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CONTENTS

The Lipids of Green Beans[a]

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SUMMARY

Raw green snapbeans (*Phaseolus vulgaris* var. Slendergreen) were cut, frozen, lyophilized, and extracted with chloroform-methanol 2:1. The crude extract amounted to 9.9% of the dry weight of the beans. The lipid material in the extract composed 2.6% of the dry weight of the bean. The crude lipids were fractionated with acetone, and the acetone-soluble portion was subjected to countercurrent distribution between *n*-heptane and 95% methanol. The major lipid fractions were treated with glacial acetic acid, followed by partition between benzene and 50% aqueous acetic acid to aid in removal of non-lipid contaminants. The composition of snapbean lipids was studied by measuring the nitrogen, phosphorus, sugar, and fatty acid content of the various fractions.

The lipids of the common bean (*Phaseolus vulgaris*) have been examined by various workers during the past six decades (Grimme, 1911; Ito, 1939; Kosutany, 1900; Ott and Ball, 1944; Takahashi *et al.*, 1949; Trier, 1913; Wittke, 1916). For the most part these studies have entailed examination of fatty materials extracted from dried mature seeds with ether, benzene, or unspecified non-polar solvents. The composition of bean lipids has been reported mainly in terms of classical "fat constants," including some information on the qualitative and quantitative distribution of fatty acids, nitrogenous bases (Trier, 1913), and sterols (Ito, 1939; Ott and Ball, 1944; Takahashi *et al.*, 1949).

The probable implication of lipids in off-flavor formation during frozen storage of green snapbeans served as the impetus for the present work on bean lipids. Here we are concerned with the whole fruit, in which the lipid contribution of the edible pod portion is nearly on a par with that of the immature seeds contained therein (McHargue and Roy, 1931). To ensure complete removal of the lipids from the green snapbeans, following lyophilization, the solvent employed was chloroform-methanol 2:1 (v/v). This solvent mixture is widely accepted for use in extraction of lipids from both animal and plant sources (Folch *et al.*, 1951; Wagenknecht, 1957).

EXPERIMENTAL

Slendergreen snapbeans, grown on Experiment Station plots, were harvested, size-graded, snipped, and cut mechanically. The size 4 beans (43.9 kg cut to one-inch lengths) were frozen, lyophilized in a Stokes cabinet-type freeze dryer, and stored at −17.8°C in friction-lid 30-lb cans. The fresh raw beans contained 11.49% total solids and 6.54% of alcohol-insoluble solids. The seeds content was 10.64% by weight; the pods contained 11.98% total solids.

Analytical procedures were the same as those employed in the study of green pea lipids (Wagenknecht, 1957). Nitrogen was determined with both Kjeldahl and Dumas methods.

PREPARATION OF CRUDE SNAPBEAN LIPIDS

Lyophilized raw green beans were ground to pass 20-mesh in a Wiley mill (No. 1 size) and extracted twice with chloroform-methanol 2:1 for 3 hours at a solvent-to-solids ratio of 5:1 (Wagenknecht, 1957). The solvent was removed by vacuum distillation in a stream of nitrogen at temperatures not exceeding 40°C. The yield of crude bean lipids was 491 g from 4.94 kg of lyophilized beans (9.93%). The crude lipid was stored under nitrogen at −17.8°C (Fraction I).

The scheme for solvent fractionation of crude bean lipids into several major fractions, along with certain analytical data, is shown in Fig. 1 and Table 1.

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Table 1. Composition of green snapbean lipids.

	% of total lipid	N (%)		P (%)	Molar N/P	Long-chain base N (%)	Sugars (%)	Fatty acids (%)	Unsaponifiable matter (%)
		Kjeldahl	Dumas						
Crude snapbean lipid (I)	100	0.84	.....	0.44	4.22		39.8		
Acetone-soluble (II)	24.5								
Heptane-soluble (III)	10.1								
Benzene-soluble (V)	10.0	0.34	0.71	0.09	8.35	0.171	2.2	22.0	55.1
50% acetic-acid-soluble (VI)	0.06	0.16	0.23	2.53	0.14		2.1		
95% methanol-soluble (IV)	14.4								
Glacial-acetic-acid-soluble (VII)	.....								
Benzene-soluble (IX)	5.8	0.52	0.60	0.46	2.48	0.169	13.0	46.7	16.8
50% acetic-acid-soluble (X)	7.7	0.66	0.69	0.004	365		80.4	2.2	0
Glacial-acetic-acid-insoluble (VIII)	.....								
Benzene-soluble (XI)	0.01	0.38	0.52	0.42	2.03		15.7		
50% acetic-acid-soluble (XII)	0.8	0.58	0.67	0.002	642		100	5.1	2.7
Acetone-insoluble (XIII)	75.5								
Benzene-insoluble (XV)	59.4							3.6	0.6
Benzene-soluble (XIV)	16.2								
Glacial-acetic-acid-insoluble (XVII)	.....								
Benzene-soluble (XX)	2.6	0.47	0.47	3.58	0.29	0.075	2.1	60.8	8.4
50% acetic-acid-soluble (XXI)	0.8	4.96	5.51	3.60	3.05		8.7	9.0	3.8
Glacial-acetic-acid-soluble (XVI)	.....								
Benzene-soluble (XVIII)	7.8								
95% ethanol-soluble (XXII)	5.2	0.91	0.90	3.37	0.60	0.060	3.4	60.8	2.9
95% ethanol-insoluble (XXIII)	2.6	1.16	1.28	2.68	0.96	0.071	5.6	59.9	9.5
50% acetic-acid-soluble (XIX)	5.1	1.85	1.83	0.33	12.5		30.6	3.3	0.2

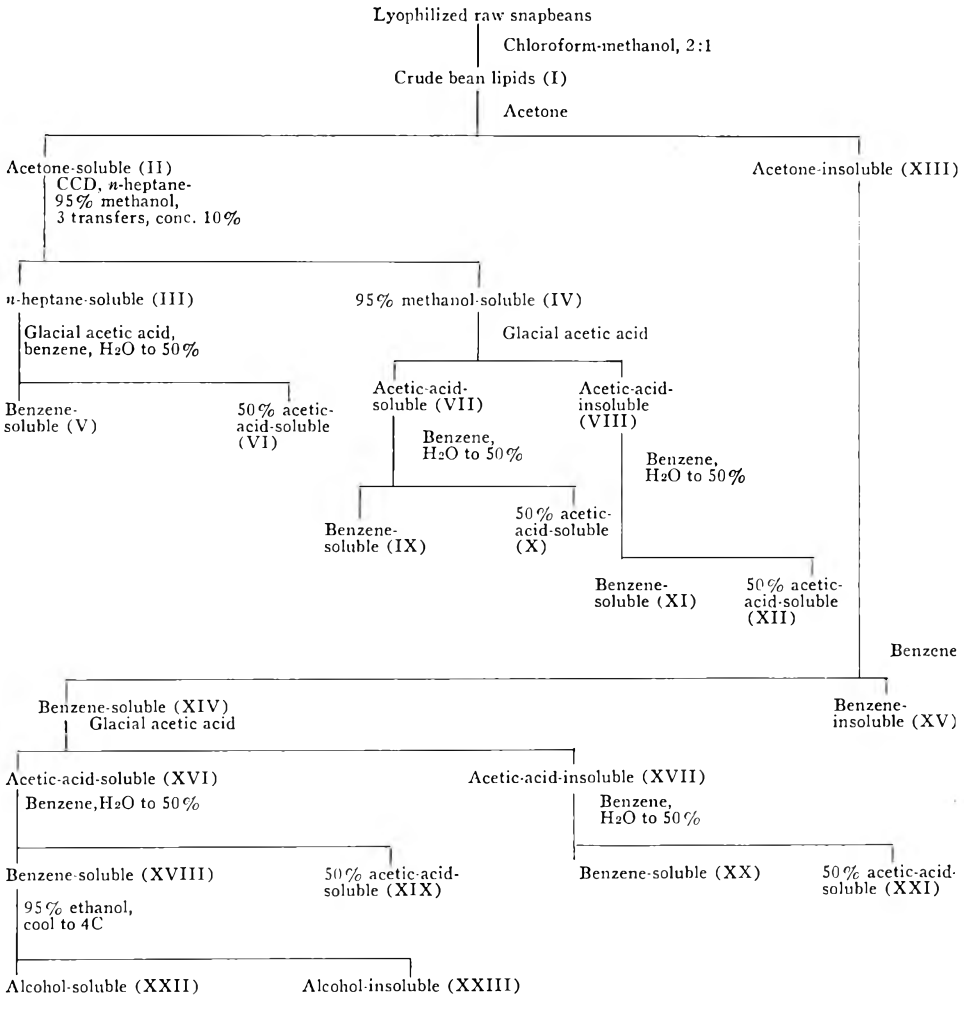

Fig. 1. Solvent fractionation of snapbean lipids.

SOLVENT FRACTIONATION OF CRUDE BEAN LIPIDS

Preparation of acetone-insoluble lipids (Fraction XIII). A sample of 477.9 g of crude bean lipid was stirred with 2 L of acetone in a nitrogen atmosphere and allowed to settle. The supernatant liquid was decanted and filtered. The insoluble residue was treated as above with 11 additional portions of acetone. The total volume of acetone extracts was 13 L. Acetone was removed from the insoluble matter in a vacuum desiccator by means of a water pump.

The acetone-insoluble lipids were treated with 500 ml of benzene, allowed to stand 4 hours to soften the hard mass, stirred thoroughly, and centrifuged for ½ hr at low speed. After decantation of the supernatant liquid the insoluble residue was worked up with benzene 4 additional times and centrifuged as above. The benzene was removed from the combined extracts by vacuum distillation. The yield of benzene-soluble acetone-insoluble bean lipids (Fraction XIV) was 75.5 g. The benzene-insoluble material (Fraction XV) amounted to 267.5 g, or 59.4% of the total lipid extract. This material was essentially non-lipid in nature, as shown by the low levels of fatty acids and unsaponifiable matter, respectively 3.64 and 0.58%.

Acetone-soluble lipids (Fraction II). The solvent was removed from the acetone extract of snapbean lipids by evaporation to a small volume under reduced pressure in a stream of nitrogen, and the process was completed in a rotary evaporator, followed by vacuum desiccation over P_2O_5.

The feasibility of using countercurrent distribution as a means of fractionation was indicated

Table 2. Properties of snapbean lipids.

Fraction	Saponification value	Acid value	Ester value	Soluble acids	Insoluble acids (Hehner No.)	Saponification equivalent
Acetone-soluble (II)	212.4	....	....	24.4	43.2	261.1
Heptane-soluble (III)	201.1	15.2	185.8	27.7	38.3	279.1
Methanol-soluble (IV)	225.0	28.9	196.1	28.7	25.8	249.3
Acetone-insoluble						
Benzene-soluble (XIV)	264.5	26.5	238.0	22.4	40.8	212.2
Benzene-insoluble (XV)	357.8	21.3	336.5	47.6	3.9	156.9

when 2.5 g of acetone-soluble bean lipids showed a distribution pattern similar to that obtained when pea lipids were partitioned between *n*-heptane and 95% methanol (Wagenknecht, 1957).

Accordingly, a sample of 107 g of acetone-soluble bean lipids was fractionated by means of countercurrent distribution between *n*-heptane and 95% methanol in 4-L separatory funnels at a concentration of 10% with respect to each solvent. Three transfers were employed, with no withdrawal of phases. After separation of the phases, all of the heptane fractions were combined, the heptane removed by vacuum distillation and in a rotary evaporator, and the dark-colored oil dried *in vacuo* over P_2O_5 (Fraction III). The methanol fractions were pooled and treated in a similar manner (Fraction IV). These lipids were then stored under nitrogen at −17.8°C.

Table 2 gives some of the properties of the four major subfractions of crude snapbean lipids (Fractions III, IV, XIV, and XV). To facilitate removal of non-lipid impurities, these major subfractions were treated with glacial acetic acid, and the soluble portion was partitioned between benzene and 50% aqueous acetic acid (Wagenknecht, 1957). After removal of solvents, samples of the subfractions were saponified by refluxing with 4% aqueous KOH.

Heptane-soluble fraction (Fraction III). The heptane-soluble fraction of acetone-soluble bean lipids was completely soluble in glacial acetic acid. The free fatty acid content of this fraction was 5.80%. Gas-liquid chromatography of the methyl esters of the free fatty acids disclosed the following mol percentage composition: C6-2.6, C8-2.7, C10-0.06, C11-0.06, C12-0.2, C13-0.1, C14-0.6, C15-0.3, C16-39.9, $C18^0$-11.3, $C18^1$-8.4, $C18^2$-16.6, $C18^3$-15.9, C20-1.2. The fatty acid content of the heptane-soluble fraction was unusually low, 22.0%, whereas unsaponifiable matter was 55.1%.

The saponification mixtures were characterized by a pungent, minty odor, which was found to emanate from the unsaponifiable portion. A similar odor was detected in the unsaponifiable portion of the methanol-soluble fraction (Fraction IX). A sample of 2.17 g of unsaponifiable matter from Fractions V and IX was steam distilled, and the distillate was collected in a receiver cooled in an ice bath. Oily droplets were observed on the surface of the distillate, which was extracted with ether. The ether was removed and the sample dried. This material, when examined by gas-liquid chromatography on Craig 1,4-butanediol polyester succinate, was found to contain one major component, five minor components, and traces of 14 other materials in the region where terpenes and sesquiterpenes were expected to be found. The orange-colored residue remaining after steam distillation, when shaken with ether, gave an emulsion that broke upon the addition of 95% ethanol. The aqueous layer was extracted three times with ether. The ether extract, containing finely divided white particles, along with the interfacial material was taken to dryness. Yield: 1.48 g of a waxy solid.

Methanol-soluble fraction (Fraction IV). The methanol-soluble fraction of acetone-soluble bean lipids was not completely soluble in glacial acetic acid. This material (Fraction VIII) was washed three times with glacial acetic acid and partitioned between benzene and 50% acetic acid. Most of the material was soluble in 50% acetic acid and was very rich in carbohydrate. The benzene-soluble portion, when compared with Fraction XX, was apparently too low in phosphorus content to be an inositol phosphatide.

The free fatty acid content of this fraction was 1.88%; the methyl esters had the following mol percentages: C6-9.6, C8-1.0, C9-0.2, C10-3.5, $C11^0$-10.8, $C11^1$-7.9, $C12^0$-0.4, $C12^1$-0.4, C13-0.6, C14-1.2, $C16^0$-31.7, $C16^1$-4.0, $C18^1$-4.0, $C18^2$-7.7, $C18^3$-17.3.

The amount of unsaponifiable matter, although considerably less in this fraction than was found in the heptane-soluble fraction, probably was not qualitatively different from that found in the heptane-soluble fraction.

Benzene-soluble acetone-insoluble fraction (Fraction XIV). A considerable portion of this fraction of bean lipids was insoluble in glacial acetic

acid. This material (Fraction XVII), after purification by extraction with benzene from an aqueous acetic acid suspension, gave, upon lyophilization of the benzene solution, 10.8 g of a fluffy solid (Fraction XX) similar in composition to the inositol phosphatide fraction of peas (Wagenknecht *et al.*, 1959). The iodine number of the intact phosphatide was 109.7. Five fatty acids were found, in the following mol percentages: palmitic-29.5, stearic-5.7, oleic-9.6, linoleic-27.9, linolenic-27.3. This would provide an average molecular weight of 273 for the fatty acids.

The glacial-acetic-acid-soluble portion was partitioned between benzene and 50% aqueous acetic acid. The benzene-soluble fraction (Fraction XVIII) was further fractionated with 95% ethanol at 4°C to give alcohol-soluble (Fraction XXII) and alcohol-insoluble (Fraction XXIII) phosphatides.

DISCUSSION

The use of a relatively polar solvent system resulted in the extraction of large amounts of non-lipid matter as well as most of the pigments from the dried green beans. Tanret (1912) reported that dry beans *(P. vulgaris)* contain 2.1% stachyose and 3.9% sucrose. The majority of the non-lipid contaminants were carried over into the acetone-insoluble fraction, and were largely eliminated at this stage by virtue of insolubility in benzene. Furthermore, by partitioning the various lipid fractions between benzene and 50% aqueous acetic acid, additional quantities of non-lipids, consisting essentially of sugars and nitrogenous compounds, were removed. However, all of the lipid fractions contained varying amounts of chlorophyll and its degradation products. These highly colored materials apparently possess distribution coefficients very similar to those of the major lipid classes encountered in the snapbean lipid extracts. Separation and complete removal of chlorophyll pigments was not achieved by any of the solvent fractionation steps employed in this study.

Thus, the total lipid extracted amounted to 2.60% (dry weight basis). McHargue and Roy (1931), from a study of the lipids of Kentucky Wonder beans, reported a fat content of 1.37% in the dry beans and 1.41% and 1.29% in the immature beans and pods, respectively (dry-weight basis). Other workers have reported lipid contents of 1.2 to about 2% for the seeds of several varieties of beans, these lipids containing 5–7% of unsaponifiable matter (Grimme, 1911; Ito, 1939; Ott and Ball, 1944; Takahashi *et al.*, 1949; Wittke, 1916).

The suitability of the Dumas method for determining the nitrogen content of lipid materials has been questioned at times. Accordingly, the nitrogen content of the various fractions obtained in this study was determined by both the micro-Kjeldahl and Dumas methods, with a semiautomatic nitrogen analyzer used for the latter. In general, the Dumas method showed higher nitrogen values than the Kjeldahl method; discrepancies were greatest for samples consisting primarily of triglycerides, such as Fractions V and XI. The possibility that CH_2 groups are converted to methane, which is then measured as nitrogen gas, has been advanced as an explanation of the erroneously high results obtained by this method.

The distribution of long-chain base nitrogen in the various lipid fractions was very similar to that in pea lipids (Wagenknecht, 1955), being about twice as abundant in the acetone-soluble portion as in the acetone-insoluble portion. This material was evenly distributed between heptane and methanol by countercurrent distribution.

The presence of glycolipids is suggested by the finding of relatively large percentages of sugars in certain of the benezene-soluble lipid fractions following partition between benzene and 50% aqueous acetic acid. The latter treatment is considered quite effective for removal of non-lipid impurities from lipid samples.

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Lipid Alterations During the Fermentation of Vegetables by the Lactic Acid Bacteria[a]

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SUMMARY

Qualitative and quantitative analyses of the fatty acids of the various lipid fractions of raw and fermented cabbage and Brussels sprouts were made by gas-liquid chromatography of the methyl esters.

The amount of free or non-esterified fatty acids increased considerably in the lipid fractions of both vegetables as a result of fermentation. The unsaponifiable matter, and the fatty acids of both the acetone-soluble and acetone-insoluble fractions of the vegetables, decreased during the fermentations.

Free or non-esterified palmitic acid increased significantly during fermentation—from 0.63 to 6.87% of the total fatty acid lipid in the cabbage fermentation. Results were similar in Brussels sprouts. During fermentation, the unsaturated C_{18} fatty acids decreased while the shorter-chain fatty acids increased. As a result of fermentation, a change was observed in the waxy or shiny appearance of the cabbage. The presence of longer-chain saturated fatty acids in the non-esterified fatty acid fraction of the fermented material has been attributed to the changes in the unsaponifiable fraction. These longer-chain fatty acids were notably absent from the fatty acids of the acetone-soluble and acetone-insoluble lipids of the unfermented vegetables.

The data suggest that the changes in the lipid fraction during fermentation were effected primarily through the metabolic activities of the various lactic acid bacteria. Since it is known that many of the lactic acid bacteria require certain fatty acids for growth, it has been postulated that the changes in the lipid fraction could influence the growth and sequence of the various bacterial types that are observed in a particular fermentation.

INTRODUCTION

The preservation of foods by fermentation antedates recorded history. In many foods, the effects of fermentation are mediated through the metabolic activities of the various lactic acid bacteria. It has been well established (Pederson, 1960) that the conversion of carbohydrates to acids, carbon dioxide, and alcohol by this group of microorganisms constitutes the primary reaction during the fermentation of vegetables such as cabbage. Very little is known, however, about the biochemical reactions that occur among the minor constituents of the vegetables and their resultant effect upon flavor. It is apparent that the characteristics of sauerkraut, which are produced through complex reactions during fermentation, are difficult to analyze and have not been duplicated artificially.

The nutritional requirements of the lactic acid bacteria are complex and exacting. Besides an energy source, a variety of essential growth factors must be made available to the cell. In contrast to a completely synthetic, chemically defined medium, in cabbage it would appear that the bacteria may invoke mechanisms whereby the various factors essential for growth would become available to the cell in a usable form.

Previous work at this laboratory (Vorbeck *et al.*, 1961b) has demonstrated the

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presence of lower-molecular-weight fatty acids in an abnormal cabbage fermentation characterized by a "cheese-like" off-odor. These findings have led to a study of the metabolic fate of the lipids of vegetables during normal fermentation by the lactic acid bacteria. The amount of lipid material in cabbage and related vegetables is small; however, modification of the fatty acid distribution could have a considerable bearing upon the flavor changes induced in fermentations by the lactic acid bacteria.

The vegetables used were cabbage and Brussels sprouts. Although Brussels sprouts are rarely fermented, they were nevertheless included in this study.

EXPERIMENTAL

Fermentation of vegetables. Approximately 400 lb of shredded cabbage, obtained from a nearby sauerkraut factory, were packed with 10 lb of salt in a 52-gallon polyethylene-lined steel drum fitted with a false bottom of perforated plexiglass. The cabbage was tightly packed and covered with a sheet of perforated plexiglass. Paraffin-coated blocks of wood were placed on top of the plexiglass. The height of the blocks was so arranged that the pressure exerted on the plexiglass, when the drum lid was in place, was sufficient to force the brine level up and thereby cover the surface of the cabbage. The drum was equipped, by means of "Swagelok" fittings, with a ⅜-inch I.D. stainless-steel tube for withdrawing samples of brine for chemical and bacteriological analyses. The fermentation was allowed to proceed 14 weeks at a constant temperature of 68–70°F.

Brussels sprouts, harvested from Experiment Station plots at the stage of maturity suitable for use as a fresh vegetable, were trimmed and washed to remove gross adhering dirt. They were packed in a 5-gallon glass jar and covered with a brine of sufficient strength to give 2.5% salt based on the combined weight of the Brussels sprouts and the brine. The Brussels sprouts were held below the surface of the brine by a paraffin-coated block of wood.

Analyses during fermentation. Samples of brine were withdrawn at intervals during the fermentation to determine acidity, hydrogen-ion concentration, and salt concentration (Pederson, 1930, 1932; Pederson and Albury, 1954). Samples of the fermenting brine were also used for bacteriological analyses. The total count and relative populations of each species during the various stages of fermentation were determined.

Cultures isolated during fermentation were identified on the basis of morphology and physiology. Gram-positive rods producing 1.0% or more acid, and little or no gas in a tomato juice, tryptone, glucose, yeast extract broth were considered strains of *Lactobacillus plantarum*. Gram-positive rods producing 0.4% or more acid with marked gas production in the broth were considered strains of *Lactobacillus brevis*. Gram-positive cocci exhibiting the characteristic tetrad appearance in an acid environment, and producing 0.5% or more acid and little or no gas in the tomato glucose broth were considered strains of *Pediococcus cerevisiae*. Gram-positive coccus to short rod forms producing copious gas, and an acidity of 0.25% or more in the tomato glucose broth were presumed to be strains of *Leuconostoc mesenteroides*. To confirm this, they were inoculated into sucrose gelatin stab tubes and incubated at 25°C. Dextran production, characterized by a mucoid appearance in the stab and on the surface, was considered sufficient evidence to classify these isolates as strains of *L. mesenteroides*. The gram-positive coccus forms failing to produce gas, and producing 0.35% or less acid in the tomato glucose broth were transferred to litmus milk and incubated at 32°C. The characteristic gram-positive streptococcus morphology, coupled with the production of acid curd, and litmus reduction indicated strains of *Streptococcus faecalis*. A few strains of *Streptococcus* sp. did not respond to the above characterization, at least when first isolated. The detailed procedure used for identification of the various species has been given (Pederson, 1930, 1932; Pederson and Albury, 1954).

Lipid analyses. Samples used for lipid extraction and analyses were dried by lyophilization in a Stokes freeze dryer. To inactivate lipolytic enzymes, control samples of the fresh vegetables were blanched in boiling water for 1 min before lyophilization. Crude vegetable lipid was extracted with chloroform-methanol (2:1, v/v, Wagenknecht, 1957) by mechanical agitation using a Teflon-covered magnetic stirrer. All solvents were analytical or reagent grade. Two 3-hr extractions were used with a 10:1 solvent ratio (based on the dry weight of the lyophilized material). The solvent was removed from the combined extracts in a stream of nitrogen under reduced pressure at temperatures not exceeding 40°C.

Crude lipid was fractionated on the basis of acetone solubility according to the procedure of Lee and Mattick (1961) and is outlined in the flow diagram in Fig. 1.

The fatty acids from the various lipid fractions were converted to the corresponding methyl esters

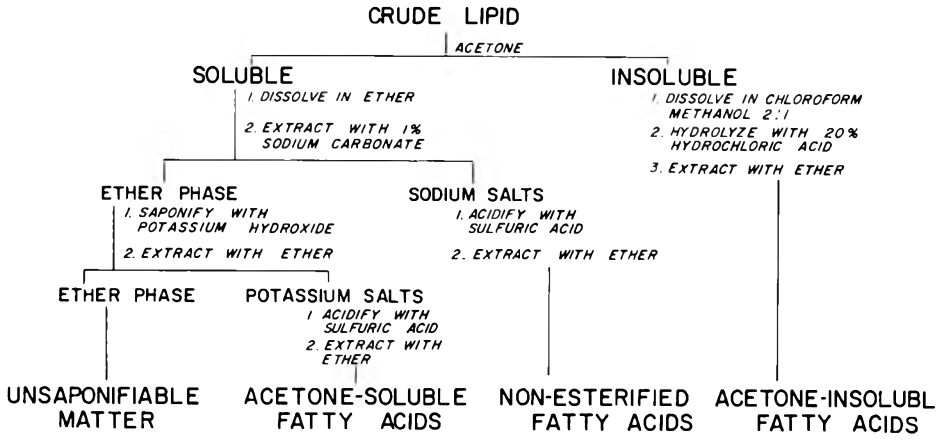

Fig. 1. Flow diagram for solvent fractionation of crude lipid.

by reaction with diazomethane (Vorbeck *et al.*, 1961a). A Barber-Colman Model 10 gas chromatograph, employing a beta-ray ionization detection cell containing 56 μc of radium-226, was used for qualitative and quantitative determinations of the methyl esters of the fatty acids. The stationary phases used were Apiezon L, a non-polar saturated paraffin hydrocarbon, and diethylene glycol succinate, a polar polyester of succinic acid. The preparation of the columns and the operating parameters for the instrument have been described in detail (Vorbeck *et al.*, 1961a).

Since retention data on a single stationary phase can be misleading, two chemically distinct stationary phases were used to define the chain length and structure of the unknown acids. The tentative identity of the various peaks on the gas chromatograms was established using a grid prepared from relative retention data obtained with known acids (James, 1959). To obtain relative retention data for polyunsaturated fatty acid methyl esters, the highly unsaturated fatty acids of menhaden oil were used (Vorbeck *et al.*, 1961a). Unsaturation was verified by observing the chromatographic behavior of the sample before and after bromination (James and Martin, 1956).

RESULTS AND DISCUSSION

Table 1 gives chemical and bacteriological analyses of the cabbage, showing the progress of the fermentation. The data are consistent with those reported for normal fermentations (Pederson, 1930, 1932; Pederson and Albury, 1954). The changes in the acidity and hydrogen-ion concentration were similar for the Brussels sprouts fermentation, and after 32 days of fermentation the

Table 1. Development of acid and change in bacterial flora in sauerkraut fermentation.

Time (days)	Total acid	pH	Total plate count × 10^5 per ml	Estimated no. of each type × 10^5 per ml				
				Aerobes	*Leuconostoc mesenteroides*	*Lactobacillus brevis*	*Lactobacillus plantarum*	*Pediococcus cerevisiae*	Yeasts
5/6	0.15	4.48	1320	1	1319				
1 1/6	0.30	4.23	4400		4400				
1 5/6	0.74	3.93	9650		9150		500		
2 1/6	0.77	4.00	4660		4660				
2 5/6	0.97	3.87	4490		4440	50			
4	1.18	3.67	1250		687	62	313	188	
5	1.19	3.71	970			40	436	145	
6	1.16	3.63	1410			70	1270	70	
8	1.42	3.68	4670			750	3170	250	
10	1.57	3.59	2300			1035	690		
13	1.65	3.59	1200			200	1000		
17	1.61	3.58	546				546		
21	1.78	3.51	251				248		3

acidity (calculated as lactic acid) was 1.71% and pH was 3.77. These data indicate normal fermentation by the lactic acid bacteria for both vegetables.

Tables 2 and 3 show quantitative changes in the total fatty acids of the various lipid fractions and in the unsaponifiable material during the fermentation of cabbage and Brussels sprouts, respectively. In both fermentations there is an increase in total free or non-esterified fatty acids with concomitant decreases in the fatty acids of both acetone-soluble and acetone-insoluble lipids.

Tables 4 and 5 show qualitative and quantitative changes in the distribution of fatty acids as a result of fermentation of cabbage and Brussels sprouts, respectively. Acetic acid and, if present, the trace amounts of other lower-molecular-weight fatty acids in the non-esterified fatty acid fraction of the fermented vegetables may have been lost during lyophilization. Both fermentations are characterized by marked changes in the qualitative and quantitative nature of the fatty acids of the various fractions analyzed. A comparison of the fatty acid distribution of the raw and the fermented product for both vegetables indicates that the effects of fermentation were not so drastic on the fatty acids of the acetone-soluble and acetone-insoluble lipids as on the non-esterified fatty acids.

During the fermentation of cabbage, modification of the fatty acid distribution was observed in addition to significant increases in total non-esterified fatty acids (Tables 2 and 4). The most striking change was the increase in non-esterified palmitic acid. This increase in palmitic acid appears to be a rather general phenomenon, having been observed in fermentation by a mold and also during enzymatic changes in vegetable production. Wagenknecht *et al.* (1961) observed increases in palmitic acid during fermentation of tempeh, and Lee and Mattick (1961) and Mattick and Lee (1961) observed similar increases during storage of peas and spinach.

In raw cabbage, the non-esterified palmitic acid (Table 4) represented 0.63% of the total fatty acid lipid; following fermentation, it accounted for 6.87%. The loss of 2.61% palmitic acid from the acetone-soluble and acetone-insoluble lipids does not completely account for the increase in non-esterified palmitic acid, indicating that the increases in the non-esterified fatty acids are not merely the result of hydrolysis of the complex lipids of the cabbage. Also, the increase in non-esterified shorter-chain acids cannot be completely accounted for by the simple hydrolysis of combined lipid. Before fermentation, the non-esterified acids from C_8 to C_{14} accounted for 0.16% of the

Table 2. Gross changes in the various lipid fractions during cabbage fermentation.

Fraction	Mg per 200 g dry weight		Percent increase or decrease
	Cabbage	Sauerkraut	
Non-esterified fatty acids	86	649	+ 657
Acetone-soluble lipid fatty acids	526	193	− 63
Acetone-insoluble lipid fatty acids	978	504	− 49
Unsaponifiable matter	321	183	− 43

Table 3. Gross changes in the various lipid fractions during Brussels sprouts fermentation.

Fraction	Mg per 200 g dry weight		Percent increase or decrease
	Raw	Fermented	
Non-esterified fatty acids	118	1745[a]	+ 1382
Acetone-soluble lipid fatty acids	1961	1367	− 30
Acetone-insoluble lipid fatty acids	1108	850	− 23
Unsaponifiable matter	302	208	− 31

[a] The non-esterified fatty acid fraction of the fermented material contained some pigment impurities which could not be removed.

Table 4. Distribution of fatty acids in the lipid fractions of cabbage and sauerkraut.

		% of total fatty acids					
		Cabbage			Sauerkraut		
Acid	Shorthand designation	Non-esterified fatty acids	Acetone-insoluble	Acetone-soluble	Non-esterified fatty acids	Acetone-insoluble	Acetone-soluble
Caprylic	8:0	0.02	....	Tr	0.99	....	0.01
Capric	10:0	0.01	....	Tr	1.08	Tr	....
Lauric	12:0	0.05	0.36	0.05	0.43	0.47	0.03
Dodecoic	12:br	0.01	Tr	....	Tr	....	....
Dodecenoic	12:1	....	0.86	0.03	0.22	0.08	Tr
Tridecoic	13:br	0.03	....	....	Tr	....	....
Myristic	14:0	0.04	0.45	0.20	0.13	0.15	0.13
Pentadecanoic	15:0	0.03	0.02	0.23	0.13	0.13	0.25
Pentadecoic	15:br	0.03	0.07	0.03	Tr	0.08	....
Pentadecenoic	15:1	0.01	0.07	0.04	0.69	0.06	0.03
Palmitic	16:0	0.63	9.49	5.03	6.87	8.96	2.95
Hexadecenoic	16:1	0.01	0.09	0.03	0.04	0.04	Tr
Hexadecadienoic	16:2	0.03	0.04	0.13	0.13	0.04	0.15
Margaric	17:0	Tr	0.28	0.09	0.09	0.04	Tr
Heptadecoic	17:br	0.03	0.69	0.46	0.39	0.44	0.16
Heptadecenoic	17:1	....	0.09	0.02	Tr	Tr	0.03
Stearic	18:0	0.06	0.32	0.32	0.60	1.04	0.30
Octadecenoic	18:1	0.46	5.99	3.31	3.72	3.97	1.57
Octadecadienoic	18:2	1.50	17.74	8.30	9.77	9.82	3.74
Octadecatrienoic	18:3	2.42	20.96	12.56	8.73	9.35	4.16
Arachidic	20:0	Tr	0.16	Tr	Tr	Tr	1.80
Eicosatetraenoic	20:4	....	0.14	0.20	0.01	0.01	....
Higher saturated acids		....	0.20	....	2.50	....	....
Higher unsaturated acids		....	3.46	0.20	6.41	5.71	....

Table 5. Distribution of fatty acids in the lipid fractions of raw and fermented Brussels sprouts.

		% of total fatty acids					
		Raw Brussels sprouts			Fermented Brussels sprouts		
Acid	Shorthand designation	Non-esterified fatty acids	Acetone-insoluble	Acetone-soluble	Non-esterified fatty acids	Acetone-insoluble	Acetone-soluble
Caprylic	8:0	0.08	0.33	....	1.17	....	....
Capric	10:0	0.02	0.01	....	0.03	....	....
Lauric	12:0	0.01	0.04	....	0.11	....	....
Myristic	14:0	0.07	0.11	....	....	....	....
Pentadecanoic	15:0	....	0.04	0.09	0.20	Tr	....
Pentadecenoic	15:1	....	0.01	0.06	0.03	Tr	....
Palmitic	16:0	0.77	3.57	17.33	0.45	4.38	7.38
Hexadecenoic	16:1	0.04	0.46	Tr	0.67	0.42	0.43
Hexadecadienoic	16:2	....	Tr	1.03	0.49	0.37	0.58
Margaric	17:0	0.03	Tr	0.07	0.14	....	....
Heptadecoic	17:br	....	0.20	0.94	Tr	0.08	0.04
Stearic	18:0	0.01	0.31	0.75	0.44	0.47	0.43
Octadecenoic	18:1	0.18	3.44	4.24	3.18	1.88	2.99
Octadecadienoic	18:2	0.68	7.75	8.23	8.13	5.11	7.55
Octadecatrienoic	18:3	1.74	15.22	28.39	4.15	8.25	14.20
Arachidic	20:0	0.01	0.08	Tr	Tr	0.07	0.33
Eicosatetraenoic	20:4	....	0.26	....	0.72	Tr	0.07
Higher saturated acids		....	....	....	0.74	....	....
Higher unsaturated acids		....	2.50	....	2.88	1.28	....

total fatty acid lipid; whereas, following fermentation, they represented 2.85% of the total. The data also show significant losses of unsaturated C_{18} acids from the acetone-soluble and acetone-insoluble fractions of raw cabbage. In the acetone-soluble and acetone-insoluble fractions of raw cabbage, the unsaturated C_{18} acids represented 68.9%; however, in sauerkraut they accounted for only 32.6% of the total fatty acid lipid. Although the non-esterified unsaturated C_{18} acids increased from 4.38 to 22.2% of the total fatty acid lipid, the loss of 36.3% C_{18} unsaturated acids from the complex lipid fractions cannot be explained entirely by the increase of only 17.8% in non-esterified fatty acids.

The unsaponifiable material decreased during fermentation. Although notably absent from the fatty acid lipid of raw cabbage, longer-chain saturated and unsaturated acids accounted for 8.91% of the non-esterified fatty acid fraction of sauerkraut. These factors indicate hydrolysis of waxy material, which may account for the presence of the longer-chain unesterified fatty acids in the sauerkraut. In addition, the change in the appearance of the waxy or shiny surface of the cabbage observed during fermentations may be the result of hydrolysis of waxy material. Camien and Dunn (1957), from studies on the role of lipid material in the metabolism of *Lactobacillus casei*, suggested that sterols might be a source of essential lipid intermediates; however, whether any unsaponifiable material is utilized by the lactic acid bacteria during the fermentation of vegetables is not known.

The changes in distribution of fatty acid lipid as a result of fermentation of Brussels sprouts are similar to those observed with the fermentation of cabbage (Table 5). The weight of the non-esterified fatty acids of the fermented Brussels sprouts may be somewhat high since this fraction was contaminated with green pigments that could not be removed. Although the total weight of non-esterified fatty acids may be high, the relative proportions of the various fatty acids would not be affected. Non-esterified fatty acids increased as a result of fermentation. As with cabbage, the non-esterified palmitic acid showed the greatest change, increasing from 0.77% of the total fatty acid lipid to 10.45% of the total. The shorter chain, non-esterified fatty acids also showed an increase that could not be accounted for by simple hydrolysis of the combined lipids of the Brussels sprouts. The change in the C_{18} unsaturated fatty acids was not so drastic as in the cabbage. The total C_{18} unsaturated fatty acids in the acetone-soluble and acetone-insoluble fractions accounted for 67.3% of the total fatty acid lipid before fermentation, and only 40.0% after fermentation. As in sauerkraut, the longer-chain fatty acids were also observed in the non-esterified fatty acids of the fermented Brussels sprouts.

The studies of the physiology of the lactic acid bacteria in general and also the studies of their role during the fermentation of vegetables have centered predominantly on their carbohydrate metabolism. The lactic acid bacteria in general are considered non-lipolytic, and no active extracellular lipases have been reported for this group. Unlike in a report on cheese (Peterson and Johnson, 1949), the activities of the lactic acid bacteria during fermentation are ordinarily not associated with changes in the lipid fraction of vegetables during normal fermentation.

The results of this study have revealed significant changes in the lipid fraction of vegetables during normal fermentation. Whether these changes were brought about solely through the growth of the various lactic acid bacteria is unknown. An increase in non-esterified fatty acids has also been observed in this laboratory during the fermentation of cucumbers (Pederson *et al.*, unpublished observations). The activity of plant lipolytic enzymes may have caused some of the alterations of the lipid fraction during fermentation, especially in the cabbage. The shredding of the cabbage and the subsequent release of cellular material may have liberated lipolytic enzymes. Except for the trimming of the outer leaves and stem ends, the Brussels sprouts were not cut or broken apart. Since comparable significant changes were also observed as a result of fermentation of the Brussels sprouts, it does not appear that all the lipid changes can be ascribed to the liberation of plant enzymes.

In addition, plant lipases, in general, are active under alkaline conditions. The rapid increase in hydrogen-ion concentration during fermentation would limit considerably the activity of enzymes having optimum activity at low hydrogen-ion concentrations.

A phospholipase that hydrolyzes the ester linkage between the nitrogenous base and the phosphoric acid group of cabbage lecithin producing a phosphatidic acid has been observed in cabbage (Hanahan and Chaikoff, 1948). Although this enzyme could release choline for the formation of acetyl and lactyl choline, it does not liberate non-esterified fatty acids. Solvent-activated phospholipases that could be activated during the extraction procedure must also be considered. These enzymes would be inactivated in the raw vegetable by the blanching procedure. Since the concentration of water in the material to be extracted was very low, it would appear that the contribution of these enzymes during the extraction of the fermented material would be limited.

An increase in non-esterified fatty acids and losses of certain fatty acids were observed during the fermentation of both cabbage and Brussels sprouts. Williams and Fieger (1949) reported the fundamental observation that a number of biotin-requiring lactobacilli grow normally in the absence of this factor when supplied with certain fatty acids. In addition, for some strains of lactic acid bacteria, certain fatty acids are known to be indispensable growth factors (Nieman, 1954). On the basis of the data presented, it is suggested that the increase in non-esterified fatty acids is brought about primarily by a lipase-like activity of one or more of the species of bacteria involved in the fermentation. The physiology of these organisms cultured in a natural environment has received limited attention, and the possible existence of lipolytic activity in this group cannot be eliminated on the basis of results obtained with cells grown in laboratory media. Segal and Bloch (1956), for example, observed that cells of *Mycobacterium tuberculosis* grown in lung tissue differed from cells of the same strain grown in laboratory medium with respect to both utilization of various fatty acids and relative lipid content. If the role of the lipase-like activity would be to provide essential growth factors, the subsequent increase in non-esterified fatty acids would be a secondary effect. Further study is necessary to determine the nature of this lipase-like activity and whether it is liberated during the growth of the bacteria or released by autolysis of the cells. Studies of Peterson and Johnson (1949) on *Lactobacillus casei* with milk fat have indicated a release of lipase activity by autolysis of the cells.

