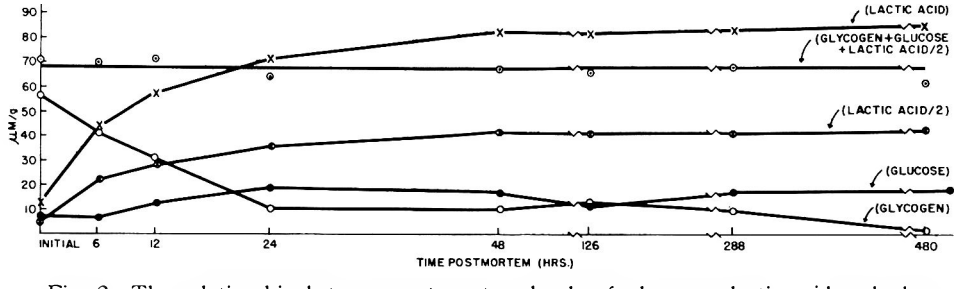

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Thermal Conductivity of Freeze-dried Model Food Gels

SUMMARY

The thermal conductivities of freeze-dried slabs of starch, gelatin, pectin, cellulose gum, and egg albumen gels were determined under a variety of conditions, using a guarded hot-plate apparatus. The effects of temperature, gas pressure, and gas composition on a starch gel were studied in detail. The thermal conductivities of dry materials at atmospheric pressure varied from 0.921×10^{-4} cal cm^{-1} $°C^{-1}$ sec^{-1} (gelatin) to 1.337×10^{-4} (cellulose gum). In vacuum this variation was from 0.218×10^{-4} (starch) to 0.467×10^{-4} (cellulose gum). The difference between atmospheric pressure and vacuum was equal to the thermal conductivity of air for all the materials except cellulose gum, which gave a greater difference. The thermal conductivity of starch gel increased linearly with increasing temperature from 0 to 70°C, and decreased with decreasing pressure, as porous materials normally do, to a constant value below 0.1 mm Hg. A helium atmosphere gave a higher thermal conductivity than air or nitrogen. The thermal conductivity was higher in all freeze-dried gels containing adsorbed water than after the removal of all the water. The thermal conductivity of pectin gels increased with the density. Thermal conductivity was affected by the type and size of pores of the dried materials. In general, changes in thermal conductivity were significant with pressure, type of gas, and nature of the material, particularly the fibrous structure, hut less important with temperature and amount of adsorbed water.

INTRODUCTION

The freeze-drying rates of some food-like gels were recently studied under a variety of experimental conditions (Saravacos, 1965). Comparatively high drying rates were obtained in normal vacuum when adequate heat was supplied to the test materials. Model gels can be used as experimental materials for the study of heat and mass transfer in freeze-drying, because of the uniform composition, structure, and dimensions of the samples. It is thus possible to make more useful measurements than on actual food materials, which inherently have a more complex composition and structure.

In normal freeze-drying, after the sublimation of surface ice, heat must be supplied to the interior through a porous dried layer. It has been known that porous materials are poor conductors of heat, particularly at the low pressures usually employed in freeze-drying. Harper (1962) used heat-flow transducers to measure the conductivity of freeze-dried peach, apple, and beef. More recently, Harper and El Sahrigi (1964) studied the thermal conductivity of freeze-dried beef, apple, pear, and a polyurethane foam in various gas atmospheres and pressures. Thermal conductivity was found to decrease from a constant value at atmospheric pressure to a lower value at pressures below 0.1 mm Hg, the difference between the two values being close to the thermal conductivity of the gas in the pores of the material. Lusk *et al.* (1964) calculated the thermal conductivities of freeze-dried fish from drying-rate data, and reported similar values. Little work has been reported on the effect of temperature, moisture content, and sample composition on the thermal conductivity of freeze-dried food materials. The present work was done to show the effect of a variety of experimental conditions on the thermal conductivity of freeze-dried food gels, using apparatus of high accuracy.

EXPERIMENTAL MATERIALS AND METHODS

Freeze-dried gels. The following gels were used: potato starch, gelatin, pectin, pectin and glucose (1/1), cellulose gum (carboxy-methyl-cellulose), and egg albumen. The gels were prepared by dispersing 25 g of the dry powder in 250 ml of distilled water and heating until boiling, when a viscous solution was formed. The hot gels were poured into a circular brass mold of 17 cm diameter. The resulting slabs had a thickness close to 1 cm. The egg albumen gel was prepared by heating a solution of separated egg whites in the mold until gelation. Pectin gels of different concentration

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(5%, 10%, and 5% pectin–5% glucose) were prepared in order to obtain dried gels of various densities.

The gels were frozen in the mold by contact with powdered dry ice; then they were quickly freeze-dried in a laboratory glass freeze-dryer. The freeze-dryer consisted of an 8-L vacuum desiccator connected to a 500-ml dry ice–ethanol condenser and a vacuum pump maintaining an average pressure of 0.1 mm Hg. The heat of sublimation was supplied by radiation from the environment, and drying was completed within 48 hr. The samples shrank slightly during drying, and the freeze-dried gels had an average water content of 2%. Before thermal conductivity was measured, all the dried gels except the starch were equilibrated to a constant moisture content by placing the samples in an evacuated desiccator containing a saturated solution of sodium dichromate. This solution maintained 52% RH at room temperature, and the corresponding water content was 12–15%, as indicated by the sorption isotherms of the freeze-dried gels (Saravacos, 1965). The starch gels were equilibrated to normal summertime room humidity, and the quantity of adsorbed water was approximately the same as that of the other gels.

The density of the freeze-dried gels was calculated from the weight of the circular slabs (equilibrated at 52% RH) and their dimensions. The porosity, defined as the ratio of the volume of pores to the volume of the dried sample, was determined by a simple air-displacement procedure (Harper, 1962): a measured air pressure (380 mm Hg, above atmospheric) was introduced into a 100-cc glass cell containing the sample; the pressure was then released, and the volume of the air discharged was measured. This procedure was repeated on the empty cell, and the porosity was obtained by a simple calculation. The pore size of the freeze-dried gels was measured with a microscope (×400) equipped with a calibrated eye-piece and incident illumination. The range of the pore diameters was determined by examining several microscopic fields.

Apparatus and procedure. The thermal conductivities were measured under steady-state conditions in a guarded hot-plate apparatus (Hoge *et al.*, 1962), shown schematically in Fig. 1. A sample 16.5 cm in diameter and about 1 cm thick was placed between the hot and cold plates. The guard ring, concentric with the hot plate, and a shield above it were maintained at the same temperature as the hot plate, so the only heat flow from the hot plate was through the sample. The bottom of the cylindrical vacuum-tight container served as the cold plate. In the cover of the container were means for changing the position of the hot-plate assembly to bring it in contact with the sample, connections to evacuate the container, and connections for electrical leads. The cold-plate temperature was maintained constant by immersing the whole apparatus in a thermostated oil bath. The temperatures were measured with copper-constantan thermocouples, and a precision potentiometer was used for both temperature and power measurements. To ensure steady-state conditions, a single thermal-conductivity measurement lasted about 6 hr. The absolute accuracy of the apparatus is estimated to be within 2%. This was confirmed by measuring standard materials calibrated by the National Bureau of Standards. Reproducibility for a single sample is within ½%. The largest observed difference between different samples of the same model food gel measured under the same conditions was 3.6%.

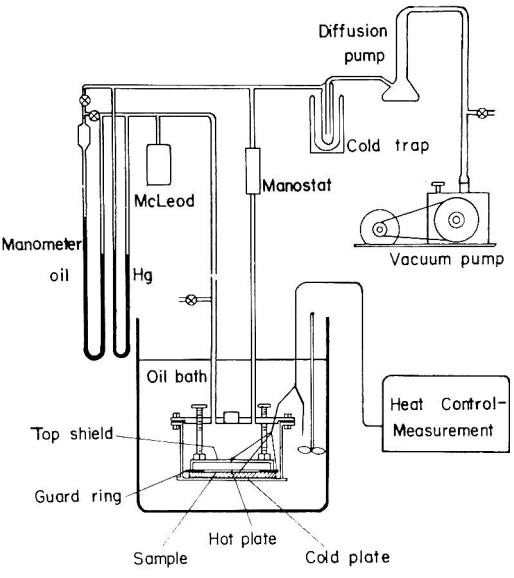

Fig. 1. Thermal-conductivity apparatus.

The thermal conductivity is given by the relation,

$$k = \frac{W}{A} \times \frac{\Delta X}{\Delta T}$$

where W = the power input, watts; A = the hot plate area, sq cm; ΔX = the thickness of sample, cm; ΔT = the temperature difference, °C.

Thus, k is obtained in watt cm^{-1} $°C^{-1}$, and multiplying by 0.2389 converts it to cal cm^{-1} $°C^{-1}$ sec^{-1}. Except for the temperature curve (Fig. 2) with the starch gels, all measurements were made with the cold plate at about 36°C. This temperature was chosen as the lowest that could be accurately controlled at any possible room temperature without cooling. A 10°C temperature difference was maintained across the sample.

After a sample had been measured at atmospheric

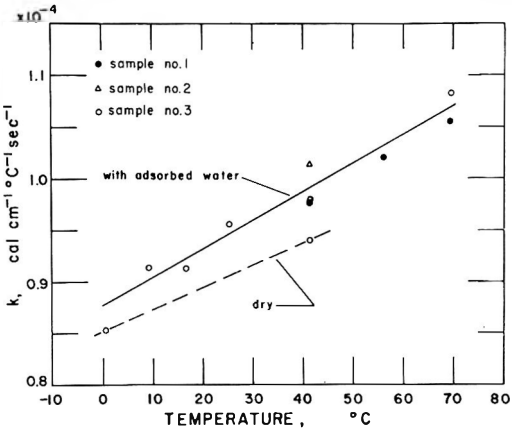

Fig. 2. Effect of temperature on the thermal conductivity of freeze-dried starch gel (atmospheric pressure).

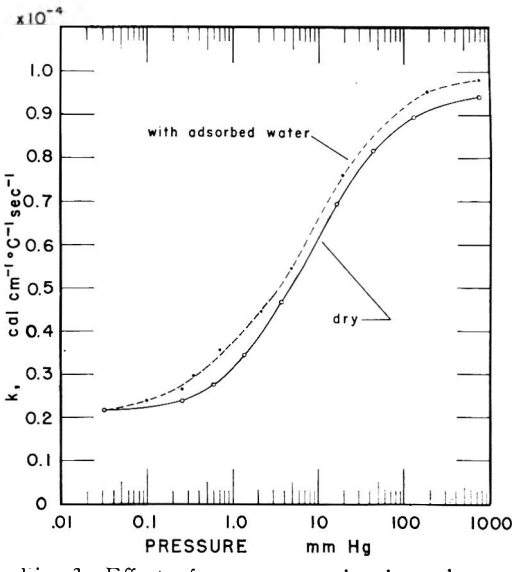

Fig. 3. Effect of pressure on the thermal conductivity of freeze-dried starch gel (mean temperature, 41°C).

pressure the apparatus was evacuated and pumped continually for at least 24 hr with the sample at about 60°C. It was assumed that this treatment effectively removed all adsorbed water from the sample. The cold-plate temperature was then dropped to 36°C, and k was measured while maintaining the low pressure. Because of traces of water vapor or other volatiles, the lowest pressure that could be maintained was about 0.03 mm Hg. Dry air was then admitted to the apparatus, and a final measurement of k was made at atmospheric pressure.

Investigation of the effects of temperature, pressure, and sample preparation on starch gels was more detailed. Three different samples were prepared separately, and the thermal conductivity was measured at various temperatures (Fig. 2). The samples were either equilibrated to room relative humidity or dried completely. The sample of starch gel which was measured at the greatest number of temperatures (No. 3) was used for the measurements involving pressure variations. The sample was first equilibrated to normal room relative humidity, and the thermal conductivity was measured at a series of different pressures, evacuating a part of the remaining air after each measurement (Fig. 3). There was some uncertainty in these measurements because of drying out of the sample and the presence of water and possibly other vapors as well as dry air in the atmosphere of the apparatus. The pressures given were observed with manometers, using mercury for the higher pressures and oil for the lower. At the lowest pressures even the oil manometer did not give very high accuracy, but the curve had leveled off so the exact pressure value was not critical. A McLeod gauge was also used, but it gave somewhat lower pressures, possibly because of the condensation of water vapor in the gauge. At the lowest possible pressure the temperature was raised to above 60°C, and pumping was continued for at least 24 hr to remove as much of the water as possible. The pressure was then increased in steps by the introduction of dry air, and the thermal conductivity was measured at each step. The thermal conductivity in a gas atmosphere was measured by introducing dry gas (nitrogen or helium) from a cylinder into the evacuated sample container.

RESULTS AND DISCUSSION

Table 1 shows the results of measurements of thermal conductivity of seven different freeze-dried gels at a mean temperature of 41°C, both at atmospheric pressure and in vacuum. The thermal conductivities of the bone-dry materials at atmospheric pressure varied from 0.915×10^{-4} cal cm^{-1} °C^{-1} sec^{-1} (5% pectin) to 1.337×10^{-4} (cellulose gum). In engineering units this variation is from 0.0221 to 0.0323 Btu ft^{-1} °F^{-1} hr^{-1}. The thermal conductivity of all samples increased to a small degree when water vapor (12–15%, dry basis) was adsorbed by the gels. This increase was lowest (2%) in the 5% pectin gel and highest (12%) in cellulose gum. The values of the thermal conductivity of freeze-dried gels were somewhat lower than those of freeze-dried foods (Harper, 1962; Lusk *et al.*, 1964). The difference

Table 1. Thermal conductivity of freeze-dried model food gels (mean temperature 41°C).

Freeze-dried slab (prepared from gels containing 10% solids)	Thickness (cm)	Density at 52% RH (g/cc)	Thermal conductivity (cal cm^{-1} $°C^{-1}$ sec^{-1}) Atmospheric pressure At 52% RH ($\times 10^{-4}$)	Atmospheric pressure Bone dry ($\times 10^{-4}$)	Vacuum Bone dry ($\times 10^{-4}$)
Potato starch	0.813	0.109	0.980[a]	0.940	0.218
Gelatin	0.943	0.083	0.987	0.921	0.352
Cellulose gum	0.796	0.150	1.499	1.337	0.467
Egg albumen	1.119	0.077	0.999	0.938	0.309
Pectin 10%	1.310	0.066	1.133	1.048	0.416
Pectin 5%	0.980	0.039	0.935	0.915	0.287
Pectin 5%–glucose 5%	0.931	0.110	1.196	1.150	0.428

[a] At room RH.
Note: To convert to Btu ft^{-1} $°F^{-1}$ hr^{-1}, multiply the above values by 241.9.

may reflect the lower total solids content in wet gels (10%) than in raw foods (meat, fruit). Another possible factor may be the variation in porous structure encountered in freeze-dried gels and foods. This is discussed later in this paper.

A significant decrease in thermal conductivity was found when the pressure was lowered from atmospheric to 0.03 mm Hg. Except with cellulose gum, the difference between the high and low pressure values was close to 0.653×10^{-4} cal cm^{-1} $°C^{-1}$ sec^{-1}, which is the thermal conductivity of air at the same temperature. In cellulose gum this difference was significantly higher. Table 2 shows the effect of gas atmosphere on the thermal conductivity of a freeze-dried starch gel. Air and nitrogen gave about the same thermal conductivity, while helium resulted in a significant increase. The difference of the k values between atmospheric pressure and vacuum was close to the thermal conductivity of the gas contained in the pores for both air and nitrogen, but it was significantly less for helium. Similar results were obtained on freeze-dried foods by Harper and El Sahrigi (1964), who developed a semi-theoretical relationship expressing the thermal conductivity as a combination of the individual solid and gas conductivities.

Table 2. Thermal conductivity of freeze-dried starch gel (bone dry) in various gases at 1 atm. pressure (mean temperature, 41°C).

Gas	Thermal conductivity (cal cm^{-1} $°C^{-1}$ sec^{-1}) Gas-filled gel ($\times 10^{-4}$)	Difference from vacuum ($\times 10^{-4}$)	Pure gas ($\times 10^{-4}$)
Air	0.940	0.722	0.653[a]
Nitrogen	0.935	0.717	0.648[a]
Helium	3.571	3.353	3.684[b]
Vacuum	0.218	.....	.....

[a] Hilsenrath *et al.*, 1955. Tables 2-9, 7-9.
[b] Nuttall, 1950.

Fig. 2 shows that, within the accuracy of the measurements, the thermal conductivity of starch gels increased linearly with the temperature between 0 and 70°C. The agreement for the three samples was good, considering the possible effects of sample preparation (smoothness, uniform thickness) and the accuracy of the apparatus.

The thermal conductivity of freeze-dried starch gel at various pressures is shown in Fig. 3. At all pressures the thermal conductivity of the gel containing adsorbed water (dotted line) was higher than the k of the bone-dry gel (solid line). The thermal conductivity had an almost constant value between 760 and 100 mm Hg, and a lower almost constant value at pressures lower than 0.1 mm Hg. A sharp decrease in k was found as the pressure was reduced from 100 to 1 mm Hg. The variation of the thermal conductivity of the dried gels with pressure is related to the change of k of the gas contained in the pores of the material. The sigmoid shape of the thermal conductivity-pressure curve has been found to be characteristic of gas-filled porous solids, as, for example, in textile materials (Hoge and Fonseca, 1964).

The thermal conductivity of pectin gels was found to increase as the density was increased (Fig. 4). The sugar-containing pectin gel had a higher density and a higher

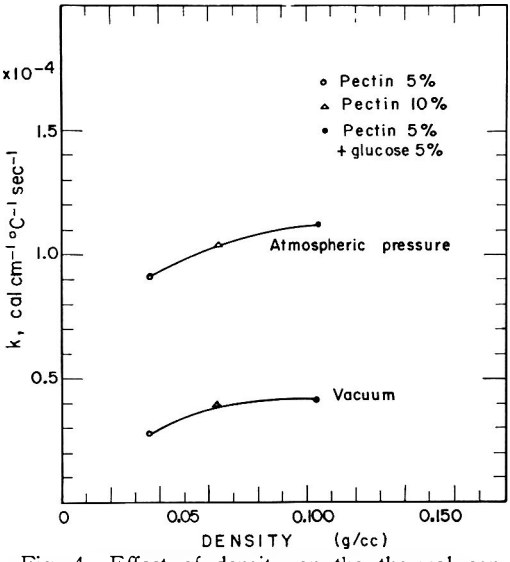

Fig. 4. Effect of density on the thermal conductivity of freeze-dried pectin gels (mean temperature, 41°C).

thermal conductivity than the simple pectin gel which initially contained the same amount of total solids (10%). The porosities and the pore dimensions of the dried gels are shown in Table 3. The porosities varied from 0.89 to 0.98, considerably higher than those of freeze-dried foods (0.76 to 0.87) reported by Harper and El Sahrigi (1964). At the same time the average pore diameters were much smaller in the gels (1–15μ) than in freeze-dried foods (30–300 μ). It is possible that these differences are due to the low initial gel concentration and the rapid freezing rate before drying. Luyet (1962) found that freezing meat rapidly, produced pores of an average diameter less than 10 μ in the freeze-dried material. Rapid freezing produces small crystals, which upon sublimation give rise to a microporous structure in the freeze-dried material. Starch, gelatin, and egg albumen gels had more or less spherical small pores, while cellulose gum and the pectin gels consisted of bundles of thin fibers running across the sample. The relatively high thermal conductivity of cellulose gum can be explained on the basis of the high density of the freeze-dried gel, and the fraction of the microscopic fibers which were oriented parallel to the heat flow. The fibrous structure of pectin gel (10% sample) was possibly responsible for its higher thermal conductivity than starch and gelatin gels, both of which had higher densities. Freeze-dried beef has been found to have a thermal conductivity higher than apple or peach, and this may be attributed to its lower porosity and more fibrous structure.

The freeze-drying rates which were observed on model gels (Saravacos, 1965) can be partially explained on the basis of the thermal conductivities of the dried materials. Cellulose gum dried more rapidly than any of the other gels, which can be related to the high thermal conductivity of the dried material. Starch, gelatin, and pectin gels dried at approximately the same rate and had similar thermal conductivities. Egg albumen was the slowest-drying gel, but its thermal conductivity was not particularly low. In this case the limiting factor may be the slow transfer of water vapor during freeze-drying.

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Table 3. Porous structure of freeze-dried gels (samples equilibrated at 52% RH).

Gel	Porosity [a]	Size of pores (μ)		k (cal cm^{-1} °C^{-1} sec^{-1}) ($\times 10^{-4}$)
Starch	0.93	2–10	pores of various shapes	0.980
Gelatin	0.93	3–15		0.987
Egg albumen	0.97	3–15		0.999
Cellulose gum	0.89	2–10		1.499
Pectin 10%	0.97	1–10	bundles of fibers across the sample	1.133
Pectin 5%	0.98	1–10		0.935
Pectin 5%–glucose 5%	0.96	1–8		1.196

[a] The ratio: $\frac{\text{volume of pores}}{\text{volume of sample}}$.

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Effect of Temperature and Pressure on the Sorption of Water Vapor by Freeze-Dried Food Materials

SUMMARY

The equilibrium isotherms and the rates of adsorption and desorption of water vapor by selected freeze-dried food materials were studied between −20 and 50°C with a spring-balance sorption apparatus. In simple freeze-dried gels (starch, gelatin) the quantity of adsorbed water at equilibrium increased as the temperature was lowered from 50 to 0°C, and it remained almost constant below 0°C. In freeze-dried foods (potato, peach, and raw beef) and sugar-containing starch gel, adsorption was maximum at between 10 and 30°C. The equilibrium vapor pressure of simple gels followed the Clausius-Clapeyron equation throughout the temperature range studied, and the same relationship applied to the foods at the high and low temperature regions, with a change of slope at about 20°C. The rates of adsorption and desorption at a pressure of 0.1 mm Hg remained constant at temperatures below 0°C, and they increased linearly as the temperature was increased from 0 to 50°C. As the pressure was raised from 0.1 mm Hg to atmospheric, the rates of adsorption at 30°C decreased by a factor of about 100. The adsorption rates at atmospheric pressure increased exponentially with the temperature between 0 and 50°C. The results are discussed in relation to the freeze-drying operation.

INTRODUCTION

In the freeze-drying of foods and biological materials, a portion of water must be desorbed during drying so as to obtain the low moisture content required. It is therefore of interest to know both the equilibrium water content and the rates of desorption (and adsorption) in various freeze-dried materials. The sorption of water vapor by freeze-dried model food gels at 30°C was studied recently (Saravacos, 1965). It was shown that the equilibrium adsorption isotherms were similar to those of air-dehydrated materials, but the rates of adsorption and desorption in vacuum were relatively high, even in the region of low water content. Since the temperature of a product may vary from at least −20 to 50°C during the entire process of freeze-drying, sorption information should be available over the same temperature range.

Stitt (1958) has reviewed the sorption of water vapor by dehydrated foods. Görling (1956) presented a family of water-sorption isotherms for potato between 0 and 100°C, and he found that as the temperature was increased the equilibrium water content decreased. The sorption isotherms of dehydrated foods have been studied in connection with quality and chemical stability during storage. These studies were made at room temperatures, or over a short range near room temperature (Bushuk and Winkler, 1957; Salwin, 1963; Karel and Nickerson, 1964). But no literature references have been found for temperatures below 0°C.

Little work has been done on the effect of pressure on the rates of adsorption and desorption of water vapor by freeze-dried materials. Harper (1962) has found that the permeability of freeze-dried foods to air and gases increased considerably as the pressure was decreased from atmospheric to 1 mm Hg.

The present work was done to establish the equilibrium adsorption isotherms of selected freeze-dried food materials from −20 to 50°C, and to study the rates of sorption of water vapor as affected by changes in temperature and pressure.

EXPERIMENTAL MATERIALS AND METHODS

Apparatus and methods. The sorption apparatus described in a previous paper (Saravacos, 1965) was modified to allow the maintenance of different vapor pressures in the sorption apparatus over a wide range of sample temperatures. An essentially similar apparatus was used by Karel and Nickerson (1964). A McBain quartz spring balance was used to sense the changes in weight of the sample. The spring extension was calibrated as 3.3 mg/mm, and measured with a cathetometer. The sorption chamber was kept at a constant temperature by circulating water or

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refrigerated alcohol. An aluminum foil–glasswool jacket was used to reduce heat losses. The desired water vapor pressure in the sorption chamber was obtained from distilled water or ice contained in a 100-ml flask; this flask was maintained at a constant temperature by using a water bath, or by frozen aqueous ethanol solutions in a Dewar flask. The temperature of water or ice in the flask was always lower than the sample temperature, except that the two temperatures were equal at saturation measurements. The vapor pressure at equilibrium was measured with a differential oil manometer, or it was taken from the temperature-pressure tables of ice in the low-temperature region. The relative humidity was expressed by P/P_0 with P the equilibrium vapor pressure, and P_0 the saturation vapor pressure of water at the temperature of the sample chamber.

Desorption was generally studied by connecting the sample chamber with a dry ice–ethanol trap (−78°C) and continuous vacuum-pumping. The effect of pressure on the adsorption rate at 30°C and 52% RH was measured by placing a tube containing saturated sodium dichromate solution close to the sample in the sorption chamber. Adsorption at different temperatures at atmospheric pressure was measured with a saturated sodium chloride solution in the sorption chamber, since the relative humidity maintained by this salt is not affected significantly by temperature.

Freeze-dried samples. The following freeze-dried materials were used: starch gel, gelatin gel, starch-glucose gel, blanched potato, peach, and raw beef. Freeze-dried discs of gels 15 mm in diameter and about 1 mm thick were prepared as described by Saravacos (1965). Discs of the same dimensions were cut with a cork borer from pieces of blanched potato, clingstone peach, and lean raw beef. The discs were frozen rapidly at dry-ice temperature and freeze-dried at a pressure of 0.1 mm Hg and an ambient temperature of 30°C. The dried materials had a final moisture content of about 2% as determined by the vacuum-oven method. Five freeze-dried discs were used for the determination of each adsorption isotherm. Before starting the adsorption measurements, the sample was dried 6 hr in the vacuum chamber at 50°C using the dry ice–ethanol trap, and the equilibrium weight was taken as that of the bone-dry solids. The adsorption isotherms at various temperatures were measured on the same samples of starch and gelatin gels, potato, and beef. However, it was necessary to use different samples of peach and starch-glucose gel at each temperature because of a significant shrinkage of the discs at high relative humidities. Duplicate measurements on different samples were made for all materials.

RESULTS AND DISCUSSION

Adsorption isotherms. The adsorption isotherms of the freeze-dried materials are shown in Figs. 1–6. In the starch and gelatin gels, temperature had the normal effect predicted by the theory of physical adsorption, i.e. the quantity of adsorbed water, at a given relative humidity, increased as the temperature was decreased from 50 to 0°C, and below 0°C remained almost constant. The sigmoid shape of the isotherms indicates that water is adsorbed in multimolecular layers according to the Brunauer-Emmet-Teller (B.E.T.) theory (Salwin, 1963). In starch and gelatin gels, equilibrium was

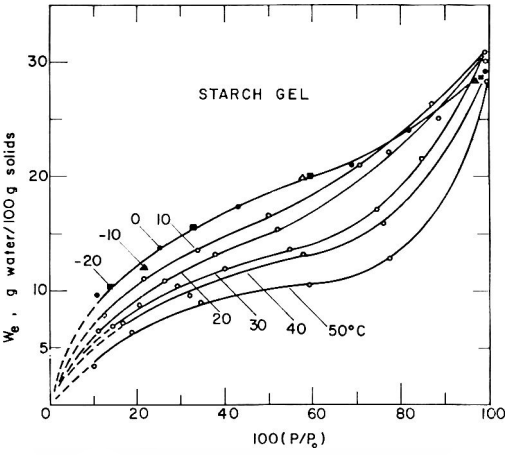

Fig. 1. Equilibrium adsorption isotherms of freeze-dried starch gel.

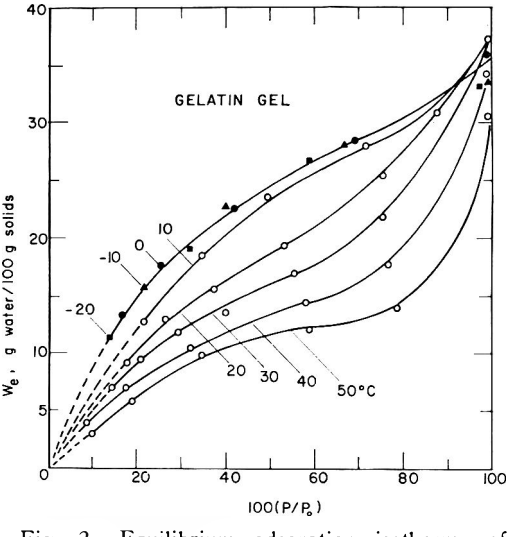

Fig. 2. Equilibrium adsorption isotherms of freeze-dried gelatin gel.

reached in vacuum within 1 hr except at 100% RH, when adsorption was completed within 2 hr. No measurements were made at relative humidities less than 10%; and the isotherms were extrapolated to zero water content (dotted lines).

Adsorption isotherms changed significantly when glucose was incorporated in the starch gel (Fig. 3): the quantity of adsorbed water increased to a maximum as the temperature was decreased from 50 to 30°C, and then decreased as the temperature was lowered from 30 to −20°C. A similar effect was found on a freeze-dried starch-sucrose (1:1) gel. The isotherms of freeze-dried

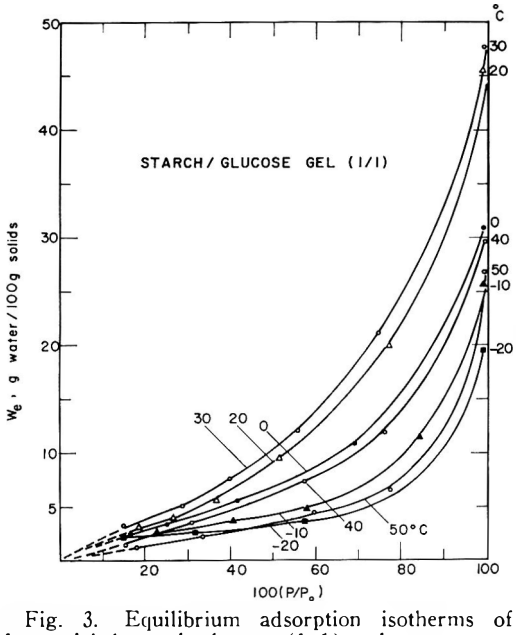

Fig. 3. Equilibrium adsorption isotherms of freeze-dried starch-glucose (1:1) gel.

peach (Fig. 6) were similar to those of the starch-sugar gels. Freeze-dried potato and beef (Figs. 4, 5) showed a maximum adsorption between 10 and 20°C. Compared to the starch and gelatin gels, all freeze-dried foods adsorbed less water vapor at low humidities but more at high humidities. At saturation the sugar-containing materials (peach, starch-glucose gel) adsorbed water continuously, and no equilibrium could be reached at temperatures above 0°C; the saturation points of the isotherms were taken arbitrarily as the water contents after 2 hr of adsorption in vacuum.

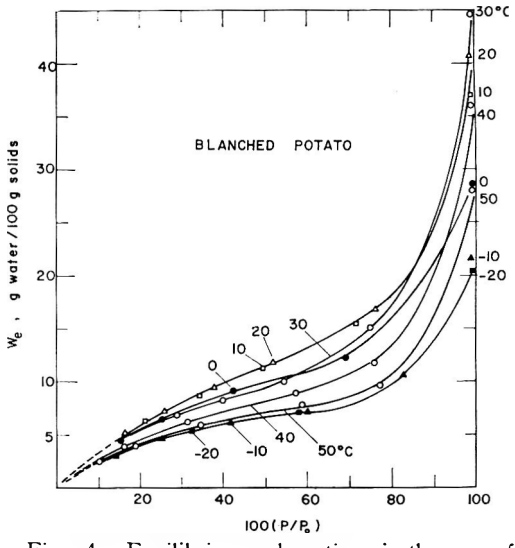

Fig. 4. Equilibrium adsorption isotherms of freeze-dried potato.

The adsorption isotherms of the foods can be interpreted on the basis of their basic components, i.e. polymeric materials (proteins, starch, pectin) and soluble solids (sugars, acids, minerals). The soluble solids adsorb very little water at low humidities, and adsorption is mainly due to the polymers. As vapor pressure is increased

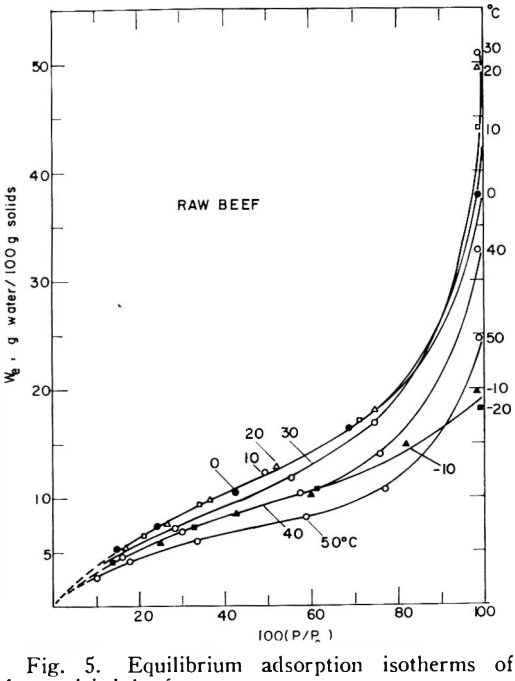

Fig. 5. Equilibrium adsorption isotherms of freeze-dried beef.

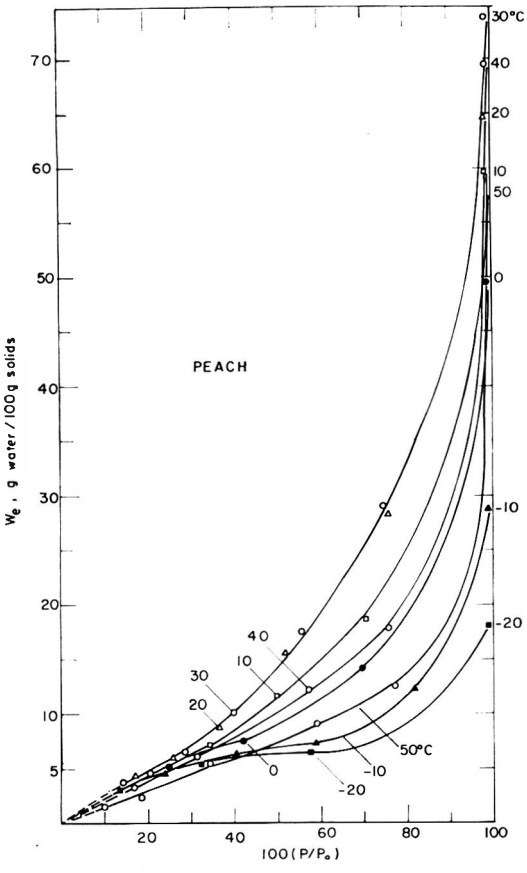

Fig. 6. Equilibrium adsorption isotherms of freeze-dried peach.

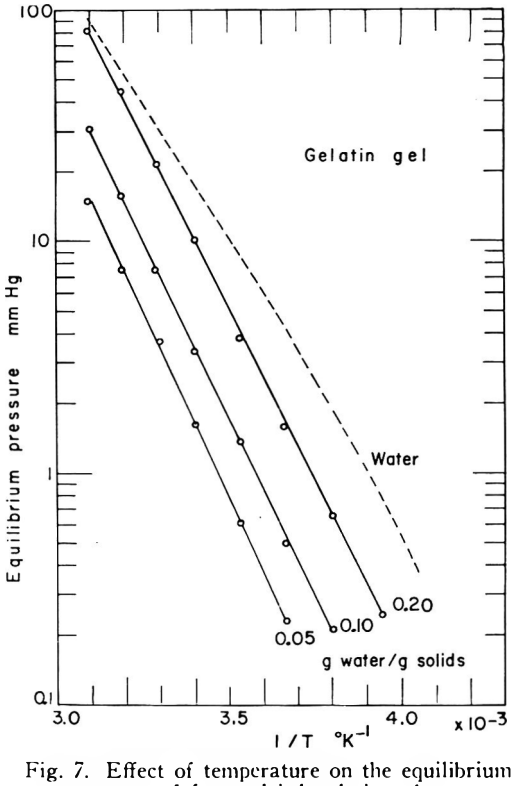

Fig. 7. Effect of temperature on the equilibrium vapor pressure of freeze-dried gelatin gel.

above the vapor pressure of the saturated solution of the soluble solids, adsorption increases considerably and a solution is produced. The maximum adsorption of foods between 10 and 30°C may he due to a combination of two opposing effects, i.e. the dissolving of soluble solids, and the normal effect of temperature on the physical adsorption of water vapor by polymeric materials.

The equilibrium vapor pressures (P) at various specific water contents were taken from the adsorption isotherms and plotted versus the inverse of absolute temperature. For both starch and gelatin gels (Fig. 7) the data followed the Clausius–Clapeyron equation: $\ln P = -\Delta H/RT$, where ΔH is the heat of adsorption, R is the gas constant, and T is the absolute temperature. The heat of adsorption, ΔH, is defined as the sum of the heat of condensation of water vapor and the differential heat of wetting (Stitt, 1958).

It was noted that the slopes of the straight lines increased as the water content decreased. Above 20% water content the plots were almost parallel to the line of pure water, which represents the vapor pressure of liquid water above 0°C, and the vapor pressure of ice below 0°C. The heats of adsorption of water vapor in the gelatin gel were calculated graphically: 14.3 Kcal/g mole at 5% water content; 13.8 at 10%; and 12.3 at 20%. The heat of condensation of water at 0°C is 10.8 Kcal/g mole.

The equilibrium vapor pressures in beef and peach followed the Clausius-Clapeyron equation from −20 to 20°C, and from 20 to 50°C (Figs. 8, 9). Results were similar in freeze-dried potato and starch-glucose gel. The slopes of these plots (and, therefore, the heats of adsorption) were higher at temperatures above 20°C than at lower temperatures. At temperatures below 20°C the plots were almost parallel to the line of water, and they did not change much as the water content was increased; the heats of adsorption in this region of temperatures

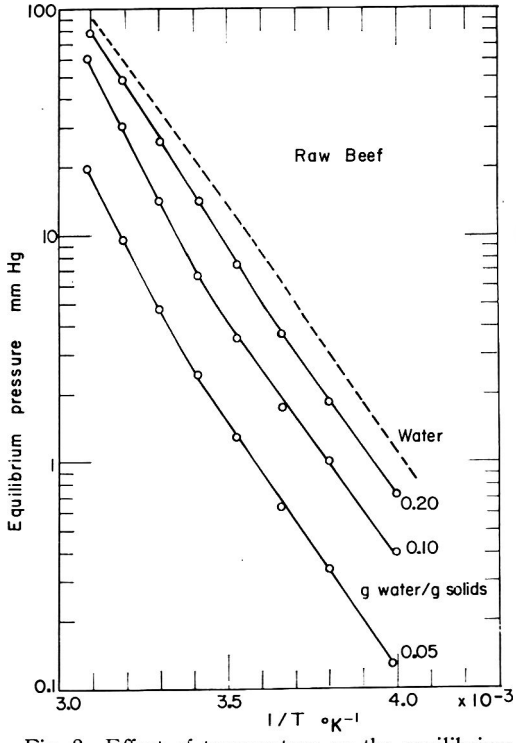

Fig. 8. Effect of temperature on the equilibrium vapor pressure of freeze-dried beef.

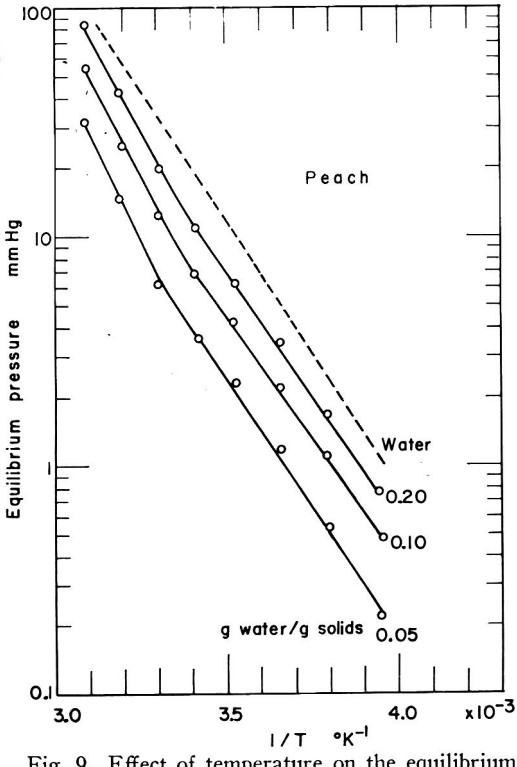

Fig. 9. Effect of temperature on the equilibrium vapor pressure of freeze-dried peach.

were close to the heat of condensation of water. Görling (1956) found that the Clausius-Clapeyron equation was applicable to the adsorption of water vapor by air-dried potato at temperatures between 0 and 100°, but our results on freeze-dried potato show that there is a change of slope below 20°C.

Some of the adsorption data were plotted according to the B.E.T. equation (Salwin, 1963). This equation was applicable only between 0 and 40% RH. At higher humidities the experimental data deviated significantly from the straight line. This method of interpreting adsorption data was not applied any further, because it did not cover the whole isotherm.

Sorption rates. The adsorption and desorption rates of water vapor at various temperatures were studied in detail, since this information is useful in understanding the freeze-drying of the food materials. Figs. 10 and 11 show the results of some measurements in vacuum (absolute pressure approximately equal to water vapor pressure) and for two water-content regions. It was necessary to use different relative humidities for obtaining the same equilibrium water content at various temperatures. The

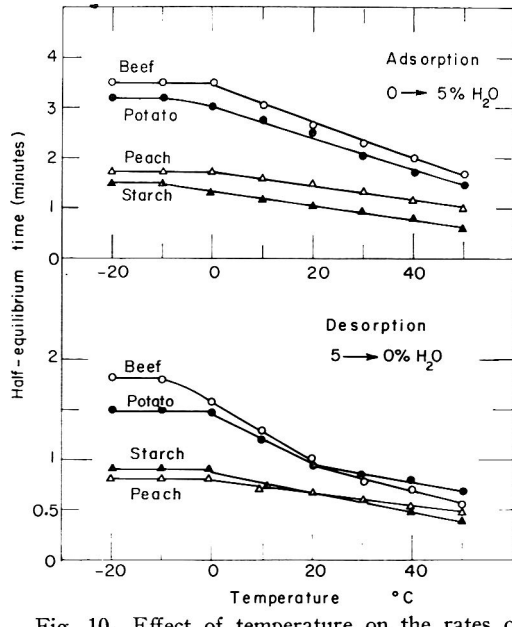

Fig. 10. Effect of temperature on the rates of water sorption of freeze-dried food materials in vacuum (0–5% water content).

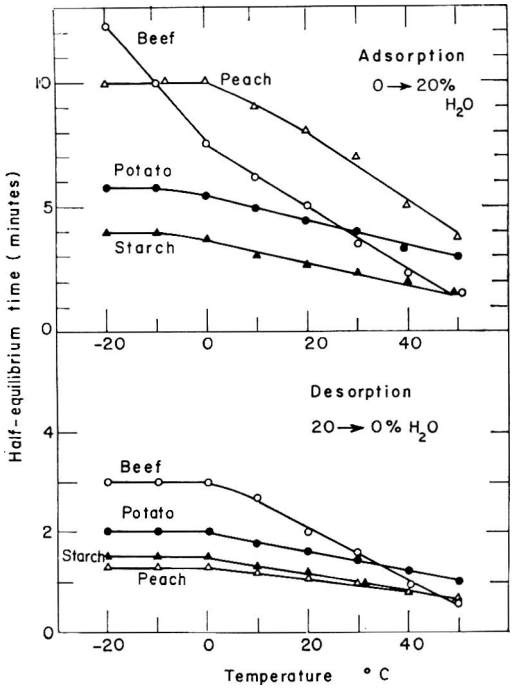

Fig. 11. Effect of temperature on the rates of water sorption of freeze-dried food materials in vacuum (0–20% water content).

sorption rate is inversely proportional to the half-equilibrium time, which is defined as the time required to reach one-half the final equilibrium water content of a freeze-dried sample of 15 mm diameter and 1 mm thickness. The desorption rate in vacuum was always higher than the adsorption rate, and both rates were higher in the low water-content region (Saravacos, 1965). Both the adsorption and the desorption rates increased almost linearly as the temperature of the material was increased from 0 to 50°C, and they were approximately constant from 0 to −20°C; the temperature effect was small compared to the exponential increase of the diffusion rate observed at atmospheric pressure, as discussed below.

Pressure had a significant effect on the rate of adsorption of water vapor by freeze-dried starch gel and potato (Fig. 12). The equilibrium water content was not affected by pressure. The half-equilibrium time in starch gel at 30°C increased from about 2 to 200 min as the air pressure in the sorption chamber was increased from 0.1 mm Hg to atmospheric. The same pattern of change

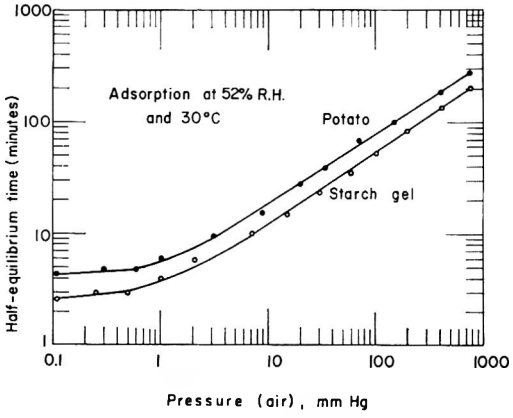

Fig. 12. Effect of pressure on the rates of water adsorption of freeze-dried starch gel and potato, at 30° C and 52% RH.

was found in the desorption rates. It is known that the diffusivity of water vapor (D) in air increases as the pressure (P) is reduced. The diffusivity of water at atmospheric pressure is 0.26 sq cm sec^{-1} at 25°C, and the product $P \times D$ has been found constant up to 20 atm pressure (Reid and Sherwood, 1958). It seems reasonable to assume that the changes in rate of adsorp-

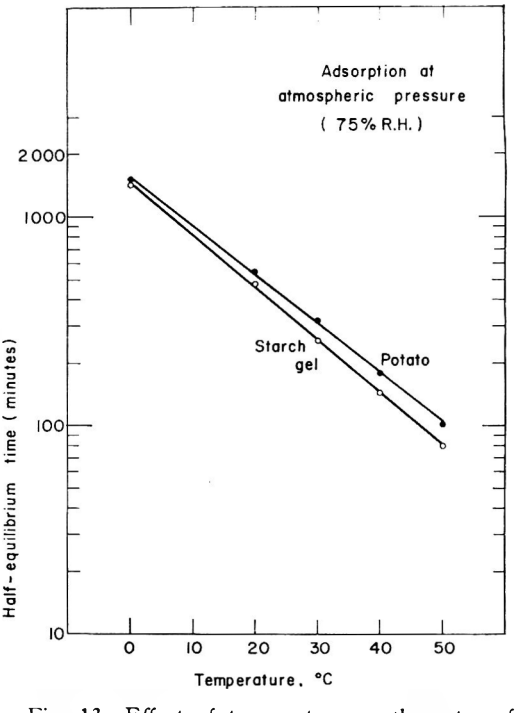

Fig. 13. Effect of temperature on the rates of water adsorption of freeze-dried starch gel and potato, at atmospheric pressure and 75% RH.

tion due to pressure are related to the effect of pressure on the diffusivity of water vapor in the air contained in the pores of the freeze-dried material.

The adsorption rates of water vapor by freeze-dried starch gel and potato at atmospheric pressure increased exponentially as the temperature was increased from 0 to 50°C (Fig. 13). This effect of temperature is characteristic of activated diffusion, which has been found to be the main mechanism of water transfer in the air-dehydration of foods. Assuming that molecular diffusion is controlling the transfer of water vapor at atmospheric pressure, it was possible to calculate the diffusivities of water vapor in some freeze-dried food materials, using the adsorption rate data. During the adsorption of water vapor the unsteady-state diffusion equation can be applied, assuming that the diffusivity is constant in the particular region of water content. The diffusion equation has been solved for various shapes of solids, and the simplified solution for thin slabs given by Fish (1958) at half-equilibrium adsorption becomes,

$$D = 0.049 \times \frac{L^2}{t_{1/2}}$$

where D = diffusivity, sq cm sec^{-1}; L = thickness of the slab, cm; and $t_{1/2}$ = time to reach one-half equilibrium, sec.

Table 1. Diffusivity of water vapor in freeze-dried food materials (calculated from adsorption data on discs of 1.5 cm diameter between 0 and 10% moisture content, at atmospheric pressure and 30°C).

Sample	Thickness of sample (cm)	Time to reach one-half equilibrium (min)	D sq cm sec^{-1} $\times 10^{-7}$
Starch gel	0.145	150	1.15
Starch-glucose gel	0.130	270	0.52
Blanched potato	0.140	200	0.83
Peach	0.125	150	0.85
Raw beef	0.175	260	1.00

The thin discs used in this work were assumed as infinite slabs, i.e. the diffusion from the sides was neglected. Table 1 shows the data for the calculation of D at 30°C in freeze-dried starch gel, starch-glucose gel, potato, peach, and beef, between 0 and 10% water content. The diffusivities were close to 1×10^{-7} sq cm sec^{-1}, which is approximately 10 times as high as the diffusivity of water in air-dried starch gel, at the same temperature and water content (Fish, 1958).

Freeze-drying considerations. The results obtained in this work indicate that in normal vacuum-freeze-drying (0.1 to 1.0 mm Hg) water vapor is desorbed and transferred through the dried food material at a relatively high rate, even at low temperature, provided sufficient heat can be supplied for the sublimation of inner ice. It seems possible that desorption may take place at the same time as the sublimation of ice, and this combination may be responsible for the high drying rates observed for food-like gels. The equilibrium water content of freeze-dried materials at saturation and at temperatures below 0°C may be related to the "non-freezable water," which is the portion of water remaining unfrozen in several frozen foods (Kuprianoff, 1958). This water can be desorbed at a relatively high rate at temperatures below 0°C. Raising the temperature of the product would have only a small effect on the desorption rate. The only advantage of a higher temperature in the last stage of drying would be a lower relative humidity in the chamber, and, therefore, a lower equilibrium water content of the dried material.

In a series of experiments with freeze-dried starch gel and potato, the rates of adsorption and desorption at a given temperature in vacuum decreased linearly as the thickness of the samples was increased from 0.5 to 2.5 mm. A similar effect of thickness has been found for the freeze-drying rates of starch and gelatin gels (Saravacos, 1965).

It has been suggested that, in normal vacuum freeze-drying, water vapor is transferred through the porous dried layer of the material by a combination of hydrodynamic and diffusional, or slip, flow (Harper, 1962). At low pressures the permeability of the freeze-dried material to water vapor represents more accurately the flow properties than diffusivity. Measurements of flow of air and other gases through freeze-dried foods have shown that the permeability in-

creases rapidly as the average pressure is reduced from atmospheric to about 1 mm Hg, and it approaches a constant value at lower pressures (Harper, 1962). The order of change of permeability with pressure and the shape of the plots were similar to the change of adsorption rate with pressure. (Fig. 12).

Freeze-drying at atmospheric pressure in a stream of desiccated cold gas has been found to be very slow, and this can be attributed to the small values of the diffusivity of water vapor, which decreases exponentially as the temperature is lowered.

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Influence of Prerigor, Rigor and Postrigor Freezing on Drip Losses and Protein Changes in Chicken Meat

SUMMARY

Tests made with chickens frozen before rigor, during rigor, and after rigor showed that the amount of drip exuded on thawing was greatest in poultry frozen during rigor. The loss of nitrogenous constituents and ribose increased proportionally with the amount of drip. Protein solubility was minimum, and cooking losses maximum, in poultry frozen during rigor. The factors responsible for drip loss in poultry appear to be similar, regardless of the stage of rigor mortis at which the meat was frozen. It is probable that more water froze out of muscle during the state of rigor, causing higher solute concentrations in the tissue. The higher solute concentration in the muscle tissue frozen during rigor may affect the solubility of proteins and their ability to reabsorb water on thawing, and thus affect tenderness and loss of drip.

INTRODUCTION

It is well known that meat frozen without aging loses considerable water on thawing (drip) and tends to become dry and unpalatable. This phenomenon has been studied in relation to the mechanism of contraction in isolated rabbit and lamb muscle and has been shown to be the result of thaw-rigor contracture (Bendall, 1960; Marsh and Thompson, 1957). This phenomenon, besides its general biochemical interest, is also of importance in the commercial quick-freezing of poultry because the poultry processors are progressively reducing the aging time in order to cut the processing time and operating cost. Since freezing meat "on the bone" rarely gives rise to thaw contracture (Marsh and Thompson, 1958), the results obtained with isolated muscle may not apply to poultry meat, which is seldom removed from the skeleton in commercial practice. Tests were therefore made to determine the effect of freezing poultry at three different stages of post-mortem aging on protein solubility, amount and composition (total nitrogen, nonprotein nitrogen, ribose, and phenol-reagent-positive materials) of drip exuded on thawing, and cooking losses. The three stages of post-mortem aging were prerigor (within 15 min of slaughter), during rigor (after 4 hr of aging), and postrigor (after 24 hr of aging). These times were selected on the basis of biochemical tests (Khan and van den Berg, 1964) and by judging the firmness and elasticity of the flesh manually. To obviate the effect of bird-to-bird variability in initial and ultimate pH of the muscle, which influences the course of rigor mortis with time (Bate-Smith and Bendall, 1949), comparisons were made between left and right halves of the same bird.

EXPERIMENTAL

Samples were obtained from male chickens (broilers, Ottawa Meat Control strain) raised under similar environmental and nutritional conditions. The birds were killed in the laboratory by cutting the jugular vein and carotid arteries, bled (2 min), scalded at 53–54°C (2 min), plucked by hand (about 3 min), eviscerated, washed, and cut into halves (about 4 min). During cutting into halves, care was taken not to tear or cut the muscle away from the bones. To prevent moisture absorption during aging, half birds were vacuum packaged in plastic bags and either frozen within 15 min of killing or after aging in ice for the desired length of time. To obtain conditions necessary for thaw contracture the samples were frozen quickly by immersing in a methanol–dry ice mixture and thawed quickly by placing in water at 30°C.

For analysis the drip was collected by suspending samples after thawing inside their plastic bags to allow the exudate to drain from the meat. The volume of the drip obtained was measured and made to a known dilution with KCl-borate buffer, pH 7.0. The drip was analyzed for total nitrogen by the standard micro-Kjeldahl procedure. Nonprotein nitrogen, phenol-reagent-positive materials, and total ribose were determined in the soluble phase, obtained by treating drip solution with equal volume of trichloroacetic acid solution (20% aqueous, w/v). The analytical procedures have been described (Khan, 1964).

The amount of buffer-extractable nitrogen in pectoralis major and quadriceps femoris muscles was determined with KCl-borate buffer, pH 7.5, ionic strength 1.0. Preparation of the sample, and the extraction and protein fractionation techniques, have been described (Khan, 1962).

Studies on cooking losses were made with pectoralis major and biceps femoris. These muscles were removed from the carcass after thawing and were freed from skin and visible fatty tissues before cooking. The liquid exuded during cooking was determined by the following method, modified from Downing (1963). A 30–40-g sample of muscle with a thermocouple inserted in its center was placed inside a hollow Lucite cylinder, 4 inches long, equipped with a perforated Lucite disc fitted crosswise at ⅓ of its length from the bottom to separate exudate from the meat. The cylinder was fitted inside a standard stainless-steel centrifuge cup of 200 ml operating capacity. The centrifuge cup was closed with a rubber stopper equipped with a capillary tube which also served as an outlet for thermocouple wires. The samples were cooked to an internal temperature of 82–85°C (about 1 hr in boiling water). The samples were then cooled and centrifuged for 15 min at 4000 × G. The volume of liquid collected during centrifugation was measured, and the weight lost during cooking was determined.

RESULTS

The amount of drip exuded on thawing as well as the amount of nitrogenous constituents and ribose lost in drip were maximum in chickens frozen during rigor (Table 1). Statistical calculations based on F test indicated that the differences were highly significant (1% level). Composition of the drip as indicated by nitrogenous constituents, phenol-reagent-positive materials, and ribose contents remained relatively constant regardless of the state of rigor mortis at which the muscle was frozen. These results are in agreement with findings of Sair and Cook (1938) on frozen minced chicken meat. Since all the birds were raised and processed under similar conditions, between-bird variability in quantity of drip suggests that drip loss may depend in part on the physiological condition of the bird, as affected by fatigue, emotional stress, and struggle at the time of death.

Freezing during rigor resulted in the reduction of buffer-extractable nitrogen content of both breast and leg muscles (Table 2). Quantitative fractionation of muscle proteins showed that the reduction in buffer-extractable nitrogen content occurred mainly as a result of loss of solubility of the myofibrillar proteins and suggests that freezing during rigor denatures this fraction of muscle proteins.

Cooking losses were maximum in muscle frozen during rigor (Table 3). The modified centrifuge cup method used in determining cooking losses gave results reproducible within ±3% and has an advantage over other methods described in the literature in that six samples weighing up to 100 g each can be analyzed simultaneously with full control of temperature and centrifugation speed.

Table 2. Buffer-extractable nitrogen in breast and leg muscles of poultry frozen before, during, and after rigor (values are averages of five samples).