It is known that the growth of the bacterial types in a natural fermentation is controlled by certain imposed environmental factors such as salt concentration, acid concentration, and temperature (Pederson, 1960); however, the availability of essential growth factors would also influence this progression. It appears reasonable to postulate that the growth of each bacterial type in such a sequence either produces, or makes available in a usable form, some factor or factors essential to the growth of the successive bacterial type. The release and utilization of fatty acids could have an influence on the naturally regulated progression of bacterial types observed during a fermentation.

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Age-Associated Changes in Muscle Composition. The Isolation and Properties of a Collagenous Residue from Bovine Muscle [a]

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SUMMARY

Age-associated changes in the chemical composition of bovine biceps femoris muscle were studied. Veal muscle had significantly lower Kjeldahl nitrogen and higher moisture contents than muscle from the three older age groups studied. Muscle from veal and from the oldest group (cows, 10 years) possessed less fat than muscle from the two intermediate groups (steers, 1–2 years, and cows, 5 years). A modified procedure for determination of hydroxyproline and its use directly on mean hydrolysates are described. Use of this technique failed to reveal any significant differences in the hydroxyproline content, and presumably the connective-tissue content, of muscle from the four groups. Warner-Bratzler shear-force values of cores from biceps femoris steaks from the three oldest groups indicated that tenderness decreased with age. A method is given for isolation of large quantities of connective tissue from biceps femoris. Chemical analyses of these connective-tissue residues are presented, and the possibility is discussed that the veal connective tissue contains large amounts of reticulin.

Chronological age has generally been considered a factor in meat tenderness. Mackintosh *et al.* (1936) and Mitchell and Hamilton (1933) found that meat from young steers contained less collagen and was more tender than meat from older animals. Hiner and Hankins (1950) and Jacobson and Fenton (1956) reported that Warner-Bratzler shear-force values tended to increase, representing decreased tenderness, during the maturation of bovine animals. Subjective tenderness measurement of over-all panel evaluation has also detected significant differences among the age groups of bovine animals (Dunsing, 1959; Lohse, 1959; Simone *et al.*, 1959; Tuma *et al.*, 1961b). Beef from younger animals was consistently judged more tender than beef from older animals.

Other studies have revealed age-associated changes in the chemical composition of muscle. Lohse (1959) noted highly significant differences in the fat and moisture content of bovine muscle from different age groups. Veal possessed a significantly higher moisture and a lower fat content than steers or cows. In a study involving 56 Hereford males and females 6, 18, 42, and 90 months of age, Tuma *et al.* (1961a) noted that the 6-month-old animals possessed a significantly higher moisture content than the other three groups, which differed little among themselves in moisture content of the longissimus dorsi. Ash and protein contents did not appear to be influenced by animal age.

Early studies failed to reveal any consistent age-associated changes in the connective tissue content of muscle (Bate-Smith, 1948; Mitchell *et al.*, 1928). More recently, Wilson *et al.* (1954) reported that veal longissimus dorsi contained more collagen

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and elastin nitrogen and less fat than the corresponding muscle in either steers or cows. Loyd and Hiner (1960), however, found no significant difference between veal and beef in hydroxyproline content.

Investigators working with human lung connective tissue also obtained contradictory results regarding the effect of age. Pierce and Hocott (1960) found no change in the collagen content of human lung with increasing age using the Lowry method (Lowry *et al.*, 1941), but Briscoe *et al.* (1959) reported a significant increase in the hydroxyproline content of lung tissue with increasing age. Chvapil (1957) and Boucek *et al.* (1961), however, have reported a decrease in the collagen and elastin content of human lung tissue with increasing age as measured by the Lowry method.

This study investigates some of the age-associated chemical changes in bovine muscle. A modified hydroxyproline technique is described which circumvents the need for extraction of the muscle proteins prior to collagen determination. Reports by Adams *et al.* (1960), Loyd and Hiner (1960), and Parrish *et al.* (1961) indicate a negative association between collagen content and meat tenderness. The paradox presented by these reports, together with the results of Wilson *et al.* (1954) showing a lower collagen content in the muscle of older animals, may indicate a structural change in collagen as an animal matures. Also described is a technique for the isolation of collagen from large amounts of muscle tissue as a preliminary step to the study of its structure.

METHODS

Eleven animals of differing genetic and nutritional backgrounds were divided into four age groups: Group I) three Holstein veal calves 40–49 days old; Group II) three Hereford steers 403–495 days old; Group III) three cows (Guernsey, Hereford, and Holstein) 4 years and 8 months to 5 years and 5 months old; and Group IV) two Holstein cows 10 years and 2 months old, and 10 years and 5 months old.

The entire biceps femoris muscle was removed from the right side of each animal within 20 minutes of death, chilled on dry ice, freed of adhering fat and connective tissue, and minced 5 minutes with dry ice in a Buffalo silent cutter, Model 23. Prior to mincing, a cross-sectional slice approximately two inches thick was removed from the muscle for Warner-Bratzler shear tests. Samples of the minced tissue were taken for fat, moisture, Kjeldahl nitrogen, and hydroxyproline analyses, and the remainder was placed in 6-L Erlenmeyer flasks for extraction of the muscle protein by a modification of Helander's method (1957).

Procedure for the isolation of connective-tissue residues. The extraction was performed in the cold room at 0–4°C with precooled 1.1*M* KI buffered at pH 7.4 with 0.1*M* potassium phosphate (0.09*M* K_2HPO_4 and 0.01*M* KH_2PO_4). Solvent-to-tissue ratios of 10:1 (v/w) were used. The tissue was extracted three times with continuous stirring, the first two times for 3 hr each, and a final extraction of 2 hr duration. After the third extraction, the residue was placed in 20 volumes of deionized water and allowed to sit overnight at 0–4°C. The water was then strained through cheesecloth, and 10 volumes of acetone were added to the residue and stirred occasionally for 12 hr. The acetone was also removed through cheesecloth, and 10 volumes of a 3:1 chloroform-methanol solution added to this residue. The chloroform-methanol extraction continued for 4 hr at −21°C. After being filtered through cheesecloth, the residue was again treated with acetone for 12 hr at 0°C. The residue from this extraction was washed thoroughly with deionized water and lyophilized. The lyophilization required 48–72 hr for completion. Then the dried samples were powdered in a small precooled Wiley mill at 0°C and stored in 250-ml Erlenmeyer flasks at −30°C. Samples were taken of the powdered residue for fat, ash, Kjeldahl nitrogen, and hydroxyproline determinations.

Analyses on biceps femoris tissue. Routine fat, moisture, and Kjeldahl nitrogen analyses were conducted on portions of the minced biceps femoris tissue. The two-inch cross-sectional slices removed from the biceps femoris prior to mincing were broiled to an internal temperature of 165°C, and triplicate cores 1 inch in diameter were removed from each slice for Warner-Bratzler shear tests. Shear values could not be obtained for the veal age group since the cross-sectional area of the veal muscle was too small for removal of an inch core.

Hydroxyproline analysis on the minced biceps femoris tissue was carried out with a modification of the Prockup-Udenfriend technique (1960). Three to five grams of minced biceps femoris tissue were weighed directly into flasks and autoclaved 9 hr with 6*N* HCl at 16–19 lb pressure (122–126°C). Previous trials had shown that the yield of hydroxyproline under these conditions approached a maximum after 9 hr but decreased

after 12 hr of hydrolysis. The samples were cooled and decolorized with 2 g of a 1:2 w/w mixture of charcoal and Dowex 1-X8 ion-exchange resin. The filtrate was neutralized to a phenolphthalein endpoint with NaOH and made up to 1 L.

The hydroxyproline analysis was carried out as outlined under Procedure II by Prockup and Udenfriend (1960) with the following exceptions: 1) 10 ml of sample were used instead of 8 ml, and 2) the color reagent was changed from 20% *p*-dimethylaminobenzaldehyde and 6.85% concentrated sulfuric acid in ethanol to 10% *p*-dimethylaminobenzaldehyde and 4.5% concentrated sulfuric acid in 1-propanol. The pH did not need readjustment after addition of the KCl and one preliminary extraction with toluene was sufficient, even when large amounts of contaminating material were present. NaOH could be substituted for KOH in neutralization of the samples with no effect upon the analysis. After addition of the color reagent, the samples were allowed to set at room temperature for 1 hr and the light absorbancy read at 5600 Å in a Zeiss spectrophotometer.

Use of the above-described color reagent increased the sensitivity of the analysis to less than 0.5 μg per ml and resulted in the formation of a color that was stable for 4–5 hr. The standard curve was linear from the origin up to a concentration of 2.5 μg hydroxyproline per ml, but the slope decreased in the range of 2.5–5 μg/ml. Higher concentrations of *p*-dimethylaminobenzaldehyde corrected this, but such concentrations were difficult to mix into the toluene and generally proved less satisfactory for routine use. Higher concentrations of sulfuric acid resulted in more rapid development of the color, but also more rapid fading. Centrifugation after the extraction of the pyrrole into the toluene layer was necessary to prevent rapid color fading due to small amounts of thiosulfate trapped in the toluene. Standard curves made on separate days with this analysis generally agreed within 5%.

The hydroxyproline content of the minced biceps femoris tissue was converted to collagen with a factor of 7.25, assuming that the collagen-to-elastin ratio is 3:1 (Wilson *et al.*, 1954) and that the hydroxyproline content is 13.3% on a weight basis (Eastoe and Leach, 1958), eight times the hydroxyproline content of elastin.

Analyses on the collagenous residues. Ash analyses on the powdered connective-tissue samples were performed by weighing out 400-mg samples in duplicate into platinum crucibles that had been brought to a constant weight. The samples were pre-ashed for 3–4 hours on a hot plate under heat lamps and then placed in a muffle furnace at 690°C for 8 hr. After cooling, the crucibles and ash were weighed and the percent ash computed on the basis of the original sample weight.

Lipid analyses were conducted by weighing 300 mg of the powdered residue into 125-ml Erlenmeyer flasks and adding 75 ml of a 3:1 chloroform-methanol solution. The flasks were shaken, covered with aluminum foil, and allowed to stand at room temperature for 72 hr. The suspension was then filtered through No. 42 Whatman filter paper and the filtrate made up to 100 ml. Five-ml portions were taken, evaporated to dryness, and subjected to an organic solids analysis according to the procedure of Johnson (1949). A blank Erlenmeyer flask was subjected to the same procedure, and the percent lipid in the samples was calculated on the sample weight basis after the organic-solids content of the samples had been corrected for the blank. Semimicro-Kjeldahl analyses were performed on 30–70-mg samples, and percent nitrogen expressed on a sample weight basis.

Hydroxyproline analyses on the collagenous residues isolated from the minced biceps femoris were performed according to a modification of the procedure outlined by Woessner (1961). Samples of 20–60 mg were hydrolyzed for 9 hr with 6*N* HCl at 16–19 lb pressure (122–126°C). No humin formation was noted in the flasks, because of the lack of tryptophan in the residues. Therefore the acid hydrolysates were neutralized with NaOH to a phenolphthalein end point and made up to 1 L.

The Woessner (1961) procedure outlined under method I was used with the following exceptions: 1) 0.5 ml of the pH 6.5 buffer was added separately to the samples prior to addition of the oxidant using an automatic pipette; 2) 0.5 ml of a 3.00% chloramine T solution in 2-methoxyethanol was used as the oxidant; 3) 1 ml of a 1.49*N* perchloric acid solution was used to destroy the chloramine T; and 4) the *p*-dimethylaminobenzaldehyde reagent used in the modified Prockup-Udenfriend procedure was employed as the color reagent. The samples were cooled in water and allowed to stand at room temperature for 1 hr after removal from the 60°C water bath, and the optical density was read at 5570 Å in a Zeiss spectrophotometer. The color was stable for 4 hr and the analysis gave practically the same sensitivity and standard curve as the modified Prockup-Udenfriend procedure already described. This analysis agreed closely with results from the modified Prockup-Udenfriend analysis when used on samples containing less than 1% of their amino acids as hydroxyproline. The hydroxyproline content of the collagenous residues was again converted to collagen by using a factor of 7.25.

RESULTS AND DISCUSSION

Analyses of variance of the data showed significant differences among animals of the same age group for every variable measured. This was not unexpected, because no effort was made to select or standardize the animals used and individual animal variation is expected. Variations in the chemical analyses among animals of the same age group are presented in Tables 1 and 2. This study was designed to study intensively the muscle connective tissues of animals varying widely in age. Thus the number of animals per age group was kept small. Table 3 shows the mean values of the chemical composition of bovine biceps femoris for the four age groups studied. Although the numbers involved are small, the findings are in general agreement with those of Wilson *et al.* (1954), Tuma *et al.* (1961a), and Lohse (1959).

Duncan's multiple-range test revealed that muscle from Group I possessed significantly ($p < 0.01$) less protein and more moisture than muscle from the other three age groups. Groups I and IV possessed significantly less fat ($p < 0.01$) than Groups II and III, but there was no significant difference in fat content between either Groups I and IV or Groups II and III. Muscle from Group IV possessed significantly more moisture ($p <$

Table 1. Variations in the chemical composition of bovine biceps femoris muscle among animals of the same age group.

Animal	% protein [a, b]	% fat [c]	% moisture [a]	% other [a, d]	% collagen [a]	% collagen [e]
Veal 1	19.3±0.0 [f]	4.7±0.3	79.4±0.4	0.3±0.5	1.34±0.05	6.95±0.26
Veal 2	19.0±0.2	0.7±0.3	78.9±0.0	1.9±0.1	1.32±0.06	6.92±0.34
Veal 3	19.0±0.2	2.0±0.5	79.2±0.1	1.4±0.1	1.44±0.03	7.55±0.18
Steer 1	19.7±0.3	18.2±1.6	72.2±0.1	3.0±0.4	0.93±0.03	4.71±0.15
Steer 2	20.7±0.6	18.6±0.6	72.6±0.0	1.6±0.2	0.89±0.07	4.30±0.35
Steer 3	20.4±0.2	18.9±0.4	71.7±0.1	2.6±0.2	1.00±0.06	4.92±0.31
Cow 1	20.6±0.2	18.8±0.4	72.5±0.0	2.0±0.4	1.33±0.16	6.46±0.77
Cow 2	20.2±0.2	20.9±1.3	72.5±1.0	1.3±0.2	0.92±0.01	4.56±0.06
Cow 3	20.8±0.2	15.0±1.2	75.0±0.4	0.5±0.1	1.19±0.06	5.72±0.31
Aged cow 1	20.3±0.2	6.5±0.4	76.6±0.4	1.6±0.4	1.12±0.04	5.49±0.18
Aged cow 2	19.9±0.0	2.2±0.9	77.0±0.0	2.6±0.2	2.54±0.09	12.77±0.45

[a] Expressed on a fresh-weight basis.
[b] Calculated as Kjeldahl nitrogen × 6.25.
[c] Expressed on a dry-weight basis.
[d] Percent of fresh biceps femoris not accounted for by protein, fat, and moisture.
[e] Expressed on the basis of protein (Kjeldahl nitrogen × 6.25) content.
[f] Standard deviation.

Table 2. Variations in the chemical composition of lyophilized connective tissue residues isolated from bovine biceps femoris muscle among animals of the same age group.

Animal	% protein [a]	% fat	% ash	% collagen	% collagen [b]
Veal 1	95.7±1.6 [c]	1.00±0.03	1.462±0.094	40.6±0.4	42.4±0.4
Veal 2	95.4±2.0	1.29±0.09	0.734±0.005	53.2±2.7	55.8±2.8
Veal 3	91.6±1.0	0.57±0.01	1.495±0.088	53.0±1.9	57.9±2.1
Steer 1	82.0±1.6	3.01±0.12	0.640±0.086	65.9±0.4	80.3±0.6
Steer 2	84.6±1.8	0.96±0.04	0.888±0.071	57.5±0.7	67.9±0.8
Steer 3	87.7±1.6	3.23±0.04	0.508±0.013	54.8±0.7	62.4±2.5
Cow 1	95.7±1.3	0.50±0.05	0.640±0.029	71.3±2.6	74.4±2.7
Cow 2	94.1±2.5	0.36±0.01	0.698±0.064	64.9±1.8	69.0±1.9
Cow 3	93.4±1.9	0.81±0.01	0.838±0.057	68.7±4.1	73.5±4.4
Aged cow 1	90.5±0.4	0.45±0.01	0.790±0.128	55.6±1.2	61.4±1.3
Aged cow 2	87.9±0.8	0.61±0.05	0.958±0.054	63.8±3.4	72.5±4.0

[a] Kjeldahl nitrogen multiplied by a weighted mean of 5.55 and 6.25, depending on collagen content (see text).
[b] Expressed on a protein-content basis (see footnote *a*).
[c] Standard deviation.

Table 3. Mean values for the chemical composition of bovine biceps femoris muscle.

Age group	% protein [a, b]	% fat [c]	% moisture [a]	% other [a, d]	% collagen [a]	% collagen [e]
Veal	19.1±0.2 [f]	2.45±1.85	79.2±0.3	1.2±0.8	1.36±0.07	7.14±0.37
Steers	20.3±0.6	18.55±0.84	72.2±0.4	2.4±0.6	0.94±0.07	4.64±0.35
Cows	20.5±0.3	18.23±2.76	73.3±1.4	1.2±0.7	1.14±0.19	5.58±0.91
Aged cows	20.1±0.2	4.32±2.58	76.8±0.3	2.1±0.6	1.83±0.75	9.13±3.82

[a] Expressed on a fresh-weight basis.
[b] Calculated as Kjeldahl nitrogen × 6.25.
[c] Expressed on a dry-weight basis.
[d] Percent of fresh biceps femoris tissue not accounted for by protein, fat, and moisture.
[e] Expressed on the basis of protein (Kjeldahl nitrogen × 6.25) content.
[f] Standard deviation.

0.01) than muscle from Groups II or III, but significantly less moisture ($p < 0.05$) than muscle from Group I. There were no significant differences among the age groups in percent of fresh biceps femoris tissue not accounted for by protein, fat, and moisture.

No significant difference was found among the four age groups in collagen content of fresh biceps femoris. This is in agreement with Loyd and Hiner (1960), who also used a hydroxyproline technique for collagen determination, but disagrees with the findings of Wilson *et al.* (1954), who fractionated the protein constituents of muscle on the basis of their solubility and used a nitrogen measurement for collagen determination. The collagen content of Group IV muscle had a high value because one of the two animals in this group had an abnormally high collagen content (over 2.5% on a fresh-weight basis). The other animal in this group possessed only slightly over 1% on a fresh-weight basis. If this latter value is more characteristic of the collagen content of muscle from bovine animals of this age, it would appear that veal muscle tends to have a slightly higher collagen content than the other three age groups, although this difference approached significance only at the 10% level.

The modified Prockup-Udenfriend hydroxyproline technique offers promise as a method for the routine determination of collagen in meat. It combines the advantages of sensitivity and convenience since no prior extraction of the muscle proteins is required. The results with this method were 75–100% higher than the figures given by Wilson *et al.* (1954). Part of the difference may be due to an extraction of the soluble collagens by the 0.1*N* NaOH used in Wilson's procedure, but direct comparison is difficult since Wilson used the longissimus dorsi muscle.

Table 4 shows the mean values for the chemical composition of the lyophilized collagenous residues isolated from the biceps femoris muscle.

Duncan's multiple-range test showed that the samples isolated from Group II muscle possessed significantly less protein ($p < 0.01$) than the samples isolated from Group I or III muscle. The collagenous residues isolated from Group IV had a significantly higher ($p < 0.05$) protein content than those isolated from Group II, but a significantly lower ($p < 0.05$) protein content than those isolated from Group I or III. The factor used to convert Kjeldahl nitrogen to protein was a weighted mean of the factors used

Table 4. Mean values for the chemical composition of lyophilized connective tissue residues isolated from bovine biceps femoris muscle.

Age group	% protein [a]	% fat	% ash	% collagen	% collagen [b]
Veal	94.2±2.4 [c]	0.95±0.33	1.230±0.389	49.0±6.3	52.1±7.3
Steers	84.8±2.8	2.40±1.12	0.679±0.179	59.4±5.1	70.2±7.8
Cows	94.4±2.0	0.55±0.21	0.724±0.100	68.3±3.9	72.3±3.9
Aged cows	89.2±1.5	0.53±0.10	0.874±0.126	59.7±4.9	67.0±6.4

[a] Kjeldahl nitrogen multiplied by a weighted mean of 5.55 and 6.25, depending on collagen content (see text).
[b] Expressed on a protein-content basis (see footnote *a*).
[c] Standard deviation.

to convert collagen nitrogen to protein (5.55) and non-collagenous nitrogen to protein (6.25). The hydroxyproline content of the samples was used as a measure of their collagen content, and different factors were calculated for each animal. This procedure was necessary because the nitrogen content of collagen has been reported to be 17.8–18.2% (Eastoe and Leach, 1958).

The collagenous residues from Group II possessed significantly more fat ($p < 0.05$) than the residues isolated from the other groups. It is difficult to understand how as much as 2.4% lipid could have remained in the connective tissue samples after the rigorous fat-extracting methods used in their isolation. Evidently, the lipid is tightly bound and is released only after denaturation of the protein by chloroform extraction at room temperature. An examination of its qualitative nature might prove interesting.

Although the samples isolated from veal muscle appeared to have a higher ash content than those isolated from muscle of the other three age groups, this difference only approached significance at the 5% level of probability. Bray *et al.* (1951) have previously reported that veal connective tissue had a higher ash content than connective tissue from cows or steers.

The collagenous residues from Group I appeared to have a lower collagen content than the residues from the other three groups. The difference in collagen content between Groups I and III was significant at the 1% level when expressed on a sample weight basis. None of the other means differed significantly when expressed on this basis although the differences in collagen content between Groups I and II and between Groups I and IV approached significance at the 5% level of probability. When expressed on a protein-content basis, the collagen content of Group I samples was significantly lower than the collagen content of samples from Groups II and III at the 1% level of probability and significantly lower than the collagen content of Group IV samples at the 5% level of probability.

The low hydroxyproline content of the veal samples, coupled with their high protein content, indicates either that these samples are rich in some insoluble hydroxyproline-poor protein, possibly elastin, or that hydroxyproline content is lower in veal collagen than in collagen from older animals. From the evidence presented here, it is impossible to distinguish between these two possibilities. Boucek *et al.* (1961) found that the amount of collagen and elastin in human lung tissue as measured by the Lowry procedure decreased with increasing age. The amount of hydroxyproline in the same tissue, however, appeared to increase with increasing age. In discussing their results, Boucek *et al.* pointed out that either the presence of reticulin, which has a lower hydroxyproline content than collagen, or a strong conjugation of lipids with the protein, resulting in their ability to resist an alcohol-ether extraction, could, if it appeared only in their young group, cause the above results. The low lipid content would seem to eliminate the latter alternative as an explanation for the low hydroxyproline content of Group I. Other experiments have indicated that the non-collagenous protein of Group I is not affected by temperatures up to 70°C in 0.1*M* phosphate buffer, pH 7, but that this protein is solubilized by 100°C temperatures in the same buffer after 2 hr. This would rule out the possibility that the non-collagenous protein is elastin, but it is still possible that large amounts of reticulin were present in the Group I samples. A complete amino acid analysis of the samples might clarify the situation.

The high hydroxyproline content of the connective-tissue residues in general attests to the ability of the extraction procedure to remove the soluble muscle proteins. According to the results of Wilson *et al.* (1954), only 65–75% collagen should be expected in such samples since ⅓–¼ of the connective-tissue proteins in beef muscle are elastin, which should also be left in the residue by the extraction procedure. All but the Group I fell in this range. While it is difficult to detect denaturation in insoluble proteins without X-ray diffraction techniques, certain physicochemical tests involving shrinkage temperature have indicated that very little denaturation of the collagen occurred during the isolation procedure.

Triplicate Warner-Bratzler shear-force

values on steaks from the three older groups also indicated a significant age effect. The mean shear-force values were: Group IV, 56.0 lb; III, 36.0 lb; II, 36.7 lb. The difference in shear-force values between Groups III and IV was significant at the 1% level. Groups II and III did not differ significantly in shear-force values. These results are in accord with those reported by Simone *et al.* (1959), Dunsing (1959), Lohse (1959), and Tuma *et al.* (1961b), indicating decreased tenderness with advancing age.

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Acid-Soluble Phosphates in Cow Milk [a]

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SUMMARY

The acid-soluble organic phosphates of cow milk were obtained by trichloroacetic acid extraction or by dialysis and separated by ion exchange chromatography. Recovery in the eluate was 90–100% of the phosphates adsorbed by the resin. Approximately 90% of the phosphate fraction was sugar monophosphate. Small amounts of possible nucleotide monophosphates and of several more acidic phosphates were detected. *N*-Acetylglucosamine-1-phosphate, fructose-6-phosphate, galactose-1-phosphate, and glucose-6-phosphate were identified and their quantity determined.

The acid-soluble organic phosphates of cow milk were investigated to gain knowledge of milk constituents and to evaluate their contribution to the stability of milk and milk products against gelation and browning. Sugar phosphates have been found (Schwimmer and Olcott, 1953) to be highly reactive precursors to browning reactions. Polyphosphonucleotides and inorganic polyphosphates were observed to cause stabilization of milk proteins subjected to ionizing radiations (Hoff *et al.*, 1960). In addition, knowledge of the acid-soluble organic phosphates might give insight into biosynthetic processes in the mammary gland.

McGeown and Malpress (1952) demonstrated that glucose-1-phosphate and galactose-1-phosphate were constituents of milk, and that glucose-6-phosphate, lactose-1-phosphate, and phosphopyruvic acid were probably present. Small amounts of cytidine nucleotides (Deutsch and Nilson, 1960) and of 3′,5′-cyclic adenosine monophosphate (Kobata *et al.*, 1961) have been detected in cow milk. However, these substances account for only approximately 0.3% of the total acid-soluble organic phosphorus present (about 10 mg phosphorus/100 ml).

Quantitative extraction of the organic phosphates in unchanged form from milk was attempted by two methods. The mixtures of phosphates obtained were separated by ion exchange chromatography and their components characterized.

EXPERIMENTAL METHODS AND RESULTS

Milk. Fresh milk from mixed morning production (Hood and Sons, Charlestown, Mass.) was maintained on ice until analysis was begun (within 6 hr of milking) or was stored at −40°C for later use.

Extraction of phosphorylated components from milk. (Table 1). *Trichloroacetic acid (TCA) extraction.* Ice-cold milk (100 ml) was treated with 25 ml of 50% TCA solution. The precipitated protein was immediately removed by filtration on filter aid and washed with 25 ml of 5% TCA solution. The combined filtrate and washings (about 150 ml) were immediately extracted with seven 250-ml portions of cold (−10°C) ethyl ether to remove the TCA. Residual ether was removed from the aqueous layer by evaporation in a stream of nitrogen. The resulting clear solution was passed through a Dowex 50W column (1 × 10 cm) followed by 50 ml of water to remove cations. The eluate was diluted with water to a total volume of 200 ml. It contained 81% of the inorganic phosphate originally present in the fresh milk sample.

Removal of phosphates by countercurrent dialysis in the presence of EDTA. A solution of 2 g of ethylenediaminetetraacetic acid (EDTA) disodium salt in 50 ml of water was added to 250 ml of milk. The pH was adjusted to 7.9 with ammonium hydroxide, and the mixture was dialyzed against 1 L of water in a countercurrent

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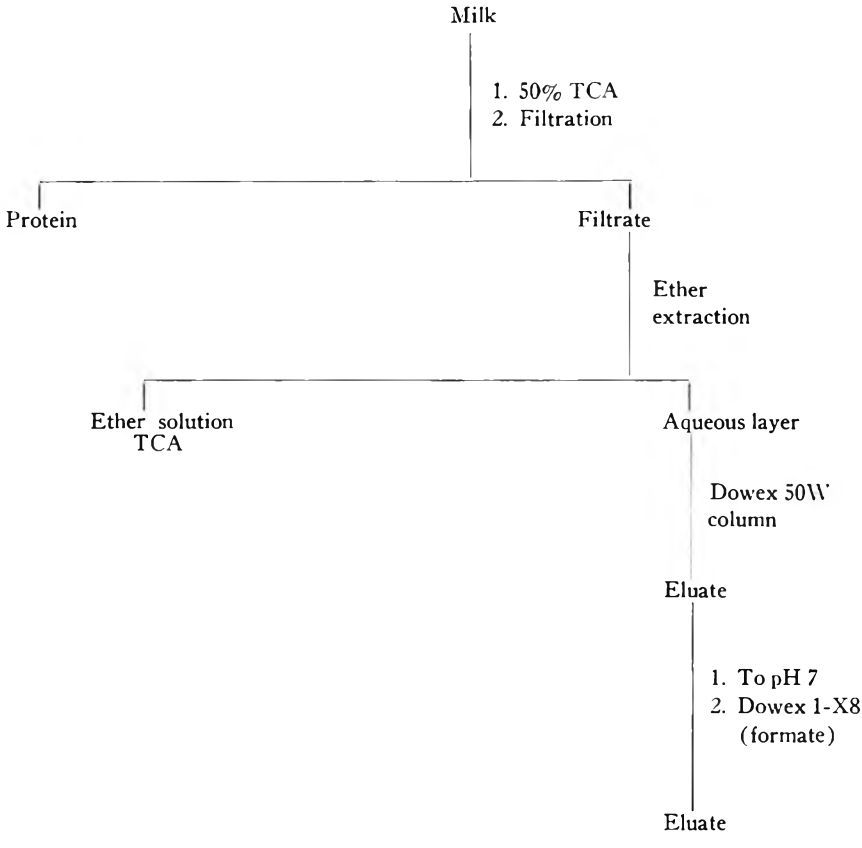
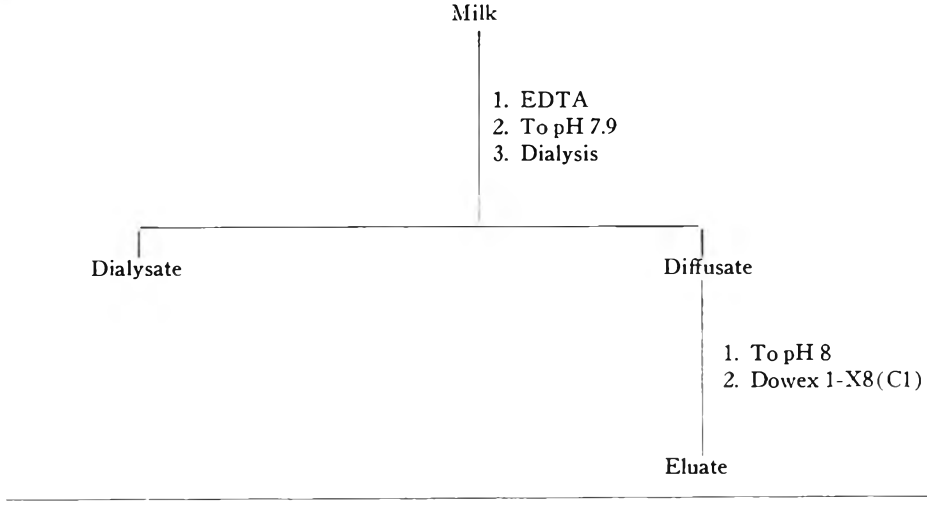
Table 1. Methods for phosphate extraction.

TCA Extraction



Dialysis



dialysis apparatus at 5°C. The apparatus consisted of an outer Tygon tube (½ in. × 20 ft) containing an inner tube of semipermeable cellulose (¼ in. × 20 ft dialysis tubing). The liquids circulated countercurrently, one (the milk) inside the inner tubing, the other (water) outside the inner tubing. Circulation was achieved with a Sigmamotor pump (Sigmamotor, Middleton, N. Y.). The water was changed six times at intervals of 1 hr. The resulting total diffusate (6 L) contained 91.5% of the inorganic phosphate originally present in the milk. The organic phosphate amounted to 10.1% of the total phosphate.

Separation and characterization of milk phosphates. *Analytical procedures.* Total and inorganic phosphorus was determined by the Fiske-SubbaRow method as modified by Bartlett (1959c). Bartlett's (1959b) modifications of the anthrone determination of hexoses and of the cysteine-carbazole reaction for fructose were carried out. Pentoses were detected by the orcinol reaction (Mejbaum, 1939), and *N*-acetylhexosamines by the modified (Reissig *et al.*, 1955) Morgan-Elson reaction.

Separation of the TCA extract. The TCA extract (200 ml) was neutralized with dilute ammonia, added to a 2.2×50-cm Dowex 1-X8, 100-200 mesh, column in the formate form, and unadsorbed solutes eluted with water (100 ml). The combined eluate (310 ml) was analyzed for total phosphorus, acid-labile phosphorus, and inorganic phosphorus, and its ultraviolet spectrum was determined (λ maximum 255 mμ at pH 1; end absorption and a shoulder at 245–270 mμ at pH 13). Approximately 64% of the organic phosphorus in the TCA extract was adsorbed on the column. If the peak at 255 mμ represented mononucleotides, these compounds were not adsorbed. They amounted to 10% of the organic phosphate.

Linear gradient elution with the formic acid-ammonium formate systems of Hurlbert *et al.* (1954) and Denamur *et al.* (1958) was carried out at 5°C with a flow rate of 2 ml/min. A total of 8 L of eluate was collected in 10-ml fractions. The fractions were analyzed for total phosphorus, ultraviolet absorption at 260 mμ, and hexoses. The elution diagram obtained is shown in Fig. 1. Concentrations of phosphorus-containing components were calculated by assuming the presence of one phosphorus atom per molecule. Ultraviolet-absorbing substances were assigned a molar absorbancy of 10,000 since most nucleotides and orotic acid possess molar absorbancies close to this value (Beaven *et al.*, 1955). Essentially 100% of the organic phosphorus adsorbed on the column was accounted for by peaks 2, 4, 5, 6, 7, 8, and 9 (Fig. 1).

Separation of diffusate. A 500-ml portion, representing 21 ml of milk, of the diffusate was brought to pH 8.0 with dilute ammonia and added to a column containing 1 × 25 cm of Dowex 1-X8 in the chloride form. Analysis of the resulting eluate for total phosphorus, inorganic phosphorus and acid-labile phosphorus showed that all inorganic phosphorus, and 52% of the organic phosphorus present in the diffusate had been adsorbed on the column. About 19% of the organic phosphorus not adsorbed could be recovered by re-

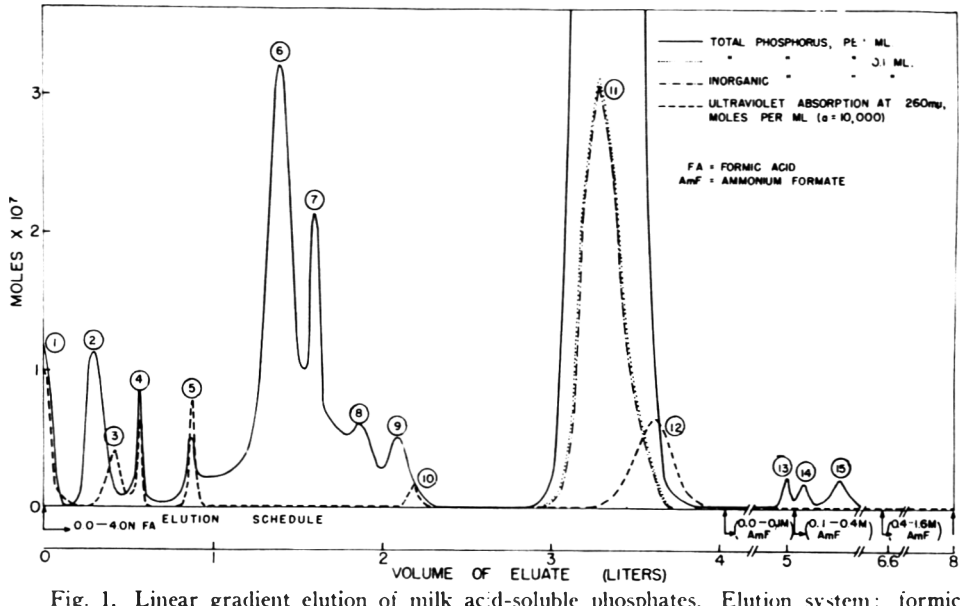


Fig. 1. Linear gradient elution of milk acid-soluble phosphates. Elution system: formic acid–ammonium formate. Dowex 1-X8.

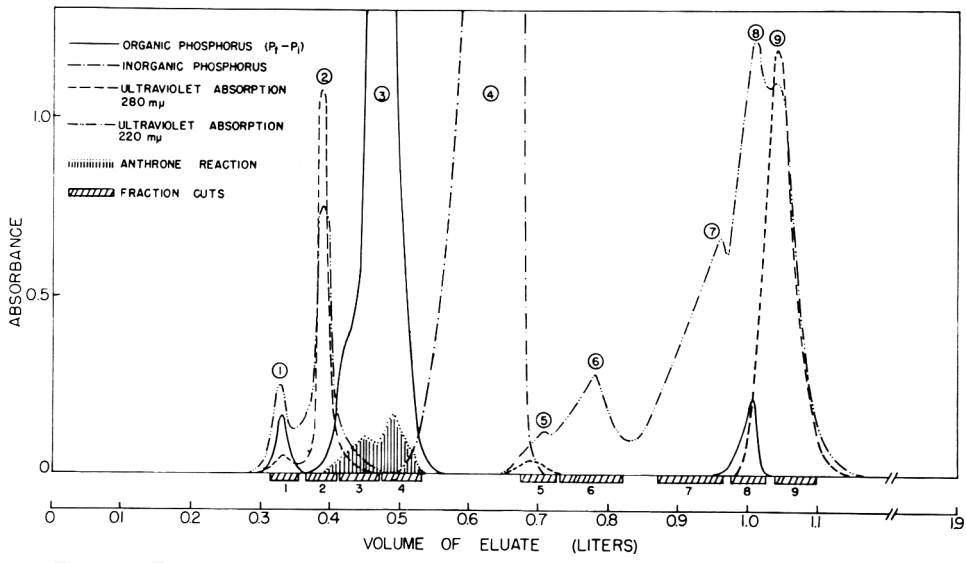


Fig. 2. Exponential gradient elution of milk diffusate (EDTA). Elution system: NH_4Cl—HCl. Dowex 1-X8.

chromatography of the eluate on the column. The remaining unadsorbed organic phosphorus was not investigated.

Gradient elution of the column with a mixture of 0.004*N* hydrochloric acid and 1*N* ammonium chloride, in which the concentration of ammonium chloride increased exponentially, was carried out at a flow rate of 1.5 ml/min. A total of 183 10-ml fractions were collected and examined for total phosphorus, inorganic phosphorus, hexoses, and ultraviolet absorption at 220 and 280 mμ. The elution diagram obtained is shown in Fig. 2. Essentially 100% of the organic phosphorus adsorbed on the column was accounted for in peaks 1 and 3. Six 500-ml portions of milk diffusate were separated in the above manner. Similar fractions were combined and stored at −40°C for subsequent investigation.

1) Sugar phosphates (Peak 3, Fig. 2). The fractions in peak 3, designated cuts 3 and 4, gave positive anthrone reactions and contained 56.2% of the total organic phosphorus eluted from the column. Inorganic phosphate (Fraction 4) was removed from the combined cuts 3 and 4 by chromatography (Bartlett, 1959a) on a 1×14-cm column of activated carbon (Barneby-Cheney No. 4422) previously treated with 500 ml 1*N* hydrochloric acid and washed free of chloride with water. Elution was carried out with 100 ml of water and 500 ml of 0.01*N* ammonium hydroxide at a flow of 2 ml/min. Fractions (10 ml) were analyzed for total phosphorus, inorganic phosphorus, hexoses, fructose, and *N*-acetylglucosamine. The elution scheme obtained is shown in Fig. 3. Organic phosphates (free of inorganic phosphate) were eluted in fractions 71–100. Fractions 71–75 contained a mixture of hexose-, fructose-, and unknown phosphates. Fractions 76–100 contained completely unknown phosphate(s).

Fractions 71–75 (Fig. 3) were combined and freed of cations on a Dowex 50W-X8 column,

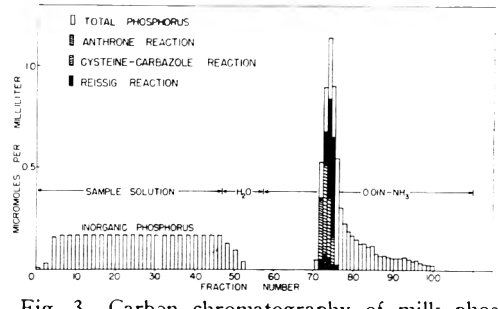


Fig. 3. Carbon chromatography of milk phosphates.

and the eluate was evaporated to dryness under vacuum. Analysis of a 1-ml solution of the resulting white solid yielded the results given in Table 2a. Acid hydrolysis of a portion of the solution was carried out for 7 min at 100°C in 1*N* hydrochloric acid. Enzymatic hydrolysis of another portion (0.1 ml) was carried out by heating 2 hr at 37°C in the presence of 1% alkaline phosphatase (calf intestinal phosphatase, Mann Research Labs, New York) in 0.1*N* sodium bicarbonate solution containing a trace of magnesium chloride. The resulting solution was deionized by ion-exchange chromatography and evaporated to dryness.

Table 2. Results of analysis of fractions 71–75 and fractions 76–90.

a. *Fractions 71–75*	*Micromoles*
Total phosphorus	25.6
Acid-labile phosphorus	20.8
Hexose (as glucose)	7.6
Triose	negative
Fructose	1.2
N-Acetylglucosamine-1-phosphate	6.8
b. *Fractions 76–90*	*Micromoles*
Inorganic phosphorus	2.3
Inorganic phosphorus (after treatment for 7 min with 1*N* H_2SO_4 at 100°C)	16.8
N-Acetylhexosamine	0.0
N-Acetylhexosamine (after hydrolysis for 5 min at 100°C in 1*N* HCl)	17.5

Descending paper chromatographic analysis (Natl. Acad. Sci., 1960) on Whatman No. 1 paper of combined fractions 71–75, of their acid hydrolysis products, and of their products of enzymatic dephosphorylation yielded the results shown in Table 3. The solvent system was *n*-butanol–pyridine–water (3:2:1.5). Reducing sugars were detected with ammoniacal silver nitrate, and phosphates were detected with molybdate solution (Hanes and Isherwood, 1949).

Fractions 76–90 (Fig. 3) were combined and freed of cations and the eluate was lyophilized. Analysis of a 1-ml solution of the resulting white solid before and after acid hydrolysis gave the results shown in Table 2b. Two-dimensional paper chromatography (Fletcher and Malpress, 1953) on Whatman No. 1 paper in: a) methanol–88% formic acid–water (80:15:5) and b) methanol–ammonia (sp. gr. 0.9015)–water (60:10:30) (Bandurski and Axelrod, 1951) was carried out. It showed the presence of a single phosphorylated compound and a trace of inorganic phosphate. One-dimensional paper (Scheicher and Sehuell, No. 589) chromatography of combined fraction 76–90 and of the mixture obtained by treatment of fraction 76–90 with alkaline phosphatase before and after chromatography (Fletcher *et al.*, 1953) in solvent b yielded the results shown in Table 4. Paper chromatography of hydrolysis products of combined fractions 76–90, *N*-acetylglucosamine, *N*-acetylgalactosamine, and glucose in *n*-butanol-pyridine-water (3:2:1.5) by the method of Pontis (1955) gave the results shown in Table 5.

2) Organic phosphate (Peak 1, Fig. 2). The fractions in peak 1 had strong end absorption in the uv. Enzymatic analysis for pyruvic acid (Bücher and Pfleiderer, 1955) was negative.

Table 3. Paper chromatographic analysis of fractions 71–75 and of certain reference compounds

	Rf values relative to galactose	
	Ammoniacal silver nitrate [a]	Molybdate reagent [b]
Fractions 71–75	0.00–0.13 (s)	0.00
	0.47 (m)	0.48
	0.97 (w)	
	1.42 (w)	
Acid hydrolysis products of 71–75	0.00–0.14 (m)	0.00
	0.48 (m)	0.49
	0.98 (s)	
	1.49 (m)	
Enzymatic hydrolysis products of 71–75	0.45–0.64 (w)	0.49 (a mixture)
	1.01 (s)	
	1.17 (s)	
	1.28 (m)	
	1.48 (m)	
Known compounds		
Lactose	0.64	
Galactose	1.00	
Glucose	1.16	
Fructose	1.27	
N-Acetylglucosamine	1.48	
Glucose-1-phosphate		0.00
Glucose-6-phosphate	0.00	0.00

(s): strong; (m): medium; (w) weak

[a] Detects reducing sugars.
[b] Detects phosphate.

3) Organic phosphate (Peak 8, Fig. 2). The fractions in peak 8 had strong end absorption in the uv. Analyses for hexose, pentose, glyceric acid (Bartlett, 1959d), and phosphopyruvate (Bücher and Pfleiderer, 1955) were negative.

Table 4. Paper chromatographic analysis of combined fraction 76–90 (Fig. 3).

Analysis	Fraction 76–90 (R_f)	Enzymatic hydrolysis products of fraction 76–90 (R_f)
Phosphate (Hanes and Isherwood, 1949)	0.37 (w) 0.58 (s)	0.37 (s) No spot
Reducing sugar Trevelyan *et al.*, 1950)	No spot	0.47
N-Acetylhexosamine (Partridge, 1949)	No spot (0.58 after phosphatase treatment)	Not determined

(s): strong; (m): medium; (w): weak

4) Nonphosphorylated components. Peak 2 (Fig. 2): The position of elution and the ultraviolet spectrum of a representative fraction of peak 2 were identical with those obtained with an authentic sample of uric acid.

Peaks 5 and 6 (Fig. 2) were not investigated.

Peak 7 (Fig. 2) yielded 25 mg of EDTA and showed uv absorption at 220 mμ.

Peak 9 (Fig. 2): The position of elution and the ultraviolet spectrum of a representative fraction of peak 9 were identical with those of an authentic sample of orotic acid. Microbiological assay for orotic acid according to the method of Wright *et al.* (1950) gave positive growth response and acid production with selected fractions.

DISCUSSION

The degree of extraction achieved by TCA treatment and by dialysis of milk in the presence of EDTA was determined by the quantity of inorganic phosphate (P_i) present in each extract. Since P_i composed about 90% of total acid-soluble milk phosphate, its determination was more accurate than the indirect determination of organic phosphate ($P_{total} - P_i$). Thus it was assumed that the quantity of P_i in each extract was a valid measure of the degree of extraction of total phosphates. On this basis the TCA extract contained 81% of total milk phosphorus. The diffusate obtained in the presence of EDTA contained 91.5% of total milk phosphorus.

The TCA extraction subjected the milk to acidic conditions (pH 0–1), which could cause hydrolysis of labile components. The dialysis provided mild conditions for relatively long periods, during which enzymatic changes might occur. Assessment of the effect of these procedures on the milk phosphates required direct investigation of the extracts.

Since ion exchange chromatography of the milk phosphates caused adsorption of only 64% of the organic phosphate in the TCA extract and of about 51–52% of organic phosphate in the diffusate, it was concluded that the unadsorbed substances must be weak acids such as diester phosphates or phosphorylated amino acids and peptides.

Comparison of the elution diagram of the TCA extract (Fig. 1) and the diffusate obtained in the presence of EDTA (Fig. 2) showed that separation was more complete in the first case. The large quantity of P_i (peak 11, Fig. 1), was well separated from the organic phosphates present in peaks 1, 2, 4, 5, 6, 7, 8, 9, 13, 14, and 15. Since peaks 1, 4, and 5 showed uv absorption at 260 mμ it was possible that they contained monophosphorylated nucleotides. The absence of uv-absorbing peaks in the area of peaks 13, 14, and 15 indicated that nucleotide di- and triphosphates were not present in the TCA extract. The major portion of organic phosphates consisted of sugar phosphates (peaks 2, 6, 7, and 8) since they contained hexose and phosphate but had no uv absorption. They accounted for approximately 90% of the organic phosphorus detected in the total eluate from the TCA extract. Peaks 3 and 12 were believed to be uric and orotic acids on the basis of their position of elution, their uv spectra, and the fact that they contained no phosphorus.

Review of Fig. 2 shows that separation of the diffusate was less efficient than in Fig. 1. The elution system, however, allowed enzymatic determinations of eluted components. For this reason, and in spite of the relatively poor resolution of the components obtained, the ammonium chloride-hydrochloric acid system was chosen for the accumulation of relatively large quantities of milk phosphates for direct investigation.

Sugar phosphates (peak 3, Fig. 2), which accounted for 56.2% of the total organic phosphorus eluted from the column, were separated from inorganic phosphorus (peak 4) by chromatography, and the elution scheme obtained is shown in Fig. 3. Analysis of the contents of fractions 71–75 (Fig. 3) showed the presence of fructose- and hexose- (determined as glucose) phosphates and *N*-acetylglycosamine-1-phosphate (see Table 2a). Acid-labile phosphorus was detected in considerable quantity (20.8μM). Paper chromatographic analysis of these fractions indicated that small amounts of galactose and free *N*-acetylglucosamine in addition to at least two organic phosphates (see Table 3) were present. This implied that certain of the organic phosphates were quite unstable. The paper chromatogram of the products of acid hydrolysis of fraction 71–75 showed that the amounts of galac-

tose and *N*-acetylglucosamine had increased and that an acid-stable sugar phosphate (R_f 0.49) and a completely unknown phosphate (R_f 0.0) were present (Table 3). The chromatogram of fraction 71–75 after hydrolysis with alkaline phosphatase showed that at least two very stable organic phosphates (R_f 0.49) were present, and that the sugar phosphates originally present had contained glucose, fructose, galactose, and *N*-acetylglucosamine. It was concluded, on the basis of the observed reactivity during hydrolysis by acid and by alkaline phosphatase, that the phosphates present in fractions 71–75 were glucose-6-phosphate, galactose-1-phosphate, fructose-6-phosphate, *N*-acetylglucosamine-1-phosphate, and at least two very stable unknown organic phosphates.

Fractions 76–90 (Fig. 3) contained a single organic phosphate and a trace of P_i. Acid hydrolysis yielded P_i and an *N*-acetylhexosamine (Table 2b). Alkaline phosphatase treatment produced increased P_i (R_f 0.37) and a reducing sugar (R_f 0.47) (see Table 4). Direct evidence that the reducing sugar was an *N*-acetylhexosamine and that it was originally present as a phosphate in fractions 76–90 was provided by application of alkaline phosphatase to the organic phosphate spot (R_f 0.58) on the chromatogram (Table 4). A positive test for *N*-acetylhexosamines was then obtained. The presence of *N*-acetylglucosamine was proved by paper chromatographic comparison (Table 5) of acid hydrolyzed fractions 76–90 with authentic reference compounds. Identification of *N*-acetylglucosamine-1-phosphate as a component of fractions 76–90 was thus accomplished.

The 4 phosphates identified accounted for 71.4% of the organic phosphorus present in the sugar phosphate mixture (peak 3, Fig. 2). Calculation of the quantity of each of these compounds in 100 ml of milk, from data presented in Fig. 3, yielded the results given in Table 6. The quantity of *N*-acetylglucosamine-1-phosphate and of fructose-6-phosphate was obtained directly (Reissig *et al.*, 1955; and cysteine-carbazole reaction, Bartlett, 1959b). Phosphate liberated by mild acid hydrolysis in excess of that accounted for by *N*-acetylglucosamine-1-phosphate was assumed to be due to galactose-1-phosphate. Glucose-6-phosphate was obtained by subtracting absorbancy values due to galactose-1-phosphate and fructose-6-phosphate from the absorbancy observed in the anthrone reaction (Bartlett, 1959b).

The organic phosphates detected in peaks 1 and 8 (Fig. 2) could not be identified on the basis of the evidence available. Their positions of elution indicated that the phosphate(s) in peak 1 must be weakly acid and the phosphate(s) in peak 8 strongly acid. Since fractions in peak 8 gave negative results for hexose, pentose, glyceric acid, and phosphopyruvate, they may, by process of elimination, contain a phosphogluconic-acid-like substance. The fact that monophosphorylated, monocarboxylic acids are eluted at approximately the same position supports this possibility. The identification of these substances and of the two very stable phosphates described above is worthy of further investigation.

Uric acid was identified in peak 2 (Fig.

Table 5. Paper chromatographic comparison of acid hydrolysis products of fraction 76–90 with certain reference compounds.

Compound	R_f[a] (relative to *N*-acetylglucosamine)
Hydrolyzed fraction 76–90	1.01
N-Acetylglucosamine	1.00
N-Acetylgalactosamine	0.38
Glucose	No spot

[a] Spots detected by reagent of Partridge (1949).

Table 6. Sugar phosphates in cow milk.

Compound	Micromoles	Mg/100 ml milk
Galactose-1-phosphate	17.3	4.5
N-Acetylglucosamine-1-phosphate	29.5	8.9
Glucose-6-phosphate	4.5	1.2
		(0.01)[a]
Fructose-6-phosphate	1.5	0.4
Glucose-1-phosphate[a]	.....	.....
		(0.1)[a]
Lactose-1-phosphate[a]	.....	.....
		(0.01)[a]
Phosphopyruvic acid[a]	.....	.....
		(0.01)[a]

[a] McGeown and Malpress (1952).

2) on the basis of the identity of its uv spectrum at pH 2.4 with that of an authentic sample, and on its position of elution. Orotic acid was identified in peak 9 on the basis of its uv spectrum at pH 4.0, its position of elution, and its microbiological assay.

The presence of galactose-1-phosphate and glucose-6-phosphate in milk has been definitely confirmed, and the presence of fructose-6-phosphate and *N*-acetylglucosamine-1-phosphate demonstrated for the first time. The fact that glucose-1-phosphate, lactose-1-phosphate, and phosphopyruvic acid (all reported by McGeown and Malpress, 1952) were not found in the EDTA diffusate may be due to the fact that the two sugar phosphates were not present in large enough quantities to allow their detection in the complex mixture present in peak 3. It is also possible that phosphopyruvic acid, which should be eluted near peak 8, was masked by the relatively large quantity of non-phosphorylated substances present. Direct examination of the fractions obtained from the TCA extract (Fig. 1) might have resulted in the detection of glucose-1-phosphate, lactose-1-phosphate, and phosphopyruvic acid. A number of the acid-soluble organic phosphates found in milk remain to be characterized. Future investigations should devote attention to the nature of the organic phosphates that were not adsorbed on the columns.

Fructose-6-phosphate has been shown to be a constituent of cow milk. Its presence may reflect the operation of the pentose cycle in mammary gland tissue. The significance of *N*-acetylhexosamine-1-phosphate in cow milk remains to be evaluated. UDP-hexosamines have been found in sheep milk (Denamur *et al.*, 1958) and in mammary glands (Manson, 1956; Smith and Mills, 1954). The immunoproteins of cow milk are reported (Larsen and Kendall, 1957) to contain hexosamine residues, and saccharides containing *N*-acetylglucosamine have been found in human milk (Gyorgy, 1958). It is noteworthy that only small quantities of nucleotides were found, and that no intermediary compounds of the Emden-Meyerhoff glycolytic pathway were detected.

The presence of lactose-1-phosphate in cow milk has been neither confirmed nor disproved. In view of the interest attached to this compound in discussions of the biosynthesis of lactose (Gander *et al.*, 1957; Malpress, 1958; Hassid, 1960; Wood *et al.*, 1958), particular attention should be paid to it in future investigations. The absence of organic and inorganic polyphosphates in the extracts studied eliminates them as possible metal-sequestering agents that aid in the stabilization of cow milk. The presence of relatively large quantities of sugar phosphates may indicate that they are important contributors to browning reactions in heat processed and in dehydrated milk products. Glucose-6-phosphate and fructose-6-phosphate may contribute particularly to such reactions since they are reducing sugars.

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Storage of Mandarin Oranges
II. Effects of Hormones and Wax Coatings

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SUMMARY

The effects of temperature, wax emulsion coating, hormone treatment, and their combination on the storage behavior of Nagpur and Darjeeling mandarins were investigated. Use of 2,4-D with wax coating was more effective in prolonging the storage life of both varieties at any storage temperature than either wax coating or 2,4-D used separately. The storage lives of Nagpur and Darjeeling oranges were respectively 50 and 35 days at normal temperature (50–85°F) and 90 and 75 days at 40 ± 1°F when treated with 2,4-D (1000 ppm for Nagpur and 2000 ppm for Darjeeling varieties) followed by wax coating. Wax-coated Nagpur and Darjeeling oranges were in a good marketable condition for 30 and 22 days, respectively, at room temperature, whereas the respective controls remained marketable for only 10 and 7 days. At 40 ± 1°F, storage lives were respectively 60 and 52 days for Nagpur and Darjeeling oranges coated with wax, and 40 and 25 days for their respective controls. Hormone-treated fruits, like the controls, lost their marketable appearance through considerable desiccation, pitting, and shriveling. Loss in weight and spoilage due to fungal attack and rind blemishes were least in fruits treated with 2,4-D followed by wax coating in both varieties and each storage temperature. Pulp-to-peel ratio, reducing sugar, sucrose, total sugar, sugar-to-acid ratio, pH value, and specific gravity in juice increased, whereas vitamin C content and titratable acidity decreased, during storage in all treatments. No off flavor was produced in any case.

INTRODUCTION

Next to the mango and banana, citrus fruits are the largest fruit crop of India, occupying about 6% of the total area under cultivation. Mandarin oranges *(Citrus reticulata)*, the largest citrus crop grown in India, is of great nutritive, protective, medicinal and industrial value. Therefore, evaluation of proper storage conditions for extending its period of availability in the market appears very useful.

Siddappa and Bhatia (1954) reported varietal differences in the composition of different mandarin oranges grown in India. Cheema *et al.* (1937) and Singh and Hamid (1942) determined the optimum temperature for the storage of Nagpur oranges. Stewart (1949) observed that wastage from fungal attack could be reduced by treating citrus fruits with 2,4-D and 2,4,5-T. Stewart *et al.* (1952) reported that post-harvest treatment of lemons with higher concentration of 2,4-D and 2,4,5-T retarded the yellowing of the fruits. Dutt *et al.* (1960) lengthened the storage life of Nagpur oranges by coating the fruits with wax emulsion.

No work has been reported, however, on the combined effect of hormone treatment and wax coating on the storage behavior of mandarin oranges. The present investigation was carried out to find the effect of hormones and wax coating, separately and in combination, on storage behavior at normal and at low temperatures.

MATERIALS AND METHODS

The varieties of oranges used in these experiments were Nagpur and Darjeeling. Fruits were brought from particular gardens, with arrangement for collection through dealers in the wholesale market of Calcutta. About three days elapsed between harvest and treatments. The fruits were in fully mature stage. Nagpur oranges were green in color, while Darjeeling oranges were orange yellow with a greenish tinge. Fresh and sound fruits were surface cleaned with a piece of cotton soaked in 1% ethanol solution.

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Wax emulsion coating. Paraffin-carnauba wax emulsion as given by Trout (1942) was prepared in the following composition (parts by weight): paraffin wax, 112; carnauba wax, 28; oleic acid, 22; $NaHCO_3$, 4.5; Na_2CO_3, 1.5; water, 392. The emulsion contained 30% solid and 25% wax. It was diluted with cold water (50:50) to obtain 12.5% total wax content emulsion, which was used in all cases. Both Nagpur and Darjeeling oranges were coated with wax emulsion by dipping in the emulsion for 2 minutes and then drying in air.

Hormone treatment. Used on Nagpur oranges were 3 hormone solutions; maleic hydrazide (MH), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) at 100, 500, and 1000 ppm. Darjeeling oranges were treated with 2,4-D and 2,4,5-T solutions of 100, 1000, and 2000 ppm.

The hormones were dissolved in alcoholic solution (1:9 80% ethanol and water) in which 0.15% Tween-80 was incorporated as a surface-active agent. Both Nagpur and Darjeeling oranges were dipped in the hormone solutions for 3 min and dried in air. About 0.04 g of hormone was required for 100 fruits when a 1000-ppm concentration was used.

Hormone treatment plus wax coating. Some hormone-treated fruits from each lot were coated with wax emulsion and dried in the open air. The wax required for 100 fruits was 4.3 g. Such treated (hormone-treated, wax-coated, and hormone-treated followed by wax coating) and control fruits were kept in rectangular wooden boxes (24 × 18 × 6 inches) lined inside with corrugated brown paper and stored at room temperature (58–80°F, 45–75% RH) and in a constant-temperature room at 40±1°F (68–82% RH). In each treatment, 50 fruits were kept for study of orange life and batches of five samples in each were weighed at regular intervals to determine physiological losses in weight. Similar lots for each treatment were also kept from which six samples were picked at intervals for analysis of the chemical constituents. The experiments were repeated in two consecutive years, in 1960 and 1961.

Extraction of juice and analysis of constituents. The rind of the fruit was removed, and the segments only were crushed in a screw-type juice extractor. The extracted juice, filtered through a fine cloth, was used for chemical analysis. Sugars were estimated by the copper reduction method of Lane and Eynon (1923). Titratable acidity was determined by titrating the diluted juice with standard 0.1N NaOH solution, with 1% phenolphthalein used as an indicator. Vitamin C was estimated by 2,6-dichlorophenolindophenol visual titration methods. pH value was determined by taking a direct reading on a Cambridge pH meter. Specific gravity was determined with a Sp. gr. bottle. Organoleptic tests were also carried out. Pulp-to-peel ratios were determined in a number of fruits. Cumulative percentage physiological loss in weight was determined by weighing a few fruits in each treatment at the beginning of the experiment and taking the weight of those fruits at regular intervals. Observations on spoilage were also made at regular intervals.

RESULTS AND DISCUSSION

Storage life, physical appearance, and spoilage. Both the ripening and development of color of both varieties were greatly delayed by higher concentrations of 2,4-D. The 2,4-D plus wax emulsion was more effective in prolonging the storage life of both varieties than was either wax or 2,4-D alone. Less effective, though of similar effect, was 2,4,5-T. At no concentration did

Table 1. Storage life of mandarin oranges under different treatments and at different temperatures of storage, based on 4% spoilage due to mold attack, rotting, and desiccation to an extent of 20% loss of moisture or more.

Variety	Treatment	Storage conditions [a]	Mean storage life (days)
Nagpur	2,4-D (1000 ppm)	C	90
	+ wax emulsion	r	50
	Wax emulsion	C	60
		r	30
	2,4-D (1000 ppm)	C	46
		r	14
	2,4,5-T (1000 ppm)	C	78
	+ wax emulsion	r	40
	MH (1000 ppm)	C	62
	+ wax emulsion	r	33
	MH (1000 ppm)	C	42
		r	9
	Control	C	40
		r	10
Darjeeling	2,4-D (2000 ppm)	C	75
	+ wax emulsion	r	35
	Wax emulsion	C	52
		r	22
	2,4-D (2000 ppm)	C	32
		r	12
	2,4,5-T (2000 ppm)	C	65
	+ wax emulsion	r	30
	2,4,5-T (2000 ppm)	C	28
		r	10
	Control	C	25
		r	7

[a] C = cold, 40±1°F, 68–82% RH; r = room temperature, 58–80°F, 45–75% RH.

Table 2. Cumulative percentage physiological losses in weight in Nagpur mandarin oranges, under different treatments, during storage. (Results in parentheses indicate those of Darjeeling mandarin oranges).

Period of storage (days)	At 40±1°F, 68–82% RH				At room temp. 58–80°F, 45–75% RH			
	Control	Waxed	2,4-D (1000 ppm [a])	2,4-D (1000 ppm [a]) with wax	Control	Waxed	2,4-D (1000 ppm [a])	2,4-D (1000 ppm [a]) with wax
3	2.20	0.83	2.04	1.07	4.15	2.22	5.00	1.76
	(1.30)	(0.20)	(1.78)	(0.61)	(4.44)	(2.09)	(3.66)	(2.17)
9	5.00	1.98	5.71	2.15	8.87	4.84	10.38	4.29
	(3.35)	(2.25)	(4.22)	(1.83)	(9.09)	(7.12)	(10.35)	(5.32)
21	9.80	3.96	11.44	3.66	....	8.97	....	7.62
	(6.89)	(4.17)	(7.33)	(3.23)	(....)	(11.68)	(....)	(9.46)
27	12.20	5.00	14.08	4.32	....	10.96	....	9.37
	(8.35)	(4.79)	(9.10)	(4.20)	(....)	(....)	(....)	(11.84)
45	....	7.50	19.15	7.10	....	....	....	14.24
	(....)	(7.11)	(....)	(7.00)	(....)	(....)	(....)	(....)
60	....	9.35	....	9.92	....	....	....	....
	(....)	(....)	(....)	(9.84)	(....)	(....)	(....)	(....)
90	....	....	....	10.55	....	....	....	....
	(....)	(....)	(....)	(....)	(....)	(....)	(....)	(....)

[a] 2000 ppm in Darjeeling mandarin oranges.

MH show any advantage over the control with either variety.

Table 1 shows that the storage life of Nagpur and Darjeeling oranges at room temperature was 50 and 35 days, respectively, compared to 10 and 7 days for the respective controls, when the fruits were treated with 2,4-D (1000 ppm in case of Nagpur and 2000 ppm for Darjeeling oranges) followed by coating with wax. The stored fruits retained their attractive color and characteristic flavor. Nagpur and Darjeeling oranges coated with wax alone had a storage life of 30 and 22 days, respectively. Fruits treated with only 2,4-D, like the controls, showed sign of desiccation, pitting, and shriveling, and had a storage life of 14 and 12 days, respectively.

At 40±1°F, the storage life of Nagpur oranges treated with 2,4-D (1000 ppm) followed by wax coating was extended up to 90 days, as against 60 days for wax-coated fruit and 40 days for the controls. When 2,4-D-treated Darjeeling oranges (2000 ppm) were wax coated, they had 75 days' storage life, whereas fruits coated with wax alone and the controls had respective storage lives of 52 and 25 days. Fruits treated with only 2,4-D lost their attractive appearance through desiccation and pitting.

The predominant types of molds found on spoiled oranges were *Penicillium digitatum* and *Penicillium italicum*, although Alternaria rot caused by *Alternaria citri* and other type of rind blemishes (stem end rot, crinkled collapse, dry spots, scaled, etc.) were observed. Mold attack is the main cause of spoilage of fruits treated with wax emulsion alone. Spoilage was quicker in Darjeeling oranges than in Nagpur oranges with corresponding treatments at both temperatures.

2,4-D not only delayed ripening of both varieties but also reduced spoilage due to fungal attack. Similar reduction in wastage of citrus fruits was also observed with the application of 2,4-D in wax emulsion by Stewart (1949). This reduced wastage was directly due to the toxic effect of 2,4-D on the organisms concerned, and indirectly to the delay in ripening.

Cumulative percentage physiological losses in weight. Physiological weight losses

Table 3. Changes in chemical constituents of Nagpur mandarin oranges under different treatments during storage at different temperatures. (The data in parentheses indicate values calculated on original fresh-weight basis).

Storage conditions	Period of storage	Pulp-to-peel ratio	Sugars (g/100 g juice)			Titratable acidity expressed as anhydrous citric acid (g/100 g juice)	Vitamin C (mg/100 g juice)	pH of the juice	Sp. gr. of the juice
			Reducing	Sucrose	Total				
40±1°F, 68–82% RH	Initial	2.56	2.65	3.32	5.97	1.10	35.76	3.11	1.0316
	40 days, control	3.51	3.82 (3.48)	6.22 (5.64)	10.04 (9.14)	0.62 (0.56)	28.58 (25.90)	3.74	1.0521
	60 days, wax	3.20	3.46 (3.35)	6.98 (6.76)	10.44 (10.11)	0.60 (0.58)	24.38 (23.60)	3.92	1.0513
	46 days, 2,4-D (1000 ppm)	4.08	3.11 (2.81)	5.40 (4.88)	8.51 (7.69)	0.80 (0.72)	26.35 (23.80)	3.48	1.0490
	90 days, 2,4-D (1000 ppm) + wax	3.30	3.52 (3.38)	7.62 (7.25)	11.14 (10.63)	0.58 (0.55)	27.52 (26.08)	3.94	1.0529
Room temp., 58–80°F, 45–75% RH	10 days, control	3.00	3.97 (3.78)	6.34 (6.05)	10.31 (9.83)	0.68 (0.65)	23.35 (22.25)	3.67	1.0524
	30 days, wax	3.62	3.44 (3.29)	6.92 (6.62)	10.36 (9.91)	0.64 (0.61)	22.15 (21.20)	3.70	1.0498
	14 days, 2,4-D (1000 ppm)	3.90	2.81 (2.64)	4.05 (3.81)	6.86 (6.45)	0.94 (0.88)	30.93 (29.00)	3.34	1.0408
	50 days, 2,4-D (1000 ppm) + wax	3.67	3.61 (3.33)	7.41 (6.85)	11.02 (10.18)	0.61 (0.56)	24.45 (22.55)	3.90	1.0528

Each figure is an average of three observations, having 6 fruits in a batch for each treatment.

Table 4. Changes in chemical constituents of Darjeeling mandarin oranges under different treatments during storage at different temperatures. (The data in parentheses indicate values calculated on original fresh-weight basis).

Storage conditions	Period of storage	Pulp-to-peel ratio	Sugars (g/100 g juice)			Titratable acidity expressed as anhydrous citric acid (g/100 g juice)	Vitamin C (mg/100 g juice)	pH of the juice	Sp. gr. of the juice
			Reducing	Sucrose	Total				
40±1°F, 68–82% RH	Initial	2.85	3.11	6.14	9.25	0.86	33.53	3.48	1.0420
	25 days, control	3.93	3.65 (3.52)	6.92 (6.67)	10.57 (10.19)	0.48 (0.46)	20.93 (20.20)	4.08	1.0520
	52 days, wax	3.40	3.59 (3.36)	6.82 (6.38)	10.41 (9.74)	0.58 (0.54)	21.38 (20.00)	3.82	1.0524
	32 days, 2,4-D (2000 ppm)	4.00	3.46 (3.25)	6.51 (6.10)	9.97 (9.35)	0.79 (0.74)	23.21 (21.80)	3.58	1.0464
	75 days, 2,4-D (2000 ppm) + wax	3.83	4.05 (3.74)	7.25 (6.70)	11.30 (10.44)	0.53 (0.49)	17.86 (16.45)	3.98	1.0525
Room temp., 58–80°F, 45–75% RH	7 days, control	3.28	3.31 (3.09)	7.12 (6.64)	10.43 (9.73)	0.59 (0.55)	26.35 (24.50)	3.98	1.0518
	22 days, wax	3.91	3.77 (3.48)	6.97 (6.43)	10.74 (9.91)	0.50 (0.46)	23.79 (21.95)	3.98	1.0489
	12 days, 2,4-D (2000 ppm)	4.12	3.24 (2.98)	6.21 (5.73)	9.45 (8.71)	0.72 (0.66)	28.34 (26.10)	3.68	1.0500
	35 days, 2,4-D (2000 ppm) + wax	4.90	4.18 (3.86)	7.42 (6.83)	11.60 (10.69)	0.40 (0.37)	18.10 (16.65)	4.04	1.0566

Each figure is an average of three observations having 6 fruits in a batch for each treatment.

(Table 2) indicate that losses in weight were greater at room temperature (58–80°F) than at 40±1°F in both orange varieties under corresponding treatments. Loss in weight was least with 2,4-D plus wax, and then in increasing order for wax-coated, control, and only 2,4-D-treated fruits, for both the varieties and storage temperatures. Darjeeling oranges lost more weight at room temperature (58–80°F, 45–75% RH) than Nagpur oranges, but the reverse was true at 40±1°F (68–82% RH) with similar treatments after same days at storage.

Pulp-to-peel ratio. The increased pulp-to-peel ratio for both the varieties under different treatments (Tables 3, 4) indicates that the loss of weight was proportionately higher from the peel than from the pulp. A similar increase in pulp-to-peel ratio during ripening was obtained by Hawkins (1921) in grapefruits and by Trout *et al.* (1938) in Washington Navel oranges.

Changes in chemical constituents. The trend of chemical changes (Tables 3, 4) was the same for both the varieties under different treatments, but the rate of changes and specific values were different for different treatments. Of the total sugar, sucrose constituted the greater portion, and the rest was shared by reducing sugars. The rate of increase of sucrose was greater than that of reducing sugars during ripening. Specific gravity of juice increases mostly due to increase in sugar content during ripening. With the increase of sugars, the titratable acidity gradually decreased. Increase in pH value is due to the decrease in effective acidity. Increase of sugar and decrease of acid content in juice led to a greater sugar-to-acid ratio during ripening. A similar relation between sugar and acid was found in mandarin oranges by Kudryavtseva (1931). The vitamin C content decreased slightly during ripening in both varieties under different treatments. Bratley (1940) reported a similar decrease in vitamin C content in oranges. It is noteworthy that the physiological break-down of the constituents was less when the fruits were treated with hormone.

The characteristic color of juice was deeper for Darjeeling oranges than for Nagpur oranges at the eating-ripe condition. No off flavor was produced in any case. Fruits stored at 40±1°F were decidedly better to the taste than fruits kept at room temperature (58–80°F).

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The Relationship of Some Intracellular Protein Characteristics to Beef Muscle Tenderness [a, b]

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SUMMARY

A procedure developed to fractionate the major nitrogen-containing components of muscle was utilized to study the relationship of intracellular muscle proteins to tenderness. The study was performed on a group of 20 yearling bulls from two lines of cattle that had been selected for differences in tenderness. The longissimus dorsi muscle contained more total nitrogen per unit of muscle tissue and almost twice as much sarcoplasmic protein as the infraspinatus muscle. The longissimus dorsi contained less fibrillar protein and non-protein nitrogen than the infraspinatus. The following factors were correlated with tenderness as measured by shear and panel: sarcoplasmic protein nitrogen/total fibrillar protein nitrogen; soluble fibrillar protein nitrogen/total fibrillar protein nitrogen; water released/total water. Fibrillar protein solubility was highly correlated with tenderness ($r = -0.69$ for shear and $r = 0.59$ for panel). An r value of 0.49, significant at the 5% level, was found between water-holding capacity and tenderness as measured by the shear.

INTRODUCTION

Since Wierbicki *et al.* (1954) investigated the possibility that the dissociation of actin and myosin was involved in the post-mortem tenderization of meat, considerable interest has been developed in the behavior of actin and myosin and their reactions during and after rigor in relation to meat tenderization. Kamstra and Saffle (1959) and Carpenter *et al.* (1961) were able to produce hams with a significant increase in tenderness by interrupting the normal course of rigor by the infusion of chelating agent. Weinburg and Rose (1960), in studies with chicken breast muscle, found that an increase in tenderness post-mortem was paralleled by an increase in extractability of the contractile proteins. Wierbicki *et al.* (1956) stated that the post-mortem dissociation of actomyosin probably played an insignificant role in meat tenderization, but Partmann (1963) stated there was little doubt that the post-mortem tenderization of meat was directly related to the dissociation of actomyosin.

The work reported in this paper was carried out to clarify and further investigate the relationship of intracellular protein composition and solubility behavior to meat tenderness. This research was confined to the study of variations in tenderness between animals that were very similar in most other respects.

EXPERIMENTAL

Experimental animals. The longissimus dorsi muscles of 20 yearling bulls raised on the Michigan State University farms were used for the tenderness study. In addition, the intracellular protein composition of the infraspinatus muscles of four of these bulls was determined for comparison purposes. These bulls were within a three-month age group and sired by bulls selected for differences in tenderness. They were fed and managed identically and yielded carcasses of the Standard and Good grades.

Tenderness determination. Tenderness was determined on the first three 1½-inch short loin steaks, taken from carcasses that had been aged seven days. At this time the fourth 1½-inch steak was removed and vacuum packaged in a Cryovac

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bag and immediately frozen and maintained in this state for approximately one month, after which time they were used in the fractionation procedure. Steaks were cooked in deep fat at 141°C to an internal temperature of 63°C. They were allowed to cool 24 hr, and one-inch cores were submitted to a 12-member taste panel for evaluation of tenderness on a nine-point hedonic scale. The scores were rated from 1 (extremely tough) to 9 (extremely tender). Tenderness of eight ½-inch cores was also measured with the Warner-Bratzler shear.

Protein fractionation. To study the protein composition of muscle, a relatively rapid method for fractionating the muscle proteins and for determining fibrillar protein solubility was developed, as outlined in Figs. 1 and 2. This procedure was adapted from procedures of Seagran (1958) and Turner and Olson (1959). The principal changes made in adapting these procedures were an increase in the volume of extracting solutions relative to sample size, and an increase in sample size and in the length of time involved in each extraction.

All fractionation procedures were carried out in duplicate at 4°C unless otherwise stated. The design outlined in Fig. 1 was utilized for the quantitative determination of sarcoplasmic protein nitrogen, non-protein nitrogen, and total fibrillar protein nitrogen. Data obtained from the design outlined in Figs. 1 and 2 were necessary for the determination of fibrillar protein solubility.

For scheme 1, a 5-g sample was weighed into a 250-ml centrifuge tube, and 70 ml of a phosphate buffer (pH 7.6, ionic strength 0.05) was added to the tube. The entire contents of the tube were transferred to a microblender jar. An attempt was made to attain a maximum degree of subdivision with a minimum of foaming. This was achieved by blending for 1 min at a blender speed of 8000 rpm (adjusted with a Powerstat transformer setting of 40). It was extremely important to control blender speed and time since any variation

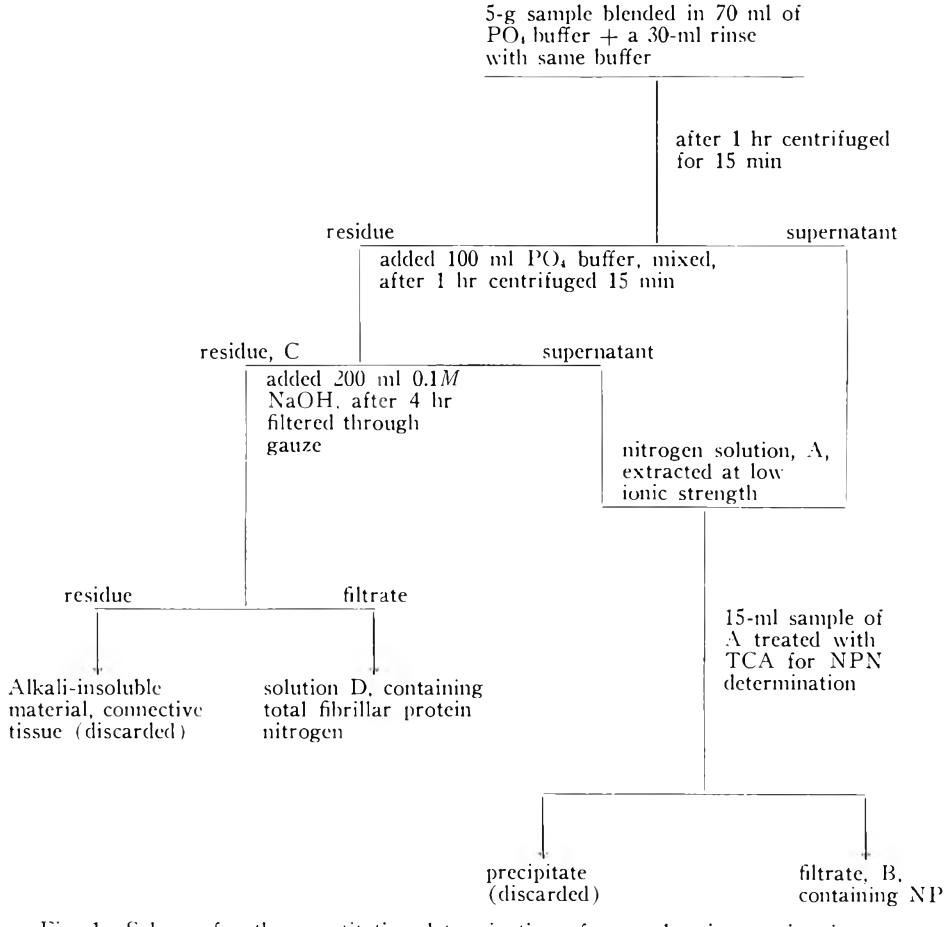

Fig. 1. Scheme for the quantitative determination of sarcoplasmic protein nitrogen, non-protein nitrogen, and total fibrillar protein nitrogen.

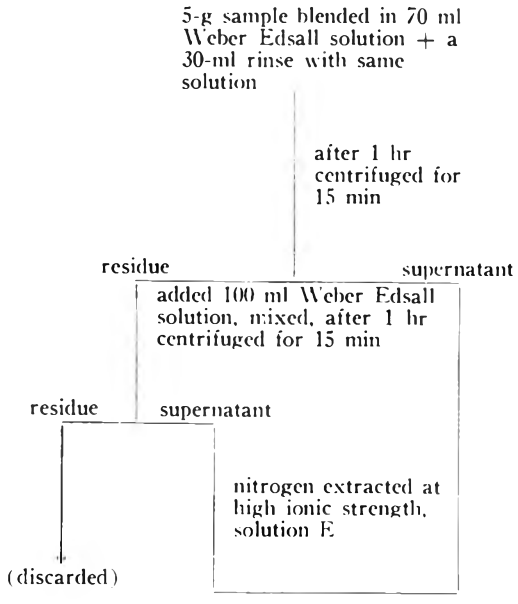

Fig. 2. Scheme for the quantitative determination of fibrillar protein nitrogen solubility (complement to Fig. 1).

in these factors caused differences in extractability. After blending, the material was transferred to its original tube. The blender jar was rinsed with 30 ml of extracting solution, and this solution was added to the centrifuge tube. After 1 hr the material was centrifuged for 15 min, the supernatant was decanted, and its volume was recorded. One hundred ml of extracting solution was again added to each tube. The tube was stoppered and shaken until dispersion of the tissue was complete (about 20 seconds). After 1 hr the material was centrifuged, the supernatant decanted, and the volume recorded as before. The two solutions obtained were combined and filtered through 8 layers of gauze to remove fat and other particulate material not removed by centrifugation. This combined solution was designated as A (solution extracted at low ionic strength). A 15-ml sample was taken for nitrogen analysis, and a 15-ml sample was used for the determination of non-protein nitrogen. The filtrate resulting from the TCA precipitation was designated as B. The residue (C) remaining from the extraction with phosphate buffer, was extracted with 200 ml of 0.1*M* NaOH for 4 hr at room temperature. The volume of the tube contents was measured and then filtered through gauze. A very small amount of residue (alkali-insoluble material, i.e., connective tissue) was retained on the gauze. An aliquot of the filtrate (D) was taken for nitrogen analysis, and the remainder was discarded. The procedure in Fig. 2 was exactly the same as the first two steps outlined in Fig. 1 except that the extracting solution was Weber Edsall solution (NaCl carbonate buffer, pH 9.0, ionic strength 0.67). The solution extracted by this scheme was designated E. Solutions A, B, D, and E were analyzed for nitrogen, and the results were designated as A^n, B^n, etc. Total nitrogen content of the fresh tissue was denoted F^n. These symbols (nitrogen contents) represented the following fractions:

B^n = non-protein nitrogen
A^n = nitrogen extractable at low ionic strength
D^n = total fibrillar protein nitrogen
E^n = nitrogen extractable at high ionic strength
$A^n - B^n$ = sarcoplasmic protein nitrogen
$E^n - A^n$ = soluble fibrillar protein nitrogen
$F^n - (D^n + A^n)$ = connective-tissue protein nitrogen

The above values were averages of duplicate analyses recorded to the second decimal place. Variation between duplicates for A^n, D^n, and E^n was normally from 0 to 0.02 mg/ml, with an extreme range in one or two cases of 0.05 mg. Variation in the second decimal place was seldom observed in the case of B^n.