State of muscle when frozen	Chicken halves used for comparison	Buffer-extractable nitrogen (% of total N)	
		Pectoralis major	Quadriceps femoris
Prerigor	Left	81.5	60.7
Rigor	Right	72.1	55.0
Prerigor	Left	76.3	50.0
Postrigor	Right	80.0	53.2
Rigor	Left	72.8	56.8
Postrigor	Right	81.8	61.6

Table 1. Volume and composition of drip exuded during thawing of poultry meat frozen before, during, and after rigor (values are averages of five samples).

State of muscle when frozen	Chicken halves used for comparison	Drip (ml/100 g carcass)	Drip (mg/100 g carcass)		% of total N		Phenol-reagent positive materials (μg tyrosine/mg N)
			Ribose	Total N	Nonprotein N	Protein N	
Prerigor	Left	1.1 (0.4–2.0)	0.27	8.8	33	67	32
Rigor	Right	1.7 (0.8–3.7)	0.36	11.1	34	66	32
Prerigor	Left	1.0 (0.4–2.5)	0.40	9.0	31	69	30
Postrigor	Right	0.7 (0.5–0.9)	0.29	5.9	32	68	35
Rigor	Left	1.6 (0.6–2.7)	0.43	10.2	41	59	44
Postrigor	Right	0.9 (0.1–1.1)	0.25	6.0	38	62	43

Table 3. Cooking loss (weight and volume) for breast and leg muscles of poultry frozen before, during, and after rigor (values are averages of five samples).

State of muscle when frozen	Chicken halves used for comparison	Kind of muscle	Liquid exudate (ml/100 g muscle)	Weight loss %
Prerigor	Left	P. major	35	37.2
Rigor	Right	P. major	38	37.8
Prerigor	Left	B. femoris	29	36.9
Rigor	Right	B. femoris	32	38.3
Prerigor	Left	P. major	31	36.8
Postrigor	Right	P. major	33	36.6
Prerigor	Left	B. femoris	29	37.7
Postrigor	Right	B. femoris	31	38.2
Rigor	Left	P. major	34	38.0
Postrigor	Right	P. major	30	36.1
Rigor	Left	B. femoris	32	36.6
Postrigor	Right	B. femoris	28	33.1

DISCUSSIONS

The results indicated that the freezing of poultry during rigor decreases the solubility of muscle proteins and increases the drip loss, and that protein changes occurring during aging improve the water-holding capacity of poultry meat. Since it has been shown that the solubility of myofibrillar proteins increases during the postrigor aging period concomitantly with an increase in tenderness (Khan and van den Berg, 1964), protein extractability results in the present study suggest that muscle frozen prior to rigor is likely to be more tender than that frozen during rigor. By shear force tests de Fremery and Pool (1963) have also shown that chicken muscle is more tender if cooked immediately after killing, than if aged for 1 hr. It appears that components of muscle responsible for post-mortem tenderization are more subject to damage when meat is frozen during rigor mortis. In taste-panel tests Rose and Lentz (1956) showed that short aging periods caused toughness in frozen turkeys. Klose *et al.* (1959) and Pool *et al.* (1959) have shown that thawing time required for tenderizing prerigor-frozen turkeys and chickens is the same as the aging time before freezing. In commercial practice, however, prefreezing tenderization or aging would appear to be preferable because of the rapid onset of rigor mortis in chicken muscle and widely variable thawing in domestic practice.

Factors which may influence the differences in drip loss on freezing and thawing in poultry frozen prerigor, during rigor, and postrigor, are pH, rapid rate of depletion of adenosinetriphosphate (ATP) during rapid thawing of prerigor-frozen meat (Bendall, 1960), thaw shortening in muscle frozen during rigor (Marsh and Thompson, 1957), mechanical disturbance of the fibrillar system due to the violent thaw-rigor contraction (Cassens *et al.*, 1963), and the size of the ice crystals formed as a result of slow freezing (Empey, 1933; Moran, 1932). It is not known whether drip results from only one or a combination of these possible factors, but our results on the composition of drip suggest that the factors responsible for drip losses are the same regardless of the state of rigor mortis at which the muscle was frozen. Since rigor begins when the ATP content of the muscle is depleted and a steady pH is attained (de Fremery and Pool, 1960) neither lower pH of the muscle nor a rapid rate of depletion of ATP during thawing appear to be responsible for quantitative differences in drip obtained from poultry frozen during rigor and after rigor. Differences in initial pH of the meat (Bendall, 1960) may cause between-bird variations in the amount of drip exuded, how-

ever, and the elevated pH found in poultry frozen before rigor (van den Berg, 1964) may be responsible for the smaller amount of drip produced from meat frozen in that state. The composition, particularly ribose content, of drip indicated that freezing poultry meat "on the bone" did not cause thaw contracture and cell rupture.

The fact that the water-holding capacity of muscle frozen during rigor was minimum may be helpful in understanding the cause of drip and protein denaturation. A decrease in water-holding capacity may increase the amount of water available for freezing during rigor and give rise to higher solute concentrations in muscle during and after freezing. These higher concentrations of solute may cause damage by affecting the solubility of muscle proteins and the ability of cell constituents and proteins to reabsorb water on thawing. Since the amount of bound water also governs the migration of free water and the size of ice crystals formed during freezing and subsequent storage (Fennema and Powrie, 1964), the possibility of increased damage due to larger ice crystals in muscle frozen during rigor cannot be excluded.

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Automated Analysis of Hypoxanthine

SUMMARY

Hypoxanthine concentration is a useful index of the quality of flesh foods. A rapid manual assay of the purine was automated by modifying commercially available proportioning and spectrophotometric equipment. Extracts are sampled serially and mixed with xanthine oxidase and buffer. After reaction at 37° in a time-delay coil, the mixture passes through a low-volume flow-through cell, where its absorption at 290 mμ (and hence uric acid production from hypoxanthine) is monitored continually. Concentrations are estimated by reference to standards and blanks.

INTRODUCTION

Recent observations (Jones *et al.*, 1964; Spinelli *et al.*, 1964) have confirmed preliminary reports from this laboratory that the specific determination of hypoxanthine affords a good index of quality and potential shelf-life in chill-stored fish. The concentration of the purine is related to storage change also in meats (see Kassemsarn *et al.*, 1963, for literature coverage). From a comparison of currently available manual procedures for hypoxanthine determination, Jones *et al.* (1964) concluded that a modification of the xanthine oxidase assay (Kalckar, 1947) had decided advantages, in terms of speed, specificity, and accuracy, over heavy-metal precipitation techniques and chromatography. The present paper describes successful automation of the enzymic assay with commercially available equipment.

EXPERIMENTAL

The manual xanthine oxidase assay, upon which these developments are based, is carried out by treating a diluted, neutralized, perchloric acid extract of the tissue with a commercial preparation of the enzyme. The reaction-mixture is buffered with phosphate. Hypoxanthine concentration is evaluated, after subtraction of enzyme and substrate "blanks," from optical density at 290 mμ, which monitors uric acid formation.

In its essentials, the automated procedure described in this paper represents a direct translation of the manual procedure. Its operation is based on the AutoAnalyzer system (Technicon Instruments Co., Ltd., Chertsey, Surrey), but we believe that a number of points that have arisen in the study should be applicable also to any other system that can sample sequentially and mix closely defined ratios of reactants accurately in an air-segmented stream.

The basic arrangement used throughout is illustrated in Fig. 1. In the preliminary experiments,

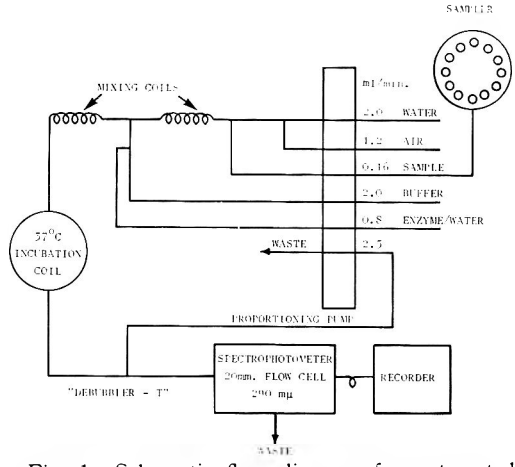

Fig. 1. Schematic flow diagram for automated hypoxanthine analysis. The concentrations of solutions used are given in the text.

standard solutions were used. These were mixed with reagents and segmented with conventional AutoAnalyzer equipment. The segmented mixture passed through a 52-ft spiral of glass tubing (3-4 mm ID) at 37°, taking 25 min to pass through.

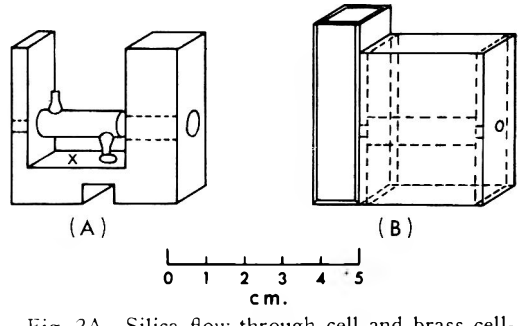

Fig. 2A. Silica flow-through cell and brass cell-holder. A simple spring clip screwed to the holder at point *X* holds the cell in position and alignment.

Fig. 2B. Reference assembly. A drilled metal block with soldered end plates attenuates the reference light beam. Coarse adjustment of recorder baseline is obtained by using uric acid solutions of varying strengths in the standard 1-cm-path-length silica cell.

The stream was then split at a T piece: an aliquot and the segmenting air were passed back through the proportioning pump to "waste," and the remainder of the reaction-mixture flowed through a cell (Fig. 2A) for continuous spectrophotometric monitoring at 290 mμ.

Initially, we tried out a flow-through cell 1 cm (path-length) × 1 cm × 1.5 cm in a single-beam spectrophotometer modified for continuous single-wavelength recording (Cahn, 1956; Shrewsbury, 1959). This arrangement yielded poor "washes" between samples, and also a drifting baseline. Subsequently, and in the work described in this paper, we have used a cell of considerably greater path-length-to-volume ratio, in an arrangement fabricated to fit into the housing of an inexpensive double-beam spectrophotometer (Beckman DB). This yielded drift-free traces and considerably better "washes." For the purposes of this assay, the assembly offers advantages, in terms of ratio of path-length to volume, over that offered commercially with the instrument.

No serious difficulties were encountered in operation of the analysis. Since commercial enzyme preparations vary in strength and purity, however, it may be as well to point out that certain characteristic recorder trace patterns have been observed that relate to inhibitory effects of high hypoxanthine concentration on the production of uric acid in the reaction. Conditions of operation in the manual procedure (Jones *et al.*, 1964) and in the method detailed below are such that the relative proportions of enzyme to substrate are considerably in excess of those associated with such inhibition. The following observations are presented as a cautionary note to anyone considering analysis, with xanthine oxidase of unknown activity, at substrate ranges of which he has no prior experience.

Fig. 3A illustrates the changing pattern of trace observed with a stock enzyme concentration of 0.0036 International Units/ml reacting with increasing hypoxanthine concentrations. Above 4 μg hypoxanthine/ml reaction mixture, uric acid production fell progressively and the geometry of the peaks also changed. As compared with those for hypoxanthine concentrations below that yielding maximum 290 mμ absorption, the peaks for higher hypoxanthine concentrations showed pronounced "blips" at the tops of the trailing edges. This resulted from substrate dilution at the end of the sampling period. Similarly, a lesser "blip," on the top of the leading edge of a peak, is often observable under such conditions. This rise and fall of absorption within the peak results from passage through the optimum substrate concentration during sampling, as a result of dilution of the sample reaction mixture with the inter-sample wash. Curve *A* in Fig. 3B is a plot of hypoxanthine concentration against percent transmission (log) for the data contained in Fig. 3A. Curve B contains values obtained from a similar experiment, but with twice the amount of enzyme present. The region of linearity has in this case been extended to approximately 9.6 μg/ml of reaction mixture.

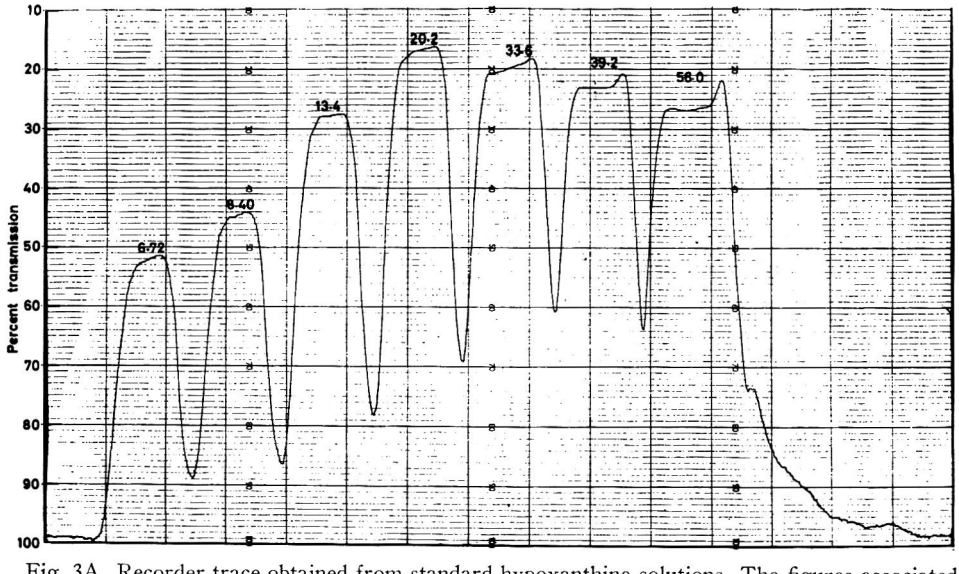

Fig. 3A. Recorder trace obtained from standard hypoxanthine solutions. The figures associated with each peak refer to the amount of hypoxanthine present in the total reaction mixture per test (i.e. 4.96 ml).

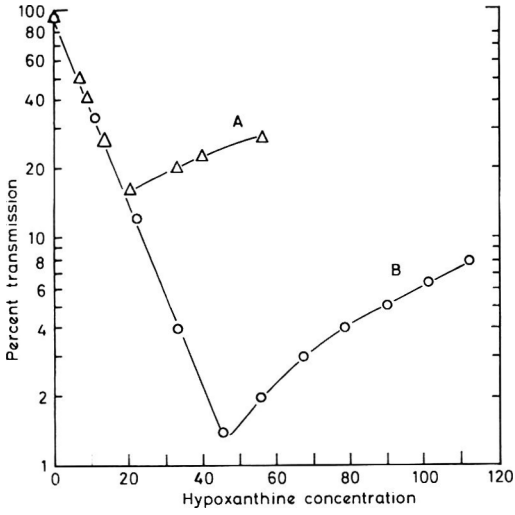

Fig. 3B. Plot of hypoxanthine concentration (μg/4.96 ml reaction mixture) against percent transmission. For experimental details, see text.

Such traces indicate a necessity either to increase enzyme concentration or decrease the concentration of hypoxanthine in samples and standards.

In practice, we found it necessary in our early work to run all standard and extract "blanks" through the system before introducing xanthine oxidase into the reaction train. Otherwise some oxidation of the purine (small, but sufficient to lead to serious "blank" errors at low hypoxanthine levels) resulted from contamination by residual enzyme even after a continuous 40-min water wash. This appeared to be due to trapping at tubing junctions and sorption on the plastic tubing.

Replacement of such tubing with glass as far as possible, and the careful butting of joints end-to-end, has almost eliminated this trouble—except occasionally at the very lowest purine levels. Such enzymic contamination decays to zero overnight.

RECOMMENDED PROCEDURE

Apparatus. *Automatic sampler and proportioning pump. Heated incubation coil.* A total of 52 ft of glass tubing (3–4 mm ID) is immersed in a bath accurately thermostated at 37°. In our assembly, the tubing was wound in 2 concentric coils of diameters 6 inches and 9 inches, respectively 20 and 32 ft long.

Recording double-beam spectrophotometer, operable continuously at 290 mμ, is fitted with a silica flow-through cell of dimensions similar to those in Fig. 2A. The spectrophotometer should be operated in a slightly tilted position to allow ready escape from the cell of any small air bubble that may elude the "debubbler-T" piece.

Reagents. *Phosphate buffer*, 0.25*M*, pH 7.6.

Xanthine oxidase. Dilute commercially available enzyme with cold distilled water to an activity of 0.022 International Units/ml. The solution should be chilled in an ice bath. It should be free from activity toward guanine, inosine, or inosine 5′-monophosphate under the conditions of assay (*cf.* Jones *et al.*, 1964).

Preparation of extracts. Tissue is extracted with perchloric acid and neutralized with potassium hydroxide as described by Jones *et al.* (1964).

Operation of assembly. Initially, water is fed into all lines other than the air line. The "debubbler-T" position is then adjusted for proper functioning if necessary (it is sometimes advantageous to "pinch" the line between the "T" and the flow-through cell temporarily to initiate the correct streaming). Buffer solution is then introduced.

"Blank" values are then determined, as optical-density change, on duplicated cups in the sampler, which holds a sequence such as 4 standards: 1 water, 6 extracts; 1 water, 6 extracts; 1 water, 6 extracts; 1 water,— — repeating, at a sampling rate of 40/hr. The reading for the second of each pair of samples is noted (this eliminates any possible error resulting from sudden transitions from high to low concentrations, or vice versa, in the train). Water values provide the baseline for evaluation of the "blanks." Usually, this baseline is steady; but any minor variations in the running of the assembly can be compensated for by reference to it.

The sample line is then washed out with water, enzyme is introduced, and the concentration of uric acid in the reference cell (Fig. 2B) is increased to give a high transmission reading. The sequence of extracts, water, and standards is now resampled.

Hypoxanthine concentrations in extracts are evaluated against standards after deduction of relevant blanks, with reference to the water base as above.

RESULTS AND DISCUSSION

Fig. 4 compares hypoxanthine concentrations in extracts of cod (*Gadus callarias*) muscle of various degrees of freshness, as determined by automated and manual xanthine oxidase assays. There was little scatter about a median line. As measured by the manual procedure, concentrations averaged some 2 μg/ml higher, irrespective of concentrations. The addition of this factor would be necessary for any quality appraisal on the freshest fish (Jones *et al.*, 1964; Spinelli *et al.*, 1964).

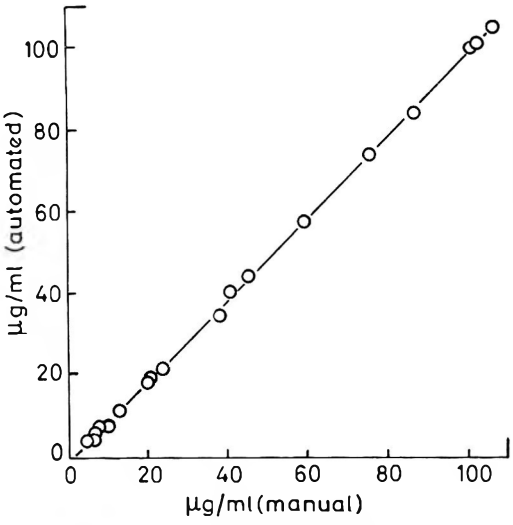

Fig. 4. Comparison of hypoxanthine concentrations in neutralized perchloric acid extracts of cod muscle determined by manual and automated procedures.

After the initial priming period, complete analyses (inclusive of "blanks," baseline waters, and standards) are obtained at the average rate of one every 8.7 min with the sampling sequence described above. Where material has a fairly uniformly high concentration of the purine, and "blank" values are known to be fairly low, it may be acceptable for some purposes to double the rate of analysis by eliminating the initial determination of "blanks." Speed of analysis may also be increased, at the loss of little accuracy, by raising sampling rates to 60/hr and by less frequent interpolation of standards into the sequence of samples.

Screening of the enzyme preparation to avoid activity toward inosine 5′-monophosphate, etc., represents a considerable saving of analysis time in that it is then not necessary to introduce a step for the removal of nucleotide (*cf.* Spinelli *et al.*, 1964) from the extract. In practice, we have found that generalized interferences by such compounds have not been a source of error in either our manual or automated analyses.

Together with the recent introduction of electronic quality assessment (Hennings, 1963) and the automation of the trimethylamine assay (Murray and Burt, 1964), it may well be that the present development can go some way toward removing the drudgery from routine objective quality control in the fish processing industry.

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The Effect of Selected Anions of Potassium Salts on the Gel Strength of Carrageenan High in the Kappa Fraction

SUMMARY

Breaking-force-deformation curves and total energy data were obtained for kappa-carrageenan gels prepared with increasing concentrations of CH_3COOK, KBr, KCl, $K_3C_6H_5O_7$, KNO_3, $K_2C_2O_4$, and K_2SO_4. Increasing the concentrations of each potassium salt, used in combination with kappa-carrageenan, produced gels which gave significantly higher breaking-force and total energy values and lower deformation readings. Moreover, significant differences for all three measures of gel strength occurred among kappa-carrageenan gels prepared with each normality of the various potassium salts; therefore the anion as well as the cation used as a gelling agent affected the characteristics of the kappa-carrageenan gels. In general the effectiveness of the potassium salts in producing gels from kappa-carrageenan, in decreasing order, were: chloride, acetate, sulfate, nitrate, bromide, citrate, and oxalate.

INTRODUCTION

Carrageenan, a naturally-occurring polysaccharide with a strong negative charge, has been used extensively in the food, drug, and cosmetic industries as a gelling, thickening, and stabilizing agent. It consists of two components, designated as kappa- and lambda-carrageenan, gelling and non-gelling respectively. Composition of commercial carrageenan preparations varies in the proportions of these two fractions, depending on the natural source and on the procedures used in processing. Because of the interaction of the gelling and thickening properties of these fractions, carrageenans can be produced with widely different properties that can be adapted to a variety of uses.

Smith and Cook (1953) fractionated the carrageenan macromolecule by selective flocculation of the kappa-carrageenan with potassium salts, using lower concentrations than required for gel formation. Although both lambda- and kappa-carrageenan contain ester sulfate groups, lambda-carrageenan contains substantially more. Lambda-carrageenan is considered to be a straight-chain polymer of 1,3-α-D-galactopyranose-4-sulfate (Glicksman, 1962). Kappa-carrageenan is composed of 3,6-anhydro-D-galactopyranose units linked through C_4 to D-galactopyranose-4-sulfate units with a glycosidic linkage at C_3. A backbone structure of alternating β-D-galactopyranose-4-sulfate and 3,6-anhydro-α-D-galactopyranose units has been proposed (Glicksman, 1962). Contradictory reports concerning the ratio of galactose to anhydrogalactose occur in the literature. Smith *et al.* (1955) stated that these components were present in nearly equal amounts. However, from X-ray diffraction studies Bayley (1955) concluded galactose and anhydrogalactose units occur in the carrageenan macromolecule in a 2:1 ratio.

Gel formation with kappa-carrageenan is a precipitation phenomenon which involves ionic bonding between certain metallic cations and the negative charge of the ester sulfate group. Calcium, potassium, ammonium, rubidium, and cesium salts have been reported to gel kappa-carrageenan sols (Smith and Cook, 1953; Stoloff, 1959). The bivalent calcium cation probably forms a cross link between carrageenan molecules, whereas the mechanism of the monovalent cations is regarded as a zipper arrangement between aligned sections of linear polymer sulfates with the ion forming the lock between alternating sulfate radicals from each section. The four monovalent ions reported to be effective gelling agents have similar ionic diameters; however, sodium and lithium, which do not have the ability to gel kappa-carrageenan sols, are thought to have ionic diameters too large to fit into the crystal lattice of the carrageenan molecule.

Gel strength increases with increasing concentrations of potassium ions until the solubility of the carrageenan is affected (Stoloff, 1959). Although potassium chloride is most commonly used as a gelling agent (Baker, 1949, 1954; Campbell, 1962; Standard Brands, 1960), other potassium salts may also be used. Several patents suggest the inclusion of gums, such as locust

bean, to improve the characteristics of the carrageenan gels (Baker, 1949; Campbell, 1962; Standard Brands, 1960).

The use of different anions in association with potassium might conceivably affect the characteristics of the carrageenan gels. This study was made to determine the effect of selected anions on the gel strength of carrageenan gels prepared with potassium as the gelling cation. Since the kappa fraction is responsible for gelation, a carrageenan sample with as high a proportion of this component as possible was selected for use in the study.

EXPERIMENTAL

Lot No. A020-0586-2 kappa-carrageenan, used in this investigation, was reported by personal communication to be 72% kappa and 28% lambda when estimated in terms of Marine Colloids, Inc., true reference kappa- and lambda-carrageenan standards (Stoloff, 1963). The complete chemical analysis is included in Table 1. All the chemicals used were Fisher Certified reagents. Water used in the preparation of the gels and the preparation of all pH samples was distilled and then deionized with a Universal Model Illco-Way Ion exchange column.

One percent dispersions of carrageenan were gelled with potassium acetate, bromide, citrate, chloride, nitrate, oxalate, and sulfate, respectively, over the range 0.025–0.15*N*. Concentrations of each salt used in the study are shown in Table 2.

Table 1. Chemical analysis of kappa-carrageenan sample Lot No. A020-0586-2.

	Av. (%)	Meq/100 g	
		Cations	Anions
Moisture [a]	12.3		
Moisture [b]	11.8		
Total sulfate	25.1		
Free sulfate	0.0		0.0
Ester sulfate [c]	25.1		261.0
Ester sulfate [d]	28.7		299.0
KCl	0.17		2.0
Na^+	0.68	30	
K^+	7.64	195	
Ca^{++}	0.35	18	
Total ions		243	263 or 301 [e]
3-6-anhydro-galactose	22.4		
Galactose	41.8		

[a] Vacuum oven at 70°C.
[b] Vacuum drying over P_2O_5 at 72°C in an Abderholden pistol.
[c] Calculated by difference.
[d] Determined by cetylpyridinium chloride method.
[e] Uses ester sulfate calculated by CPC method.

The carrageenan solutions were prepared by mixing the carrageenan, salt, and water for 1 min at 80°C in a Waring blender. This solution then stood for 1 min allowing entrapped air to escape, and was then poured into three 50-ml aluminum dishes. The 53-mm outside diameter of the dishes gave ample surface area for the plunger to puncture the gel without the readings being influenced by side effects. The 27-mm height of these containers had been extended an additional 15 mm with adhesive tape. After the gels were poured, they were held in a constant temperature bath at 25±0.2°C for 2 hr prior to testing. Pretesting established that gel strength did not change with continued holding up to 6 hr after the gel had formed. Gelling time varies, but all samples gelled within 1 hr.

Immediately preceding testing, the adhesive tape extending above the container top was carefully removed; with a fine wire the gel was cut level with the top edge of the dish, and this top section was slipped off. Each gel was carefully loosened from the container with a ¼-inch stainless-steel spatula blade and then inverted on a level aluminum sheet. This sheet containing the gel sample was then placed on the movable platform of a Baldwin-Emery SR-4 testing machine, Model FGT, which moved the gel upward at a constant speed of 0.51 cm per minute against a polished stainless steel plunger.

The plunger (Fig. 1) was firmly attached to a special transducer which adapted the load capacity of the testing machine to a range of 50–200 g full scale. Figs. 2 and 3 are respectively schematic and electrical diagrams of this adaptation. The instrument was calibrated by detaching the transducer, inverting it, and placing known weights on the cantilever beam. The calibration curve is given in Fig. 4.

The testing machine, which is normally used for the determination of breaking strength of metals, was chosen for this study since none of the commonly used instruments in the food industry record continuous time-force curves for extremely low force readings. Since completion of the study, modifications of the Allo-Kramer shear-press have made possible its use for determination of gel strength (Kramer, 1964).

Breaking force and deformation were recorded for each gel, and the area under each of these curves was measured with a planimeter for calculating the total energy required to break each gel. At least nine breaking-force-deformation curves were obtained for each normality of every salt. For this study breaking-force was defined as the maximum force required for the plunger to rupture the gel, deformation as the distance the plunger

Table 2. Summary of treatment means [a] and significant differences for breaking-force, deformation, and total energy data.

Gel strength measure	Normality of salt	Potassium salt used							Significant differences [b]	
		Acetate	Bromide	Chloride	Citrate	Nitrate	Oxalate	Sulfate	At 1%	Additional at 5%
Breaking-force (g)	0.025	52.1	43.6	47.8		43.8	32.4	44.5	Ac > Cl > SO_4 NO_3 Br > C_2O_4	None
	0.038				46.5				None	None
	0.05	91.4	74.9	94.7		77.5	70.0	86.8	Cl Ac SO_4 > NO_3 Br C_2O_4	NO_3 > C_2O_4
	0.075	133.9	100.4	145.6	94.3	109.6			Cl Ac > NO_3 Br Cit	Cl > Ac; NO_3 > Br
	0.10		[c]	173.6			141.6	151.3	Cl > SO_4 C_2O_4	SO_4 > C_2O_4
	0.15			[c]	[c]		[c]	[c]		
Deformation (cm)	0.025	0.84	0.83	0.84		0.85	0.87	0.87	C_2O_4 SO_4 NO_3 Ac Cl Br	C_2O_4 > NO_3 SO_4 > NO_3 & AC
	0.038				0.83				None	None
	0.05	0.68	0.69	0.70		0.71	0.75	0.76	SO_4 C_2O_4 > NO_3 Cl Br Ac	NO_3 > Ac
	0.075	0.65	0.66	0.70	0.69	0.68			None	Cl Cit NO_3 Br Ac
	0.10		[c]	0.68			0.67	0.73	SO_4 > Cl C_2O_4	None
	0.15			[c]	[c]		[c]	[c]		
Total energy	0.025	15.5	13.0	13.9		12.7	9.6	13.5	Ac > Cl SO_4 Br NO_3 > C_2O_4	Cl > NO_3
	0.038				14.0				None	None
	0.05	25.0	20.7	25.5		21.3	21.0	24.8	Cl Ac SO_4 > NO_3 Br C_2O_4	None
	0.075	33.3	28.4	42.7	26.0	32.0			Cl > Ac NO_3 Br Cit	Ac > Br
	0.10		[c]	49.4			40.1	43.9	None	Cl SO_4 C_2O_4
	0.15			[c]	[c]		[c]	[c]		

[a] Based on at least 9 values.
[b] Anions connected by one continuous line did not produce significance differences.
[c] Preparations attempted but samples jelled too quickly to mold suitable gels.

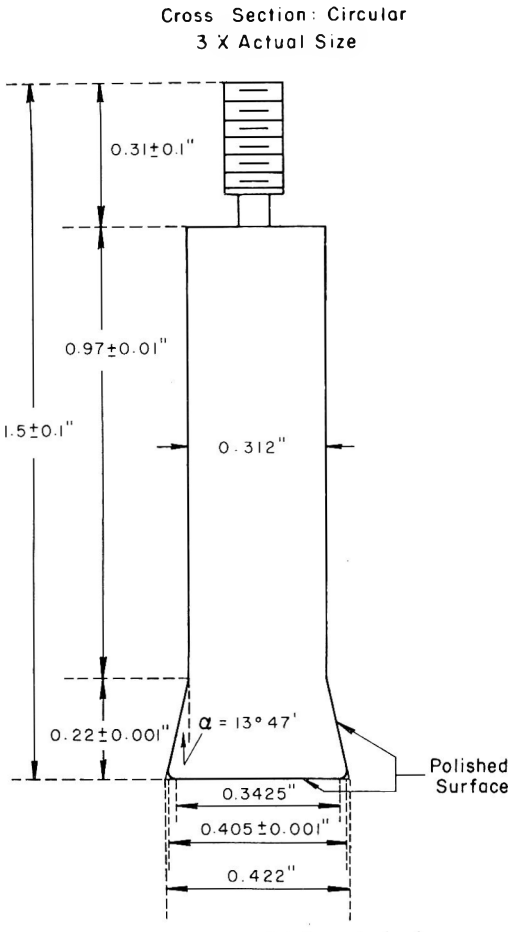

Fig. 1. Diagram of stainless-steel plunger.

depressed the surface of the gel from the point of contact until the gel ruptured, and total energy as the area-under-the-curve.

After the breaking-force-deformation data had been collected, a 15-g portion of each gel was thoroughly blended with 60 ml of water. The pH of these slurries and of the water to prepare the gels was determined with a Zeromatic pH meter.

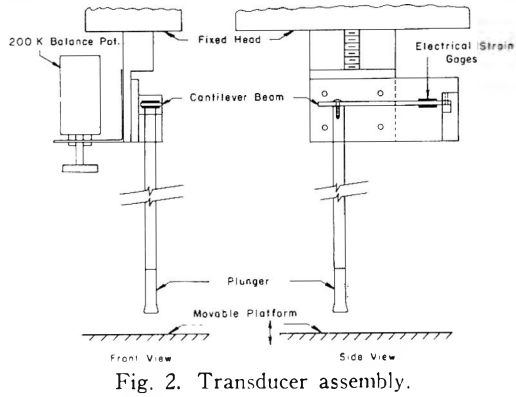

Fig. 2. Transducer assembly.

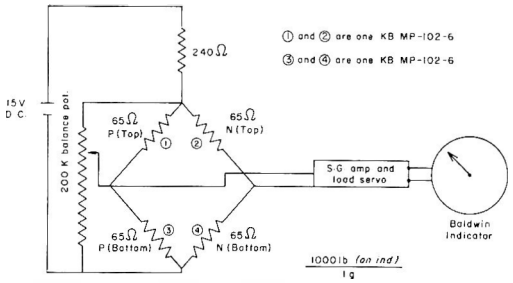

Fig. 3. Electrical bridge used with transducer assembly.

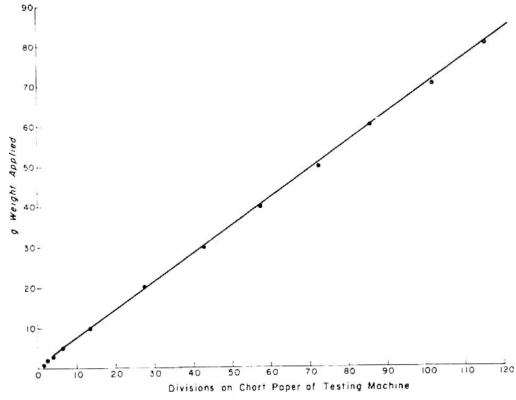

Fig. 4. Calibration curve for modification of Baldwin Emery SR-4 testing machine.

RESULTS AND DISCUSSION

Table 2 summarizes data concerning the breaking-force, deformation, and total energy required to break the gels. It was not possible to obtain these data for gels prepared with 0.10N potassium bromide, 0.15N potassium citrate, chloride, oxalate, and sulfate, because they gelled too quickly for suitable sample molds to be prepared.

Increasing concentrations of each potassium salt produced kappa-carrageenan gels with significantly higher ($p < 0.01$) breaking-force and total energy and smaller deformation. However, one exception occurred among the significant differences for deformation values; no significant difference was found between the deformation data for the gels prepared with 0.05 and 0.07*N* potassium chloride.

Although increasing the concentration of each potassium salt produced stronger kappa-carrageenan gels, the degree of increase differed between salts. Ratios of increase of breaking-force and total energy data for gels prepared with potassium acetate, bromide, or

nitrate were quite similar and were substantially less than double or triple with doubled or tripled concentrations of salt. Similar ratios were obtained for kappa-carrageenan gels prepared with potassium chloride or sulfate, and both showed double and triple values for breaking-force when double and triple concentrations of salt were used. However, the linearity of increase dropped off at the 0.10*N* level of salt. Kappa-carrageenan with potassium oxalate produced gels which gave data with higher ratios of breaking-force and total energy than double or quadruple when the concentration of salt was doubled or quadrupled.

The increased strength of the kappa-carrageenan gels prepared with increasing concentration of potassium salts agreed with results reported by Stoloff (1959). It is possible that the smaller increase in gel strength for samples prepared with 0.10*N* potassium chloride or sulfate was caused by a partial reduction in solubility of the carrageenan in solutions of this high salt concentration. Stoloff (1959) reported that hydration of carrageenan is prevented by 0.25*M* potassium chloride and that, in general, any additional solute will retard the hydration.

Variations also occurred among breaking-force data, deformation, and total energy data for kappa-carrageenan gels prepared with one normality of potassium. Therefore, it appeared that the particular anion influenced not only the degree of increase in gel strength but also the breaking-force, deformation, and total energy values for gels prepared with each concentration of potassium salts.

Analysis of variance indicates highly significant differences among the strength of gels prepared with the different potassium salts at each level of concentration. The Studentized range test (Duncan, 1955) was used to identify significant differences revealed by the analyses of variance. These significant differences for all three measures of gel strength are also given in Table 2.

Breaking-force data and total energy values show the same trends for the effects of the various anions although fewer significant differences occur among the total energy values than among breaking-force data. Among all salts used at 0.025*N*, potassium acetate produced the strongest gels. However, at all other concentrations, potassium chloride, the most commonly used gelling salt in the food industry, produced gels stronger than or as strong as gels from the other potassium salts. Potassium oxalate produced the weakest gel structure; potassium sulfate, nitrate, and bromide produced carrageenan gels of intermediate strength.

In general, the weaker gels had higher deformation values than did the stronger gels. One marked exception was noted: the weak potassium bromide gels had lower deformation values.

The ratios of the breaking-force data for carrageenan gels with potassium chloride to carrageenan gels with the other potassium salts for each concentration used (Table 3) give a relative indication of the respective strengths obtained and may be an indication of the degree of increase required to obtain gels of comparable strength. With the exceptions of the reverse effect of acetate and chloride at the 0.025*N* concentration, the effectiveness of the following potassium salts in producing carrageenan gels, in decreasing order, were: chloride, acetate, sulfate, nitrate, bromide, citrate, and oxalate.

Correlation coefficients were calculated for all three measures of gel strength. Highly significant correlation coefficients existed between breaking-force and total energy data for all four normalities of potassium salts used (Table 4). No highly significant corre-

Table 3. Breaking force ratios of potassium chloride gels to gels made with other potassium salts at each concentration.

Normality of salt	Potassium salt used						
	Acetate	Bromide	Chloride	Citrate	Nitrate	Oxalate	Sulfate
0.025	1.09	0.91	1.00		0.92	0.68	0.93
0 05	0.97	0.79	1.00		0.82	0.74	0.92
0.075	0.92	0.69	1.00	0.65	0.75		
0.10			1.00			0.82	0.87

Table 4. Correlation coefficients for the three measures of gel strength.

Normality	Breaking force vs. total energy	Breaking force vs. deformation	Total energy vs. deformation
0.025	+ 0.95 **	− 0.04	− 0.20
0.05	+ 0.93 **	− 0.05	+ 0.15
0.075	+ 0.87 **	+ 0.15	+ 0.37 *
0.10	+ 0.87 **	+ 0.13	+ 0.38 *

** Significant at 1% level of probability.
* Significant at 5% level of probability.

lations were found between deformation data and breaking-force or total energy data.

The pH of the solutions, the dissociation constants of the respective acids, the ionic diameters of the salts, the solubility of the salts, and the relative acid strength of each salt were examined to determine whether or not any relation existed between two or more of these factors which might explain the effects of the anions of the potassium salts on the characteristics of the kappa-carrageenan gels investigated. There is no apparent relation of any of these factors which explains the results of this study. However, if the potassium does fit into the crystal lattice of the carrageenan to cause gelation, then the anions must be affecting the availability of the potassium. Further investigations of the actual structure of the carrageenan gels are necessary to elucidate the role of the anion on the gel strength of kappa-carrageenan gels.

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Meat Flavor. I. Fractionation of Water-Soluble Flavor Precursors of Beef

SUMMARY

Water-soluble extracts of beef contain flavor precursors of compounds responsible for a characteristic broiled steak aroma. Dialysis separated the extract into a nondialyzable high-molecular-weight fraction having a brothy odor during boiling, and a low-molecular-weight fraction having a broiled-steak odor when pyrolyzed. The fraction responsible for the broiled-steak aroma was separated on ion-exchange resins. An aromatic fraction was obtained containing amino acids and peptides. Although sugars have been found in previously described flavor fractions, no sugars were found in this material.

Precursors of meat flavor are among the water-soluble components of raw-meat extracts. Although a number of studies have attempted to elucidate development of the characteristic odors, neither specific precursors nor aroma components have yet been identified completely.

In a series of investigations involving commercial meat extracts (heated concentrates of water extracts) as well as cold-water extracts of beef, Wood and Bender (1957) and Wood (1956, 1961) identified an extensive series of nitrogenous compounds (purines, amino acids, and peptides), carbohydrates (free sugars and sugar phosphates), and organic acids. Wood (1961) postulated that the development of the brown color and flavor in the extracts is due to a Maillard reaction between the amino acids and sugars. In model experiments, browning and a "meat extract" flavor were obtained by heating glucose with a synthetic extract, while browning and a bitter flavor resulted from the interaction of glucose with various amino acids at elevated temperatures. Ribose and ribose-5-phosphate were implicated in the browning reaction in aged or fresh ox-muscle extracts. Certain of the meat flavor and aroma precursors are low-molecular-weight compounds and can be separated by dialysis through cellulose membranes. Hornstein and Crowe (1960) found that the diffusate yielded a fraction similar to one obtained from the whole extract when both were pyrolyzed under vacuum. When free amino acids were separated from the diffusate and heated, unpleasant odors were obtained. They concluded that free amino acids, as such, were not flavor precursors. Recently, Macy *et al.* (1964a,b) determined quantitatively that heating the diffusate of a dialyzed water extract resulted in large losses of amino acids and peptides. A corresponding loss was noted among the carbohydrate components, particularly in the glucose content.

All of these studies were done with water extracts or with diffusates of dialyzed extracts. These extracts contained a large number of components, among which it is difficult, if not impossible, to determine the compounds and reactions actually involved in producing aroma. Batzer *et al.* (1960, 1962), however, fractionated a water extract, carrying it through two dialysis steps, Sephadex gel filtration, and separation by ion-exchange chromatography. They isolated a glycoprotein fraction which, when heated with glucose and inosinic acid, resulted in a meaty aroma. A mixture of glucose, inosine, phosphate, and the amino acid components of the glycoprotein also yielded meaty odors after heating. Those authors (1962) stated that ". . . only certain of the amino acids in the glycoprotein are necessary precursors of meat flavor." The particular amino acids and related compounds involved were not determined.

In the work described herein the fractionation procedure of Batzer *et al.* (1960, 1962) was carried out to obtain more information about the separated fractions. Additional data, varying significantly from published results, have provided further information about the composition of flavor precursors.

MATERIALS AND PROCEDURES

Meat fractionation. Bottom round of beef, purchased in a commercial meat market, was cut into pieces and the fat removed as completely as pos-

sible. The meat was ground; suspended in a volume of cold, distilled water approximately equal to 1.5 times the weight of the meat; and extracted for 18 hr, with continuous stirring, at 4°C. The red-colored extract was clarified by filtration through cheesecloth and then by centrifugation to remove finely suspended particles. The extract was fractionated by the method of Batzer *et al.* (1960, 1962) with some modifications. A cellulose dialysis tube containing 300 ml of distilled water was suspended in the meat extract and maintained with agitation for 18 hr at 4°C. (The contents were then designated extract A.) The slightly colored contents of the dialysis tubing were transferred to Visking No-Jax sausage casing and dialyzed for 18 hr at 4°C against 500 ml of distilled water. The dialysate remaining in the sausage casing is designated *Aa*, and the diffusate as *Ab*. The solutions were freeze-dried. The resultant yellow powders were very hygroscopic, so they were dissolved in small quantities of water and stored at −18°C until used. Fraction *Aa* was separated on a column of Sephadex G-25, the elution being carried out with distilled water. One hundred 3-ml fractions were collected, and the absorbance at 290 mμ was determined with a Bausch and Lomb Spectronic 505 spectrophotometer. Three fractions, designated Aa_1, Aa_2, and Aa_3, were obtained.

Fraction *Ab* was further separated on a column of Dowex 50W [H^+] ×8, 50–100-mesh. Following addition of *Ab* to the column, 150 ml of water was passed through the column followed by 150 ml of *2M* NH_4OH. The eluates were collected in 3-ml fractions, and absorbance was determined at 290 mμ. Three fractions, designated Ab_1, Ab_x, and Ab_2, were obtained.

Identification of fraction components. *Carbohydrates.* The presence of carbohydrate in the various fractions was determined qualitatively by a simple spot test with 0.5% anthrone in glacial acetic acid (3 drops of sample, 3 of anthrone solution, and 9 of conc. H_2SO_4). Specific identification was made with paper chromatography on Whatman No. 1 paper using *n*-butanol–acetic acid–water (65:15:25) solvent. Silver nitrate and aniline hydrogen phthalate (Block *et al.*, 1952) were used as general sprays for the carbohydrates. A modified Dische spray reagent (0.5% cysteine HCl in conc. H_2SO_4; heat 5–10 min at 85°) was used to identify deoxyribose.

Acids. Organic acids were determined by paper chromatography with the same solvent as above. Bromphenol blue was used as the spray.

Purine compounds. Purines, nucleosides, and nucleotides were separated on paper chromatograms with the butanol–acetic acid–water solvent and identified by their absorption in ultraviolet light at 253 mμ. These compounds also gave blue spots when treated with the silver nitrate-bromphenol blue (SB) reagent of Wood (1955). Further identification of the bases and nucleic acid derivatives was made by their absorption spectra in the ultraviolet (Beaven *et al.*, 1955).

Amino acids. Amino nitrogen was located qualitatively in the various fractions by spotting on Whatman No. 1 paper, spraying with 0.2% ninhydrin in alcohol, and heating for 5 min at 90°. Amino acids were identified by two-dimensional paper chromatography according to the method of Rockland and Underwood (1954) and Underwood and Rockland (1954). Further identification and quantitative data were obtained by analyzing the amino acids before and after hydrolysis for 18 hr in constant-boiling HCl at 110°. The method of Spackman *et al.* (1958) was followed, using a 150-cm column for the neutral and acidic amino acids and a 15-cm column for the basic amino acids. The analyses were done on the automatic analyzer of the Phoenix Precision Instrument Co.

Aroma. Fractionation of precursors of meat aroma was followed by the development of aroma in 0.1-ml samples heated to dryness in small beakers on a hot plate. The temperature at the bottom of the beaker was 150–160°C, as determined with a chromel-alumel thermocouple. Odor identification was made by laboratory personnel, then confirmed by other members of the staff.

RESULTS AND DISCUSSION

Water extraction of ground beef left a grayish-white fibrous residue. When the residue was formed into a hamburger patty and grilled, no browning or other color change occurred, and there was no development of the characteristic meaty aroma and flavor. The hamburger was tough and tasteless. All of the aroma-producing components were in the water extract. On heating of the solution, a profile of odors was noted beginning with a serumy or blood-like odor in the cold extract. As the extract boiled, accompanied by a flocculent precipitation of protein, the aroma formed was described as brothy and buttery, or oleaginous. After complete evaporation of the water, the remaining constituents underwent pyrolysis at higher temperatures. Browning occurred with the development of various odors, culminating in an aroma resembling that of broiling steak.

The scheme for the dialysis and fractionation of the extract is shown in Fig. 1. The first dialysis into the cellulose tubing sepa-

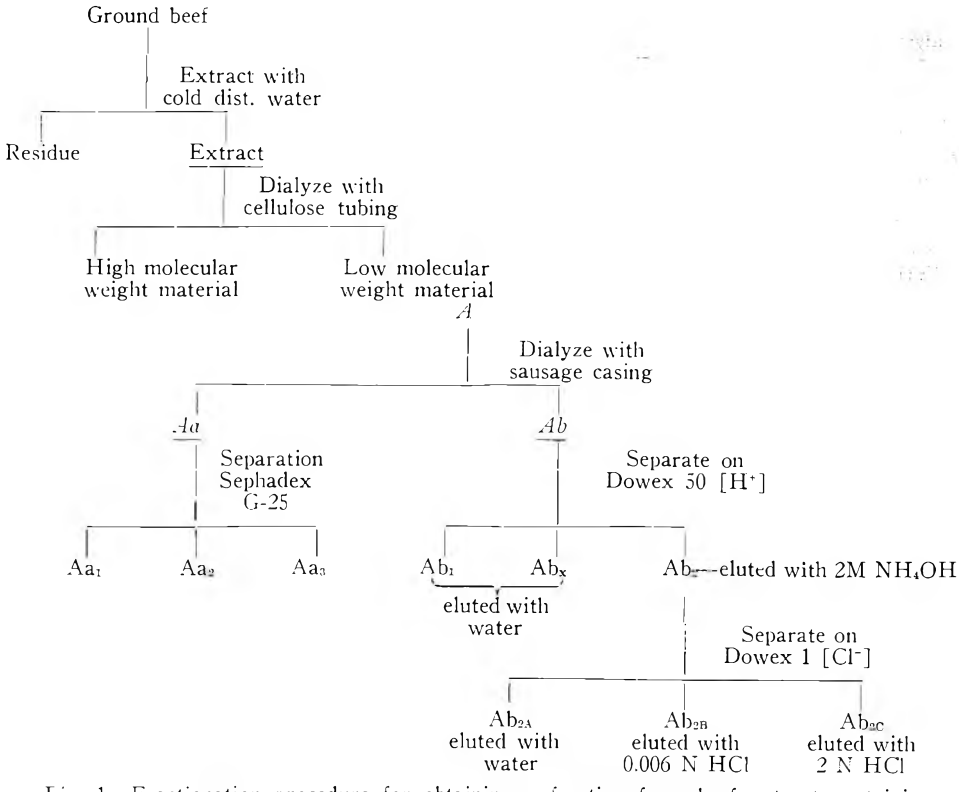

Fig. 1. Fractionation procedure for obtaining a fraction from beef extract containing a meaty aroma.

rated the low-molecular-weight compounds—such as sugars, acids, amino acids, small peptides, and salts—from the proteins. This dialysis step also resulted in separation of the aroma components of the extract. In a thoroughly dialyzed preparation the dialysate retained the substances responsible for the brothy or buttery aroma while the solution was boiling but did not have a characteristic meaty aroma on pyrolysis. The diffusate lacked the full aroma in the boiling stage, achieving at best a mild brothy odor; however, when pyrolysis occurred, a steak-like aroma could be discerned. In all further fractionations using the diffusate fraction of the extract, only the meaty aroma on pyrolysis was observed.

The dialysis of Fraction *A* was carried out in Visking sausage casing. Batzer *et al.* (1960) reported a further fractionation as a result of this procedure. In Table 1, however, the data for *Aa* and *Ab* (columns 1 and 5) indicate that there was essentially an equal distribution of the sack contents across the membrane. It should be noted that the previous authors stated that only particular rolls of Visking sausage casing were able to effect the separation, as a result of small differences in the pore size of the membrane. Reproduction of the reported separation under normal laboratory conditions may be difficult to attain.

The composition of the three fractions obtained by fractionating *Aa* on Sephadex G-25 is shown in Table 1, columns 2, 3, and 4. Since neither Aa_1 nor Aa_3 produced a meaty odor on heating, they were not investigated further; Aa_2 (which did have the characteristic aroma) was freeze-dried. The material was hygroscopic and began to absorb moisture immediately, so the powder was dissolved in a small quantity of water. The solution began to darken, and within a few hours was dark brown. The solution developed an aroma reminiscent of commercial meat extract.

Fraction *Ab* was separated on Dowex 50 [H^+]. Examinations of the fractions

Table 1. Composition of fractions obtained by the separation of meat extract through dialysis and Sephadex or ion-exchange chromatography.

	1 Aa	2 Aa_1	3 Aa_2	4 Aa_3	5 Ab	6 Ab_1	7 Ab_x	8 Ab_2	9 Ab_{2A}	10 Ab_{2B}	11 Ab_{2C}
Meat aroma	+[a]	±	+	−	+	−	−	+	+	+	−
Anthrone test	+	−	+	−	+	+	−	−	−	−	−
Glucose	+		+		+	+	−	−			
Deoxyribose	+		+		+	+	−	−			
Acids	+		+		+	+	−	−	−	−	−
Succinic	+		+		+	+	−	−			
Unknown	+		−		+	−	−	−			
Ninhydrin test	+	+	+	+	+	±	+	+	+	+	−
Alanine[b]	+		+		+	−	−	+	+	+	
Arginine	+		+		+	−	−	+	+	−	
Glutamic	+		+		+	−	−	+	+	−	
Glutamine	+		+		+	−	−	+	−	+	
Glycine	+		+		+	±	+	+	−	−	
Hydroxyproline	+		+		+	−	−	−	−	−	
Leucine (iso)	+		+		+	±		+	+	−	
Methionine	+		+		+	−	−	+	+	−	
Hypoxanthine	+		+		+	−	−	+	−	+	+
Inosine	−		±		±	−	−	±			
Abs. max., mμ	248 400		248 406		248	253	246	248			
Unknown UV absorb. spot	−		−		+	−	−				

[a] + = present; − = absent.
[b] The amino acids were determined by paper chromatography.

at 290 mμ showed three peaks: Ab_1 and Ab_x had been eluted from the resin with water, while fraction Ab_2 came off with $2N$ NH_4OH; Ab_1 contained carbohydrates while fractions Ab_x and Ab_2 were ninhydrin-positive. After freeze-drying, the meaty aroma was found only in pyrolyzed solutions of Ab_2. The composition of the three fractions is shown in Table 1, columns 6, 7, and 8. Glucose, deoxyribose, succinic acid, and traces of leucine and glycine were found in Ab_1, and only glycine in Ab_x. Fraction Ab_2 contained only amino acids, hypoxanthine and a trace of inosine. Large sheets of Whatman No. 1 filter paper (18¼ × 22½ inches) were streaked heavily with a solution of Ab_2 and chromatographed overnight. Strips were cut from the edge of the sheet and sprayed with ninhydrin reagent to locate the amino acids. Another set of strips was observed under ultraviolet light, and absorbing spots marked; the strips were then sprayed with the SB reagent. Amino acid areas were cut out, eluted with water, and rechromatographed for identification after concentration. Two of the spots that reacted with the ninhydrin spray could not be identified. Two areas absorbed in ultraviolet light. The R_f of one spot coincided with a hypoxanthine standard that was run concomitantly on the filter paper. Both the hypoxanthine standard and its corresponding component in Ab_2 reacted positively with the SB reagent, but the second spot absorbing in the ultraviolet light did not react with this reagent. The R_f value of this second spot did not coincide with those of inosine or inosinic acid. The ultraviolet-absorbing areas corresponding to hypoxanthine standard and the two components of Ab_2 were eluted and their ultraviolet absorbance spectra determined. One component of Ab_2 was identified as hypoxanthine, by its spectral characteristics and its behavior on paper chromatography. The second component, although absorbing in uv at 253 mμ, did not show a characteristic spectrum and was not identified.

Hypoxanthine and inosine have been demonstrated in meat extract fractions, and

a requirement for inosinic acid (or inosine plus phosphate) has been reported for the development of meat aroma (Batzer *et al.*, 1962). However, in the fractions obtained in these experiments, only hypoxanthine, with occasional traces of inosine, was found. The hypoxanthine results from the degradation of inosine and inosinic acid, either enzymatically during the storage of the meat or chemically during the procedures to which the extract was subjected. Hypoxanthine itself has not been implicated in flavor development. Fraction Ab_2 consisted of amino acids and the purine; it also had a meaty aroma.

Attempts failed to collect sufficient pure fractions of Ab_2 by paper chromatography to allow the study of aroma development on pyrolysis. Inconclusive odors were obtained with the various components. Ab_2 was then separated on an ion-exchange resin column, Dowex 1 [Cl^-], and eluted with water, 0.006N HCl, and then 2N HCl. The composition of these fractions is shown in Table 1, columns 9, 10, 11. The water eluate (Ab_{2A}) contained alanine, glutamine, glycine, arginine, methionine, and leucine. The dilute HCl fraction (Ab_{2B}) contained hypoxanthine, alanine, and glutamine. The more concentrated HCl eluate (Ab_{2C}) contained material that absorbed in the ultraviolet light but did not react with the SB reagent. On pyrolysis of these solutions, both the water and dilute acid eluates (Ab_{2A} and Ab_{2B}) had an aroma described as meat-like. Recombining the two fractions did not enhance the odor.

In identifying the amino acid components of the various fractions, shown in Table 1, two-dimensional paper chromatography was used and only qualitative results were obtained. The ion-exchange procedure of Spackman *et al.* (1958) was then used for two purposes: 1) to obtain quantitative data about the amino acid components; and 2) to determine the absence or presence of peptides and proteins by acid hydrolysis of the material. Analyses were made before and after hydrolysis of Fraction Ab_2. The data are shown in Table 2. It was immediately apparent that, instead of the seven amino acids consistently found in this fraction, or the eight amino acids found as a maximum in the meat extract, by paper chromatography, eighteen components were identifiable by the ion-exchange procedure. The discrepancy in the number of amino acids found cannot be explained. Further study with paper chromatography, using different solvents and larger sheets of paper to improve resolution, have not led to the separation of more than eight amino acids by these techniques.

Table 2. The amino acid composition of meat extract fraction Ab_2[a]

	μmoles/mg		
	Unhydrolyzed	Hydrolyzed	Δ
Aspartic acid	.0156	.0933	.0777
Threonine	.0483	.070	.0217
Serine	.186	.105	−.081
Glutamic acid	.082	.357	.275
Proline	.0184	.071	.0526
Glycine	.101	.412	.311
Alanine	.300	.319	.019
Valine	.0695	.104	.0345
Methionine	.0277	.0319	.0042
Isoleucine	.047	.054	.007
Leucine	.092	.107	.015
Tyrosine	.0352	.053	.0178
Phenylalanine	.0375	.048	.0105
Unknown No. 1	[b]	.....	.....
Lysine	[b]	.110	.....
Unknown No. 2	[b]	.....	.....
Histidine	[b]	1.141	.....
NH_3	.437	1.38	.943
Arginine	.0388	.0495	.0107
Tryptophan	.0076	.....	−.0076

[a] Determined with the automatic amino acid analyzer.
[b] Unknown No. 1 and lysine were unresolved; Unknown No. 2 and histidine were unresolved.