Nitrogen analysis. All nitrogen analyses were performed by the micro-Kjeldahl method as outlined by the American Instrument Co. (1961), except in the case of total tissue nitrogen, which was determined by the macro-Kjeldahl method outlined in AOAC (1960). All nitrogen contents were reported as mg of protein nitrogen or non-protein nitrogen per ml of solution, or per g of tissue. Nitrogen analyses were made in duplicate.

pH measurements. All pH measurements were made with a Beckman Model G pH meter. The electrodes were placed directly into the ground sample or protein solution, and the observed values were recorded to the nearest one-tenth unit.

Non-protein nitrogen determination. Non-protein nitrogen was determined by mixing 15 ml of protein solution with 5 ml of 10% TCA. After 15 min this material was filtered through Whatman No. 1 filter paper. The filtrate was analyzed for nitrogen. This value was multiplied by 1.33 (necessary because of the TCA dilution) to give non-protein nitrogen per ml of original solution.

Centrifugation. A model PR-2 Refrigerated International Centrifuge was used throughout the experiment. Centrifuging was done at 2500 rpm (1400 × G) with the exception of water-holding-capacity determinations.

Water-holding-capacity determinations. Water-holding capacity was determined according to the centrifugal method of Wierbicki *et al.* (1957). Ground samples of 25 g were heated for 30 min at 70°C and centrifuged for 10 min at 1000 rpm

(250 × G). The percent of total water released was reported as free water. Triplicate determinations were made.

RESULTS AND DISCUSSION

The fractionation procedure developed in this research facilitated a relatively rapid and reasonably accurate determination of nitrogen components in muscle. The procedure could be carried out in duplicate in one day. To obtain complete data, analysis for connective tissue would have been desirable. Connective tissue was determined by difference in this procedure, and such a determination reflects all the errors incurred in all the separate determinations of the other components (Table 1). Ritchey *et al.* (1963) reported values for collagen determined by the hydroxyproline method of approximately 1.3 g collagen nitrogen/100 g total nitrogen for beef longissimus dorsi muscles (1.3%). This differed considerably from the average value for the same muscle from 20 animals in this study (0.5%). Accurate measurements of such small amounts of connective tissue by difference would be almost impossible.

Table 1. Average nitrogen composition of two muscles from yearling bulls.

Muscle	Nitrogen-containing fraction (% of total N)			
	Sarcoplasmic	Fibrillar	NPN	Stroma
Longissimus dorsi (20 animals)	31	62	6.5	0.5
Infraspinatus (4 animals)	18	68.5	9.0	4.5

The four bulls from which the infraspinatus muscle was fractionated were from the group of 20 bulls from which the longissimus dorsi muscle was fractionated. There was little similarity between the nitrogen composition of these two muscles from the same animals. The longissimus dorsi contained almost twice as much sarcoplasmic protein, and almost 1 mg more total nitrogen/g of tissue, than the infraspinatus. Respective total nitrogen contents for the longissimus dorsi and infraspinatus were 34.3 and 33.35 mg/g. It was possible that the value for sarcoplasmic proteins was somewhat higher for the longissimus dorsi than the actual sarcoplasmic protein content, since these muscles were frozen for a short time before being fractionated. Mechanical damage to the intimate muscle structure by freezing could have allowed a greater dissolution of actin and myosin in the 0.05 ionic strength buffer, ultimately resulting in a slight overestimation of the sarcoplasmic proteins. Considerably less of the fibrillar protein fraction and the non-protein nitrogen compounds occurred in the longissimus dorsi than in the infraspinatus.

Trautmann and Fiebiger (1952) stated that the ratio of sarcoplasm to fibrils was proportionate to the amount or kind of work that a muscle performs. This would indicate that the ratio of sarcoplasmic protein to fibrillar protein has a similar relationship. The corresponding ratios obtained in this research were 18/68.5 for the infraspinatus and 31/62.0 for the longissimus dorsi. If such a relationship existed between this ratio and muscle activity, be it direct or inverse, there was also the possibility that this ratio could be related to tenderness. Consequently, this aspect was also investigated.

Table 2 shows the tenderness measurements, water-holding capacity, and amounts of the more important nitrogen-containing fractions of the longissimus dorsi of the 20 bulls involved in this study. The only large variations between animals were the values for tenderness and fibrillar protein solubility. Total nitrogen, sarcoplasmic protein, fibrillar protein, water-holding capacity, and non-protein nitrogen were remarkably constant from animal to animal, as shown by small standard deviations. The mean, standard deviation, and standard error for non-protein nitrogen, were respectively 2.22, 0.10, and 0.02.

Simple correlation coefficients were calculated between tenderness (measured by shear and panel) and sarcoplasmic protein nitrogen/total fibrillar protein nitrogen, soluble fibrillar protein nitrogen/total fibrillar protein nitrogen, and free water (Table 3). Tenderness, as measured by both shear and panel, was highly correlated with fibrillar protein solubility ($r = -0.69$ and 0.59, respectively). Correlations of the other factors

Table 2. Tenderness, water-holding capacity and nitrogen composition of the longissimus dorsi of 20 bulls.

Bull no.	Tenderness		Water-holding capacity (% released)	Nitrogen composition (mg N/g)			
	Shear	Panel		Sarcoplasmic	Fibrillar	Soluble fibrillar	Total nitrogen
247	10.60	5.4	47.6	10.1	22.2	8.2	34.5
23	12.30	4.1	46.5	10.4	21.5	10.6	34.3
3	9.05	7.3	45.3	10.2	20.4	13.4	34.1
37	9.27	7.4	45.4	10.1	20.9	15.0	33.5
32	12.50	4.7	46.3	10.7	21.3	8.2	35.3
15	9.68	6.0	46.6	11.3	20.7	14.6	34.8
25	9.82	6.8	43.9	11.1	20.7	8.0	34.4
50	7.33	6.9	42.1	11.9	21.3	17.4	35.3
20	10.64	5.4	43.6	10.2	20.6	11.2	35.4
700	7.41	7.7	46.3	10.8	19.6	11.6	34.5
361	8.29	7.6	44.3	10.1	20.9	12.4	32.3
22	8.37	6.3	44.1	10.1	20.5	14.2	34.1
21	7.78	6.8	43.3	10.7	21.5	15.0	34.1
672	9.40	5.8	45.6	10.7	23.3	9.6	33.3
47	7.30	7.3	45.0	11.5	21.3	18.2	34.8
6	9.58	5.8	46.2	10.2	21.4	12.2	33.9
720	9.38	6.5	46.7	11.2	22.2	10.2	35.2
29	8.83	6.5	44.7	11.8	21.0	14.0	35.0
42	10.91	3.5	47.1	10.4	21.6	8.2	33.4
12	8.57	7.0	47.1	10.4	21.4	11.4	33.8
Mean	9.35	6.24	45.4	10.7	21.2	12.2	34.3
Std. dev.	1.51	1.16	1.48	0.56	0.80	3.04	0.80
Std. error	0.34	0.26	0.33	0.12	0.18	0.68	0.18

Table 3. Correlation coefficients for various factors related to tenderness (tenderness measured by shear and panel).

Factor	Tenderness	
	Shear	Panel
Sarcoplasmic N/ total fibrillar N	−0.43	0.41
Soluble fibrillar N/ total fibrillar N	−0.69**	0.59**
Water released (free water/ total water)	0.49*	−0.40
Tenderness as measured by panel	−0.83**	.....

* $p < 0.05 = 0.44$.
** $p < 0.01 = 0.56$.

with tenderness were all very close to the $p < .05$ level of significance; however, only one other r value was significant at this level. This was the correlation between free water and tenderness as measured by the shear ($r = 0.49$). Hamm (1960) reported that a relationship existed between water-holding capacity and tenderness, but that the relation appeared only if the differences in water-holding capacity of meat were relatively great. In other words, a high correlation between water-holding capacity and tenderness will usually be found only in extreme cases. The highly significant correlation coefficient between tenderness as measured by panel and shear is in agreement with previous work reported in this area.

The only significant correlation in Table 4 is that between free water and percent soluble fibrillar protein. Such a correlation would be expected, since both free water

Table 4. Correlation coefficients of some factors determined from protein fractionation in relation to free water.

Factor	Free water
Total nitrogen	−0.12
Percent soluble fibrillar protein	−0.53*
Total fibrillar protein	0.27
Sarcoplasmic protein	−0.32

* $p < 0.05$.

and soluble fibrillar protein are correlated with tenderness.

It has been made evident from the results of this study that the variation in tenderness between similar animals is closely related to the tendency of the fibrillar protein fraction to be dissolved or extracted by salt solution. The difference may or may not be attributed to the same mechanism as the increase in solubility or extractability of the muscle proteins during post-mortem aging. The possibility remains that the differences in extractability may be associated with indirect mechanisms, i.e., dependent upon another factor or factors, such as a variation in fragility of the sarcolemma, which would allow more of the contractile proteins to be extracted.

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Changes in Total Carbonyl Content of Orange Juice and Concentrate During Storage

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SUMMARY

A method is described for the recovery of volatile organic components of citrus juices by a continuous, reverse-phase, liquid-liquid extraction of the juice and distillation of the extract. The distillate may be used directly for gas chromatography. Six aldehydes have been identified. Recovery of added aldehyde exceeded 90%. Assay was by a colorimetric 2,4-dinitrophenylhydrazone method. Total carbonyl content of concentrated orange juice rose sharply shortly after processing, followed by a slower decline. The changes were qualitatively similar whether a pure aldehyde or cold-pressed orange oil was added prior to frozen storage. Carbonyls in chilled orange juice followed a roughly similar pattern, with a final rise as microbial spoilage became apparent.

INTRODUCTION

The study reported was made to develop a method for the extraction in nearly quantitative yield of the volatile carbonyl components of orange juice and to obtain preliminary data on changes in the total carbonyl content of frozen orange concentrate and of chilled orange juice. The importance of the volatile oils, and particularly of their carbonyl components, in both the aroma and flavor of foods, is widely recognized, but methods for their isolation, characterization, and estimation are poorly developed for fruit juices in general and citrus juices in particular.

In an earlier study of volatile components of orange juice, Kirchner and Miller (1957) distilled 3000 gal. of fresh juice and further concentrated the distillate by solvent extractions and fractional distillations to characterize a large number of carbonyls and other classes of compounds. They found acetaldehyde to be the principal carbonyl, with only fractional parts per million of higher aldehydes.

Gaddis *et al.* (1959) described chromatographic methods for the investigation of steam-volatile carbonyls from rancid fats as their 2,4-dinitrophenylhydrazones. Steam distillation of orange juice was found in this laboratory to be impractical because of long-continued evolution of carbonyl compounds and a consequent necessity of handling large volumes of distillate containing only a few parts per million of the carbonyls.

Pippen *et al.* (1958) described a distillation technique for the separation of volatile carbonyl compounds of cooking chicken. The carbonyls were isolated as their 2,4-dinitrophenylhydrazones, and estimation of their concentrations was obtained by weighing the chromatographically separated fractions. Their method proved slow and cumbersome and detected only those carbonyl compounds forming insoluble chromatographically separable hydrazones. It was also found in this laboratory that yields of carbonyls from orange concentrate depended upon distillation temperature. When distillation was carried out at atmospheric pressure the yield of hydrazones was approximately five times that obtained under mechanical-pump vacuum. Furthermore, the hydrostatic head of the reagent traps resulted in a boiling temperature high

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enough to make it possible that volatile carbonyl artifacts might he formed during the necessarily prolonged distillation.

Investigations of the composition of cold-pressed orange peel oil have been stimulated in recent years by the development of gas-liquid chromatography. Bernhard (1961) reported an orange peel oil to contain over 50 components, of which 10 were carbonyls. Wolford *et al.* (1962) detected a similar number of components in a commercially prepared orange essence. The development of simple column chromatographic methods of separating citrus oils into terpene and oxygenated fractions (Bernhard, 1960; Kirchner and Miller, 1952) simplified examination of the carbonyl components by concentrating them manyfold in the oxygenated fraction.

For investigation of changes in the volatile organic components of orange concentrates or juices during processing and storage studies it was desirable that a method be developed for separation of the volatile from the nonvolatile components. It was essential that the method he reasonably rapid and require only a small sample. It was desirable that mild conditions be used to minimize changes during analysis and that the isolated components be amenable to examination by gas chromatography.

METHODS

Solvent extraction of orange juice. A modified continuous, reverse-phase, countercurrent, liquid-liquid extractor (Fig. 1) similar to that described by Kolfenbach *et al.* (1944) proved suitable for extraction of citrus juices. The small amount of oil-in-water emulsion that tended to form broke readily on standing. A 250-ml bulb at the base of the column was adequate to prevent emulsion build-up. The 25-mm-ID extraction column was 1 m long from the 0.40-mm jet orifice to the inlet for the extractant.

A sample of 500–600 g of thawed frozen concentrate (one No. 2 can or three 6-oz cans) was convenient. It was diluted with 3 volumes of 50% ethanol. Two-liter samples of single-strength juice were diluted with an equal volume of 95% ethanol. The mechanically mixed juice was vacuum filtered on 100-mesh stainless-steel screen held in a Kreuger filter to remove coarse pulp particles that could clog the jet.

The juice was forced from the 4-L separatory funnel used as a reservoir through the jet by air

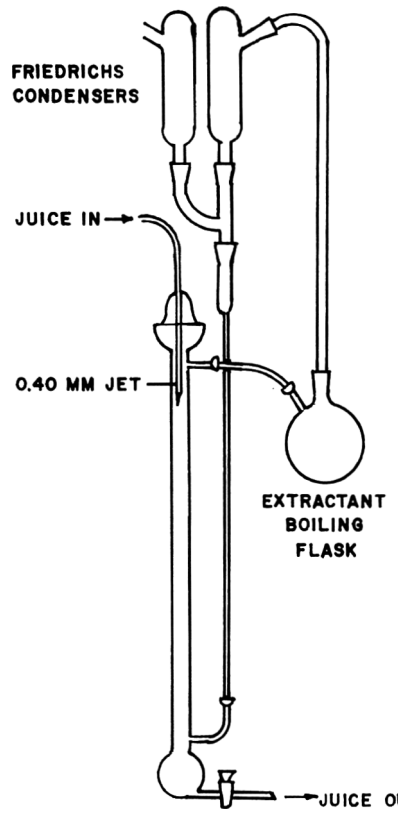

Fig. 1. Continuous, countercurrent, liquid-liquid extractor.

pressure at 3–5 psi at a rate of about 3 L per hr. The extracted juice was collected and returned to the reservoir for a second extraction with the same batch of solvent.

Pentane was used as the extractant. The boiling flask was heated with steam, and the condensers were cooled with water at approximately 7°C. The extractant flow rate, of about 60 ml per minute, was limited by the capacity of the condensers.

For the recovery experiments, solutions of nonanal in ethanol were added, with mechanical stirring, before filtration.

Preparation of extracts for analysis. Water-soluble materials, such as small amounts of sugars that had been mechanically carried over, were removed by washing the extracts twice with water in a separatory funnel and drying over anhydrous sodium sulfate.

The sodium sulfate was filtered off and washed once with fresh pentane, and the filtrates were concentrated to about 2 ml by distillation at atmospheric pressure through a 15 × 100-mm fractionating column packed with number 3013 Helipak packing. The condenser was replaced

with a trap cooled in liquid nitrogen. The pressure was reduced to approximately 15 mm. The still pot was totally immersed in a boiling-water bath, and the fractionating column and connecting tube were heated with electrical heating tape to drive all volatile materials over into the cold trap. Carotenoids, lipids, and other nonvolatile materials remained in the still pot and were discarded. After 15 min the vacuum was released with nitrogen through a sidearm on the still pot.

Determination of total carbonyls. The distillate was quantitatively recovered from the cold trap and diluted to an appropriate volume, usually 25 ml, with carbonyl-free methanol. Total carbonyls were determined as nonanal by the colorimetric 2,4-dinitrophenylhydrazone method of Lappin and Clark (1951) as modified by Mattick and Robinson (1960). It was found necessary to add 1 ml of water to each tube before heating to prevent occasional precipitation of KCl during subsequent manipulations. Evelyn colorimeter readings with a 440 filter were taken at intervals and extrapolated to zero time, and total carbonyls were determined from a standard curve. Nonanal was selected because it is a minor component of orange oil of intermediate retention time whose peak is well separated from the peaks of the major hydrocarbons under the conditions established. The peak absorption for nonanal 2,4-dinitrophenylhydrazone against a reagent blank was found to be 430 mμ with a Cary Model 14 spectrophotometer.

Identification of carbonyls. Several batches of commercial frozen orange concentrate were extracted in the usual manner and the distillates pooled. The oxygenated components were separated from the predominating hydrocarbons on a 10 × 150-mm column, using florisil as adsorbent, eluting the hydrocarbons with hexane and the oxygenated components with carbonyl-free methanol. The methanol eluate was diluted with 1 volume of distilled water and extracted repeatedly with *n*-pentane in a separatory funnel. The several epiphases were dried over anhydrous sodium sulfate, filtered, and evaporated to a small volume in a stream of nitrogen at room temperature.

Gas chromatographic analysis was carried out on a ⅜-in. × 10 ft column of coiled copper tubing packed with 30% Carbowax 1540 on Chromosorb. Helium flow rate was 120 ml per min at 150°C. A hot-wire thermal-conductivity detector was used. Effluent fractions containing carbonyls were located by attaching a glass capillary to the exhaust port of the instrument and bubbling the gas through a few drops of 2,4-dinitrophenylhydrazine reagent as suggested by Walsh and Merritt (1961). These peaks were collected on subsequent runs, along with two of the major hydrocarbon peaks, and identified by retention time and comparison of infrared spectra with authentic compounds. Each sample was checked for purity on a 0.02-in. × 250-ft capillary column coated with Carbowax 20M at 80° with a helium flow rate of 30 ml per min. A flame ionization detector was used.

Preparation and storage of concentrates. Only two representative examples of 10 studies are discussed in this report. In the first, a sample of 55° Brix concentrated orange juice without cutback juice or added peel oil was obtained from a processing plant. Cold-pressed orange oil was added at a level of 0.05% on the concentrate basis, mechanically mixed without appreciable aeration, sealed in No. 2 plain tin cans, and stored at −20°C. One-can samples were withdrawn at intervals, thawed 1 hr in running tap water, and extracted. One sample was run immediately after packing. Total carbonyls were determined.

A similar sample was obtained and treated in the same fashion, except that 0.4 mg per 100 g (0.0004%) nonanal (Eastman, practical grade, without purification) was added instead of cold-pressed orange oil.

Preparation and storage of chilled juice. Ten gal. of Valencia orange juice was obtained from a commercial plant prior to pasteurization, and was stored in bulk in a covered stainless-steel container at 4.5°C. At intervals, 2-L samples were withdrawn after thorough mixing, diluted with 2000 ml 95% ethanol, and extracted and assayed as were the concentrate samples. The microbial plate count at the conclusion of storage was made on orange serum agar after dilution with distilled water. Flavor observations were by the author.

RESULTS

Recovery. Recovery data for several levels of nonanal added to a number of different commercial frozen orange concentrates are given in Table 1. A blank without added nonanal was run with each determination, and the yield of carbonyls is ex-

Table 1. Recovery of *n*-nonanal added to frozen orange concentrate.

n-nonanal (ppm)		Recovery (%)
added	found	
0.45	0.44	98
.84	.82	98
.85	.80	94
.85	.74	87
1.74	1.69	97
1.71	1.62	95
1.77	1.76	100
6.45	6.22	97

pressed as nonanal. The blank value was subtracted from the experimental value to obtain the data for nonanal found. These blank values ranged from 1.66 to 3.15 ppm. In each case the blank and the experimental values were obtained with the same lot of concentrate and at the same time in a duplicate apparatus.

Gas chromatography. Gas chromatography of the distillate from commercial concentrates without the dilution necessary for quantitative carbonyl determination gave traces strikingly similar qualitatively to those obtained with whole cold-pressed Valencia orange oil. Fig. 2 presents the recorder traces obtained on the capillary column for whole oil and a representative extract. These traces were obtained with an Aerograph Hy-Fi Model 600, manually programmed from 50° to 225°C over about 45 min. The chart speed was 15 in. per hr on a 1-mv recorder.

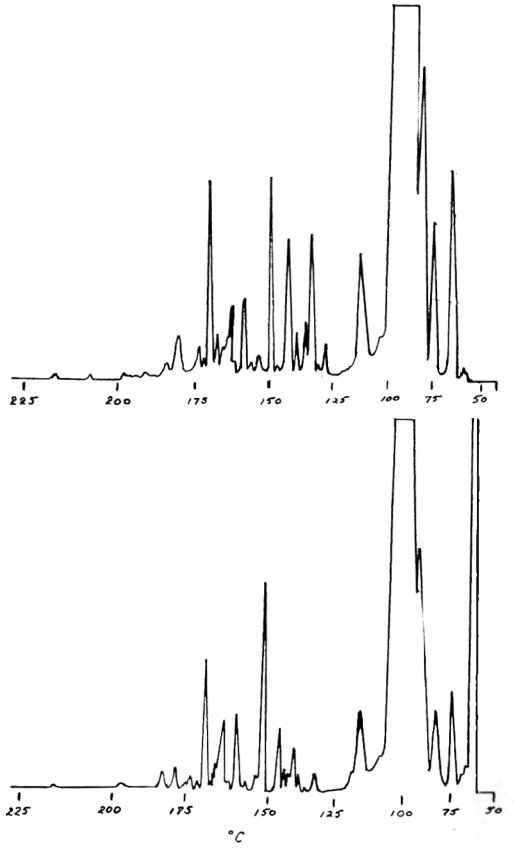

Fig. 2. Gas chromatographic traces of cold-pressed orange oil (upper) and of an extract of orange concentrate (lower). Column 250 feet by 0.02 inch, coated with Carbowax 20M. Hydrogen flame detector. Helium flowrate: 30 ml per minute. Sample size: 0.2 μl. Attenuation 8×. Recorder: 1-mv full scale. Manually programmed from 50° to 225°C in about 45 min.

The carbonyl peaks and two of the hydrocarbon peaks separated on the larger column were identified as heptanal, octanal, nonanal, decanal, citronellal, undecanal, limonene, and myrcene. Decanal and citronellal were only partially resolved on the preparative column, but were completely separated on the capillary column. Except for this C_{10} aldehyde peak, only minor impurities accompanying the major component were detected with the capillary column.

Changes in total carbonyls. A marked and rapid rise in total carbonyls was observed in all three storage studies reported (Figs. 3, 4, 5), followed, in the concentrates, by a relatively slower drop. Fig. 3 gives the results for concentrate with added orange oil. The carbonyls are on the basis of weight of concentrate. Plotting the logarithm of the carbonyl content against time (Bull, 1951) gave the curve of Fig. 6, which suggests a monomolecular, probably pseudo first-order, reaction

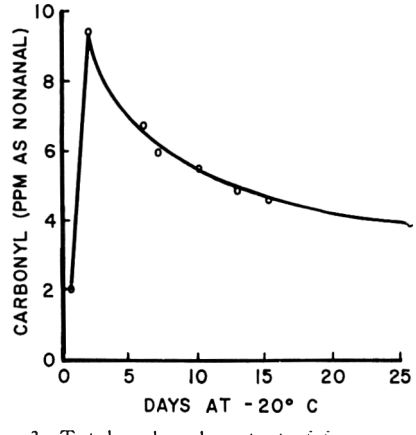

Fig. 3. Total carbonyl content of frozen concentrated orange juice with added cold-pressed orange oil.

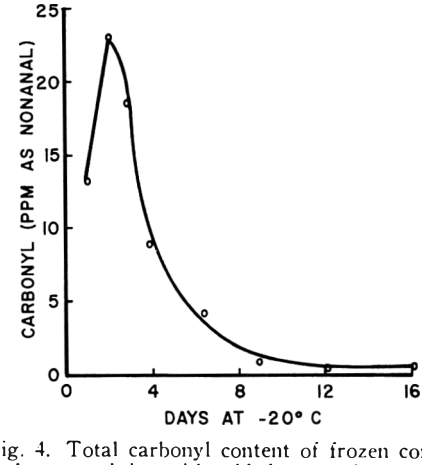

Fig. 4. Total carbonyl content of frozen concentrated orange juice with added nonanal.

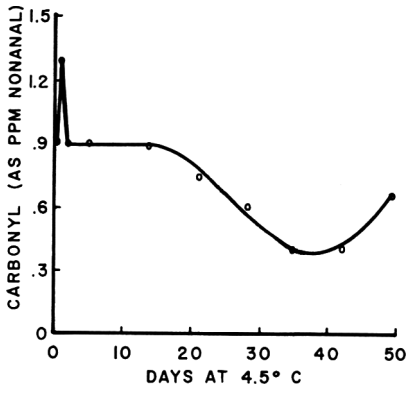

Fig. 5. Total carbonyl content of chilled orange juice.

with a specific reaction velocity of about 0.045 $days^{-1}$, or a half life of about 15 days.

Fig. 4 presents the total carbonyl data on a concentrate weight basis for the samples with added nonanal. Total carbonyl decreased more rapidly in this pack than when orange oil was added. The disappearance of carbonyl did not closely follow first-order kinetics, but gave a reaction velocity curve of gradually decreasing slope.

The chilled juice pack (Fig. 5) showed a more complex carbonyl history. Following the initial rapid rise, carbonyl content returned to the original value and remained there for several days, after which a slow decline was observed. The flavor was characterized as "good" when received, "fair" at one day, and "flat" until a slightly spoiled flavor was detected, at 6 weeks, and a distinctly spoiled flavor, at 7 weeks. The final rise in carbonyl content is correlated with the development of the spoiled flavor. At 7 weeks the microbial plate count was 7 million organisms per ml.

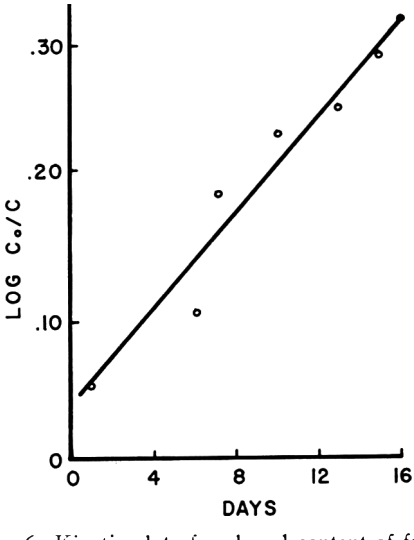

Fig. 6. Kinetic plot of carbonyl content of frozen concentrated orange juice with added cold-pressed orange oil.

DISCUSSION

Previously published methods for the separation of volatile carbonyls from very dilute aqueous systems (Kirchner and Miller, 1957; Pippen *et al.*, 1958) required large samples, were slow and cumbersome, and subjected the sample to conditions that might cause changes in relatively labile carbonyls. The present method requires only a relatively small sample which is subjected to elevated temperatures only briefly. It is rapid enough to yield a total carbonyl analysis and a gas chromatographic trace of the total volatile organic constituents from a frozen sample within a single working day. The utility of the method was demonstrated by the first-reported identification from orange juice on a laboratory scale of several aldehydes known to be constituents of cold pressed orange oil (Stanley *et al.*, 1961; Bernhard, 1961) and of commercial orange essence (Wolford *et al.*, 1962).

In the exploratory phases of the work it was found that the continuous recycling system of Kolfenbach *et al.* (1944) could not be used with orange juice since the systems stabilizing the dispersion of fine pulp were disrupted by the first passage through the extractor. During subsequent residence time in the juice reservoir the fine pulp tended to agglomerate as very soft curds, and the clear serum tended to channel between them. This resulted in unequal extraction of the soluble and insoluble phases. For this reason a batch-type extraction was used.

Extraction of untreated orange juice gave recoveries of less than 50% of the added nonanal on extraction with ether or pentane. Recoveries were increased to over 90% by saturation of the reconstituted concentrate with sodium chloride or sodium sulfate or by partial saturation with ammonium sulfate. Ether extraction resulted in recovery of larger amounts of nonvolatile material than did pentane extraction. The smallest amount of nonvolatile material, coupled with nearly quantitative recovery of added non-

anal, was obtained by extraction of a dilute ethanol solution of the juice with pentane, and this latter method was adopted. The ethanol is believed to increase recovery by increasing the solubility of the carbonyls in the aqueous phase. Only trace amounts of carbonyl were found in an ethanol-water blank. The bulk of the extractable carbonyls was obtained on the first pass. Usually, small additional amounts were obtained on a second pass, with only trace amounts, if any, detected after additional passes through the extractor.

Changes in total carbonyl content during storage of frozen orange concentrate and of chilled orange juice were followed. The initial rapid rise and decline in total carbonyls was observed in all experiments, but has not been explained. This sharp change occurs at the same time as the initial decrease in flavor of chilled orange juice, but it is not necessarily true that the carbonyls are of primary importance in this flavor change. The mechanism of this initial rise and the nature of the precursors have not been clarified, nor has the mechanism or nature of the products formed during the succeeding decrease in carbonyls.

The relatively rapid changes in carbonyls that would be expected at 4.5°C compared with those found at −20°C were not observed. The explanation may be similar to that advanced by Lamden *et al.* (1960) to explain the greater retention of ascorbic acid in fermenting orange juice over that in orange juice preserved with iodoacetate. Consumption of dissolved oxygen by respiring yeasts may have limited oxidation of aldehydes. The true explanation will require quantitative data on the individual carbonyl compounds and of their precursors and products throughout a storage history.

The similarity of the gas chromatographic trace to that of cold-pressed orange oil suggests that the extraction method, coupled with suitable analytical techniques, would be applicable to the study of changes in classes of compounds other than carbonyls during processing and storage of citrus juice products.

ACKNOWLEDGMENT

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Co-oxidation of the Sulfur-Containing Amino Acids in an Autoxidizing Lipid System

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SUMMARY

Oxidation of the sulfur amino acids by autoxidizing lipids was studied in a model system consisting of an amino acid dispersed in cold-pressed, molecularly distilled menhaden oil (20–80% w/w). Under all conditions investigated, cysteine was oxidized completely to cystine. Preliminary results suggest that at 110°C the oxidation follows first-order kinetics for at least the first 8 hr. A specific reaction rate constant of 0.25 per hour was calculated. When fatty acids were added to the system, cystine was oxidized to its thiosulfinate ester. When the fatty acid-cystine ratio was 1:2, oxidation of cystine was a maximum. No oxidation of cystine occurred unless either a fatty acid, volatile organic acid, or ethanol was added. Under the conditions investigated, methionine was not oxidized to either its sulfoxide or its sulfone.

INTRODUCTION

When oxidative rancidity develops in foods, it is accompanied by the destruction of vitamins A and E, thiamin, and biotin (Cummings and Mattill, 1930; Pavcek and Small, 1942; Holman, 1949) and a decrease in protein quality (Carpenter *et al.*, 1962; Tappel, 1955). Two interactions have been noted: direct oxidation by peroxides and free radicals, or reactions between carbonyls produced by the autoxidizing oil and free amino groups in proteins. Friend (1958) showed that β-carotene is co-oxidized in an autoxidizing lipid system, the products being epoxides. However, most of the investigations on the interactions of proteins and amino acids with autoxidizing lipids have been in terms of the Maillard reaction (Carpenter *et al.*, 1962; Tappel, 1955). The sulfur-containing amino acids are important constituents of proteins and are known to be easily oxidized. Classically, it has been shown (Greenstein and Winitz, 1961) that the following oxidations are possible: Mercaptans such as cysteine are oxidized easily to disulfides and, with more difficulty, to thiolsulfinates and then to thiolsulfonates. Thiolsulfinates and thiolsulfonates can be hydrolyzed to sulfinic and sulfonic acids. Organic sulfides, such as methionine, can be oxidized to sulfoxides and sulfones.

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METHODS AND MATERIALS

A finely powdered amino acid was dispersed (20–80% w/w) in cold-pressed molecularly distilled menhaden oil of iodine number 207 and acid value of 0.33 mg KOH per gram of oil. In some of the experiments an organic acid was added to the system. In the controls, tristearin was substituted for the menhaden oil. The mixture was allowed to autoxidize at 75 or 110°C for 24 or 48 hr, by which time the oil had become polymerized. Temperatures both above and below 100°C were chosen for these experiments, since hydroperoxides decompose more rapidly above 100°C. The suspension was stirred continuously to keep the solid particles suspended.

After the oil had been allowed to autoxidize for the desired period, either warm methyl acetate or chloroform was added to dissolve as much of the polymerized oil as possible. The solvent-oil-sample mixture was then centrifuged to separate the solid particles of sample. The solid material was washed several times with warm methyl acetate or chloroform until the solvent remained clear, and then the residue was washed with several volumes of 95% ethanol. The final product was placed in a vacuum at 40°C to remove the last traces of solvent. In most cases the solvent extraction procedure did not yield a sample pure enough for analysis. For further purification, the residue was dissolved by wetting with a few

drops of 95% ethanol and then for each gram of amino acid in the original reaction mixture either 100 ml of 0.1*N* HCl or distilled water was added. It was necessary to decolorize the solution with Norit A to remove traces of residual oil and other unidentified colored compounds. These colored products were not studied, and are probably similar to those reported by Okada (Okada *et al.*, 1961).

The controls were treated in essentially the same manner except that benzene was substituted for chloroform and methyl acetate in the extraction procedure, since it is a better solvent for tristearin. Cysteine and cystine were subjected to the complete solvent extraction and alcohol purification procedures. Under these conditions there was no detectable oxidation of cysteine or hydrolysis of cystine.

The samples were chromatographed by thin-layer chromatography. The supporting medium was silicic acid and the solvent system was phenol saturated with water. Known compounds were used to obtain R_f values to aid in identification of the oxidation products.

Infrared spectra were obtained with a Perkin-Elmer model 21-C instrument with a NaCl prism. A Nujol mull of the sample was prepared and placed in a demountable KBr cell employing a path length of 0.25 mm. A Nujol blank was placed in the reference beam of the instrument.

Thiol groups were determined with the amperometric titration method of Siggia (1954), except that the solvent used was distilled water instead of ethanol. The diffusion current was followed with a model 354 Simpson microammeter, and the equivalence point determined graphically.

Thiolsulfinates were determined by the method of Barnard and Cole (1959). Thiolsulfonates interfere in this determination and were determined separately, using a method suggested by Lavine (1936).

The observation of Toennies and Kolb (1939) that methionine sulfoxide is not precipitated with $HgCl_2$ or $Cu(Ac)_2$ served as a basis for the method developed to determine the quantity of oxidized methionine formed. The crystallized sample was dissolved in distilled water, and an excess of saturated aqueous $HgCl_2$ was added. The resulting precipitate of unoxidized methionine was separated by centrifugation, and the sulfoxide or sulfone remaining in the solution was determined by the ninhydrin method of Harding (Harding and MacLean, 1916).

RESULTS AND DISCUSSION

Cysteine. Oxidation products of cysteine recovered from the autoxidized samples were insoluble in distilled water but were soluble in 0.1*N* HCl. The infrared spectrum of the oxidation product was identical to that of L-cystine; thiol groups and thiolsulfinate groups were absent. These data indicate that cysteine was completely oxidized to cystine. In the controls, about one-half of the cysteine was oxidized to cystine. This latter oxidation could be accounted for as air oxidation. When palmitic acid was added to the tristearin control samples, 95% of the cysteine was oxidized to cystine. There were no differences between samples allowed to autoxidize at 75 and at 110°C.

Preliminary results indicate that the rate of formation of cystine from cysteine at 110°C is approximately first order for at least the first 8 hr of the autoxidation (Fig. 1). The specific rate constant was found to be 0.25 per hour at 110°C, and thus the half life of cysteine in the autoxidizing oil was about 2.5 hr.

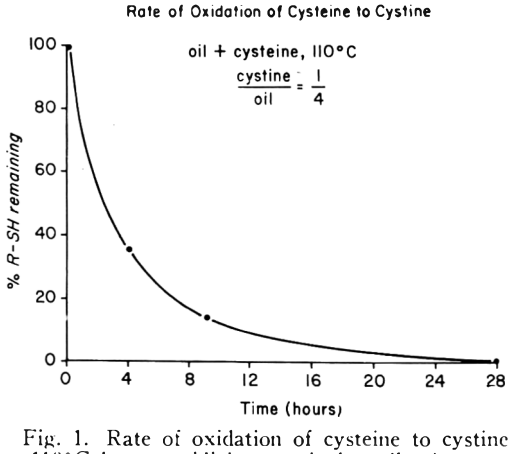

Fig. 1. Rate of oxidation of cysteine to cystine at 110°C by autoxidizing menhaden oil. Average of duplicates.

Cystine. When cystine was dispersed in the menhaden oil system and allowed to autoxidize at 110°C, the infrared spectrum of the recovered product was indistinguishable from that of a cystine standard. In addition, no thiolsulfinate groups or thiol groups could be found. In contrast, when acetic or palmitic acid was added to the system the isolated product exhibited several new infrared bands. One was at 1040 cm^{-1}, the thiolsulfonate region. Consequently the samples were analyzed for thiol,

thiolsulfinate, and thiolsulfonate groups. Only thiolsulfinate groups were found. No thiolsulfinate groups were detected when no fatty acid was added. These data indicate that cystine was oxidized to cystine thiolsulfinate when free fatty acids were present. Samples with a free fatty acid-cystine ratio of 0, 1:4, 1:2, 1:1 and 2:1 were allowed to autoxidize 24 hr at 110°C (Fig. 2). The amount of cystine thiolsulfinate increased to a maximum of 12% when the palmitic acid-cystine ratio was 1:2, and then decreased to only about 6% as the acid ratio was increased to 1:1. If only solvent extraction was used as a purification procedure, about twice the above yields were found, which indicates that cystine thiolsulfinate is unstable in aqueous solution.

A possible explanation for these oxidative changes lies in the reaction mechanism postulated by Bateman and Hargrave (1954) and summarized in Fig. 3 for the oxidation of sulfides by hydroperoxides in aqueous systems.

The acid serves as a hydrogen bonding agent, facilitating the nucleophilic reaction of sulfur with the hydroperoxide.

Another possible explanation, although not a very probable one, would be the formation of peroxy acids from the reaction between added free fatty acids and the hydroperoxides in the oxidizing oils. The reaction mechanism postulated by Bateman and Hargrave (1954) is given in Fig. 3. This mechanism differs from the previous one in that hydrogen bonding is internal and that there is no acid catalysis.

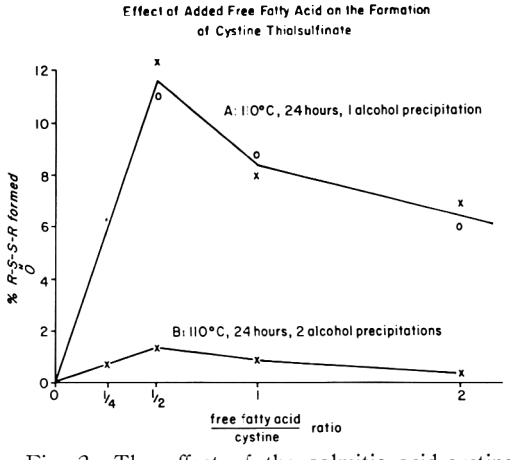

Fig. 2. The effect of the palmitic acid-cystine ratio on the formation of cystine thiolsulfinate.

PROPOSED MECHANISMS FOR SULFUR OXIDATION

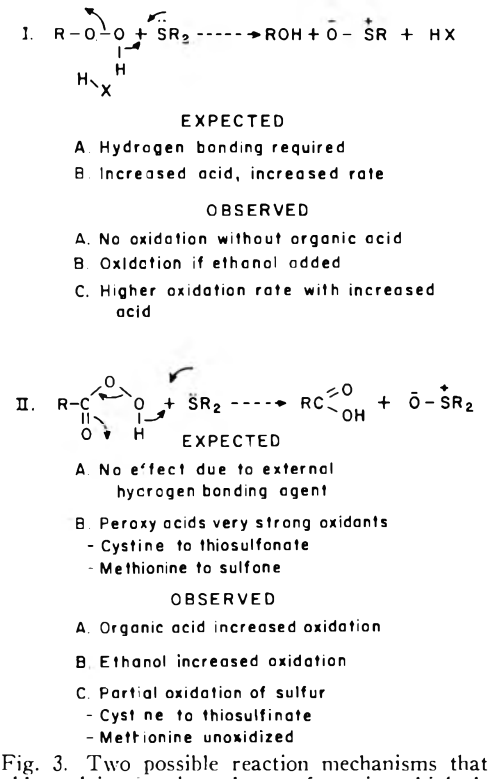

Fig. 3. Two possible reaction mechanisms that could explain the dependence of cystine thiolsulfinate formation on added fatty acid or other hydrogen-bonding agent.

The results of these experiments favor the previous mechanism in that: 1) no thiolsulfinate was formed unless an organic acid or ethanol was present; 2) increasing the free fatty acid-cystine ratio increases the thiolsulfinate yield; 3) peroxy acids are unstable at elevated temperatures; 4) peroxy acids are such strong oxidants that more highly oxidized forms of sulfur would be expected than were found; 5) the formation of peroxy acids requires fairly strong acids and concentrated hydrogen peroxide and so it not likely to occur in this system.