The concentration of lysine and histidine in the unhydrolyzed material could not be calculated, because these peaks were not resolved from the peaks of two unidentified components. Histidine, which appeared to be present in low concentration, was unresolved from a peak which may contain the greatest concentration of amino acid material.

After hydrolysis, significant concentration changes occurred in a number of amino acids. Histidine, while not completely resolved from the unknown material, increased considerably in concentration, whereas its associated unresolved peak decreased in its concentration. Lysine and its unresolved unknown peak separated after hydrolysis; the unknown is still not identified. It was not

possible to determine what changes occurred in the lysine component as a result of the treatment. Aspartic acid, glutamic acid, and glycine increased in concentration after hydrolysis. Serine concentration decreased after hydrolysis. It has been shown that asparagine and glutamine can be eluted with serine (Canfield and Anfinsen, 1963). After acid treatment these dibasic amino acids are converted to aspartic acid and glutamic acid, respectively. This would account for the apparent loss of serine and some of the increase of aspartic and glutamic acids.

The fraction Ab_2 appears to contain peptides or low-molecular-weight water-soluble protein in addition to free amino acids.

The interaction of amino acids with glucose and ribose (or ribose-5-phosphate) by way of the Maillard reaction has been implicated in the development of the meat aroma (Wood, 1961; Hornstein and Crowe, 1964). Batzer *et al.* (1962) isolated a glycoprotein containing glucose that appeared to be part of the aroma-precursor complex. The aromatic fraction Ab_2, described above, however, had no sugar detectable by paper chromatography. The material was further tested for glucose by a specific glucose oxidase procedure (White, 1964) before and after hydrolysis. Fraction Ab_2 was hydrolyzed in 2-ml volumes of 1*N* H_2SO_4, refluxing in a boiling-water bath for 3½ hr. The $SO_4^=$ was precipitated as $BaSO_4$ and the supernatant liquid evaporated to dryness. The hydrolyzed material was redissolved in 2 ml of water for assaying. No glucose was detected in 10-mg samples of Ab_2, either before or after hydrolysis, by this procedure, which is sensitive to 1–5 μg of sugar.

The presence of glucose, and other sugars, was also investigated by the gas-liquid chromatographic method of Sweeley *et al.* (1963). The trimethylsilyl derivatives of the components of Ab_2 were prepared and compared with similar derivatives of glucose, ribose, and deoxyribose. The one small peak present in the Ab_2 GLC chromatogram did not correspond to the peaks for the sugar standards. The peak was not further identified, but since the reagent reacts with other compounds containing −OH groups and amino acids the component is not necessarily a carbohydrate. Thus, on the basis of several types of assays, a fraction of water extract of meat which retains a meat-like aroma does not contain glucose or ribose sugars associated with the Maillard reaction.

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Reactivity of Malonaldehyde with Food Constituents

SUMMARY

Malonaldehyde and/or substances closely resembling it occur in foods as decomposition products of oxidizing polyunsaturated fatty acids. The reaction of malonaldehyde with various food constituents was studied. In the presence of water, malonaldehyde exists mainly as its nonvolatile enolate anion. As such, it can react with amino acids, proteins, glycogen, and other food constituents to form products in which the malonaldehyde exists in bound form. Aqueous malonaldehyde can be converted to its volatile isomer by acidification only but acid and heat are necessary to release malonaldehyde from its bound state in proteins. These combined observations throw light on problems connected with the quantitative recovery of 2-thiobarbituric-acid-reactive substances (TBRS) in moist foods. Proteins prepared from frozen tuna fish contained appreciable amount of bound TBRS.

INTRODUCTION

Malonaldehyde (MA) and/or substances closely resembling it occur in foods as end-products of oxidation of polyunsaturated fatty acids. 2-thiobarbituric acid (TBA) has been a useful reagent for measuring this product. A recent statistical analysis (Zipser *et al.*, 1964) indicated that rancidity of animal foods correlated better (Spearman $rs = 0.92$) with the content of 2-thiobarbituric-acid-reactive substances (TBRS) than it did with peroxide determinations. However, the exact relationship between the actual total content of TBRS and the amount determined by the TBA reaction (Tarladgis *et al.*, 1960) is still not completely understood.

When the present investigation of the reaction of MA with food constituents was initiated, it was generally believed that MA was itself the TBRS. Several observations which will be discussed below now suggest that MA is only one of the TBRS. Nevertheless the TBRS from oxidized lipids and foods have many properties very similar to those of MA, and hence the reactions of MA may be very closely related to those of TBRS.

This investigation was designed to obtain information on the reactions of MA with food constituents. MA was reacted with aldolase, as an example of a water-soluble protein; myosin, as an example of a salt-soluble protein; a water-soluble protein-free fraction from fish muscle; water-soluble protein fractions from fish and chicken muscle; and glycogen. In addition, the presence of protein-bound TBRS in frozen fish muscle was demonstrated.

EXPERIMENTAL

Reagents and material. TBA (Sigma), glycogen (Fisher), and soluble starch (Allied Chemical & Dye), were used without further purification. MA in aqueous solution was prepared from its bis-(diethyl acetal) (Kay Fries) by acid hydrolysis (Kwon and Watts, 1963). Dialysis tubing (La-Pine) was heated with 1% $NaHCO_3$ solution on a steam bath for 2 hr and rinsed thoroughly with glass-distilled water to remove impurities.

Aldolase and myosin were prepared from yellowfin tuna (*Neothunnus macropterus*) white muscle following the methods described by Kwon and Olcott (1965) and Connell (1960), respectively.

Extracts of tuna and chicken white muscle were prepared as follows: 10 g of muscle were homogenized in a Waring blender for 1 min with 100 ml of water and allowed to stand 24 hr at 6°. The mixtures were then centrifuged and extensively dialyzed against glass-distilled water at 6°. The products contained mixtures of the water-soluble proteins of muscle.

A water-soluble protein-free fraction was prepared from tuna white muscle as follows: 10 g of muscle were homogenized in a Waring blender for 1 min with 30 ml of water. After centrifugation, the supernatant was heated in a boiling water bath for 15 min, then filtered through Whatman No. 42 filter paper.

Ultraviolet absorbance of MA at different pH levels. Aliquots of $2.3 \times 10^{-5} M$ MA solution were mixed with equal volumes of $0.05 M$ citrate-phosphate buffers, pH 2.6 to 6.8, and absorbances were recorded at 245 and 267 mμ, with a Cary recording spectrophotometer, model 15.

Reaction conditions. Equal volumes of MA (1×10^{-3} to $2 \times 10^{-3} M$) and of protein (1–10 mg/ml) or other solutions were mixed and incubated at 30 or 6° at neutral pH. At intervals, the

reaction mixtures were dialyzed for at least 72 hr against glass-distilled water at 6°. The water was replaced each 24 hr. This procedure removes free MA (Kwon *et al.*, 1965). Since it was not applicable to the study of interaction of MA with the water-soluble protein-free fraction, this reaction was followed by changes in the absorption spectra of the reaction mixture.

Protein, glycogen, and MA were determined by biuret (Layne, 1957), anthrone (Morris, 1948), and TBA tests, respectively.

Isolation of TBRS-protein products formed in situ. Frozen tuna red muscle, which had been held 8 months at −18°, was used to isolate the TBRS-protein products formed *in situ*. The water-soluble protein fraction, prepared as described above, was dialyzed until TBRS could no longer be detected in the external solution. Following the procedure of Whitaker (1963), a column (2 × 98 cm) was prepared from Sephadex G-100 (beaded form, Lot No. 103B-1140, Sigma) which had been equilibrated with 0.01*M* sodium acetate buffer containing 0.1*M* NaCl ($\mu = 0.11$), pH 6.0. The tuna protein fraction was dialyzed against the acetate buffer and 2 ml of the dialysate (10 mg protein/ml) were placed on the column. Protein was eluted by the addition of more of the same buffer at a rate of 15 ml/hr (Spinco Accu-flo pump) at 4°. The absorbance of each of the 3 ml fractions was measured at 280 and 406 mμ, and at 532 mμ after the TBA reaction.

RESULTS AND DISCUSSION

Interaction of MA with water. MA occurs mainly as its enolic tautomer in aqueous solution. Although it is known that the ultraviolet spectrum is pH-dependent (Mashio and Kimura, 1960; Kwon and Watts, 1963; Saunders and May, 1963), precise data between pH 2.5 and 7, where the absorbance changes gradually, are lacking. Such data were collected in the present study and are shown in Fig. 1. Dissociation of the enolic aldehyde starts at pH 2.8 and is complete at pH 6.5. Kwon and Watts (1964) showed the effect of pH on the recovery of MA by a distillation procedure (Tarladgis *et al.*, 1960). The shape of the recovery curve was similar to that of the absorbance curve at 245 mμ in Fig. 1. This correlation therefore is confirmation that MA exists completely as a volatile species only below pH 2.8. Volatility is attributable to intramolecular hydrogen bond formation. Under acidic conditions, 65% (Kwon and Watts, 1964) or 68% (Tarladgis *et al.*, 1960) of the total

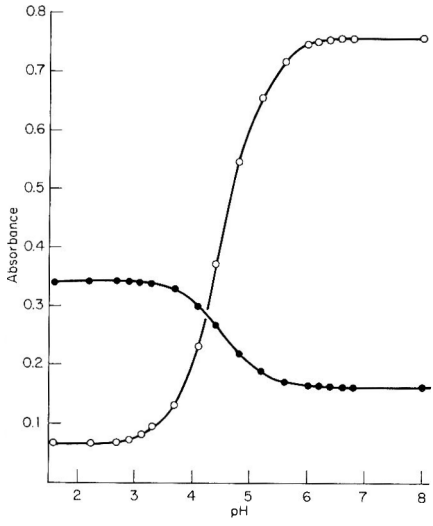

Fig. 1. Effect of pH on the ultraviolet absorbance of MA. Final concentration of aldehyde in aqueous solution, $2.3 \times 10^{-5}M$. Absorbance at 245 mμ, closed circles; at 267 mμ, open circles.

aldehyde was recovered in 50 ml of steam distillate from 100 ml of aqueous MA solutions. On the other hand, above pH 6.5, no volatilization of the aldehyde occurred. At this pH, the aldehyde is totally dissociated into its anion, as shown by the absorbances at 267 mμ (Fig. 1).

It is possible that the anion is prevented from vaporization by intermolecular hydrogen bonding and hydration of the center carbon atom through electrostatic forces. In moist food systems at neutral pH, substances such as MA would be present as hydrated anions which are not volatile. They would therefore accumulate and be available for further interaction with other food constituents.

Interaction of MA with proteins. The water-soluble protein fraction (1 mg protein/ml) prepared from chicken muscle was incubated with an equal volume of 2×10^{-3} *M* MA at pH 7.1 and 6° for 15 days. After dialysis, the soluble product contained 0.2 μg MA per mg protein (Table 1). Under similar conditions, the water-soluble protein fraction (1 mg protein/ml) prepared from tuna muscle yielded a product which contained 2.6 μg MA per mg protein at 25° and 1.3 μg MA per mg protein at 6° after 7 days incubation at pH 6.5. Yellowish precipitates obtained in both cases were also

Table 1. Relative reactivity of malonaldehyde toward different food constituents.

Reactant	Amt. of malonaldehyde per mg protein in reaction mixtures (mg)	Incubation temp. (°C)	Incubation pH	Incubation time (days)	Bound malonaldehyde (μg per mg reactant)
Water-soluble protein fraction (chicken)	140	6	7.1	15	0.2
Water-soluble protein fraction (tuna)	140	25	6.5	7	2.6
Water-soluble protein fraction (tuna)	140	6	6.5	7	1.3
Aldolase (tuna)	15	6	6.6	19	1.2
Myosin (tuna)	15	6	7.0	19	0.3
Glycogen	...	30	7.0	4	2.6

highly TBA-reaction positive, indicating that MA was bound to the precipitate. The ultraviolet absorption spectra of the soluble complexes showed shifts in the maxima from 279 or 280 to 282 mμ. This was also obtained by the reaction of MA with bovine serum albumin (Kwon *et al.*, 1965).

The rates of reaction of MA with tuna aldolase and myosin were observed at 6° and at neutral pH (Fig. 2). The reaction mixture of MA and aldolase was dialyzed against glass-distilled water; that of MA and myosin was dialyzed against 0.04*M* sodium pyrophosphate buffer, pH 7, at 6°. Bound MA per mg of protein increased as incubation time increased. Bound aldehyde per mg

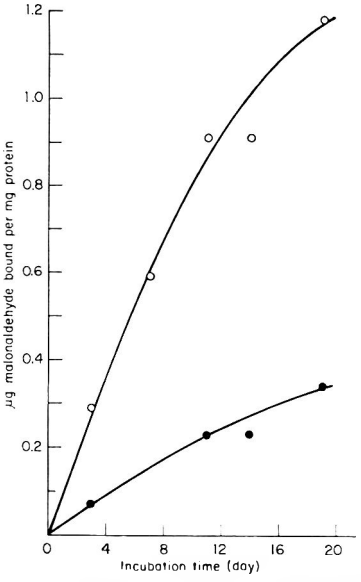

Fig. 2. The rate of reaction between MA and proteins at 6°. Closed circles, myosin at pH 7; open circles, aldolase at pH 6.6.

aldolase was about 4 times more than that with myosin. During incubation at 6°, the reaction mixture of MA and myosin remained clear for 19 days, whereas the control myosin solution became turbid after 5 days. With aldolase, the reaction mixture became turbid after 5 days and after 14 days a precipitate had formed.

Interaction of MA with water-soluble protein-free fractions. The absorption spectrum of the total water-soluble fraction (about 10 mg of protein per ml) has an absorption maximum around 247 mμ with a shoulder around 280 mμ (Fig. 3A). The protein-free solution showed a broader absorption maximum at about 247 mμ (Fig. 3A). A mixture of equal volumes of the protein-free fraction and $10^{-3}M$ MA solution at pH 6.6 initially had the characteristic MA carbonyl absorption at 350 mμ; however, this absorbance almost completely disappeared after 24 hr at room temperature (Fig. 3B). Also, with time the absorbance at about 250 mμ after 1:50 dilution decreased gradually and a new absorption peak was formed around 277 mμ. The spectrum after 47 hr at room temperature is shown with a control solution in Fig. 3C. The disappearance of absorption at 350 mμ indicated that the carbonyl group of MA was involved in the reaction (Kwon *et al.*, 1965). A component of the fraction having an absorption maximum at 250 mμ had reacted with MA to produce a new molecular species absorbing at around 277 mμ. The compounds involved are not yet identified.

Interaction of MA with glycogen. Saturated glycogen and starch solutions were al-

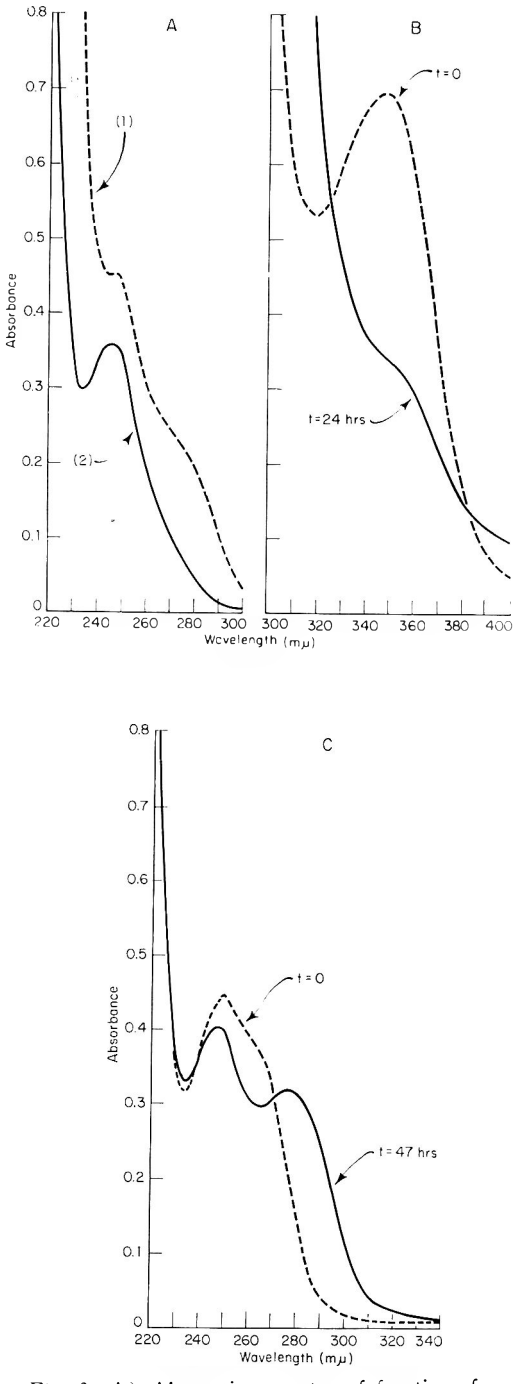

Fig. 3. A) Absorption spectra of fractions from tuna white muscle. 1) Total water-soluble fraction; 2) protein-free fraction; B) reduction of absorption of MA at 350 mμ during incubation of a mixture of protein-free muscle extract and MA; C) absorption spectra of a mixture of a protein-free muscle extract and MA before and after incubation. The spectra were obtained after dilution of the reaction mixtures 1:50.

lowed to react with equal volumes of $10^{-3}M$ MA for 4 days at 30°. After extensive dialysis, only the glycogen showed a positive TBA reaction. The amount of MA bound per mg of glycogen was comparable to that bound by proteins (Table 1). The difference of reactivity between glycogen and starch suggests that this binding requires a certain type of organized structure.

Isolation of TBRS-protein products formed in situ. The concentration of TBRS in the original water extract of frozen tuna red muscle was the equivalent of $5.6 \times 10^{-4}M$ MA; after extensive dialysis this was reduced to 0.4×10^{-4}M (equivalent to 0.5 μg MA per mg dry matter in the extract). The water-insoluble fraction of the red muscle was collected by centrifugation and extensively washed with water until no free TBRS was present. This preparation contained 2.1 μg MA equivalent per mg of dry matter, a much greater amount than was present in the water-soluble fraction. This finding coincides with the observation mentioned above, that water-soluble proteins were precipitated on extensive reaction with MA.

The dialyzed water-soluble extract was chromatographed on a Sephadex G-100 column. The absorbance at 280 mμ of the fractions coincided with the amount of TBRS (absorbance at 532 mμ) and furthermore coincided with the Soret absorbance for the main peak (Fig. 4). This showed that the water-soluble fraction consisted mainly of metmyoglobin and small amounts of other proteins, all of which contained bound aldehyde. The amount of bound TBRS was calculated to be about 0.4 μg MA equivalent per mg protein for metmyoglobin and about 0.15 μg per mg protein for the other protein peaks. The recovery of bound TBRS from the column was 100%; however, the recoveries of the total absorbances at 280 and 406 mμ were about 80%, possibly due to the changes with time of the TBRS-protein absorption spectra.

CONCLUSION

Free MA is readily converted into its volatile form by acidification only, whereas both acidification and heating are necessary to volatilize bound MA from its reaction

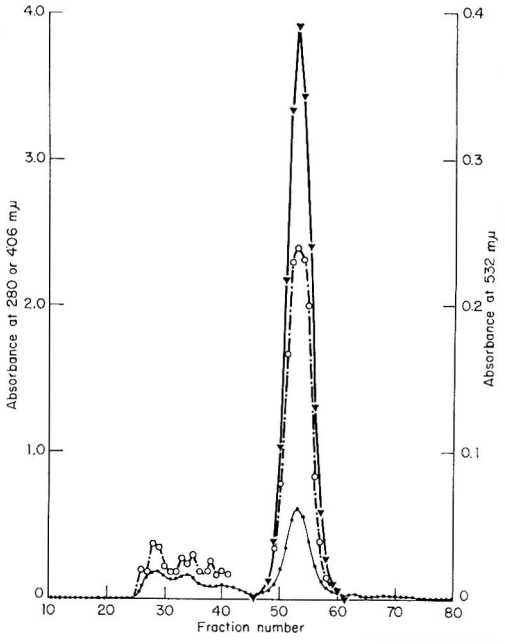

Fig. 4. Column chromatography of the TBRS-protein reaction products extracted from frozen tuna red muscle (2 × 98-cm Sephadex G-100 column at 4°). Absorbance at 280 mμ, closed circles; at 406 mμ, arrows; at 532 mμ, open circles.

products with proteins (Kwon *et al.*, 1965). Maximum recovery of TBRS in the steam distillate from oxidized food was obtained only at acidic pH (Tarladgis *et al.*, 1960). Tarladgis *et al.* (1964) prepared simple aqueous extracts from foods for the TBA test. Such a procedure may be useful only in the initial stages of lipid oxidation when insoluble TBRS-protein products are unlikely to occur. From our experiments, water extraction cannot be considered satisfactory, since aqueous extracts contain only the free TBRS and water-soluble products, but would not include TBRS bound to water-insoluble proteins and other food constituents.

In the pH range of moist foods, especially animal tissues, free TBRS produced from lipid oxidation would not volatilize. Probably hydration of the TBRS alone, as with MA anion, may lead to the accumulation of the compound at low concentration. When the TBRS concentration in foods increases with advanced lipid oxidation, the anion may react further with amino acids, peptides, proteins, glycogen and other food constituents. If the TBRS concentration is further increased, binding sites of the proteins may be saturated by the compound. Eventually cross-linking with other protein molecules could occur with resultant loss of solubility and recoverable TBRS. The ability of the compound to combine with proteins even at −18°, as indicated by the *in situ* formation of TBRS-protein products in frozen tuna, accounts for the fate of some of the compound in the moist food systems.

The absolute magnitude of the TBA number produced during oxidative rancidity of a foodstuff depends upon the composition of the lipid contained therein. For the proper use of this index of rancidity one must take into account the reactivity of TBRS with the food constituents enumerated above and the conditions necessary for its quantitative recovery. Indeed, the accumulation of the compound through its interaction with food constituents makes this test more desirable than others in assessing rancidity in foods.

The above discussion is predicated on the supposition that the reactions of the TBRS are similar to those of MA. However several lines of evidence suggest that the two are not the same. Saslaw and Waravdekar (1965) present evidence from thin-layer chromatography studies of extracts of irradiated fatty acids that none of the TBRS was MA. These and our own observations suggest strongly that the prevailing concept that MA is the sole end product of lipid oxidation needs careful re-evaluation.

Nevertheless MA and TBRS are alike in being water-soluble, dialyzable, TBA-reactive, able to react with proteins, and having a pH-dependent volatility. Studies on the nature of the TBRS are in progress.

With respect to the final evaluation of TBRS as an index of rancidity in foods, the possibility that it may be formed from an unknown precursor by acidic steam distillation, or that it may be formed by some hitherto unrecognized biochemical mechanism, unrelated to oxidation of unsaturated fats, must be taken into account.

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Steam-Volatile Components from Freeze-Dried Beef Stored at Room Temperature

SUMMARY

Volatiles obtained from freeze-dried beef by distillation with steam at ordinary pressure aided by a stream of nitrogen were collected in cooling traps. An attempt to classify the volatile components and to follow the changes in the spectrum of volatiles was carried out with both gas-liquid chromatography and chemical analysis. Some of the components resolved by gas-liquid chromatography were tentatively identified as acetaldehyde, propanal, pentanal, hexanal, acetone, methyl-mercaptan, and methyl disulfide. Hydrogen sulfide was identified by chemical analysis.

INTRODUCTION

The volatile carbonyl compounds which are believed to be associated with food flavor have been studied by converting carbonyls to their dinitrophenylhydrazone derivatives. The carbonyls have been identified by paper chromatography and melting-point determination of the isolated compounds. This technique has been employed by several investigators (Burnett *et al.*, 1955; Hornstein *et al.*, 1960; Yueh and Strong, 1960) to study the volatiles of meat.

Yueh and Strong (1960) studied the volatile fraction from fresh lean beef cooked in boiling water. Hornstein *et al.* (1960) isolated acetone, propanal, acetaldehyde, hexanal, and 2,4-dienals from a cold-water extract of lean beef.

Increasing interest has been shown in the use of gas-liquid chromatography in the separation and identification of volatile compounds contributing to the flavor of foods. In the present work, studies were carried out to isolate and identify some of the volatile compounds developed in freeze-dried beef during storage in the raw state.

EXPERIMENTAL

The semimembranosus, semitendinosus, and biceps femoris muscles of Shorthorn steers were closely trimmed of external fat and sliced into pieces of 1 × 1 × ½ in. The meat was then quick-frozen at −40°. The frozen meat was dried for 24 hr in a Stokes freeze-dryer, Model 2003-F2, at 35° and 0.15 mm Hg. The moisture content of the dried meat was 2.85%.

Approximately 120 g of the dried meat were packed in No. 2 lacquered cans. The cans were hermetically sealed. The end seams were protected by a continuous film of Wood's metal to ensure gas tightness of the containers. The cans were stored for six months at room temperature in a nitrogen atmosphere containing 0 and 2.0% oxygen. The cans were punctured and placed in a chamber, re-evacuated, and flushed with required gas as described in a previous paper (El-Gharbawi and Dugan, 1965).

Fig. 1 shows the equipment used for isolation and trapping of the volatile compounds of freeze-dried beef. Three hundred grams of ground dry material and 1500 ml of distilled water were placed in a 3-L two-neck round-bottom Pyrex flask fitted with an adapter that mounted an inlet glass tubing extending to about 1 inch from the bottom of the flask, whereby highly purified nitrogen could be bubbled through the slurry. The other neck was fitted with a reflux condenser. Heading from the condenser were a series of cooled traps *d* or gas-washing tubes *e* containing the following reagents: lead acetate, 4% mercuric chloride solution, 4% mercuric cyanide, acidic hydroxylamine, and basic hydroxylamine. The distributor *f* permitted the diversion of volatiles separately or concurrently into the gas-washing tubes containing reagents for functional groups. Also, the traps and the washing bottles were arranged so as to collect the total volatiles or, in succession, the volatiles remaining after certain types of compounds were removed. The traps were immersed in Dewar flasks containing methanol-dry ice or liquid nitrogen. Nitrogen gas was passed through the system before cooling with liquid nitrogen, to avoid condensation of oxygen, which may block the system.

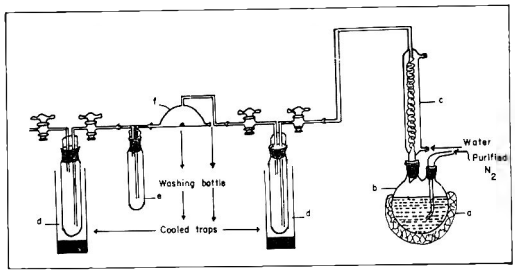

Fig. 1. The set-up used for isolation and trapping the flavor components of freeze-dried raw beef. a) heating mantle; b) round-bottom two-neck flask; c) condenser; d) Dewar flask; e) washing bottle; f) distributor.

The collection of volatiles was accomplished while the meat slurry was heated at boiling for 4 hr. At the end of the process, nitrogen gas flow was stopped, and the washing reagents were removed for further chemical analysis. The cold traps were disconnected, and the stopcocks were closed and placed aside in the cooling reagent for subsequent fractionation of the volatile components by gas-liquid chromatography.

A gas-liquid chromatograph (Model 500, F and M Scientific Co., Avondale, Pa.) with flame ionization detector was used for separation and detection of the volatile compounds obtained from freeze-dried raw beef. A ¼-inch-OD × 13-ft coiled copper column packed with 15% by weight Apiezon L grease adsorbed on the 60–80-mesh Chromosorb W was used. The following operating conditions were used during these analyses: column T, 100°C; injection T, 225°C; carrier gas, helium; carrier gas flow rate, 100 ml/min; hydrogen flow rate, 35 ml/min; air flow rate, 250 ml/min; attenuator setting, 16; sensitivity 1×10^{-6} amps.

Each cold trap was removed from the cooling reagent and warmed up to room temperature, and a few crystals of anhydrous sodium sulfate were introduced into it. A sample of 1–2 ml of trapped volatiles was withdrawn with a gas-tight syringe and injected into the gas chromatograph column. Five rinsings of the syringe with trapped volatiles were necessary to obtain reproducible results. At least three runs were carried out for analysis of the components from each trap.

The peaks resolved by gas chromatography were tentatively identified by retention times for each unknown peak compared with the retention time resulting from the gas chromatographic analysis of known substances. Identification of the peaks as groups was also attempted by using the selective qualitative reagent technique suggested by Bassette *et al.* (1962). Selective qualitative reagents were used to eliminate or retain only certain classes of compounds, and thus the peaks could be classified according to functional groups. Peaks corresponding to carbonyls were eliminated when the steam-volatile components were bubbled through a tube containing acidic hydroxylamine prior to collecting them in the cooled trap. Esters as well as carbonyls were absorbed by basic hydroxylamine. Hydrogen sulfide was absorbed by lead acetate. Mercuric cyanide was employed to react with mercaptans, and mercuric chloride was used for mono- or disulfides (Challenger, 1959). Portions of the precipitate from the mercuric cyanide trap were treated with 4*N* HCl to detect the presence of H_2S and/or mercaptans. Portions of the precipitates from the mercuric chloride absorption trap were treated with either 4*N* HCl or 10% NaOH to detect the presence of disulfide and monosulfides, respectively.

RESULTS AND DISCUSSION

At least 15 components were resolved by gas-liquid chromatography when the steam volatiles of freeze-dried raw beef which had been stored under nitrogen at −40° were injected directly into the fractionation column. Tentative identification was made of some of those peaks. Acetaldehyde, propanal, pentanal, hexanal, acetone, and methyl disulfide were identified, as shown in Fig. 2. Selective qualitative reagents were used to eliminate certain classes of compounds, and thus they could be classified into groups. Both acidic and basic hydroxylamine reagents were needed to establish that esters were not among the components of volatiles from freeze-dried beef as detected by gas chromatography. All peaks resolved by gas chromatography except the second and third either were removed or decreased in size when the volatiles were bubbled through acidic or basic hydroxylamine.

Hydrogen sulfide is not detected by gas-liquid chromatography when the hydrogen flame detector is used. Chemical analysis was carried out for hydrogen sulfide as well as other sulfur compounds which might be present. A black precipitate was obtained in the tubes containing lead acetate, thus indicating the presence of H_2S. On the other hand, a white precipitate formed in mercuric chloride solution was an indication of the presence of mono- or disulfide among the volatile compounds obtained by steam distillation. When mercuric cyanide

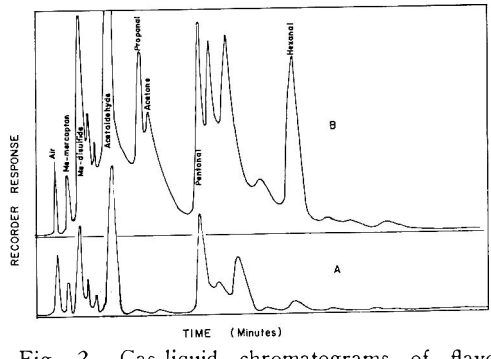

Fig. 2. Gas-liquid chromatograms of flavor volatiles from freeze-dried raw beef stored six months under purified nitrogen at −40°C (A) and room temperature (B).

was used for washing the volatiles, a yellowish-green color was produced. Challenger (1959) showed that when both hydrogen sulfide and mercaptan are present, the color of the precipitate with aqueous mercuric cyanide could be yellow, yellowish, or black, depending on the relative quantities of the two components. When a portion of the precipitate from the mercuric cyanide washing tube was warmed up with 4*N* HCl, a faint sulfurous odor was detected. This may indicate the presence of mercaptans among the steam volatiles of freeze-dried beef.

Results in Figs. 2 and 3 indicate that the volatile compounds of freeze-dried beef were increased in concentration during storage. Also, the number of the volatiles in all the samples stored under purified nitrogen or oxygen-nitrogen at room temperature was increased. Moreover, the volatile components were increased in number and concentration as the initial oxygen concentration in the storage atmosphere was increased. One of the identified compounds was *n*-hexanal which increased greatly during storage. The autoxidation products of linoleate have been reported (Gaddis *et al.*, 1961; Johnson *et al.*, 1953), and a fairly well supported mechanism has been proposed (Evans, 1961) to explain the formation of hexanal from linoleate. It has been reported recently (Buttery and Teranishi, 1963) that the rates of increase in *n*-hexanal concentration in the volatiles of food products can be correlated with susceptibility to oxidation of dehydrated products. However, it is not implied here that hexanal is the main carbonyl compound which may be used as a measure for prospective deterioration of dried meat or that it is the product mainly responsible for the characteristic odor of rancidity. Moreover, further work is needed to identify more of the volatile compounds produced during storage. The identification of such compounds qualitatively and quantitatively may lead to a better understanding of the mechanism of their formation.

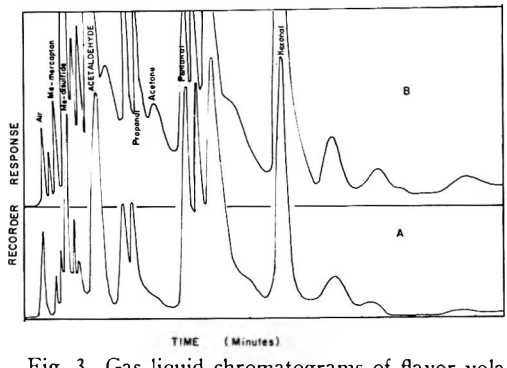

Fig. 3. Gas-liquid chromatograms of flavor volatiles from freeze-dried raw beef stored at room temperature and under an atmosphere of 2% oxygen for four (A) and six (B) months of storage.

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Stability of Nitrogenous Compounds and Lipids During Storage of Freeze-Dried Raw Beef

SUMMARY

The solubility of the nitrogenous compounds of freeze-dried raw beef was observed to change during storage in cans with controlled oxygen-nitrogen atmospheres. Generally, total soluble nitrogen, soluble protein nitrogen, and soluble nonprotein nitrogen decreased with increasing storage time and initial concentration of oxygen in the can atmosphere. Free amino groups also decreased during storage, and this change was dependent on both initial oxygen content of the storage atmosphere and time.

Dried beef tissue lipids were fractionated into neutral fat and phospholipids by silicic acid. The fatty acid composition of each fraction, determined by gas-liquid chromatography, was observed to change from oxidation during storage. Oxidation of tissue lipids seems to occur in two stages: the phospholipids are oxidized first, and the neutral fat autoxidizes later. Also, loss of the unsaturated fatty acids was more pronounced in the phospholipid fraction than in the neutral fat.

INTRODUCTION

The proteins and fats of meats are subject to deterioration during handling, processing, and storage. The changes occurring may result in toughness, discoloration, or off-flavor.

The goal of food preservation is to maintain a product as nearly like the original as possible. One of the most fully preservative processes in terms of maintaining the original characteristics of certain foodstuffs is freeze-dehydration. Although there have been improvements in the quality of freeze-dried beef as determined by taste panel, insufficient knowledge exists regarding the type or extent of changes in proteins and lipids in freeze-dried raw beef during storage.

It is generally agreed that the deteriorative reactions of dried foods are kept at a minimum if the foods are stored under nitrogen. However, some investigators have reported that in certain cases browning deterioration is greater in nitrogen or *in vacuo* than in air atmosphere (Kline *et al.*, 1951; Tarr, 1950). This study was aimed at determining changes in the nitrogenous substances and in the lipid fractions of freeze-dried raw beef during storage under different oxygen-nitrogen atmospheres.

MATERIALS AND METHODS

The semimembranosus, semitendinosus, and biceps femoris muscles of 14–16-month-old Shorthorn steers were the raw material. The carcasses were aged ten days at 34°F. The muscles were dissected from the rounds, closely trimmed of external fat, wrapped in laminated freezer paper, and chilled 30 min at 28°F. The meat was then cut into pieces of 1 × 1 × ½ in. All cutting processes were carried out in the cold room at 34°F. Under these conditions of preparation, tissue fluids were retained and no expressed fluid was noticed on the surface. The meat cubes were then quick-frozen and stored at −40°F until freeze-dried.

A Stokes freeze-dryer, Model 2003-F2, was used at a pressure of approximately 0.15 mm Hg and a plate temperature of 35°C. The moisture content of the dried meat after 24 hr was 2.85% as determined in a vacuum oven. All results in this investigation are reported on a dry basis.

The dried meat was packed in No. 2 lacquered cans which were hermetically sealed at room temperature and at atmospheric pressure. The end seams of all cans were protected by a continuous film of Wood's metal to assure gas tightness of the containers.

To obtain samples with different gas content, a drop of solder was placed on the top of each can and a small hole was punched through the solder and the lid. The cans were placed in a vacuum chamber which was then evacuated and subsequently filled with the desired gas or gas mixture. The process was repeated three times for each sample. After the last filling with the desired atmosphere, the chamber was opened and the cans were sealed while still in the presence of the desired gas. These samples were stored for six months in nitrogen atmosphere designed to contain 0, 0.1, 0.5, and 2.0% oxygen at room temperature of 75–80°F. The designated gas mixtures were obtained in cylinders from the Matheson Co.

At intervals of 4–6 weeks, samples were removed from storage and analyzed or placed at −40° with control samples until analysis could be made. Prior to analysis, the freeze-dried raw beef was passed through a Wiley Mill equipped with a 10-mesh screen. Small batches of meat were ground at a time to overcome any heating effect of the grinding operation on meat components.

Nitrogenous compounds. *Water extract.* One gram of freeze-dried beef was rehydrated for 10 min with 5.0 ml of water at room temperature. The rehydrated meat was then minced for 3 min in a Virtis 45 blender with an additional 20 ml of water. The slurry was then poured into a 50-ml centrifuge tube and centrifuged 15 min at 1500 × G. The supernatant liquid was decanted and the precipitate re-extracted with 15 ml of water by macerating with a glass rod. The mixture was centrifuged again, and the supernatant was removed, combined with the first extract, and brought to 50 ml with water.

Soluble protein and nonprotein nitrogen. One gram of ground dry beef and one gram of glass powder were placed in a mortar. Enough 0.5*N* KCl was added to wet the material thoroughly and the mixture was ground. The suspension was then transferred to a test tube with three 25-ml portions of KCl for extraction by occasional shaking. After 2 hr the extraction mixture was then centrifuged and the supernatant was removed and diluted for determination of total soluble nitrogen.

Soluble proteins were precipitated with TCA, and the soluble nonprotein nitrogen was determined in the supernatant. The soluble protein nitrogen was calculated by difference from the total soluble nitrogen of the same extract.

Nitrogen was determined by the micro-Kjeldahl method.

Amino nitrogen. Free amino nitrogen was determined by formol titration with modifications described by Regier (1956).

Lipids. *Extraction.* The extraction of tissue lipids was essentially that of Folch *et al.* (1957), using cold 2:1 chloroform-methanol (v/v). The extract was washed with cold water overnight, dried over anhydrous sodium sulfate, and then concentrated in a Rinco rotary evaporator connected to a dry-ice-cooled trap and a positive-displacement vacuum pump. The dried concentrate was designated a crude lipid extract.

Fractionation. The crude lipids were fractionated to neutral and phospholipids on a 2.5 × 60-cm silicic acid column. The crude extract, dissolved in chloroform, was applied to the top of the column. The column was then washed successively with 300 ml chloroform, 200 ml acetone, and 300 ml methanol. A flow rate of approximately 5 ml/min was obtained by applying about 3 lb of nitrogen pressure to the top of the column. Chloroform and methanol fractions were concentrated and dried by the technique described above, and were respectively designated neutral and phospholipids. This method was adapted from Kuchmak and Dugan (1963) who found that effective and complete separation into neutral and phospholipid fractions could be accomplished.

Esterification. A sample containing 200–300 mg of dried lipids was taken to dryness in a 50-ml round-bottom flask, and 25 ml of 0.5*N* alcoholic KOH were added. The mixture was refluxed for 6 hr, acidified, and extracted with petroleum ether. The petroleum ether extract was then mixed with 10 g Amberlite IRA 400 (Mallinckrodt Chemical Works). The fatty acids were converted to their methyl esters directly on the resin, using 6% anhydrous HCl in methanol, as described by Hornstein *et al.* (1960, 1961). The methyl esters were then extracted with petroleum ether, dried over anhydrous sodium sulfate, and concentrated by evaporation under nitrogen.

Gas chromatography. An aliquot containing 600–800 μg of total esters was used for gas chromatography analysis. An F and M gas chromatograph, Model 500, was used. A 5-ft coiled copper column ¼-inch in diameter was packed with 20% by weight Lac-2-R-446 on Chromosorb W, 60–80-mesh (Applied Science Lab., Inc., Pa.). The thermal conductivity detector was used for methyl ester analysis. The operating conditions used for this investigation were: column temperature, 210°; injection port temperature, 225°; detector block temperature, 225°; carrier gas, helium; carrier gas flow rate, 100 ml/min; reference gas flow rate, 180 ml/min; attenuator setting, 1.

The methyl ester peaks recorded were identified by the retention time for each unknown peak compared with the retention time resulting from gas chromatographic analysis of known methyl esters under the same operating conditions.

RESULTS AND DISCUSSION

The semimembranosus, semitendinosus, and biceps femoris muscles were chosen for this investigation because their physical and chemical properties are representative for beef muscles and have been studied extensively. The freeze-dried raw beef obtained was light pink when it came out of the freeze-dryer.

The water extract of the dried beef contains the sarcoplasmic group of proteins which consists, in addition to myoglobin and hemoglobin, of all of the enzymes of the glycolysis and citric acid cycles. Fig. 1

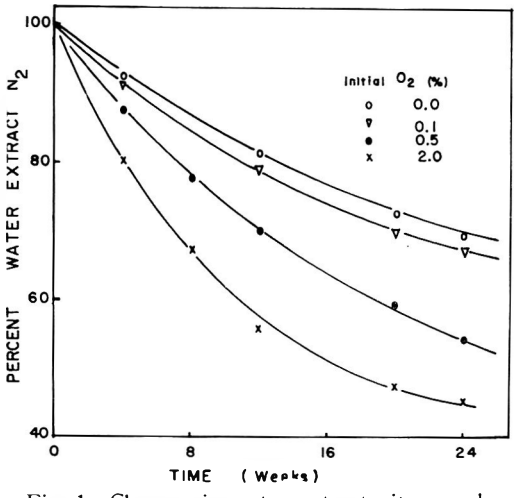

Fig. 1. Changes in water extract nitrogen during storage of freeze-dried raw beef at room temperatures and under different oxygen-nitrogen atmospheres.

shows that the total nitrogen of the water extract of the dried meat decreased as a result of two functions: time of storage and the initial concentration of oxygen in can atmospheres.

Figs. 2, 3, and 4 show the changes in solubility of total soluble nitrogen, soluble protein nitrogen, and nonprotein nitrogen of freeze-dried raw beef during storage at room temperature and under different ratios of oxygen to nitrogen in the gaseous atmosphere. Generally, the solubility of all the

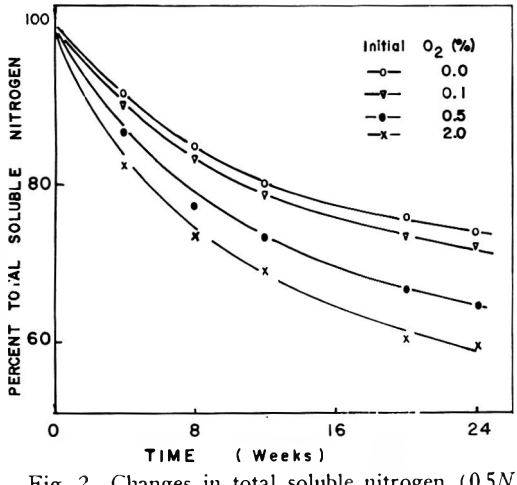

Fig. 2. Changes in total soluble nitrogen (0.5*N* KCl) during storage of freeze-dried raw beef at room temperature and under different oxygen-nitrogen atmospheres.

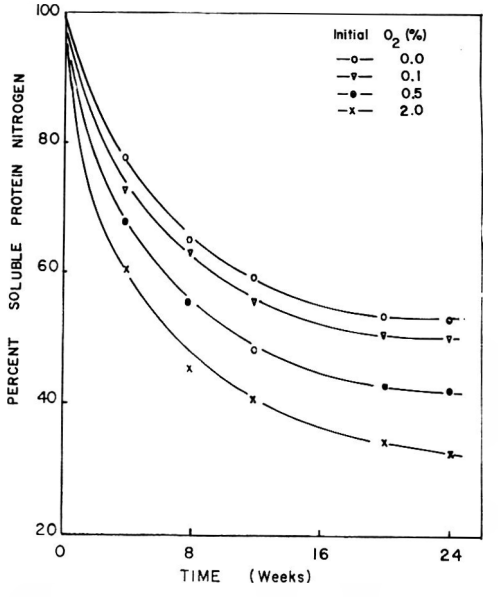

Fig. 3. Changes in soluble protein nitrogen (0.5*N* KCl) during storage of freeze-dried raw beef at room temperature and under different oxygen-nitrogen atmospheres.

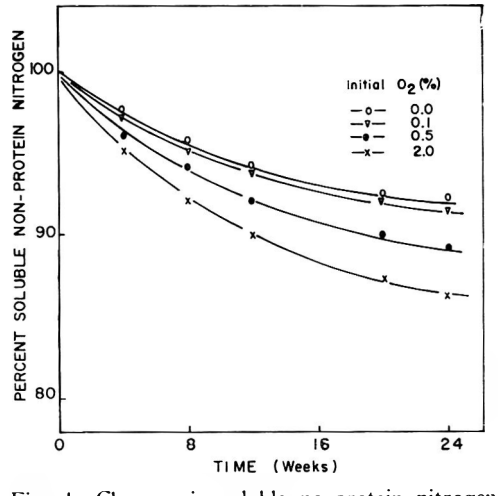

Fig. 4. Changes in soluble nonprotein nitrogen (0.5*N* KCl) during storage of freeze-dried raw beef at room temperature and under different oxygen-nitrogen atmospheres.

nitrogenous extracts decreased. Although the changes were at different rates, all were dependent on both time and concentration of oxygen in the storage atmosphere.

The decrease in the solubility of the nitrogenous compounds of freeze-dried raw beef during storage was probably due in part to denaturation. That not all proteins are de-

natured by this process is shown by the fact that some enzymes may maintain activity function in freeze-dried fresh meat and fish products. Adenosine triphosphate and lipoxidase have been identified among others (Cole and Smithies, 1960; Hunt and Matheson, 1958). Cole and Smithies (1960) have recovered practically all the original ATP-ase of beef muscle in freeze-dried products. Storage of freeze-dried beef progressively reduces its ATP-ase activity and the measurement of this has actually been proposed as a criterion of quality.

The free amino groups of freeze-dried beef decreased during storage. This change was dependent on both the initial concentration of oxygen in storage atmospheres and time, as shown in Fig. 5. Since amino

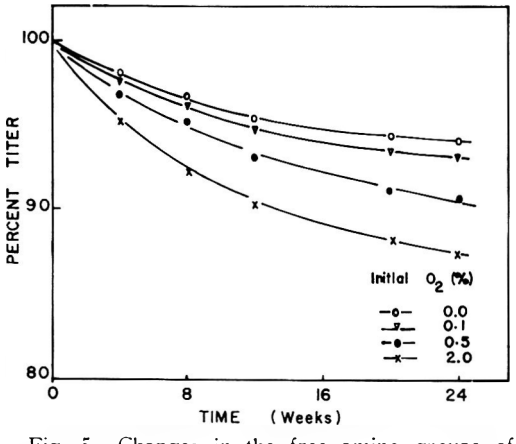

Fig. 5. Changes in the free amino groups of freeze-dried raw beef, determined by formol titration, during storage at room temperature and under different oxygen-nitrogen atmospheres.

groups are available as reactants for the browning reaction in freeze-dried beef, the decrease in free amino nitrogen during storage may be due to amine-carbonyl compound formation.

Table 1 shows the fatty acid composition of neutral and phospholipids of freeze-dried raw beef. It was found that the polyunsaturated acids make up 6.22 and 33.81% of the neutral and phospholipid fractions, respectively. However, the actual weights of polyunsaturated acids contributed by the two fractions are similar since the ratio of triglycerides to phospholipids is 5:1 in freeze-dried beef. The major quantitative

Table 1. Analysis of methyl esters of fatty acids of neutral and phospholipid fractions of freeze-dried raw beef stored at −40°F and under an atmosphere of nitrogen.[a]

Fatty acids[b]	Area percent[c] Neutral lipids	Phospholipids
12:0	0.21	0.32
14:0	3.72	2.13
14:1	2.29	1.63
16:0	22.24	17.27
16:1	6.13	4.96
18:0	16.20	12.41
18:1	42.98	27.44
18:2	3.15	15.29
18:3	0.97	2.45
20:3	.....	2.95
20:4	.....	9.47
22:3	2.10	3.65
Total saturated acids	42.37	32.13
Total monounsaturated acids	51.40	34.03
Total dienoic acids	3.15	15.29
Total trienoic acids	3.07	9.05
Total tetraenoic acids	.....	9.47
Total polyunsaturate acids	6.22	33.81

[a] 10 days' storage
[b] Indicates carbon chain length and degree of unsaturation.
[c] Area calculated by disc integrator.

difference is the large amount of arachidonic and docosatrienoic acid contributed by the phospholipid fraction and which has no counterpart in the neutral lipid fraction.

Since oxidative degradation of unsaturated fatty acids was expected to exert the most damaging effects during storage, lipid fractionation and fatty acid analysis was used to follow the change in the composition of tissue lipids. The changes in composition of the polyunsaturated acids in both neutral and phospholipids were followed during this investigation.

Figs. 6 and 7 show that the oxidation of tissue lipids seems to occur in two successive steps or at different rates for each general lipid class. The phospholipids are oxidized first, and the neutral fat is oxidized somewhat later. It was noticed that there was a very marked loss of the unsaturated fatty acids in the phospholipids fraction after a short time of storage, whereas these components disappeared more gradually in the

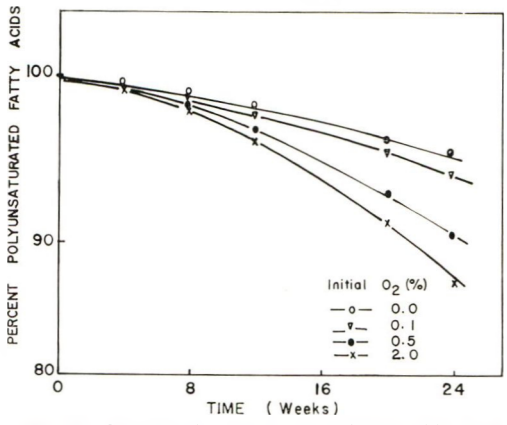

Fig. 6. Changes in unsaturated fatty acids (two or more double bonds) in the neutral lipid fractions of freeze-dried raw beef during storage at room temperature and under different oxygen-nitrogen atmospheres.

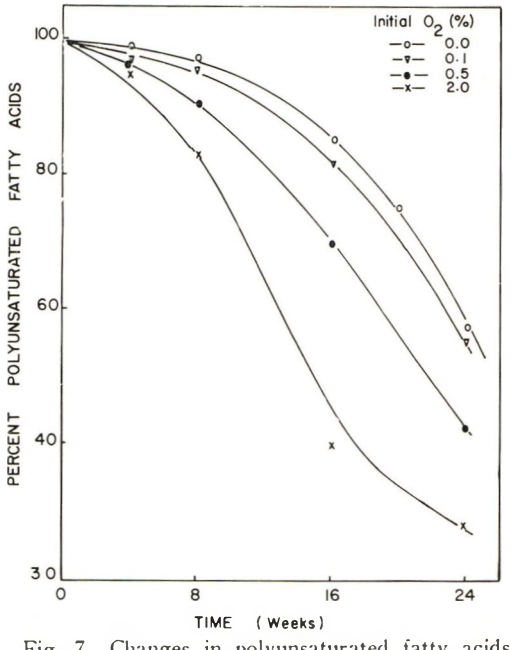

Fig. 7. Changes in polyunsaturated fatty acids in the phospholipid fractions of freeze-dried raw beef during storage at room temperature and under different oxygen-nitrogen atmospheres.

neutral lipids of dried beef. This is consistent with the observation by Younathan and Watts (1960) that the tissue lipids remaining after extraction of cooked pork with chloroform-methanol mixture were more readily oxidized than the extracted lipid.

The amount of arachidonic acid decreased from 28.12% of the polyunsaturated fatty acids in phospholipids in the sample at the beginning of storage, to 16.25% of polyunsaturated fatty acids in phospholipids in samples stored six months at room temperature under purified nitrogen. The trienoic acids obtained from the control sample and the sample stored six months at room temperature decreased from 26.76 to 16.56%, respectively. On the other hand, the changes in the unsaturated fatty acids of the neutral fractions were considerably less than those of the phospholipid fractions. The decrease in the trienoic acids was equivalent to 40% of the changes which occurred in the phospholipid fraction for the same samples stored six months under purified nitrogen.

It is not known why the phospholipids are more susceptible to oxidation than neutral fats, but a possible explanation may lie in the fact that the fatty acids of the complex lipids are more unsaturated than those of triglycerides. Also, the former may be more closely associated with the iron-containing heme compounds of meat tissue, which may act as prooxidants.

It seems clear that lipid oxidation of freeze-dried raw beef is a very real problem even if storage is under purified nitrogen. Also, some of the protein denaturation, which resulted in an appreciable decrease in solubility, was associated with a pronounced oxidation of the polyunsaturated fatty acids.

The changes noted in nitrogenous matter and lipids were apparently oxygen-dependent to varying degree. The changes observed for the samples with an atmosphere of nitrogen with no added oxygen cannot be assumed to be completely free of an oxygen effect. The chamber of the freeze-dryer was permitted to come to atmospheric pressure, at the end of each drying process, by admitting air from the room. The technique utilized to remove the air from the samples and incorporate an atmosphere of known composition undoubtedly removed the interstitial air but would probably be inadequate for complete removal of all adsorbed oxygen. For this reason, it is believed that all effects are increased to an indeterminate degree by the possibility that adsorbed oxygen may have interposed an added effect.

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Isolation of Proteins from Plant Material

SUMMARY

Proteins were extracted with a yield of up to 90% from wheat flour and finely powdered untoasted soya flour, with a 3*M* urea solution at 4°C. The urea-extracted protein contained, on hydrolysis, higher amounts of ammonia than a commercial soya protein isolate. Amino acid analysis by ion-exchange chromatography of soya proteins isolated with 3*M* urea showed that, on an ammonia-free basis, the urea-extracted proteins were comparable to a commercial soya protein isolate. Both had an amino acid composition comparable to that of a commercial solvent-extracted 50%-protein soya meal. The urea-extracted proteins from wheat flour and soya flour retained their functional properties in bread-making. Extraction with 3*M* urea was lowered in coarsely ground soya meals and was reduced substantially by heat-treatment of soya products. Dispersibility in 3*M* urea at 4°C was found to be a useful parameter to evaluate the extent of heat damage to plant proteins.

INTRODUCTION

A worldwide shortage of protein and a relative abundance of high-quality, low-cost plant proteins has increased interest in new methods of producing vegetable protein concentrates (Altschul, 1958). Cereals are among the most important foods in the world because they supply most calories per acre, can be stored for long times, and processed into a large number of foods that are acceptable throughout the world. However, cereal grains are low in protein, which, moreover, contains a poor balance of essential amino acids and is especially low in lysine. The outstanding feature of soybeans is their relatively high content of excellent-quality protein. Specifically, soya proteins serve very effectively to supplement cereal proteins properly (Hayward and Diser, 1961). Raw soya flour contains, however, certain "anti-growth" factors that have an adverse effect on utilization of proteins by animals. Proper heat-treatment destroys the "anti-growth" factors.

Recently, isolated plant proteins have come into great demand; these protein concentrates contain 90–100% protein on a dry-matter basis. They are free of color, taste, odor, and objectionable minor constituents that interfere with growth and are found to some extent in many vegetable sources of protein (Liener, 1962). Commercial isolation of soybean protein is based on extraction of dehulled and undenatured soybean flakes with water or dilute alkali solution and precipitation of the extracted protein at low pH (Smith and Wolf, 1961).

Recently, Jankiewicz and Pomeranz (1965) found it is possible to solubilize, almost quantitatively, wheat flour proteins with urea solutions.

Work reported here evaluates the possibility of isolating plant proteins by hydrogen-bond-disrupting agents such as urea. It also enumerates the factors affecting the isolation, and compares properties of the isolated proteins.

MATERIALS AND METHODS

Wheat flour. The flour used was untreated flour milled in an experimental Allis mill from a composite grist of several winter wheat varieties grown in 1963 at a number of locations throughout the Great Plains. Certain chemical and baking properties of the flour composite on a 14% moisture basis follow: ash, 0.4%; protein, 12.8%; 100 g flour gave a loaf volume of 950 cc. The baking method involved the optimum mixing time and the following "rich" formula: flour 100 g; sugar 6 g; salt 1.5 g; shortening 3 g; yeast 2 g; nonfat milk solids 4 g; 120°L malt syrup 0.25 g; water (61.7%); mixing time (3⅛ min), and potassium bromate (3 mg) as needed to produce optimum loaf volume and crumb grain. Doughs were fermented for 3 hr and proofed for 55 min at 86°F. Finney and Barmore (1945) described baking and rheological dough testing methods in greater detail.

Soya flour samples. Twenty-four samples of soya flour, obtained from four manufacturers, were chosen to represent typical products marketed for use by food and feed industries. In addition, a sample of soya flour with a protein dispersibility index 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.

Analytical methods. Moisture, ash, and protein were determined by the official AOAC (Horwitz, 1955) method. Percent nitrogen was converted to percent soya protein with the factor 6.25, and to percent wheat flour protein with the factor 5.7. Estimation of protein by the biuret method was made according to the procedure of Pinckney (1961). Orange-G binding was determined with the Udy Analyzer according to the manufacturer's instructions.

Extraction of protein. Extraction of the proteins from the composite flour or from a soya flour with a protein dispersibility index of 90%, concentration by ultrafiltration, dialysis, and fractionation were all performed at 4°C. The extractant used was 3.0*M* urea in 0.01*M* sodium pyrophosphate buffer, pH 7.0. Concentrations of urea above 3.0*M* solubilized part of the carbohydrates; below 3.0*M*, protein yields were low. Wheat flour proteins were also extracted with 0.05 acetic acid. For each extraction, a sample containing 10 g protein was homogenized in a Waring blender with 250 ml of solution. The suspension was shaken for 1 hr and centrifuged in an International Centrifuge for 25 min at 3,500 rpm. The residue was returned to the blender and the whole procedure repeated three more times. Combined centrifuged extracts were made up to 1000 ml with extractant, and the solution was allowed to stand overnight at 4°C. The extract was clarified by centrifuging for 15 min at 12,000 rpm in a Servall centrifuge and concentrated for 72 hr to about 250 ml with an ultrafiltration technique employing a device similar to that described by Siegelman and Firer (1962). The concentrated extract was dialyzed for 48 hr in a continuous procedure against about 10 L 0.005*M* sodium acetate buffer, pH 4.1. The dialyzed extract was concentrated to 100–150 ml in a small ultrafiltration unit and lyophilized. Adding 3.0*M* urea in 0.01*M* sodium pyrophosphate buffer, pH 7.0, solubilized about 90% of the protein. No additional protein was extracted from wheat flour by subsequent use of 0.05*M* acetic acid. Protein content was 103% in the wheat flour isolate, and 114% in the soya flour isolate. The abnormally high protein content probably resulted from using too high N-to-protein conversion factors and from traces of urea in the extracted, dialyzed, and lyophilized isolates.

To compare the effects of variations in chemical composition, particle size, and heat treatment on protein extraction from soya flours, a simplified urea extraction procedure was used. Relative urea dispersibility index was determined by shaking 200 mg soya flour in a stoppered 125-ml Erlenmeyer flask with 20 cc 3.0*M* urea in 0.01*M* sodium pyrophosphate buffer, pH 7.0, for 2 hr on a laboratory shaker at 4°C. The supernatants were cleared by centrifugation at 4°C for 1 hr at 5,000 rpm in a Servall angle centrifuge. Ten ml of the clarified solution was mixed with 10 ml of double-strength biuret reagent and shaken for 90 min at room temp. (about 26°C), and absorbance was measured at 550 mμ in the Beckman DU spectrophotometer.

Amino acid composition. Lyophilized soya-flour proteins were prepared for amino acid analyses by acid hydrolysis. A sample was placed in a test tube and weighed on a micro-balance. After the mouth of the tube was narrowed, 6*N* HCl was added at a concentration of approximately one ml per 10 mg protein. The contents of the tube were frozen by placing the tube in an alcohol-dry ice bath, sealed under vacuum, and hydrolyzed for 22 hr at 110°C. The hydrolyzed sample was filtered through a fritted-disc funnel. Samples were then evaporated to dryness under partial vacuum. Five ml of a 0.2*N* sodium citrate buffer, pH 2.2, were added; and the samples were stored at −30°C until analyzed. The hydrolysates were practically clear and were light tan, indicating little if any formation of humin. Amino acid analyses were made by the ion-exchange column chromatography technique of Spackman *et al.* (1958) on a Beckman amino acid analyzer, model 120. No correction was made for possible destruction of amino acid during hydrolysis, since nitrogen recovery following chromatography of hydrolysates was 100±5%.

RESULTS AND DISCUSSION

The effects of supplementing wheat flour with wheat flour proteins on bread quality are shown in Fig. 1. The urea-extracted protein increased the loaf volume to an extent comparable to that obtained with high-quality undamaged wheat protein. It also considerably improved crumb grain and texture, slightly shortened dough mixing

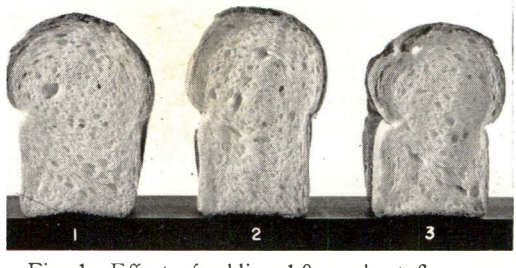

Fig. 1. Effect of adding 1.0 g wheat flour protein on bread quality: 1) control (853); 2) with urea-extracted protein (900); 3) with acetic-acid-extracted protein (880). Figures in brackets denote loaf volume in ml. Baked by the complete formula less milk solids.

time, and had no adverse effects on water absorption or rheological dough properties. The results seem to indicate that extraction with urea impaired the functional bread-making properties of wheat proteins less than did extraction with 0.05*M* acetic acid. The effects of supplementing wheat flour with soya products on bread quality are shown in Fig. 2. Again, the two urea-extracted soya proteins were at least comparable to commercially available soya flours or soya-protein isolates manufactured for use in bread-making.

Data in Table 1 compare the amino acid composition of the urea-extracted soya flour isolate with that of a commercially available soya protein isolate and that of a solvent-extracted commercial soya meal. Results are expressed as amino acid residues per 100 g protein, recovered from the column, exclusive of ammonia. Except for the two basic amino acids histidine and arginine, which are present in higher concentration in the commercial isolate, and of glutamic acid, concentrated more in urea-extracted protein, the amino acids in both isolates are almost identical. However, the urea-extracted protein hydrolysate contained more ammonia than the hydrolysate of the commercial isolate (25.4 and 19.6 g amino acid residues per 100 g protein recovered from the column). The high ammonia content of urea-extracted protein probably resulted from the presence of residual urea, despite prolonged and continuous dialysis and ultrafiltration.

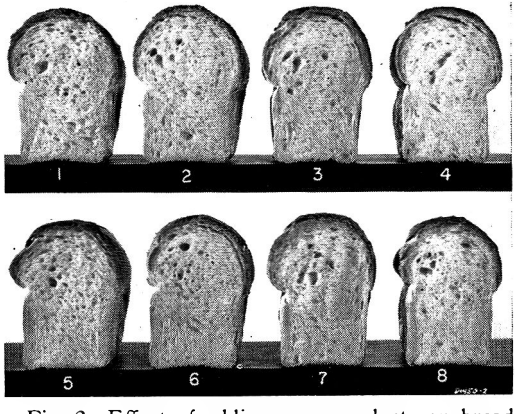

Fig. 2. Effect of adding soya products on bread quality; 1) control (853); 2) with 1 g urea-extracted isolate A (882); 3) with 1 g urea-extracted isolate B (860); 4) with 2 g commercial soya flour A (888); 5) with 2 g commercial soya flour B (833); 6) with 1 g commercial protein isolate A (843); 7) with 1 g commercial protein isolate B (840); 8) with 1 g commercial isolate C (848). Figures in parentheses denote loaf volume in ml. Baked by the complete formula less milk solids.

Table 1. Amino acid composition of soya products.

Amino acid	Amino acid residues/100 g protein[a]		
	Soybean	Commercial isolate	Urea isolate
Lysine	5.4	7.9	7.8
Histidine	2.1	3.2	2.3
Arginine	5.6	9.6	6.0
Aspartic acid	12.2	11.1	12.0
Threonine	4.4	3.2	3.5
Serine	6.6	6.0	6.1
Glutamic acid	16.9	17.9	19.7
Proline	6.1	5.7	5.7
Glycine	7.7	6.6	6.8
Alanine	6.7	5.0	5.3
Half cystine	0.6	0.7	0.9
Valine	5.6	4.9	4.9
Methionine	0.9	0.8	0.8
Isoleucine	4.7	4.3	4.4
Leucine	8.2	7.0	7.2
Tyrosine	2.3	2.4	2.5
Phenylalanine	4.1	3.9	4.2

[a] Excludes ammonia.

The effect of particle size of soya meals on protein extraction is summarized for two series of samples from two manufacturers, in Table 2. As expected, the amount of protein extracted with 3*M* urea was higher in finely powdered than in coarsely ground samples.

Table 2. Effect of particle size of defatted toasted soya meals on protein extraction by 3*M* urea.

Soya sample no.	Particle size[a]	Moisture (%)	Total Kjeldahl protein[b]	Biuret absorbance of 3*M* urea extract
1	14	8.4	51.0	0.185
2	18	8.7	51.2	0.165
3	25	8.8	51.5	0.195
4	50	9.2	51.0	0.198
5	100	5.5	52.9	0.270
1	8	8.2	52.5	0.246
2	28	7.1	52.9	0.277
3	64	7.2	54.1	0.273
4	100	6.8	52.6	0.338

[a] Products passing through given no. of mesh per inch.
[b] Nitrogen × 6.25.

Table 3. Effect of heat treatment of soya products on protein extraction by 3*M* urea and dye binding.

		Total Kjeldahl protein (%)	Biuret absorbance of 3*M* urea extract	Orange-G transmission of product
A. Samples autoclaved at 10 psi (soya flours)	1	51.9	0.455	41.00
	2	51.9	0.445	40.00
	3	51.9	0.425	40.00
	4	51.9	0.417	39.75
	5	51.9	0.388	39.50
	6	51.9	0.248	39.00
	7	51.9	0.163	37.75
	8	51.9	0.071	33.50
	9	51.9	0.065	29.25
B. Commercial samples (soya flours)	1	51.8	0.455	43.00
	2	51.3	0.371	42.75
	3	51.0	0.210	38.75
C. Commercial samples (coarse soya meals)	1	54.1	0.350	44.50
	2	52.5	0.246	43.00
	3	52.1	0.198	41.75
D. Commercial samples (soya flours)	1	52.9	0.443	43.75
	2	52.6	0.338	43.25

Table 3 shows that heat treatment of soya products substantially decreased the dispersibility of soya proteins in 3*M* urea, in samples heated in an autoclave for increasing periods, and in three series of commercially processed soya products. In each series the increasing sample number indicates progressively increased heat severity. In each case, protein dispersibility in 3*M* urea decreased much faster than Orange-G binding decreased. The latter has been proposed as an index of heat treatment of soya flours (Moran *et al.*, 1963). Dispersibility in 3*M* urea therefore seems to be a sensitive index of heat denaturation of soya products. Dispersibility in 3*M* urea could not be measured in soya flours containing 14.5–19.0% fat, as the extracts were turbid and difficult to clear. The biuret determination in such samples should be made after lipid is removed to avoid turbidity from unextracted lipid material (Torten and Whitaker, 1964). Table 4 compared the urea dispersibility of proteins from mildly heated soya flours ground to pass a 100-mesh sieve with biuret determinations of commercial soya protein isolates. The data indicate that up to 90% of the proteins are extracted from soya flour provided it has been pulverized to a fine flour and has not been denatured by heat treatment. The two desugared samples, however, show lowered extraction in 3*M* urea. The limited data seem to indicate that the proteins were denatured, as assessed by dispersibility in 3*M* urea, during the process of removing soluble sugars, nonprotein nitrogen, and other low-molecular-weight components.

Table 4. Comparison of 3*M*-urea-extracted proteins from mildly heated soya flours and from soya protein isolates.

Description	Total Kjeldahl protein (%)	Biuret absorbance of 3*M* urea extract
Soya flour	51.8	0.455
Soya flour	52.9	0.443
Desugared soya flour	65.3	0.148
Desugared soya flour	63.9	0.385
Sodium proteinate	82.3	0.585
Sodium proteinate	94.5	0.650
Isolated protein	96.6	0.608
Isolated protein	83.3	0.567

Current outlets for isolated soya protein are mostly industrial, such as paper-coating and sizing, lamination of fiberboard, fire-foam liquids, inks, leather finishing, adhe-

sive formulations, and insecticide preparations. With increasing interest in soya flour and isolated protein for foods, it is anticipated that such uses will soon equal or surpass industrial uses. Edible or food-grade proteins must be produced under sanitary conditions and isolated by a procedure that impairs the proteins as little as possible.

Procedures available for increasing the protein concentration of soya meal by extraction of nonprotein components with alcohol or for isolating proteins by acid precipitation of extracted nitrogen are likely to modify the proteins. Isolating proteins with urea at low temperatures seems to give a product comparable in functional properties to that in the source plant material. The extraction should be performed at low temperatures since protein dispersibility is lowered and denaturation may take place at higher temperatures. The need for high concentrations of urea, and the difficulty of removing residual urea from the isolate, are disadvantages of the proposed method. Data are not yet available on actual feeding tests on the nutritional value of the isolate, and feasibility predictions of large-scale operations would be premature. The procedure is of theoretical interest because it is based on solubilization of proteins as a result of disaggregation believed to be caused by disruption of hydrogen bonds. The method seems to have wide application possibilities in isolating plant proteins. Solubility in urea is affected by, and related to, heat denaturation; consequently, it is a useful parameter in following heat treatment. The isolated preparations contain high concentrations of protein whose functional properties are affected only slightly.

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Aldehyde-Amine Condensation Reaction: A Possible Fate of Carbonyls in Foods

SUMMARY

The reaction rate of L-tyrosine ethyl ester with n-heptanal was determined at 10, 20, 30, and 40°C by following the change in optical rotation. The reaction-rate data indicate that two consecutive first-order reactions occur. The apparent energies of activation for the first and second reactions were calculated to be 5.8 and 6.4 kcal, respectively. Infrared spectral data suggest the presence of the imine linkage in the product. Colored pigments of the reaction of glycine with n-heptanal were fractionated, but the fractions were not pure enough for further study. Alk-2-enals were the only class of carbonyls noted in the nitrogen-free pigments. A mechanism consistent with the available experimental information is proposed for the removal of carbonyls with simultaneous formation of unsaturated-polymeric pigments in foods.

INTRODUCTION

The carbonyl compounds resulting from lipid oxidation have been well documented as off-flavor components in many foods. The distribution of the carbonyls into classes and the composition of each class appear to affect the type of off-flavors observed. Factors in addition to the fatty acid composition of the native lipids that can affect the carbonyl distribution are the oxidation of the carbonyls (Lillard and Day, 1964) and the reaction of the carbonyls with other functional groups in the food material. Burton *et al.* (1963) showed the relative effect of various types of carbonyls on the degree of browning with glycine; the α,β-unsaturated aldehydes were most effective. The initial reaction in this case is presumably the condensation of the carbonyl with the amino group to yield the imine (Schiff base) and water:

$$R-CHO + R'-NH_2 \longrightarrow R-\underset{\displaystyle H}{\underset{|}{C}}=N-R' + H_2O.$$

From a flavor viewpoint, the role of the aldehydes would be significantly reduced once they react to form imines. This investigation was made to study the initial steps in the carbonyl-amine reaction. Tyrosine ethyl ester, glycine, and *n*-heptanal provided the functional groups for the study.

EXPERIMENTAL

Determination of reaction rates. L-Tyrosine ethyl ester (Calbiochem) was recrystallized from ethyl acetate. *n*-Heptanal (K&K Laboratories) was purified by preparative-scale gas chromatography. The ester (approximately 96 mg) was weighed into a 3-ml volumetric flask and made to volume with aldehyde. Within 1 min the ester dissolved and the solution was immediately transferred to a water-jacketed polarimeter tube having a light path of 20 cm and a volume of 2.5 ml. Using a sodium-vapor lamp, the change in optical rotation was followed from 4 min after mixing until no further change could be detected. Reaction rates were determined by this procedure at 10, 20, 30, and 40°C. The data were calculated and plotted by the procedure of Daniels *et al.* (1956).

Isolation of the reaction product. Five ml of *n*-heptanal were saturated with L-tyrosine ethyl ester (0.85 g), and the mixture was allowed to stand at room temperature for 3 hr. The layer of water that had formed was then removed by addition of anhydrous Na_2SO_4. After removal of the Na_2SO_4 by filtration, 100 ml of petroleum ether were added to the clear liquid, and the solution was stored for 12 hr at 4°C. A yellow oily liquid settled to the bottom of the vial, and the petroleum ether layer was decanted. The last traces of petroleum ether were removed at reduced pressure, and the infrared spectra of the oily liquid and *n*-heptanal were obtained with the sandwich cell technique. The infrared spectrum of L-tyrosine ethyl ester was obtained from a KBr micro pellet.

Fractionation of the colored pigments of the browning reaction. Equal molar quantities of glycine and *n*-heptanal were stored for 9 days in a sealed vial at 55°C. The glycine did not dissolve completely, but the solution became light brown within a few hours and dark brown at the end of 9 days. The glycine was removed from the reaction mixture by filtration after addition of petroleum ether. Analysis of the brown oily liquid by the micro-Kjeldahl and sodium fusion methods both gave negative results for nitrogen.

The 2,4-dinitrophenylhydrazones of the brown oily liquid were prepared by the method of Wyatt and Day (1963) and chromatographed on a Seasorb column (Schwartz *et al.*, 1960). As indicated by the characteristic color on the Seasorb column and an absorption maximum at 376 mμ in chloroform, the only class of carbonyls present appeared to be alk-2-enals.

One-half ml of the brown oily liquid was placed on a column containing 20 g of packing (5:1 silicic acid and Celite 545). The pigments were separated and eluted with a continuous gradient, starting with chloroform and ending with 30% ethanol in chloroform. Five colored fractions were obtained with colors varying from yellow to pink. The brown oily liquid was also fractionated by thin-layer chromatography using Silica Gel G as the adsorbent and 2% ethanol in chloroform as the solvent. Seven to eight components were visible when the plates were sprayed with 50% H_2SO_4 and heated 2 min at 100°C.

The thin-layer procedure was used to determine the purity of the fractions from the column procedure. Each of the five fractions from the column was found to contain at least four components, and some as many as eight. The multiplicity of the components in the fractions from the columns may have been due to the formation of other pigments within the fractions after elution from the column. Because of the heterogeneity of the column fractions, molecular-weight determinations and elemental analysis were not attempted.

RESULTS AND DISCUSSION

Fig. 1 shows a typical plot of the reaction rate data (30°C) when treated as a simple first-order reaction (Daniels *et al.*, 1956). Plots of data obtained at 10, 20, and 40°C differed only in the slope of the curves. These data indicate that two consecutive first-order reactions occur, which is consistent with the mechanism proposed by Bergel *et al.* (1959) in studies on cyclohexanone and L-tyrosine ethyl ester. The reaction of *n*-heptanal and tyrosine would be:

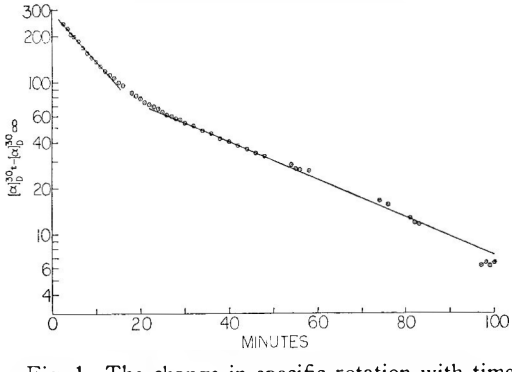

Fig. 1. The change in specific rotation with time at 30°C of L-tyrosine ethyl ester in *n*-heptanal (c = 3.21). $[\alpha]_D^{30}$ t is the specific rotation of the solution at time *t*. $[\alpha]_D^{30}$ ∞ is the specific rotation when no further change could be detected (164 min).

$$RCH_2CHO + H_2NCHR'R'' \xrightarrow{k_1}$$

$$RCH_2C(OH)H - NHCHR'R''$$

I

$$RCH_2C(OH)H - NCHR'R'' \xrightarrow{k_2}$$

$$RCH_2CH = NCHR'R'' + H_2O$$

II

where R is $-C_5H_{11}$, R′ is $-COOC_2H_5$, and R″ is $-CH_2C_6H_4OH$. Since the aldehyde concentration was essentially constant, the initial pseudo-first-order reaction would account for formation of the amino alcohol (I). The subsequent first-order reaction would represent dehydration of the amino alcohol to yield the imine (II) and water. However, Hine (1956) suggested that the dehydration reaction in the formation of semicarbazones is faster than the amino alcohol formation. If this is the case in the present system, the initial pseudo-first-order reaction could account for the imine (II) formation, and the second first-order reaction might be the addition of another aldehyde molecule to the imine by a reaction similar to that reported by Patrick (1952). This reaction would be:

$$RCH_2CHO + H_2NCHR'R'' \xrightarrow{k_1} RCH_2CH = NCHR'R'' + H_2O$$

II

$$RCH_2CH = NCHR'R'' + RCH_2CHO \xrightarrow{k_2} RCH_2CH = CRCH = NCHR'R'' + H_2O$$

III

where R, R′, and R″ are the same as indicated above. The data available are not suffi-

cient to indicate which reactions are observed in Fig. 1, but evidence presented below indicates the formation of the imine (II).

Assuming that the reactions are not reversible and do go to completion, the apparent first-order rate constants were calculated and are presented in Table 1. From an Arrhenius plot of these data, the apparent energies of activation for the first and second reactions were calculated to be 5.8 kcal and 6.4 kcal, respectively. Price and Hammett (1941) found heats of activation to range from 1.1 kcal to 4.6 kcal for the formation of semicarbazones of various carbonyl compounds.

Table 1. Apparent first-order rate constants for imine formation.

Temperature (°C)	k_1[a] (min^{-1})	k_2[b] (min^{-1})	Concentration[c]
10	0.0417	0.0165	3.23
20	0.0593	0.0243	3.16
30	0.0753	0.0285	3.21
40	0.127	0.0627	3.22

[a] k_1 is apparent rate constant for first reaction.
[b] k_2 is apparent rate constant for second reaction.
[c] Concentration is g of L-tyrosine ethyl ester/100 ml solution in *n*-heptanal.

The infrared spectrum of the reaction product of *n*-heptanal and L-tyrosine ethyl ester is compared with the spectra of the starting compounds in Fig. 2. The benzene

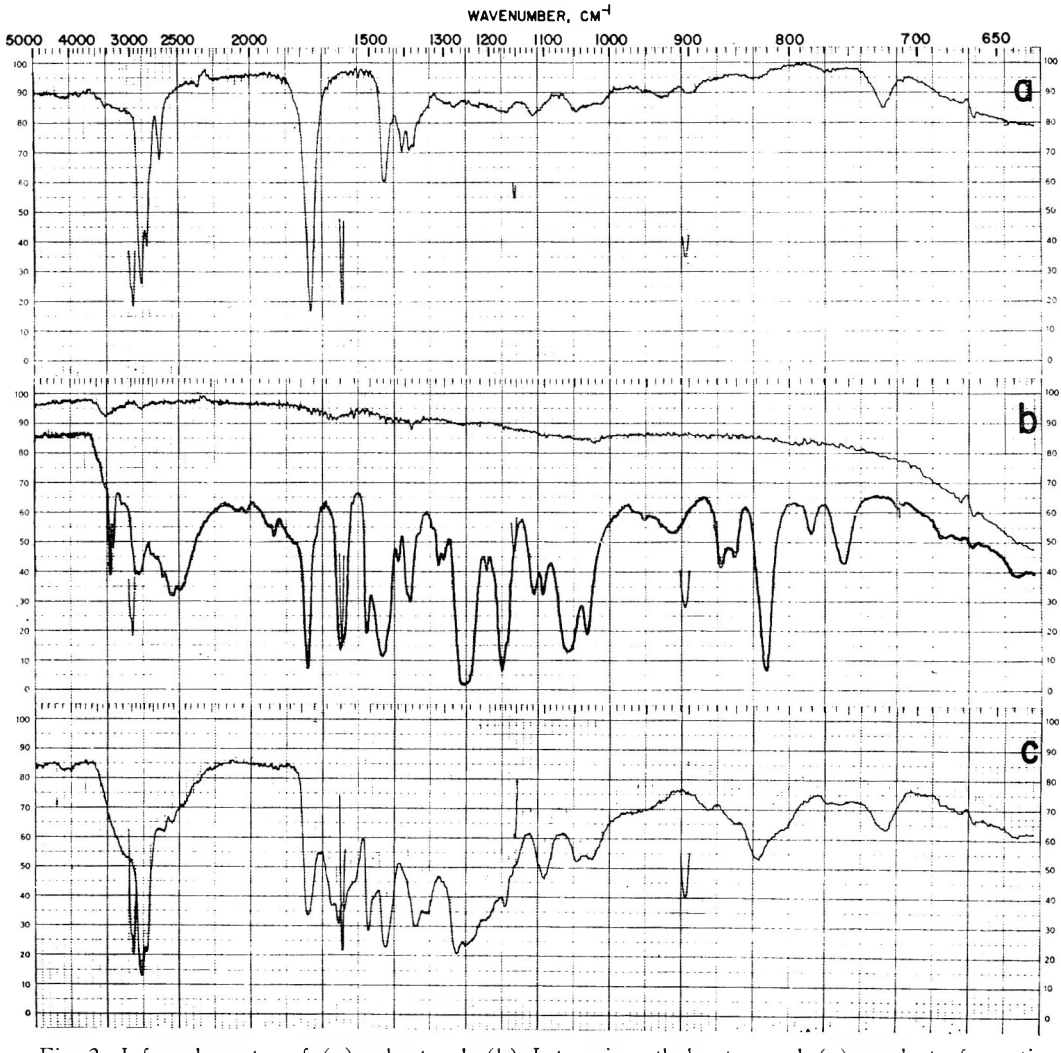

Fig. 2. Infrared spectra of (a) *n*-heptanal, (b) L-tyrosine ethyl ester, and (c) product of reaction (see text). Upper spectrum in *b* is of the KBr micro pellet blank.

ring from tyrosine is suggested in the reaction product (spectrum *c* of Fig. 2) by the presence of the benzene stretching bands at 1500 and 1600 cm^{-1} (Bellamy, 1959), while the seven-carbon chain contributed by *n*-heptanal is suggested by the bands at 2800 and 2900 cm^{-1} (Bellamy, 1959). However, the absence of the sharp bands at 3200 and 3300 cm^{-1} for NH_2 and NH stretching (Bellamy, 1959) in the product, which are shown in the spectrum of tyrosine ethyl ester (spectrum *b* of Fig. 2), indicates that the amino group was removed in the reaction. Since any unreacted aldehyde was probably removed with the petroleum ether extraction, the band at 1730 cm^{-1} suggests that the carbonyl of the ester remains intact. The presence of the C=N linkage in the product is indicated by the band at 1640 cm^{-1}, which exists as a shoulder on the benzene stretching band at 1600 cm^{-1}. This value (1640 cm^{-1}) for the C=N linkage is close to the 1682 cm^{-1} reported by Bergel *et al.* (1959) for cyclopentylidenetyrosine ethyl ester and is within the range of those reported by Bellamy (1959) and Suydam (1963).

Attempts to purify the pigments resulting from reaction of *n*-heptanal and glycine were not successful. Apparently, the pigments represent various stages in the polymerization reaction. Since the pigments did not contain nitrogen, it would appear that the amino group serves as a catalyst in the reaction and is regenerated in a manner similar to that observed in the browning of protein-reducing sugar systems (Keeney and Bassette, 1959). Alk-2-enals were the only class of carbonyl compounds found in this reaction system. These data indicate that the amino group serves as a basic catalyst in the aldol condensation of the carbonyls, followed by dehydration of the condensation product to yield the alk-2-enals. Therefore, the presence of alk-2-enals is explainable by a reaction between the imine and aldehyde, similar to that reported by Patrick (1952), with subsequent hydrolysis of the imine and regeneration of the amino acid. The general reaction would be:

$$RCH_2CHO + H_2NCH_2COOH \longrightarrow RCH_2CH{=}NCH_2COOH + H_2O$$

$$RCH_2CH{=}NCH_2COOH + RCH_2CHO \longrightarrow RCH_2CH{=}CRCH{=}NCH_2COOH + H_2O$$

$$RCH_2CH{=}CRCH{=}NCH_2COOH + H_2O \longrightarrow RCH_2CH{=}CRCHO + H_2NCH_2COOH$$

where R is $-C_5H_{11}$ of the aldehyde molecules. Repetition of this reaction scheme would lead to the formation of unsaturated-polymeric pigments in foods of low moisture content. The carbonyl reactants normally arise via lipid oxidation. Hence, the above reaction scheme furnishes a means for depletion of carbonyl flavor components in various food systems.

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Composition of Orange Juice Cloud

SUMMARY

Reported for the first time are analyses of materials identified with and contributing to the cloud of orange juice. Hexane-soluble materials provided up to one-fourth of the cloud-producing components. In contrast to the composition of structural components of the fruit, cloud components insoluble in alcohol, acetone, and hexane were shown to be rich in nitrogen (*ca.* 7%), pectins (*ca.* 80%), and phosphorus (*ca.* 1.5% as the pentoxide) and to be very low in cellulosic components (*ca.* 2–5%). This is evidence that cloud originates in the juice cells rather than from mechanical disintegration of structural tissue. No appreciable difference in cloud composition was observed between fruit varieties or between freeze-damaged and normal fruit.

INTRODUCTION

The retention of cloud in orange juice during storage and distribution is of prime importance to the citrus processing industry. Factors affecting loss of cloud and empirical methods for stabilizing cloud have been given much attention, but only limited studies on its characterization and formation have been reported (Higby, 1961; Kiefer, 1961; Rouse *et al.*, 1954, 1956, 1958). Cloud has generally been considered a heterogeneous mixture of cellular materials, and perhaps emulsoids, held in suspension by pectin. The nature and relative proportions of the various components have not previously been investigated. This study was undertaken to determine the composition and physical properties of cloud, with the expectation that this knowledge would be of help in evaluating the various steps in the production of citrus juices, concentrates, and powders.

EXPERIMENTAL

Sampling procedures. Sampling of Valencia (late-season) orange juices covered three years spanning the December, 1962, freeze, resulting in two seasons of normal fruit (8 samples) and one of freeze-damaged fruit (2 samples). Midseason juices represented one season each of damaged (2 samples) and undamaged (2 samples) fruit, while early-season juices were obtained in the two-week period preceding the freeze (2 samples) and a year later (4 samples).

Fresh juices, without heat stabilization, were obtained from nearby commercial processing plants and used as promptly as possible. The two major types of extraction equipment, Brown and FMC, were represented. All samples were strained through 16-mesh stainless screen to remove particles too large to be properly considered as suspended matter.

In addition to the samples of commercially extracted juices, there were obtained eight samples of water-extract of finisher waste, which concentrators use for the recovery of additional fruit solids and add to evaporator-feed juice. Also, three samples of Valencia oranges were dissected by hand to provide reasonably pure fractions of albedo, rag, pulp, and pulp-free juice.

Isolation of cloud for analysis. One of the first problems was that of recovering cloud for analysis. Filtration was not considered, since the required filter-aid would interfere with subsequent analyses. Dialysis was considered as a method for separating solubles and insolubles. Objections to this were the resultant dilution and the excessive time required for the separation, during which enzyme activity would possibly change the character of the cloud-producing constituents. The method finally adopted was the classification of suspended solids according to sedimentation rate, accomplished by using a laboratory-model steam-driven supercentrifuge equipped with a clarifier bowl.

Screened juice was first passed through the centrifuge at a flow rate of about 1000 ml/min and with 8 lb gauge pressure on the steam turbine. This produced the minimum rotational speed that could be maintained. Separation achieved was comparable to that obtained in the "free and suspended solids" test used in control laboratories, in which 50-ml tubes of juice are spun in a centrifuge for 10 min at about 600 × G and the suspended solids estimated by volume of compacted material in the bottom of the tubes. In this procedure the supernatant liquid is often used to estimate cloud by placing it in a colorimeter and determining light transmission or optical density with a red filter. Solids recovered from the clarifier bowl, corresponding to "free and suspended solids," are designated here as "Sediment A."

Effluents from the first centrifuging had an

average optical density of 1.20 (Lumetron colorimeter, model 410, 650 filter, 22-mm tubes), which indicates that most of the cloud remained in suspension. They were promptly centrifuged again, this time for 3 min at a flow rate of about 100 ml/min and at a bowl speed of 50,000 rpm, or at an applied force of about 60,000 × G. Solids recovered under these extreme conditions represented much of the fine cloud and are designated here as "Sediment B."

Since effluents from the second centrifuging had an average optical density of 0.51, which is still fairly turbid, they were stored overnight at 40°F and re-run at maximum centrifugal force. The additional solids recovered, representing very fine cloud, are designated as "Sediment C." The optical density of the final effluents averaged 0.15, which is fairly clear. These effluents were usually discarded.

Classification of components of centrifuge sediments. Sediments as recovered from the centrifuge were far from homogeneous, since the heavier particles tended to settle out immediately upon entering the lower end of the bowl, and the sludge cake became progressively thinner and wetter toward the top exit. To permit representative sampling, all of each sediment was creamed with water to a rather thin homogeneous slurry. Components of the sediments were classified into insolubles, lipids, and alcohol-solubles as follows:

Insolubles. To a weighed portion of slurry, usually about 250 ml, four volumes of 95% ethanol were added and heated to 160°F with constant stirring, then allowed to settle overnight, and filtered through sintered glass. The filter cake was comminuted and extracted twice with 80% ethanol, twice with acetone, and twice with *n*-hexane. The remaining insolubles, upon air drying and grinding in a Wiley Mill, produced a fluffy white powder.

Lipids. Filtrates from above were evaporated under vacuum to remove solvents, then extracted with *n*-hexane. Extracts were dried over anhydrous sodium sulfate, and the solvent removed under vacuum. The nonvolatile hexane-soluble residue constituted the lipid fraction. The composition of orange juice lipids has been adequately described (Swift and Veldhuis, 1951).

Alcohol-solubles. The aqueous residue from the hexane extractions included all the juice solids that were soluble in the original 80% ethanol mixture and not extractable with hexane. Since they are composed largely of sugars and acids, and obviously do not contribute to cloud, they were not considered further.

Peel-oil. The above method of classification of solids does not provide opportunity for a study of peel oil as an individual component of cloud. Because of its volatility, peel oil had to be studied principally by its relationship to the more predominant oil material, lipids. Recoverable oil determinations were made by the Clevenger method (Agr. Marketing Service, 1955) on centrifuge effluents and on sediments, to find out if peel oil: 1) retains its identity as relatively light droplets or emulsion; 2) enters into solution unaffected by gravity; or 3) becomes associated with the relatively heavy lipid fraction. This information should help to evaluate its role.

Fruit parts. To determine the source of materials contributing to cloud, oranges were dissected by hand and the various parts subjected to analyses similar to those applied to centrifuge sediments. Albedo and rag were comminuted with water in a blender to prepare a slurry for extraction as above. Orange sections were gently kneaded by hand in a bag formed of 4 thicknesses of cheesecloth in order to obtain juice free of cell structure. Residual pulp was comminuted with water in preparation for extraction. An aliquot of the juice was treated directly with 4 volumes of 95% ethanol as above, and the resulting precipitate extracted similarly.

Water extract of pulp. Commercial pulp extracts were too viscous for efficient centrifuging, so solids were recovered by direct treatment of an aliquot with ethanol, just as they were from the hand-prepared juice samples. Solids were similarly classified into insolubles, hexane-solubles, and alcohol-solubles.

Analyses of insolubles. Official methods of the AOAC (1960) were used for the determination of ash (29.013), phosphorus (20.030), and nitrogen (micro-Kjeldahl, 38.011).

Carbohydrates were classified into pectins, hemicellulose, and cellulose by the method of Jermyn and Isherwood (1956) modified as follows:

Pectins. About 0.4 g of solvent-insoluble material was treated with 80 ml of 0.05*N* NaOH and stirred occasionally for 2 hr at room temperature to solubilize pectins, transferred to graduated 50-ml centrifuge tubes, and centrifuged. Pectin-containing supernatants were decanted, and the sediments extracted twice with hot water to complete the recovery of pectins. Combined supernatants were adjusted to pH 6 with acetic acid and treated with 4 volumes of 95% ethanol to precipitate pectins. The alcoholic mixture was heated to 160°F with constant stirring, allowed to cool overnight, then filtered through paper discs. The precipitate was rinsed with acetone, vacuum dried, and weighed.

Hemicellulose. As is the case with pectins and cellulose, hemicellulose is not a specific compound. It is a type of carbohydrate material insoluble in 0.05*N* NaOH but soluble in 4*N* NaOH. Sediments remaining in the centrifuge tubes were made

4*N* with respect to NaOH by treating with 2 volumes of 6*N* solution. They were stirred occasionally during 2 hr at room temperature, then filled to volume with water, and centrifuged. Hemicellulose was recovered in solution by decanting and extracting the sediment twice with hot water, then precipitated with alcohol at pH 6, dried, and weighed just as was the pectin above.

Cellulose. Sediments remaining in the centrifuge tubes following extraction of hemicellulose were washed out of the tubes with 80% ethanol, filtered, rinsed with acetone, vacuum dried, and weighed.

RESULTS AND DISCUSSION

Average yield on a dry-solids basis of Sediment A was 0.19% of the juice weight, of Sediment B was 0.34%, and of Sediment C was 0.14%. Average dry-solids content of sediments recovered by centrifugation from each juice was 0.67% of original juice weight.

Relationship between lipids and insolubles. Proportions of lipid material and insolubles, expressed as percent of total light-scattering components (lipids plus insolubles), are shown in Table 1. Results shown are averages for all samples examined. Attention is called first to the low lipid content of rag and albedo. These structural components were quite well separated from juice in the dissecting process. The pulp was higher in lipid content probably because of the large amount of juice associated with this fraction. As prepared, the pulp showed composition very similar to that of Sediment A, which contained a large proportion of the larger and more readily sedimented cell fragments and considerable juice due to light compaction in the centrifuge. Highest lipid content was found in the pulp-free juice, where care was taken to prevent maceration of structural tissue. In commercially extracted juices, true cloud, as represented by Sediments B and C, was composed of about one-fourth lipid and three-fourths insoluble materials. Cloud from pulp extract had about the same lipids-to-insolubles ratio as that found in Sediments B and C.

Table 1. Ratio of lipids to insolubles in centrifuge sediments, pulp extract, and fruit parts, expressed as percent of total light-scattering components.

	Lipids	Insolubles
Sediment A	10	90
Sediment B	25	75
Sediment C	27	73
Pulp extract	26	74
Albedo	7	93
Rag	3	97
Pulp	11	89
Juice (pulp-free)	35	65

The high lipid content of cloud, in comparison with that of cellular structure, is an indication that cloud comes principally from the juice itself rather than from the lipid-deficient structural tissues.

Composition of insolubles. The average nitrogen and ash contents, and phosphorus content calculated as the pentoxide, of insolubles from the various classifications of materials are shown in Table 2. Of particular interest is the low nitrogen content of solids from the structural tissues (albedo and rag) and the high nitrogen content of solids from juice and from Sediments B and C. The 7% nitrogen in the latter is rather surprising since the commonly recognized nitrogenous components, amino acids and phospholipids, were removed as water-, alcohol-, or hexane-solubles. If all the nitrogen were from protein, use of the usual 6.25 conversion factor would indicate the presence of 45% protein, which is highly improbable. A brief study was made to determine the nature or source of this nitrogen. Several samples were dispersed in 0.05*N* NaOH for 1 hr, then examined for amino acids by ninhydrin (Ting and Deszyck, 1960). The values indicated an average of only 1% amino-nitrogen, distributed about equally between ninhydrin-blue acids and proline. The biuret test indicated very low protein content. This leaves about 6% nitrogen not classified.

Relatively high ash values were observed in insolubles from pulp extract and from juice. This may be due to the different treatment given these samples. They were the only samples precipitated directly with alcohol and therefore contained all the original soluble pectin as well as any accompanying minerals. In all other samples this pectin and accompanying minerals were eliminated by previous treatment.

The phosphorus contents of Sediments

Table 2. Inorganic composition of insolubles.

	Percent by weight		
	Nitrogen	Ash	P_2O_5
Sediment A	4.2	3.5	0.4
Sediment B	7.6	2.6	1.3
Sediment C	7.1	3.0	1.6
Pulp extract	4.0	10.1	1.4
Albedo	0.7	3.1	tr.
Rag	0.7	3.5	0.1
Pulp	2.8	4.1	0.3
Juice (pulp-free)	6.0	9.4	2.0

B and C, pulp extract, and juice were much higher than in the other classes of material. These are the same four that were rich in lipids, and similar logic as to origin of these components applies. Since phosphorus is apparently not readily available from structural tissue, this cloud constituent must originate in the juice, as does the lipid material.

The carbohydrate content of insolubles is shown in Table 3. The principal point of interest is the high proportion of the two cellulosic components in the structural tissues: albedo, rag, and pulp. The probability that portions of these tissues were mechanically disintegrated and dispersed in the juice by extraction and finishing, concentrated by mild centrifuging, and recovered in Sediment A is evidenced by the higher cellulosic content of this fraction. Apparently some hemicellulose and cellulose were picked up in the multiple extraction process of preparing pulp extract as well. Insolubles from juice and Sediments B and C were largely pectinous.

Effect of variety. It is well recognized in the industry that there are differences in the stability, and to a less extent in the appearance of cloud, among juices of different varieties. Accordingly, data on the composition of the three classes of sediment summarized above were re-evaluated by variety. Table 4 shows the average composition of Sediment B, which was most typical of true cloud, from early, midseason, and Valencia juices. There was very little variation between varieties in any of the components measured. While the hemicellulose content of the midseason varieties approximately doubled that of the early and late varieties, they were still of the same order of magnitude, and low. Actually, there was often more variation among samples of the same variety than between varieties.

Table 3. Carbohydrate content of insolubles.

	Percent by weight		
	Pectins	Hemicellulose	Cellulose
Sediment A	63	19	11
Sediment B	83	3	2
Sediment C	77	1	1
Pulp extract	71	8	5
Albedo	44	13	32
Rag	52	16	32
Pulp	57	19	19
Juice (pulp-free)	80	1	2

Table 4. Effect of variety on cloud composition—Sediment B averages.

	Early	Midseason	Valencia
Insols/lipids ratio	2.9	3.0	3.0
Percent of insolubles			
Ash	2.6	2.5	2.7
P_2O_5	1.3	1.2	1.4
Nitrogen	7.7	7.5	7.7
Pectin	84.5	86.5	81.6
Hemicellulose	2.6	5.3	2.8
Cellulose	1.9	2.3	2.6

Effect of freeze-damage. Comparison of orange juice cloud from freeze-damaged versus normal fruit is shown in Table 5. Freeze damage resulted in appreciably higher insolubles, slightly lower lipid and insoluble nitrogen, and slightly higher ash. Differences between average values for clouds from freeze-damaged fruit were less than some differences between individual samples of each class, so should not be overemphasized.

Effect of peel oil. Immediately following extraction of orange juice, peel oil can be recovered from, or concentrated in, the lightest phase of centrifuge effluents. But when juice was held for a few hours and then centrifuged, oil was found principally in the heavy fraction. Centrifuge sediment, which amounted to 2–3% of the juice weight, was found to contain as much recoverable oil as all the effluent. For example, upon centrifugation of 1 L of juice, 25 g sediment

Table 5. Comparison of orange juice cloud from freeze-damaged versus normal fruit.

	Normal [a]	Damaged [b]
Insolubles (% of LSC [c])	71.0	79.0
Lipids (% of LSC [c])	29.0	21.0
Nitrogen (% of insolubles)	7.6	6.9
P_2O_5 (% of insolubles	1.5	1.5
Ash (% of insolubles)	2.7	3.2

[a] Average of 2 samples from midseason and 8 from Valencia.
[b] Average of 2 samples from midseason and 2 from Valencia.
[c] Total light-scattering components.

yielded oil equivalent to that yielded by 975 ml of effluent. This indicates that peel oil combines with lipids, thus becoming associated with cloud-producing components, whether or not it contributes to increased light scattering. It further indicates that no more than a slight amount of the oil goes into solution in the aqueous phase. This combination of oil and lipids, with attendant decrease of volatility, may be a partial explanation of the rather rapid loss of fragrance of fresh orange juice following extraction.

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Some Effects of Added Dietary Fats on the Lipid Composition of Hen's Egg Yolk

SUMMARY

The fatty acid composition of the total lipid of eggs produced by hens on a control diet agreed closely with the compositions reported by other workers. The neutral glyceride fraction was more unsaturated than the phospholipid fraction. Addition of 10% of linseed, cottonseed or coconut oil to the diet altered the fatty acid composition of the egg lipids. Neither linseed nor cottonseed oil affected the total lipid content of the yolk, but coconut oil feeding brought about a small increase. In general, the acids of the neutral glyceride fraction were more susceptible to dietary manipulation than those of the phospholipid fraction. The effects of dietary fat on yolk lipid composition were compared for two types of bird.

INTRODUCTION

Considerable attention has been paid to the influence of diet on the composition of the total lipids of the hen's egg (Reiser, 1951; Fisher and Leveille, 1957; Murty and Reiser, 1961; Wheeler *et al.*, 1959; Feigenbaum and Fisher, 1959; Machlin *et al.*, 1962; Evans *et al.*, 1961; Evans *et al.*, 1963; Skellon and Windsor, 1963; Coppock and Daniels, 1962). Information on the effects of dietary fats on the glyceride and phospholipid fractions of the yolk is relatively limited (Reiser, 1950; Choudhury and Reiser, 1959; Murty and Reiser, 1961; Evans *et al.*, 1961).

The only reported studies of the influence of type or strain of bird on responses to dietary lipids of various compositions appear to be those of Edwards *et al.* (1960) and Edwards (1964). Those workers observed significant differences in response as between different strains with respect to some fatty acids, but they also observed significant differences between individual birds of a given strain with respect to other fatty acids.

The present paper describes a study of some effects of certain dietary fats on the compositions of the total lipids and also of the neutral glyceride and phospholipid fractions of the yolk. The influence of type of bird on the responses of the egg yolk lipid composition to different dietary fats was also investigated.

EXPERIMENTAL

Feeding experiments. Twenty-four Single Comb White Leghorns (laying type) and twenty-four Arkansas Silvers (meat type) were taken at random from flocks of birds in their second year of laying. The birds were allotted at random to eight groups. The four groups of Leghorn hens were designated Groups IA-IVA, and the Arkansas Silvers Groups IB-IVB. Each group was housed in a separate pen on deep litter and provided with water and feed *ad libitum* for the duration of the experiment.

The birds were maintained for a standardization period of two weeks on a control diet consisting of a commercial laying ration containing 3.8% fat to which ground oat hulls were added at a level of 10% as a nutrient diluent. Eggs were not collected during this period.

Following the two-week standardization period, eggs were collected for two days in the middle of each week over a period of three weeks. At the end of the third week, the experimental diets were given as follows:

Groups IA and IB—control diet

Groups IIA and IIB—control diet + 10% linseed oil

Groups IIIA and IIIB—control diet + 10% cottonseed oil

Groups IVA and IVB—control diet + 10% coconut oil

Eggs were collected each mid-week for the following six-week period. At the end of this time all the birds were returned to the control diet and eggs were collected for a final four-week period.

Separation and storage of egg yolks. The eggs were stored at 5°C. Six eggs were selected from each group, and the whites were separated from the yolks. The yolk was allowed to drain completely into a freeze-drying flask and dried in a Virtis freeze-drying apparatus. The dried yolks were stored in polyethylene bottles at −20°C.

Extraction of lipids. The total lipids were extracted by the following modification of the method of Bligh and Dyer (1959): Five g of dried yolk

was placed in a Waring blender flask, and 80 ml of water was added. The mixture was homogenized for 30 seconds. Chloroform (100 ml) and methanol (200 ml) were added, and the mixture was homogenized for 2 min. Another 100 ml of chloroform was added, and the mixture was homogenized for 30 seconds. A further 100 ml of water was added, and the mixture was homogenized for a final 30 seconds and then filtered through filter paper (Whatman No. 1) under gentle suction. The filter paper and blender flask were rinsed with 40 ml chloroform, which was filtered and added to the original filtrate. The filtrate was transferred to a 500-ml separatory funnel, and the suction flask was rinsed with 10 ml chloroform, which was added to the filtrate. The mixture was allowed to separate (approximately 2 hr), after which the lower chloroform layer was evaporated in a rotary evaporator in a water bath at 60°C and the residue weighed. The lipid was taken up in ethyl ether, the solvent evaporated in a stream of nitrogen, and the recovered lipid stored under nitrogen at −20°C.

Fractionation of the total lipid. The total lipid was fractionated into neutral glyceride and phospholipid fractions by the following modification of the method of Hawke (1959):

Approximately 1 g of the total lipid was weighed into a 15-ml centrifuge tube, and 10 ml of acetone was added. The mixture was brought to a boil with stirring and left to stand overnight, after which it was centrifuged and the supernatant acetone decanted into another 15-ml centrifuge tube. The procedure was repeated with 4 ml of acetone, the supernatant solutions were combined, and the acetone was evaporated under nitrogen. Both fractions were stored under nitrogen in a vacuum desiccator at −20°C.

The phosphorus content of each fraction was determined by the method of Harris and Popat (1954).

Preparation of methyl esters. The method was a modification of that described by Stoffel *et al.* (1959).

Thirty mg of lipid was dissolved in 0.5 ml of dry benzene and 5 ml of 5% hydrogen chloride in anhydrous methanol in a micro-sublimation tube. The mixture was refluxed, with frequent shaking, in a sand bath at 80–100°C until only one phase was observed (approximately 2–3 hr). The mixture was then cooled to room temperature, and 2 volumes water (11 ml) were added. The esters were extracted with redistilled petroleum ether (3 × 3 ml), and the extract was neutralized and dried for 1 hr over a mixture of anhydrous Na_2SO_4 and $NaHCO_3$ (4:1). The esters were transferred to a 5-ml test tube and the solvent evaporated under nitrogen. The esters were stored under nitrogen in a vacuum desiccator at 5°C.

Gas chromatography. The esters were analyzed in a Beckman GC-2 chromatograph (thermal conductivity detector; 1,4-butanediol succinate polyester column; mobile phase, helium; flow-rate, 100 ml/min; column temperature, 210°C; sample inlet temperature, 245°C). The fatty acids were identified by comparison with chromatograms of samples of standard mixtures of known composition (Hormel Institute, University of Minnesota) and by homologous series plots. Peak areas were determined by triangulation.

RESULTS AND DISCUSSION

Fatty acid compositions of the dietary fats. The compositions of the control diet and the added dietary oils are shown in Table 1. The cottonseed oil had a slightly lower linoleic acid content (46.4%) than that (55%) reported by Wheeler *et al.* (1959) and by Evans *et al.* (1961) and small traces of lauric and capric acids were present, acids which have not usually been reported as present in this oil. Each group consumed approximately the same quantity of food for the duration of the experiment, hence addition of the oils to the control diet did not affect palatability. No effect on the egg production of the different groups was evident.

Fatty acid composition of egg yolk lipids from control diets. *Composition of the total lipids.* The fatty acid composition of the total lipids of eggs laid by hens fed on the control diet is shown in Table 2. The

Table 1. Fatty acid compositions[a] of the dietary fats as determined by GLC.

Fatty acid	Control diet	Linseed oil	Cottonseed oil	Coconut oil
8:0[b]	0	0	0	3.4
10:0	0	0	0.2	6.7
12:0	0	0	0.5	52.3
14:0	0.8	0	1.4	18.7
16:0	17.9	6.0	26.8	9.0
16:1	1.0	0	2.1	trace
18:0	3.1	2.7	2.7	2.5
18:1	31.6	17.5	19.9	5.9
18:2	43.9	14.6	46.4	1.5
18:3	1.7	58.9	0	0

[a] Expressed as percentage of total fatty acids.
[b] Number of carbon atoms: number of double bonds.

Table 2. Fatty acid compositions[a] of the total lipid of egg yolk as determined by GLC.

Fatty acid	Control diet		Control diet + 10% linseed oil		Control diet + 10% cottonseed oil		Control diet + 10% coconut oil	
	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver
12:0[b]	0	0	0	0	0	0	0.8±0.2	0.7±0.2
14:0	0.4±0.1	0.4±0.1	0.3±0.1**	0.3±0.1**	0.4±0.1	0.5±0.1	5.4±0.6**	5.9±0.8**
14:1	0	0	0	0	0	0	1.1±0.3	1.2±0.3
16:0	27.4±1.1	28.6±0.9	20.5±1.2**	20.6±1.3**	28.2±0.5	28.4±1.5	27.6±0.8	28.4±0.6
16:1	3.5±0.5	3.7±0.3	2.1±0.2**	2.4±0.1**	1.6±0.2**	1.5±0.1**	3.6±0.2	4.0±0.2
18:0	8.6±0.9	8.2±0.5	10.4±0.6**	9.4±0.7	13.9±1.2**	13.5±1.1**	8.6±0.7	7.7±0.6
18:1	44.6±1.5	43.3±1.1	38.7±1.6**	39.1±1.1**	29.7±0.9**	31.2±0.3**	39.4±1.6**	40.0±1.2**
18:2	15.6±1.2	16.0±1.1	17.0±1.7	16.8±1.1*	26.3±1.6**	25.0±1.5**	12.6±1.1**	12.2±1.2**
18:3	0	0	11.3±1.3	11.5±1.6	0	0	0	0
20:4	0	0	0	0	0	0	0	0

[a] Expressed as percentage of total fatty acids ± standard deviation.
[b] Number of carbon atoms: number of double bonds.
*Differences from control significant at 5% level.
**Differences from control significant at 1% level.

proportions of the more abundant acids agreed closely with the results of Wheeler *et al.* (1959) and Skellon and Windsor (1962). In contrast with the findings of those workers, linolenic acid was not identified, and a small peak in the expected position of arachidonic acid was observed but was too small to permit measurement. The egg fat contained approximately 64% unsaturated fatty acids (mainly oleic and linoleic) and 36% saturated acids (mainly palmitic and stearic).

Composition of the neutral glyceride and phospholipid fractions. Fractionation of the total lipids gave 75.3% neutral glycerides and 24.7% phospholipid. The neutral glyceride fraction contained 0.25% P, and the phospholipid fraction contained 2.44% P. These results indicate that the fractionation was relatively crude and that the fractions obtained were not completely pure.

The fatty acid composition of the neutral glyceride fraction (Table 3) was similar to that of the total lipid, except for a lower proportion of stearic acid. The results were similar to those reported by Privett *et al.* (1962) except that linolenic, arachidonic, and other polyunsaturated acids were not observed. Furthermore, the observed palmitoleic acid content was lower than that reported by Privett *et al.* (1962). The myristic acid in the egg lipid was concentrated in the neutral glyceride fraction. The fatty acids of the neutral glyceride fraction

Table 3. Fatty acid composition[a] of the neutral glyceride fraction of egg yolk as determined by GLC.

Fatty acid	Control diet		Control diet + 10% linseed oil		Control diet + 10% cottonseed oil		Control diet + 10% coconut oil	
	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver
12:0[b]	0	0	0	0	0	0	0.9±0.2	0.8±0.3
14:0	0.5±0.2	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.6±0.1	7.2±0.6**	7.2±0.5**
14:1	0	0	0	0	0	0	1.2±0.4	1.7±0.3
16:0	26.0±0.8	21.1±0.7	19.1±0.4**	19.3±0.3**	21.8±0.8**	28.4±0.9*	27.0±0.5**	27.7±0.4*
16:1	3.9±0.3	4.1±0.3	2.6±0.2**	3.0±0.2**	1.7±0.2**	1.8±0.1**	3.9±0.2	4.8±0.3**
18:0	6.0±0.6	5.6±0.5	7.4±0.9**	5.6±0.3	11.6±1.5**	10.4±0.8**	6.4±0.2	5.2±0.4
18:1	48.3±1.2	47.0±1.2	40.5±2.0**	39.8±0.8**	30.5±0.8**	31.8±0.7**	41.1±1.1**	40.9±0.3**
18:2	15.2±1.1	15.6±1.3	16.9±1.1*	17.7±0.9**	27.6±1.8**	27.2±0.9**	12.0±0.8**	12.0±0.6**
18:3	0	0	13.1±0.9	14.2±0.6	0	0	0	0
20:4	0	0	0	0	0	0	0	0

[a] Expressed as percentage of total fatty acids ± standard deviation.
[b] Number of carbon atoms: number of double bonds.
*Differences from control significant at 5% level.
**Differences from control significant at 1% level.

were found to comprise 67% unsaturated and 33% saturated acids.

The composition of the crude phospholipid fraction (Table 4) was similar to that reported by Privett *et al.* (1962). This was the only fraction in which a measurable amount of arachidonic acid was observed (6.9%). Despite the presence of this acid and the absence of myristic acid, the phospholipid fraction was more saturated than the neutral glyceride fraction. This agrees with results reported by others (Evans *et al.*, 1961; Privett *et al.*, 1962).

Effects of added dietary fats on the total lipid content of the hen's egg. The total lipid content of the eggs produced by birds on the control diet was *ca.* 67%. The linseed-oil- and cottonseed-oil-supplemented diets did not influence the total lipid content of the eggs, but the coconut-oil-supplemented diet produced an increase (69%) which was significant ($P = 0.01$). Reiser (1950) and Wheeler *et al.* (1959) reported that oils such as cottonseed, safflower, soybean, and linseed, when added to the hen's diet, did not affect the total lipid content of eggs. A search of the literature has not revealed any report on the effects of a highly saturated fat, such as coconut oil, on the total lipid content of hen's egg yolk.

Effects of added dietary fats on the fatty acid composition of the lipids of egg yolk. *Effects of linseed oil on composition of total lipids.* Addition of linseed oil (10%) to the diet resulted in significant changes in almost all the fatty acids of the yolk lipids of eggs (Table 2). The contents of myristic, palmitic, palmitoleic, and oleic acids decreased, whereas there was a significant increase in stearic acid. In addition, linolenic acid (11%) appeared in the egg yolk. The alteration in the lipid composition of the eggs produced on this diet was maximum by the second week. When hens were returned to the control diet, the effect of the linseed oil lessened gradually, but even four weeks thereafter linolenic acid was still detectable in the egg yolk.