Methionine. Under the conditions investigated, methionine was not oxidized to its sulfoxide or sulfone even when palmitic acid was added to the mixture in ratios of 1:2 or 2:1.

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Volatiles from Oranges. I. Hydrocarbons. Identified by Infrared, Nuclear Magnetic Resonance, and Mass Spectra

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(Manuscript received April 11, 1963)

SUMMARY

Investigation of the lower-boiling fraction of orange volatiles showed the following hydrocarbons: limonene, myrcene, alpha-pinene, alpha-thujene, camphene, alpha- and gamma-terpinene, alpha-phellandrene, *p*-cymene, and *p*-isopropenyltoluene. Four sesquiterpenes were isolated from the higher-boiling fraction. The major sesquiterpene found has not been related to any known compound. Of those present in less amounts, one has been identified as farnesene, and another as ylangene.

The volatile flavoring constituents of orange have been the object of study for many years (Attaway and Wolford, 1962; Bernhard, 1961; Calvarano, 1959; Guenther, 1949; Kefford, 1959; Kirchner and Miller, 1957; Stanley, 1958; Wolford *et al.*, 1962). A systematic investigation of orange volatiles using advances now available in analytical methods and physical measurements has been initiated in our laboratory.

Since it is considered that most of the characteristic flavor and aroma of oranges are associated with compounds that are oil-soluble (Kirchner and Miller, 1957), the preliminary study is concerned with the composition of the cold-pressed and condensate oils from commercial sources. Fractions separated from these relatively stable oils are being used for composition studies and are being added to bland, reconstituted juice to determine desirability as to organoleptic qualities.

This paper reports on investigation of the lower- and higher-boiling hydrocarbon fractions found in the condensate and cold-pressed oils.

EXPERIMENTAL

The condensate oil, which was centrifuged from the condensate from the concentration of orange juice, was kindly given to us by R. W. Kilburn, Florida Citrus Canners Cooperative, Lake Wales, Florida. The cold-pressed oil was obtained from Sunkist Growers, Ontario, California. These oils are blends from the varieties processed during the whole season and obtained under usual factory conditions.

The fractionation scheme of both types of orange oil is summarized in Fig. 1. The oils were separated into three fractions by a simple vacuum distillation at vapor temperatures never exceeding 40°C and at pressures less than 1 mm Hg. The three fractions were designated low, intermediate (principally limonene), and high boiling. Recombination of these fractions gave a product that was not distinguished from the original oil by taste-panel analysis. The residue in the still pot (high-boiling fraction) was about 1% of the total condensate oil and 5% of the total cold-pressed oil.

The lower-boiling material was subjected to further vacuum distillation through a column packed with stainless-steel helices (9 theoretical plates with total reflux at atmospheric pressure). The forerun of this distillation was separated on

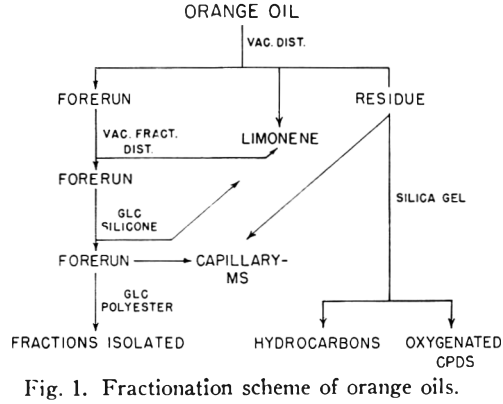

Fig. 1. Fractionation scheme of orange oils.

a preparative GLC column packed with firebrick impregnated with silicone oil SF 96–50 as the stationary phase. The material emerging from this column before limonene, was collected and fractionated on another preparative GLC column, with polyneopentylglycol succinate as the stationary phase. The fractions were collected and analyzed by NMR, IR, and MS methods. In addition, the fraction before limonene from the silicone oil preparative column was analyzed directly by the capillary GLC fast-scan mass-spectrometry method (Cap-MS) described by McFadden *et al.* (1963).

The higher-boiling material from the condensate oil and that from the cold-pressed oil were fractionated by liquid-solid adsorption chromatography on silica gel columns into various hydrocarbon and oxygenated-compound fractions. The hydrocarbon fractions were rechromatographed on silica gel and further purified by preparative GLC. Four sesquiterpenes were isolated. The entire residue fraction and the oxygenated-compound fractions have been analyzed by the Cap-MS method. The separation and identification work on the oxygenated compounds is being continued and will be reported in later publications.

RESULTS AND DISCUSSION

Identifications of the isolated hydrocarbons were based on IR, NMR, and MS data by interpretation and by comparison with data from known authentic samples. Used as additional evidence were the GLC retention times.

Lower-boiling fraction. The lower-boiling hydrocarbons have been reported in part (McFadden *et al.*, 1963) and are listed in Table 1. Other terpenes were detected but not identified.

Not enough alpha-thujene was obtained to isolate a pure sample of this compound. Enough of the alpha-pinene fraction was obtained, however, to show by NMR analysis of this entire fraction that alpha-thujene was present. The samples for NMR analyses were 10% terpene in carbon tetrachloride with 1% tetramethylsilane as internal reference. The two protons on the methylene in the cyclopropane ring in alpha-thujene show very characteristic multiplets centered at 9.30 and 10.00 τ units. The higher-field multiplet provides a simple means of distinguishing alpha-thujene from sabinene since the latter has no such high field baud. Another absorption peak for identifying alpha-thujene is from the endocyclic olefinic proton. This band at 5.14 τ units is very convenient for determining the amount of alpha-thujene in the presence of alpha-pinene since the corresponding band for alpha-pinene is at 4.84 τ units. Also, the sharp resonance at 8.74 τ units from the protons on the gem-dimethyls is very useful for determining the presence of very small amounts of alpha-pinene.

Table 1. Hydrocarbons found in orange oils.

Lower-boiling fraction	Higher-boiling fraction
Limonene (major)	Sesquiterpene I (major)
Myrcene (minor)	Sesquiterpene II (minor)
alpha-Pinene (trace)	Farnesene (trace)
alpha-Thujene (trace)	Ylangene (trace)
Camphene (trace)[a]	
alpha-Terpinene (trace)	
gamma-Terpinene (trace)	
alpha-Phellandrene (trace)	
p-Cymene (trace)	
p-Isopropenyltoluene (trace)[a]	

[a] GLC retention and M.S. identification only.

The presence of alpha-thujene in the alpha-pinene fraction was also shown by capillary GLC with silicone oil Dow 710 as the stationary phase. Separation of these two terpenes with this stationary phase has been reported (McFadden *et al.*, 1963). With Tween-20 as the stationary phase, these two terpenes could not be separated even with column efficiency of over 100,000 theoretical plates (calculated with terpene hydrocarbons). This observation further demonstrates the need for different stationary phases in spite of the very high theoretical plate values achieved with capillary columns. Excellent quantitative data as to retention times of terpenes chromatographed with different stationary phases have been reported by Klouwen and ter Heide (1962), and Bernhard (1962) has reported some analyses of terpenes with capillary GLC. Application of these reported data is certainly helpful in analyses of fractions containing terpenes difficult to separate.

The capillary GLC chromatogram (Fig. 2) is an analysis of a crude fraction obtained from a total cold-pressed orange oil fractionation on silica gel. The IR analysis showed a strong absorption at 5.8 μ and a medium absorption at 3.7 μ, indications that this was an aldehyde fraction. How-

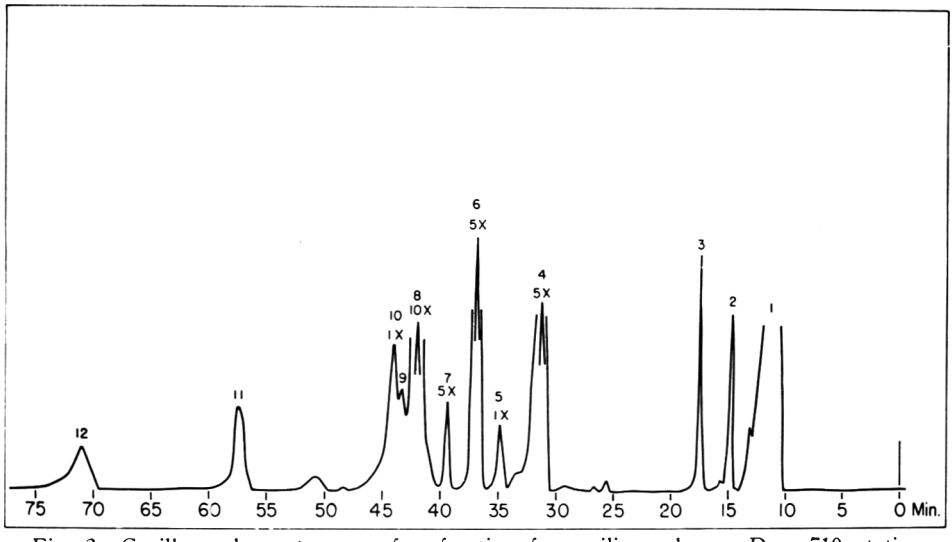

Fig. 2. Capillary chromatogram of a fraction from silica column. Dow 710 stationary phase, 264 ft long, 0.01 in. I.D., isothermal at 95°C.

Peak no.	Compound	Peak no.	Compound
1.	heptane and octane (solvent?)	7.	unknown
2.	toluene (solvent?) and an aldehyde	8.	n-octanal
3.	nonane (solvent?)	9.	unknown
4.	myrcene	10.	gamma-terpinene
5.	unknown	11.	*p*-isopropenyltoluene
6.	limonene	12	n-nonanal

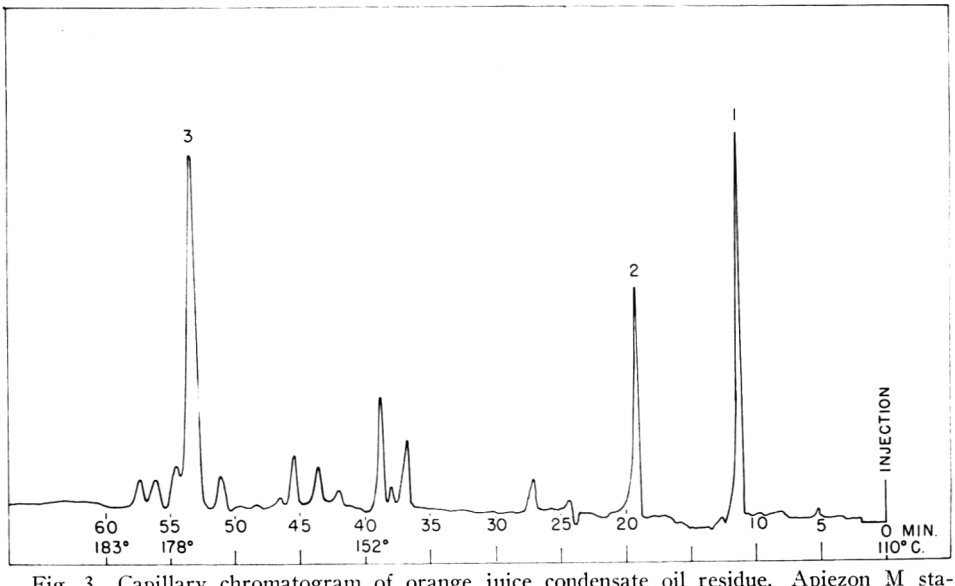

Fig. 3. Capillary chromatogram of orange juice condensate oil residue. Apiezon M stationary phase, 50 ft long, 0.01 in. I.D., programmed temperature.

Peak no.	Compound
1.	limonene
2.	ylangene
3.	sesquiterpene I

ever, the MS analysis of this material fractionated by capillary GLC showed that hydrocarbons were present in appreciable amounts. Also, by this method of analysis, the material represented by peak 11 was found to be a $C_{10}H_{12}$ compound. The structure of *p*-isopropenyltoluene has been tentatively assigned to this material by comparison of its MS fragmentation pattern and its GLC retention time with those of a synthesized sample of this material. This example of finding hydrocarbon compounds in a sample that appears to be predominantly an aldehyde fraction shows the danger of drawing conclusions as to constituents from gross properties. It also shows the advantages of the MS monitoring of materials fractionated by the capillary GLC to find compounds that are difficult to separate and identify in small amounts by the usual methods.

Higher-boiling fraction. The sesquiterpenes found are listed in Table 1. A capillary GLC chromatogram of the higher-boiling fraction of the condensate oil is shown in Fig. 3. The first peak was due to limonene, which had not been completely removed. Peaks 2 and 3 were both due to sesquiterpene compounds having molecular weight 204 (by MS), i.e., $C_{15}H_{24}$. The IR spectrum of the material represented by peak 2 corresponds with the ylangene spectrum reported by Pliva *et al.* (1960), but no structure has been proposed for this sesquiterpene. The sesquiterpene I represented by peak 3 has not been related to any reported compound, i.e., IR and NMR data are not compatible with any known sesquiterpene.

The results of monitoring the silica gel fractionations by GLC are shown in Fig. 4. Curve *A* is an isothermal chromatogram of the same higher-boiling residue as in Fig. 3, but with a larger sample and with Carbowax 1540 instead of Apiezon M as the stationary phase. Curve *B*, a chromatogram of the first fraction from the silica gel column, shows that this fraction is predominantly a component that was shown to be ylangene. Curve *C* shows a chromatogram of a transition fraction and shows trace amounts of a number of other materials, primarily sesquiterpenes. Curve *D* is a chromatogram of a following fraction in which the sesquiterpene I is predominant.

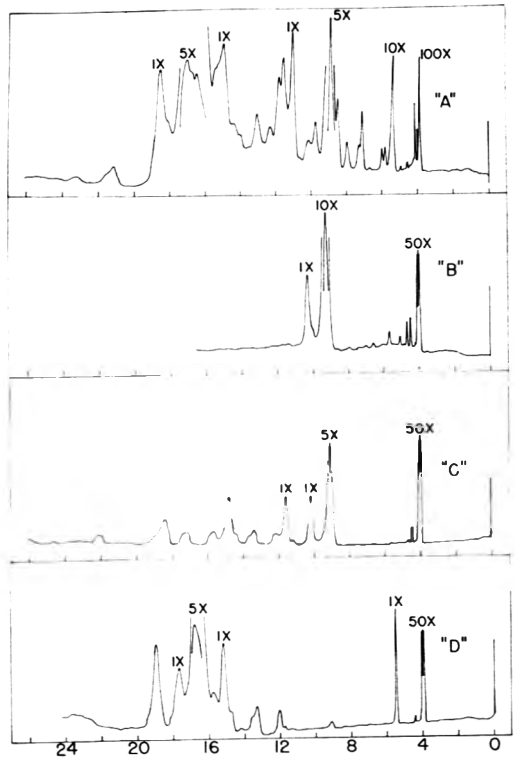

Fig. 4. Capillary chromatograms of residue and of sesquiterpene fractions eluted from a silica gel column. Carbowax 1540 stationary phase, 100 ft long, 0.01 in. I.D., isothermal at 180°C.

Curve	Chromatogram
A	orange juice condensate oil residue
B	ylangene fraction
C	transition fraction
D	sesquiterpene I fraction

Sesquiterpenes from cold-pressed orange oil were similarly separated, purified, and analyzed. Sesquiterpene I was found to be predominant in this oil also, and three other sesquiterpenes present in lesser amounts were isolated. Of these in lesser amounts, one was identified as ylangene by IR spectra (Pliva *et al.*, 1960); another, as farnesene by IR spectra (Pliva *et al.*, 1960) and by interpretation of NMR spectra. Another sesquiterpene, designated sesquiterpene II, has been isolated but has not been related to any reported compound.

Although the sesquiterpenes have only a very delicate and mild aroma and are not the major contributors to the orange aroma, knowledge of their presence, structure, and

chemistry will be helpful in further studies of the volatiles from the orange.

ACKNOWLEDGMENTS

The authors thank W. L. Stanley for suggestions and encouragement, and D. C. Patterson for help.

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

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Optimum Conditions for the Interaction of Food Gums with the Anthrone Reagent

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SUMMARY

Optimum conditions for the reaction of food gums with the anthrone reagent vary from one gum to another. The sulfated, carboxylic acid type and mixed polysaccharides produced colors with absorption maxima at 620 mμ. The uronic acid type of polysaccharides, pectin and sodium alginate (Kelco Gel HV), produced colors exhibiting absorption maxima at 520 mμ. In all cases, color intensity increased with increasing temperature. The heating time necessary for maximum color development at 99°C varied from 4 to 10 min, depending on the hydrocolloid in question. With pectin and sodium alginate, although color intensity increased with heating time at 99°C, no definite heating time for maximum color development was observed up to 60 min. In most cases, aging led to a rapid decrease in color intensity, except for pectin and sodium alginate, in which cases color intensity increased.

INTRODUCTION

Anthrone in sulfuric acid has been used widely for the determination of carbohydrates of various sorts (Morris, 1948; Helbert and Brown, 1957; Haas and Fleichmann, 1958; Richards, 1959; Shields and Burnett, 1960). However, as a distinct class, the food gums, particularly many of the natural plant hydrocolloids, have not been investigated in detail. Yaphe (1960) determined 3,6-anhydrogalactose and galactose in marine algal polysaccharides using the anthrone reagent. He presented data on the concentration of these sugars in agar, lambda- and kappa-carrageenan, and kappa-furcellaran. Morris (1948) mentioned that plant polysaccharides and gums responded to the anthrone test. However, no data were given on the optimum conditions necessary for color formation. Sodium carboxymethylcellulose (Black, 1951) and methylcellulose (Samsel and DeLap, 1951) have also been determined by this reagent.

Optimum conditions for quantitative determination of carbohydrates by the anthrone method differ from sugar to sugar and from polysaccharide to polysaccharide (Helbert and Brown, 1955, 1957, 1961). Since most of the natural plant hydrocolloids are mixtures of sugars or mixtures of sugars and uronic acids, no single set of conditions will hold for their quantitative determination. While applying the anthrone method to the determination of hydrocolloids separated by the use of long-chain quaternary ammonium detergents, it was found necessary to establish optimum conditions for each hydrocolloid with respect to heating time, temperature of heating, and time of measurement of the color developed. This article reports on these and other variables.

EXPERIMENTAL

Materials. The hydrocolloids used are listed in Table 1. All stock solutions were prepared and dialyzed as described by Graham (1960).

Sulfuric acid was reagent grade, 95–98%, sp. gravity 1.8407–1.8437.

Anthrone reagent was prepared by dissolving 0.15 g of anthrone in 100 ml of 76% sulfuric acid. The solution was stored at 4°C for at least 4 hr before use, and all solutions were discarded after 24 hr.

Equipment. The equipment used was Coleman Universal spectrophotometer, model 14; volumetric pipettes; Pyrex glass-stoppered test tubes; dialysis membrane, 27/32 dialysis tubing (Visking Com-

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Table 1. Interaction of hydrocolloids with anthrone reagent.

Hydrocolloid tested	Color produced with anthrone reagent	Wavelength of maximum absorption (mμ)	Heating time (99°C) for maximum color development (min)
Group I. Sulfated polysaccharides			
Furcellaran	Green	620	6
Lambda-carrageenan	Green	620	6
Gelcarin	Green	620	6
Seakem type 5	Green	620	4
Seakem type 402	Green	620	4
Euchema cottonii	Green	620	4
Kappa-carrageenan	Green	620	6
Agar	Green	620	4
Gelcarin	Green	620	4
Hypnean	Green	620	4
Group II. Carboxylic acid type or mixed type of polysaccharides			
Gum karaya	Green	620	4
Gum arabic	Green	620	6
Gum tragacanth	Green	620	8
Gum ghatti	Green	620	4
Tamarind seed mucilage	Green	620	6
Sodium alginate	Reddish brown	520	No max.
Quince seed mucilage	Green	620	10
Sodium carboxymethylcellulose	Green	620	8–10
Okra gum	Green	620	6
Mucin	Green	620	6–8
Pectin	Reddish brown	520	No max.
Group III. Neutral polysaccharides			
Locust bean gum	Green	620	6
Gum guar	Green	620	4
Potato starch	Green	620	6
Group IV. Proteins			
Lactoglobulin	Reddish	530	.. [a]
Casein	Reddish	530	.....
Gelatin	Green	620	.....
Group V. Other compounds			
Polygalacturonic acid	Reddish	520	None
Tween 20	Brownish yellow	None	None
Cetylpyridinium chloride	No color change	.....	None
Polyglucose sulfate	Green	620	.....
Chondroitin sulfate	Reddish	530	.....
Mepesulfate	Reddish	525	.....

[a] ..... = not determined.

pany, Chicago, Illinois), and a constant-temperature water bath or oil bath.

General procedure. One ml containing 250–1,000 μg of the particular hydrocolloid was placed in Pyrex glass-stoppered test tubes. The tubes were placed in the freezer compartment of the refrigerator for at least 4 hr. Nine ml of a freshly prepared, and of an aged and cooled batch, of anthrone reagent was added to each tube. The tubes were kept submerged in an ice-water bath. They were then placed in a boiling-water bath (99°C) for 10 min, cooled immediately in an ice-water bath for 10 min, taken out, and allowed to stand at room temperature for 10 min, and the optical density of the colors developed was measured against a reagent blank at the appropriate wavelength of maximum absorption.

To establish the wavelength of maximum absorption of the colors produced by each hydrocolloid with the anthrone reagent, the anthrone-carbohydrate mixtures were treated according to the general procedure. The color produced by each hydrocolloid was measured over the wavelength range of 350–800 mμ with sulfuric acid as

the blank. A plot of the absorbance vs. wavelength established the wavelength of maximum absorption as shown in Fig. 1.

To quickly gain some idea of the color reactions of several hydrocolloids used in the food industry with the anthrone reagent, a screening procedure was devised. For this, 5–10 mg of each of the hydrocolloids was placed in Pyrex glass-stoppered test tubes. Nine ml of the anthrone reagent was added to each tube and the tubes were shaken gently and then placed in a boiling-water (99°C) bath for 10 min. The color developed was observed visually. The results are summarized in Table 1.

Influence of time and temperature on color intensity. Several investigators (Helbert and Brown, 1955, 1956, 1957; Richards, 1959; Yaphe, 1960) have shown that both the time and temperature of heating of the anthrone-carbohydrate mixture profoundly influenced the intensity of the color produced. Therefore, a detailed study was made of this variable employing the conditions outlined in the general procedure except that the time and temperature of heating were varied as shown in Table 2 and Fig. 2. All absorbance readings were made at the appropriate wavelength of maximum absorption of the particular hydrocolloids, and a reagent blank was included for each time-temperature experiment.

Effect of acid concentration on color. To determine the effect of acid concentration on color intensity, the general procedure was followed, using solutions respectively containing 70, 85, 90% and concentrated sulfuric acid and 250 μg of each hydrocolloid.

Effect of anthrone concentration on color. Differences in color intensity due to variations in the concentration of anthrone in the reagent were assessed by using 250 μg of each hydrocolloid and anthrone levels of 0.05–1.0% (W/V).

Effect of aging on color developed. Although it has been reported that the green color developed with sodium carboxymethylcellulose and anthrone is stable for several hours (Black, 1951), with some hydrocolloids, especially sodium alginate, and pectin, the color intensity, especially for the shorter periods of heating, increased considerably. This effect was investigated by heating 250–1,000 μg of the hydrocolloids for varying periods and measuring the color intensities produced by the particular hydrocolloids after various time intervals.

Absorbance-concentration relations of interaction between food gums (hydrocolloids) and the anthrone reagent. After the influences of the several variables were delineated, the quantitative response of the interactions was investigated. For this, 1 ml containing 10–500 μg of the particular

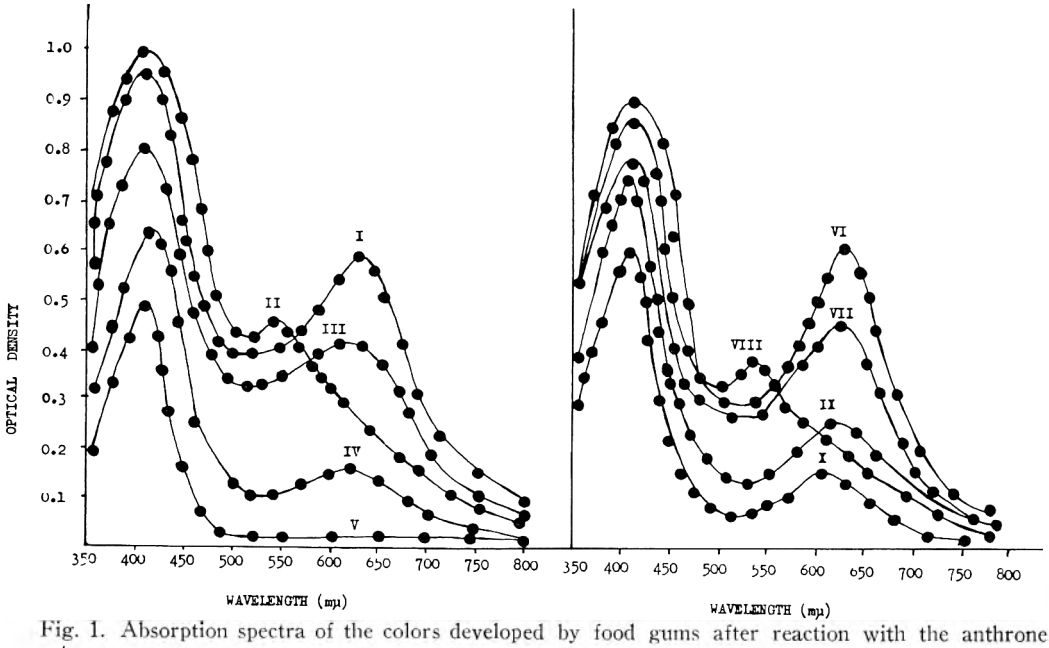

Fig. 1. Absorption spectra of the colors developed by food gums after reaction with the anthrone reagent.

I. Gum arabic
II. Pectin
III. Gum tragacanth
IV. Gum guar
V. Reagent blank
VI. Carrageenan (Seakem type 5)
VII. Locust bean gum
VIII. Sodium alginate
IX. Sodium carboxymethylcellulose
X. Agar

Table 2. Influence of heating for various periods on color development.

Temp. (°C)	Time heated (min)	Carrageenan (Seakem type 5)	Sodium carboxymethyl-cellulose	Gum tragacanth	Gum guar	Locust bean gum	Sodium alginate	Pectin
30°	30	0.20	0.01	0.02	0.01	0.01	0.05	0.02
	60	0.27	0.02	0.02	0.02	0.02	0.05	0.02
40°	10	0.20	0.00	0.01	0.01	0.00	0.00	0.02
	30	0.26	0.01	0.09	0.02	0.03	0.04	0.03
	60	0.35	0.03	0.15	0.02	0.05	0.05	0.03
50°	10	0.25	0.01	0.02	0.01	0.00	0.02	0.00
	20	0.30	0.03	0.04	0.01	0.02	0.03	0.00
	30	0.33	0.03	0.08	0.02	0.07	0.04	0.04
	40	0.34	0.04	0.09	0.03	0.08	0.06	0.04
	60	0.37	0.05	0.12	0.03	0.12	0.11	0.04
70°	5	0.33	0.04	0.13	0.02	0.10	0.01	0.05
	10	0.42	0.17	0.18	0.02	0.27	0.01	0.08
	15	0.47	0.22	0.20	0.03	0.39	0.15	0.19
	30	0.50	0.36	0.27	0.16	0.72	0.24	0.38
	60	0.55	0.37	0.26	0.21	0.85	0.29	0.41
99°	2	0.43	0.13	0.30	0.10	0.51	0.12	0.13
	4	0.47	0.27	0.46	0.29	0.90	0.14	
	6	0.44	0.30	0.68	0.28	0.94	0.16	
	8	0.39	0.31	0.72	0.25	0.84	0.14	
	10	0.36	0.30	0.70	0.21	0.76	0.19	0.23
	12	0.35	0.29	0.64	0.21	0.72	0.21	
	14	0.34	0.28	0.60	0.20	0.66	0.22	
	16	0.33	0.27	0.57	0.19	0.65	0.26	
	20	0.32	0.26	0.58	0.20	0.62	0.29	0.44
	24			0.58	0.21	0.58		
	25		0.24					
	30		0.21				0.33	0.43
	40						0.44	0.65
	60						0.66	0.72

hydrocolloid was placed in a Pyrex glass-stoppered test tube. Nine ml of the anthrone reagent in 76% concentrated sulfuric acid was added, and the color was developed as outlined in the general procedure. The colors developed were measured at the appropriate wavelength of maximum absorption. For pectin and sodium alginate, anthrone in concentrated sulfuric acid was used. The results are shown in Fig. 3.

Effect of various food ingredients. If the method is applied to the determination of hydrocolloids isolated from formulated foods or pharmaceutical preparations, it is important to know the extent of possible interference from such additives as inorganic salts, sodium benzoate, sorbic acid, vanillin, etc. This aspect of the problem was studied by adding varying amounts of the excipients to a constant amount (500 μg) of carrageenan (Seakem type 5) and developing the color as described under the general procedure. The intensity of the color developed under these circumstances was then determined, and the maximum amount of the added ingredient that would not cause an interference appreciably greater than possible experimental error was calculated. The results are shown in Table 3.

The "tolerance level" was taken as that maximum final concentration (the concentration of salt or other excipient in the 10 ml of reaction medium) that did not cause a significant difference in absorbance as compared to a control tube to which no salt or other excipient was added.

RESULTS AND DISCUSSION

From Table 1, it is evident that at 99°C, the heating time necessary for maximum color development differs from one hydrocolloid to another. Most of the food gums are polymers of different sugars, and this variation in optimum heating time is a reflection of the differences in heating time necessary for maximum color development. The sulfated hydrocolloids and the neutral polysaccharides all seemed to produce maximum color intensity after 4–6 min of heat-

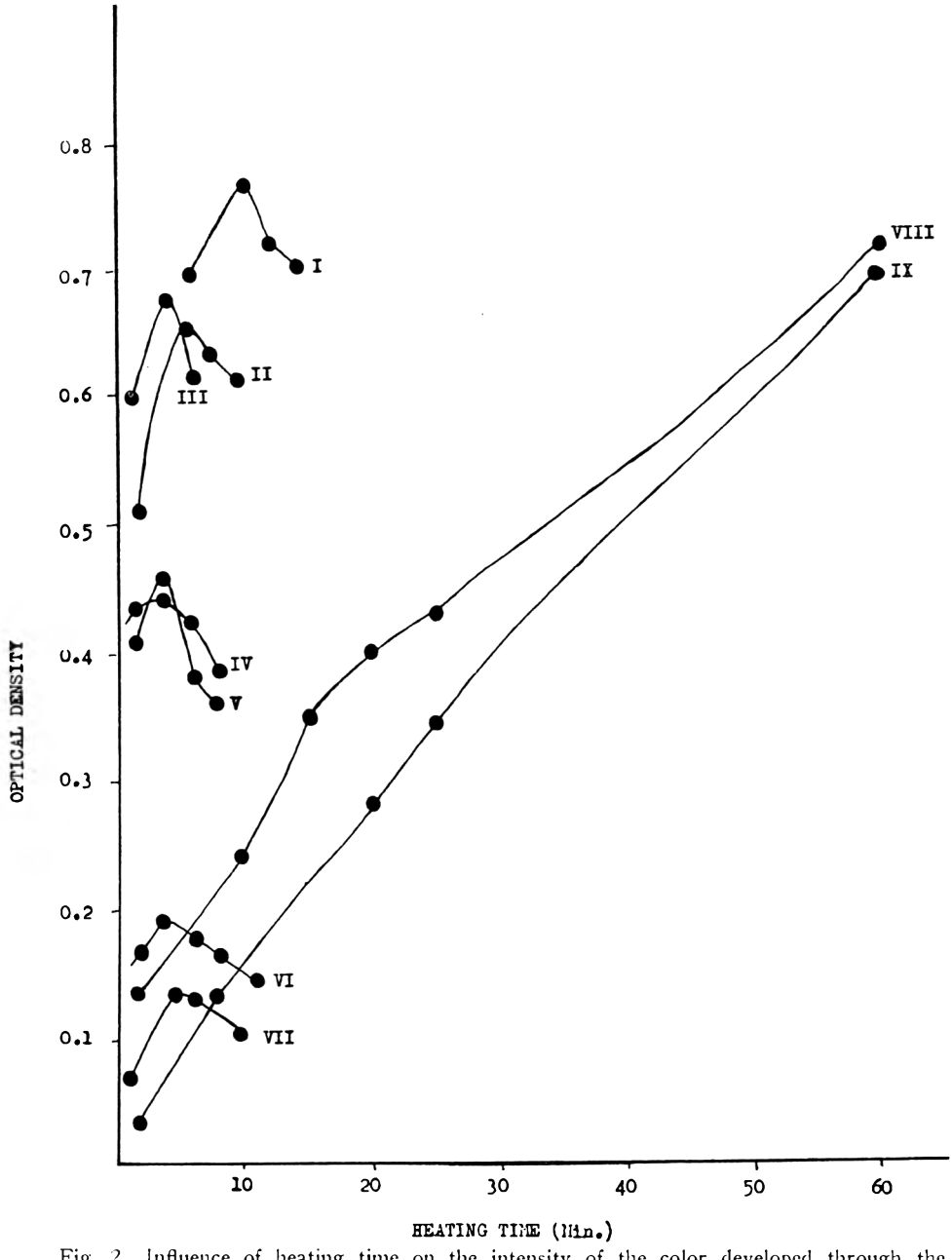

Fig. 2. Influence of heating time on the intensity of the color developed through the interaction of hydrocolloids with the anthrone reagent.

I. Sodium carboxymethylcellulose
II. Gum tragacanth
III. Furcellaran
IV. Carrageenan (Seakem type 5)
V. Gum ghatti
VI. Agar
VII. Gum arabic
VIII. Pectin
IX. Sodium alginate

ing. Quince seed mucilage and sodium carboxymethylcellulose gave maximum values after 10 min and 8–10 min, respectively, and mucin gave a maximum value after 6–8 min. The uronic acid polysaccharides, sodium alginate and pectin, when heated in anthrone in concentrated sulfuric acid, showed no optimum time after heating for

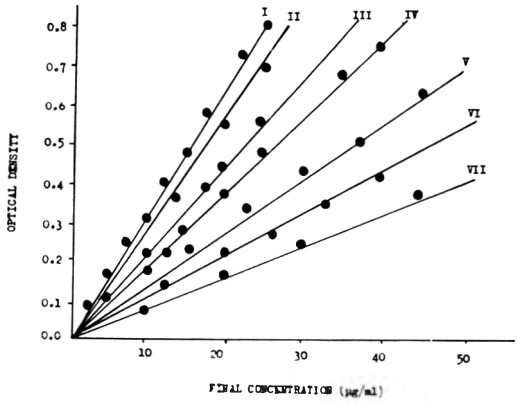

Fig. 3. Quantitative response of food gums in the anthrone method.

I. Sodium carboxymethylcellulose
II. Carrageenan (Seakem type 5)
III. Gum arabic
IV. Locust bean gum
V. Gum guar
VI. Pectin
VII. Sodium alginate

2–60 min. This is consistent with observations noted for uronic acids and their polymers (Helbert and Brown, 1956, 1957, 1961). Several proteins tested gave color, and the optimum conditions for their interaction with the anthrone reagent will be reported in another communication.

Color development was greatly influenced by the temperature of heating (Table 2). Up to 50°C, the hydrocolloids produced little color, even if heated for as long as 60 min. However, at 70°C, appreciable color development was noted, especially at the longer heating times for carrageenan, sodium carboxymethylcellulose, gum tragacanth, gum guar, and locust bean gum.

Since pectin and sodium alginate produced color so slowly and feebly at 70°C or below, even when heated for extended periods, this feature could be exploited to minimize interference by these hydrocolloids when determinations of the other hydrocolloids are being carried out. Carrageenan and sodium carboxymethylcellulose developed more color when heated at 70°C for 60 min than when heated at 90°C for shorter periods.

With the sulfated, neutral, and mixed polysaccharides, color intensity decreased with the time of aging. With the uronic acid type of polysaccharides, however, color intensity increased. This latter phenomenon was noted for uronic acids singly and in

Table 3. Influence of added inorganic salts and other excipients on the intensity of the color produced by carrageenan (Seakem type 5) in the anthrone reaction.

Inorganic salt added as chloride	Tolerance level (M)	Other excipients	Tolerance level (μg/ml)
Monovalent			
K^+	3.8×10^{-4}	Sodium benzoate	3.0
Li^+	1.6×10^{-4}	Spans 20, 40, 60, 65	4.0
Na^+	3.0×10^{-4}	Tweens 20, 21, 40, 60, 61, 80	3.2
Divalent			
Ca^{++}	1.0×10^{-4}	Glycerol	0.1
Mg^{++}	2.2×10^{-4}	Ethylene glycol	3.6
Ba^{++}	2.5×10^{-4}	Polyoxyethylene glycols	3.1
Cd^{++}	2.8×10^{-4}	Propylene glycol	4.2
Cu^{++}	1.3×10^{-4}	Polyoxypropylene glycols	3.2
Hg^{++}	1.0×10^{-4}	Olive oil	2.9
Mn^{++}	2.0×10^{-4}	Coconut oil	4.2
Ni^{++}	1.1×10^{-4}	Linseed oil	3.6
Sr^{++}	1.3×10^{-4}	Cottonseed oil	2.9
Zn^{++}	1.4×10^{-4}	Stearic acid	4.6
Trivalent			
Al^{+++}	1.0×10^{-10}	Oleic acid	3.8
Fe^{+++}	1.1×10^{-10}	Sugar alcohols	
		Sorbitol	5.0
		Mannitol	5.0
		Dulcitol	5.0
		Erythritol	7.5

polymers (Helbert and Brown, 1961). Some relationship was found between the heating period and aging and between the heating temperature and aging and their influence on color intensity. Generally, with all the hydrocolloids, if the heating period at 99°C was less than necessary for maximum color development, color intensity increased with aging, at least up to 12–24 hr of aging. With pectin and sodium alginate, heating below 60°C, especially for 15–40 min, led to considerable increase in color density upon aging. However, if the mixtures were heated for 60 min at 99°C, the aging effect resulted in less drastic increase in color density. With the other polysaccharides, heating for their optimum periods at 99°C led to no outstanding increase or, in many cases, a decrease in color density on aging.

Mixtures heated at low temperatures (50–70°C) for 10 min or less led to little increase in color density or aging. Above 70°C, however, all aged hydrocolloid-anthrone mixtures showed an increase in color density. Up to 24–48 hr of aging this was particularly outstanding with pectin and sodium alginate and is in agreement with previous findings (Helbert and Brown, 1956, 1957).

Acid concentration was of primary importance. With the sulfated and neutral polysaccharides, a slight difference in optical density was noted as the acid concentration of the anthrone reagent increased beyond 70%. Above 90%, slight decreases were observed. With the uronic acid type of polysaccharides the opposite was observed: as acid concentration increased, great increases were observed in the optical density of the color produced.

Of the various substances that may be found in food and pharmaceutical preparations, the most common are the inorganic salts. With carrageenan (Seakem type 5) these may interfere with color development at rather low concentrations (Table 3). This same relationship would most likely hold for all those hydrocolloids that produced maximum color intensity within a few minutes of heating at 99°C (boiling-water bath). The tolerance levels for the uronic acid type of hydrocolloids, pectin and sodium alginate, were somewhat higher, probably because these hydrocolloids had to be heated for as long as 60 min, at which times the maximum color intensity was not even achieved. Interference from the inorganic salts was probably due to competition for the binding sites on the hydrocolloid molecule or by interaction with the chromogen produced. The organic compounds interfered because most of them produced some color with the anthrone reagent. With carrageenan (Seakem type 5) the tolerance levels were lower than those for hydrocolloids, which required longer heating periods for maximum color development. The uronic acid type of hydrocolloids, pectin and sodium alginate, would be most affected since the longer heating times required here will lead to color production by glycerol, the Spans and the Tweens, polyglycols and the sugar alcohols, in particular. Moreover, the colors produced by most of these excipients absorb rather strongly in the region of maximum absorption of the colors produced by pectin and sodium alginate with the anthrone reagent.

Color production through the interaction of carbohydrates with the anthrone reagent has been attributed to the formation of furfural or some derivative thereof. For this, it has been postulated that there must be a possibility of dehydration in the 2,3-position (Sattler and Zerban, 1948). With the hydrocolloids investigated, hydrolysis of the polysaccharide to the component sugars was rapid. These sugars are then subsequently converted to furfural and other furfural derivatives, which react with anthrone to give the final green or other colors.

These studies have provided pertinent data on the reaction of hydrocolloids (food gums) with the anthrone reagent. The variations with respect to heating time, acid concentration of the anthrone reagent, and the effects of aging clearly indicate that for quantitative assay the specific optimum conditions for each hydrocolloid must first be established.

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Volatile Carbonyl Constituents of Dairy Butter

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SUMMARY

Volatile constituents of fresh butter were separated from fat and other components of high molecular weight by vacuum steam distillation. The carbonyl compounds in the aqueous distillate were converted to their 2,4-dinitrophenylhydrazones. Separation of these by column and paper chromatography gave the derivatives of formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, *n*-hexanal, *n*-nonanal, phenylacetaldehyde, acetone, 2-heptanone, 2-nonanone, diacetyl and (−)-acetoin, all identified by comparison with authentic samples. Possible biosyntheses of these flavor constituents are discussed.

Butter is undoubtedly one of the most important foods of man. Its dietary value is due to a high content of fats, vitamins, and minerals. Although a number of other edible fats, particularly margarine, resemble butter in general composition and appearance, they lack its unique and pleasant aroma. The nature of this flavor has long intrigued chemists and flavorists, and the patent literature abounds with recipes for the production of "artificial" butter aromas. The majority of these are not the result of chemical analysis but the creations of imaginative flavorists. In the early days of flavor chemistry, butyric acid was believed to be solely responsible for the aroma of butter, but later studies indicated that diacetyl plays a more important, and possibly a major, role. Refined analyses, with modern techniques, of the carbonyl constituents of fresh and autoxidized butter revealed the presence of additional substances. Stearyl and palmitylaldehyde were identified positively, and a number of other aldehydes, both saturated and unsaturated, were identified tentatively (van Duin, 1958). Further, it seems pertinent to mention that Patton and co-workers (Day *et al.*, 1957) found formaldehyde, acetaldehyde, *n*-hexanal, acetone, and butanone in gamma-irradiated skim milk. Of these, only acetaldehyde and acetone seemed to be native constituents, the others being produced during irradiation. In contrast, Bassette (1960) detected formaldehyde, acetaldehyde, isovaleraldehyde, hexanal, and nonanal in dry milk.

This paper describes our own investigations of the volatile carbonyl constituents of fresh dairy butter.

METHODS AND PROCEDURES

The success of any flavor analysis depends largely upon proper preparation of a concentrate. Preliminary, but nevertheless careful, investigations, made in collaboration with Dr. L. Bernardi, indicated that a mild and fairly complete separation of volatile flavor constituents from butterfat was possible by steam distillation under reduced pressure of a butter-water emulsion. This method was definitely superior to vacuum distillation of butterfat at elevated temperatures, which caused the appearance of many pyrolytic artifacts.

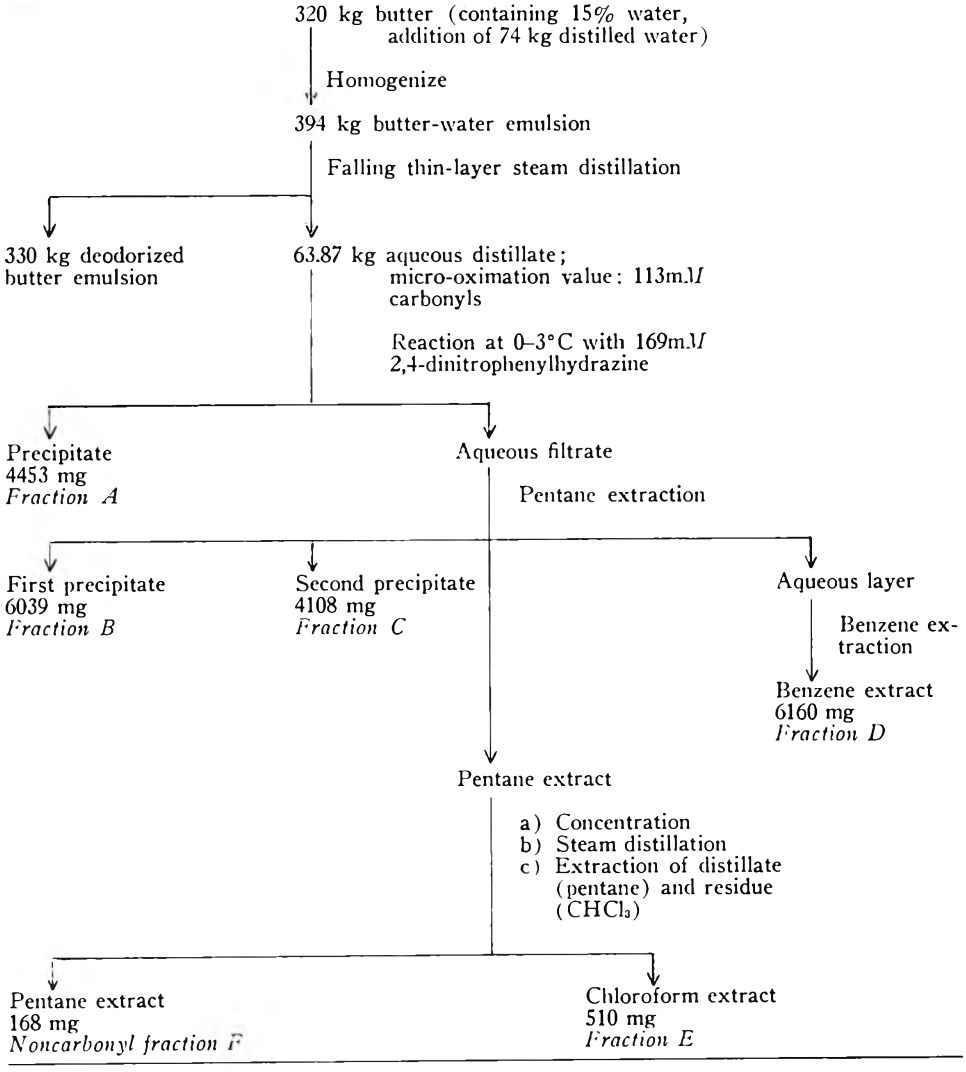
Preparation of a butter steam distillate. By falling film distillation, the volatile butter constituents were first separated from the butterfat. Fresh butter was melted overnight in a water bath at 38–42°C. The melted butter was then transferred to a blender, where it was homogenized with 20% of its weight of oxygen-free distilled water. The butter-water emulsion was allowed to flow through two falling-film thin-layer distillation columns, which were under reduced pressure of 18–20 mm Hg and heated by a water jacket to 29–32°C. The aqueous distillate was condensed in ice-water-cooled glass condensers and collected in ice-water-cooled glass flasks. All these operations were performed under a nitrogen atmosphere. The homogeneous distillates after dilution developed a characteristic butter flavor.

The equipment was entirely home-built (Winter *et al.*, 1962), and the distillation columns consisted

of two precision Pyrex glass tubes (interior diameter 97.2–97.35 mm; length 800 mm) surrounded by a circulating hot-water jacket (85–90°C). A well adjusted 4-blade rotor turning at 1700–2000 rpm produced a uniform film of the butter emulsion in the columns. With this equipment, 16–18 kg of butter emulsion could be distilled per hour. For our investigation, we used fresh French butter (Savoie, France) made from unpasteurized cream, which contained about 15% of water.

Isolation of the carbonyl compounds as 2,4-dinitrophenylhydrazones. The total carbonyl content in the fresh aqueous distillates was determined by a micro-scale oximation (Winter *et al.*, 1962). Immediately after it, the carbonyls were treated at 0–3°C with a 50% excess of an acidic 2,4-dinitrophenylhydrazine solution (prepared by dissolving 1 g of 2,4-dinitrophenylhydrazine in 20 cc of concentrated H_2SO_4 and diluting with 80 cc of water; 1 cc of solution contains 0.05m*M* of reagent). Deposition of the crystalline 2,4-dinitrophenylhydrazones (2,4-DNPs) proceeded slowly, and after 15 hours' reaction time at 0–3°C, most of the butter fragrance had disappeared. Filtration yielded *2,4-DNP-fraction A* (4453 mg) (see Table 1). To isolate slightly water-soluble 2,4-DNPs, the filtrates were extracted with pentane three times at 0°C. During this operation a yellow crystalline substance precipitated, which was collected by filtration, yielding *fraction B* (6039 mg). A new crystalline precipitate was formed in the clear pentane-extracted filtrate on

Table 1. Isolation of the butter 2,4-dinitrophenylhydrazones.



storage for several days at 0°C, yielding, after filtration, *fraction C* (4108 mg). After concentration of the clear pentane extract, there remained an odorous residue, which was separated by a vacuum steam distillation (distillation temperature 40–50°C) in a volatile (steam distillate) and a nonvolatile part (residue). The latter was extracted with chloroform, yielding *fraction E* (510 mg). This fraction should contain pentane-soluble and slightly water-soluble 2,4-DNPs. The steam distillate was extracted with pentane, yielding after concentration the *pentane extract F* (168 mg).

Aqueous distillates partitioned with pentane were subsequently extracted with benzene in order to isolate slightly water-soluble but pentane-insoluble 2,4-DNPs. Concentration of this benzene extract yielded *fraction D* (6160 mg).

Separation and identification of the 2,4-dinitrophenylhydrazone fractions. The 2,4-DNP fractions A, B, C, and D were extracted repeatedly with boiling benzene in order to separate benzene-soluble material from benzene-insoluble parts (see Table 2).

Table 2. Pre-separation of the 2,4-DNP fractions A, B, C, and D by benzene extraction.

2,4-DNP		Benzene-soluble		Benzene-insoluble	
fraction	mg	mg	%	mg	%
A	4453	2282	49	2271	51
B	6039	5435	90	604	10
C	4108	911	22	3179	78
D	6180	5360	87	800	13

Analysis of the benzene-insoluble 2,4-DNPs. Preliminary analyses (paper chromatography, infrared spectroscopy, hydrolysis) revealed that the benzene-insoluble fractions consisted essentially of diacetyl-bis-2,4-DNP. A pure sample was isolated by partition chromatography on perlon powder (Winter, 1958). Besides diacetyl-bis-2,4-DNP, only traces of diacetyl-mono-2,4-DNP could be detected, and no homologs of diacetyl were found.

Analysis of benzene-soluble 2,4-DNPs. The benzene-soluble material was first chromatographed separately on a mixture of Bentonite and Kieselguhr (4:1 by weight), as recommended by Elvidge and Whalley (1955). The water content of Bentonite was adjusted to 11–13%, and, to ensure homogeneity, the mixture of Bentonite and Kieselguhr was passed through a 100-mesh sieve. It was then packed into a vertical column as a slurry in a mixture of benzene-hexane or in pure benzene. The ratio of support to derivative varied between 50 and 100. Used for elution were benzene-hexane mixtures, benzene, benzene-chloroform mixtures, chloroform, and chloroform-methanol mixtures. Table 3 illustrates the first chromatographic separation of 1215 mg of benzene soluble 2,4-DNPs of fraction A on 70 g of Bentonite-

Table 3. Column chromatography of fraction A (1215 mg).

Fraction	Solvent	Proportion	Cuts		Total mg
			ml	mg	
1–2	Bz		125	111	111
3			100	113	224
4			100	48	272
5			100	24	296
6			100	16	312
7			50	8	320
8			150	44	364
9			50	14	378
10	Bz/$CHCl_3$	1:1	100	17	395
11			50	54	449
12			100	19	468
13			100	10	478
14			100	7	485
15			25	3	488
16			25	2	490
17			50	5	495
18			50	8	503
19			25	2	505
20			25	2	507
21			25	5	512
22	$CHCl_3$		25	5	517
23			25	6	523
24			25	2.5	525.5
25			25	4.5	530
26			25	5	535
27			50	8	543
28	$CHCl_3$/MeOH	98:2	50	10	553
29			50	103	656
30			50	82	738
31			50	11	749
32			50	8	757
33			50	11	768
34			50	11	779
35			50	13	792
36			50	12	804
37			50	10	814
38			100	19	833
39			25	3	836
40		95:5	50	5	841
41			50	1	842
42		90:10	50	5	847
43			50	8	855
44		50:50	50	130	985
45			50	77	1062
46			50	...	...

Total eluted: 1062 mg = 87.5% (not eluted = diacetyl-bis-2,4-DNP).

Kieselguhr (12.5% water content; column dimensions: 26 × 120 mm).

Eluted fractions were analyzed by paper chromatography in two systems: dimethylformamide/decalin (Horner and Kirmse, 1955) and dimethylformamide-cyclohexane/cyclohexene (5:3) (Sundt and Winter, 1958). Schleicher & Schüll papers No. 2043 B were used for paper chromatographic separations. In preparative chromatograms (see below), the derivatives were applied in chloroform solutions in quantities up to 10 mg on 18-cm-wide sheets of solvent-impregnated papers. After development, the papers were dried for a day or two at room temperature, or for 1 hr in an oven at 50–60°C. The different zones were then cut apart and extracted separately with methylene chloride or chloroform in a small Soxhlet apparatus. The solvent extracts were filtered and evaporated to dryness at 50–60°C/30–70 mm Hg. Traces of dimethylformamide were removed at 70°C/0.04 mm Hg, and the crude derivatives were purified by conventional methods.

Fig. 1 shows a paper chromatogram obtained with the dimethylformamide/decalin system from fractions mentioned in Table 3.

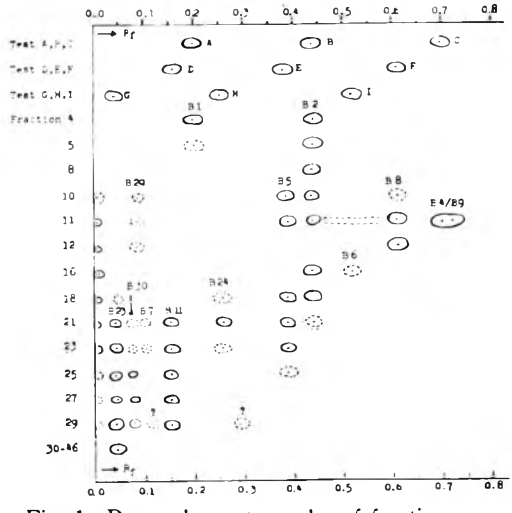


Fig. 1. Paper chromatography of fractions mentioned in Table 3.

	2,4-Dinitrophenylhydrazone of:	R_f
A	Phenylacetaldehyde	0.20
B	Isovaleraldehyde	0.44
C	Nonanaldehyde	0.70
D	Acetaldehyde	0.16
E	Isobutyraldehyde	0.38
F	2-Heptanone	0.61
G	Acetoin	0.04
H	Acetone	0.26
I	Hexanal	0.52

Tentative identifications were possible by comparison of the R_f values with those of authentic samples and by observing the colors of the spots before and after spraying the dried papers with 10% aqueous sodium hydroxide solution. With this reagent, all 2,4-DNPs showed a color change from yellow to orange. Furthermore, some derivatives developed characteristic color reactions; for instance, 2,4-DNPs of diacetyl-mono → rose-red; diacetyl-bis → intense yellow center with circular violet surrounding zone; acetoin → intense brown. Some spots did not give any color reaction, and consequently were not 2,4-DNPs. We considered them to be artifacts. Some of these artifacts showed intense colors under ultraviolet light.

Fractions judged to be mixtures were rechromatographed on Bentonite-Kieselguhr and/or separated by preparative paper chromatography in either of the above systems. For example, fractions 9 to 17 (Table 3; 132 mg) were recombined and the 2,4-DNPs applied on 15 sheets of paper. After development, 8 separated zones appeared (R_f 0.0 trace; R_f 0.08, B29, trace; R_f 0.38, B5, strong; R_f 0.44, B2, strong; R_f 0.52, B6, very weak; R_f 0.61, B8, weak; R_f 0.70, B4, very weak; R_f 0.72, B9, very weak), the separation of the two last zones (B4 and B9) being incomplete. The individual 2,4-DNPs were extracted according to the general method given above. Finally, chromatographed fractions that appeared to be pure were recrystallized to constant melting points.

For definite identification of the purified 2,4-DNPs the following criteria were used: comparison of R_f value, melting point and mixed melting point determination with authentic derivatives (taken on a Kofler hot stage microscope, uncorrected), C, H micro-analyses (by the Scandinavian Microanalytical Laboratory, Copenhagen, and M.I.T. Microchemical Laboratory, Cambridge, Massachusetts), ultraviolet and infrared studies according to Jones *et al.* (1956), and finally comparison of infrared spectra with authentic 2,4-DNPs (taken in KBr discs and/or in $CHCl_3$ solution).

Analysis of fraction E. Fraction E was first investigated by qualitative and preparative paper chromatography in the systems mentioned above. Comparison with the previously analyzed fractions A, B, C, and D indicated no new derivatives, so no further separation was undertaken.

RESULTS AND DISCUSSION

The results of our analyses are summarized in Tables 4 and 5. We do not claim to have isolated and identified all carbonyl compounds in butter, and the presence of other volatile substances in *trace quantities* is probable.

As anticipated, fraction A represented the most complex mixture of carbonyl derivatives. The major constitutents in this frac-

Table 4. 2,4-DNP derivatives isolated from butter distillate.

Spot	R_f[a]	2,4-DNP[b] of	Melting points (°C, uncorrected) Natural	Authentic	Mixed	Further structure proofs	Fractions A	B	C	D	E	F
	0.0	Diacetyl-bis				R_f, IR	+	+	+	+		
	0.10	Diacetyl-mono				R_f, IR	+	+	+	+	+	
B1	0.20	Phenylacetaldehyde	120.5–124	119–120 and 122.5–127	123.5–127	R_f, UV[c]: λ max.: 357 mμ	+					
B2	0.44	Isovaleraldehyde	123–124	123–124	123–125	R_f;[d]	+				+	
B4	0.70	n-Nonanal	100–102	102	100–102	R_f, UV[c]: λ max. 358 mμ	+					
B5	0.38	Isobutyraldehyde	184.5–186	185–186	184.5–186	R_f	+				+	
B6	0.52	n-Hexanal	98–102	103–104	98–103	R_f	+					
B7	0.10	Formaldehyde[e]				R_f	+					
B8	0.61	2-Heptanone	72–74	73–74	72–74	R_f, UV[c]: λ max. 363.5 mμ	+					
B9	0.72	2-Nonanone[e]	about 30°	39–40		R_f, UV[c]: λ max. 364 mμ (ketone)	+					
B11	0.16	Acetaldehyde	146–147[f]	167[f]	146–154[f]	R_f	+		+		+	
B23	0.04	(−)-Acetoin	114.5–116	108–109[g]	108–111–114	IR (KBr and $CHCl_3$), UV[c]: λ max. 356.6 mμ[h]	+	+	+	+	+	
B24	0.26	Acetone	126–126.5	126–126.5	126–126.5	R_f	+	+	+	+	+	
B25	0.20	not identified						+				
B29	0.08	not identified					+					
B30	0.08	not identified					+					
Artifacts[i]:												
	0.0/0.11	2,4-Dinitrophenylhydrazine				R_f			+			
B26	0.18	2,4-Dinitrochlorobenzene	41–42	42–43	42–43	R_f, mass spectral analyses					+	
B28	0.08	2,4-Dinitroaniline	177–178	177–178	177–178	R_f		+	+			
Several spots not identified with R_f 0.0; 0.11; 0.32												

[a] The R_f values are corrected to the medium R_f of phenylacetaldehyde = 0.20.
[b] 2,4-Dinitrophenylhydrazone.
[c] In chloroform solution.
[d] C,H analysis. Calc. for $C_{10}H_{10}O_4N_4$: C 49.62, H 5.30, N 21.04. Found: C 50.08, H 5.64, N 21.03.
[e] Tentative identification.
[f] Two forms of acetaldehyde-2,4-DNP are known (Ross, 1953). The derivative isolated from butter was the low-melting form.
[g] Melting point of d,l-acetoin-DNP.
[h] Further structure proofs for B23 were: 1) C,H analysis. Calc. for $C_{10}H_{12}O_5N_4$: C 44.78, H 4.51, N 20.89%. Found: C 44.86, 44.77, H 4.67, 4.51, N 20.85%. 2) Absence of a carbonyl absorption in the infrared spectra (KBr and chloroform). 3) Conversion of B23 to diacetyl-bis-DNP by treatment with excess of 2,4-dinitrophenylhydrazine in acidic alcoholic solution. 4) Optical activity of B23: $[\alpha]_D^{25} = -12°$ ($c = 2.765$ in $CHCl_3$).
[i] These spots show no characteristic color reaction with NaOH.

tion were diacetyl (50%) and (−)-acetoin (28%).

The composition of fractions B, C, and D was much simpler. Fraction B contained 80% of (−)-acetoin and 10% of diacetyl derivatives. On the other hand, fraction C was rich in diacetyl and poor in (−)-acetoin, and was accompanied by about 10% of 2,4-dinitroaniline. It was surprising to note that fractions B and C were precipitated during extraction of the aqueous filtrates with pentane (Table 1). This may be explained by assuming (subsequently confirmed by experiments) that acetoin-2,4-

Table 5. Carbonyl compounds and their concentrations in butter distillate.

Compound	Concentration [a]
Formaldehyde	sm[b]
Acetaldehyde	sm
Isobutyraldehyde	sm
Isovaleraldehyde	m
n-Hexanal	tr
n-Nonanal	tr
Phenylacetaldehyde	sm
Acetone	sm
2-Heptanone	sm
2-Nonanone	tr
Diacetyl	450 mg/100 kg butter
(−)-Acetoin	1882 mg/100 kg butter

[a] The concentrations were evaluated according to the intensities of the 2,4-DNP spots on the paper chromatograms and according to quantitative evaluation of the column chromatograms.

[b] Scale: m (medium), 10–100 mg/100 kg butter; sm (small), 1–10 mg/100 kg butter; tr (trace), <1 mg/100 kg butter.

DNP has a rather high solubility in water (13 mg of derivative in 100 ml of water at 0°C), and that its solubility is lowered by adding pentane to the distillate. At the same time, soluble (−)-acetoin-2,4-DNP was converted by excess of 2,4-dinitrophenylhydrazine to insoluble diacetyl-bis-2,4-DNP. The isolation of large amounts of 2,4-dinitrophenylaniline in fractions C and D, and only small amounts of unreacted 2,4-dinitrophenylhydrazine used in excess agrees with this postulate.

Fraction E, according to its origin, should have contained small amounts of slightly water- and pentane-soluble 2,4-DNPs. This was confirmed by the presence of the following substances (in order of decreasing amounts): 2,4-DNP of acetaldehyde, acetone, (−)-acetoin, isovaleraldehyde, and isobutanal.

(−)-Acetoin-2,4-DNP has not yet been reported in the literature. Our preparation melted at 114.5–116°C and in a mixture with d,l-acetoin-2,4-DNP at 108–114°C. The ultraviolet spectra of the pure enantiomer and of the racemate were identical [λ max. 243.3 mμ, $E = 18572$ and 356.6 mμ, $E = 33366$ (in chloroform solution)] and the infrared spectra in chloroform solution were superimposable (Fig. 2). Infrared spectra in KBr discs, however, were clearly different (Fig. 3). Very recently, Lindsay *et al.* (1962) reported rather different properties for d,l-acetoin-2,4-DNP (melting point 178–179°C, λ max. 259, 327 mμ). Such an ultraviolet absorption is unusual for 2,4-DNPs, hence their substance must have a structure different from that of acetoin-2,4-DNP.

According to the literature (Schormüller, 1961), fresh butter contains 1–2 mg/kg of diacetyl and up to 70 mg/kg of acetoin. It is obvious that much of the diacetyl-2,4-DNP isolated in this work has been formed from (−)-acetoin-2,4-DNP by excess of 2,4-dinitrophenylhydrazine, as mentioned previously.

We might add that the non-carbonyl part of butter distillate (fraction F) revealed none of the characteristic butter notes, and, especially, no lactone odor could be detected. According to a preliminary investigation (analysis by column chromatography and mass spectrometry), extract F contained different artifacts, 80% of which was 2,4-dinitrochlorobenzene, apparently present as an impurity in commercial 2,4-dinitrophenyl-

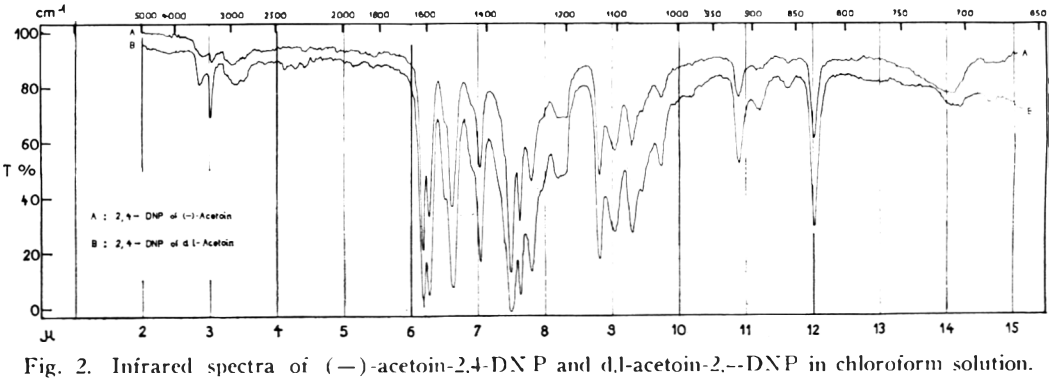


Fig. 2. Infrared spectra of (−)-acetoin-2,4-DNP and d,l-acetoin-2,4-DNP in chloroform solution.

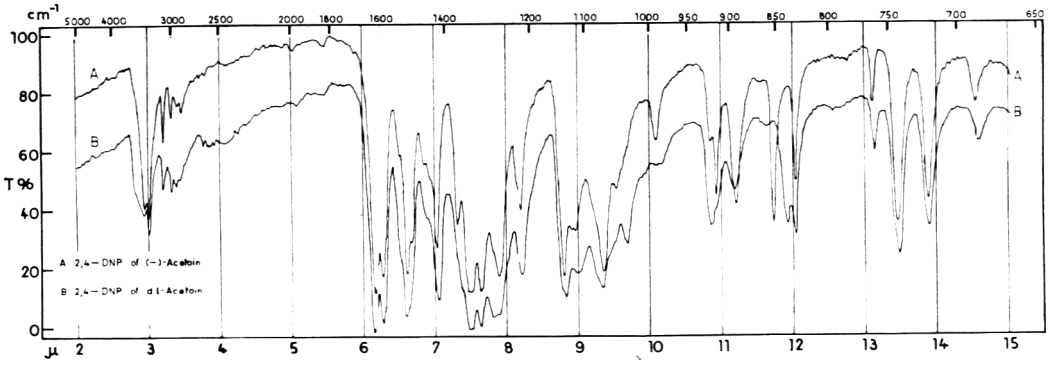
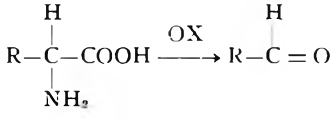


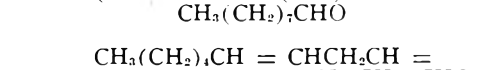
Fig. 3. Infrared spectra of (−)-acetoin-2,4-DNP and d,l-acetoin-2,4-DNP in KBr discs.

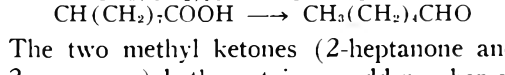
hydrazine. The remainder was a mixture of phthalic esters and of tributylphosphates, which were probably extracted from synthetic rubber tubes used erroneously to transfer the melted butter.

With the techniques already discussed, it was possible to identify the twelve substances mentioned in Tables 4 and 5. By what processes are these aldehydes and ketones synthesized in nature? It is to be noted first that the two branched-chain aldehydes cannot be derived from straight-chain polyketo acids by subsequent methylation. Their structures, however, do suggest that they result from enzyme-catalyzed oxidative decarboxylation of amino acids during the manufacture of butter. This well known biochemical process is entirely analogous to the oxidation of amino acids to the next-lower aldehydes by means of alkali hypochlorites.

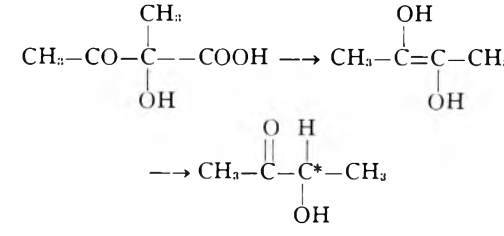


If this hypothesis is correct, formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, and phenylacetaldehyde would originate from glycine, alanine, valine, leucine, and phenylalanine, all amino acids known to be present in milk. In support of this theory tracer studies demonstrated that part of the acetaldehyde present in evaporated milk did indeed originate from alanine (Dutra *et al.*, 1959). *n*-Nonanal and *n*-hexanal may be products of oxidative degradation of oleic and linoleic acids, respectively.





The two methyl ketones (2-heptanone and 2-nonanone) both contain an odd number of carbon atoms, and we favor the possibility that they are produced by decarboxylation of β-keto acids, known to be involved in fatty acid synthesis and degradation. (See also Patton and Tharp, 1959.) Consequently, the ketones should be present in milk, and 2-pentanone and 2-heptanone were indeed found in evaporated milk (Dutra *et al.*, 1959; Wong *et al.*, 1958). Homologous methyl ketones are present in milk fat (Patton and Keeney, 1958). In this study they must have remained in the nonvolatile fraction, which was not analyzed. The formation of acetoin by decarboxylation of α-acetolactic acid is discussed elsewhere (de Man, 1959), and the isolation of optically active acetoin in this study suggests the intervention of an enzyme delivering a proton to the adsorbed enediol in a stereoselective manner.



According to this hypothesis, acetoin should be the precursor of diacetyl rather than vice versa, and recent experimental work (Dolezalek, 1958) agrees with this.

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Inhibiting Action of Oxidized Pork Fat on the Germination of Spores of Bacillus Subtilis [a]

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SUMMARY

Rancid lard, as determined by peroxide number, iodine number, and TBA values, inhibited the germination and/or growth of *Bacillus subtilis* spores. As peroxide numbers and TBA values increased and iodine numbers decreased, the number of *B. subtilis* spores which germinated and produced visible colonies decreased at constant incubation time. This inhibitory effect may be used as a criterion of pork fat rancidity.

INTRODUCTION

Roth and Halvorson (1952) found that rancid corn oil and lard inhibited the germination of spores of certain microorganisms, including *Bacillus subtilis*, whereas nonrancid samples at the same concentrations had little or no effect. Rancid and nonrancid unsaturated fatty acids and their methyl esters gave similar results. However, little effort was made to characterize the degree of rancidity of the fats and fatty acids, other than designation as Kreis + or Kreis −, or to determine the effects of varying degrees of rancidity on spore germination. This study was undertaken to determine the effects of uncured pork fat, oxidized for varying periods, on the germination and colony formation of spores of an aerobic microorganism, *B. subtilis*.

MATERIALS AND METHODS

Preparation of fat samples. Uncured composite fat trim of swine was dry-rendered in a steam-jacketed kettle and the lard stored in 50-lb cans under refrigeration. To obtain samples of varying rancidity, a 3-L quantity was heated to 96±2°C and oxidized by bubbling dry air at the rate of approximately 14 ft^3 per hour through the lard. A total of 13 samples were taken at half-hour intervals for 6 hr and stored in desiccators under nitrogen in a refrigerator until used.

Chemical tests. Each fat sample was analyzed in duplicate for iodine number by the Hanus method (AOAC, 1955); TBA value by a modification of the method of Turner *et al.* (1954); free fatty acid content, calculated as percent oleic acid, as outlined by the AOAC (1955); and in quadruplicate for peroxide number by the Wheeler method as outlined by Lea (1939).

Bacterial medium and spore suspension preparation. An agar medium composed of 0.3% beef extract, 0.5% peptone, 1.0% dextrose, 2.0% agar, 0.0025% bromothymol blue indicator, and a buffer ($Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4) was used in all studies. Plates of this medium were streaked with a 24-hr broth culture of a strain of *B. subtilis* obtained from the Microbiology Department of the University of Kentucky. After incubation, slants were streaked with a selected colony. After incubation of the slants, cells were harvested by washing the agar surface with distilled water. The resulting suspension was incubated 24 hr and heated 20 min at 80°C to kill vegetative growth. After this treatment, the cells were spun down in a centrifuge, washed, and stored in autoclaved sand. Spore suspensions were prepared by adding a loopful of spore-containing sand to the phosphate buffer solution. The approximate number of viable spores in the suspensions was determined by serial dilution and plating on the above-described agar medium. A dilution that gave approximately 1000 viable spores per ml was selected.

Addition of lard to agar, plating, and counting. To obtain an emulsion of lard in the agar medium, lard at 70°C and the agar medium at approximately 95°C were blended 1½ min in a Waring blender. The resulting emulsion was

[a] The investigation reported in this paper relates to a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

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poured into test tubes and autoclaved. The test tubes were then placed in a 45°C water bath before inoculation with 0.1 ml of the standardized spore suspension. The tubes were removed from the water bath and shaken vigorously, and the contents poured into sterile petri plates. This procedure gave a uniform distribution of bacterial colonies throughout the medium and retained the fat-agar emulsion. The colonies present after incubation were reasonably easy to count with the aid of the bromothymol blue indicator and a modified Spencer colony counter. At a pH near 7, the agar was light green, and the acid-producing *B. subtilis* colonies were yellow with a yellow surrounding zone. Modifications of the counter were: 1) removal of the baffle plate between the light source and glass window; 2) replacement of the lined glass window with an unlined glass window; 3) substitution of a 60-watt blue light bulb for the white light bulb; 4) use of a rheostat to vary the light intensity. The modifications produced a direct light that could be varied to provide optimum conditions for counting colonies in media of differing translucence.

RESULTS AND DISCUSSION

The effects of varying the pH of the agar medium from 5.8 to 8.2 are shown in Fig. 1.

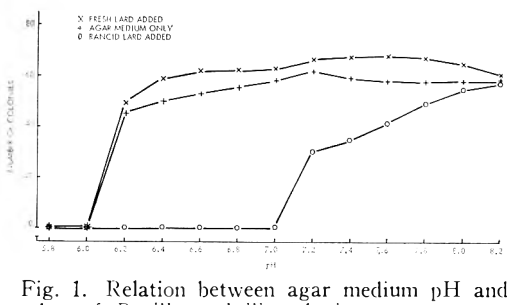

Fig. 1. Relation between agar medium pH and number of *Bacillus subtilis* colonies.

When the agar medium alone was used, no *B. subtilis* colonies developed below pH 6.2 after incubation for 20 hr at 37°C. The number gradually increased to pH 7.2 and then varied little, from pH 7.2 to 8.2.

When a 5.5% by weight concentration of fresh lard (iodine number = 61.5 and peroxide number = 2.5) was emulsified with the agar medium after pH adjustments, colony numbers were slightly higher than those obtained in the agar medium alone. Thus, fresh lard enhanced the germination of the spores, their outgrowth, and /or vegetative growth.

Rancid lard (iodine number = 55.5 and peroxide number = 155) that had been vigorously oxidized for 6 hr decidedly affected the number of colonies which developed from the spores. This sample was obtained from the same can as the fresh sample. No colonies developed in plates containing this rancid sample (emulsified with the agar medium) when the pH was below 7.2. The number of colonies increased rather rapidly as the pH rose from 7.2 to 8.2. At pH 8.2, both lard samples and the agar medium alone allowed development of almost the same number of colonies. The pH of the agar medium was not measurably changed by addition of the lard samples to the agar medium and subsequent emulsification. Therefore, the large differences in number of colonies which developed among plates containing fresh lard, rancid lard, and no lard cannot be attributed to a change in pH caused by lard additions.

An agar medium pH of 6.8 was used in subsequent studies. At this pH no colonies developed in plates to which very rancid lard has been added. However, colony numbers were near the maximum in plates containing fresh lard.

Fig. 2 depicts the relation between number of colonies and degree of lard oxidation. Also shown are the effects of incubation at 37°C for varying periods. Each value is an average of colony counts in 11 plates. After a 14-hr incubation period, an average of 88 colonies were visible in plates containing the agar medium plus a fresh lard sample (0 hr of oxidation). Colony numbers decreased as lard oxidation in-

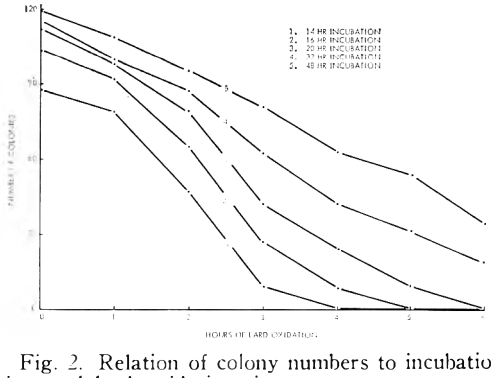

Fig. 2. Relation of colony numbers to incubation time and lard oxidation time.

creased until no colonies were visible in plates containing a sample oxidized for 4 hr. The number of colonies increased with incubation time. After 32 hr of incubation, colonies were present in plates containing even the most rancid lard samples. Increases in colony numbers tended to be greater in the more rancid samples than in the less rancid samples as incubation time increased. The decrease in number of spores that produced visible colonies in the presence of the more rancid fat samples appeared to be the result of a delay in germination and /or outgrowth rather than a spore-killing effect. Spores were able to overcome this inhibition after a sufficient period elapsed. However, the rancid fat affected vegetative growth also. Colonies in plates containing the more highly-oxidized fats did not become nearly as large as colonies in plates containing the less rancid samples or the agar medium alone. This size differential existed even after several days of incubation. Since the 20-hr incubation period gave complete inhibition during the preliminary work in the most rancid samples, and since it would fit more conveniently into a working schedule, it was chosen for subsequent work.

Table 1 and Figs. 3, 4, and 5 show chemical rancidity test results and *B. subtilis* colony counts for lard samples oxidized from 0 to 6 hr. Ten Petri plates were poured for each lard sample, and the colonies were counted after 20 hr of incubation at 37°C. There was no apparent relation

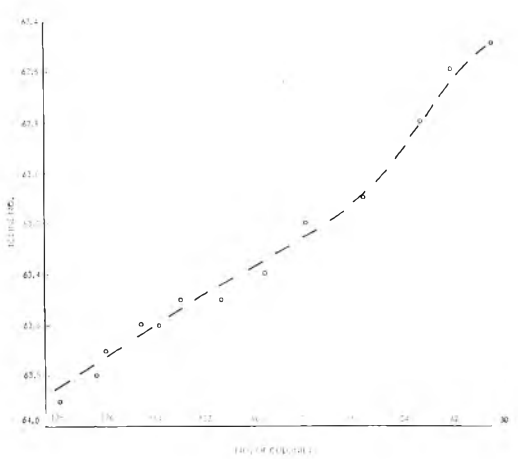

Fig. 3. Relation between iodine number and number of colonies.

between free fatty acid content and other chemical tests or number of colonies. Free fatty acid values and colony numbers showed a nonsignificant negative associa-

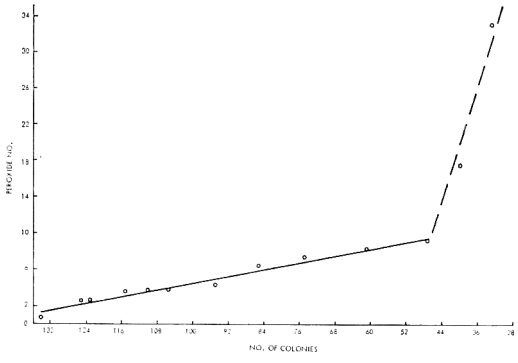

Fig. 4. Relation between peroxide number and number of colonies.

Table 1. Results of tests on lard samples oxidized 0–6 hours.

Hours oxidized	Peroxide no.	Iodine no.	Free fatty acid content	TBA value	No. of colonies
0	0.8	63.9	.146	.09	134
0.5	2.4	63.8	.225	.11	125
1.0	2.5	63.7	.212	.11	123
1.5	3.6	63.6	.244	.14	115
2.0	3.7	63.6	.245	.15	110
2.5	3.9	63.5	.304	.16	105
3.0	4.1	63.5	.380	.26	95
3.5	6.3	63.4	.208	.28	85
4.0	7.2	63.2	.157	.30	75
4.5	8.2	63.1	.267	.34	61
5.0	9.1	62.8	.262	.41	47
5.5	17.5	62.6	.328	.68	40
6.0	32.8	62.5	.284	1.20	33

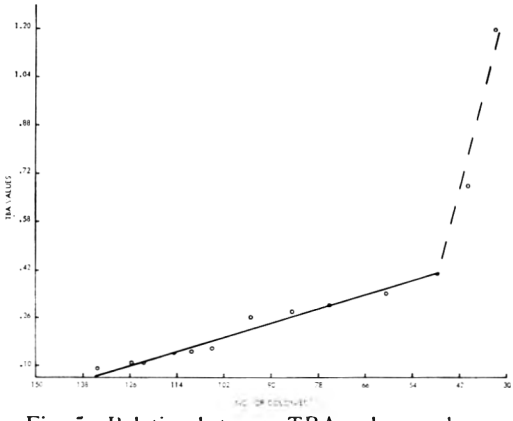

Fig. 5. Relation between TBA values and number of colonies.

tion ($r = -.40$). Iodine numbers and colony numbers had an almost straight-line relation (Fig. 3) with a significant ($p < .01$) positive correlation ($r = .99$). An almost straight-line inverse relation existed between peroxide number and number of colonies (Fig. 4) and between TBA values and number of colonies (Fig. 5) through the 5-hr oxidation period. Beyond this oxidation period, peroxide numbers and TBA values increased greatly, but the number of colonies continued to decrease at approximately the same rate. No exact explanation of these results is known. However, it is possible that the 5-hr oxidation period in this study was the critical period above which additional chemical rancidity, as measured by peroxide numbers and TBA values, had a diminishing effect on germination of *B. subtilis* spores, outgrowth and/or vegetative growth. The negative associations between peroxide numbers and colony numbers ($r = -.81$) and between TBA values and colony numbers ($r = -.84$) were significant at the 0.01 level of probability.

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The Recovery of Enterococci from Food Using KF Streptococcus Media

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SUMMARY

KF Streptococcus media were examined as to suitability for isolation and enumeration of enterococci from foods. Recovery of *Streptococcus faecalis*, *Streptococcus faecalis* var. *zymogenes*, *Streptococcus faecalis* var. *liquefaciens*, and *Streptococcus durans* was excellent with either a pour-plate technique or the liquid medium in an MPN technique. *Streptococcus bovis*, *Streptococcus equinus*, and *Streptococcus mitis* can be isolated with these media, but low recovery levels are obtained in the broth. Food constituents in concentrations as high as one part in five do not appear to affect the specificity of these media. The development of red colonies in the agar medium is indicative of the presence of *S. faecalis* or one of its varieties. The development of pink colonies in the medium or turbidity in the broth requires isolation and further study to determine the presence of enterococci.