No increase in the linoleic acid content was observed. This is in contrast with the reports of others (Cruickshank, 1934; Fisher and Leveille, 1957; Wheeler *et al.*, 1959) who have observed that added dietary linseed oil increased the linoleic acid content. The fat component of the diet used in the present study had a relatively high linoleic acid content (43.9%) and this could account for the relatively high linoleic acid content (15%) of eggs produced by hens on the control diet. In addition, the linoleic acid content of the linseed oil used in the present experiment was unusually low (14.6%), and this circumstance may have contributed to the failure of the added linseed oil to increase the amount of this acid in the yolk. Fisher and Leveille (1957) and Wheeler *et al.* (1959) observed an in-

Table 4. Fatty acid composition[a] of the phospholipid fraction of egg yolk as determined by GLC.

Fatty acid	Control diet		Control diet + 10% linseed oil		Control diet + 10% cottonseed oil		Control diet + 10% coconut oil	
	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver	S.C.W. Leghorn	Arkansas Silver
12:0[b]	0	0	0	0	0	0	trace	trace
14:0	0	0	0	0	0	0	2.0±0.2	2.3±0.2
14:1	0	0	0	0	0	0	0	0
16:0	32.2±0.7	33.7±0.9	27.6±0.9**	27.5±0.4**	32.4±0.2	31.3±0.9**	33.0±0.4**	33.1±0.4
16:1	1.5±0.2	1.5±0.2	1.1±0.1**	1.1±0.2**	0.8±0.1**	0.7±0.2**	1.6±0.3	1.6±0.2
18:0	18.7±0.9	18.2±0.7	22.9±0.9**	23.4±1.4**	20.3±1.1*	21.5±1.1**	17.0±1.5*	17.8±0.6
18:1	25.9±1.2	25.3±0.7	26.2±1.4	26.5±0.8*	18.4±0.7**	19.0±0.8**	25.4±1.0	25.9±1.1
18:2	14.8±1.0	14.7±0.6	16.7±0.9**	15.0±0.5	21.5±1.5**	20.8±0.7**	15.0±1.1	13.6±0.3**
18:3	0	0	2.6±0.5	2.6±0.9	0	0	0	0
20:4	6.9±0.7	6.6±0.6	3.1±0.2	3.9±0.4	6.6±0.5	6.8±1.0	6.1±0.8	6.0±0.5

[a] Expressed as percentage of total fatty acids ± standard deviation.
[b] Number of carbon atoms: number of double bonds.
*Differences from control significant at 5% level.
**Differences from control significant at 1% level.

crease in the linoleic acid content of eggs due to linseed oil added to the diet. However, their control diets contained very little linoleic acid, which is probably the reason that their control eggs contained less linoleic acid (about 9%) than was observed in the present study (15%). The values for linoleic acid obtained by those workers for eggs laid by birds on a linseed-oil-supplemented diet were comparable with those observed in the present study. The foregoing observations suggest that any change in the linoleic acid content of eggs produced by birds on a linseed-oil-supplemented diet should be attributed to the linoleic acid content of the total diet and not to any particular factor present in the linseed oil.

The observed increase in stearic acid was surprising, since the stearic acid content of the added dietary linseed oil was very low (2.7%). Wheeler *et al.* (1959) reported a slight increase in stearic acid content when a linseed-oil-supplemented diet was fed, but they did not consider this small change to be significant. There have been no other reports on the effect of linseed oil on the stearic acid content of egg yolk lipids.

There may be a relation between linolenic acid and palmitic acid, since linseed oil was the only oil which caused any decrease in palmitic acid. Wheeler *et al.* (1959) observed that dietary soybean (8.8% linolenic acid) and linseed oils decreased the palmitic acid content of egg yolks. Murty and Reiser (1961) observed that trilinolenin, but not trilinolein, decreased the palmitic acid content of the neutral glycerides of egg yolk. The mechanism involved is not known.

The over-all effect of the linseed oil was an increase in the total unsaturation of the fatty acids of the egg lipid, which was due mainly to the deposition of linolenic acid and to the decrease of palmitic acid.

Effects of linseed oil on compositions of neutral glyceride and phospholipid fractions. The addition of linseed oil to the diet resulted in the deposition of linolenic acid in both the neutral glyceride and phospholipid fractions (Tables 3, 4). Linolenic acid was deposited to a greater extent in the neutral glyceride fraction (13%) than in the phospholipid fraction (2%).

The observed increase of stearic acid in the total lipid of the eggs laid by birds on a linseed-oil-supplemented diet was mainly in the phospholipid fraction. Murty and Reiser (1961) observed that added dietary trilinolenin caused a sudden but transient increase in the stearic acid content of the phospholipid fraction of egg lipids.

Decreases in the palmitic and palmitoleic acid of the total lipid were observed in both fractions, but a decrease in oleic acid was observed only in the neutral glyceride fraction. This latter observation supports the view that decreases in oleic acid are due to increases in linoleic and linolenic acids, since the contents of these latter acids were greatly increased in the neutral glyceride but not in the phospholipid fraction.

Arachidonic acid in the phospholipid decreased, which may be related to the appearance of linolenic acid in the egg lipid. Murty and Reiser (1961) observed a similar effect with trilinolenin but not with trilinolein. There is no obvious explanation for this result, but it would seem either that: a) linolenic acid competes with arachidonic acid insofar as incorporation into the egg fat is concerned; or b) linolenic acid interferes with the deposition of arachidonic acid into the egg lipid. Reiser (1951) has suggested, however, that dietary trienoic acids are converted to tetraenoic acids and are deposited as such in the egg lipid.

Although the levels of almost all of the individual fatty acids in the phospholipid fraction were modified in some way, the ratio of saturated to unsaturated acids was not affected. This concurs with Reiser's (1951) results.

Effects of cottonseed oil on the composition of total lipids. Although the cottonseed oil contained about 70% unsaturated acids, it increased the saturated acids of the egg yolk from 36.4 to 43.4%. The levels of myristic and palmitic acids were unchanged even though the cottonseed oil contained almost 27% palmitic acid. Palmitoleic and oleic acids decreased, whereas linoleic and stearic acids increased. Linolenic acid was not detected. The large increase in linoleic acid was expected because the cottonseed

oil had a very high linoleic acid content (46.4%).

The most interesting change was the increase in stearic acid content. The refined cottonseed oil used contained only some 3% stearic acid, so this increase, obviously, was not due to added dietary stearic acid. Work by others (Murty and Reiser, 1961; Wheeler *et al.*, 1959; Evans *et al.*, 1961) has ruled out the possibility that this effect is attributable to the linoleic acid content of cottonseed oil. The similar effect of *Sterculia foetida* seeds has led to the suspicion that the cyclopropenoid acids (sterculic and malvalic) may be responsible for some interference in the fatty acid metabolism. Deutschman *et al.* (1964) recently reported that incorporation of small amounts of synthetic sterculene in the diet resulted in a sharp increase of the stearic acid and an equally sharp decrease in the oleic acid of egg lipid. Although the cottonseed oil used in the present study gave a positive Halphen test (AOAC, 1960), a peak corresponding to malvalic acid was not observed on chromatograms of the methyl esters of this oil. The total lipid of the egg yolk from birds on a cottonseed oil diet gave a weakly positive Halphen test, so that at least some cyclopropenoid acid had been deposited in the egg.

Effects of cottonseed oil on the compositions of neutral glyceride and phospholipid fractions. The cottonseed-oil-supplemented diet produced quantitatively greater changes in the neutral glyceride fraction than in the phospholipid fraction. The saturated acids of this fraction increased from about 33 to 40%, whereas those of the phospholipid fraction only increased from about 51 to 53%. These findings agree with those of Evans *et al.* (1961), except that the latter workers reported much greater increases in the saturated acids of the neutral glyceride fraction. This is attributable to the fact that the increase in linoleic acid obtained in the present investigation was much greater than the increase reported by Evans *et al.* (1961).

The neutral glyceride fraction exhibited a small but significant increase in palmitic acid. Both fractions showed increases in stearic acid, but the effect was more pronounced in the neutral glyceride fraction. The latter fraction gave a weakly positive Halphen test, but the phospholipid fraction did not.

Linoleic acid was deposited to a greater extent in the neutral glyceride fraction than in the phospholipid fraction, resulting in a correspondingly greater decrease in oleic acid in the neutral glyceride fraction. Murty and Reiser (1961) obtained similar results when trilinolein was fed to hens.

Surprisingly, the arachidonic acid content of the phospholipid fraction was not affected by the dietary cottonseed oil. Other workers (Reiser, 1951; Murty and Reiser, 1961; Choudhury and Reiser, 1959) have claimed that linoleic acid was converted to arachidonic acid and that the addition of linoleic acid to the diet generally resulted in an increase in the arachidonic acid content of the egg lipid. However, none of those workers used cottonseed oil as their source of linoleic acid.

Effects of coconut oil on composition of total lipids. The addition of coconut oil increased the saturated acids and decreased the unsaturated acids, as expected. This agrees with results of Machlin *et al.* (1962) but disagrees with results of Cruickshank (1934) and Fisher and Leveille (1957). However, the latter workers observed only the gross effect of the diet on the degree of saturation or unsaturation, not the effects on the individual fatty acids.

Myristic acid was increased substantially, from an average value of 0.4 to about 6%. Only a very small amount of lauric acid was found in the egg lipid, although the dietary coconut oil contained 50% of this acid. Chung *et al.* (1964) reported that the myristic acid content of lipids from egg yolk was higher than the lauric acid content when hydrogenated coconut oil was consumed. That result agrees with the findings now reported. In the present work, myristoleic acid was found in the egg lipids of birds fed coconut oil, a point of interest since neither the coconut oil itself nor the lipid of the control diet contained detectable amounts of myristoleic acid. The appearance of myristoleic acid in the egg lipid may therefore have been due to desaturation of dietary myristic acid.

Linoleic and oleic acids were decreased significantly, but the stearic and palmitic acid contents did not change. The saturated fatty acids increased from 36.4 to 43.4%, and the unsaturated fatty acids decreased from 63.6 to 56.6%. These results indicate that the saturated acids are less susceptible to dietary manipulation. The detailed fatty acid compositions of the egg yolk did not provide any further information why the total lipid content of eggs laid was higher for hens on this diet than for hens on the other diets.

Effects of coconut oil on neutral glyceride and phospholipid fractions. The foregoing discussion and observations have shown that unsaturated oils, such as linseed and cottonseed oils, effect changes in both fractions. When a highly saturated oil (coconut oil) was fed, however, the changes observed in the total lipid were due mainly to changes in the neutral glyceride fraction. The fatty acids of the phospholipid fraction did not, in fact, undergo any major changes. It therefore seems likely that the fatty acids of the phospholipid fraction are not susceptible to manipulation by addition of a highly saturated fat to the diet. The shorter-chain acids appear not to be readily incorporated into the phospholipid fraction of egg yolk. The changes observed in the neutral glyceride fraction were analogous to those observed in the total lipid. A search of the literature did not reveal any report on the effects of dietary coconut oil on the neutral glyceride and phospholipid fractions of egg yolk.

Influence of type of bird on responses of egg yolk lipid composition. This part of the present investigation was designed to examine possible differential responses of the egg yolk lipid of different strains of hen to various dietary fats. The two strains chosen were a laying type (S. C. White Leghorn) and a meat type (Arkansas Silver). Attention was directed to the following comparisons: 1) the possible influence of type of bird on the fatty acids of eggs laid when the control diet was fed; and 2) the possibility that the two strains might differ in regard to the response of their egg yolk lipid composition to different dietary fats.

The comparisons may be summarized as follows: The differences in fatty acid composition of egg lipid exhibited by the two different bird types were not always the same in each individual fraction of the egg lipid. When the diet was supplemented with linseed oil or coconut oil, the total fatty acids of the eggs laid by the two strains did not differ significantly but the individual lipid fractions did display significant differences in some fatty acids. The effects of the cottonseed-oil-supplemented diet on the yolk lipid composition did not differ significantly between the two strains, except that the oleic acid content of the total lipid was slightly but significantly ($P = 0.05$) lower for the White Leghorns. The neutral glyceride and phospholipid fractions showed no significant differences. Edwards (1964) reported that there is a certain amount of variation between eggs laid by hens of the same breed, hence differences due to the breed would appear to be of relatively minor importance with regard to the fatty acid composition of the egg lipid.

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Quantitative Determination of Sugar Alcohols by the Komarowsky Reaction

SUMMARY

When heated in a reagent consisting of a cyclic aldehyde, thiourea, and concentrated sulfuric acid, the sugar alcohols, including glycerol and inositol, give orange, pink, or red colors whose intensities follow Beer's law. The reagent is stable for 6 weeks or more in an amber bottle under refrigeration. Of the aldehydes tested, *p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde were the most sensitive. Generally, the reactivity of the sugar alcohols was as follows: sorbitol > dulcitol > mannitol > erythritol > xylitol > arabitol > ribitol > glycerol > inositol. Carbohydrates and other interferences were removed by acid hydrolysis followed by degradation with hot alkali and treatment with ion-exchange resins. Hydrolysis, degradation, and removal of degradation products can also be done in a single operation with a mixture of ion-exchange resins. Important factors for reproducible quantitative results are the concentration of acid, thiourea, and aldehyde in the reagent, the time and temperature of heating, and the particular aldehyde used. Recovery from complex milieu was good (95–101.8%).

INTRODUCTION

Usually, the sugar alcohols are determined by subjecting them to oxidation with periodate in alkaline solution to produce formaldehyde, which is then measured by reaction with chromotropic acid to give a red or wine color with an absorption maximum at 570 mμ (Snell *et al.*, 1961). Because of their increasing importance in the food and pharmaceutical industries (Merck and Co., 1960; Genest and Chapman, 1962) and their physiological and metabolic roles in animals and higher and lower plants (Carr and Krantz, 1945; Hajny 1964a,b; Anderson *et al.*, 1961, 1962; Touster and Shaw, 1962), simple, rapid, and easily executed tests for this class of compounds are needed. It was recently shown that the anthrone reagent could be used for the colorimetric determination of sugar alcohols (Graham, 1963). However, both this and the chromotropic acid reagent are rather unstable in sulfuric acid and must be prepared fresh each day.

The Komarowsky reaction has been used for the determination of aliphatic alcohols (Coles and Tournay, 1942). Although it is thought that the reaction is not given by polyalcohols, 2-ethyl-1,3-hexanediol was determined by the use of *p*-dimethylaminobenzaldehyde in strong sulfuric acid (Bowman *et al.*, 1959), and Komarowsky-type spray reagent has been used for the detection of polyols (Godin, 1954). Preliminary investigations showed that, with proper control of reaction time, reaction temperature, and the concentrations of sulfuric acid and of the aldehyde used, the sugar alcohols can be determined quantitatively by this reaction. The addition of 2.5–5% thiourea to the acid-aldehyde mixture provides a reagent which is stable for as long as 6 weeks if kept in an amber bottle and refrigerated.

Carbohydrates constitute the most common interference in the determination of sugar alcohols. Adcock (1954) removed interferences through degradation in sodium carbonate and treatment with ion-exchange resins. White (1958) suggested preliminary hydrolysis of nonreducing carbohydrates such as sucrose to facilitate their degradation and removal. Anderson *et al.* (1961) proposed a similar method using ion-exchange resins only. In the procedure presented here, carbohydrates were removed by preliminary heating in hydrochloric acid followed by treatment with hot dilute sodium hydroxide and, finally, filtration over a bed of ion-exchange resins.

EXPERIMENTAL

Materials. The sugar alcohols (Nutritional Biochemicals Corp., Cleveland, Ohio) and aldehydes (Eastman Chemicals, Rochester, N.Y.) and the concentrations used are shown in Table 2. Sulfuric acid was reagent grade, 95–98%, sp. gr.

1.8407–1.8437. Thiourea (Baker Chemical Co.), was reagent grade, m.p. 171.5–172.5°C. The concentrations used are shown in Table 1. Ligroin, b.p. 66–75°C, highest purity (Fisher Scientific Co.), was used.

Aldehyde-thiourea-sulfuric acid reagents. Thiourea was placed in a 100-ml volumetric flask, and 80 ml of concentrated sulfuric acid was added. After the thiourea was dissolved (with heating, if necessary), the desired amount of the particular aldehyde was added and the mixture was heated for 10 min at 80°C. After cooling to room temperature (29±1°C), the volume was made up to 100 ml with concentrated sulfuric acid and the mixture transferred to an amber bottle and stored in the refrigerator when not being used.

Equipment. The equipment used was a Beckman DU spectrophotometer, borosilicate ground-glass-stoppered test tubes, 250-ml ground-glass-stoppered volumetric flasks, and ion-exchange columns. The columns were made from glass tubings, 15-mm inner diameter and 60 cm long. They were packed to a height of 10 cm with a 1:1 mixture of AG50 W-X8 resin (H^+ form, 100–200-mesh) and AG1-X8 resin (OH^- form, 100–200-mesh) obtained from Bio-Rad laboratories, Richmond, California. The resin was held in place by a plug of glass wool.

General procedure. One ml containing 2.5–1000μM of the sugar alcohol in water was placed in borosilicate ground-glass-stoppered test tubes. The water was removed by drying for 8 hr at 105°C. After cooling, 10 ml of the reagent was added to each test tube and the tubes were heated for 60 min in a water bath (99±1°C). The tubes were cooled for 5 min in an ice-water bath and allowed to stand at room temperature (29±1°C) for 30 min, after which the color developed was measured against a reagent blank at the wave length of maximum absorption.

The wave length of maximum absorption for each sugar alcohol-reagent mixture was determined by treating the mixtures as described above. The absorbance of the color developed was measured over the wave-length range of 380–700 mμ against distilled water. A plot of absorbance as a function of the wave length established the wave length of maximum absorption. The results for *p*-hydroxybenzaldehyde are shown in Fig. 1.

Factors influencing the reaction. Preliminary investigations showed that the time and temperature of heating, the concentration of acid, and

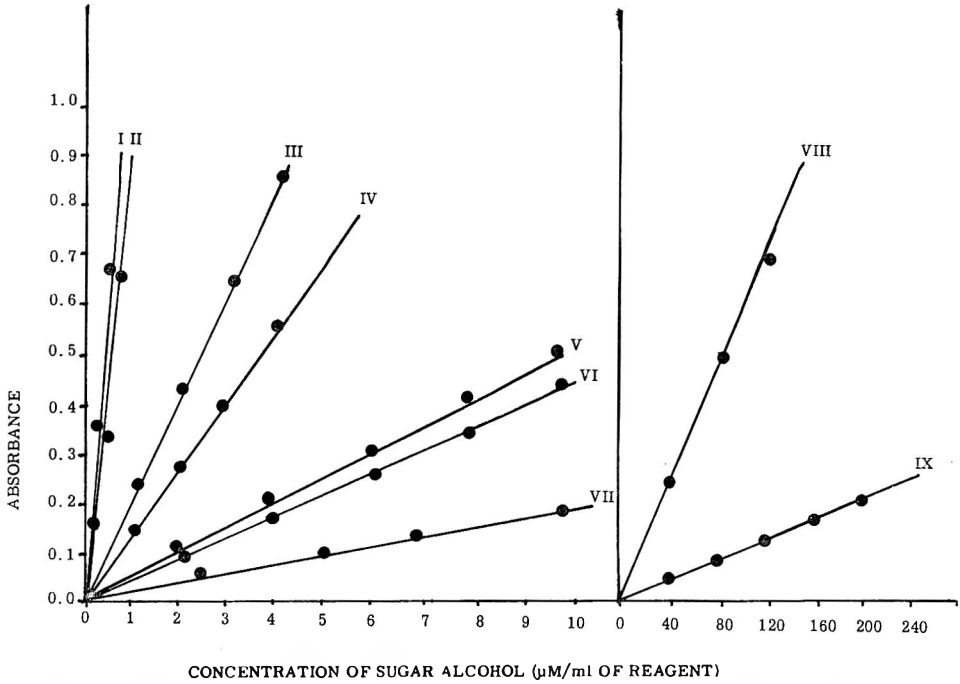

Fig. 1. Absorption spectrum of sugar alcohols in *p*-hydroxybenzaldehyde-H_2SO_4-thiourea reagent.

I = Dulcitol
II = Mannitol
III = Sorbitol
IV = Ribitol
V = Glycerol
VI = Erythritol
VII = Xylitol
VIII = Inositol
IX = Reagent blank

other factors were critical for maximum color development. Therefore, these and other variables were investigated under the conditions described in the general procedure. The results are shown in Table 1.

Comparative sensitivity of the various aldehyde reagents. To assess the sensitivity of the various aldehydes used, a fixed amount of each sugar alcohol was treated with each of the aldehyde reagents according to the general procedure. The results are shown in Table 2.

Quantitative response of the sugar alcohols. After the limits of the variables were established, the quantitative response of the various sugar alcohols was tested. For this, increasing quantities of each sugar alcohol were treated according to the general procedure, and the colors developed were measured at the wave length of maximum absorption against the appropriate reagent blank. The results, using *p*-hydroxybenzaldehyde, are shown in Fig. 2.

Removal of carbohydrates. Since sugars will interfere, these were removed by a modified version of the procedure outlined by Anderson *et al.* (1961). An aqueous mixture of 10 ml (500μM) of sorbitol and 10 ml (500μM) of sucrose was placed in a 250-ml Erlenmeyer flask, and 20 ml of 1N hydrochloric acid was added. The mixture was heated for 30 min in a boiling-water bath. Then 2 ml of 10N NaOH was added and heating was continued for another 20 min. Three g of the cationic resin and 3 g of the anionic resin were added and the mixture was shaken and allowed to stand for 10 min at room temperature ($29 \pm 1°C$) and finally filtered over the ion-exchange column. The column was washed with 200 ml of double-distilled water, the filtrate was collected in a 250-ml volumetric flask and made up to volume with double-distilled water, and one ml was used for assay according to the general procedure. Mixtures contained only sorbitol and only sucrose were treated similarly. The unmodified method of Anderson *et al.* (1961) was also tried for the removal of interferences.

Recovery of sorbitol from complex mixtures. Sorbitol (500μM) in 5 ml of distilled water was added to separate 100-ml glass-stoppered centrifuge bottles containing the materials shown in Table 3. The mixtures were heated for 30 min at 100°C and cooled, and 50 ml of ligroin was added. The mixtures were shaken for 30 min and centrifuged for 10 min at 2500 rpm and the ether layer was removed by careful siphoning. The ether extraction procedure was repeated, and the combined ether layers were washed twice with 5-ml portions of distilled water. The aqueous layers were removed and added to the residues. The residues were heated over a steam bath to remove residual ether, and after cooling, 50 ml of absolute methanol was added. The mixture was heated for 15 min on a steam bath, with a small funnel placed in the mouth of the bottle to minimize evaporation, and filtered while still hot into a 250-ml volumetric flask. The centrifuge bottles were washed twice with 10-ml portions of hot 90% methanol which was poured over the residue on the filter paper. The filtrate was evaporated almost to dryness on a steam bath,

Table 1. Influence of variables on color development in the interaction of sugar alcohols in the Komarowsky reaction.

Variable	Range investigated	Limits for reproducibility		Value or condition selected	
		Max	Min	Rgt A[a]	Rgt B[b]
Temperature of heating (°C)	60-100	100	95	100	100
Time of heating at 100°C (min)	15–120	60	60	60	60
Normality of H_2SO_4 in reagent	18–36	36	36	36	36
Amount of aldehyde in reagent (mg/100 ml of reagent)	25–400	200	200	200	250
Amount of thiourea in the reagent (% w/v)	0–10	5.0	2.5	5.0	2.5
Stability of color developed (hr)	0-240	4.0	0.5	0.5	0.5
Stability of reagent itself (days)	0–70–	56	42	42	42
Diluent for color developed	Water and several organic solvents tried			Concentrated H_2SO_4	

[a] Reagent A, *p*-Dimethylaminobenzaldehyde + thiourea in conc. H_2SO_4.
[b] Reagent B, *p*-Hydroxybenzaldehyde + thiourea in conc. H_2SO_4.
For aldehydes 3, 5, 6, 7, 8, and 9 (legend in Table 2), 2.5% thiourea was used and a level of 1% for aldehyde No. 4.

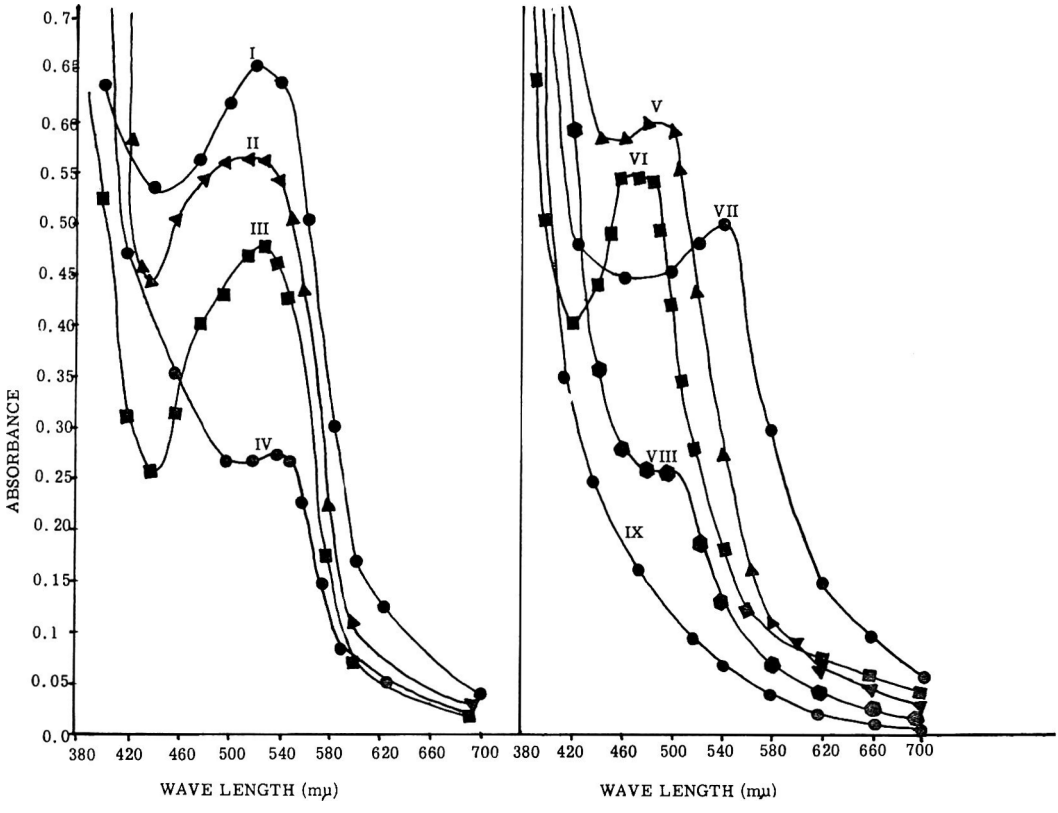

Fig. 2. Quantitative response of the sugar alcohols when treated with the *p*-hydroxybenzaldehyde-H_2SO_4-thiourea reagent.

I = Sorbitol	IV = Erythritol	VII = Ribitol
II = Dulcitol	V = Xylitol	VIII = Glycerol
III = Mannitol	VI = Arabitol	IX = Inositol

the residue was treated for the removal of interferences as outlined previously, and then 1-ml portions of the filtrate were assayed for sorbitol according to the general procedure. The results are shown in Table 3. The above procedure served well for materials which are low in proteins.

Deproteinization of milk and high-protein foods. Milk and other materials high in protein were deproteinized by adjusting the pH of the medium to 3.5 with glacial acetic acid. The pH was followed with a Beckman Zeromatic pH meter. Any precipitate formed was concentrated by centrifugation for 15 min at 1500 rpm and the supernatant was poured over Whatman No. 2 filter paper. The filtrate was collected, made up to 50 ml with distilled water, and 10 ml of freshly prepared 50% (w/v) trichloroacetic acid was added. The mixture was heated at 70°C for 10-15 min, cooled, filtered and treated for the removal of carbohydrates as described in the general procedure. Then, 1-ml portions of the filtrate were assayed for sorbitol according to the general procedure.

Instead of trichloroacetic acid, 2 g of sodium hexametaphosphate may be added and the mixture allowed to stand for 30 min at room temperature (29 ± 1°C). The remainder of the procedure remains the same.

Reproducibility. Ten separate determinations were done on solutions of sorbitol made up in distilled water, and 5 determinations on sorbitol recovered from complex milieu. The standard deviations were calculated from the data obtained.

RESULTS AND DISCUSSION

Of the aldehydes used, *p*-dimethylaminobenzaldehyde and *p*-hydroxybenzaldehyde were the most sensitive. The latter is preferable since, generally, the colors produced with the sugar alcohols have maximum absorption at higher wave lengths. *p*-Chloro-

Table 2. Relative sensitivity of various aldehydes in the Konarowsky reaction of sugar alcohols.

Aldehyde no.[b]	Amt. of aldehyde used (mg/100 ml)	Sugar alcohol (μM/ml of reagent).[a] Sorbitol 0.5	Dulcitol 0.5	Mannitol 4.0	Erythritol 4.0	D-arabitol 10	Xylitol 10	Glycerol 40	Inositol 40
1	200	0.66 (450)	0.55 (450)	0.72 (450)	0.52 (480)	0.60 (490)	0.58 (540)	0.14 (490)	0.22 (490)
2	250	0.59 (520)	0.56 (520)	0.86 (520)	0.56 (460)	0.45 (540)	0.50 (540)	0.24 (490)	0.05 (490)
3	200	0.56 (530)	0.47 (530)	0.57 (530)	0.13 (540)	0.10 (540)	0.31 (540)	0.14 (540)	0.17 (540)
4	50 (ml/100 ml) (of reagent)	0.24 (510)	0.24 (510)	0.29 (500)	0.215 (510)	0.29 (500)	0.48 (500)	0.45 (510)	0.07 (510)
5	0.01	0.03 (480)	0.05 (480)	0.12 (480)	0.52 (460)	0.01 (460)	0.38 (460)	0.00 (470)	0.06 (480)
6	0.05	0.36 (480)	0.25 (480)	0.65 (480)	0.33 (460)	0.06 (460)	0.09 (460)	0.00 (480)	0.20 (480)
7	0.01	0.00 (480)	0.00 (480)	0.00 (480)	0.30 (460)	0.00 (460)	0.0 (460)	0.00 (480)	0.16 (480)
8	0.02	0.09 (480)	0.06 (480)	0.18 (480)	0.40 (460)	0.26 (460)	0.05 (460)	0.00 (480)	0.00 (480)
9	0.01	0.05 (480)	0.05 (480)	0.12 (480)	0.78 (460)	0.03 (460)	0.40 (460)	0.00 (480)	0.14 (480)

[a] Numbers in parentheses represent wave length of maximum absorption of the sugar alcohol-aldehyde reagent mixture.
[b] Aldehydes: 1. *p*-Dimethylaminobenzaldehyde; 2. *p*-Hydroxybenzaldehyde; 3. *p*-Chlorobenzaldehyde; 4. Vanillin; 5. Furfural; 6. Benzaldehyde; 7. Cinnamaldehyde; 8. Salicylaldehyde; 9. *p*-Anisaldehyde.

benzaldehyde and vanillin gave colors with absorption maxima at 500–510 mμ, but the sensitivity was always much lower than with *p*-dimethylaminobenzaldehyde or *p*-hydroxybenzaldehyde. The intensities were always much less with the liquid aldehydes than with the solid ones. Since the solid aldehydes can be more readily purified (by recrystallization) and weighed out than the liquid aldehydes, these are to be preferred, with *p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde, respectively, the first and second choices.

The reactivity of the sugar alcohols can be listed as sorbitol > dulcitol > mannitol > erythritol > xylitol > arabitol > ribitol > glycerol > inositol.

Since color development decreased considerably as the acid concentration decreased, concentrated sulfuric acid was used throughout the experiment. Inadequate drying of the sample will lead to low results, and alcohol and other solvents must be absent.

A heating time of 1 hr or more is needed for maximum color development. Although heating for 90 min gave slightly higher absorbance, the color was somewhat less stable than when heating was done for 60 min at 100°C, the selected condition.

For consistent results, the temperature must be maintained at 99±1°C.

If the concentration of the aldehyde is too low, maximum color development will not occur, while if it is too high the reagent will be too highly colored, and, as a consequence, high reagent blanks will be obtained.

As the concentration of thiourea was increased, the stability of the reagent also increased. A concentration of 5% (w/v) was chosen since little added stability was achieved beyond this level and higher levels caused too intense yellowing and decreased sensitivity of the reagent. The thiourea serves as an antioxidant to prevent or retard color development in the aldehyde-H_2HO_4 mixture.

Aging of the colors affects the absorbance. Generally, at the shorter heating times (15–30 min) the absorbance increased considerably with aging. Since the minimum change in absorbance with aging occurred after heating for 45–75 min, a heating time of 60

min at 100°C was selected. Under these conditions, no change in absorbance occurred after aging for 60 min at room temperature (29±1°C), and after 6 hr the change was only 0.05 absorbance unit.

Recoveries from complex milieu were good (Table 3). With materials which are low in lipids, for example, some leaf extracts, the preliminary ether extraction can be omitted. The removal of proteins in blood will be more complete if $ZnSO_4$-$Ba(OH)_2$ (Somogyi, 1945) is used. With milk and materials high in protein the acetic acid-trichloroacetic acetic or acetic acid-sodium hexametaphosphate procedures are preferable. Complete deproteinization is essential since tryptophane will give a positive reaction.

The procedure of Anderson *et al.* (1961) for removal of interferences is quite suitable and is somewhat less destructive to the sugar alcohol than the direct acid-alkali treatment. However, the latter procedure is less time consuming. In both cases the recovery of pure sorbitol must be ascertained since, if destruction occurs, erroneous results will be obtained. The completeness of the removal of carbohydrates can be readily checked by heating a 1-ml portion of the filtrate for 5 min at 100°C with the anthrone-thiourea reagent (Graham, 1946). If a bluish-green color appears, destruction is incomplete. In this case, another sample should be treated carefully according to the procedure outlined until a negative anthrone test is obtained. Alternately, 50 ml of the same sample may be treated with 0.5 ml of 2*N* NaOH and heated for 6-10 min in a boiling-water bath, treated with the mixed resin, filtered over the ion-exchange column, the filtrate collected, made up to volume with distilled water and an aliquot assayed as described under the general procedure. Materials like tryptophane, ascorbic acid, sorbic acid, lactic acid, piperine, etc., are either destroyed or removed during the preliminary treatment to rid the solution of interferences. However, ethylene glycol, propylene glycol, and the polyoxyethylene and polyoxypropylene esters and glycols are not removed. When these are present, they can be separated by chromatographic procedures (Moore *et al.* 1960; Genest and Chapman, 1962) prior to elution and colorimetric determination of the sugar alcohol. The proposed method does not distinguish between individual sugar alcohols and, if the material being examined contains more than one sugar alcohol, chromatographic separation prior to elution and colorimetric determination would also be nec-

Table 3. Recovery of sorbitol from complex mixtures.[a]

Sorbitol added (μM)	Other material present	Method for removal of interferences: Sorbitol recovered (percentage)	
		Method 1[b]	Method 2[c]
0.0	Carrageenan (200 mg)	0.0	0.0
0.0	Starch (200 mg)	0.01	0.00
0.0	Sodium carboxymethyl-cellulose (200 mg)	0.01	0.01
0.0	Gum acacia (200 mg)	0.00	0.01
0.0	Pectin (200 mg)	0.02	0.0
0.0	Sucrose (500 μM)	0.0	0.1
500		98.0	100.6
0.0	Spinach leaf extract	0.0	0.05
500	Spinach leaf extract	97.5	99.9
0	Milk (3 g)	0.1	0.12
500	Milk (3 g)	101.1	101.8
0	Biscuits (2 g)	0.05	0.09
500	Biscuits (2 g)	95.0	99.2

[a] Average of 3 different determinations.
[b] Method 1, mixture heated in hydrochloric acid followed by hot alkali and ion-exchange treatment.
[c] Method 2, as per Anderson *et al.* (1961).

essary. Possible interference from seaweeds and other products which contain sugar-alcohol moieties (Touster and Shaw, 1962; Lohmar and Goepp, 1949), when these products themselves are not being analyzed, should not be ignored.

The mechanism of the Komarowsky reaction is still not fully known. Dehydration of the compound by strong sulfuric acid to produce olefinic-type intermediates which condense with the aldehyde to give characteristic colors, is thought to occur (Nogare and Mitchell, 1953). With alcohols, a mechanism involving carbonium ion formation and aldol condensation to form colored products has been proposed (Duke, 1947). The following sequence of reactions was postulated:

$$R_2CHOH + H^+ \longrightarrow R_2HC^+ + H_2O \qquad [1]$$

$$R_2HC^+ + RCHO \longrightarrow R-\overset{H}{\underset{+}{C}}-OCR_2 \quad (H) \qquad [2]$$

$$\left[R-\overset{H}{C}-O-CR_2 \quad (H,\ C=O)\right] \longrightarrow RCH^+_2 + R_2 \qquad [3]$$

$R_2C = O + RCHO \longrightarrow$ colored aldol condensation products. In acid solution, the condensation product resonates, resulting in color formation. The elimination or inhibition of color formation by added water, observed here, indicates that dehydration is involved. As in the reaction with the anthrone reagent (Graham, 1963), chain length seems to be an important factor in color production since the hexitols (sorbitol, dulcitol, and mannitol) were the most reactive, followed by the pentitols (xylitol, arabitol, and ribitol). The intermediate reactivity of erythritol indicates that configuration may also be important. Since glycerol was rather inactive, this probably reflects the opinion of Duke (1947) that, with primary alcohols, the equilibrium concentration of primary carbonium ion is extremely small, and since the equilibrium concentration of secondary carbonium ions is relatively higher than that of primary carbonium ions, oxidation of secondary alcohols goes on more readily than that of primary alcohols. Additionally, aldol condensation products involving ketones (from secondary alcohols) are more stable to polymerization than are the aldehyde products (from primary alcohols). This supports the finding that, generally, the greater the number of secondary alcohol groups possessed by the sugar alcohol, the greater the reactivity, as with the hexitols. The refractory nature of the cyclohexane ring probably accounts for the rather low activity of inositol.

The procedure has the distinct advantage of employing highly stable reagents, and it allows for much flexibility since one of several aldehydes may be used. Although the sugar alcohols can be determined by the anthrone reagent (Graham, 1963) and the chromotropic acid method (Snell *et al.*, 1961), both of these reagents are highly unstable in concentrated sulfuric acid. This imposes a severe limitation on routine analyses where many samples must be handled.

The method has good precision and reproducibility. For *p*-hydroxybenzaldehyde, the aldehyde of choice, the standard deviation was found to be 0.98% for pure sorbitol and 1.06–1.26% for the assay of sorbitol added to complex mixtures.

A linear response was obtained when the absorbance of the color produced was plotted against the concentration of the sugar alcohol present. For *p*-hydroxybenzaldehyde and sorbitol, this relationship can be expressed by the equation: $X = 0.8203\ Y\text{-}0.0034$

where X = concentration (μM) of sorbitol per ml of reagent

Y = absorbance at 540 mμ

$$\text{Percent sugar alcohol in sample} = \frac{X\ (\text{mol. weight} \times 10^{-3})\ (\text{dilution factor})}{\text{sample weight (mg)}} \times 100$$

Similar equations can be derived for any other sugar alcohol-aldehyde mixture.

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The Measurement of Monocarbonyl Classes in Cocoa Beans and Chocolate Liquor With Special Reference to Flavor

SUMMARY

Monocarbonyl compounds in unroasted cocoa beans and chocolate liquor were converted to 2,4-dinitrophenylhydrazones and then separated into methyl ketone, saturated aldehyde, 2-enal, and 2,4-dienal fractions. In most comparisons the aldehydes were more concentrated in liquor than in unroasted beans, which contained more ketone. Over 30 derivatives, several within each class, were detected on thin-layer plates. Spot densities were most intense in the region between the C_4 and C_7 standards. Differences among Accra, Arriba, and Bahia varieties involved relative concentrations of individual compounds rather than the presence or absence of specific carbonyls. Regeneration of carbonyls revealed each class to be a potent reservoir of aroma-emitting compounds. Methyl ketones were cheese-like; saturated aldehydes, waxy and fruity; 2-enals, oxidized, painty, and like old meat; 2,4-dienals, nutmeg and spicy. From the quantitative data it was concluded that each carbonyl class is an important contributor to chocolate aroma.

Although there has been no lack of speculation about the chemical nature of chocolate flavor, only a few odorous compounds have been actually identified, and none by themselves could be described as "like chocolate" (Forsyth and Quesnel, 1963). That chocolate flavor, especially the aroma portion, is a composite of a vast number of compounds is evidenced by the fact that over 50 peaks appear on gas chromatograms of flavor concentrates even when relatively insensitive separating conditions are employed (Patton *et al.*, 1964).

While it is highly unlikely that chocolate flavor will ever be traced to a single class of compounds, current knowledge points to carbonyls as being of great importance. Gas chromatography of headspace samples from ground roasted beans and liquor revealed saturated aldehydes as the dominant class of compounds in the aroma fraction (Bailey *et al.*, 1962). 3-Methyl butanal was found in greatest concentration, followed in order by 2-methyl propanal, propanal, ethanal, and butanal. Other carbonyls identified in various cocoa bean and chocolate flavor extracts include 2,3-butanedione and 3-hydroxy-2-butanone (Schmalfuss and Barthmeyer, 1932), 2-furancarbinal (Mohr, 1958), propanone (Bailey *et al.*, 1962), 6-methyl-5-heptene-2-one, and 3,7-dimethyl-6-octenal (Van Elzakker and Van Zutphen, 1961).

The research reported herein concerns the relative concentrations of the various monocarbonyl classes and how they are influenced by variety and source of cocoa bean, and by processing variables involved in the manufacture of chocolate liquor. Data were collected using the elegant techniques developed by Schwartz *et al.* (1963) whereby carbonyls are converted to 2,4-dinitrophenylhydrazones (DNP-hydrazones) and separated into the following classes: methyl ketone, saturated aldehyde, 2-enal, and 2,4-dienal. Dicarbonyls and oxygenated compounds were not considered. Thin-layer and gas chromatography were used to assess the complexity of each carbonyl class.

EXPERIMENTAL

Samples. Raw unroasted beans and liquor representing Accra, Arriba, and Bahia varieties were provided by several member companies of the Chocolate Manufacturers' Association of the USA. Each raw bean sample was a composite from several bags to ensure a representative product. The corresponding liquor sample also came from this blend. Samples were stored at 35°F until analyzed.

Extraction of fat. Shelled raw beans were converted to semi-fine powders with a food mill, and chocolate liquor was pulverized in a mortar. Thirty grams of liquor and 40 g of beans were needed to ensure the recovery of approximately 15 g of fat.

An equal weight of Celite 545 (Fisher Scientific Company, Pittsburgh), dried for 24 hr at 150°C, was ground in a mortar with the pulverized sample. Ten milliliters of water were added during the grinding to increase the moisture level to at least 15%. Water increases the extractability

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of fat and facilitates firm packing in the chromatographic column. The resulting mixture was packed into a chromatographic column and extracted with purified hexane rendered carbonyl-free by passage through a DNP-hydrazine reaction column (Schwartz and Parks, 1961). The 300 ml of eluate contained practically all of the lipid material in beans and liquor measured by Official Methods of Analysis (AOAC, 1960).

Reaction of carbonyls with DNP-hydrazine. The hexane extract was passed through a Celite column impregnated with DNP-hydrazine, H_3PO_4, and H_2O (Schwartz *et al.*, 1963) to convert monocarbonyls to DNP-hydrazones. The column was flushed with hexane until the effluent had the same spectral properties as pure hexane passed through the reaction column. This usually required the collection of a 500-ml sample.

The total concentration of monocarbonyl DNP-hydrazone was estimated by finding the absorbancy of the solution compared to hexane at 340–345 mμ and converting the reading to micromoles using $E = 22{,}500$.

DNP-hydrazones were freed of lipid material by passing the effluent from the reaction column through a Celite 545-Sea Sorb 43 column (I. No. 80; Fisher Scientific Company, Pittsburgh). Celite had been activated for 24 hr at 150°C. The column was essentially fat-free after flushing with 200–250 ml of hexane. Adsorbed hydrazones of monocarbonyls were eluted with 50–75 ml of 3:1 chloroform-nitromethane, and after evaporation of the solvent under N_2 the residue was dissolved in hexane.

DNP-hydrazone class separation. Monocarbonyl derivatives were separated from ketoglyceride DNP-hydrazones on a column of weak alumina (Schwartz *et al.*, 1963). Depending upon the amount of fat involved in the analysis, it was sometimes necessary to put the sample over a second alumina column to realize good separation.

After solvent evaporation the monocarbonyl residue was dissolved in hexane and applied to a 10-g Celite–Sea Sorb (1:1) column. Separation of the four DNP-hydrazone classes was obtained using the following sequence of solvents: 50-ml quantities of 25, 40, 60, and 80% chloroform in hexane, 100 ml of chloroform, 50-ml quantities of 2, 4, 6, 8, and 10% methanol in chloroform, and, finally, 50 ml of 25% nitromethane in chloroform.

The chromatographic fractions were evaporated to dryness under N_2, the residues were dissolved in chloroform, and spectrophotometric measurements were made for each carbonyl fraction. Class authenticity was established on the basis of the following absorption maxima: methyl ketones, 365; saturated aldehydes, 355; 2-enals, 373; 2,4-dienals, 390. With application of the proper molar extinction coefficient (Jones *et al.*, 1956), quantitative values for each class were calculated. Class authenticity was also verified by determining the stability of each fraction in alcoholic base (Jones *et al.*, 1956) and by thin-layer chromatography (Schwartz and Parks, 1963).

Separating individual compounds within a class. Kieselguhr-G (Brinkman Instruments, Inc., Great Neck, New York) thin-layer plates impregnated with Carbowax-400 (Badings and Wassink, 1963) with methyl cyclohexane as the solvent were used to separate the components and assess the complexity of each carbonyl class.

RESULTS AND DISCUSSION

Differences among varieties. Concentrations of carbonyl classes in raw beans of Accra, Arriba and Bahia varieties are recorded in Table 1. The most striking difference was found in the saturated aldehyde fraction of Arriba, which had ten times the concentration of the other varieties. This value is reflected in the relatively high "total carbonyl" figure for Arriba raw beans.

Table 1 shows that DNP-hydrazones other than those of the listed monocarbonyl classes contribute greatly to the figures for "total carbonyls." That the descrepancies are caused, at least in part, by ketoglycerides, was proved by gas chromatography of methyl esters prepared from the DNP-hydrazone fraction after the removal of lipids with the Celite-Sea Sorb column. Retention times were identical to those of known esters, and relative concentrations were similar to those recorded in a methyl ester analysis of the hexane extracted lipids of raw beans. After the monocarbonyl classes are accounted for, it is evident that Accra beans do not contain as much ketoglyceride and other DNP-hydrazine reacting compounds as Arriba and Bahia varieties.

The data for chocolate liquor in Table 1 reflect differences caused by variables other than those associated with the manufacture of liquor since all samples were processed in the same equipment and received similar temperature treatments. Bean temperatures were approximately 150°C on leaving a Burns Thermalo roaster, and chocolate liquor was at about 88°C from a Lehmann 48-inch Stone Mill. Fineness of liquor was reported

Table 1. Concentrations of monocarbonyl classes in raw beans and liquor of Accra, Arriba, and Bahia varieties.

Sample	Micromoles per 100 g fat				
	Total carbonyl	Methyl ketone	Saturated aldehyde	2-enal	2,4-dienal
Accra raw	175	15.6	10.7	0.7	1.6
Accra liquor	119	23.8	33.0	5.3	5.9
Arriba raw	354	10.7	102.0	0.9	0.7
Arriba liquor	266	7.8	42.0	3.0	3.7
Bahia raw	262	16.7	14.0	1.4	1.7
Bahia liquor	292	11.0	44.0	2.6	3.2

at 95–97% through a 200-mesh screen. The results are presented as differences due to variety *per se*. However, before reliable conclusions can be made, many samples will have to be analyzed so as to balance distorted values in individual samples caused by abnormalities in agronomic and fermentation practices. These variables which are associated with the cocoa bean producing countries are themselves worthy of study in regard to their influence on carbonyl patterns.

The 2-enal and 2,4-dienal levels in liquor were elevated several-fold over those in raw beans, with the greatest increase in the Accra sample. The identity and origin of these heat-generated unsaturated aldehydes remain to be elucidated. Odor observations of regenerated carbonyls suggest the presence of typical lipid oxidation compounds in the 2-enal fraction.

In Accra and Bahia liquors the saturated aldehyde concentrations were three times those of raw beans. These aldehydes logically arise through deamination and decarboxylation of amino acids via Strecker degradation reactions (Baily *et al.*, 1962). In Arriba liquor a net reduction from 102 to 42 micromoles was found, indicating a loss of aldehyde that was faster than the accumulation of Strecker reaction compounds. It is not known whether reduction is a result of volatilization into the exhaust gas during roasting or a degradation of heat-labile compound(s). The unusually high concentration of saturated aldehyde in unroasted Arriba beans cannot be attributed to an analytical error, since duplicate analyses were in good agreement. It remains to be determined whether this value is characteristic of the variety or is the result of an environmental abnormality.

The origin of the methyl ketones in raw bean is not known, although it is tempting to suggest mold activity as a factor to be considered. In all but one of the analyses for methyl ketone recorded in Tables 1 and 2, liquor showed a lower concentration than raw bean. A loss of low-molecular-weight ketone produced by mold on exterior surfaces would be expected to occur during roasting and grinding. While some ketone is removed during processing, others, especially long-chain methyl ketones, are formed and accumulate in roasted beans. These ketones logically arise from ketoglycerides under the influence of heat and moisture, as has been demonstrated in other lipid systems (Keeney *et al.*, 1962).

Variations among several samples of Accra beans and liquor. Table 2 lists data collected on Accra raw beans and liquor supplied by four different chocolate manufacturers. The differences among raw bean samples were greatest in the total carbonyl and methyl ketone fractions.

Major differences were found among the liquor samples. However, it is not possible to relate these differences to the carbonyl pattern of raw bean, since the suppliers did not use identical roasting and grinding conditions in either temperatures or equipment. The significant facts to emphasize about the data in Table 2 are: a) except for methyl ketone, carbonyl values for raw beans did not show striking differences; and b) the relatively large variations detected among liquors reflect the dominance of the manufacturing process in controlling

Table 2. Concentration of various monocarbonyl classes in raw Accra beans and chocolate liquor supplied by four manufacturers.

Company	Sample	Micromoles per 100 g fat				
		Total carbonyl	Methyl ketone	Saturated aldehyde	2-enal	2,4-dienal
A	raw	175	15.6	10.7	0.7	1.6
A	liquor	119	23.8	33.0	5.3	5.9
B	raw	169	4.6	14.4	2.0	3.3
B	liquor	225	3.8	26.0	3.2	3.2
C	raw	229	8.9	19.0	1.3	1.3
C	liquor	236	5.0	32.0	1.3	trace
D	raw	175	12.9	15.0	0.6	1.6
D	liquor	232	6.1	17.0	3.3	1.1

the monocarbonyl pattern in chocolate.

Separation of derivatives. Fig. 1 is a typical thin-layer chromatogram of separated DNP-hydrazones within each class. The complex nature of the carbonyl fractions of chocolate liquor is still evident, even though considerable detail was lost during reproduction. Conclusive identification of individual compounds was complicated by the fact that many of the more than 30 regularly appearing spots did not coincide exactly with available known derivatives. Chromatographic behavior suggests that some of the spots were derivatives of branched chain carbonyls.

At least five methyl ketones were regularly observed, with the spots being most intense near locations of the C_5 through C_7 reference compounds. The presence of ketones larger than C_{13} accounted for a spot at the solvent front.

Eight to ten saturated aldehydes appeared on most plates at positions near C_4 through C_6 and C_8 through C_{10}, and at the solvent front. The most prominent spots were in the C_4 to C_6 region.

Six to eight 2-enals and almost as many 2,4-dienals were found in liquor. Most of the former were concentrated between the C_3 and C_7 enals, and the latter were clustered primarily in the C_5 to C_8 dienal region. But-2-enal, propenal, and octa-2,4-dienal were tentatively identified.

The information obtained from thin-layer chromatography suggests that the relative and total concentrations of individual carbonyls are probably more closely related to variety than is the presence of or absence of a particular compound. Variables associated with the manufacturing process are at least of equal importance, and studies will have to be conducted under standardized manufacturing conditions before final conclusions can be drawn.

Odor evaluation. To assess odor character, DNP-hydrazone fractions from several analyses of liquor were combined and treated with H_2SO_4 (Bassette and Day, 1960) to

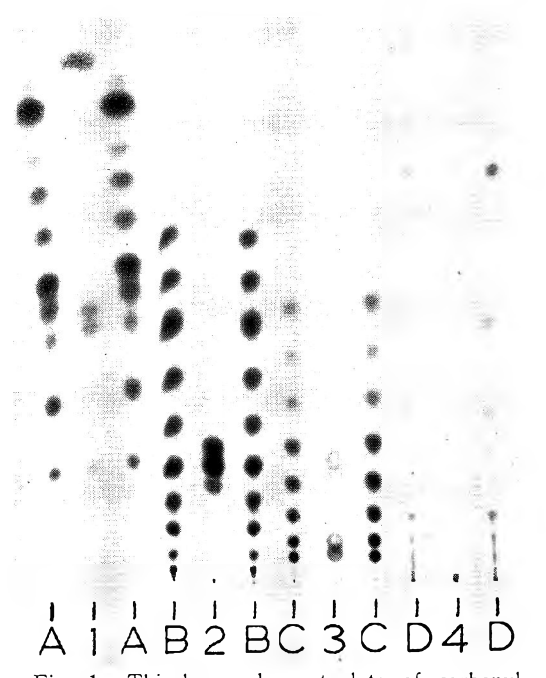

Fig. 1. Thin-layer chromatoplate of carbonyl compounds as their 2,4-dinitrophenylhydrazones comparing reference derivatives with those from liquor (Accra). A) alk-2-ones C_3 through C_{13}; B) alkanals C_1 through C_{10}; C) alk-2-enals C_3 through C_{10}; D) alk-2,4-dienals C_3 through C_{10} (less C_8, C_{13} and C_{15}). 1, 2, 3, and 4 are respectively the fractions from liquor corresponding to the reference mixtures A, B, C, and D.

regenerate carbonyls. Descriptions given by the authors were as follows:

Methyl ketone—cheesy, cheese crackers, suggestive of 3-methyl butanal

Saturated aldehyde—waxy, fruity, like C_8—C_{10} aldehydes

2-enal—oily, oxidized, painty, old meat

2,4-dienal—strong nutmeg, spicy, suggestive of octadienal.

When DNP-hydrazones from each class were combined and the monocarbonyls regenerated, the odor was described as being more reminiscent of chocolate than any of the individual classes.

The odor-assessment work yielded convincing evidence that the monocarbonyl fractions of chocolate liquor contain potent aroma-emitting compounds. If data in Tables 1 and 2 are estimated in ppm, carbonyl concentrations are found ranging from a minimum of 1 ppm, for some dienal fractions, to as much as 50 ppm, for saturated aldehydes in liquors. A statement to the effect that monocarbonyls are of critical importance in chocolate flavor seems irrefutable when it is remembered that enals and dienals may have flavor thresholds expressed in ppb, and some ketones and saturated aldehydes are detected at concentrations of 1 ppm or less. Recent evidence (Day *et al.*, 1963) that some carbonyl combinations give detectable responses even when individual compounds are present at subthreshold levels lends additional support to this general statement. Furthermore, a hexane extract of liquor has a distinct chocolate aroma but the intensity is diminished and the character of the aroma is changed when the extract is reacted with DNP-hydrazine.

Because of the limitations of the analytical procedures, aromatic carbonyls, especially phenolic derivatives, oxygenated compounds, and dicarbonyls, were not considered in this investigation. The demonstrated importance of polyphenols as flavor precursors (Forsyth and Quesnel, 1963), and the fact that roast temperatures are high enough to cause carbohydrate and protein degradation, suggest that these carbonyls also contribute to chocolate flavor.

The critical role of DNP-hydrazine reacting components has been stressed. However, it must be emphasized that chocolate is one of the most complex natural flavors, with a vast number of compounds from several major functional group classes being involved. Characterizing chocolate flavor represents one of the most challenging of food research problems.

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Fatty Acid Content of Pork Cuts and Variety Meats as Affected by Different Dietary Lipids

SUMMARY

The fatty acid concentrations of different pork cuts and variety meats are presented as affected by diets supplemented with peanut oil, hydrogenated coconut oil, or hydrogenated coconut oil plus cholesterol. With the higher linoleate diet, greater proportions of linoleic acid were deposited in all parts, and with the higher oleate diet greater proportions of oleic acid were deposited in the liver, kidney, ham, skin, boston butt, fat back, picnic, tongue, and bacon but not in the heart and chop. Only small proportions of lauric and myristic acids were present in all tissues studied although large proportions were consumed. Cholesterol feeding increased the liver linoleate and arachidonate contents and decreased the heart and kidney linoleate and arachidonate contents. Also, the liver and kidney stearate content increased, the liver palmitate content decreased, and the kidney palmitate content increased. With the same diet, the vital organs contained large proportions of arachidonate and were richer sources of linoleic acid than the depot fats and muscles.

INTRODUCTION

The texture, flavor, shelf-life, and nutritional value of meat depend to a large extent on the chemical composition of the meat product. It has been shown that the fatty acid concentration of meats is influenced by the type of fat consumed by the animal. Sink (1962) and Sink *et al.* (1964) found that there were great changes in the fatty acid content in the loin area of pig back fat. The rump region showed the least changes while the outside layer of the back fat was more dynamic than the inside layer. The heart and liver tissues increased in polyunsaturated fatty acid concentrations when pigs were fed linoleate-supplemented diets (Hill *et al.*, 1961). High levels of trienoic acids and low levels of tetraenoic acids also occurred with the unsupplemented diet. Oleic, palmitic, stearic, and linoleic acids were found in decreasing order of magnitude in the back fat and liver lipids of pigs given a potato diet (Jaarma, 1964).