The commercially available dehydrated media appear to give excellent results, comparable to those obtained with batches compounded in the laboratory; however, there is some indication that extended storage of the dehydrated form may lead to deterioration.

INTRODUCTION

The isolation and enumeration of enterococci in foods is an important aspect of food microbiology, because of their possible significance as indicators of fecal pollution, as well as their suspected role in food-poisoning outbreaks. A number of media have been used for this purpose, including: SF medium (Hajna and Perry, 1943); BAGG broth (Hajna, 1951); enterococci presumptive and confirmatory media (Winter and Sandholzer, 1946); the media of Barnes (1956); and azide dextrose broth (Roth) as recommended by Mallman and Seligmann (1950), with confirmation in ethyl violet azide broth of Litsky *et al.* (1953).

Kenner *et al.* (1960, 1961) described 2 media, KF Streptococcus agar and broth, which gave excellent results in the quantification of streptococci in feces and in surface waters. Because of these results, a study was initiated to determine the applicability of their media to food problems. This report describes results obtained with these media in the isolation and enumeration of enterococci from foods.

MATERIALS AND METHODS

The KF Streptococcus media used in this study were Difco KF Streptococcus agar (0997-01), Difco KF Streptococcus broth (0496-01), and similar media prepared in the laboratory according to directions by Kenner *et al.* (1961). The characteristic reactions in these media of enterococci isolated from water and feces are given by those researchers as follows: in KF Streptococcus agar plates, red colonies 0.3–2 mm in diameter are produced by *Streptococcus faecalis*, *Streptococcus faecalis* var. *zymogenes*, and *Streptococcus faecalis* var. *liquefaciens*, whereas pink colonies of similar size are produced by *Streptococcus durans*. In the KF Streptococcus broth, turbid growth, with an acid reaction demonstrated by a change to a yellow color, is typical for all enterococci.

The foods studied included specimens from food-poisoning outbreaks, meats purchased over the counter, and laboratory-prepared chicken-pot-pie filling containing chicken meat, carrots, potatoes, onions, peas, and celery in natural gravy. For recovery studies, 50 g of food were blended with 450 ml of buffered dilution water (APHA, 1960) for 2 min at slow speed in a Waring blender (no endorsement by the Public Health Service implied).

When enterococci and other organisms were

added to the food before blending, the inocula were prepared in buffered dilution water by making suitable dilutions of trypticase soy broth cultures that had been incubated 24 hr at 35°C.

The cultures of enterococci and other organisms used included fresh isolates of enterococci from fecal specimens, stock cultures from the Food Microbiology stock culture collection, and cultures kindly supplied by Mr. Bernard A. Kenner of the Microbiology Section, Division of Water Supply and Pollution Control, Robert A. Taft Sanitary Engineering Center. Stock cultures were maintained at room temperature in stock culture medium (formula kindly supplied by Dr. James C. Humphries, Department of Microbiology, University of Kentucky). To prepare a broth inoculum, growth from the stock-culture-medium tube was streaked on a slant of trypticase soy agar incubated 24 hr at 35°C, and a tube of trypticase soy broth was inoculated from the growth on the agar slant.

Plate counts were made with the KF Streptococcus agars by preparing suitable dilutions of the food blend in buffered dilution water, adding 1-ml volumes of each dilution to each of 2 plates, and pouring approximately 15 ml of melted and cooled (50°C) agar to each plate. Cover layers (not used in this study) may be used if only subsurface colonies are desired. When very low levels of recovery were anticipated, 10 ml of the 1:10 dilution were distributed evenly between 3 plates, and the total number of colonies on all 3 was recorded as the number of organisms per gram of food. Colony counts with the plates containing dilutions were made according to directions given by the APHA (1960). Counting was done after 48 hr of incubation at 35°C. The KF Streptococcus broths were used in 10-ml volumes in either screw-capped tubes, 150 × 15 mm, or metal-capped tubes, 250 × 20 mm. One-milliliter volumes of suitable dilutions of the food blend were added to each of 5 tubes, and the Most Probable Number (MPN) was calculated as directed by the APHA (1958) after 48 hr of incubation at 35°C.

The number of organisms per gram added to the foods was determined by preparing suitable 10-fold dilutions of the broth cultures used as inocula and plating suitable dilutions in duplicate in either brain-heart infusion agar or Eugonagar. The plates were incubated 24 hr at 35°C. The appropriate volume of the diluted broth culture was added to the food and blended as described above. This procedure was employed, primarily, to obtain an accurate estimate of the concentration of each group of organisms used in the mixed-culture experiments. Some loss of accuracy was experienced, however, in the pure-culture work. It is assumed that blending the food after inoculation resulted in chain disruption, and recoveries greater than the calculated addition level were obtained.

RESULTS AND DISCUSSION

To determine the efficiency of the KF Streptococcus media in terms of quantitative recovery of pure cultures of enterococci in foods, a series of experiments was carried out in which various numbers of organisms were added to chicken-pot-pie filling and the levels of recovery determined in KF Streptococcus agar and broth.

The enterococcal species *S. faecalis*, *S. faecalis* var. *zymogenes*, *S. faecalis* var. *liquefaciens*, and *S. durans* were added at levels from fewer than 10 to tens of millions per gram of food. In addition, similar studies were made with *Streptococcus faecium* and streptococci freshly isolated from feces. Representative results of these experiments are shown in Table 1. Although preliminary studies had shown that equivalent recovery of these species was obtained when dilutions of broth cultures of them were plated in brain-heart infusion agar, Eugonagar, or KF agar, the recovery from homogenates indicated that a greater disruption of the chains of cocci had occurred than had been anticipated. Consequently, it was found that in almost every instance the recovery levels were slightly higher than had been expected.

The results of these experiments indicated that both of the KF Streptococcus media recovered the streptococcal strains tested quantitatively in the range from fewer than 10 to several millions of organisms per gram. Furthermore, the recovery was not affected by the addition of the chicken-pot-pie filling homogenate at either high or low dilutions. These results also confirmed the findings of preliminary studies with broth cultures, indicating that the inhibitory substances present in the medium to render it specific had no deleterious effect upon the enterococci.

The colony characteristics of the various strains of streptococci in KF Streptococcus agar agreed with the descriptions of Kenner *et al.* (1961) in that *S. durans* and *S. faecium* produced pink colonies, and the other

Table 1. Recovery of pure cultures of streptococci from chicken-pot-pie by means of KF Streptococcus agar and broth.

Organisms	Calculated no. organisms added per gram (plate count of broth cultures)	Dilution of food plated	No. organisms recovered/gram KF *Streptococcus* agar (plate count)	No. organisms recovered/gram KF *Streptococcus* broth (MPN)
S. faecalis	3×10^7	1 ml of 10^{-6}	4×10^7	1×10^7
S. durans	3×10^4	1 ml of 10^{-3}	3×10^4	5×10^4
S. faecium	1×10^3	1 ml of 10^{-2}	3×10^4	3×10^3
S. faecalis var. *zymogenes*	4×10^2	1 ml of 10^{-1}	7×10^2	5×10^2
S. faecalis var. *liquefaciens*	3×10	10 ml of 10^{-1}	4×10	5×10
S. faecalis var. *liquefaciens*	3	10 ml of 10^{-1}	5	5
Fecal streptococci [a]	2×10^5	1 ml of 10^{-3}	2×10^5	5×10^5
Fecal streptococci [a]	2×10	1 ml of 10^{-1}	1×10	5×10

[a] Fresh isolates from fecal specimens.

species produced dark-red colonies. Furthermore, the reactions of these organisms in KF Streptococcus broth, even in the presence of 1 part in 10 of food homogenate, also corresponded to that described for water and fecal specimens. Acceptance of those researchers' criteria for typical enterococcal reactions in these media therefore appears to be justified.

The possibility that the addition of food homogenate to the medium might decrease its specificity was also examined. A series of experiments was carried out in which enterococci and other organisms were simultaneously added to chicken-pot-pie filling and recovery of the enterococci made in KF Streptococcus agar and broth. Relatively low levels of enterococci were used in these experiments, so that a maximum amount of food homogenate was added. Representative results of such experiments are shown in Table 2.

It was apparent from the results that the addition of 1 part in 5 of chicken-pot-pie homogenate (10 ml of a 1:10 dilution of homogenate distributed between 3 plates—3.3 ml of homogenate to approximately 15 ml of medium) did not affect the specificity of either the KF Streptococcus agar or broth. In only one instance, when *Escherichia coli*, *Pseudomonas aeruginosa*, and *Clostridium perfringens* were added with *S. faecalis*, was there any indication of interference with the usual activity of the media. In this instance, the KF broth yielded an excessively high MPN (20,000). The broth tubes containing the 10^{-4} and 10^{-5} dilutions of the homogenate were turbid, indicating growth; however, the color change was atypical in that a complete change to yellow did not occur. Gram-stained smears revealed the presence of many Gram-positive bacilli. It appears probable that this combination of organisms allowed the *C. perfringens* to grow and produce a slight amount of acid; however, subculture at the end of 48 hr failed to reveal the presence of viable bacteria other than enterococci.

In further substantiation of this lack of effect upon the specificity of the medium by food substances, the examination of 38 different foods from 10 outbreaks of foodborne disease during the past two years has failed to indicate any adverse influence. Included in this group of foods were fresh and cured meats, both cooked and raw, raw and cooked vegetables, pickles, bread, pastry, and fruit. As in our experiments with prepared food and with broth cultures of enterococci, each time red or pink colonies were picked and the cells biochemically tested, they were, without exception, found to be fecal streptococci. In addition, the red colonies were invariably identified as *S. faecalis* or one of its varieties.

Experiments were also carried out to determine the action of the KF Streptococcus media on the following species of organisms:

Table 2. Recovery of fecal streptococci from mixed flora in chicken-pot-pie filling by means of KF Streptococcus agar and broth.

Streptococci		Other bacteria				
Species or strain	No. added per gram (plate count of broth culture)	Species or strain	No. added per gram (plate count of broth culture)	Dilution of food plated	No. of streptococci recovered per gram in KF *Streptococcus* agar (plate count)	No. of streptococci recovered per gram in KF *Streptococcus* broth (MPN)
Fecal streptococci [a]	13	*E. coli*	2×10^3	10 ml of 1:10 [b]	10	7
		S. aureus	5×10^2			
		P. vulgaris	5×10^2			
S. faecalis	2×10^3	*E. coli*	2×10^3	1 ml of 1:10	2×10^3	2×10^4
		P. aeruginosa	1×10^4			
		C. perfringens	2×10^5			
Fecal streptococci [a]	16	*E. coli*	3×10^5	10 ml of 1:10	11	49
		S. marcescens	1×10^3			
		B. cereus	6×10^2			
S. durans	1×10^3	*E. coli*	22	1 ml of 1:10	1×10^3	2×10^3
		S. aureus	5×10^2			
		P. vulgaris	5×10^2			

[a] Fresh isolates from fecal specimens.
[b] Ten ml distributed among three plates.

Escherichia coli, *Staphylococcus aureus*, *Proteus vulgaris*, *Proteus rettgeri*, *Pseudomonas aeruginosa*, *Pseudomonas* sp., *Paracolobactrum intermedium*, *Alkaligenes faecalis*, *Clostridium perfringens*, *Clostridium bifermentans*, *Serratia marcescens*, *Bacillus cereus*, *Bacillus subtilis*, *Salmonella typhimurium*, and *Shigella flexneri*. One-milliliter volumes of undiluted 24-hr-old trypticase soy broth cultures and 1:10 dilutions in food of these same broth cultures were inoculated into KF Streptococcus agar and broth. No growth of any strains tested developed in either medium as the result of inoculation of the undiluted broth cultures of 1:10 dilutions of food.

Kenner *et al.* (1961) reported that *Streptococcus bovis*, *Streptococcus equinus*, *Streptococcus mitis*, and *Streptococcus salivarius* would grow in their media, producing acid and turbidity in the broth and pink colonies in the agar. To determine whether recovery of these organisms from food would also occur, a series of experiments was carried out in which strains of *S. bovis*, *S. equinus*, *S. mitis*, and *Streptococcus agalactiae* were added to chicken-pot-pie filling at levels of 10^2 to 10^3 organisms per gram of food (Table 3). *S. agalactiae* was recovered in KF Streptococcus broth but not in the solid medium. The remaining 3 species were recovered in both media. The recovery was not quantitative, however, and marked inhibition was observed in the KF Streptococcus broth. The growth characteristics of these species corresponded to the descriptions of Kenner *et al.* (1961) in every case.

In all of the previous studies, the enterococci were recovered immediately after addition to the food. It also appeared desirable to determine whether reasonably quantitative recovery could be obtained after the enterococci had been subjected to freezing, frozen storage, and thawing. Chicken-pot-pie filling was inoculated with various levels of freshly isolated enterococci, and recovery after 24 hr at 4°C and at −15°C was compared with recovery immediately following addition of the inoculum. Representative

Table 3. Recovery of Streptococcus species from food by means of KF Streptococcus agar and broth.

Species of *Streptococcus*	Estimated addition (plate count of broth cultures)	Recovery in KF Streptococcus agar (plate count)	Recovery in KF Streptococcus broth (MPN)
S. agalactiae	9×10^2	0	23
S. bovis	2×10^2	3×10^2	33
S. equinus	2×10^3	2×10^3	2×10^2
S. mitis	1×10^3	1×10^3	8×10

results of these experiments are shown in Table 4. The only variation in the technique used in the initial examination of the specimens was the tempering of the frozen specimens at room temperature until they could be completely blended in 2 min. The results indicated that recovery levels did not change appreciably, either after cold storage or freezing and thawing, and explained the consistent isolation of large numbers of enterococci from foods involved in food-poisoning outbreaks that had to be shipped in the frozen state to our laboratory for analysis.

It would appear that most, if not all, viable enterococci present in frozen-food specimens may be capable of initiating growth either in KF Streptococcus agar or in broth, and that these media may be useful in the analysis of frozen foods.

Since the primary purpose of this study was to determine whether the KF Streptococcus media could be used to isolate and enumerate enterococci from food substrates, exhaustive comparisons between media were not carried out. During the study, however, some comparisons of a general nature were made. In pure-culture studies, the recovery in KF Streptococcus agar was equivalent to that obtained with brain-heart infusion agar, azide dextrose agar, and Eugonagar. Barnes (1956) and mitis-salivarius agar media (Chapman, 1946) were used in spread plates, so no direct comparison of the limits of recovery can be made; but the specificities of the Barnes and KF Streptococcus agars were equivalent when tested with mixed cultures in food, and each was superior to mitis-salivarius and azide dextrose agar media.

Recovery with KF Streptococcus broth was equivalent to or better than that obtained with azide dextrose, the AD-EVA combination, or BAGG broths. In specificity, it exceeded all but BAGG broth, which appeared to be about equivalent.

In general, it appeared that the KF Streptococcus media were comparable, both in their limits of recovery and in specificity, to other media used in the examination of foods for enterococci, but more detailed studies would be required to establish its exact position in this respect.

Although the majority of the studies were made with dehydrated media, each type of study was also carried out at least once with media compounded in the laboratory. The formulation as described by Kenner *et al.* (1960) differs from the listed formulation of the dehydrated product in that it contains 0.636 g per liter of sodium carbonate. The adjustment of the pH of the dehydrated product, however, is made with sodium carbonate; therefore, it too contains the salt.

In some instances, media compounded in the laboratory were found to be somewhat more inhibitory to streptococci other than the enterococci (*S. salivarius*, *S. agalactiae*), and in one instance, strains of *Staphylococcus aureus*, *Proteus*, and *Bacillus* species grew in the dehydrated agar medium but not in the medium prepared in the laboratory. Since it has been shown by Gerencser and Weaver (1959) that sodium azide in a medium gives rise to hydrazoic acid, which volatilizes even at pH values as high as 7.6, it is probable that the shelf life of all azide-containing media should be carefully considered. In the above-mentioned instance, the bottle of dehydrated powder from which the batch of agar was prepared was nearly empty and had been in use for several months. It is possible that some loss of

Table 4. Recovery of fecal streptococci after exposure to refrigerator and deep-freeze temperatures.

Organism	Initial recovery		After 24 hr at 4°C		After 24 hr at −15°C	
	KF agar (plate count)	KF broth (MPN)	KF agar (plate count)	KF broth (MPN)	KF agar (plate count)	KF broth (MPN)
Fecal streptococci [a]	2×10^8	1×10^8	1×10^8	2×10^8	1×10^8	1×10^8
Fecal streptococci	2×10^6	1×10^6	1×10^6	2×10^6	1×10^6	1×10^6
Fecal streptococci	11	49	9	17	1	4

[a] Fresh isolates from fecal specimens.

sodium azide had occurred in the dehydrated product and suggests that care should be taken in resealing bottles of azide-containing media to prolong their specificity.

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Detection of Staphylococcus Enterotoxin
I. Flotation Antigen-Antibody System

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SUMMARY

Staphylococcus enterotoxin B has been concentrated and removed from aqueous solution by froth flotation. An anionic wetting agent and an enterotoxin B antiserum labeled with Rhodamine are added to the toxin solution. Compressed air is blown through the mixture and a foam is produced. The dye-toxin complex, located in the top layer of the foam, separates from the remainder to give a distinctly red-colored fraction. With a slight increase in air pressure, the red fraction is removed. The toxin is identified by taking one drop of the foam fraction, one drop of buffer at pH 4.5, and one drop of latex polystyrene suspension. Within 1 min a heavy agglutination occurs.

INTRODUCTION

Food poisonings of the type attributed to the Staphylococci are estimated as the cause of 500,000–1,000,000 cases of sickness each year. A simple and effective test for the presence of the enterotoxin in foods would be helpful as a preventive measure.

This paper reports a new approach to such a test. It consists of the flotation technique of mining technology combined with the antigen antibody technique of microbiology. The method is described in its application to aqueous samples known to contain enterotoxin B (Casman *et al.*, 1963).

The flotation method has been used for the separation and concentration of small particles from aqueous media (Hopper and McCowen, 1952; Hopper, 1945; Hopper and Khoobyarian, 1956). Gaudin (1957) points out that "in flotation the separation takes place in water in which the solid particles are suspended, and results from the adhesion of some species of solids to gas bubbles which are generated or introduced in the pulp." Excellent reviews of flotation may be found in the texts by Taggart (1951) or Sutherland and Wark (1955).

Application of the method to industrial wastes is described by Vrablick (1959), and a mathematical interpretation of flotation for solids-liquid separation is given by Howe (1958).

Flotation of the solids adhering to the bubbles permits their removal from the pulp as a froth of different composition from the pulp. Although the process is used in the mining industry to selectively adsorb certain minerals, its use in biological research has been more general in nature. For example, the cationic wetting agent used in water purification removed the anionic particles such as bacteria, mud, amoebic cysts, and viruses. The method had not been developed whereby one could separate or selectively adsorb a given organism from a mixture of bacteria. With development of the fluorescent antibody (FA) technique, it occurred to the author that a combination of the FA technique with flotation might have considerable merit for the concentration and identification of specific bacteria or a particular metabolic product. With this in mind, the following hypothesis was formulated with respect to the detection of Staphylococcus enterotoxin B.

Enterotoxin B antiserum reacts specifically with its homologous antigen. If the antiserum were labeled with a dye, one would expect the same reaction to occur. In addition to this, it might be possible to use a dye that would combine with a foaming agent similar to those used in flotation. Then, using the flotation procedure, it should be possible to concentrate and collect the dye in the froth or foam. If the dye has previously been attached to the antiserum, and if the antiserum has reacted with its antigen, then the froth should con-

tain surface-active agent plus dye-antiserum-antigen complex. In effect, it is suggested that a particular antigen antibody mixture can be concentrated from an aqueous solution by flotation. Furthermore, it is hypothesized that this procedure is selective in nature.

MATERIALS AND METHODS

Enterotoxin. Staphylococcus enterotoxin B, from strain S-6, is described as a 20% product because it appears to contain one-fifth of the amount of enterotoxin in the 100% product based on monkey feeding, gel-diffusion tests, and nitrogen content.

It is a cell-free partially purified lyophilized product of the liquid culture filtrate of a strain (S-6) of *S. aureus* that has not been known to cause natural outbreaks of food poisoning. The organism was first isolated from shrimp not implicated in foodborne disease. The 20% product will stimulate the production of several antibodies, as shown by multiple bands in gel-diffusion tests. A highly purified preparation (94%) can be made that will produce a single band. In the cruder preparation, dermonecrotizing and hemolytic toxins can be easily demonstrated. The dry material is hygroscopic and readily soluble in water.

Antiserum. Rabbit anti-enterotoxin serum prepared by Bergdoll *et al.* (1959) was labeled with Rhodamine B Extra Conc., a cationic dye, using the method of Moody (personal communication, M. D. Moody, Communicable Disease Center, U.S.P.H.S., Atlanta, Ga.). The carbonate buffer noted in Part II of the following outline was prepared by the method of Cherry *et al.* (1960).

LABELING SERUM WITH LISSAMINE RHODAMINE B-200 OR RHODAMINE B. EXTRA CONC.

I. Preparation of RB200 sulfonyl chloride
   1. Weigh 1 g RB200
   2. Weigh 2 g PCl_5 (handle with care)
   3. Grind (1) and (2) together in a mortar in fume hood for 5 min.
   4. Add 10 ml dry acetone (dried over $CaSO_4$) or Spectro Grade.
   5. Stir gently 5 min.
   6. Filter through no. 1 filter paper. Filtrate is the acetone solution of RB200 sulfonyl chloride. Stopper tightly to prevent evaporation of the acetone. Filter into a separatory funnel.

II. Preparation of serum
   1. Combine 1 volume of serum, 1 volume of 0.85% saline, and 1 volume of carbonate-bicarbonate buffer pH 9.0 (0.5*M*).
   2. Place in ice bath

III. Labeling
   1. Keep serum solution in ice bath during entire labeling process
   2. Add teflon-covered magnetic stirring bar to serum solution
   3. Place on Mag-Mix and stir briskly, but not fast enough to cause frothing
   4. Add RB200 sulfonyl chloride a drop at a time, over a 15-min period (0.1 ml sulfonyl chloride per ml original serum volume). Check pH during the period of addition with litmus paper. If solution becomes acid, add enough carbonated buffer to keep it alkaline
   5. Stir for 15 min after all sulfonyl chloride has been added. This completes the conjugation
   6. Place labeled serum in dialysis tube and dialyze in buffered saline (pH 7.2–7.5) for 6–8 days with daily changes. Use large volume (4–5 liters) of saline and dialyze at 5°C (41°F)

Feeding experiments. For feeding experiments, the Macacca mulatta (Rhesus) monkey was used. The animals varied from 2 to 4 kg in weight. All enterotoxin solutions were made by dissolving one mg of the crude material in 100 ml distilled water. Since this was a 20% product, the concentration of enterotoxin was 200 μg per 100 ml. Each animal was given 50 ml intragastrically. The animals were divided into groups of 6 each, were used for one feeding only, and were observed for 6 hr.

Flotation. For the flotation procedure, 1% Aerosol MA was used. This is dihexyl sodium sulfosuccinate, an anionic surface-active agent. It is used at the rate of 1 ml/L, though this may vary. The apparatus consists of 500-ml graduated cylinders, spargers, and a source of compressed air (Hopper and McCowen, 1952). A brief outline of the flotation procedure is as follows:

1. Label antiserum with Rhodamine B extra conc. by Moody method employing phosphorus pentachloride.
2. Add 1 ml labeled antiserum to 250–300 ml aqueous solution of enterotoxin in a 500-ml graduated cylinder.
3. Add 1 ml of 1% Aerosol MA to this system and bubble air through it. Do this slowly so as to get a dry foam
4. The foam produced contains the Aerosol plus the Rhodamine B antibody antigen complex as a layer of pink bubbles on top of a column of white bubbles
5. The pink foam fraction is collected, and two drops placed on a slide

6. One drop of an approximately 1:30 suspension of latex particles in phosphate buffer at pH 4.5 is added to the two drops of foam fraction on the slide
7. Mix these reagents with an applicator stick and observe for agglutination
8. A positive reaction is typified by a very rapid granular and thorough clumping (within 5–40 seconds of mixing) which results in the formation of both white and pink clumps suspended in a clear liquid phase. The length of time needed for clumping varies with the concentration of enterotoxin in μg per ml
9. A negative reaction is typified by a very slow formation (within 2–5 min) of only a few pink clumps floating in turbid liquid phase.

RESULTS AND DISCUSSION

Table 1 summarizes the results of monkey feeding tests following flotation of an enterotoxin solution. With Rhodamine-labeled specific antiserum, tests 1 and 2, the foam fraction was removed and the residue was fed after it had been treated with charcoal and filtered. This was repeated without treatment of the residue with charcoal. None of the animals vomited, indicating that treatment of the residue with charcoal had no effect. Since the residue did not cause vomiting, the enterotoxin had either been removed in the foam fraction or neutralized. In test No. 3 the residue was fed and no emesis occurred. Analysis of this residue by the Kjeldahl method was negative for ammonia or organic nitrogen. This was interpreted to mean that the enterotoxin is removed from the solution by flotation. For test No. 4 the foam fraction was reconstituted and fed, and no emesis occurred. In a series of control tests, starting with No. 5, a Rhodamine nonspecific serum (McCowen) was used in the flotation and the residue was fed. Four of six animals vomited. Thus, the enterotoxin was not removed from the solution by a labeled nonspecific serum. In test No. 6, an aqueous solution of Rhodamine was used, and the residue was toxic. In test No. 7, the solution of enterotoxin caused 5 of 6 animals to vomit. It was unnecessary to feed an aqueous solution of the Aerosol MA, since it was used in too small an amount to cause any sickness (Hopper *et al.*, 1959). Thus, the enterotoxin is removed from aqueous solution by means of flotation and a specific labeled antiserum.

Table 1. Monkey feeding tests following flotation with Rhodamine-labeled antisera.

Type of treatment	No. vomiting/no. fed
Remove foam fraction	
1. B crude antiserum. Treat residue with charcoal Filter, feed filtrate	0/6
2. B crude antiserum. Filter, feed filtrate	0/6
3. B purified antiserum. Feed residue	0/6
4. B purified antiserum. Reconstitute foam fraction and feed	0/6
5. Nonspecific serum Feed residue	4/6
6. Aqueous Rhodamine. Feed residue	5/6
7. Aqueous Enterotoxin B	5/6

It was obvious that the foam fraction was composed of material in which an antigen-antibody reaction had taken place. The identification of the enterotoxin in the foam fraction posed a difficult problem since it was felt that the method to be employed would have to be simple, would not involve expensive apparatus, or require advanced training in physicochemical methods. The latex slide agglutination procedure was adopted, and the investigation of its mechanism is summarized as follows:

1. Anion plus latex in buffer at pH 4.5—general dispersion
2. Rhodamine plus latex—heavy ppt., very large clumps
3. Rhodamine, anti-enterotoxin antiserum plus latex—moderate clumping, well distributed
4. Anti-enterotoxin antiserum plus latex—general dispersion, no clumping
5. Anion-Rhodamine-antiserum — good dispersion, some small clumps
6. Rhodamine plus buffer at pH 4.5 and no latex suspension—some particulate matter

7. Anion-Rhodamine-labeled antiserum, toxin, plus latex in buffer—excellent clumping in less than 1 min.
8. Anion-Rhodamine-labeled antiserum plus latex in buffer—general dispersion. If toxin added, excellent clumping.
9. Anion-antienterotoxin antiserum plus latex—small amount of clumping.
10. To reduce the effect of Rhodamine, take the foam fraction of No. 7 above and dilute it 1:10. Add latex and buffer—large clumps within 3 min
11. Take a drop of latex suspension and add one drop of 1% ceepryn (a cationic wetting agent). Clumping occurs, hence latex suspension is anionic
12. Take a drop of 0.5% egg albumen plus latex in buffer—moderate clumping and also a general dispersion of particles. Therefore, the clumping, or agglutination reaction is a nonspecific reaction

The general conclusion from this series of tests is that an anionic-cationic reaction is taking place in the slide agglutination procedure, and that this reaction is nonspecific in nature. This, however, has no importance as a diagnostic criterion, since the preceding technique (flotation) is specific.

Because egg albumen gave a positive reaction in the slide agglutination test, an experiment was done in which egg albumen was added to the original sample, which was used for the flotation procedure. In this case, no separation of Rhodamine-labeled antiserum took place. That is, a pink layer did not collect at the surface of the column of bubbles. This lack of separation into a distinct foam fraction is due to the action of the added protein. In mining technology, materials that interfere with flotation are known as depressants. With protein present, the entire reaction is self-limiting since, if no fractionation of separation into two distinct foam layers is produced, then there is nothing to concentrate or collect for the subsequent slide agglutination test.

To be more nearly sure that the flotation technique is specific, a series of tests were done with various antigen antibody systems. These are summarized in Table 2.

Table 2. Flotation antigen antibody systems.

Antigen antibody system	Slide agglutination
1. Rhodamine, aqueous 1%	negative
2. Rhodamine-labeled anti B purified serum	negative
3. Rhodamine-labeled anti B purified serum with:	
Sac culture filtrates 33,196E, 8 Dack, or 196E crude enterotoxin	negative
With 243, S-6 (20% B), or B purified	positive
4. Rhodamine-labeled anti B crude (20%) serum with 2463A	negative

Note that positive results occurred only with preparations containing Enterotoxin B. These were: 243 sac culture supernate, S-6 (20% B), and the purified B. All other tests were negative, since they contained either enterotoxin A, or none at all. A brief description of the reactants used above is as follows:

1. Antisera

B purified antiserum—a monovalent rabbit antiserum produced in response to a highly purified enterotoxin B.

Anti B crude antiserum—rabbit antiserum produced in response to a crude form of enterotoxin B formed by *Staphylococcus aureus* S-6. This is a polyvalent antiserum and not specific for enterotoxin B (see description of S-6 enterotoxin below).

2. Enterotoxins

B purified—a highly purified form of enterotoxin B produced by *Staphylococcus aureus* S-6. The purity of this enterotoxin is estimated at above 99%.

196E crude—a crudely purified form of enterotoxin A produced by *Staphylococcus aureus* 196E. The purity of this enterotoxin is estimated at approximately 20% and contains a number of antigenic materials (media components, metabolites, lytic products from cells, etc.) in addition to enterotoxin A.

S-6 crude—a crudely purified form of enterotoxin B produced by *Staphylococcus aureus* S-6. The purity of this enterotoxin is estimated at approximately 20% and contains a number of antigenic

materials (media components, metabolites, lytic products from cells, etc.) in addition to enterotoxin B.

3. Cultures

Sac cultures—these are very turbid suspensions of staphylococci in a few ml of buffer in the bottom of a 1-L Erlenmeyer flask. Suspended in the flask is dialysis tubing containing 100 ml of double-strength brain heart infusion broth. The tubing lies in intimate contact with the suspended cells. The system is incubated 48 hr at 37°C on a rotary shaker. Under these conditions, it is possible to obtain a strong concentration of toxin in a small volume of buffer. After incubation, the dialysis tube is removed from the flask, and the buffer solution containing the toxin and cells is filtered or centrifuged off, leaving a small volume of material containing a strong concentration of enterotoxin.

33 sac culture filtrate—sintered-glass (UF) filtrate of sac culture of *Staphylococcus aureus* 33. This strain has never been demonstrated to produce enterotoxin.

S-6 sac culture supernate—supernate from a sac culture of *Staphylococcus aureus* S-6. This strain characteristically produces large quantities of enterotoxin B and a very little enterotoxin A.

196E sac culture filtrate—sintered-glass (UF) filtrate of *Staphylococcus aureus* 196E. This strain characteristically produces only enterotoxin A.

2463A sac culture filtrate—sintered-glass (UF) filtrate of sac culture of *Staphylococcus aureus* 2463A, which characteristically produces only enterotoxin A.

243 sac culture supernate—supernatant from a sac culture of *Staphylococcus aureus* 243. This strain produces only enterotoxin B.

8 Dack sac culture supernate—supernatant from a sac culture of *Staphylococcus aureus* 8-Dack. This strain characteristically produces only enterotoxin A.

Flotation for the concentration and removal of solid particles from an aqueous suspension is a well established industrial practice. However, this report represents the first description of a combination of this technique in the mining industry being used with one from the field of immunology. That it works is quite evident from the experimental results reported. The surface-active agent Aerosol MA is only one of a number of anionic surfactants that can be employed. No extensive survey or series of tests were done to determine if this is the only one to use. It works very well, and is readily available. With respect to the use of Rhodamine, it is possible that it can be replaced by other cationic dyes. It is not necessary for the dye to be fluorescent, but it must be one that will react with the surfactant and be separated from the solution in the foam fraction during flotation. Furthermore, it is reasonable to suggest that other methods for attaching the dye to the antiserum can be used, such as diazotization.

As for specificity, the parameters of the procedure are determined by the limits of immunological technique. The use of the purified B-labeled antiserum detected the presence only of its antigen, enterotoxin B. For the detection of other enterotoxins it will be necessary to prepare their labeled antisera. Many of these materials are not available at present.

Note that this system can also be used for the recovery of specific organisms. Some preliminary experiments relating to this have been performed. With careful attention to the immunological aspects of testing, it is possible to concentrate and identify bacteria in a relatively short time. This may be useful as a possible rough screening technique in the bacteriological analysis of water, for example; in the search for salmonellae in foods; or in the detection of organisms from body fluids. Other investigators may wish to examine the usefulness of the system in these or similar areas.

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Factors Influencing Quality in Pork. B. Commercially Cured Bacon [a, b]

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SUMMARY

A total of 227 pork carcasses, varying in weight, chronological age, and loin intramuscular fat content, provided bellies for a study of some factors possibly associated with bacon quality. Tenderness scores of bacon significantly improved with a decrease in age and weight and with an increase in intramuscular fat content of the longissimus dorsi. The bacons derived from the 36–42-month packer carcasses were less tender than those from the other age and weight categories studied. Juiciness scores were not associated with carcass weight, age, or intramuscular fat content of the longissimus dorsi.

The bellies shrank approximately 13% during the curing, smoking, and skinning processes. There were differences in shrinkage due to weight and age groups, but no significant differences in shrinkage due to intramuscular fat content of the longissimus dorsi were found.

The mean value of the percentage of separable lean in bacon from the 227 carcasses was 31.7%. Average shrinkage during the cooking of the sliced bacon was approximately 66%. Lean bacon shrank significantly less than did fat bacon.

About 19% of the bacons were considered to be excessively salty, and originated from carcasses possessing a more highly unsaturated fat.

INTRODUCTION

The belly, one of the primal cuts of pork, represents about 12–15% of the carcass weight, and when cured, contributes substantially to the total value of the pork carcass.

Saffle and Bratzler (1959) demonstrated that fatness of pork carcasses had little influence on the palatability characteristics of bacon; however, increased fatness was inversely related to curing and cooking losses. White (1941) found that the tendency for bacon sides to become rancid was due primarily to an inherent characteristic of the fat, and that there was little relation between rancidity and known variations in either the processing conditions or the properties of the muscle tissue.

A study was conducted to compare the organoleptic properties of commercially cured bacon with chronological age, carcass weight, intramuscular fat as observed in the longissimus dorsi, and some other carcass traits.

EXPERIMENTAL METHODS

General design of experiment providing data for this series of publications. A total of 439 pork carcasses were selected and categorized on the basis of: 1) five degrees of intramuscular fat in the longissimus dorsi as subjectively scored on the cut surface of the muscle at the twelfth rib; and 2) according to either of five chronological age classifications designated the known-history group (4–4½, 6–7, 9–11, 15–18, and 36–42 months) or four carcass weight categories representing the unknown-history group (<125 lb, 155–170 lb, >200 lb, and 280–320 lb). Approximately

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equal numbers of barrows and gilts were selected for the three younger and the three lighter weight classifications, while packer sows were chosen for the heaviest weight group and the two oldest age categories. About eleven individuals were obtained for each butcher-type classification, and about seven carcasses were secured for each sow group. A total of 206 carcasses represented the known-history weight groups whereas 233 additional carcasses represented the unknown-history weight groups.

Over 90% of the known-history age groups originated from the University of Wisconsin swine farm. These animals received similar rations and were managed identically. During the summer months, pasture was provided, while ground alfalfa was supplemented in the winter rations.

Fatback and length measurements, in addition to several subjective scores, were determined on each chilled carcass before fabrication. Cutting procedures were similar to those outlined by Cole (1952). After fabrication, additional physical and subjective measurements were taken to estimate qualitative and quantitative carcass traits.

All left hams and a selected group of 227 left bellies were cured and smoked by accepted commercial procedures. The right hams and loins were aged five to eight days at 3°C to simulate normal marketing practices prior to sampling for chemical, physical, and taste-panel analysis.

Two 15-member semitrained taste panels evaluated both fresh and cured cooked slices obtained from the center of the ham, cooked fresh center cut loin chops, and cooked center cut slices of bacon. Flavor, juiciness, and tenderness scores were recorded on a six-point hedonic rating scale. Loin chops were seasoned with salt and baked at 163°C to an internal temperature of 74°C, while fresh ham slices were similarly seasoned, browned for five minutes, and then braised to 77°C internal temperature. The cured ham slices and bacon strips were broiled for approximately 10 min at 204°C.

Objective measurements included tenderness analyses by the Warner-Bratzler shear, denture tenderometer, wedge tenderometer, and the electric food grinder as described by Schultz (1957). Muscle firmness was measured by a modified precision penetrometer as described by Pilkington *et al.* (1960). In addition, pH, electrical resistance, expressible juice, and Hunter color-difference-meter measurements were made as defined by Kauffman (1959). Combined drip and evaporation shrinkages from curing and cooking were measured by weight loss of the product.

Proximate analysis for protein, moisture, and ether extract as prescribed by the AOAC (1955) were performed on the fresh loin and ham samples from which the subcutaneous fat had been removed.

Separate analysis of variance computations were made for the known- and unknown-history factorial designs. The analyses were used to compare intramuscular fat, carcass weight or chronological age, and sex with a number of qualitative and quantitative variables. Simple, partial, and multiple correlation coefficients were also calculated to further compare the traits studied. From means and standard deviations, differences were measured as prescribed by Student's t-test and by Duncan's new multiple range test. All values were tested for significance at the 5 and 1% levels of probability.

Specific procedure for this study. A total of 227 pork carcasses were selected from the original group of 439 and included animals of known- and unknown history. These carcasses provided bellies that were processed for the study of bacon quality. Fresh bellies from the left side of the carcasses were subjectively scored for lean-to-fat ratio on a 5-point scale (1 representing least desirable). The lean-to-fat score was based primarily on the percentage of exposed lean strips along the sides and ends of the belly.

The bellies were placed in polyethylene bags and quick-frozen in a −40°C blast freezer for 12 hr and stored at −20°C until time of curing. Prior to curing, the bellies were air-defrosted to an internal temperature of 5–7°C, and were then injecto-pumped with a commercial pickle containing sodium chloride, sugar, sodium nitrate, and sodium nitrite. The injected solution represented 5% of the original fresh weight of the bellies. The cuts were covered with a dry cure containing the same ingredients as the pickle solution. This procedure was designed to produce a finished product containing 1.75% (±0.5%) of salt. The curing required 5–7 days and was followed by smoking and heating to an internal temperature of 55°C. The bacons were subsequently skinned and chilled to a 2°C internal temperature and weighed to determine processing shrinkage. Each bacon was separated into a flank and brisket half, and a ¾-inch slice was removed from the flank half adjacent to the point of division for the subsequent physical separation of fat and lean. Twenty-five slices, each 0.15 inch thick, were removed from the mid-section of the bacon. After weighing, the slices were placed between two wire-mesh racks and cooked in a 400°F oven. The samples were rotated periodically to provide uniform heat penetration and browning during the cooking period of approximately 10 min. Each strip was blotted free of excess fat drippings and weighed to determine total shrinkage. One strip of bacon was provided for each of 15 taste pan-

elists for evaluation of flavor, juiciness, and tenderness on a 6-point hedonic scale as reported by Carpenter (1961).

Five ½-inch cores from the lean streaks of 1-inch-thick slices provided samples for the Warner-Bratzler shear measurement. Unfortunately, the muscles were not large enough to provide cores containing only muscle tissue, especially the smaller, lightweight bacons.

RESULTS

Analyses of variance showed significantly (<0.01) lower values for tenderness of bacon from the 36–42-month packer carcasses than the other age groups. In addition, tenderness score improved significantly (<0.05) with an increase in the marbling score of the longissimus dorsi. There were no differences in bacon juiciness scores due to variations in carcass weight, chronological age, or loin marbling scores.

No data were obtained for curing losses prior to smoking and skinning, because of the normal commercial procedures through which the bellies were processed. Table 1 presents the means for composite shrink due to curing, smoking, and skinning. These data, when analyzed by Duncan's new multiple range test, show that bellies from carcasses weighing under 125 lb lost significantly (<0.01) more weight during curing, smoking, and skinning than did bellies from the other unknown-history weight groups. Also, the 4–4½-month age group yielded bellies that shrank significantly (<0.01) more during processing than those from the other known-history categories. The other known- and unknown-history age and weight groups were not statistically different in the percentage of weight lost in processing. However, the known-history butcher carcasses provided bellies that shrank significantly more during processing than did the unknown-history butcher carcasses. There was no significant difference in this shrinkage due to variations in the intramuscular fat content of the longissimus dorsi muscle.

The mean value for the percentage of separable lean in bacon from the 227 carcasses was 31.7%. The standard deviations were quite large within and between age, weight, and marbling groups. There were no differences in the percentage of separable lean due to the effects of the intramuscular fat content of the longissimus dorsi. The bacon derived from the 4–4½-month age group, and the lightweight group (<125 lb) contained significantly more separable lean than the other weight and age groups.