In feeding pigs herring meal- and codliver oil-supplemented diets Notevarp *et al.* (1961) found that the triunsaturated acids seemed to have been lost or converted to other compounds, whereas these acids were deposited with a low-fat diet. Christensen (1964) observed that most of the dietary coconut oil was modified before deposition in the back fat of bacon pigs, whereas soybean oil was more directly deposited.

The fatty acid composition of body fat has been reported for steers (Edwards *et al.*, 1961; Cabezas *et al.*, 1965), sheep (Tove and Matrone, 1962), rabbits (Borgman, 1964), and chickens (Marion and Woodroof, 1963).

This investigation was undertaken to determine the fatty acid composition of pork cuts and variety meats of pigs fed diets supplemented with an unsaturated (peanut oil) and a saturated (hydrogenated coconut oil) fat. The effect of ingested cholesterol with the saturated fat was also investigated.

EXPERIMENTAL PROCEDURES

Twelve Hampshire pigs averaging 96 lb (range 80–112 lb) were allotted to three ration treatments of supplemental lipids. The treatments were diets containing 3% peanut oil, 3% hydrogenated coconut oil, or 3% hydrogenated coconut oil plus 0.5% cholesterol. These lipids were added to a basal ration of 67% ground corn, 20% hog supplement (40% protein; Cosby-Hodges Milling Co., Birmingham, Alabama) and 10% alfalfa meal (17% protein; Consolidated Blenders, Inc., Guntersville, Alabama). The cholesterol was added at the expense of ground corn.

The experimental animals were confined to concrete-floor houses until the experiment terminated. The pigs were slaughtered and cut up after 8 weeks on the experimental diets. Lipids were extracted with chloroform-methanol (2:1) from the diets, the different variety meats, and the cuts of fresh pork. To reduce fat oxidation to a minimum, atmospheres of nitrogen were maintained through the extraction and evaporation procedures. Lipid extracts were stored in 10-ml vials under nitrogen at −8°F for approximately two weeks before analysis.

After saponification with 4% potassium hydroxide in ethanol and acidification with hydrochloric

acid, the fatty acids were esterified by refluxing with absolute methanol with sulfuric acid as the catalyst. The fatty acid methyl esters were analyzed by GLC (Chung *et al.*, 1965). The gas chromatograph used was an Aerograph model A-350-B instrument with dual column and a hot wire thermal conductivity detector system. The polar liquid phase was diethyleneglycol succinate (20%, w/w) on 30-60-mesh firebrick. Ten-foot copper columns were used. Column temperature was 215°C, and helium gas flow 120 ml/min.

Fatty acid mixtures A, C, D, and F obtained from the National Institutes of Health were used to calibrate the instrument and to provide information on retention times for identification of sample components. Peak areas were calculated by a disc integrator. Peak areas of the standard mixtures were proportional to the weight percentages of the fatty acid methyl esters. The data presented do not include fatty acids with retention times greater than 20:4. Fatty acid composition is expressed as percent of total methyl esters.

Lipids were analyzed for each animal, and the results are reported as averages for 4 pigs with their standard deviations. Experimental data were analyzed by analysis of variance (Snedecor, 1959). Differences between means were located with the Newman-Keuls test (Duncan, 1955).

RESULTS AND DISCUSSION

Table 1 shows that pigs on the peanut-oil-supplemented diet consumed larger proportions of linoleic acid and smaller proportions of the shorter-chain fatty acids (caprylic, capric, lauric, and myristic) than pigs on the hydrogenated-coconut-oil-supplemented diet.

Table 1. Fatty acid composition of lipids extracted from the diets.

Fatty acid [a,b]	Peanut oil	Hydrogenated coconut oil
8:0	.....	2.6
10:0	<.1	2.8
12:0	0.4	22.9
14:0	0.4	8.7
16:0	14.0	13.5
16:1	0.6	0.4
17:0	0.1	.....
18:0	3.2	7.0
18:1	42.9	19.5
18:2	33.8	21.5
18:3	1.8	1.1
20:0	0.5	.....
20:1	0.8	.....
20:3	0.4	.....

[a] Carbon chain length: number of double bonds.
[b] Values expressed as % of total methyl esters.

The total polyunsaturated acid content was greater in the liver, heart, and kidney lipids of pigs on the peanut oil diet than those on the hydrogenated coconut oil diet (Table 2). This was accounted for, primarily, by the larger linoleate concentration with the peanut oil diet. Several workers (Hill *et al.*, 1961; Leat and Cuthbertson, 1962; Borgman, 1964; Widmer and Holman, 1950; Chung *et al.*, 1964) have shown that the linoleic acid content of the pig and other animal tissues was greater with a higher linoleic acid intake.

The heart arachidonic acid content was less with the hydrogenated coconut oil diet. The other polyunsaturated acid concentration was small in the three organ tissues. The total polyunsaturated acid content of the liver was greater than that of the heart and kidney. Although much smaller proportions of lauric and myristic acids were consumed by pigs on the peanut oil diet, the concentrations of these acids in the organ tissues were slightly less than for pigs on the hydrogenated coconut oil diet.

With cholesterol feeding, the fatty acid changes were greatest in the liver, where an increased deposition of linoleic and arachidonic acids occurred. Conversely, the proportion of the kidney linoleate and arachidonate decreased. The proportion of stearic acid increased in the liver, heart, and kidney, while that of palmitic acid decreased in the liver and increased in the kidney during cholesterol feeding. The liver oleate content showed a large increase.

The linoleic acid content of the meats in Table 3 was greater and the saturated acid (lauric, myristic, and stearic) content was less with the peanut oil diet except in the case of pork chop which showed no difference in stearate content. The oleic acid content, also, increased for all meat except pork chop.

In comparison, the organ tissues were richer sources of the polyunsaturated fatty acids, especially the essential fatty acids (linoleic and arachidonic acids). The total monoenoic acid content was less and the total saturated acid content was greater for the organ tissues than for the tissues in Ta-

Table 2. Fatty acid composition of vital organs of pigs on different lipid diets.

Fatty acid [a]	Liver [b,c]			Heart [b,c]			Kidney [b,c]		
	Peanut oil	Hydrogenated coconut oil	H. coconut oil + cholesterol	Peanut oil	Hydrogenated coconut oil	H. coconut oil + cholesterol	Peanut oil	Hydrogenated coconut oil	H. coconut oil + cholesterol
10:0	.....	.....	.....	<0.1	<0.1	0.2±0.1	0.1±0.1	0.2±0.1	<0.1
12:0	0.2±0.1	0.7±0.2	0.4±0.1	0.3±0.1	0.6±0.1	0.8±0.1	0.2±0.1	0.6±0.4	0.7±0.1
14:0	0.5±0.1	1.8±0.5	1.0±0.1	2.1±0.2	2.2±0.4	3.2±0.3	1.8±0.1	4.3±0.5	3.4±0.5
14:1	<0.1	<0.1	<0.1	0.2±0.1	0.1±0.1	0.4±0.1	.....	.....	.....
15:0	0.2±0.1	0.6±0.2	0.3±0.1	0.2±0.1	0.2±0.1	0.4±0.1	0.2±0.1	0.2±0.1	0.2±0.1
15:1	.....	.....	.....	1.1±0.1	0.7±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
16:0	20.9±0.7	21.2±1.0	17.1±0.2	20.4±0.5	21.6±0.5	21.7±1.0	23.3±0.1	23.9±3.4	26.2±0.7
16:1	1.7±0.1	2.6±0.6	1.0±0.1	2.8±0.2	2.6±0.2	2.0±0.1	2.0±0.1	2.3±0.6	1.9±0.1
17:0	0.5±0.2	0.8±0.1	1.8±0.4	0.9±0.1	0.7±0.1	0.7±0.1	0.6±0.1	0.6±0.1	0.8±0.4
17:1	0.1±0.1	0.2±0.1	0.3±0.1	0.8±0.1	1.0±0.2	0.5±0.1	0.3±0.1	0.4±0.2	0.4±0.2
18:0	20.8±1.2	26.1±2.0	30.8±1.6	13.0±0.7	13.3±0.6	15.2±0.8	13.3±0.2	14.3±0.9	22.9±1.3
18:1	25.8±1.5	19.9±1.7	10.4±0.5	32.4±0.5	35.4±0.4	36.7±1.9	34.2±2.1	30.6±1.8	28.7±0.5
18:2	15.8±0.5	11.7±0.6	14.2±0.6	16.5±0.4	13.0±0.4	10.3±1.0	14.0±0.3	12.3±0.3	7.7±1.1
18:3	0.2±0.1	0.1±0.1	0.1±0.1	0.4±0.1	0.6±0.2	0.3±0.1	0.5±0.1	0.4±0.1	0.2±0.1
20:0	<0.1	<0.1	0.4±0.1	.....	.....	.....	.....	.....	.....
20:1	0.1±0.1	0.3±0.1	0.9±0.2	0.5±0.1	0.6±0.2	1.3±0.1	0.9±0.1	0.8±0.1	0.9±0.3
20:2	0.6±0.2	0.4±0.1	0.6±0.1	0.7±0.5	0.6±0.1	0.7±0.1	0.8±0.1	0.5±0.1	0.3±0.1
20:3	0.7±0.2	1.4±0.5	1.8±0.1	0.6±0.2	0.7±0.2	0.4±0.1	0.6±0.1	0.6±0.1	0.3±0.1
20:4	11.2±0.7	12.2±0.9	18.4±0.4	6.6±0.3	3.9±0.2	2.3±0.1	6.9±0.4	7.9±0.4	5.2±0.6

[a] Same as footnote in Table 1.
[b] Mean for 4 pigs ± standard deviation of mean.
[c] Same as footnote *b* in Table 1.

Table 3. Fatty acid composition of some tissues of pigs on different lipid diets.

Fatty acid[a]	Ham[b,c]		Skin[b,c]		Boston butt[b,c]		Chop[b,c]	
	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil
10:0	0.1±0.1	0.1±0.1	0.2±0.1	0.2±0.1	<0.1	0.2±0.1	<0.1	0.2±0.1
12:0	0.1±0.1	0.7±0.1**	0.3±0.1	0.9±0.1**	0.2±0.1	0.8±0.1**	0.2±0.1	0.6±0.1*
14:0	1.7±0.1	2.9±0.1**	1.8±0.1	3.0±0.3*	1.6±0.1	2.9±0.1**	1.7±0.1	2.8±0.2**
14:1	0.3±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1
15:0	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1
16:0	23.1±0.3	24.4±0.5	21.8±0.9	24.4±0.5	23.1±0.3	24.4±0.3	24.7±0.4	26.6±0.9
16:1	3.7±0.2	4.3±0.2	4.2±0.5	4.6±0.3	3.6±0.1	3.7±0.1	4.1±0.2	4.9±0.2
17:0	0.3±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.3±0.1	0.3±0.1
17:1	0.3±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.2±0.1	0.2±0.1
18:0	7.8±0.5	9.4±0.6	6.4±0.2	7.7±0.4*	9.2±0.1	11.0±0.2**	10.5±0.2	10.4±0.7
18:1	47.2±1.1	44.6±0.8	51.6±1.5	48.1±0.9	47.1±0.2	42.8±0.9**	43.8±0.6	43.4±0.7
18:2	11.9±0.5	9.9±0.4*	10.6±0.6	7.9±0.5*	12.2±0.3	9.9±0.5**	10.5±0.6	8.1±0.3**
18:3	0.5±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.4±0.1	0.6±0.1	0.4±0.1	0.5±0.1
20:1	0.8±0.1	0.6±0.1	1.0±0.1	0.8±0.1	0.8±0.1	1.0±0.1	1.2±0.1	0.5±0.1**
20:2	0.6±0.2	.....	0.6±0.1	0.4±0.1	0.3±0.1	0.6±0.1*	0.8±0.1	0.3±0.1*
20:3	1.1±0.2	1.0±0.2	.....	.....	0.2±0.1	0.7±0.1**	1.1±0.2	0.7±0.3*

Fatty acid[a]	Back fat[b,c]		Picnic shoulder[b,c]		Tongue[b,c]		Bacon[b,c]	
	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil	Peanut oil	Hydrogenated coconut oil
10:0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	<0.1	0.1±0.1	0.1±0.1	<0.1
12:0	0.2±0.1	1.2±0.1**	0.1±0.1	0.7±0.1**	0.2±0.1	1.0±0.1**	0.1±0.1	1.1±0.1*
14:0	1.5±0.1	3.9±0.2**	1.6±0.1	3.0±0.2**	1.5±0.1	2.7±0.1**	1.5±0.1	4.0±0.3**
14:1	0.5±0.1	0.3±0.1	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1
15:0	0.1±0.1	0.3±0.1	0.2±0.1	0.3±0.1	0.1±0.1	0.2±0.1	<0.1	0.1±0.1
16:0	23.2±0.6	26.7±0.9*	22.9±0.2	24.2±0.5*	23.0±0.3	24.6±0.2**	24.6±0.4	28.0±0.1*
16:1	2.2±0.3	2.9±0.2	3.5±0.1	3.9±0.1	3.4±0.1	3.4±0.1	2.7±0.1	3.4±0.2*
17:0	0.5±0.1	0.8±0.2	0.3±0.1	0.3±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.6±0.1
17:1	0.3±0.1	0.5±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.6±0.1	0.4±0.1
18:0	10.9±0.1	12.7±0.4	8.7±0.2	9.1±0.5	8.4±0.1	7.7±0.1**	11.7±0.5	13.5±0.7
18:1	45.3±0.4	39.7±0.5**	48.1±0.3	44.8±0.9*	46.6±1.6	45.9±0.3	44.0±0.2	39.6±0.5**
18:2	13.2±1.1	8.5±0.5**	11.4±0.3	10.5±0.9	12.7±0.6	10.8±0.6*	12.5±0.5	8.3±0.1**
18:3	0.7±0.2	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.4±0.1	0.4±0.1	0.3±0.1
20:1	1.4±0.3	1.3±0.1	1.0±0.2	0.7±0.2	1.2±0.1	0.7±0.1*	<0.1	0.3±0.1
20:2	0.6±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.4±0.1	0.4±0.1	.....
20:3	.....	.....	0.6±0.1	0.7±0.2	0.7±0.2	0.8±0.2	.....	.....

[a, b, c] Same as footnotes in Table 2.
* $P < .05$.
** $P < .01$.

ble 3. The changes in the dietary fatty acids were reflected to a greater extent in the organ tissues than in the depot fats and muscles, and to a greater extent in the depot fats than in the muscles.

It is evident that the concentration of the essential fatty acid, linoleic acid, of pork will be increased by feeding pigs diets supplemented with higher proportions of this acid. In addition, it would appear that cholesterol feeding will be a factor in the variations resulting in the polyunsaturated fatty acid content of the organ tissues.

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Lemon Juice Composition. IV. Carotenoid and Sterol Content

SUMMARY

The carotenoids and sterols of lemon juice from various sources were measured. The average total carotenoid contents for reconstituted lemon juice concentrate and for fresh juice from coastal, desert, and Italian lemons were respectively 49, 50, 40, and 45 μg β-carotene/100 ml juice. The average respective sterol values were 8.28, 8.99, 9.10, and 8.46 mg β-sitosterol/100 ml juice. The major lemon juice sterol was β-sitosterol. The correlation between citric acid, carotenoids, and sterols was too low to be useful in the multiple regression analysis equation for the prediction of citric acid.

INTRODUCTION

Considerable work has been done on identification of the carotenoid pigments in highly colored citrus fruits (Curl and Bailey, 1956, 1957a, b, 1961; Curl, 1962). There are many procedures and variations for the analysis of the total carotenoids; Higby (1961) reviewed many of them and presented a modification of his own for orange juice. Until recently, however, relatively little attention has been given to the carotenoid composition of lemon juice (Yokoyama, 1964; Yokoyama and Vandercook, 1965).

Only a few workers have been concerned with the steroids of citrus. Swift (1952) found β-sitosteryl-D-glucoside in Florida Valencia orange juice, and Weizmann *et al.* (1955) found β-sitosterol in grapefruit peel oil. Weizmann and Mazur (1958), in addition to β-sitosterol, found a new sterol, citrostadienol, 4-α-methyl-$\Delta^{7,24(28)}$-stigmastadien-3β-ol (Mazur *et al.*, 1958), in grapefruit and orange peel oils. Chaliha *et al.* (1964) reported β- and γ-sitosterol in the peel of Assam lemons.

This investigation was made to measure the carotenoids and sterols in lemon juice, and to determine whether there is a correlation between the citric acid content and the carotenoids and/or sterols that might help to characterize lemon juice chemically.

EXPERIMENTAL

Materials. The commercial lemon juice concentrates tested were a broad sampling of California-Arizona lemons obtained over several growing seasons and from different processors. The fresh lemon juice samples were from approximately 30 lb of lemons picked from the same three trees at 2–3-week intervals. The Pryor Lisbon lemons from Yuma, Arizona (desert), were picked from the 1963 and 1964 seasons (October through December). The Frost Nuceller lemons from Ventura, California (coastal), were picked over the period of November 1963 to January 1965. The fresh Italian lemons were obtained from November 1963 to February 1965 through commercial channels in Europe.

Isolation of carotenoids and sterols. The extraction of carotenoids was essentially that of Curl and Bailey (1957a). These extracts also contain the sterols. Separation of the sterols as the digitonide precipitate has long been known (Windhaus, 1909), and is the basis of the AOAC (1960) method for sterols.

Since the carotenoids are light-sensitive, all solutions were kept out of direct light whenever possible. To 100 ml lemon juice was added 5–6 g of Celite and 7 g of $CaCO_3$. The mixture was filtered through a Buchner funnel and washed with water. The filter cake was transferred to a Waring blender with 75–100 ml absolute ethanol and blended. The ethanol slurry was filtered and the filter cake blended two more times with ethanol. To the combined alcohol extract was added 10 g KOH. After the KOH dissolved, the flask was covered with a towel and the mixture kept warm (40–50°C) for 30 min. The solution was cooled, transferred to a separatory funnel, and mixed with 150 ml ethyl ether (or about ½ of the volume of alcohol used). Saturated NaCl solution was added slowly and gently swirled. More water was added, and the aqueous phase separated. The ether phase containing the carotenoids and sterols was washed several times with water. The aqueous phase plus the washings was extracted with ether until the ether layer was colorless. The ether was washed with water and combined with the original ether phase. The ether solution was dried over $MgSO_4$ and evaporated to dryness under vacuum. The residue was dissolved in 10–15 ml absolute ethanol

and filtered through a sintered-glass filter into a tared 40-ml centrifuge tube. Four ml of 1% digitonin (in 80% ethanol) and water (20% of total volume) were added. After several hours the mixture was centrifuged. The carotenoid supernatant was decanted and the precipitate was washed and centrifuged first with 80% ethanol and then with ethyl ether. The washings and the supernatant solution were mixed and evaporated to dryness under vacuum. The residue was dissolved in ethanol and diluted to 25 ml, and the spectrum was measured in a 1-cm cell from 500 to 380 mμ in a Cary spectrophotometer, model 14. The total carotenoid content (Goodwin, 1955) as μg β-carotene/100 ml juice is equal to the absorbance at 447 mμ × 100.

The outside of the centrifuge tube containing the digitonide precipitate was wiped clean with an alcohol-moistened tissue. The tube was dried for 1 hr under vacuum and then weighed. It was re-weighed after another 30 min under vacuum. The sterol content as β-sitosterol/100 ml juice equals 0.248 times the weight of the digitonide precipitate.

Regeneration and identification of β-sitosterol. The sterols were regenerated from their digitonides with dimethyl sulfoxide by the method of Issidorides *et al.* (1962). The sterol and sterol acetate (prepared from acetic anhydride-pyridine) were recrystallized from diethyl ether. Melting points were determined on an Electrothermal melting-point apparatus. Sterol or sterol acetate solutions (1% in ether) were spotted on activated silica gel thin-layer plates developed with benzene-ethyl acetate (5:1) or cyclohexane-ethyl acetate (4:1) by the ascending technique at room temperature. When the solvent front had traveled about 16 cm in 3–4 hr, development was stopped and the plate was air-dried for 1 hr. After being sprayed with a 20% ethanolic solution of phosphomolybdic acid (Merck) the plate was heated about 5–10 min at 100°C. Blue-green spots appeared on a light-green background.

Infrared spectra of the samples in KBr pellets were measured on a Perkin-Elmer infrared spectrophotometer, model 137. The NMR spectra in CCl_4 solution were measured on a Varian spectrometer, model A-60, with tetramethyl silane as internal standard.

RESULTS AND DISCUSSION

To compare the data between lemon juice and lemon concentrates, some point of reference is necessary because the amount of fresh juice that went into the concentrate is impossible to determine. Normally, juice is concentrated to one of several constant citric acid values. Thus, when it is reconstituted to an average single-strength value it may or may not correspond to the original juice strength. For the sake of comparison, the sterol and carotenoid data in Table 1 were mathematically adjusted to a constant citric acid value of 100 meq/100 ml. The composition of the individual samples varied about twofold. However, the averages for each group, except the carotenoids from Yuma fruit, were quite close. The average carotenoid value for the Yuma samples is significantly lower at the 95% confidence level than are the other groups of samples.

The measurement of total carotenoids by means of absorbance at one peak has certain inherent limitations because of the different extinction coefficients and absorption maxima. However, it is a convenient way of estimating the total carotenoid content (Goodwin, 1955). Table 2 shows the recovery of β-carotene and β-sitosterol from lemon juice. The coefficient of variation for replicate samples was 8.9% for the carotenoids and 8.6% for the sterols. Recoveries ranged from 89 to 104%.

The β-sitosteryl glucoside which Swift

Table 1. Average carotenoid and sterol values of commercial concentrate, and fresh Yuma, Ventura, and Italian lemon juice samples mathematically adjusted to 100 meq citric acid/100 ml juice.

Source	No. of samples	Total carotenoids[a]			Sterols[b]		
		Av.	Std. dev.	Range	Av.	Std. dev.	Range
Commercial concentrate	26	49	9.9	29–71	8.28	1.50	4.94–11.34
Fresh Ventura	25	50	7.9	37–74	8.99	1.18	7.26–10.48
Fresh Yuma	12	40	10.5	29–66	9.10	1.39	5.92–10.25
Fresh Italian	10	45	14.8	24–75	8.46	0.82	7.52–10.17

[a] μg β-carotene/100 ml lemon juice.
[b] mg β-sitosterol/100 ml lemon juice.

Table 2. Recovery of β-carotene and β-sitosterol added to lemon juice.

	Total carotenoids[a]		Sterols[b]	
Sample	β-Carotene added	Carotenoids found	β-Sitosterol added	Sterols found
1	0	32	0	6.27
	0	37	0	6.93
	0	33	0	7.13
	0	39	0	5.98
	0	37	0	7.54
	0	40	0	7.13
		Av. 36		Av. 6.83
	22	54	2.44	9.28
	22	57	2.44	9.46
		Recovery 89%		Recovery 104%
2	0	38	0	9.60
	0	38	0	9.85
	0	35	0	9.18
	26	59	0.96	10.27
	26	62	0.96	10.66
	26	68		Recovery 97%
	26	67		
		Recovery 104%		

[a] μg β-carotene/100 ml lemon juice.
[b] mg β-sitosterol/100 ml lemon juice.

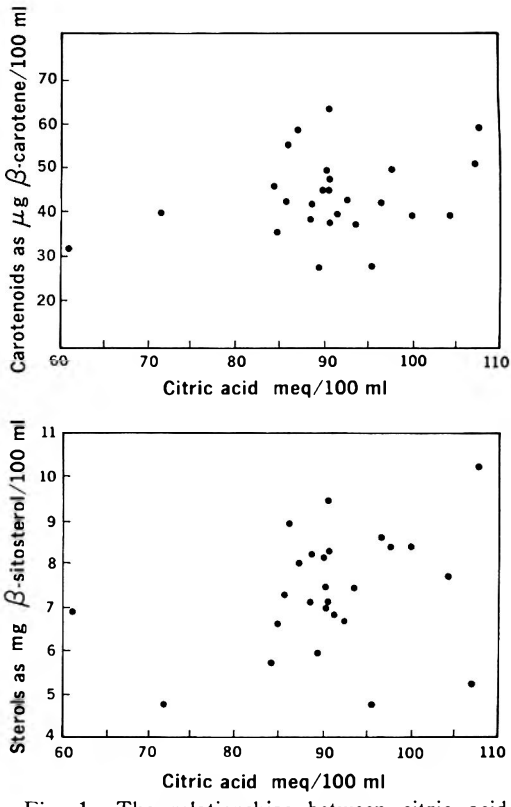

Fig. 1. The relationships between citric acid, carotenoids, and sterols of commercial lemon concentrate.

(1952) found in orange juice was also demonstrated in lemon juice by thin-layer chromatography at this laboratory (unpublished results). The glucosides are not hydrolyzed by the base, and can be observed at the ether-water interface in the extract following saponification.

The sterols, regenerated from their digitonides with dimethyl sulfoxide, were separated by thin-layer chromatography in several solvent systems along with β-sitosterol. In every case, the major lemon juice sterol (about 95% of the total) corresponded to the β-sitosterol spot. The melting points of crystallized samples of the lemon juice sterol and its acetate were identical with those of β-sitosterol and β-sitosteryl acetate, respectively 138.5–9.5° and 127–8°C (corr). The IR and NMR spectra were also the same.

One purpose of this investigation was to determine whether any useful correlation existed between the citric acid content and the carotenoid and/or sterol content of lemon juice samples. Fig. 1 illustrates the lack of a direct relationship between citric acid, the sterols, and the carotenoids. Furthermore, the low correlation coefficients (0.290 between citric acid and total carotenoids; 0.300 between citric acid and sterols) make it impractical to use these analyses to expand the multiple-regression equation (Rolle and Vandercook, 1963) for the prediction of citric acid. However, the analyses might be useful in helping to chemically characterize citrus juices in general. The data may also be useful in comparing the carotenoid and sterol contents of unknown or questionable lemon juice samples.

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Reference to a company or product name does not imply endorsement.

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Isolation and Spectral Characterization of Coumarins in Florida Grapefruit Peel Oil

SUMMARY

Nine coumarins were isolated from Florida grapefruit peel oil. Some of their physical properties are given. Bergamottin, limettin, bergapten, and two formylated coumarins found during this investigation have not been reported previously in grapefruit.

INTRODUCTION

This investigation is part of a comprehensive study of the composition of citrus products and the relationship of composition to quality. During this work, a number of regions on thin-layer chromatoplates showed under UV light a fluorescence characteristic of coumarins. Stanley and Vannier (1957a, b), Peyron (1963), Stanley (1963), and Stanley *et al.* (1965) have described the separation and characterization of many coumarins in citrus.

This paper reports the isolation of nine coumarins found in Florida grapefruit (Table 1), five of which, bergamottin, limettin, bergapten, 5-[(3,6-dimethyl-6-formyl-2-heptenyl)oxy]-psoralen and 7-methoxy-8-(2-formyl-2-methylpropyl)-coumarin, have not been reported previously in grapefruit. Proof of structure of the above formylated coumarins, accompanied by spectral means, will be published elsewhere (Fisher and Nordby, 1965).

EXPERIMENTAL

Spectroscopic measurements. All nuclear magnetic resonance (NMR) spectra were obtained with a Varian A-60 spectrometer, with tetramethylsilane used as an internal reference. IR spectra were obtained in $CHCl_3$ solutions or KBr with a Perkin-Elmer infracord spectrophotometer, model 137. Ultraviolet comparisons were made in absolute ethanol on a Cary 14 spectrophotometer. Fluorescence spectral data were obtained in absolute ethanol with an Aminco-Keirs spectrophosphorimeter operated as a spectrophotofluorometer. Mass spectra were obtained with a Bendix (TOF) mass spectrometer, model 12-100. The source was operated at 70 e.v. and recorded on an EAI X-Y Variplotter.

Reagents and materials. $FeCl_3$—$K_3Fe(CN)_6$ spray reagent was prepared by mixing equal volumes of 1% $FeCl_3$ in methanol and 1% aqueous $K_3Fe(CN)_6$.

Solvent systems for development of thin-layer chromatograms.

1) Ethyl acetate

2) Toluene-ethyl acetate-acetic acid (5:4:1) solution (v/v/v)

Table 1. Coumarins and psoralens separated on silica gel and obtained from the various hexane-ethyl acetate eluate fractions.

Fractions combined	Total fraction volume (L)	% ethyl acetate in eluate	Compound isolated	Comp. no.
18–24	6.0	2.5	bergamottin	I
25–59	40.0	2.5	7-geranyloxycoumarin	VI
60–70	12.0	2.5	osthol	IV
89–99	8.0	5.0	limettin	V
100–110	12.0	5.0	bergapten	III
128–136	9.0	7.5	5-[(3,6-dimethyl-6-formyl-2-heptenyl)oxy]-psoralen[a]	
159–167	10.0	10.0	7-methoxy-8-(2-formyl-2-methylpropyl)-coumarin[a]	
189–190	2.5	100	bergaptol	II
191	1.0	100	7-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]-coumarin	

[a] Proof of structure to be published elsewhere.

3) Toluene-dioxane-water (1:1:1) upper layer, solution (v/v/v)

4) Cyclohexane-ethyl acetate (3:1) solution (v/v)

5) Tetrahydrofuran-hexane (3:1) solution (v/v).

The thin-layer chromatoplates of silica gel with rice starch as a binder were prepared according to Smith and Foell (1962).

Separation and extraction of coumarins. Five gallons of cold-pressed Florida grapefruit peel oil were concentrated at a ratio of 12:1 with an Arthur F. Smith rotafilm molecular still, at 60–65° (6 mm), to a residue of 1200 ml. This residue was extracted with acetonitrile. The acetonitrile was removed with a rotary evaporator at 60° (6 mm), affording the crude coumarin extract.

Chromatographic separations were achieved in a manner similar to a published procedure using silica gel with hexane and hexane-ethyl acetate (Stanley and Vannier, 1957a) and on 100 g of neutral alumina (80–200-mesh) in a 25 × 520-mm column employing hexane and hexane-benzene (1:1) solution (v/v). Aliquots of the fractions were spotted on thin-layer chromatoplates and developed with solvent systems 1, 2, or 3. The chromatoplates were examined under ultraviolet light and sprayed with $FeCl_3$-$K_3Fe(CN)_6$ reagent, which instantly detected phenolic compounds as dark blue areas. Nonphenolic psoralens were observed as light-blue areas after the application of heat. Under ultraviolet light, coumarins fluoresced violet to blue whereas psoralens were reddish-brown to yellow. Various fractions were combined according to their R_f values and fluorescence on thin-layer chromatoplates. Table 1 lists the fractions as combined off the silica gel column and the coumarins isolated.

Melting points are uncorrected and were obtained with a Thomas-Hoover uni-melt apparatus.

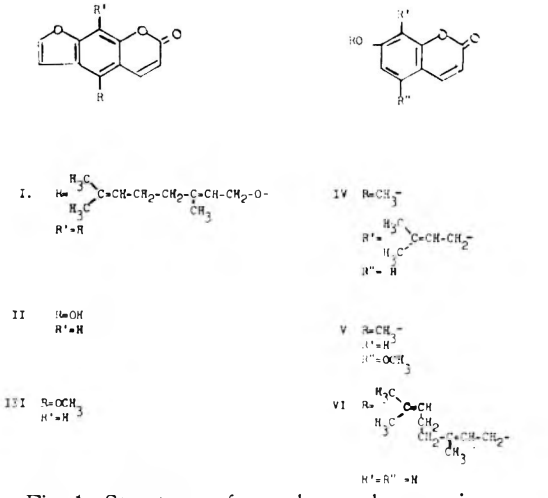


Fig. 1. Structures of psoralens and coumarins.

RESULTS

The coumarins, 7-geranyloxycoumarin VI and osthol IV (mass spectrum, Barnes and Occolowitz, 1964) and the psoralen bergaptol II, all previously reported in grapefruit (Stanley, 1963) were identified by IR, UV, NMR, and mass spectrometry, as well as R_f values as seen in Table 2.

Bergamottin, 5-geranyloxpsoralen (I). Concentration of fractions 18–24, from the silica gel column, followed by further purification on the alumina column, separated a compound which fluoresced yellow under ultraviolet light. Mild acid hydrolysis afforded bergaptol, which was identified by spectroscopic means. The NMR spectrum (Table 3) of compound I is consistent with the structure of bergamottin. Compound I was identified as bergamottin by comparing its infrared, ultraviolet, and mass spectra, as well as its R_f values, with those of authentic bergamottin.

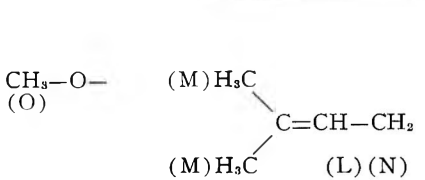
Limettin, 5,7-dimethoxycoumarin (V). Fractions 89–99 afforded solid material from which a

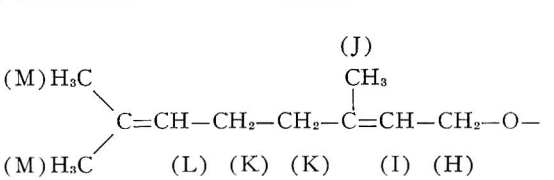
Table 2. Fluorescence and R_f values of coumarins and psoralens.

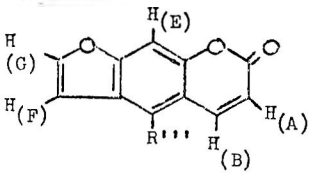
Substance no.	Substance	Fluorescence under UV light (3660 Å)	Solvent systems 1	2	3	4	5
I	bergamottin[a,b]	yellow	0.67	0.86	0.94	0.49	0.58
II	bergaptol[a]	reddish-brown	0.48	0.48	0.61	0.05	0.40
III	bergapten[a,b]	yellow	0.46	0.57	0.88	0.12	0.41
IV	osthol[a,b]	purple	0.52	0.71	0.93	0.23	0.51
V	limettin[a,b]	blue	0.47	0.57	0.89	0.12	0.44
VI	7-geranyloxycoumarin[a]	purple	0.63	0.83	0.93	0.41	0.58

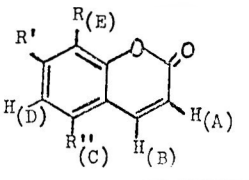
[a] Isolated.
[b] Authentic.

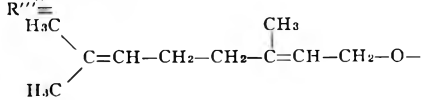
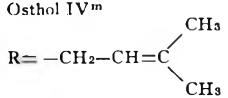
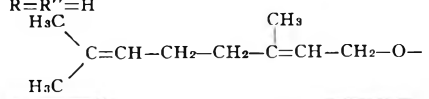
Table 3. NMR data of coumarins and pasoralens showing positions[a] at proton assignments.









Compound	Assignments															J-Values cps				
	A[b,c]	B[b,c]	C	D	E	F[b,c]	G[b,c]	H[c,d]	I[b,h]	J[g]	K[f]	L[h]	M[g]	N[c,d]	O[e,g]	J_{AB}	J_{CD}	J_{FG}	J_{HI}	J_{LN}
Bergamottin I[m] R'''= 	6.22	8.12			7.05[b,g]	6.96	7.56	4.94	5.55	1.66[e] or 1.59[e]	2.12	5.10	1.66[e] or 1.59[e]			10		2.5	7.0	
Bergaptol II[n] R'''=OH	6.28	8.28			7.16[b,g]	7.24	7.92									9.5		2.5		
Bergapten III[m] R'''=OCH_3	6.24	8.11			7.10[b,g]	7.00	7.56								4.24	10		2.5		
Osthol IV[m] R=  R'=OCH_3 R''=H	6.25	7.61	7.32[b,e]	6.84[b,e]								5.30[k]	1.69[e] and 1.88	3.56	3.95	9.5	9.0			7.0
Linettin V[m] R=H R'=R''=OCH_3	6.30	7.95		(6.02–6.45)[l]											3.85 and 3.88	10				
7-geranyloxycoumarin VI[m] R=R''=H 	6.25	7.65	7.38[j]	6.88[b,c,j]	6.84[j]–6.94			4.64	5.52	1.78[e]	2.14	5.17[l]	1.65[e] and 1.70			10	9.0		6.5	

[a] Values with reference to TMS ($\delta = 0$).
[b] Relative intensity = 1 proton.
[c] Doublet.
[d] Relative intensity = 2 protons.
[e] Relative intensity = 3 protons.
[f] Relative intensity = 4 protons.
[g] Singlet.
[h] Triplet.
[i] Multiplet.
[j] See spectrum no. 294, High Resolution NMR Spectra Catalog, Varian Associates, Palo Alto, California (1962).
[k] Weak signal.
[l] Center of broad signal.
[m] Spectrum obtained in $CDCl_3$.
[n] Spectrum obtained in dimethyl sulfoxide.
[o] Relative intensity = 6 protons.

blue fluorescent compound was isolated by preparative thin-layer chromatography using solvent system 5. The IR, UV, and mass spectra of V, as well as R_f values, were identical with those of authentic limettin.

Bergapten, 5-methoxypsoralen (III). Fractions 100–110 afforded crystals which were repeatedly recrystallized from acetone to a constant melting point of 188–189°. The admixture melting point with authentic bergapten remained unchanged. The R_f values of III and authentic bergapten were identical, as were their IR, UV, NMR, and mass spectra (Barnes and Occolowitz, 1964).

DISCUSSION

The ions formed from the α-pyrone system under electron impact are discussed in papers by Barnes and Occolowitz (1964) and Vul'fson *et al.* (1963). A m/e 191 ion ($M-CH_3$) present in the mass spectrum of 6,7-dimethoxycoumarin (Barnes and Occolowitz, 1964) is absent, as expected (Shapiro and Djerassi, 1965), in the mass spectrum of 5,7-dimethoxycoumarin V (Fig. 2). The same basic reasoning employed by Shapiro and Djerassi (1965) would explain the fragmentation of V. The absence of a parent ion at m/e 338 for I and the small parent peak observed at m/e 298 for VI is a reflection of the facile fragmentation of the allylic aliphatic side chain (Barnes and Occolowitz, 1964). The ions from m/e 137 to 41 for I and VI in Fig. 2 are characteristic of the geranyl group fragmentation (von Sydow, 1963).

The downfield shift of the C-4 proton to about 8, which is beyond the usual position of about δ7.6 (Bhacca *et al.*, 1962; Stanley *et al.*, 1965; Bottomley, 1963; Nielsen and Lemmich, 1964), as seen with compounds I, II, III, and V in Table 3, occurs in other C-5 alkoxyl-substituted coumarins such as 5,8-dimethoxypsoralen and 5-prenoxypsoralen (Sheinker, 1964). The proposal is therefore made that this shift can serve as a diagnostic aid in the structural definition of coumarins.

The bathochromic shift observed in the emission spectra of both I and III over that seen for IV, V, and VI is ascribed to the

Table 4. Fluorescence of coumarins and psoralens.

Compound no.	Coumarins	Wave lengths (mμ) Excitation	Emission
IV	osthol	353	390
V	limettin	338	420
VI	7-geranyloxycoumarin	358	390
	Psoralens		
I	bergamottin	320	485
III	bergapten	335	482

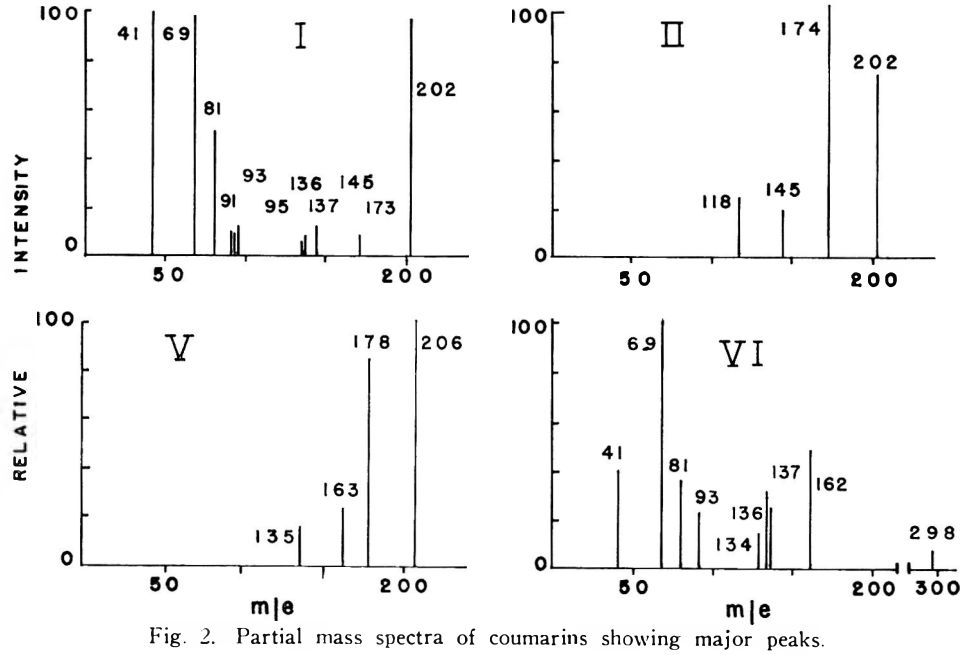


Fig. 2. Partial mass spectra of coumarins showing major peaks.

furano structure of psoralens (Ichimura, 1960).

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Mention of brand names does not imply endorsement.

RESEARCH NOTE

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Citrus Bitter Principles IV. Occurrence of Limonin in Grapefruit Juice

The bitterness of grapefruit juice has been ascribed to certain flavonoid glycosides, especially naringin, whereas the bitterness of Navel orange juice is caused by limonin (Higby, 1938; Emerson, 1948; Kefford, 1959). For studies on the flavonoid composition of grapefruit peel, see, for example, Horowitz and Gentili (1961) and Dunlap and Wender (1962). In view of the widespread interest in the bitterness of grapefruit (Thomas *et al.* 1958; Ting, 1958; Griffiths and Lime, 1959; Nomura, 1963) it is important to know whether limonoids are also present in the juice of that fruit. It has now been found that limonin occurs in significant amounts in grapefruit juice at levels above its taste threshold. Although limonin is known to occur widely in seeds of citrus fruits it has been reported previously to occur in the juice of only the sweet orange.

Examination of chloroform extracts of grapefruit juice and pulp by thin-layer chromatography (silica gel–calcium sulfate; benzene-ethanol-water-acetic acid; 200:47:15:1) (Hay *et al.* 1963) using Ehrlich's reagent as a selective spray for limonoids (Dreyer, 1965) showed limonin to be present in all samples examined. [Both TLC and isolation work show obacunone and deacetylnomilin (Dreyer, 1965), in addition to limonin, to be constituents of grapefruit and Navel orange seeds. These minor limonoids were not observed in either grapefruit or Navel juice extracts.] These included commercially canned juice, frozen concentrate, and crater-dried powder, and laboratory-prepared juice and pulp (endocarp tissue) from fresh fruit grown in Arizona, California, Florida, and Texas. The fresh fruit samples were of the Marsh seedless, pink, and Ruby Red Varieties. Pulp from the fresh fruit was carefully prepared so that all albedo and seeds (including undeveloped ones) were excluded.

To confirm these results further, limonin was isolated in crystalline form from a large batch of commercial grapefruit concentrate and from laboratory-extracted juice. [A small amount of β-sitosterol, β-sitosterol-D-glucoside (Ma and Schaffer, 1953) and 16.2 ppm of bergaptol (5-hydroxypsoralen) were isolated in crystalline form from grapefruit juice. Each was identified by color tests, R_f values, melting points, infrared, and ultraviolet spectral (bergaptol only) comparison with the authentic compounds.] The limonin was identical in all respects to an authentic sample (IR, mp, R_f values, color reaction, and taste). It was found by isolation to occur in amounts up to 9.5 ppm (on a reconstituted-juice basis). Since the bitter taste of limonin is detectable at 0.75 ppm and since orange juice is distinctly bitter at 2 ppm (Kefford, 1959), limonin was present in sufficient quantity to contribute to the bitterness of the grapefruit juice.

All samples of grapefruit tested were mid- and late-season fruit (February through April). The presence of limonin in late-season fruit is especially significant because limonin decreases and disappears from Navel orange juice as the fruit ripens beyond commercial maturity. In the late-season grapefruit tested, limonin was still present six months after the fruit had reached commercial maturity. Apparently, limonin persists longer after commercial maturity is reached in the grapefruit than it does in the Navel orange.

Limonin was found in all three major parts of grapefruit: peel, endocarp, and seeds. In the endocarp of late-season fruit it is located primarily in the carpellary membranes and the central vascular bundle. The limonin content of this tissue was estimated by isolation to be about 140 ppm (on a wet-weight basis). Since limonin or its pre-

cursor probably enters commercially prepared juice by diffusion from the pulp tissue during extraction and processing (Kefford, 1959, p. 351) the particular methods used in preparing the juice will influence its limonin content.

The limonin content of grapefruit may also prove to be dependent on a number of factors, such as variety, rootstock, environment, cultural practices, etc. Some of these factors are known to have an influence on the limonin level in the sweet orange (Kefford and Chandler, 1961; Marsh, 1953). While much remains to be learned about limonin in grapefruit it is clear that its presence, in addition to the bitter flavonoid glycosides, must be considered when dealing with the problem of bitterness in grapefruit.

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Conversion of Valencene to Nootkatone

SUMMARY

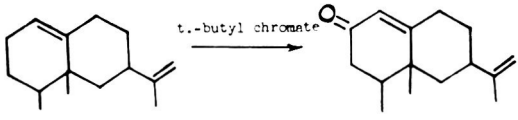
Nootkatone, an important flavoring constituent in grapefruit, has been synthesized from the sesquiterpene hydrocarbon valencene. Valencene, which is isolated from orange oil, was oxidized with tert.-butyl chromate to yield a sesquiterpene ketone having the same physical properties as the natural nootkatone.

INTRODUCTION

A new sesquiterpene hydrocarbon, valencene (I), recently reported to be a constituent of citrus oils (Hunter and Brogden, 1965b), has now been converted to the sesquiterpene ketone, nootkatone (II). Valencene has been shown to have the same parent structure as nootkatone (Hunter and Brogden, 1965a), the structure of the latter having been elucidated more recently by MacLeod (1965). Since nootkatone is an important flavoring constituent in grapefruit (MacLeod and Buigues, 1964), the conversion of valencene to nootkatone provides an additional source of this flavoring material.

A suitable quantity of valencene for conversion to the ketone was isolated from orange condensate oil.

Conversion of valencene into nootkatone was accomplished in good yield with tert.-butyl chromate, an oxidative reagent which reacts selectively at the allylic position. The synthetic nootkatone thus obtained was identical to that isolated from grapefruit essential oil.



Isolation of valencene (I). Five gallons of orange condensate oil was obtained from an orange concentrate plant. This oil was prepared by condensing the vapors removed from orange juice in the first stage of the evaporator during the preparation of orange juice concentrate. The oil was separated from the aqueous phase by centrifuging. The oil was distilled at 80°C and a vacuum of 1.0 mm Hg in a Swissco Rotavac evaporator to remove most of the limonene. The residue (450 ml) was steam-distilled to give 150 ml of limonene, which came over first, followed by 200 ml of valencene. The oxy-

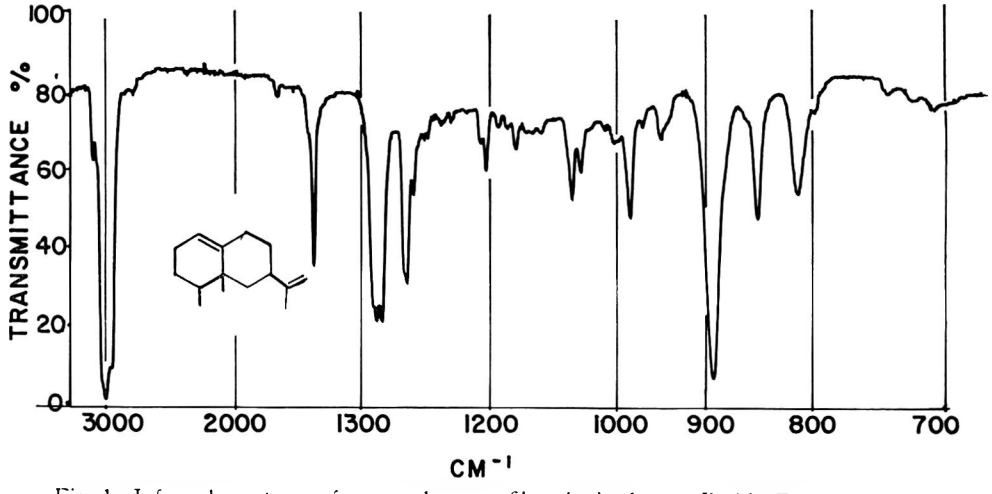


Fig. 1. Infrared spectrum of pure valencene, film obtained on a Perkin-Elmer spectrograph, model 137.

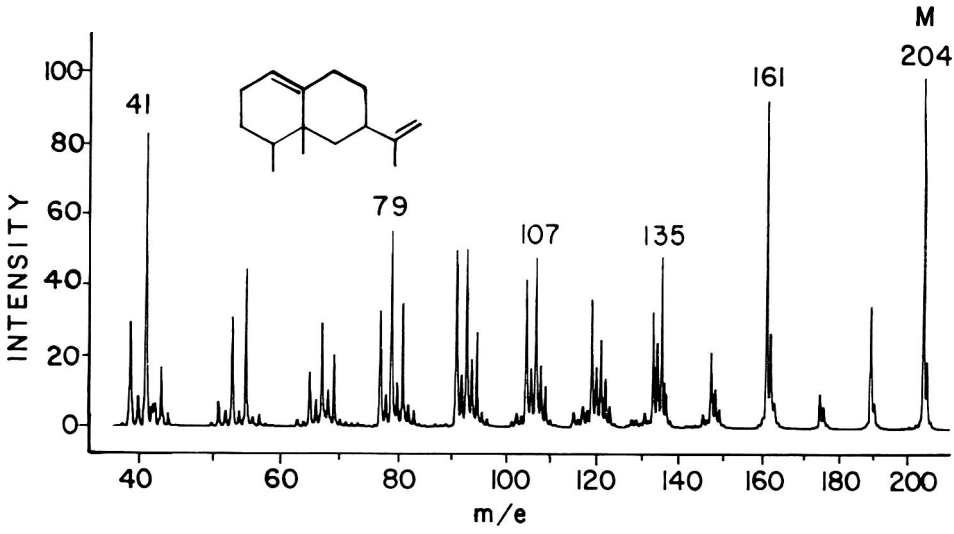


Fig. 2. Mass spectrum of valencene obtained on a Bendix Time-of-Flight mass spectrometer, model 12107, with source operated at 70 e.v. in the pulsed mode.

gen-containing materials in the crude valencene were removed by percolation though 1 lb of basic alumina with hexane used as the eluant. The solvent was removed and the residue rectified in a 3-ft spinning band column at 108°C and 5.0 mm Hg to yield 150 ml of valencene. Gas chromatographic analysis by the method of Hunter and Brogden (1965a) showed the valencene to be of high purity. Its physical properties were: bp 123°C (10 mm Hg), N°_{20} 1.5075, d_{28} 0.914, $[\alpha]_D$ + 112.8°. The infrared and mass spectra are respectively shown in Figs. 1 and 2.

Tert.-butyl chromate. Tert.-butyl chromate was prepared by the cautious addition of 20 ml of tert.-butanol to 6.8 g of CrO_3 while maintaining the temperature at 30°C with cooling according to the method of Oppenauer and Oberrauch (1949). Upon complete addition the solution was stirred for 15 min, after which 60 ml of CCl_4 was added. The resulting solution was washed three times with water and dried over Na_2SO_4. Ten ml of acetic acid and 2.6 ml of acetic anhydride were added to the dried solution of tert.-butyl chromate, completing preparation of the reagent.

Oxidation of valencene (I) to nootkatone (II). The tert.-butyl chromate reagent was added dropwise to 2.5 g of I in 60 ml of CCl_4, 10 ml of acetic acid, and 2.6 ml of acetic anhydride over a period of 45 min as described by Marshall *et al.* (1957). During the addition the temperature was maintained at 50°C, then raised to 60°C and stirred for 22 hr. The reaction products were cooled to 20°C, diluted with 180 ml of a 10% oxalic acid solution, and stirred for 30 min. The CCl_4 layer was separated from the mixture, washed twice with water, once with a 10% solution of K_2CO_3, again with water, and dried over Na_2SO_4 to yield 1.8 g (67%) of the crude ketone. Nootkatone semicarbazone was prepared, recrystallized twice from ethanol and water, and melted at 194–195°C (dec.), $[\alpha]_D$ + 364° (C = 0.39 in $CHCl_3$). A mixed melting point of synthetic and natural nootkatone semicarbazones showed no depression. Erdtman and Hirose (1962) reported a melting point of 196–197°C (dec.) and $[\alpha]_D$ + 384° (C = 0.51 in $CHCl_3$). The infrared and mass spectra of natural and synthetic nootkatone were identical.

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Mention of brand names does not constitute endorsement.

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Radiation Resistance of Spores of Clostridium Species In Aqueous Suspension

SUMMARY

Data are presented enabling survivor curves under gamma rays to be constructed for unheated spores of the following clostridia: *Cl. aerofoetidum*; *Cl. bifermentans*; *Cl. botulinum* types A, B, C, D, E, and F; *Cl. butyricum*; *Cl. caloritolerans*; *Cl. chauvoei*; *Cl. histolyticum*; *Cl. fallax*; *Cl. oedematiens* types A, B, and C; *Cl. septicum*; *Cl. sordellii*; *Cl. sphenoides*; *Cl. sporogenes*; *Cl. subterminale*; *Cl. tertium*; *Cl. tetanomorphum*; *Cl. welchii* types A, B, C, E, and F. After irradiation at 18–23°C, curves comprised a "shoulder" extending to about 0.25–0.35 Mrad, followed by an exponential kill over 6–8 log cycles with a decimal reduction value from 0.08 to 0.25 Mrad.

INTRODUCTION

In the field of radiation microbiology, there is a serious lack of detailed information on the resistance of anaerobic spores other than *Clostridium botulinum* types A and B, and *Cl. sporogenes*.

It is now clear that radiation sterilization of food for human consumption is difficult because it requires a dose large enough to cause objectionable organoleptic changes. Work is therefore concentrated on "pasteurizing" doses of radiation (0.1–0.6 Megarad) coupled with a second process, such as refrigerated storage or mild heating to destroy autolytic enzymes. It is at these relatively low radiation dose levels that surviving bacteria must be carefully considered.

The purpose of this work is simply to ascertain the resistance to radiation of a wide selection of clostridial spores under standard conditions. From this can be made a fairly reliable estimate of the resistance of these organisms under a variety of conditions, and it becomes possible to estimate the likelihood that they might survive particular irradiation treatments.

MATERIALS AND METHODS

Aqueous suspensions of unwashed spores which had not been heat-shocked of as many species of clostridia as possible were prepared and stored at 1°C. One-ml samples were irradiated in sealed freeze-drying ampoules at normal ambient temperatures.

Organisms. The organisms tested were:

Cl. aerofoetidum (NCTC 505); *Cl. bifermentans* (NCIB 506)
Cl. botulinum type A (NCTC 7272, 1192Y, 62)
type B (NCTC 751, 213B)
type C (NCTC 8264) type D (NCTC 8265);
Cl. butyricum (NCIB 7423);
Cl. caloritolerans (NCIB 9360); *Cl. chauvoei* (NCTC 8070);
Cl. fallax (NCTC 8380); *Cl. histolyticum* (NCIB 503);
Cl. oedematiens (novyi) type A (NCTC 538); type B (NCTC 9691; type C (NCTC 9746); type D (NCTC 9692);
Cl. septicum (NCIB 547);
Cl. sordellii (NCIB 2914); *Cl. sphenoides* (NCTC 507);
Cl. sporogenes (NCTC 532; PA 3679/S_2); *Cl. subterminale* (NCIB 9384);
Cl. tertium (NCIB 541); *Cl. tetanomorphum* (NCTC 2909);
Cl. welchii (perfringens) type A (NCTC 8237), type B (NCTC 3110), type C (NCTC 3181), type E (NCTC 8084), type F (NCTC 8081).

Media. The media used were reinforced clostridial medium (RCM) ("Oxoid" Oxo Ltd., London S.E.1.); reinforced clostridial agar (RCA) ("Oxoid"); cooked meat medium (CMM) comprising Hartley's digest broth (Mackie and McCartney, 1960) with twice the usual amount of meat; TPG (Schmidt *et al.*, 1962; Roberts, 1965); TPAY (TPG with added ammonium sulfate 1% (w/v) and yeast extract (Difco) 0.1% (w/v)).

Preparation of spore suspensions. Sporulation at 37°C was observed and followed by phase-contrast microscopy. At optimal spore formation, spores were removed by centrifugation, resuspended in distilled water, and stored at 1°C. Suspensions were not heat-shocked during preparation.

Irradiation. One-ml samples of aqueous spore suspension in freeze-drying ampoules sealed under

air were gamma-irradiated at ambient temperature (18–23°C) in a 4,000-curie cobalt-60 source (Nuclear Chemical Plant Ltd., London) at 1.05 Megarad (Mrad) per hour. Suspensions contained 3.58×10^7 to 7.33×10^8 spores per ml, with three exceptions: *Cl. botulinum* type C (2.65×10^6), *Cl. botulinum* type A (4.98×10^6), and *Cl. subterminale* (2.36×10^6).

Viable counts. Decimal dilutions of spore suspensions were made in RCM within 1 hr of termination of radiation. Duplicate 1/40-ml samples of each dilution were placed in 4-cm-diam. sterile plastic Petri dishes (Sterilin Ltd., Richmond, Surrey), and 10–12 ml of molten (⩾50°) RCA was added to give pour plates. Where inactivation was too great to give colonies after dilution, 1/40-ml samples of the irradiated spore suspension were placed in the dishes. Similarly, 1-ml samples of irradiated suspension in 9-cm plastic dishes ("Oxoid") increased further the observable range of inactivation. Plates were incubated in anaerobic jars (Baird & Tatlock Ltd., Chadwell Heath, Essex) at 37°C. Results were calculated using counts at two or more dilutions with weighting for dilution, as described by Farmiloe *et al.* (1954).

RESULTS

Counts of viable survivors were expressed as a percentage of an unirradiated control, and are tabulated as $\log_{10}$ of this value in Table 1. Typical survivor curves are presented in Figs. 1 and 2.

Ideally, spores should have been obtained from only one medium, but this was not possible, although almost all species sporulated to a greater or lesser extent in CMM. *Cl. histolyticum* sporulated in CMM, TPG, and TPAY. Three survivor curves were therefore constructed (Fig. 1), and two each for *Cl. bifermentans* and *Cl. tetanomorphum* (Fig. 2).

DISCUSSION

The data presented have a bearing on several aspects of radiation processing of food, which are discussed in the following order:

1) desirable degree of inactivation; 2) radiation resistance of clostridia other than *Cl. botulinum*; 3) the importance of the "shoulder" on survivor curves; 4) indicator organisms; and 5) proteolysis and toxin production.

1) A large amount of information on the radiation resistance of *Cl. botulinum* and *Cl. sporogenes*, generally the strain referred to as PA 3679, has been published (e.g. Morgan and Reed, 1954; Schmidt and Nank, 1960; Schmidt *et al.*, 1962; Denny *et al.*, 1959; Pratt *et al.*, 1959; Anellis and Koch, 1962; Kempe *et al.*, 1957). *Cl. botulinum* has been studied because of its importance to the food industry as a fairly commonly occurring pathogen which must be eliminated by processing or prevented from growing and producing toxin by storage control. Hence, a cooking process was developed sufficient to reduce the count of *Cl. botulinum* by a factor of about 10^{12}, which was based on the heat resistance studies of Esty and Meyer (1922), although grounds for justification of this factor are vague. In the case of canned cured meats, an inactivation of about 10^4 has been found effective and safe. In the interest of public health, radiation processing must be no less safe than heat processing. Similar inactivation requirements should presumably be demanded of irradiated foods.

Table 2 indicates that, assuming a protec-

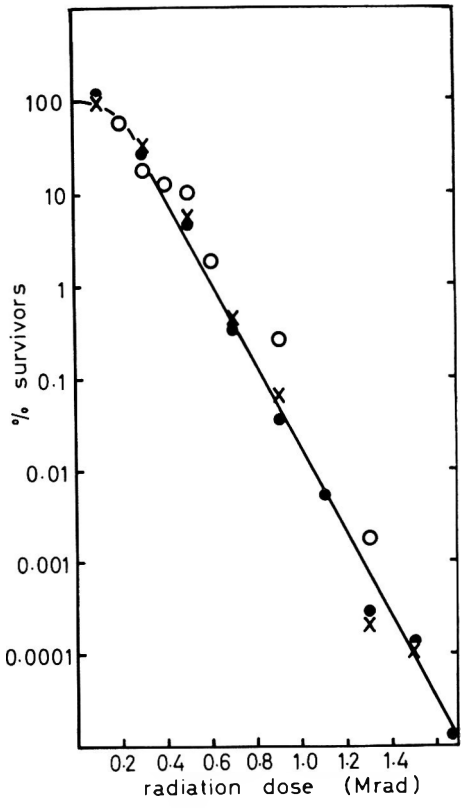

Fig. 1. The effect of sporulation medium on the radiation resistance of spores of *Cl. histolyticum*.
●, cooked meat medium; ○, TPG*; ×, TPAY*
* see text.

tion factor of ×2–3 in food products (see later), pasteurizing doses of radiation are unlikely to reduce the clostridial count by more than a factor of ×10, and not until about 1 Mrad might a reduction of a hundredfold or more be anticipated. Although evidence is accumulating that the numbers of clostridia that contaminate food products are of a relatively low order, the fact that it is possible to isolate these organisms suggests that there may well be sufficient numbers to survive low-dose radiation processing with significant frequency if the total quantity of material to be processed is considered. Heat processing equivalent to 10^{12} inactivation of *Cl. botulinum* spores has been shown to give an acceptable margin of safety. Pasteurizing doses of radiation in the absence of complementary processes would give comparable safety only if the original level of contamination by anaerobes is about 10^{10} times smaller than the contamination in foods ordinarily preserved by heat processing. In this context, curing agents can probably be classed as a complementary process, although data are lacking. Since anaerobes have already been isolated from food products, the above level seems unrealistically low, and the survival of anaerobic spores after pasteurizing doses of radiation seems certain. Storage of irradiated food products at refrigeration temperatures prevents toxin production, although the ability of *Cl. botulinum* type E to grow and produce toxin at about 3°C makes it necessary to enforce such storage control rigidly. The successful distribution of heat-pasteurized foods stored under refrigeration suggests such rigid storage control to be possible. Only large-scale studies will determine the relative frequencies of toxic and nontoxic postirradiation spoilage at various storage temperatures.

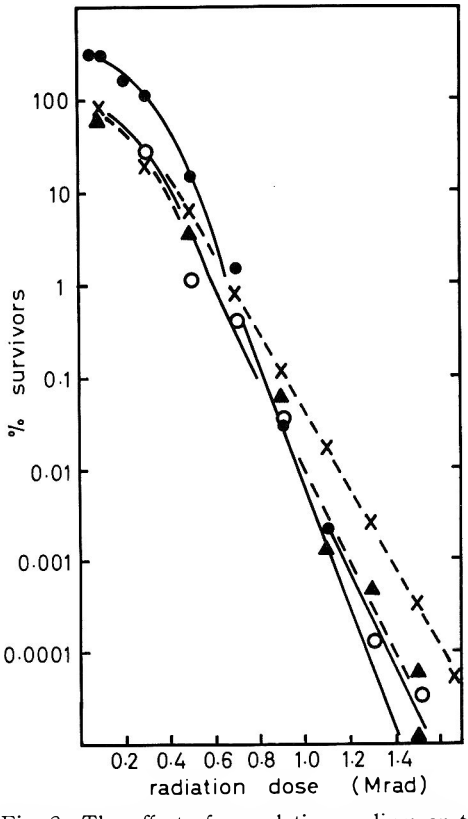

Fig. 2. The effect of sporulation medium on the radiation resistance of spores of *Cl. bifermentans* and *Cl. tetanomorphum*.

Cl. bifermentans: ●, cooked meat medium; ○, TPG *; *Cl. tetanomorphum*: ▲, cooked meat medium; ×, TPAY *
* see text.

2) Little account seems to have been taken in the published literature of other anaerobic spores which might be present. Pepper *et al.* (1956) irradiated *Cl. tetani* (2 strains), *Cl. sporogenes* (2 strains), *Cl. aerofoetidum*, *Cl. novyi (oedematiens)*, and an unnamed species on spore papers, but used only 3–4 replicates, and only "inactivation doses" were discussed. Darmady *et al.* (1961) irradiated *Cl. tetani* and *Cl. welchii* in a similar manner, using adequate replication (100 spore papers). Using their data, Bridges (1964) calculated decimal reduction (D) values, but it is doubtful if the data warrant such treatment. Table 2 lists two "inactivation doses," a D-value for the exponential part of the survivor curve, and a dose to give a 10^6 reduction in count. It is evident that the range of resistance to radiation over the species of clostridial spores examined is relatively small.

3) In publications of Pepper *et al.* (1956) and Darmady *et al.* (1961) it is not possible to evaluate the contribution of the shoulder of the survivor curve, which was evident with every organism examined in this study. It must be emphasized that such a shoulder may almost totally nullify the practical effects of low doses of radiation.

Table 1. Radiation resistance of anaerobic spores (log% survivors + 7.00).

	Radiation dose (Mrad)									
	0.1	0.3	0.5	0.7	0.9	1.1	1.3	1.5	1.7	2.0
Cl. aerofoetidum	8.95	8.08	7.11	6.01	4.29	3.55	....	2.29	0.51	....
Cl. bifermentans	9.37	9.03	8.16	7.17	5.44	4.33	....	<1.14	....	....
Cl. botulinum type C (TPAY)	8.67	8.20	7.24	5.78	4.18	....	....	....	....	....
Cl. botulinum type D	8.87	8.44	7.48	6.57	5.54	4.27	4.10	3.12	2.35	....
Cl. butyricum	8.99	8.11	7.21	5.79	4.52	....	....	....	....	....
Cl. caloritolerans (BA)	8.83	8.15	6.98	5.32	4.11	2.42	1.39	....	....	....
Cl. chauvoei	9.03	8.25	7.07	5.81	5.18	4.12	3.38	2.29	1.81	....
Cl. fallax	8.75	8.30	7.53	6.42	5.85	5.06	4.72	3.52	....	....
Cl. histolyticum	9.01	8.47	7.71	6.53	5.54	4.73	3.45	3.09	2.09	....
Cl. histolyticum (TPAY)	8.99	8.53	7.77	6.67	5.83	....	3.33	3.06	....	....
Cl. oedematiens type A	8.97	8.60	7.86	6.94	5.82	4.84	....	2.93	0.85	....
Cl. oedematiens type B	8.77	8.51	7.92	6.84	5.56	3.90	3.20	2.72	0.99	....
Cl. oedematiens type C	8.81	8.15	7.18	5.96	4.69	....	2.31	....	....	....
Cl. septicum	8.94	8.50	7.37	6.60	5.48	4.04	3.24	2.33	1.66	....
Cl. sordellii (TPAY)	8.77	8.05	6.85	5.35	3.35	1.79	....	....	....	....
Cl. sphenoides	8.97	8.60	7.93	7.07	6.01	5.04	3.98	2.68	2.56	1.48
Cl. sporogenes	8.89	8.43	7.33	6.24	4.68	3.58	3.04	2.56	....	....
PA3679/S_2	8.78	8.42	7.64	6.76	5.71	4.77	4.21	2.97	2.09	....
Cl. subterminale (BA)	8.93	8.65	7.33	6.00	5.53	....	....	....	....	....
Cl. tertium	8.94	8.35	7.11	5.36	3.92	....	2.51	....	....	....
Cl. tetanomorphum	8.76	8.44	7.53	6.52	5.77	4.10	3.67	2.73	1.32	....
Cl. tetanomorphum (TPAY)	8.91	8.25	7.79	6.89	6.03	5.20	4.40	3.49	2.70	....
Cl. welchii type A (TPAY)	9.10	8.29	7.09	5.48	....	<2.45	....	....	....	....
Cl. welchii type B	8.96	8.40	7.18	6.19	4.83	3.63	3.17	2.89	....	....
Cl. welchii type C.	8.42	8.11	7.10	5.82	4.45	3.17	2.91	1.91	....	....
Cl. welchii type E (TPAY)	8.71	7.98	6.55	5.71	3.73	3.73	1.73	....	....	....
Cl. welchii type F (TPAY)	8.67	8.26	7.22	6.18	5.48	4.76	3.65	2.36	1.18	....

	Mrad										
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.9	1.1	1.3	1.5
Cl. histolyticum (TPG)	....	8.78	8.26	8.11	8.03	7.28	....	6.43	....	4.28	....
Cl. bifermentans (TPG)	8.53	8.53	8.43	7.98	7.02	7.05	6.60	5.50	....	3.08	2.48

	Mrad						
	0.26	0.31	0.51	0.55	0.73	0.83	0.92
Cl. botulinum 1192A	8.84	....	....	7.24	....	5.48	4.87
Cl. botulinum 62A	....	8.46	7.96	7.79	5.83	....	....
Cl. botulinum 213B	....	8.51	7.85	7.47	5.94	....	....
Cl. botulinum NCTC 751B	8.98	....	....	8.52	....	5.92	5.85

Sporulation medium CMM unless otherwise stated.
CMM, TPG, TPAY; see text.
BA, blood agar.

In the case of *Cl. botulinum* types A and B the "shoulder" of the curve may be large, extending nearly to 1 Mrad. Most of the remaining anaerobic spores examined have a shoulder extending to about 0.25–0.35 Mrad (in aqueous suspension) followed by an exponential kill over at least 6–8 log cycles. *Cl. bifermentans* was unusual in showing an increased count after small doses of radiation, probably equivalent to "heat-shock" in triggering the germination process of otherwise dormant spores. It is anticipated that this will be the subject of a separate study.

It must be borne in mind that the results are for an unaerated aqueous suspension. Their resistances under more realistic surroundings can be estimated by comparing the curve for *Cl. botulinum* type A NCTC-7272 with results of Ingram and Thornley (1961), who irradiated spores of the same

Table 2. Radiation resistance of anaerobic spores in aqueous suspension.

	Sporulation medium[a]	Dexponential (Mrad)	Dose (Mrad) to give 10^6 inactivation
Cl. aerofoetidum	CMM	0.16	1.16
Cl. bifermentans	CMM	0.14	1.26
Cl. bifermentans	TPG	0.20	1.34
Cl. botulinum type A NCTC7272	CMM	0.12	1.41
Cl. botulinum type A 1192Y	CMM	0.14	1.18
Cl. botulinum type A 62	CMM	0.10	1.01
Cl. botulinum type B NCTC 751	CMM	0.10	1.18
Cl. botulinum type B 213	CMM	0.11	1.05
Cl. botulinum type C	TPAY	0.14	1.05
Cl. botulinum type D	CMM	0.22	1.50
Cl. botulinum type E Beluga	TPG	0.08	0.77[b]
16/63	TPG	0.16	1.17[b]
Cl. botulinum type F	CMM	0.25	1.96
Cl. butyricum	CMM	0.15	1.13
Cl. caloritolerans	blood agar	0.15	1.06
Cl. chauvoei	CMM	0.20	1.32
Cl. fallax	CMM	0.25	1.65
Cl. histolyticum	CMM	0.22	1.50
Cl. histolyticum	TPG	0.18	1.50
Cl. histolyticum	TPAY	0.18	1.44
Cl. oedematiens type A	CMM	0.19	1.45
Cl. oedematiens type B	CMM	0.18	1.40
Cl. oedematiens type C	CMM	0.16	1.17
Cl. septicum	CMM	0.16	1.31
Cl. sordellii	TPAY	0.15	0.95
Cl. sphenoides	CMM	0.21	1.53
Cl. sporogenes NCTC 532	CMM	0.16	1.19
Cl. sporogenes PA3679/S_2	CMM	0.22	1.53
Cl. subterminale	blood agar	0.16	1.21
Cl. tetani	CMM	0.24	1.63[c]
Cl. tertium	CMM	0.16	1.11
Cl. tetanomorphum	CMM	0.18	1.34
Cl. tetanomorphum	TPAY	0.23	1.63
Cl. welchii type A	TPAY	0.12	1.00
Cl. welchii type B	CMM	0.17	1.20
Cl. welchii type C	CMM	0.18	1.23
Cl. welchii type E	TPAY	0.12	1.04
Cl welchii type F	TPAY	0.20	1.39

[a] See text.
[b] Roberts and Ingram (1965).
[c] Shoesmith, J. G. (pers. commun.).

strain in cans of meat. The resistance in such cans was greater by a factor of the order ×2–3.

Fig. 3 is a spectrum of the resistance of spores of *Cl. botulinum* in aqueous suspensions. For clarity, experimental points are not included in Fig. 3, but the data are included in Table 1. It is evident that *Cl. botulinum* type E are most sensitive, strain 16/63 being the most resistant of nine strains previously examined (Roberts and Ingram, 1965). Most of the other clostridia studied are within the over-all range in Fig. 3, but extrapolation of the different survivor curves beyond an inactivation of 10^6 indicates several clostridia to be more resistant than the most resistant *Cl. botulinum* type A strain studied (*Cl. histolyticum*, from CMM and TPG; *Cl. tetanomorphum* (TPAY); *Cl. fallax*; *Cl. sphenoides*; and PA 3679/S_2).

4) The heat resistance of PA3679 is

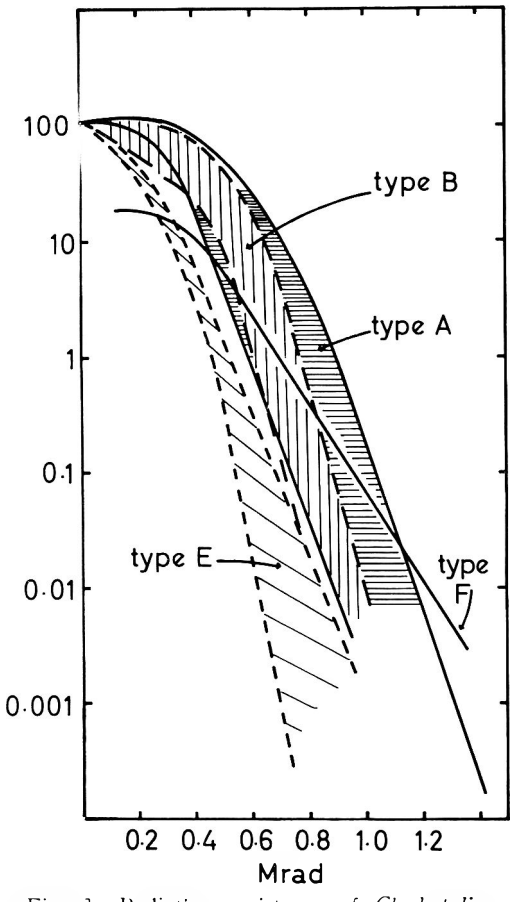

Fig. 3. Radiation resistance of *Cl. botulinum* types A, B, E and F.

greater than that of *Cl. botulinum*. Elimination by heat of *Cl. sporogenes* has therefore been taken to imply the absence of *Cl. botulinum*. A similar state of affairs, that is, the greater resistance of a common contaminant, might have been advantageous in respect of radiation processing, but it has been established that *Cl. sporogenes* is not substantially more resistant to gamma radiation than *Cl. botulinum* types A and B, and is indeed more sensitive than some strains of *Cl. botulinum* screened by Anellis and Koch (1962). On the basis of Table 2, however, it seems premature to conclude that no common *Clostridium* will be found which is more resistant to radiation than *Cl. botulinum*: the existence of heat-resistant "indicators" like PA3679 was revealed only as a result of practical experience on a large scale. Since the difference in radiation resistance is relatively small, and the size of the shoulder varies from one species to another (and from one strain to another) the question of whether any of these organisms is likely to be a suitable indicator organism in radiation processing will inevitably depend on the relative numbers present in foods, and such information is not yet available.

5) Growth of *Cl. botulinum* types A and B is generally accompanied by proteolysis, thereby making ingestion of toxin less likely. However, *Cl. botulinum* type E is nonproteolytic, and there have been instances of types A and B toxins being produced in the absence of proteolysis. Further, account should be taken of situations where ingestion of food which has undergone proteolysis is common (e.g. Scandinavia and Japan). In these instances, thorough cooking would probably inactivate the toxin, but such "fermented" foods are frequently eaten uncooked (Dolman, 1960; Dolman and Iida, 1963). The survival of *Cl. botulinum* under these latter circumstances is a severe risk, and the presence of an indicator organism would offer no increased safety factor.

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Comparative Susceptibility of Chicken, Duck and Turkey Eggs to Microbial Invasion

SUMMARY

Duck eggs were the most susceptible to bacterial spoilage, and turkey eggs were at least as resistant as chicken eggs and in some experiments appeared significantly more resistant. Under storage for 6 weeks at room temperature, however, the ability to resist bacteria was essentially unchanged in duck eggs and very markedly lowered in chicken eggs. Similarly, duck eggs lost very little quality (Haugh units) upon prolonged storage, and chicken eggs lost interior quality much faster. Bacterial penetration studies of chicken and duck exterior structures with model systems demonstrated that the outer shell membrane was the least resistant, followed by the shell and then the inner shell membrane. No consistent or significant differences in bacterial penetration were found between chicken and duck eggs, although the inner shell membrane of the former was thicker than that of the latter. Egg albumen was implicated as a major barrier to bacterial egg spoilage; conalbumin was an important inhibitor in egg white.

INTRODUCTION

Because of the economic and public-health aspects of microbial egg spoilage, a considerable volume of literature has accumulated on this topic. The egg's natural defenses against microbial invasion include the cuticle or bloom, the shell, the shell membranes, and inhibitory proteins (conalbumin, lysozyme, avidin, ovomucoid) in the albumen. The relative importance of these barriers has been assigned different emphasis by different investigators. That the shell is not the most resistant barrier to bacterial penetration has been demonstrated repeatedly (Garibaldi and Stokes, 1958; Stuart and McNally, 1943; Haines and Moran, 1940; Zagaevsky and Lutikova, 1944). Stuart and McNally (1943) and Zagaevsky and Lutikova (1944) recognized that the shell membranes became contaminated long before isolations could be made from albumen. The filtration experiments of Walden *et al.* (1956) and Garibaldi and Stokes (1958) demonstrated the resistance of shell membranes to bacterial penetration. Lifshitz *et al.* (1964), using model systems to evaluate each individual exterior egg structure as a barrier to bacterial penetration, found the outer shell membrane to be more permeable to bacteria than the shell. They reported that the inner shell membrane was the only exterior egg structure with any great resistance to microbial penetration. The inhibitory proteins, especially conalbumin, in egg albumen were stressed by Garibaldi (1960) for gram-negative bacteria. It has long been recognized that the lysozyme content of chicken albumen severely restricts gram-positive (lysozyme-susceptible) microorganisms as important egg spoilers. The protective role of albumen was further stressed by Board (1964), who surmised that the egg contents did not become grossly contaminated by organisms inoculated onto the inner shell membrane until the yolk touched this structure.

The present investigation was initiated to determine whether there were any differences between chicken, duck, and turkey eggs in susceptibility to contamination by *Pseudomonas aeruginosa* or *Salmonella derby* and whether such variations could be related to some fundamental difference in the properties of the eggs.

MATERIALS AND METHODS

The chicken eggs used were from a single strain of Single Comb White Leghorns. The duck and turkey eggs were selected from single strains of Khaki Campbells and Empire Whites. All eggs were washed for 2–3 min with an egg sanitizer-detergent at 45°C in a commercial-type washer. They were then thoroughly rinsed with 45°C water and dried on sterile egg flats. Unless stated to the contrary they were used within 4 hr of being washed.

Cultures. *Ps. aeruginosa,* isolated in this laboratory from a spoiled egg, was maintained on glucose yeast extract (GYE) agar. *S. derby* was maintained on tryptose agar. Cultures for egg contamination studies were grown in GYE or tryptose broth for 24 hr, sedimented by centrifugation, and resuspended in sterile distilled water.

Egg spoilage by the laboratory strain of *Ps. aeruginosa* was detected by ultraviolet (UV) candling or by bacteriological examination. Bac-

teriological examination of eggs was accomplished by streaking albumen or yolk material on GYE agar plates. UV candling was reported by Lorenz *et. al.* (1952) to compare satisfactorily with bacteriological isolation procedures, and work in this laboratory (unpublished) has shown better than 96% agreement.

S. derby was detected by streaking SS agar plates, followed by inoculation of triple sugar iron agar slants for confirmation.

Exposure of eggs to bacteria. Eggs were exposed to bacteria by 1 of 3 methods (A, B, C):

Method A. Eggs were warmed to 40–45°C in running tap water and were completely immersed for 10 min in a distilled-water suspension of bacteria (1×10^6 bacteria/ml) at 23–25°C. Bacterial contamination of the egg content was detected by UV candling or by periodically breaking a number of eggs into sterile dishes and sampling the yolk bacteriologically.

Method B. This method was similar to *A* except that only the lower 2/3 of the egg was immersed in the bacterial suspension. For bacteriological sampling a small hole was drilled in the upper 1/3 of the egg and the egg was placed in a sterile covered casserole dish. The albumen was sampled daily with a bacteriological transfer loop.

Method C. The egg was swabbed over the entire shell surface, or any desired portion of the surface, with a cotton swab dipped into a suspension of bacteria (1×10^9 bacteria/ml). Both the egg and the culture were at room temperature. Following surface contamination, all eggs were held at room temperature during the test period.

Physical properties of inner and outer shell membranes. Lifshitz *et al.* (1964) reported that the inner shell membrane of the chicken egg was a much more efficient barrier to microorganisms than the outer shell membrane, although the latter was heavier and thicker. Using the same procedures as Lifshitz and Baker (1964), the thickness and weights of duck and turkey egg shell membranes were measured and compared to the value of the aforementioned authors.

Bacterial penetration using model systems. Models of shell, shell with outer membrane, shell with inner plus the outer membrane, inner membrane alone, outer membrane alone, and inner and outer membranes were prepared for duck and chicken eggs, as described by Lifshitz *et al.* (1964). The models used were rinsed 3–4 times with sterile water and placed in individual containers. Nutrient broth was the test medium.

RESULTS

Bacterial penetration. *Fresh eggs.* The comparative resistance of fresh chicken, duck, and turkey eggs to *Ps. aeruginosa* and *S. derby* is shown in Table 1. In all experiments, duck eggs were the first to evidence bacterial penetration into the internal part of the egg. In 4 of 6 trials there was a significant difference between chicken and duck eggs at the 5% level by the Duncan's new multiple-range test (Steel and Torrie, 1960). Turkey eggs were at least as resistant to spoilage as chicken eggs, and in trials 1 and 2 they were significantly more resistant. In all experiments, the differences between duck and turkey eggs were significant.

Fresh versus aged eggs. Chicken, duck, and turkey eggs 1 day after lay, after 3 weeks of storage at room temperature and 6 weeks of storage at room temperature were exposed to *Ps. aeruginosa* (1×10^6 organisms/ml) by Method *A* and were observed daily for fluorescence. Chicken eggs were the most adversely affected by prolonged storage (Fig. 1) and they became progressively more susceptible to bacterial decompo-

Table 1. Summarization of bacterial penetration studies with fresh chicken, duck and turkey eggs using *Pseudomonas aeruginosa* and *Salmonella derby* as the test organisms.

Trial	Test organism	No. eggs per treatment	Egg infection method	Detection of spoilage (method)	Mean resistance time (days)			Level of significance
					Chicken	Duck	Turkey	
1	*Pseudomonas*	16	B	Bacteriol.	16.0	11.2	23.7	.005
2	*Salmonella*	16	C	Bacteriol.	26.7	24.2	32.3	.025
3	*Pseudomonas*	16	C	Bacteriol.	10.2	6.8	8.9	.005
4	*Pseudomonas*	17	B	Bacteriol.	18.6	16.8	20.3	.005
5	*Salmonella*	18	B	Bacteriol.	27.2	19.5	27.7	.005
6	*Salmonella*	17	C	Bacteriol.	20.8	13.6	.....	.005
7	*Pseudomonas*	15	A	Candled	27.6	11.4	.....	.025
8	*Pseudomonas*	50	A	Bacteriol.	13.0	10.0	16.0	[a]

Mean resistance time, average number of days for contents of eggs to become contaminated. The numbers which are underlined were not significantly different at the 5% level.

[a] Test of significance not conducted.

sition with age. Turkey eggs (Fig. 2) were also affected, though less so. Duck eggs, contrarily, were as resistant to bacterial invasion after 6 weeks of storage as they were 1 day after lay (Fig. 3).

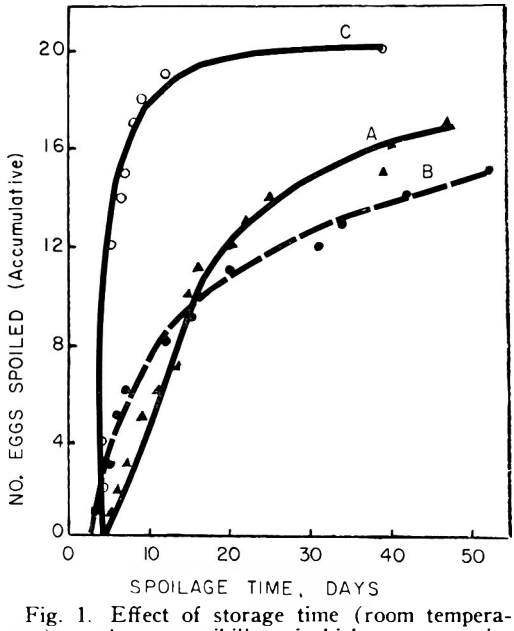

Fig. 1. Effect of storage time (room temperature) on the susceptibility of chicken eggs to infection with *Pseudomonas aeruginosa*. A, fresh eggs; B, eggs stored 3 weeks; C, eggs stored 6 weeks. Twenty eggs were used for each treatment.

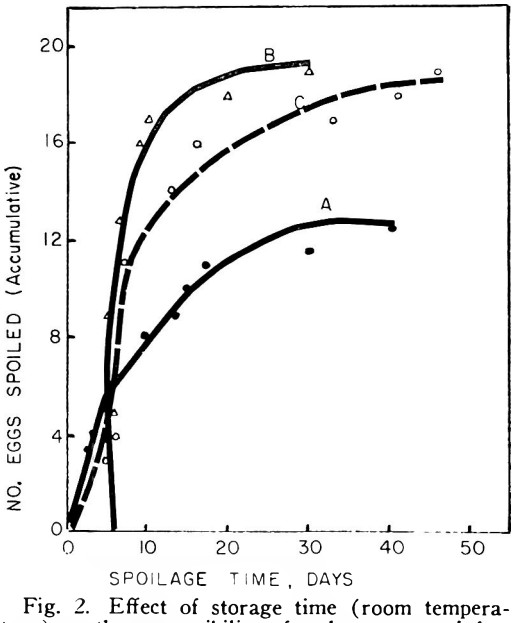

Fig. 2. Effect of storage time (room temperature) on the susceptibility of turkey eggs to infection with *Pseudomonas aeruginosa*. A, fresh eggs; B, eggs stored 3 weeks; C, eggs stored 6 weeks. Twenty eggs were used for each treatment.

When eggs were stored for similar periods and used for egg quality measurements (Haugh, 1937), chicken and turkey eggs exhibited a rapid loss of interior egg quality. Duck eggs maintained their high quality even after prolonged storage (Fig. 4).

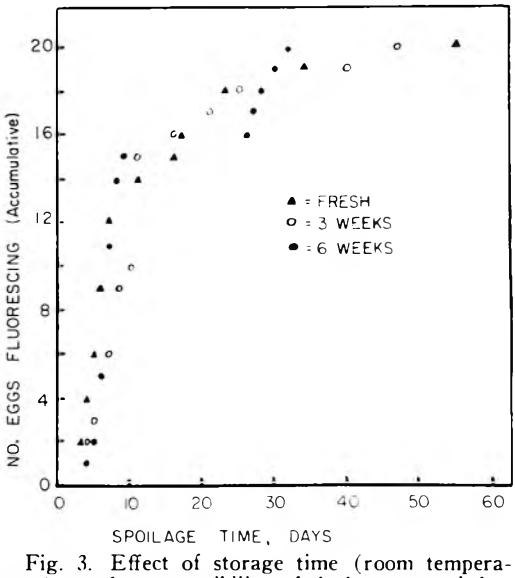

Fig. 3. Effect of storage time (room temperature) on the susceptibility of duck eggs to infection with *Pseudomonas aeruginosa*. Twenty eggs were used for each treatment.

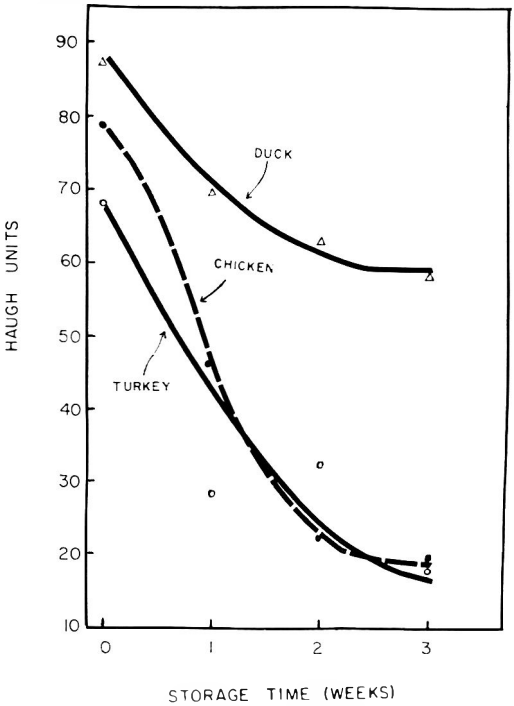

Fig. 4. Effect of storage time (room temperature) on the albumen quality of chicken, duck, and turkey eggs.

Physical properties of shell membranes. Because a number of investigators have reported that the shell membranes present a major obstacle to potential egg spoilage bacteria, it was thought advisable to study these structures in more detail. Table 2 shows that duck eggs had the thinnest inner shell membrane and chicken eggs the thickest. This is fairly consistent with the results of spoilage tests, and on the surface would tend to confirm the role of the inner shell membrane as a major barrier to bacterial contamination of shell eggs.

Bacterial penetration through models of exterior structures. A typical experiment with models of exterior structures of duck and chicken eggs is shown in Table 3. These results, in essence, confirmed work of Lifshitz *et al.* (1964) on chicken eggs in that the rate of penetration was outer shell membrane alone > shell > inner membrane alone ≥ shell and outer membrane > shell + both membranes. These results were similar for duck and chicken eggs, and no consistent or significant differences in penetration rates were noted between chicken and duck eggs. It must be concluded, therefore, that this experiment failed to implicate differences in the exterior structures of chicken and duck eggs as the limiting factor in the bacterial penetration differences noted in Table 1.

Studies on albumen. *Effect of iron.* Because of the highly erratic response elicited when low numbers of bacteria were injected directly into the albumen of chicken eggs, and in light of studies of Garibaldi (1960) on conalbumin and iron, several experiments were devised to test the effect of iron on the response to bacterial exposure.

Experiment I. Twenty-six chicken eggs and a similar number of duck eggs were injected with 0.1 ml of a solution of $FeSO_4$ (5,000 ppm Fe^{++}). The eggs were sealed with paraffin and observed daily for fluorescence. The results (Fig. 5) revealed a dramatic increased rate of fluorescence in chicken eggs in the presence of iron. All those receiving the iron fluoresced before the eggs without iron had started to show fluorescence. The effect of iron on duck egg contamination was similar, though not quite so pronounced.

Table 3. Comparison of bacterial penetration through exterior structures of duck and chicken eggs.

Model	Average penetration time (hr)	Standard deviation	No. of models
C-O	9.8	± 2.8	10
D-O	5.4	± 2.6	10
C-S	12.0	± 4.4	10
D-S	16.0	± 1.4	10
C-I	18.7	± 8.5	10
D-I	19.0	± 6.6	10
C-OS	19.0	± 9.2	10
D-OS	21.2	±10.3	10
C-IOS	27.8	± 5.3	10
D-IOS	29.7	± 7.1	10

C, chicken; D, duck; O, outer shell membrane; I, inner shell membrane; S, shell.

Experiment II. In a similar experiment, 3 groups of 10 eggs were used. For the first group, eggs were broken-out and the albumens and yolks from individual eggs were placed in sterile screw-capped specimen jars. In the second group, the albumen from each egg was separated aseptically from the yolk and placed in individual jars. The third group was identical to the second group except that 0.1 ml of a 5,000-ppm iron solution was added to each albumen. All 3 groups were then inoculated with 6,000 *Pseudomonas* organisms and observed daily for fluorescence. The results (Fig. 6) showed that adding iron to egg albumen or leaving the unbroken yolk in the albumen permitted more rapid growth of the bacterium than did just the albumen alone. In all 3 treatments, fluorescence was much more rapid than in whole eggs.

Experiment III. Iron (0.2 ml of 0.5% $FeSO_4$) was inoculated into the albumen of 23 eggs. Sterile water (0.2 ml) was similarly injected into an additional 23 eggs. The injection sites were sealed with hot paraffin and a circle 1.5 cm in diameter was marked around each injection site. The complete shell surface of all eggs, except for the area circled, was swabbed with a cotton swab dipped into a centrifuged washed culture of *Ps. aerugi-*

Table 2. Physical measurements of chicken, duck, and turkey egg shell membranes.

Species	Weight (g)			Thickness (μ)			Relative density (R_1/R_2)
	I	O	R_1	I	O	R_2	
Chicken	.0252	.1482	5.9	6.58	15.04	2.3	2.56
Duck	.0321	.2323	7.2	4.41	26.12	5.9	1.22
Turkey	.0462	.2718	5.8	5.30	29.77	5.6	1.02

O = outer membrane; I = inner membrane; $R_1 = \frac{\text{weight } O}{\text{weight } I}$; $R_2 = \frac{\text{thickness } O}{\text{thickness } I}$.

Relative density of outer to inner = R_1/R_2.

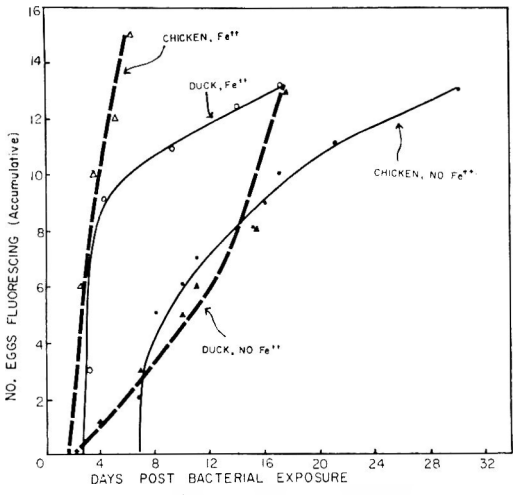

Fig. 5. The effect of iron on spoilage by low numbers of *Pseudomonas aeruginosa* injected into the albumen of chicken eggs.

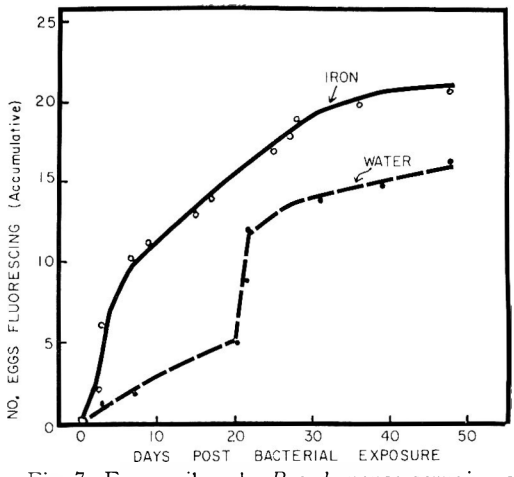

Fig. 7. Egg spoilage by *Pseudomonas aeruginosa* applied to the shell surface of chicken eggs previously injected with iron or water via albumen.

nosa. The eggs were stored at room temperature and candled daily with a UV light source. Eggs injected with iron spoiled almost twice as fast (av. 17.8 days) as eggs injected with water (av. 32 days) (Fig. 7).

The results of these 3 experiments indicate that the albumen plays a significant role in the protection of shell eggs from bacterial decomposition. In particular, the inhibitory properties of conalbumin exert a significant effect. The effect of iron was previously reported by Garibaldi and Bayne (1962a,b), Schade and Caroline (1944), and Brooks (1960). Garibaldi and Bayne (1960) noted a stimulatory effect of adding iron to bacterial suspensions applied to the exterior structures of shell eggs, and Hartung and Stadelman (1962) reported more rapid penetration of inner shell membranes *in vitro* by *Ps. fluorescens* in the presence of Fe^{++} or Mn^{++}. The present experiment is the only one reporting the effect of injecting iron directly into the albumen on bacterial deterioration of whole shell eggs.

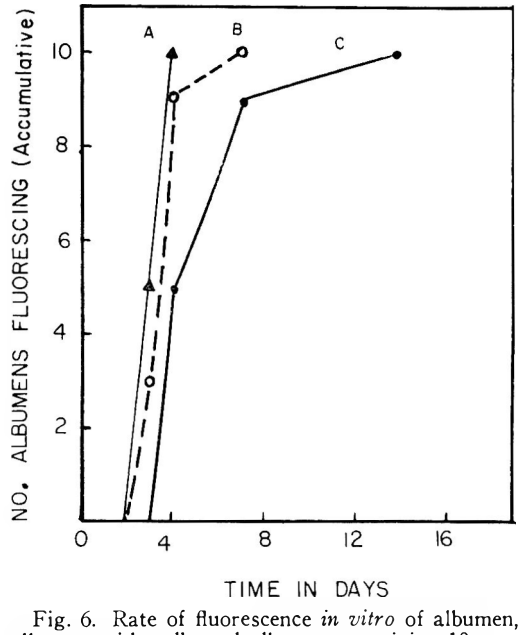

Fig. 6. Rate of fluorescence *in vitro* of albumen, albumen with yolk, and albumen containing 10 ppm Fe^{++} following contamination with *Pseudomonas aeruginosa*. A, albumen with yolk; B, albumen containing Fe^{++}; C, albumen alone.

Effect of replacing albumen with nutrients. A portion of the albumen (25 ml) was removed from the small end of 15 duck and 15 chicken eggs with a sterile 30-ml syringe and a 15-gauge needle. The albumen was replaced with an equal volume of nutrient broth supplemented with 22% gelatin. The gelatin was used to simulate the viscosity of fresh egg white. These 30 eggs, plus 30 control eggs, were swabbed with *Ps. aeurginosa*. The results (Table 4) demonstrated that the exterior

Table 4. Comparison of penetration rate of *Pseudomonas aeruginosa* into normal chicken and duck eggs with eggs in which 25 ml of nutrient broth supplemented with 22% gelatin was substituted for 25 ml of egg albumen.

Type of egg	Treatment	No. of eggs	Av. time required for eggs to fluoresce
Chicken	None	15	27.6 days
Chicken	Albumen replaced	15	4.4
Duck	None	15	11.4
Duck	Albumen replaced	15	4.4

structures, if they function purely as physical barriers, do not give extensive protection to the egg contents. In both chicken and duck eggs, when the nutrient solution was substituted for albumen, fluorescence occurred quite rapidly (4.4 days in both instances). In contrast, the control duck eggs required on the average 11.7 days for spoilage and the control chicken eggs required 27.6 days. This experiment demonstrated once more the importance of the inhibitory properties of egg white in protecting eggs from bacterial invasion.

DISCUSSION

The present paper represents the first attempts to compare the bacterial resistance of eggs from different avian species. Fresh duck eggs were found to be less resistant to spoilage by *Ps. aeruginosa* and *S. derby* than fresh chicken or fresh turkey eggs. This coincides with a report by Romanoff (1942) that duck incubator eggs spoiled much faster than similarly treated chicken eggs. During storage, however, the susceptibility of duck eggs remained essentially unchanged (Fig. 3), while chicken eggs became much more susceptible to bacteria (Fig. 1). The maintenance of interior egg quality and resistance of duck eggs to bacterial spoilage over prolonged storage times, in comparison to the loss of these qualities in chicken eggs, lends support to the concept presented by Board (1964) that spoilage does not usually occur until the yolk has migrated to the inner membrane or, at least, that egg quality changes are in some way associated with the susceptibility of eggs to bacterial deterioration. It is presumed that the lower concentrations of conalbumin and lysozyme in duck eggs permitted an earlier invasion of the albumen and yolk than occurred in chicken eggs, where these inhibitory proteins are in higher concentrations (Rhodes and Feeney, 1957; MacDonnell *et al.*, 1955, 1954; Rhodes *et al.*, 1959).

It is possible that the thinner inner membrane of duck eggs also permitted more rapid microbial infection of the egg. The studies on the permeability of the individual egg structures (Table 3), the albumen infection studies (Fig. 5, 7), and the albumen replacement experiments (Table 5) lend little credence to the concept that the inner shell membrane serves as the primary factor in the preservation of bacteriologically clean eggs. When the inner membrane was studied *in vitro* in the absence of albumen, the membranes resisted bacteria for 2–3 days at the most. The majority of them were penetrated within 1–2 days. Similarly, when a portion of the albumen of shell eggs was replaced with a nutrient solution and the shell was swabbed with *Ps. aeruginosa,* the membranes presented very little resistance to the invading bacteria. Both duck and chicken eggs so treated fluoresced under a UV candler in an average of 4.4 days. When small numbers of bacteria were injected into fresh eggs via the albumen, the eggs did not spoil immediately or even at one time. Most of the eggs spoiled between 7 and 20 days postinoculation. However, some eggs did not show fluorescence until as late as 30 days.

When the conalbumin in chicken and duck eggs was supplemented with iron (10 ppm) and the albumen was subsequently injected with 6,000 *Ps. aeruginosa* organisms (Fig. 5), the chicken eggs all fluoresced within 6 days. On the other hand, chicken eggs receiving the same bacterial challenge but no iron showed no fluorescence until the seventh day, and even after 30 days only 13/15 had shown fluorescence. The duck eggs injected with iron initially fluoresced at a faster rate than those not receiving the iron, but the effect of iron was not as complete or as consistent as it was with chicken eggs. It may be that conalbumin is not the only major inhibitor in duck albumen. Rhodes *et al.* (1959) reported that ovomucoid in duck eggs had twice the specific activity of chicken ovomucoid. In addition, duck ovomucoid was active against trypsin, chymotrypsin, and a bacterial proteinase, whereas chicken ovomucoid was active against trypsin alone. Perhaps, duck ovomucoid is more effective as an antibacterial substance than chicken ovomucoid.

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Effect of Irradiation Temperature on the Radiosensitizing Activity of Vitamin K_5

SUMMARY

***Salmonella typhimurium* was irradiated in phosphate buffer, pH 7.6, containing various levels of vitamin K_5 over a temperature range of 32 to 120°F either in air or under vacuum. Vitamin K_5 exerted a radiosensitizing effect under vacuum and a radioprotective effect in air. The radiosensitizing effect decreased with increasing temperature. Vitamin K_5 had no radiosensitizing activity, or possibly a slight protective effect, in whole egg irradiated either in air or under vacuum.**

INTRODUCTION

Various processing methods have been proposed for reducing the salmonella content of infected eggs and thereby decreasing the possibility of salmonellosis from food products containing these eggs.

Goresline *et al.* (1951) recommended a 3-min heat treatment at 140°F. Proctor *et al.* (1953) demonstrated the efficacy of ionizing radiation. Lloyd and Harriman (1957) were issued a patent on the use of hydrogen peroxide coupled with a mild heat treatment, and Bruch and Koesterer (1962) described a treatment involving the use of beta-propiolactone. Although these various processing techniques effectively destroy salmonellae, they do have some shortcomings. For example, egg protein, particularly albumen, is sensitive to heat; radiation treatment at high doses causes off flavors; and use of beta-propiolactone is not sanctioned by the Food and Drug Administration.

In recent years, El-Tabey Shehata (1961) and Silverman *et al.* (1962) reported on the radiosensitizing effect of vitamin K_5 on certain microorganisms. The present study was undertaken to determine the feasibility of destroying salmonellae in liquid egg through the combined use of vitamin K_5 and gamma irradiation.

METHODS

Preparation of stock cell suspension. The test organism used was *Salmonella typhimurium* ATCC 6994. Cells were grown on the surface of nutrient agar in Roux flasks and were washed off with phosphate buffer (pH 7) after 4 days of incubation at 97°F. After several washings with phosphate buffer, the cells were suspended in a small volume of buffer and stored at 36–40°F in a screw-cap jar containing glass beads.

Preparation of sample for irradiation in air. Two different substrates were used: liquid whole egg (pH approximately 7.6) and phosphate buffer (pH 7.6).

The liquid whole egg was prepared by blending freshly broken out eggs at a slow speed. A specific volume of the stock cell suspension was added and blending was continued. The egg magma was divided into three portions. One served as the control, while the other two portions had 4-amino-2-methyl-1-naphthol (vitamin K_5; Synkamin; Parke Davis and Co.) added in such amounts that the final concentration was 40 or 160 ppm. Portions of 0.05 ml of the different samples were filled into capillary melting-point tubes (100 × 1.5–2.0 mm OD) by means of a micrometric syringe, and the open end of the capillary tube was sealed in the flame of a micro-burner.

A similar procedure was followed with phosphate buffer, pH 7.6, used as substrate.

Preparation for irradiation under vacuum. The whole egg sample was prepared in a manner described previously except that all blending of egg with cells and vitamin K_5 was carried out under vacuum in a modified Servall Omnimixer (Coris *et al.*, 1962). Portions of the egg homogenate were filled into 1-ml ampoules and the ampoules were sealed under vacuum (1 mm Hg).

Some ampoules were filled with phosphate buffer, pH 7.6, and sealed under vacuum.

Irradiation of samples. Capillary tubes or 1-ml ampoules to be irradiated were placed in water at a given temperature in a Thermos flask. The flask, in turn, was encased with Styrofoam insulation and contained in a steel chamber which was lowered into the radiation field. Cobalt-60 was the source of radiation, and the intensity of the source was approximately 225,000 rad/hr.

Samples were kept chilled prior to and immediately following irradiation.

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Bacterial plating procedure. Capillary tubes were rinsed in acetone, placed in chromic acid for 2 min, and then rinsed with sterile distilled water. The capillary tubes were then transferred to culture tubes containing 10 ml of chilled phosphate buffer (pH 7) and were crushed with a sterile glass rod.

The culture tubes were shaken vigorously 50 times, and decimal dilutions were made.

Ampoules were opened by breaking the sealed top portion. One ml was extracted, and decimal dilutions were made with chilled buffer.

One-tenth-ml portions of the appropriate serial dilution were spread by bent glass rods over the surface of pre-poured plates of trypticase soy agar (supplemented with 10 g yeast extract/liter). Plates were incubated 48 hr at 97°F prior to counting colonies.

RESULTS AND DISCUSSION

The slopes of the irradiation survival curves of *S. typhimurium* are plotted in Fig. 1 as a function of irradiation temperature when the cells were suspended in phosphate buffer, pH 7.6, and irradiated in air. These slopes were determined by inspection and are corrected for pure temperature effect, that is, the cells were heated for various times at some given temperature and the slope of this survival curve was subtracted from the slope of the irradiation survival curve for the same temperature. The protective effect of vitamin K_5 during irradiation is rather obvious. Silverman *et al.* (1963) reported a similar protective effect of vitamin K_5 during irradiation of certain bacterial spores in an air atmosphere. Thus, during irradiation in air in a simple system such as phosphate buffer, vitamin K_5 behaves like any other complex organic molecule by competing for free radicals formed. It is also of interest to note the pronounced effect of temperature during irradiation above 100°F on the survival rate of the cells. This phenomenon was also found by Licciardello (1964) for *S. typhimurium*.

When irradiation was performed under vacuum, a genuine radiosensitizing effect was obtained (Fig. 2). Other investigators have likewise found that, with the majority of microorganisms tested, vitamin K_5 exerted a radiosensitizing action only under conditions of low oxygen tension. It has been previously demonstrated that, in aqueous solution, vitamin K_5 is rapidly oxidized to 1,4-naphthoquinone. Glew (1964) found

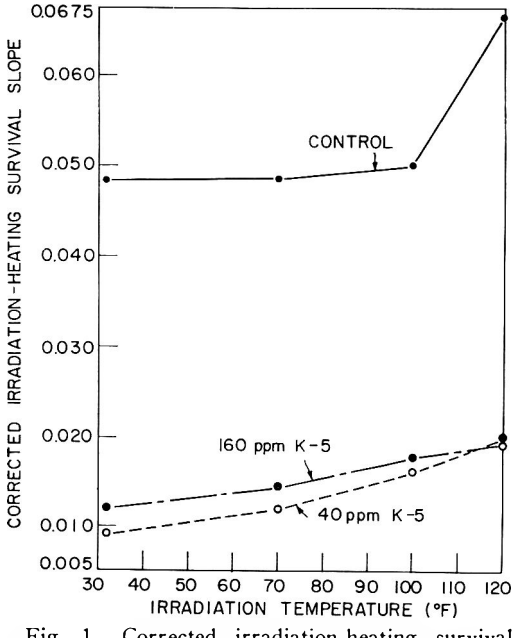

Fig. 1. Corrected irradiation-heating survival slopes of *S. typhimurium* as a function of irradiation temperature in phosphate buffer (pH 7.6) in air.

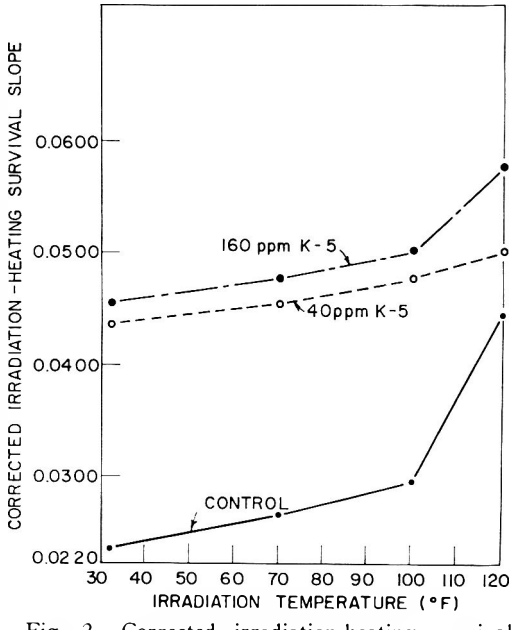

Fig. 2. Corrected irradiation-heating survival slopes of *S. typhimurium* as a function of irradiation temperature in phosphate buffer (pH 7.6) in vacuum.

that 1,4-naphthoquinone also had radiosensitizing activity and was destroyed by irradiation to a greater extent in air than in nitrogen. The breakdown products, however, were different in the two atmospheres. He raised the possibility that the radiosensitizing action in anoxia might be due to specific breakdown products.

Alexander (1962) suggested another theory to explain the radiosensitizing effect only under anaerobic conditions. He postulates that the primary radiation damage to a cell is the abstraction of a hydrogen atom from some vital molecule with the subsequent formation of a radical, R·. Under anaerobic conditions, the naturally present sulfhydryl compounds (PSH) will repair many damaged sites and thus offer protection in the following manner:

$$R \cdot + PSH \longrightarrow RH$$

However, these sulfhydryl compounds are not present in sufficient concentrations to prevent peroxidation in aerobic conditions. That is,

$$R \cdot + O_2 \longrightarrow RO_2 \cdot$$

proceeds at a greater rate than

$$R \cdot + PSH \longrightarrow RH$$

On this basis, if a sulfhydryl poisoner, such as vitamin K_5, is added, it will tie up sulfhydryl groups and thus prevent their protective action under anaerobic conditions. This becomes a radiosensitizing effect.

The sulfhydryl poisoner will also tie up the sulfhydryl groups under aerobic conditions, but no effect will be noted after irradiation because the naturally present sulfhydryl compounds are not protective in aerobic conditions.

Some bacteria are radiosensitized by sulfhydryl poisoners under both aerobic and anaerobic conditions. It may be that these organisms possess a high concentration of sulfhydryl compounds so that protection is afforded aerobically and anaerobically.

To determine whether there was any synergistic effect between irradiation temperature and vitamin K_5, the slopes of the treatment curves in Fig. 2 were divided by the slope of the control curve. The quotient thus obtained is designated the dose enhancement factor. If the value exceeds one, the treatment is radiosensitizing and if the value is less than one, the treatment is protective. The results shown in Table 1 indicate that the maximum radiosensitizing effect occurred at an irradiation temperature of 32°F. As the irradiation temperature increased, the radiosensitizing effect decreased. There was a considerable decrease at the highest irradiation temperature employed, 120°F. A probable explanation is that vitamin K_5, or the sensitizing factor, was being rapidly destroyed at the high irradiation temperature, and thus there was less available for sensitizing.

Table 1. Dose enhancement factors for two levels of vitamin K_5 in phosphate buffer with gamma irradiation carried out under vacuum at various temperatures.

Irradiation temperature (°F)	Dose enhancement factor	
	40 ppm K_5	160 ppm K_5
32	1.86	1.95
70	1.73	1.81
100	1.62	1.70
120	1.10	1.30

The graphs of the irradiation survival slopes are plotted in Figs. 3 and 4 as a

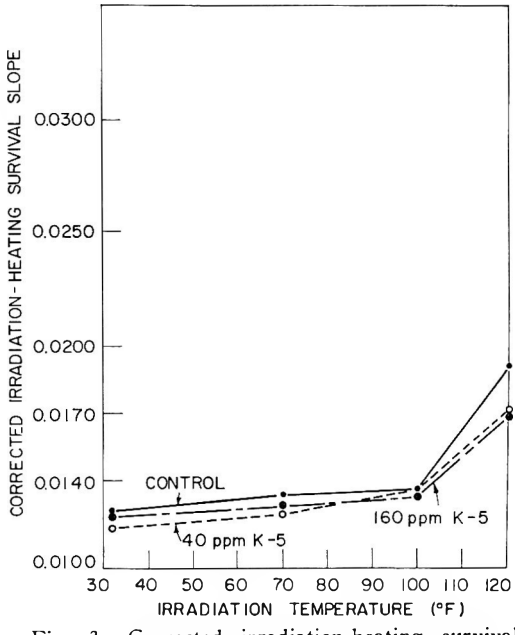

Fig. 3. Corrected irradiation-heating survival slopes of *S. typhimurium* as a function of irradiation temperature in whole egg in vacuum.

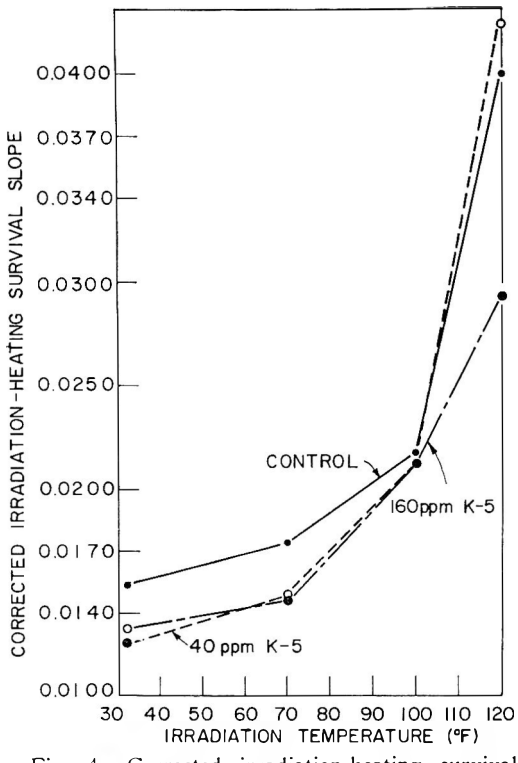

Fig. 4. Corrected irradiation-heating survival slopes of *S. typhimurium* as a function of irradiation temperature in whole egg in air.

function of irradiation temperature when irradiation was carried out in liquid whole egg either in air or in vacuum. These slopes are corrected for pure thermal effects. If the survival slopes for irradiation in air are compared with those for irradiation under vacuum, it will be noted that the former are greater than the latter, particularly at the higher temperatures. This would indicate that *S. typhimurium* in whole egg is destroyed by irradiation more easily in an air atmosphere than under low oxygen tension.

There was no difference in radiation survival slopes between 40 and 160 ppm vitamin K_5.

Vitamin K_5, in whole egg irradiated in air or vacuum, either had no effect on the radiosurvival of *S. typhimurium* over the temperature range investigated, or possibly a slight protective effect. El-Tabey Shehata (1961) and Silverman *et al.* (1963) also found that the radiosensitizing action of vitamin K_5 was not as effective in food systems such as orange juice or raw milk as in model buffer systems. El-Tabey Shehata (1961) suggested that this decrease in the sensitizing action of vitamin K_5 might be due to a reaction between the vitamin and sulfhydryl-containing compounds. Silverman *et al.* (1963) found that cysteine or glutathione added to a model system neutralized the toxic effect of vitamin K_5.

Those investigators suggested that beta-lactoglobulin, which is rich in free sulfhydryl groups, was responsible for inactivating vitamin K_5 in milk. If this theory is correct, it is conceivable that sulfhydryl-containing proteins such as ovalbumen of whole egg reacted with the vitamin K_5 or possibly 1,4-naphthoquinone and thus made it unavailable for promoting its radiosensitizing action on the bacteria present.

It was fully realized that dissolved or occluded air might not be fully removed by merely evacuating the headspace of the vials containing the whole egg sample. To determine the effect of dissolved or occluded air in the egg magma during irradiation under vacuum, an experiment was conducted in which glucose and glucose oxidase were added to consume any oxygen that might be present. The vials were sealed under vacuum as usual and were held at room temperature for 2 hr prior to irradiation. The result (not shown) was comparable to the case in which no glucose oxidase was added. It was not believed that the 2-hr holding period at room temperature caused any change in the sensitizing activity of vitamin K_5, because a previous experiment had shown there to be no difference in survival rate of *S. typhimurium* when irradiated immediately following addition of vitamin K_5 and after a 3-hr holding period at room temperature.

To determine the effect of temperature during irradiation under vacuum on the radiosensitivity exerted by vitamin K_5 in whole egg, the dose enhancement factor was calculated for the two levels of vitamin K_5 at the various irradiation temperatures. The results, which are presented in Table 2, show that the dose enhancement factor remained relatively constant over the temperature range of 32 to 100°F, but decreased at 120°F, indicating a protective effect.

Table 2. Dose enhancement factor for two levels of vitamin K_5 in liquid whole egg with gamma irradiation carried out under vacuum at various temperatures.

Irradiation temperature (°F)	Dose enhancement factor	
	40 ppm K_5	160 ppm K_5
32	0.95	0.99
70	0.94	0.96
100	1.00	0.98
120	0.90	0.88

It is concluded from this investigation that vitamin K_5 lacks promise as a radiosensitizing agent toward salmonellae in egg.

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Evaluation of Toughness Differences in Chickens in Terms of Consumer Reaction

SUMMARY

An untrained panel of approximately 100 people was used to evaluate the importance of differences in fryer chicken toughness that result from differences in chilling time before freezing. The panel discriminated and criticized toughness that could arise in commercial practice. At least 4 hr of aging is desirable even for birds that are thawed before being cooked. Shear resistance of fried meat measured with Warner-Bratzler and L.E.E.-Kramer shear apparatus correlated well, and correlated with untrained-panel evaluation. Adverse comments on toughness and dissatisfaction with the quality of the chicken increased with increase in shear resistance of the meat.

INTRODUCTION

Consumers of chicken and producers of chicken for the prepared food market continue to report occasional tough young birds. Pool *et al.* (1959) reviewed numerous studies of the effects of processing on tenderness. These studies, however, did not show what degree of toughness in chickens is serious enough to cause consumer complaints. The close relation found between consumer reaction to toughness in turkeys and shear resistance of the meat (White *et al.*, 1964) has made possible the interpretation of objective measurement of toughness in terms of consumer reaction. Adverse comments by untrained consumers increased consistently with increases in the shear resistance of turkey meat.

This study was designed to obtain information to permit similar interpretation of the correlation that has been demonstrated between shear resistance and trained-panel evaluations of tenderness of chicken meat (Koonz *et al.*, 1954; Shannon *et al.*, 1957; Paul *et al.*, 1959). Chicken meat ranging from very tender to the toughness that might be encountered in birds subjected to improper chilling and processing was obtained by varying the chilling time before freezing. Half of each bird was cooked from the frozen state and measured for shear resistance; subsequently the other half was assigned to one of three groups according to shear resistance, cooked from the frozen state, and submitted to taste-panel evaluation. The large untrained panel of laboratory personnel was not aware of the purpose of the test and had not participated in our similar test on turkeys.

Additional birds were cooked, one-half from the frozen state and the other half after thawing, and tested for shear resistance to determine the effect of thawing on tenderness. The results establish the value of aging before freezing for optimum consumer satisfaction and the benefit to be derived from a thawing period before cooking.

METHODS

Chicken processing and shear measurement. Approximately 250 9-week-old meat-type-cross mixed-sex chicken fryers were purchased from a homogeneous commercial lot, transported 50 miles, electrically stunned, slaughtered with an outside cut, bled for 2 min, scalded 1 min at 129–130°F, picked for 20 sec in a cyclic free-floating picker, pinned by hand, warm eviscerated, chilled in ice slush for 0.5, 1, 2, 4, 6.3, or 24 hr, drained for 10 min, packed minus neck in polyethylene bags (evacuated to conform), frozen in a −25°F 1300-ft/min blast freezer, and stored at −10°F until evaluation was completed (2½ mo). About 35 min elapsed between slaughter and immersion in chill tanks.

The birds were sawed in half parallel to the breast bone as close to the middle as possible. Halves averaged slightly over 1 lb. Preliminary tests on twelve birds aged 0.5 to 4 hr revealed no significant differences in shear between halves cut slightly on one side or the other of the keel bone. Each half was fried from the frozen state in peanut oil in a deep-fat fryer (Star Manufacturing Co., model 5). The oil was preheated to 140°C. The temperature dropped to 130°C when the frozen halves were added, and cooking continued at 130°C to an internal thigh temperature of 85°C. Breast and thigh muscles were removed after halves were cooled on racks for ½ hr.

Strips of meat one inch wide and the thickness of the muscle were cut parallel to the longitudinal grain from the outer breast (pectoralis major)

and the outer thigh (biceps femoris) of each half. Each strip was weighed and tested on the Warner-Bratzler shear apparatus. Each strip was sheared five times across the grain. The cut pieces of the outer breast muscle remaining from the Warner-Bratzler shear tests were weighed to 0.1 g and placed in the standard cell of the L.E.E.-Kramer shear press so that the blades of the cell would cut across the grain. The 3000-lb proving ring was used with a range of 1000 lb and a down-stroke speed of 15 sec. The entire deboned thigh and leg meat of birds aged 0.5, 6.3, and 24 hr were also tested on the L.E.E.-Kramer apparatus. The average L.E.E.-Kramer shear values were 4.99, 4.09, and 3.63 kg/g for thigh meat of birds aged 0.5 hr, 6.3, and 24 hr, respectively. Comparable values for the leg meat were 6.81, 5.90, and 5.90 kg/g. Since the panel was not expected to distinguish such small differences, only the outer breast muscles were used for the panel tests. In these, the group average shear values ranged from 15.44 to 4.09 kg/g. The samples from the outer breast muscle of the unsheared half were allotted to the panel according to the shear values of the other half.

To supplement information on shear values of meat cooked from the frozen state, one-half of each of 34 birds aged 0.5 hr was cooked after thawing, and the other half was cooked from the frozen state to 85°C in the thigh. Cooking time was approximately 18 min for thawed halves and 30 min for frozen halves. Twenty-nine halves were thawed for 1–1¼ hr in polyethylene bags in running tap water at 18°C. Then the temperature of the thigh region was approximately 4°C, and that of the breast was approximately 10°C. Halves of the other five birds were thawed 45 min, a time that proved inadequate since 4 of the 5 were still tough.

Panel test. Cooking the halves from the frozen state to an internal thigh temperature of 85°C for the shear tests caused the breast meat to be dry. Because only the breast was to be used, the cooking time was reduced from an average of 30 min for the panel tests. Halves, averaging approximately 1 lb, were fried, as were those for shear tests, but for 13–16 min, depending on weight. The outer breast muscle was separated from skin and bone and cut across the grain into 2–3 pieces, wrapped and cooled approximately 1 hr. The pieces were shaken in flour and dipped in a batter. Batter (43% water, 15.4% waxy corn, 31% cornstarch, 1% nonfat milk solids, 5% egg yolk, 4.5% salt) was prepared by adding the dry ingredients gradually to water in a Kitchen Aid mixer, model G, with the wire whip attachment, speed 1. The bowl was then scraped with a rubber spatula, and mixing was resumed for 50 sec at speed 2. The yolk was added and mixing continued for 100 sec at speed 2.

Each piece was tagged with a numbered metal flag, excess batter was drained, and the pieces were browned 3 min in the fryer in peanut oil at 193°C. Pieces were drained and placed in insulated plastic cups fitted with transparent plastic lids. The cups were labeled with the panel member's name. The members ate the samples hot and then completed the forms (Fig. 1).

Requests for volunteers for the panel were sent to all of the staff except those in the Poultry Laboratory and those who had participated in a similar study of turkey in the previous year. The only information the panel received until after completion of the test was the following statement, on the request for volunteers: "Each person selected will be served a portion of fried chicken (light meat morsels) . . . and will be asked to give his opinion about the flavor, juiciness, and tenderness of the chicken. The information that you give us will help in determining ways to improve the quality of chicken." Of 103 people who started the study, 101 completed it.

Each person received three samples, one each week for three weeks. The panel was randomly divided into nine groups. Three of the nine groups were controls to determine whether the general reaction varied from the first to the third samples. They always received meat from one shear-resistance range. The other six groups received meat of different shear resistance each week, allowing evaluation of the influence of toughness of the preceding sample on the scores for tenderness. The plan for all groups follows, with letters indicating the L.E.E.-Kramer shear range: A, low (3.63–5.45 kg/g); B, intermediate (9.08–17.25 kg/g); C, high (17.71–29.51 kg/g).

Plan for distribution of samples

	Constant-shear-force groups			Variable-shear-force groups					
	1	2	3	4	5	6	7	8	9
1st week	A	B	C	A	A	B	B	C	C
2nd week	A	B	C	B	C	A	C	A	B
3rd week	A	B	C	C	B	C	A	B	A

CODE No. ..... NAME .....

The following questions regarding the quality of the fried chicken morsels are to be answered by each person participating in the study. We are interested in your own opinion. Please answer the questions before discussing the study with anyone.

A. GENERAL OR OVERALL QUALITY (check one)		B. TENDERNESS (check one)	
1. Like extremely well	.....	1. Extremely tender	.....
2. Like very much	.....	2. Very tender	.....
3. Like moderately	.....	3. Moderately tender	.....
4. Like slightly	.....	4. Slightly tender	.....
5. Dislike slightly	.....	5. Slightly tough	.....
6. Dislike moderately	.....	6. Moderately tough	.....
7. Dislike very much	.....	7. Very tough	.....
8. Dislike extremely	.....	8. Extremely tough	.....

C. JUICINESS (check one)		D. FLAVOR (check one)	
1. Extremely juicy	.....	1. Excellent	
2. Very juicy	.....	2. Very good	.....
3. Moderately juicy	.....	3. Good	.....
4. Slightly juicy	.....	4. Fair	.....
5. Slightly dry	.....	5. Poor	.....
6. Moderately dry	.....	6. Very poor	.....
7. Very dry	.....		
8. Extremely dry	.....		

E. Would you enjoy eating chicken of this quality again? (check one)

Yes ..... No .....

F. Please comment briefly on the chicken with respect to its overall quality, tenderness, juiciness and flavor.

.....

.....

Fig. 1. Panelists form for reporting consumer reaction to chicken.

RESULTS AND DISCUSSION

Shear resistance of meat tested. Shear resistance of the samples from the outer breast muscles ranged from 3.63 to 29.51 kg/g on the L.E.E.-Kramer shear press and from 0.91 to 15.44 kg on the Warner-Bratzler shear apparatus. The average weight of samples tested was 19 g and ranged from 18 to 22 g for the six aging-period groups. The influence of aging time on the distribution of samples within various ranges of shear values (Table 1) shows both the familiar decrease of shear resistance with aging and the variability of shear resistance between birds aged the same length of time.

L.E.E.-Kramer and Warner-Bratzler results had a correlation coefficient of 0.91. Burrill *et al.* (1962) reported a slightly lower correlation (0.84) on beef. Because of the uniform size of the birds used in the present test, adjustment of the Warner-Bratzler readings for muscle weight did not alter this correlation. The few pairs that deviated considerably from one another were from tough, light (8–11 g) muscles. As expected, the L.E.E.-Kramer values for such small muscles were relatively high (23.15–25.88 kg/g), and the Warner-Bratzler values unadjusted for muscle weights were relatively low (7.72–9.99).

Table 1. Shear resistance of outer breast muscles.

Aging time (hr)	Birds cooked[a] (no.)	Distribution of samples in shear ranges (%)[b]		
		3.63–8.63 kg/g	9.08–17.25 kg/g	17.71–29.51 kg/g
0.5	48	21	29	50
1.0	55	33	33	34
2.0	52	62	23	15
4.0	52	85	15	0
6.3	20	90	10	0
24.0	20	100	0	0
	247			

[a] Deep-fat fried from the frozen state.
[b] L.E.E.-Kramer shear press.

General quality ratings. Meat having shear resistances of 3.63–5.45, 9.08–17.52, and 17.71–29.51 kg/g averaged 2.6, 3.0, and 3.7 in panel rating of general quality (Table 2). Ratings of 2, 3, and 4 are respectively described as "like very much," "like moderately," and "like slightly." The differences between these average ratings for general quality were significant at the 1% level and undoubtedly were affected by shear resistance values, although less closely than were the tenderness ratings. The juiciness ratings averaged slightly over 4, "slightly juicy," on an 8-point scale ranging from "extremely juicy" to "extremely dry." The ratings for flavor averaged slightly less than 3, "good" on a 6-point scale ranging from excellent to very poor. These two ratings were unrelated to shear resistance.

Influence of shear resistance on tenderness rating. The average ratings for tenderness of the outer breast muscle were 3.2, 4.2, and 5.4 for samples having shear resistances of 3.63–5.45, 9.08–17.52, and 17.71–29.51 kg/g on the L.E.E.-Kramer shear press (Table 2). Ratings of 3, 4, and 5 are respectively described as "moderately tender," "slightly tender," and "slightly tough." These results show the expected increase in toughness with increasing shear resistance; the differences between groups were significant at the 1% level.

In a previous study on turkeys (White *et al.*, 1964), the tenderness rating decreased for successive equivalent samples. The tenderness of preceding samples also influenced the panelists. In the present study, neither the panel as a whole nor the control groups alone became increasingly critical of tenderness with successive samples. The preceding sample did affect their rating of tenderness. For example, they rated meat in the lowest shear resistance range more tender (2.7) when it followed a sample in the above 9.08 kg/g shear range, and tougher (3.8) when the preceding sample was in the same shear range. The difference in ratings was significant at the 1% level of probability. Rating of the meat in the range above 9.08 kg/g was slightly, but not significantly, lower (4.5) when the preceding sample was in the same range than when the preceding sample was in the lowest range (5.1).

Reaction to shear resistance was appar-

Table 2. Influence of shear resistance of outer breast muscle on tenderness, with other quality ratings for comparison.

L.E.E.-Kramer shear resistance range (kg/g)	Warner-Bratzler average shear resistance (kg)	Samples[a] (no.)	General quality[b]	Tenderness[b]	Juiciness[b]	Flavor[b]
3.63– 5.45	1.96	103	2.6	3.2	4.0	2.6
9.08–17.25	6.38	103	3.0	4.2	4.2	2.6
17.71–29.51	11.26	97	3.7	5.4	4.4	2.9

[a] Halves were cooked from the frozen state, breast was cut in 2 or 3 pieces, coated, reheated to cook batter coating.
[b] For scale, see Fig. 1.

ent in the responses to the question whether the consumer would enjoy eating chicken of this quality again. Of the panelists receiving samples in the lowest shear-resistance range, 81% said they would enjoy eating chicken of this quality again. The percentage was 77% in the middle range, and decreased to 56% in the highest range. There was no significant change in opinion during the three weeks.

The number of comments on toughness increased as the shear-resistance values increased. Adverse comments on toughness were made by 11% of those testing meat in the lowest range, 39% of those in the middle range, and 57% of those in the highest range.

The decrease in complimentary comments, and the increase in toughness comments, on samples in the higher shear groups are ample evidence that untrained consumers of chicken recognize and object to toughness. They indicate at least 6 hr of aging is needed to obtain satisfactory tenderization if the birds are to be cooked from the frozen state.

Influence of minimum thaw on shear resistance. Thawing for 45 min in running tap water reduced the average shear value of 5 halves from the 0.5-hr aging group by only 2% from that of the corresponding halves cooked from the frozen state. All were in the intermediate shear group (L.E.E.-Kramer test 20–38 kg/g).

The 27 halves thawed 1–1¼ hr had an average shear value of 8.17 kg/g, 45% lower than the shear value (14.98 kg/g) of the corresponding halves cooked from the frozen state. The shear value of the thawed halves was significantly lower than the shear value (13.17 kg/g) of those aged 1 hr before freezing and cooked from the frozen state. However, it was not significantly different from the shear value of those aged 2 hr before freezing. Fifty-nine percent of the thawed halves were in the 3.63–8.63 kg/g shear range, a proportion comparable to the 62% of the birds of the 2 hr-aging group in that range (Table 1).

Warner-Bratzler shear values generally confirmed the L.E.E.-Kramer shear values (correlations of $r = 0.83$ for birds cooked from the frozen state and 0.94 for birds cooked after thawing). Although thawing for 1–1¼ hr before cooking usually lowers shear resistance, a longer time would be needed to reduce the shear values of the toughest birds to the range that caused few consumer complaints.

These results suggest that at least 4-hr aging before freezing is desirable even for birds that are completely thawed before cooking.

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Evaluation of Sugar-Acid-Sweetness Relationships in Orange Juice by a Response Surface Approach

SUMMARY

The effects of added sugar and acid on the sweetness of orange juice were evaluated by the response surfaces estimated from a central composite, second-order design in incomplete blocks. First-order polynomials were sufficient to describe intensity responses, but a second-order polynomial was needed to describe acceptability.

Information on the chemical nature of compounds that potentially can contribute to flavor and odor has accumulated rapidly as a consequence of new analytical techniques. Such advances require consideration of the question of interrelationships among the various compounds in the production of subjective flavor responses. Many studies have been made of this question, but collation of the results has been made difficult by ambiguity in defining interactions as well as by differences in methods of measuring response and in ranges of concentrations. With respect to the first point, it has not always been clear whether the term interaction was being used in a general sense synonymous with interrelationships or in the stricter statistical sense of interactions.

The present study was planned, therefore, to explore the potential of experimental approaches suggested by Box and co-workers (Box and Wilson, 1951; Box, 1954; Box and Hunter, 1957) in the definition of such interrelationships.

These designs have had limited use in the food field (Donelson and Wilson, 1960; Kissell and Marshall, 1962; Pearson *et al.*, 1962; Wilson and Donelson, 1965), although an application was suggested in one of the early articles (Box, 1954). Basically, the designs are extensions of factorial and multiple regression procedures in which the experimental levels in the design matrix are chosen to meet certain requirements for variance structure and blocking, as well as to yield data for the estimation of appropriate coefficients.

A relatively simple system, orange juice with added sugar and citric acid, was used in the current study. Sweetness was evaluated in terms of intensity of sweetness and, in one series, by acceptability. The basic assumption was that the flavor response, whether intensity or acceptability, was a function of the amount of added sugar and acid, so that:

$$Y = f(X_1, X_2) \qquad [1]$$

where Y = flavor response
X_1 = concentration of added sugar
X_2 = concentration of added citric acid

and that the function, f, could be approximated by a polynomial of some degree.

The first-order polynomial of the form,

$$Y = b_0 + b_1x_1 + b_2x_2 \qquad [2]$$

represents the interrelationship that is implied by workers who report flavor enhancement (coefficients of the same sign) or depression (coefficients of unlike signs) without interaction effects. In the case of sugar-citric acid systems, citric acid has variously been reported to increase or decrease sweetness (Fabian and Blum, 1943; Kamen *et al.*, 1961; Pangborn, 1960, 1961; Pangborn and Trabue, 1964).

The second-order polynomial,

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \qquad [3]$$

is a form that would express, in addition to the linear effects shown in Eq. 2, both nonlinear effects and sugar-acid interactions. In the usual factorial sense, such a function describes the situation in which the magnitude of the change in response, Y, as the concentration of x_1 is changed from c_1 to c_2 depends on the concomitant level of x_2. Such interactions have been reported for some but not all of the sucrose-citric acid studies of Pangborn (1960, 1961; Pangborn and Trabue, 1964). Kamen *et al.* (1961) also reported results suggestive of such interactions.

METHOD

The basic plan of the experiment was that of the central composite second-order design in incomplete blocks given by Cochran and Cox (1957). Four experimental regions were studied. The first block in each case was a 2^2 factorial with additional center points. It consisted of the four combinations of ± 1 levels of sugar and acid, and two replications of the center (0,0) point. The -1, 0, $+1$ levels of added sugar were 0.05, 0.15, and 0.25M in all of the experimental regions; of added acid, 0.005, 0.010, and 0.015M in the first region, 0.005, 0.015, and 0.025M in the second region, 0.0059, 0.02, and 0.0341M in the third region, and 0.0073, 0.025, and 0.0426M in the fourth region. The second block consisted of the four points obtained by rotating the first block through 45°, and, again, two replications of the center points. The levels were represented in the code as ± 1.414 and 0.

The series of combinations for Block I and for Block II were scored for intensity. The order within each block was randomized for each judge. The entire design was repeated on each of four days for each region. Intensity was scored on a nine-point scale with the guide words none, slight, moderate, strong, and extreme.

The combinations for region two were also scored on each of two separate days for acceptability. The order within each block was randomized for each judge. Acceptability was scored on a seven-point scale, with guide words of unacceptable, acceptable, and highly desirable.

Sufficient frozen orange juice for an entire day's judging was reconstituted with tap water in the regular 1:3 ratio. Sugar and citric acid (C.P.) were added to aliquot portions in the amounts required by the experimental design.

The judges were undergraduate women, for the most part foods and nutrition majors. The initial group for regions one and two had participated in orange juice panels for about a year before the beginning of the experiments reported here. The group for region three included new replacements, and that for region four consisted entirely of the new group. Nine, seven, eleven, and eight judges were used for the four successive regions on intensity judgments, and six for the acceptability test.

Intensity and acceptability scores were analyzed by analysis of variance techniques. The mean squares for levels × judges interaction was used to test the significance of levels and the various components of levels. The coefficients for the fitted equations were calculated by the formulas given by Cochran and Cox for this design. The minimum response was estimated by reducing the polynomial

Table 1. Analyses of variance of intensity and acceptability scores.

Source of variation	Intensity								Acceptability	
	Expt 1		Expt 2		Expt 3		Expt 4		Expt 2	
	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Levels	9	158.7737	9	114.5799	9	226.6080	9	169.0117	9	8.3343
X_1 (Sugar)	1	1313.5347	1	893.5537	1	1657.6678	1	1087.7426	1	8.0379
X_2 (Acid)	1	102.0203	1	121.7007	1	349.8194	1	417.2317	1	11.1810
X_1^2	1	0.4443	1	1.002	1	4.2217	1	1.8970	1	28.9548
X_2^2	1	3.9932	1	2.8324	1	1.5317	1	0.9888	1	12.2785
X_1X_2	1	0.0525	1	0.7640	1	0.0288	1	2.9707	1	8.1263
Lack of fit	3	2.9432	3	2.2014	3	8.3410	3	3.1955	3	1.6690
Blocks	1	0.0904	1	4.7619	1	1.2558	1	0.6876	1	1.4092
Judges	8	12.6297	6	14.8147	10	17.1703	7	13.0513	5	1.7650
Replications	3	0.6691	3	0.6999	3	3.1403	3	1.6225	1	1.8338
Levels × judges	72	1.932	54	2.214	90	2.5058	63	3.8233	45	1.9175
Levels × replications	27	1.0817	27	1.3580	27	1.2585	27	1.1814	9	1.5613
Judges × replications	24	2.161	18	3.2339	30	2.0091	21	2.3917	5	0.9504
Levels × judges × replications	216	1.0143	162	0.9328	270	1.5409	189	0.9854	45	1.4706
Duplicate points with blocks	72	1.1614	56	0.8499	88	0.7393	64	1.0430	24	0.7241

to its canonical form by the method described by Davies (1956).

RESULTS

Analyses of variance for intensity scores for each of the four experiments are shown in Table 1. The "lack of fit" terms were not significant in experiments 1, 2, and 4, and were significant at the 5% but not 1% level in Experiment 3 when tested against the level × judges interaction. Thus, the regression model appeared to describe the experimental results quite well over most of the experimental ranges.

The linear terms, but not the quadratic and interaction terms, were highly significant in each experiment. This pattern held within each experimental range, although the contribution of the linear acid component to the total sum of squares for levels increased progressively from 7% to 27% as the upper limit of the acid range was increased from 0.017 to 0.05*M*. The significance of the first-order terms and the concomitant nonsignificance of the second-order terms indicated that the effects of acid was not dependent on the sugar level. In this respect, the results agreed with those of Pangborn (1961) with suprathreshold levels rather than those with lima bean purées (Pangborn and Trabue, 1964) since in the former, but not the latter, the citric acid × sucrose interactions were not significant. Furthermore, for the experimental ranges studied here, it was not necessary to consider the relationship between intensity and concentration as a logarithmic function, as would be suggested by a number of studies, a typical one being that of Schutz and Pilgrim (1957).

The positive effect of sugar and the negative effect of acid on the intensity of sweetness may be seen in the mean intensity scores (Table 2) and in Fig. 1, in which the fitted equations are plotted. Within each experimental range, the general pattern agreed with that of workers who have reported that the effect of citric acid is to decrease sweetness (Pangborn, 1960, 1961; Pangborn and Trabue, 1964) rather than that of studies reporting increases in sweetness (Kamen *et al.*, 1961; Fabian and Blum, 1943).

Further examination of Fig. 1 shows that some overlapping of the curves occurred at the low levels of sugar and acid, but in general the relationship between intensity and sugar and acid varied with the experimental range. The question might be raised whether this variation reflected differences in perception of intensity or in the communication of differences in terms of the rating device. However, it is axiomatic that extrapolation of a fitted curve beyond the

Table 2. Mean intensity and acceptability scores for orange juice with added sugar and citric acid.

Coded level		Actual level					Mean intensity score[a,c]				Mean acceptability score[b,c]
			Acid (*M*)								
Sugar (X_1)	Citric acid (X_2)	Sugar	1	2	3	4	1	2	3	4	2
Block I											
−1	−1	0.05	0.005	0.005	0.0059	0.0073	3.0	3.1	3.2	3.6	4.06
+1	−1	0.25	0.005	0.005	0.0059	0.0073	7.5	7.0	7.8	7.9	4.48
−1	+1	0.05	0.015	0.025	0.0341	0.0426	2.1	2.0	1.6	1.6	5.15
+1	+1	0.25	0.015	0.025	0.0341	0.0426	6.5	5.7	6.3	5.3	3.92
0	0	0.15	0.010	0.015	0.02	0.025	5.0	4.7	5.1	4.9	3.28
Block II											
−1.414	0	0.01	0.010	0.015	0.020	0.025	2.0	1.8	2.0	1.8	5.31
+1.414	0	0.29	0.010	0.015	0.020	0.025	7.7	7.7	7.7	7.8	4.25
0	−1.414	0.15	0.003	0.001	0	0	5.7	5.9	6.7	6.8	3.63
0	+1.414	0.15	0.017	0.029	0.04	0.05	3.7	3.4	3.3	2.8	5.19
0	0	0.15	0.010	0.015	0.02	0.025	5.0	4.9	5.0	4.8	3.49

[a] Means of four judgments by each of 9, 7, 11, 8 judges, respectively.
[b] Means of four judgments by each of 6 judges.
[c] Scores for zero levels, means of eight instead of four judgments.

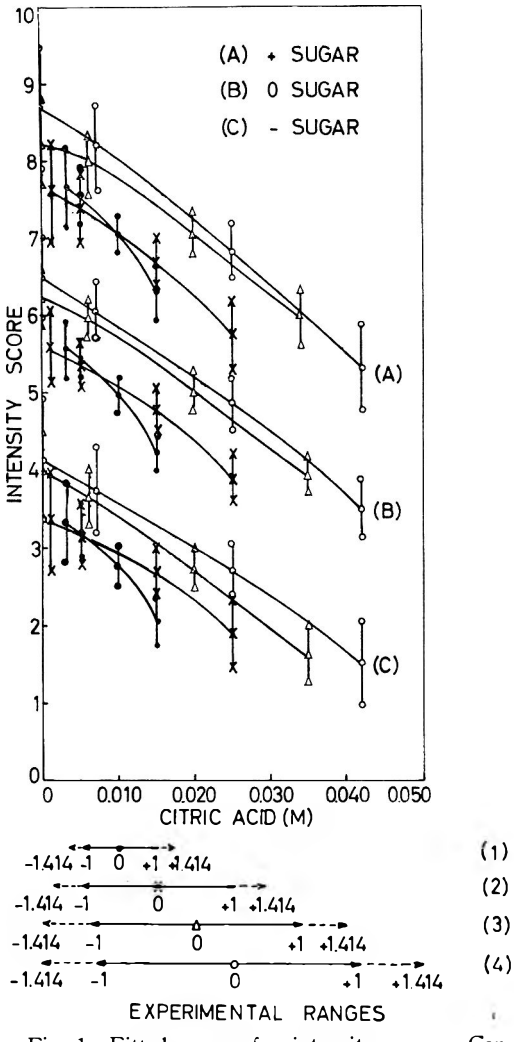

Fig. 1. Fitted curves for intensity scores. Confidence interval indicated at 5% level.

experimental region for which it was determined can lead to misinterpretation of the data.

The results for acceptability, shown also in Tables 1 and 2, indicated that the judges differentiated quite clearly between intensity and acceptability. The second-order terms of x_2^2 and $x_1 x_2$, as well as the first-order terms x_1 and x_2, were significant, and the x_1^2 term was highly significant. Consequently, the response can be shown as a series of ellipses (Fig. 2) representing combinations of sugar and acid that give equal acceptability. The minimum acceptability score, 3.28, occurred at the coded value of $+0.1, -0.3$.

The results of the evaluation of intensity and acceptability indicated the usefulness of fitted polynomials in describing flavor responses, and particularly in distinguishing between interrelationships involving linear, quadratic, and interaction effects. The variation in response with experimental range suggested, however, the need for caution in the generalization of specific results.

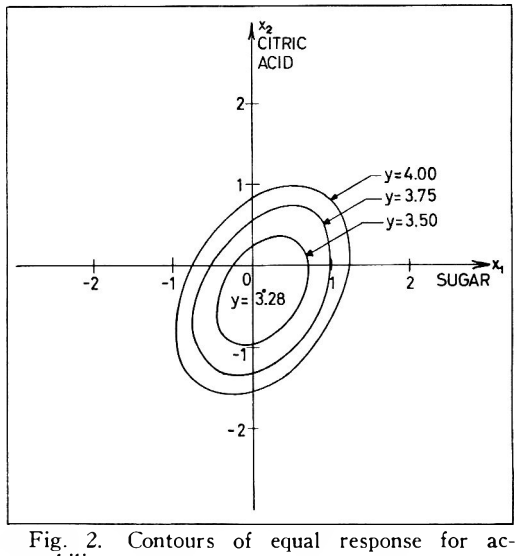

Fig. 2. Contours of equal response for acceptability scores.

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Physiological Aspects of Olfaction

To know how we perceive odors is to have solved three key problems in olfaction: How does the odorous molecule excite the receptor? How are different odors distinguished? How is the receptor output encoded and processed on its course to the higher integrative foci of the brain? And beyond perception there is the role of olfaction as a controlling device: odors can exert powerful and specific effects on behavior and on certain body processes, such as reproduction. By what mechanisms are these effects achieved?

What follows is a brief glimpse of some aspects of these problems. But to begin with, consider one property of the nose which is especially important in understanding some of the problems and phenomena in olfaction —its sensitivity.

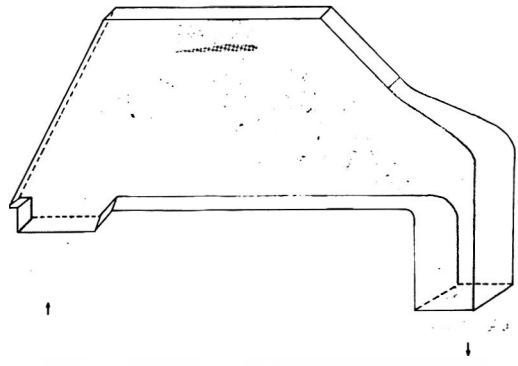

Fig. 1. Schematic diagram of lumen of the human nose. The olfactory epithelium (stippled) has an area of about 2.5 cm^2. Air enters the nostrils at lower left. The shaded area gives an approximate indication of the air flow during normal breathing as deduced by Stuiver (modified after Stuiver, 1958).

How sensitive is the nose? One approach to this question is to ask how many molecules are needed to excite a single receptor cell. This is not simply a matter of comparing the number of molecules entering the nose at threshold with the number of olfactory receptors. To excite, the molecules must first reach their targets. And these lie well recessed from the main air stream (Fig. 1). What proportion of those molecules inhaled actually reach the receptors? The first trap is the watery sheet of mucus lining the nasal cavity. One fraction of the molecules is lost by adsorption to this film. A further fraction fails to reach the receptors because it bypasses the olfactory area. To estimate this loss, Stuiver (1958) built a plexiglass model of the nose. For the airstream he substituted aluminum particles suspended in water. He could then photograph the flow and measure the volume entering the olfactory region. This suggested that only 5–10% of nasal air intake during normal respiration (250 cc per second) actually reached the olfactory area. Finally, a third fraction passes over the olfactory area but fails to hit the receptors. By deriving the size of the three fractions for threshold concentration of marcaptans, Stuiver was able to calculate the number of stimulating molecules present. This showed that out of 10^6 molecules entering the nose at each sniff only 2%—about 2×10^7 molecules—actually contacted the receptors. Assuming that those molecules were evenly distributed over 4×10^7 receptors, he further estimated that perhaps no more than eight molecules were necessary to stimulate a single receptor. The low figure suggests that the number is actually one. Neither dog, moth, nor instrument can do better.

Such estimates may involve many assumptions, not all justified. But they give an idea of the order of magnitude involved. And even when we allow for the inefficiency of the entire system in exploiting the efficiency of the receptors, the sensitivity is still impressive. For example, Kendall and Neilson (1964) compared the responses of a trained odor panel with those of a flame ionization detector of a gas-liquid chromatograph. (The odors tested were menthol, citral, methyl salicylate, and safrol.) The panel detected each odor in concen-

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trations of at least one power of ten, and—what is even more significant—contaminants in several powers of ten below chromatographic sensitivity.

When we consider the consequences of this sensitivity, several points emerge. Firstly, as is well known, conventional techniques of microanalysis cannot be relied upon to detect all odorants present in concentrations detectable by the nose. This emphasizes a major problem in flavor research—the difficulty of isolating and identifying the volatile constituents of a substance that uniquely determine its natural aroma. It is becoming increasingly apparent that trace components may be important determinants of food flavors, and, indeed, of many behavioral and physiological responses to chemicals of biological significance, such as sweat.

Secondly, this sensitivity of the nose implies that its excitability is continually being modulated by the complex "phantasmagoria" of odorants normally present in the environment. In fact, electrical recordings from the primary neurones of the rabbit, show a burst of activity with each inspiration of room air. Even in an "odorless" environment, remarkably high concentrations of volatile sulfur compounds pass through, or near, the human nasal airways. Thus, mass spectrometric analysis of fresh, early-morning mouth air show up to 9 ppm methyl mercaptan; 2–51 ppm carbon disulfide; 0–160 ppm sulfur dioxide; 3–34 ppm hydrogen sulfide, as well as 31–104 ppm ethanol (5 subjects sampled for 10–30 seconds each; Richter and Tonzetich, 1964).

Now it is claimed that methyl mercaptan can be detected at 0.002 ppm (Patton and Josephson, 1957), while only a few molecules of butyl mercaptan seem necessary to excite a single olfactory receptor (Stuiver, 1958). We may therefore ask: Is man's acute sensitivity for some mercaptans related to the continuous presence of these volatiles? Do they sensitize the receptors or otherwise influence the perception of other odorants—for example, the volatile sulfur compounds which are important flavor components of many foods (e.g. Hing and Weckel, 1964; Gumbmann and Burr, 1964). (In addition to this inescapable accompaniment of personal volatiles, experimenters in "olfaction" often contend with an atmosphere contaminated with trace odorants. It is almost as if we were to determine visual thresholds during a fireworks display.)

Clearly, more research is needed on the influence of background odors, both on the subjective appreciation of flavors, and as a factor modulating receptor response to test odorants. Work of Guadagni *et al.* (1963) confirming that subthreshold concentrations of many compounds can produce a detectable odor when intermixed, illustrates one useful approach. And no doubt the growing interest in isolating odorous counterparts to the "nucleotides" and other flavor enhancers will precipitate further work in this area.

But for our present purposes the significance of the sensitivity of the nose lies in the further problem it raises: from what properties of molecular organization it is derived?

Odorant-receptor interaction. The action of odorous molecules is to alter the receptor cell so that it fires a nerve impulse in the fiber leading to the brain. It is generally assumed that the primary events in the interaction occur in or on the receptor cell membrane. Presumably, regions of the surface are specifically organized to respond only to the appropriate stimulus—specificity being conferred by the steric arrangement at the receptor site. To understand these events we must know the detailed structure of the cell membrane. According to one model we are dealing with two lipid monolayers sandwiched between two monolayers of protein or nonlipid (e.g., hydrated polysaccharides). The total thickness of the membrane is 75–100 Å.

Modern theories regard the membrane as a dynamic structure, shifting between different phases of organization with different states of function. Thus, in going from one phase to another, pillars of lipids collapse to obliterate intervening aqueous regions (Kavanau, 1963), or a precisely ordered lattice held by water in "icelike" formation becomes disorganized when water alters to a less organized state (Hechter, 1965). Such alterations could provide temporary

channels making possible the ionic movements associated with the excitation of the membrane. However, these concepts give only a framework common to all cell membranes. The features which distinguish one membrane from another are thought by some to be associated with specialized transducing units periodically arranged in the lipoprotein matrix. These may consist of specific enzymes, pigments, or specialized electron transfer systems.

But even if such concepts find general acceptance, we are still far from knowing the organization and specific components of the transducing units which determine the unique properties of the olfactory membrane. Furthermore, the membrane is not directly exposed to the nasal cavity but is overlain by a sheet of pigmented secretions specific to the olfactory area. There is little agreement about their composition or function. Such gaps in our knowledge seriously limit the construction of useful and detailed theories of the olfactory process. In fact, there has been little real progress toward the solution of this problem in recent years. A number of analogs of the nose have been constructed, but in no case has the experimenter shown that the biological nose functions in a similar manner.

However, the inital event is probably the adsorption of the odorant molecule to the receptor membrane. According to one theory—that of Davis (1965)—the molecule then penetrates the membrane and desorbs, either into the cell or back to the secretions. He estimates a desorption rate of 10^{-8} sec for larger molecules and 10^{-11} sec for smaller molecules. But the dislocation produces a hole which does not heal immediately. By substituting estimated values in an equation derived from the theory of surface viscosity he derives a healing time of 10^{-4} seconds. With larger molecules this may be sufficiently longer than the desorption time to allow the interchange of K^+ and Na^+ through the hole. And this ionic flow is sufficient to trigger off the events that initate the nerve impulse. Small molecules, however, will not singly form a hole large enough for ionic transfer. Davies therefore supposes that a number of molecules can be concentrated on a critical area (about 60 Å) of the receptor membrane, and that they will then leave a hole large enough for ionic transfer to occur.

Enzyme theories are often favored but meet with serious objections. As Sumner (1954) has pointed out, no known enzyme system could respond to the very low concentrations of some odorants that are sufficient to excite the receptors. There is also the difficulty of having one enzyme for each of the many thousands of odorants claimed to be distinguishable by the nose. The more significant of the many remaining odor theories have been reviewed by Dravnieks (1966).

But whatever may be the mechanism in this or any other sensory system, it is conventional to look in or near the receptor cell for a slow potential immediately preceding the nerve impulse. This local, graded response—the so-called "generator potential"—is thought to trigger the all-or-none nerve discharge. Ottoson (1956) was able to record a slow potential from the surface of the olfactory epithelium in response to odor stimulation. He identified it as the generator potential and called it the electro-olfactogram (EOG). He points out, however, that it might be a complex potential in which the generator potential is only one component. But some workers question whether any part of the potential is derived from the receptor cell. In most cases its behavior parallels that of the primary olfactory neurones.

Ottoson and von Sydow (1964) connected the outlet of a gas chromatograph column to the olfactory epithelium of a frog. They then measured the response of the EOG to a concentrate of high-boiling volatiles from black currants passing through the column. No direct correlation between the amplitude of the chromatographic peak and that of the EOG appeared. For example, terpinen-4-ol gave a low chromatographic peak but a high EOG response. With car-3-ene the situation was reversed. However, when the relative stimulating efficiency of the compounds was compared with subjective evaluations of the same odors, the estimated intensities derived from the two

methods were claimed to be in good agreement.

Such applications of electrophysiological techniques to derive quantitative data will no doubt prove valuable in supplementing subjective observations. But they can also be misleading. The properties of receptor response which are important to man in evaluating an aroma need not be those which are revealed by conventional electrophysiological methods. A distinctive odor such as that of skatole or certain musks may evoke a relatively small response in the olfactory bulb of the rabbit. Furthermore, significant information about odor quality may be carried by only a small proportion of fibers in the entire organ, or by trigeminal fibers which are separate from the olfactory system and highly chemosensitive. Nor need we suppose that the properties of the receptors will be the same in man as in animals.

Differential sensitivity to odors. The simplest assumption is that the nose distinguishes between many different odors because it has different types of olfactory receptors. There is no convincing evidence that the receptors can be grouped by their appearance—at least in mammals—but differences might still exist at the molecular level. The most direct way of testing for functional differences is to compare odor-induced electrical responses, each derived from a microelectrode inside a single receptor. However, the cells are usually little more than 5 μ wide, which is too small to penetrate easily. Available records of single-unit responses are therefore mainly extracellular. The important point is that they fail to show that receptors can be classified into a small number of distinct groups.

But it might be argued that this evidence has limited significance. Since individual receptors may respond to a few molecules, standards of purity acceptable to the organic chemist are not always acceptable to the nose. In any case, it is common observation that even compounds of the highest grade of purity commercially obtainable may yield numerous peaks when fractionated by gas chromatography. Thus the simplest stimulus the experimenter uses may still be a harem of odors to the nose. Consequently, if there are a number of different types of receptors, any given stimulus might excite so many of them that differences would be obscured.

However, available techniques are capable of detecting high odor specificity at least in one instance. This occurs in a species of silkmoth (*Antheraea pernyi*). Here, Schneider *et al.* (1964) have located and recorded from single odor receptors which are sensitive to the sex attractants secreted by the females, although fruity and flowery odors also influenced their activity. Other receptors did not respond to the sex attractant. But sex attractants have unusual properties. For example, in another species of silkmoth (*Bombyx mori*), Butenandt and Hecker (1961) were able to isolate, identify, and synthesize the female sex attractant which they called Bombykol: hexadecadien-10-*trans*,13-*cis*-1-ol. Behavioral responses to this compound were obtained when it was presented in concentrations of only 10^{-12} μg/ml. Isomers were ineffective until much higher concentrations were reached.

No doubt many more examples of specific receptors for sex attractants will be found. But these are probably exceptional instances linked with the especial biological importance of such compounds. It is easier to accommodate evidence derived from other sources if we assume either that specificity is a property of receptor sites rather than types, or—less likely—that the olfactory receptors posses no intrinsic differences in odor specificity. By a receptor site we mean a region of the membrane in which a specific odorant-receptor interaction occurs. The basic concept was used by Langley as early as 1905 in relation to drug action. It is useful here because it allows us to think not only of different types of site but of different types of site on the same receptor. So long as these types are not randomly distributed, the receptor will show some degree of odor specificity. This could account for the evidence that any specificity that the receptors possess may not be of a high order.

The electrophysiological findings are not incompatible with some recent theories of odor quality such as one proposed by Davies (1965). He suggests that a "continuous spectrum of responses" may be characteristic

of the olfactory epithelium, although some compounds form relatively well defined classes. The basis for his approach is his theory of olfactory receptor excitation, outlined above. He assumes that there are different olfactory membranes with different physical properties. Some are very fluid and heal rapidly after dislocation by the odorant molecule, whereas others are resistant to penetration. Sensitivity to a small molecule such as butanol would result from its fast desorption time. It thus leaves a "clean" hole which remains open long enough for ionic transfer to occur. With a more slowly moving molecule such as musk, however, the hole would heal almost immediately behind it. A further assumption is that small molecules do not have enough energy of adsorption to penetrate some membranes which larger molecules can dislocate. Thus the significant parameters of an odorant, according to this theory, are the energy of desorption from a lipid-water interface into water, and the molecular cross-sectional area. When Davies plotted these against each other—as in Fig. 2—he obtained some distribution of compounds into classes of similar odors.

A major difference between this theory and Amoore's well-known "stereochemical theory of odors" is that the last theory emphasizes the importance of molecular shape and size rather than molecular cross-sectional area. Originally Amoore held that there are seven primary odors, five of which are distinguished by differencs in molecular shape and size, while the remaining two—"putrid" and "pungent"—depend on the electronic status of the molecule (Table 1). For each of the first five odors he advised a receptor site of complementary and specified shape and size (Amoore, 1964). However, he has now modified his views on some aspects (Amoore, 1965). In particular, he stresses the high correlation which he finds between odor similarity scores derived from a panel of judges and the similarities in molecular silhouettes of standard compounds representing the "seven" classes of the stereochemical theory. Thus, while the sitefitting concept remains an essential ingredient of the hypothesis, the precise molecular dimensions of the sites are no longer emphasized. He also considers groups to be "classes" rather than more rigidly defined "primaries" and concedes that many such classes may exist. In its present form the theory now avoids some of the many criticisms leveled against the original version and is nearer to the theory of Davies.

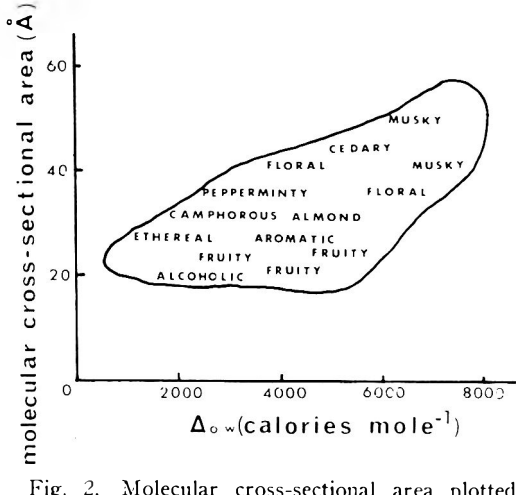

Fig. 2. Molecular cross-sectional area plotted against energy of desorption from lipid-water interface into water (after Davies, 1965).

Furthermore, the descriptive identities of several of these classes are not in dispute (Table 1). Indeed, the distinctiveness of such odor qualities as "camphorousness" (e.g. Timmermans, 1954) and "musky" has long had wide acceptance. It is therefore interesting that specific parosmias (absence of sensitivity for certain odors) have been reported for different types of "musk" (Table 1). This suggests that if odor "classes" exist, even "musky" odors may be composed of more than one "class." (Although the study of parosmias seems to offer a powerful tool in exploring the basis of odor quality, it has been given surprisingly little attention.)

However, present theories of odor quality have a major weakness which most theorists recognize. Because many of the properties of the molecule are interrelated, it is not sufficient to show that a high correlation exists between a given set of molecular properties and subjectively-derived estimates of odor quality. It must also be shown that

Table 1. Comparison of odor classes suggested by various authors with compounds for which specific parosmias have been claimed.

Zwaardemaker (1895)	Amoore (1964)[a]	Davies (1965)[a]	Parosmia[b]
			macrocyclic musks
ethereal	ethereal	ethereal	
aromatic	camphoraceous	"camphorous"	sterol musks
amber-musk	musky	musky	nitrol-musks
			methyl ionone
balsamic	floral	floral	
alliaceous	pepperminty	pepperminty	thymol
empyreumatic	pungent	almond	hydrogen cyanide
			benzyl salicylate
caprilic	putrid	cedary	
repulsive		"alcoholic"	eugenol
nauseating		"aromatic"	farnesol
		fruity	ambergris
			butyl mercaptan

[a] The terms used by Amoore and Davies are not necessarily intended to cover all odors, or to indicate sharply defined groups. They are listed here to indicate the type of categories which tend to be used in descriptions of odor quality. Similarly the list of compounds for which specific parosmias have been claimed is not exhaustive. Apart from the musks, parosmias for only two compounds—butyl mercaptan and hydrogen cyanide—have been independently confirmed (for references, see Moulton and Tucker, 1964).

[b] Odor "blindness."

a similar correlation does not exist with other properties. At the moment, few if any of the odor theories seem detailed enough to indicate how such a critical test could be made.

But there remains a further class of theory which, in contrast to the types so far considered, assumes no degree of odor specificity. Instead, it proposes that each odor sets up a different spatio-temporal pattern of excitation across the receptor sheet. The pattern depends on the fractionation of the molecules as they cross the organ, and is assumed to be determined by—for example—the relative strengths of binding of the odorant molecules to the receptors (Adrian, 1956; Beidler, 1957; Mozell, 1964; Moulton and Tucker, 1964). One advantage of this type of theory is that it can offer an explanation for the enormous area of the olfactory organ in many vertebrate animals. Thus, the rabbit's nose contains 10^8 receptors embedded in 7.4 cm^2 of highly convoluted mucosa. In the dog, the area covered by the odor detectors is more than that occupied by the receptors for vision and hearing combined. The advantages of a large area in increasing the degree of resolution of a pattern are obvious.

But if the nose fractionates and identifies odors by frontal analysis, as in gas-liquid chromatography, it is rather an unusual instrument. The analytical column is short (no more than a few centimeters); the carrier gas is air, humidified and equilibrated to body temperature by the time it reaches the detectors; the stationary phase is an aqueous saline suspension of pigmented granules overlying a lipoprotein membrane; and the flow rate of the carrier gas fluctuates rhythmically, reaching a maximum of less than a liter per minute, every few seconds. However, to achieve optimum efficiency, this rate can be increased to short bursts of a few liters per second. (The response of the system is highly flow-rate-dependent.) There is a mechanism for reversing the direction of carrier gas flow so that at least a segment of the column can be back-flushed after each analysis. (Dogs make good use of it: no doubt it helps to clear out those "high boilers" that might interfere with the next analysis.) An interesting feature in later systems is that successive increases in efficiency are achieved by successive increases in the proportion of detectors lying remote from the main carrier gas flow. They come to lie in elaborate folds and pockets in a blind-ending sac. Finally, the diameter of the injection port is considerably larger than the internal diameter of the column—an arrangement which is generally considered to decrease the efficiency of a gas chromatographic column.

But whatever may be the limitations of the nose as a gas chromatograph, there is doubtless some degree of fractionation of the incoming odorant molecules across the receptor sheet. The question is whether the resolving power of this column is only sufficient to segregate odors into a few broad categories—in which case it is unlikely to be of much significance in determining odor

quality—or whether a much finer analysis is possible. In fact, most evidence shows only a broad regional differentiation of response (e.g. Adrian, 1956; Mozell, 1964).

To search for more detailed distinctions in pattern, it is helpful to study events going on simultaneously at a number of points in the olfactory system. One way of doing this is to use alert rabbits with wire electrodes permanently implanted at different positions in their olfactory bulbs (Moulton, 1963). In this way nerve impulses ("spikes") set up by odor stimulation are picked up, amplified, and processed electronically to a smooth curve. These running averages are then exploited to modulate the intensity of dots on the face of an oscilloscope, or displayed as a series of traces on a multichannel pen recorder.

This approach shows that different odors set up patterns of excitation which are often quite distinctive, and maintain their different identities from day to day in the same animal. For example, in one experiment a group of 15 odorants were tested (Moulton, 1965). Of these, 8 were distinguishable in 80% or more of the trials, while the remainder could be assigned to one of three groups as follows: 1) *n*-propanol; 2) *n*-octane; 3) skatole; 4) 1,2-dichloroethane; 5) *n*-amyl acetate; 6) *n*-valeric acid; 7) 2-undecanone; 8) α-ionone; 9) Eugenol and nicotine; 10) *tert*-butanol, heptanol, "exaltaloid"; 11) d-limonene and butyl ether. However, compounds yielding similar patterns in one animal were often distinguishable in other animals. Since this evidence was derived from a minute fraction of the total number of olfactory cells at the rabbit's disposal, it would not be surprising if each odorant can set up a unique pattern of excitation.

In homologous series of compounds, such as the aliphatic alcohols, adjacent and subadjacent members may show similar patterns. Otherwise it is, as yet, difficult to see any system in these responses—any evidence that a consistent classification is possible. If anything, there seems to be a continuous array of distinct patterns.

Apart from gross regional differences there is no obvious relation between the position of the electrode and the nature of the pattern. The distinctions can therefore be interpreted in three ways: 1) receptors differ in their sensitivities to odors, but those of similar peak sensitivities are grouped together in the olfactory epithelium or project to the same regions of the olfactory bulb; 2) some form of fractionation of odorous molecules occurs as they pass across the receptor sheet; 3) both mechanisms are involved.

The processing and influence of olfactory information. Between the output of the receptors and the perception of an odor there intervenes a complex flow of interacting events. At the first relay station, in the olfactory bulb, the number of incoming channels is drastically reduced through convergence, by a factor of about a hundred. The olfactory information is apparently processed by action of interconnecting fibers and feedback loops so that part of its content may be suppressed. Signals from the higher centers of the brain can now exert a direct influence on the traffic of impulses ascending from the nose. It is at this stage that some of the phenomena associated with adaptation may occur.

As the modified information courses further into the brain it is split into a number of channels. One pathway—recently traced—goes to the thalamus, which is a major relay station for input from taste and and other sensory systems. Other projections lead rather directly to the hypothalamus, the amygdala, and neighboring regions of the rhinencephalon, where they could influence processes involved in emotion and feeding behavior. Possibly it is through the hypothalamus that the olfactory input is able to modify reproductive processes. For example, the odor of strange males can block pregnancy in mice.

Ultimately, input from the olfactory pathways is integrated with information derived from other sensory systems (such as the trigeminal receptors in the nasal cavity), and regions of the central nervous system (such as the reticular formation of the brainstem), to produce the perception of an odor.

Conclusion. The flavor scientist has a difficult task. He would like to determine

the compounds responsible for the aroma of a food, and derive a quick, objective means of quantifying the biological response. As a first step he may exploit gas-liquid chromatography to fractionate the volative constituents, and use mass, infrared, ultraviolet, or nuclear magnetic resonance spectrometry to identify them. This approach has yielded an impressive and rapidly accelerating growth of evidence revealing the complexity and identity of many food odorants (e.g. Wick, 1965). It is an essential and important contribution to food science. But to identify the volatile constituents of a food is not to determine the compounds which alone identify its characteristic aroma. The flavor scientist might therefore turn to the physiologist for an objective and quantitative measure of receptor response. In fact there are already reports that electrical activity associated with the olfactory system can be more effective in predicting the relative subjective intensities of the components of an odorous mixture than are the peaks derived from gas-liquid chromatography. However, such evidence could be misleading, and measuring the intensity of receptor response does not necessarily reveal all the qualitative properties of an aroma that are significant to man.

There remains a final approach: a bioassay based on the stimulation of the human nose. But the behavioral response of man is not a simple, objective index of olfactory sensitivity. It is the end product of a complex flow of interacting events, molded by the needs and experiences of the individual—by the input of many classes of information. Yet, in the last analysis, there is no adequate substitute.

The rate of future progress in flavor research will depend partly on effective evaluation and correlation of information from these various approaches. Unfortunately, too few workers are equipped to handle this task. Perhaps it is time to train a new type of flavor scientist.

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