Approximately 66% of the weight of the raw sliced bacon was lost through drippings and evaporation during cooking (Table 2). Analyses of variance of these data indicated a significantly (<0.01) higher percentage of cooking loss for bacons from butcher carcasses weighing over 200 lb than with the other unknown-history weight groups. Bacons derived from the lightweight carcass groups (<125 lb and 4–4½-months) shrank less during cooking than bacons from other weight and age groups.

The simple correlations for certain taste-panel characteristics are presented in Table 3. The bacon flavor and tenderness scores were significantly and negatively associated with bacon weight and shear force. Cooking loss was not related to flavor or tenderness, but was positively associated with bacon weight, yet negatively correlated with separable lean. Flavor was positively correlated with the percentage of separable lean, and tenderness was negatively correlated with the separable lean. Table 4 presents simple correlations between certain known-history carcass traits and the quality characteristics of bacon. The juiciness of bacon was not significantly related to

Table 1. Means and standard deviations of the percentage weight loss due to curing and skinning of bacon.

Marbling score [a]		Unknown history (weight groups)				Known history (age groups)				
		<125 lb	155-170 lb	>200 lb	280-320 lb	4-4½ mo.	6-7 mo.	9-11 mo.	15-18 mo.	36-42 mo.
1	Mean	18.8	9.0	8.9	12.0	24.7	15.2	19.7	10.8	10.9
	Std. dev.	2.4	0.0	1.0	0.0	0.6	1.3	1.1	3.1	3.4
2	Mean	16.9	14.2	8.3	12.5	23.1	14.8	18.4	19.5	8.5
	Std. dev.	1.2	2.0	0.1	2.7	1.0	0.5	4.8	6.5	1.6
3	Mean	17.1	10.9	9.2	8.2	21.9	13.3	9.1	12.4	7.2
	Std. dev.	1.6	3.4	0.4	0.9	1.4	1.7	1.1	1.1	2.7
4	Mean	14.4	12.9	9.1	12.5	24.0	13.1	9.9	16.9	15.9
	Std. dev.	2.0	2.0	0.6	2.7	0.9	2.2	1.0	7.8	4.7
5	Mean	11.7	12.8	10.4	11.6	29.9	14.2	14.3	15.3	13.6
	Std. dev.	2.8	1.2	2.1	0.5	1.4	0.8	2.1	3.7	0.0

[a] Marbling scores of 1 to 5 (5 representing loins containing abundant marbling).

Table 2. Means and standard deviations for percentage of cooking loss of bacon.

Marbling score [a]		Unknown history (weight groups)				Known history (age groups)				
		<125 lb	155-170 lb	>200 lb	280-320 lb	4-4½ mo.	6-7 mo.	9-11 mo.	15-18 mo.	36-42 mo.
		%	%	%	%	%	%	%	%	%
1	Mean	63.0	67.5	69.5	64.2	60.6	62.6	63.9	67.9	69.4
	Std. dev.	5.3	.....	4.8	5.3	4.9	2.8	3.2	1.1	2.8
2	Mean	65.6	65.6	68.4	65.1	62.8	62.3	67.9	65.4	65.3
	Std. dev.	2.7	3.6	4.2	3.3	3.0	3.4	2.2	5.1	2.0
3	Mean	65.6	66.3	68.6	66.2	55.4	64.6	66.4	66.9	69.0
	Std. dev.	7.7	2.4	2.2	5.1	1.3	2.6	1.9	3.9	7.3
4	Mean	64.8	67.4	70.7	69.6	60.5	67.8	68.3	71.3	68.0
	Std. dev.	4.6	4.3	1.7	3.4	2.9	3.4	2.0	4.3	3.7
5	Mean	68.4	70.3	71.6	68.6	55.9	63.8	70.0	72.1	71.1
	Std. dev.	2.1	3.4	3.6	7.7	2.1	3.5	3.5	9.5	.....

Total study: Mean = 65.99%; Std. dev. = 5.24%.
[a] Marbling scores ranging from 1 to 5 (5 representing abundant marbling in longissimus dorsi).

Table 3. Simple correlations between palatability characteristics of bacon from 227 pork carcasses.

	Simple correlations					
	Tenderness (score)	Juiciness (score)	Cooking loss (%)	Bacon weight (lb)	Shear force (lb/in.²)	Separable lean (%)
Flavor score	.46**	.42**	−.10	−.33**	−.21**	.13*
Tenderness score		.30**	.11	−.28**	−.17*	−.21**
Juiciness score			.00	.17*	−.06	−.09
Cooking loss (%)				.50**	−.04	−.52**
Bacon weight (lb)					.15*	−.35**
Shear force (lb/in.²)						−.03

* = P<0.05.
** = P<0.01.

Table 4. Simple correlations of carcass traits as related to quality of bacon from 120 known-history carcasses.

	Flavor (score)	Tenderness (score)	Juiciness (score)	Cooking loss (%)	Separable lean (%)
	r	r	r	r	r
Carcass weight (lb)	−.42	−.47	−.13	.54	−.25
Age (days)	−.48	−.60	−.04	.42	−.10
Loin marbling score	.03	.19	.06	.15	−.25
Lean cuts (%)	.14	−.08	−.35	−.65	.61
Belly weight (lb)	−.38	−.37	.20	.60	−.32
Bacon weight (lb)	−.40	−.38	.18	.60	−.51
Shear force (lb/in.²)	−.30	−.18	−.20	−.12	.10
Iodine number		−.37	.....	−.04	.29
Backfat thickness (in.)	−.31	−.19	.27	.63	−.49
Flavor score		.61	.49	−.13	.12
Tenderness score			.39	.01	−.13
Juiciness score				.11	−.11
Cooking loss (%)					−.51

(P < .05) r = .18.
(P < .01) r = .23.

carcass weight, chronological age, or loin marbling score, but was positively associated with backfat thickness and negatively associated with lean yield. The loin marbling score showed little association with the palatability characteristics of bacon, except for the low but significant (<0.05) positive correlation with tenderness. Age of animal adversely affected flavor and tenderness scores.

Nineteen percent of the bacons were excessively salty, according to the semitrained taste-panel. The salty bacon originated from carcasses having subcutaneous fats that were significantly (<0.05) higher in iodine number than those not described as salty.

DISCUSSION

These data suggested that the commercially cured bacon in the study was acceptable in palatability, with the possible exception of those originating from 36–42-month-old animals. This statement is applicable to the bacons that were not considered excessively salty. The extremely heavy and very light-weight bellies were difficult to process in a normal commercial procedure since machine adjustments were required prior to injection of the pickle. Also, the smoking and heating process remained standard regardless of weight of the belly; therefore, the light-weight bellies were subjected to a more rigorous heat treatment per unit of weight than were the packer bellies. This may account for the greater losses in weight of these light-weight bellies during processing. Tenderness was improved with an increase in intramuscular fat content of the longissimus dorsi; however, juiciness was not associated with marbling, weight, or age of the animal. It is generally recognized that fat influences the juiciness of a fresh product. A product such as bacon has an abundance of fat available for stimulation of the juiciness sensation, and this may account for the low associations of juiciness with marbling, weight, and chronological age. These data possibly agree with those of White *et al.* (1941), in which flavor was not related to fatness but was associated with the curing ingredients. Tenderness of the bacon depends primarily on the tenderness characteristics of the lean streaks in the bacon slice. Marbling, in the longissimus dorsi, was associated with the tenderness of this commercially cured product, as was the marbling with the loin chops as reported by Carpenter (1961). One might postulate that the muscles in the belly were likewise more highly marbled in the carcasses with highly marbled loins.

Bellies from light-weight carcasses lost a higher percentage of their weight during processing, but less weight during the cooking process. These data suggest the possibility of a higher percentage of skin on the light-weight bellies and more dehydration during the smoking and heating process. If more dehydration occurred during this process, then less dehydration would be anticipated during cookery.

With an increase in backfat thickness there was a significant decrease in the flavor and tenderness, but an increase in juiciness and percentage of cooking loss. These relations remained the same when only the butcher weight groups were considered. These data are in partial agreement with those of Saffle and Bratzler (1959) in which cooking losses increased with an increase in fatness, but are in disagreement with their report that no differences in the palatability existed in bacons from different finish groups.

The values for the iodine number analysis were significantly and negatively associated with flavor and tenderness, but positively correlated to leanness of the bacon. Also, the bacons that were salty were from carcasses possessing fat that was more highly unsaturated.

The backfat thickness was inversely associated with the subjective belly lean-to-fat scores and the percentage of separable lean from the bacon as confirmed by highly significant correlation coefficients of $-.66$ and $-.49$, respectively. Additionally, a highly significant correlation of $-.35$ was found between bacon weight and the percentage of separable lean.

The shear-force values were significantly related to the taste-panel tenderness scores, as shown by significant but low correlation coefficients. The lack of uniformity of the cores was undoubtedly responsible for the low correlations. The results indicate that the commercially cured bacon obtained from butcher carcasses was generally acceptable

in palatability regardless of age of the animal, carcass weight, or the intramuscular fat content of the loin.

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Hydrogen Bonding and the Varying Sweetness of the Sugars [a]

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SUMMARY

Sugar sweetness appears to vary with hydrogen bonding. When hydroxyl groups, which elicit sweet taste, are hydrogen bonded, ability to cause sweet taste appears to be restricted. The thesis is based upon consideration of molecular models, hydrogen-bonding measurements, and taste tests. The varying sweetness of different sugars, and the apparent anomalous sweetness of sugar anomers may be largely resolved by considering hydroxyl group bonding.

INTRODUCTION

Varying sweetness of sugars. Investigators have long been concerned with the varying and anomalous sweetness of the naturally occurring sugars. Although sweetness scores vary with method and physical state of the compounds, common sugars can be listed (Biester *et al.*, 1925; Cameron, 1947; Schutz and Pilgrim, 1957) in order of decreasing sweetness as fructose, sucrose, glucose, galactose, mannose, lactose, and raffinose.

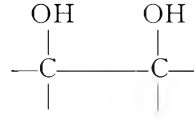
Fructose, the sweetest sugar, is 1.15–1.75 times as sweet as sucrose. Low values apparently correspond to mutarotated solutions, and high values to freshly prepared solutions, or to crystalline material. Raffinose in solution is only about 20% as sweet as sucrose.

The α-anomers of glucose, galactose and mannose are sweeter than the β-anomers. On the other hand, β-fructose and β-lactose are sweeter than their α-isomers (Tsuzuki and Yamazaki, 1953; Pangborn and Gee, 1961; Steinhardt *et al.*, 1962). Consequently phenomena which alter the equilibrium

$$\alpha \rightleftharpoons \beta$$

will also alter the apparent sugar sweetness. These include concentration and temperature (Cameron, 1947; Tsuzuki and Yamazaki, 1953). Tsuzuki (1947) pointed out that sweeter sugar anomers have a *cis* configuration between the anomeric and adjacent OH groups, but the rule does not hold for lactose. Böeseken (1949) assigned β-lactose the *trans* configuration.

The sugar —OH radical is recognized (Moncrieff, 1951) to be related to sugar sweetness. More specifically, the saporific group appears to be



Attempts to relate molecular and physical parameters to sugar sweetness include resonance energy (Tsuzuki *et al.*, 1954), vibratory hydrogen (Kodama, 1920) and solubility (Loginov, 1941). Carr *et al.* (1936) could find no relation between the number of carbon atoms, hydroxyl groups, molecular arrangement or spacial configuration and the sweet taste of sugar alcohols.

Physiological explanations for varying sugar sweetness include rate of diffusion into taste-bud receptors (Schutz and Pilgrim, 1957) and taste-bud receptor stereospecificity (Steinhardt *et al.*, 1962).

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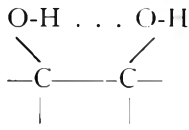
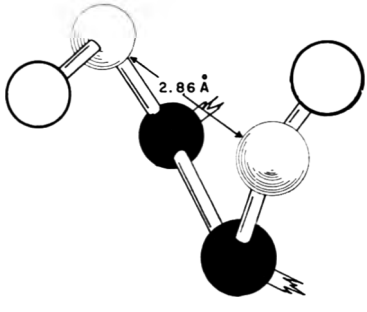
Recognizing that adjacent alcohol —OH groups may hydrogen bond (Kuhn, 1952),



it was postulated that the occurrence of the hydrogen bond might restrict the ability of the sugar saporific group to elicit the sweet taste response, and that the varying sweetness of the sugars could be largely resolved on the basis of this consideration. Evidence to support this hypothesis serves as the basis of this report.

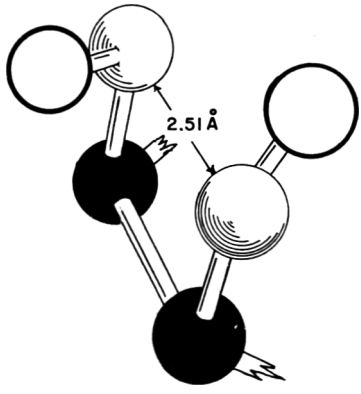
Hydrogen bonding in sugars. When the distance between two oxygen atoms is between 2.50 and 2.80 Å, a substituent hydrogen atom would be attracted to and, due to delocalization of charge, "bond" the second oxygen atom, O—H . . . O (Pauling, 1960). Certain sugar OH groups may bond in this manner. Reeves (1951) calculated that adjacent OH groups in five-membered planar or six-membered strainless rings have an O . . . O distance of 2.86 Å when the groups are *gauche*.



PROJECTED ANGLE, 60°

When the adjacent OH groups are *eclipsed*, the O . . . O distance is 2.51 Å.
It would be expected, therefore, that adjacent *gauche* sugar OH groups would hydrogen bond when the ring conformation or ring distortions cause the projected angle between OH groups to decrease; or if the adjacent groups exist in true *cis* or *eclipsed* arrangement.

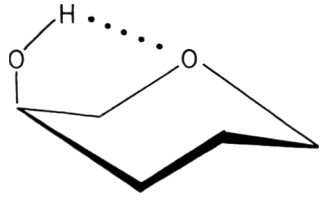
Infrared studies indicate that hydrogen bonding does occur in sugars. The strong composite OH absorption band occurring



PROJECTED ANGLE, 0°

at about 3222 cm^{-1} is associated (Kuhn, 1950) with O—H stretch. Konkin *et al.* (1958) showed that the OH groups of carbohydrates may be bonded singly, O—H . . . O, or doubly bonded, O . . . H—O . . . H. Correlation (Marrinan and Mann, 1954) of infrared with X-ray diffraction data showed that α-D-glucose has one singly bonded, and four doubly bonded hydroxyl groups.

Stereochemical studies (Brimacombe *et al.*, 1958; Barker *et al.*, 1959) based, for example, on infrared analysis of sugar-related tetrahydropyran-3-ol, have led Foster (1961) to suggest that the axial hydroxyl substituents on carbon atom No. 4 in α-galactopyranoside, and on carbon No. 2 in α-mannopyranoside (C-1 conformations) are sterically located to bond the ring oxygen.



GALACTOSE

METHODS

Molecular models. To determine probable sites of hydrogen bonding in sugars, structural molecular models (Brode and Boord) were consulted. Particular attention was paid to conformations and distortions that would result in diminishing 60° projected angles between adjacent *cis* hydroxyl groups, and also conformations leading to 0° projected angles between adjacent hydroxyl groups.

Infrared studies. Sugars were dried for 2 hr over phosphorus oxide at 3 mm Hg and 100°C. After preparing powders in a dental amalgamator, nujol mulls were prepared, and the near infrared spectra recorded with a Beckman IR-7 double-beam spectrophotometer equipped with a sodium chloride prism. Air was used as the reference cell, and a scanning speed of 40 cm^{-1}/min was employed.

Sharp infrared absorption bands in the region 3400–3600 cm^{-1} were assigned to free —OH absorption, as has been done with diols (Brimacombe *et al.*, 1958) and sucrose (Marrinan and Mann, 1954). Composite bands in the region 3200–3400 cm^{-1} were assigned to hydrogen-bonded —OH groups. The quantitative strength of the hydrogen bond was estimated as the arithmetic difference, $\Delta\nu$ (cm^{-1}), between ν (cm^{-1}) for the free —OH groups and ν (cm^{-1}) for the bonded —OH groups (Kuhn, 1952; Cole and Jefferies, 1956; Brimacombe *et al.*, 1958).

Taste tests. To compare crystalline sugars, panel members placed a few mg of one sugar on the tongue, and shortly thereafter a few mg of a second sugar. The members were then asked to score the relative sweetness on an unnumbered 10-point scale. After the tests had been randomly conducted, scores were assigned to the ratings, and "average" sweetness scores obtained. Ten to 15 persons participated in each test.

When sugar sweetness comparisons were conducted with solutions at various temperatures, the tests were again conducted as described. Care was taken, however, to ensure that reducing sugars were fully mutarotated. Sugar solution concentrations were 3%.

RESULTS

Since the β-anomer is the only known crystalline form of fructose, estimates as to why the "α-anomer" is less sweet were made from molecular models. The mutarotation of β-fructose consists mainly of a pyranose to furanose ring conversion (Isbell and Pigman, 1938). At equilibrium, the mixture is composed of 31.6% furanose form and 68.4% pyranose form (Anderson and Degn, 1962). In the furanose form, two hydroxyl groups are in a true *cis (eclipsed)* configuration. If these two OH groups should hydrogen bond, the sweetness of the compound would presumably be lowered. That they are closer is in evidence from consideration of sugar reaction rate with chemicals where the rate of reaction is governed by the proximity of the OH groups. Thus, the reaction of furanose compounds with lead tetraacetate is instantaneous (Barton and Cookson, 1956).

The chair (C-1) conformation of the pyranose ring is preferred (Barton and Cookson, 1956) either in solution, or in the crystalline lattice. As mentioned previously, axial OH groups on α-D-galacto- and mannopyranoside are sterically located to bond the ring oxygen. Should this bonding restrict the ability of the saporific groups to elicit sweet taste, it would be expected that these sugars would be less sweet than their epimer, glucose.

α-D-glucose presumably exists in a near true chair (C-1) conformation, and adjacent OH groups are *gauche*. β-D-glucose, however, exists in a couch (C-1) conformation (Lentz and Heeschen, 1961). Possibly, two OH groups approach an *eclipsed* arrangement, and should they bond, it would be predicted that β-glucose is not as sweet as α-glucose. A similar argument may hold for galactose and mannose. Interestingly, β-anomers of methylgluco- and galactopyranosides react more rapidly than the α-anomers toward cuprammonium solution (Reeves, 1951). This reaction also requires the close approach of hydroxyl groups.

Infrared studies. Infrared studies of hydrogen-bonding intensity confirm predictions based upon molecular models. Where studies were not possible with molecular models, infrared analyses indicate stronger hydrogen-bonding in the less-sweet sugars. Characteristic hydroxyl absorption spectra of sugars studied are shown in Figs. 1–3. Band positions for free and bonded OH, and the estimate of hydrogen-bonding strength, $\Delta\nu$, are shown in Table 1 where comparisons may be made to relative sweetness values. Qualitatively, the bands for the least-sweet sugars are less distinct,

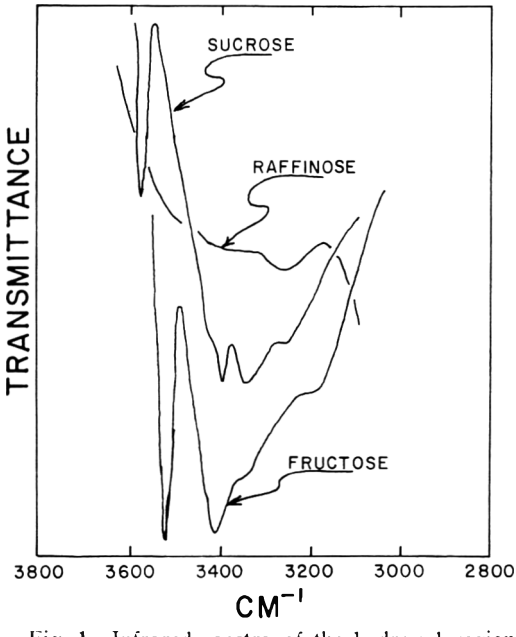


Fig. 1. Infrared spectra of the hydroxyl region of a fructose series of saccharides.

Table 1. Infrared hydroxyl band positions, ν (cm^{-1}), and hydrogen bonding strength, $\Delta\nu$ (cm^{-1}), for various crystalline sugars, and relative sweetness scores.

Sugar	Free-OH	Bonded-OH	$\Delta\nu$	Relative sugar sweetness: In solution [a]	Relative sugar sweetness: "Solid" state [b]
β-Fructose	3520	3400	120	115–175	180
Sucrose	3570	3395	175	100	100
Raffinose	.....	3180	(390) [c]	23	1
α-Glucose	3410	3315	95	64–74	74
β-Glucose	3545	3340	205	61	82
α-Galactose	3380	3210	170	32–67	32
α-Mannose	3450	3340	210	59	32
α-Lactose	3530	3360	170	16–38	16
β-Lactose	3460	3380	80	48	32

[a] Literature values; see especially Schutz and Pilgrim (1957) and Biester *et al.* (1925).
[b] Author's data.
[c] Estimated from sucrose-free OH position.

and displaced toward lower wave numbers, suggesting stronger hydrogen-bonding (Pimentel and McClellan, 1960), and $\Delta\nu$ is generally larger. The sweetest sugar, β-fructopyranose, has a very distinct free OH band. The least-sweet sugar, raffinose, apparently does not have a free OH band suggesting that all OH groups are bonded. The sucrose-free OH band position, 3570 cm^{-1}, corresponds well with the literature assignment (Marrinan and Mann, 1954).

In Fig. 2, α- and β-glucose may be compared. As indicated by the general nature of the spectra and $\Delta\nu$, it appears that the β-anomer is more strongly bonded. Since it is generally agreed that

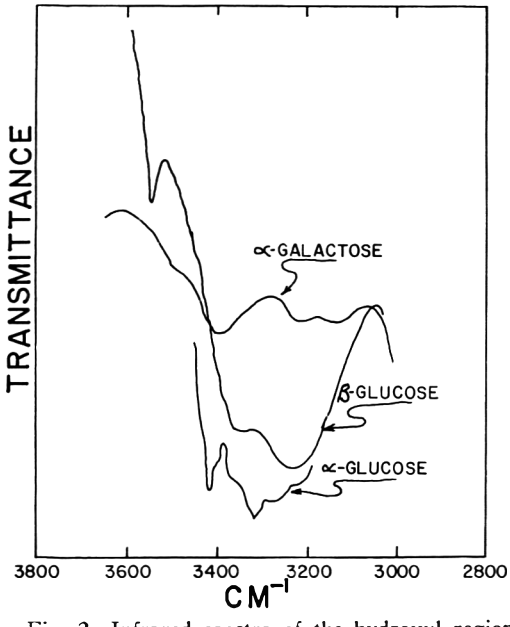


Fig. 2. Infrared spectra of the hydroxyl region of an aldose series of saccharides.

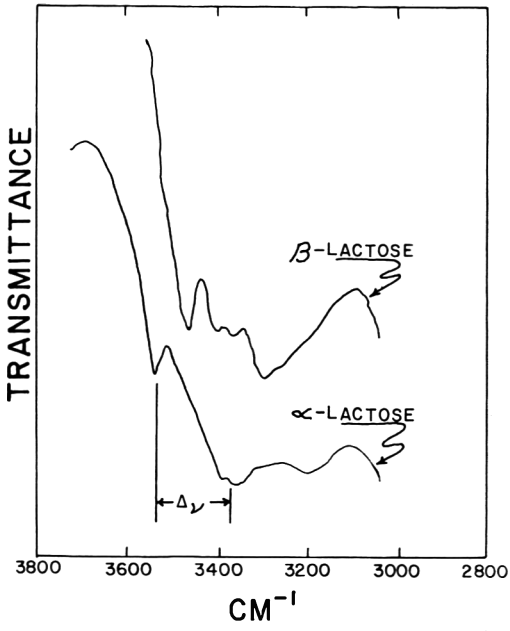


Fig. 3. Infrared spectra of the hydroxyl region of lactose anomers.

β-glucose is not as sweet as α-glucose, results with "solid" sugar (Table 1) appear contradictory. Possibly, the rapid rate of dissolution of crystalline β-glucose on the tongue makes it appear nearly as sweet as sucrose, but that final sweetness intensity is lower than that of even α-glucose.

α-Galactose gives spectral evidence of being much more strongly bonded than α-glucose. This is also true of α-mannose. Fig. 3 compares α- and β-lactose. Again the spectra suggest that the less-sweet α-form is more strongly bonded.

An empirical correlation between $\Delta\nu$ and relative sugar sweetness appears to exist. As shown in Fig. 4, the logarithm of relative sweetness scores

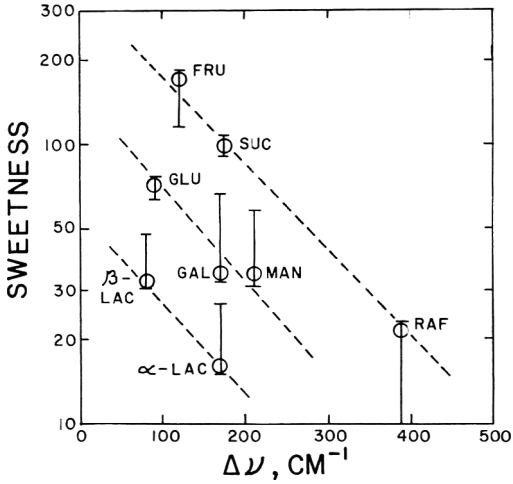


Fig. 4. Relation between the logarithm of relative sugar sweetness values and hydrogen bonding strength, $\Delta\nu$. Bars represent range of sugar sweetness values cited.

is approximately inversely proportionate to $\Delta\nu$ where comparison of one infrared spectra with another appears valid. For the fructose-series of sugars, the data of Biester *et al.* (1925) were used, whereas the author's were used for the aldohexose and the lactose-series. Substitution of other data—for example, that of Schutz and Pilgrim (1957), or the author's for the fructose-series—changes the slope of the curves.

Taste tests. It was possible to substantiate the thesis being developed with taste tests. Hydrogen bonds are severed at elevated temperatures. Therefore, a sugar that is not as sweet as a second sugar should approach the sweetness of the second sugar at elevated solution temperatures. Many comparisons were made between glucose and galactose, over the range 20–60°C. Highly significant regression equations fitted to average panel sweetness scores yielded

$$s, \text{glucose} = 0.027\,t + 3.6$$

and

$$s, \text{galactose} = 0.048\,t + 1.6$$

where s equals average sweetness score, and t equals temperature. These data suggest that the sweetness of glucose increases with increasing temperature, but that the sweetness of galactose, with reference to glucose, increased twice as fast. Both the ratio of the slopes of the curves, and the intercepts suggest that glucose is about twice as sweet as galactose.

DISCUSSION

Evidence presented suggests that the varying sweetness of different sugars and sugar anomers may be resolved by consideration of hydrogen-bonded hydroxyl groups. The most perplexing instances, nearly covering the sweetness-range of the naturally occurring sugars, have been discussed. While the concept of *intramolecular* hydrogen-bonding was the major basis of the argument presented, the role of *intermolecular* hydrogen-bonding cannot be excluded. $\Delta\nu$ for β-glucose seems much too large to be a measure of intramolecular bonding only, and there is a discrepancy concerning the sweetness of this sugar. It is possible that intermolecular hydrogen-bonding affects the rapidity with which sweetness is perceived, and that intramolecular hydrogen-bonding influences sweetness intensity.

The basis for assuming that $\Delta\nu$ is primarily a measure of intramolecular bonding is that a multiplicity of bonded OH absorption bands are not evident in the sugar spectra. Marrinan and Mann (1954) interpret carbohydrate OH spectra in this manner, with caution, however. If the OH spectra recorded were primarily inter O—H strain, raffinose pentahydrate should, perhaps, yield a very distinct OH absorption spectrum. As shown in Fig. 1, it does not.

Finally, the thesis that sugar sweetness varies with hydrogen bonding is not at variance with suggestions of others as to why sugars differ in sweetness. For example, resonance energy, solubility, and rate of diffusion into taste bud receptor sites, are probably influenced, in turn, by hydrogen bonding.

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Fluid Content and Tenderness of Three Muscles of Beef Cooked to Three Internal Temperatures

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(Manuscript received September 3, 1962)

SUMMARY

The semimembranosus, semitendinosus, and the longissimus dorsi muscles were excised from a Good-grade, and the longissimus dorsi from a Choice-grade, beef carcass, both of known history. Each muscle provided four roasts and samples adjacent to each roast for determination of total moisture in the raw meat and for tube-cooking. The meat was oven-cooked and tube-cooked to internal temperatures of 140, 158, and 176°F (rare, medium, and well-done).

When the data were analyzed statistically, no difference in the distribution of fluid in longissimus dorsi was shown between Choice grade and Good grade, nor was there a demonstrable difference between left and right sides in the muscles tested.

Total moisture content averaged 69.7, 64.7, and 60.3%, respectively, for 140, 158, and 176°F internal meat temperature, and mean press fluid as determined by the one-minute method, was respectively 54.0, 45.2, and 36.4%.

Bound water (not released by pressing but removed by vacuum oven drying) varied little when related to dry matter in the meat at each stage of cooking: 21–22 g for each 30 g of dry matter at 140, 158, and 176°F. Total fluid content (press fluid plus bound water) was respectively 76.0, 70.5, and 64.1%.

INTRODUCTION

Meat is one of the best-liked foods, having a satisfying quality and strong appetite appeal when it is well prepared. Factors determining the tenderness in cooked meat are not fully understood. When meat cooks, a portion of the connective tissue softens and becomes tender, especially in the presence of moisture. On the other hand, the lean parts may become firmer, even tougher, and the extent of this change seems to vary with animals. Just why does lean meat from one carcass become tougher than that from the same location on another carcass? Some factors are known, but not the basic reasons. Until they are, there is good reason to seek more information.

The recent interest in water relations in meat, and the indication that they are associated with tenderness in raw meat as reported by Wierbicki *et al.* (1957), led to the present study of total water in raw meat; the amount retained in meat cooked to internal temperatures of 140, 158, and 176°F; and "bound" water (the amount retained in cooked muscle after pressing under standardized conditions).

EXPERIMENTAL METHODS

The semimembranosus, semitendinosus, and the longissimus dorsi muscles were excised from a Good-grade carcass, and the longissimus dorsi muscles from a Choice-grade beef carcass, both of known history. Each muscle was cut crosswise into four parts and each part was wrapped in aluminum foil, enclosed in a polyethylene bag, labeled, and stored at 0°F or lower.

Twelve to fifteen hours before being cooked, the meat was removed from the freezer and placed in a refrigerator. While still partially frozen, a slice of meat about ½-inch thick was cut off and reserved for total moisture determina-

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Based on a study by Mabel Sanderson for the Ph.D. degree at Purdue University. Present address, Macdonald Institute, Guelph, Ontario, Canada.

tion of the raw meat and for tube-cooking. One-half-inch cores were cut in the remaining "roast" and left in place during cooking. These cores were approximately 2½ inches long.

Cutting cores in partially frozen meat before cooking was found to be satisfactory in preliminary work. Such cores were uniform throughout their length, did not dry out unduly during cooking, and were readily pushed out of a slice of cooked meat. The use of this technique shortened the time between the removal of the roast and its return to the oven for sampling at different degrees of doneness.

Oven-cooked meat. The meat was weighed and placed on a trivet in a pan with a loose-fitting cover, thermocouples were inserted, and the meat was cooked in an electric oven at approximately 220°F for the two lower temperatures. With meat to be cooked to 176°F, the oven temperature was increased to about 250°F after the internal temperature of the meat reached 158°F. Low oven temperatures were used in order that meat cooked to the different temperatures would also have cooked for appreciably different lengths of time. However, it was necessary to increase the oven temperature after the meat reached 158°F to avoid unduly long cooking.

Each piece of meat was sampled at two or three of the internal temperatures (140, 158, and 176°F), depending on the size of the roast. The sampling temperature was randomized.

The cores were located so that a thermocouple could be inserted into each, running at right angles to the core, and so that a slice of meat approximately 1½ inches thick, and cut off lengthwise, would contain at least two cores. A third thermocouple was inserted about equidistant from top and bottom and from either side of the roast—into a central core if three cores were to be taken at a given temperature, and midway between two cores if only two cores were used (Fig. 1).

When the lowest desired temperature was reached, the meat was removed from the oven and weighed, a slice was cut off, and the remainder, after weighing and inserting the thermocouples, was returned to the oven. The meat was out of the oven for about 5 min. When no sample was taken at 140 or 158°F, the meat was removed from the oven for 5 min when that temperature was reached, to equalize the conditions between different muscle portions.

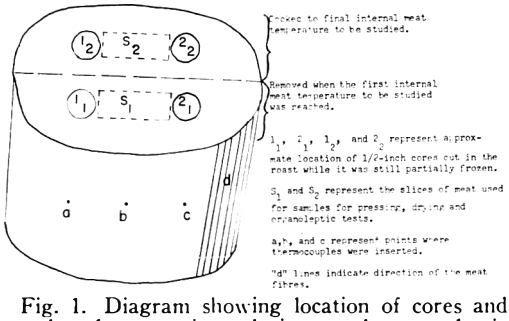

Fig. 1. Diagram showing location of cores and samples for pressing, drying, and organoleptic tests.

Cores were pushed out of the slice with a wooden plug and wrapped in foil. Samples of meat were taken from between the cores for determination of total moisture, press fluid, bound water, and organoleptic tests of tenderness and residue. The ½-in. cores were tested on the Warner-Bratzler Shear. Four or five shears were made at right angles to the fibers of each core.

Total moisture was determined by drying 15 hr in an oven at 158°F under a vacuum equivalent to 28 in. Hg. Weight losses during drying were used as total moisture content.

A strip of meat approximately ¼–⅜ in. wide, running from top to bottom of the slice and located between two cores, was removed for press-fluid determination. Four ½-g samples were taken from this strip, as near the center as possible. Press fluid was determined by the 1-min method (Sanderson and Vail, 1963). After pressing, the samples were dried as in determining total moisture, and drying loss was termed "bound" water.

A strip of meat, comparable to the one used for pressing, provided samples served to a panel of four experienced judges, who scored them for tenderness and residue. Tenderness was scored on a five-point scale, five being "very tender." A similar scale was used for residue, with the highest score representing little or no residue.

Tube-cooked meat. Tube-cooking of the meat is described elsewhere (Sanderson and Vail, 1963).

DATA STUDIED

Total moisture, press fluid, bound water, and total fluid were calculated as percent of muscle, and analysis of variance was done on the data. Percentage cooking losses were determined for the longissimus dorsi and semimembranosus when oven-cooked and taste-panel scores and shear values were obtained for all three muscles. The data were subjected to analysis of variance for a factorial experiment.

RESULTS AND DISCUSSION

Analysis of variance revealed no difference between sides of the carcass for the three muscles, or between grades for the longissimus dorsi. The data were pooled for the four longissimus dorsi, the two

semimembranosus, and the two semitendinosus muscles.

Total moisture for the different muscles and the different degrees of cooking were similar (Table 1). The respective mean values for the raw longissimus dorsi, semimembranosus, and semitendinosus were 73.5, 73.9, and 75.5%, compared to 74.2, 73.9, and 74.2% reported by Swift and Berman (1959).

The moisture content of the different muscles cooked in the oven to three internal temperatures was similar (Table 1), averaging 69.7% at 140°F, 64.7% at 158°F, and 60.3% at 176°F. This represented a marked decrease for each increase in end-point temperature. Cooking losses are known to increase with increased doneness (Cover *et al.*, 1957; Vail and O'Neill, 1937), so moisture content would be less at higher end-point temperatures.

When the values of the oven-cooked meat were compared with those for the tube-cooked meat, they were surprisingly close considering that the pieces of meat varied in weight from 228 to 922 g, for the oven-cooked meat, to about 25 g, for the tube-cooked meat. Also, the oven temperature for the lower end-point temperatures was approximately 220°F, whereas the tube sample was cooked in foil in a tube surrounded by water at 158°F, and the time required for the meat to reach 140°F was usually at least 2 hr in the oven but only thirty min in the tube.

Press fluid. Press fluid as percent of cooked muscle (Table 2) showed a highly significant difference due to internal temperature, but no effect could be attributed to muscle and no interaction was found between muscle and temperature for oven-cooked meat. There was a small but significant muscle-temperature interaction for the tube-cooked. At end-point temperatures of 140, 158, and 176°F, the mean percent press fluid was 54.0, 45.2, and 36.4, respectively, for oven-cooked meat, and 58.0, 41.6, and 33.0 for tube-cooked meat. The differences between these means emphasize the effect that increases in end-point temperature had on the amount of press fluid released.

Bound water. A highly significant temperature effect was found for bound water in oven-cooked meat, but none due to type of muscle and no interaction between muscle and temperature. The mean percent of bound water was 22.0 at 140°F, 25.2 at 158°F, and 27.7 at 176°F for oven-cooked meat, and respectively 24.2, 26.9, and 28.7% for the tube-cooked meat (Table 2). However, with increased cooking the total moisture in the meat became less and the amount of press fluid decreased. It therefore appeared that, if related to solids in the meat, the amount of bound water might be fairly constant. Calculating bound water to 30 g dry matter indicated this to be true, the respective amounts being 21.8, 21.4, and 20.9 g for 140, 158, and 176°F. The slight differences may be due to experimental error, or they may be real.

The ratio of bound water to dry matter was not as close for the tube-cooked meat, but close enough to indicate that the amount of water "bound" remained quite constant for the temperatures used.

Total fluid. Total fluid in this report refers to the fluid pressed out of the meat plus the moisture retained by the pressed tissue that was removed by drying in a vacuum oven, i.e., "press fluid plus bound water." Therefore it includes the soluble constituents and the colloidal fractions pressed out of the meat as well as the moisture. Thus, the amount or percentage

Table 1. Total moisture content of three muscles as determined by vacuum drying.[a]

Muscle	Raw	Oven-cooked			Tube-cooked		
		140°F	158°F	176°F	140°F	158°F	176°F
		%	%	%	%	%	%
Longissimus dorsi	73.5[32]	69.3	64.8	60.0	68.2	64.6	59.8
Semimembranosus	73.9[14]	68.8	62.2	60.0	68.3	62.8	58.9
Semitendinosus	75.5[16]	70.9	66.7	61.9	70.1	64.2	59.6

[a] Superscript numbers indicate no. of determinations.

Table 2. Average press fluid, bound water, and total fluid content of cooked meat (percent of cooked muscle).[a]

Muscle	Oven-cooked			Tube-cooked		
	140°F	158°F	176°F	140°F	158°F	176°F
Press fluid						
Longissimus dorsi	53.1[21]	47.1[16]	37.0[20]	50.4[21]	43.1[21]	32.0[16]
Semimembranosus	54.7[21]	43.2[21]	36.5[21]	49.5[21]	42.6[20]	31.1[12]
Semitendinosus	54.2[28]	45.9[28]	38.3[11]	50.0[24]	39.2[21]	33.2[12]
Bound water						
Longissimus dorsi	22.0[21]	24.2[16]	28.1[20]	23.8[21]	25.9[21]	27.4[16]
Semimembranosus	22.0[21]	26.2[21]	28.6[21]	24.6[21]	26.2[20]	29.6[12]
Semitendinosus	22.1[28]	24.9[28]	27.8[11]	24.3[21]	28.4[21]	29.7[12]
Total fluid content[b]						
Longissimus dorsi	75.1[21]	71.3[16]	65.1[20]	74.3[21]	69.2[24]	65.6[16]
Semimembranosus	76.7[21]	69.5[21]	65.1[21]	73.9[21]	68.8[20]	64.5[12]
Semitendinosus	76.3[28]	70.9[28]	66.1[11]	74.3[21]	67.5[21]	62.9[12]

[a] Superscript numbers indicate no. of determinations.
[b] Total fluid is press fluid plus bound water.

should be greater than the moisture content as determined by drying the unpressed meat. This proved to be true (Table 2), the mean total fluid being 76.0, 70.5, and 64.1% and total moisture 69.7, 64.7, and 62.3%, respectively, for the oven-cooked meat. The differences of 6.3, 5.8, and 1.8 for end-point temperatures of 140, 158, and 176°F are attributed to material in the press fluid other than water. The press fluid undoubtedly contained fat, which was evident as a greasy smear on the Plexiglas plate used in pressing. A small amount of minerals may also have been present, but it seems logical to assume that a certain amount of protein was also pressed out. The proteins may account for much of the differences obtained at the different end points. As meat is heated beyond 140°F, more of the proteins would be expected to coagulate, and this may account for the drop from 6.3 to 1.8% in difference between total fluid and total moisture. This would seem to agree with the findings of Child and Fogarty (1935) that the coagulable nitrogen fraction in press fluid of muscles heated to 58 and 75°C varied inversely with the interior temperature. On the other hand, tube-cooked meat did not show the same difference with the different end-point temperatures. Harrison *et al.* (1953) found that water-soluble heat-coagulable proteins actually increased between 158 and 176°F when chemical analyses were made. This is a point that seems to need further investigation.

Cooking losses. Cooking losses at 140, 158, and 176°F, determined for the longissimus dorsi and the semimembranosus muscles only, respectively averaged 9.8, 23.8, and 38.5% for the former and 10.9, 33.7 and 42.8% for the latter. There was considerable variation for each end-point temperature, which was to be expected considering the wide range in weight of roasts as well as the technique used, which required removal of the roast from the oven for sampling either once or twice during the cooking period.

Shear values and tenderness scores. Shear values (Table 3) indicate little change with degree of cooking in the force needed to shear the longissimus dorsi muscle, but a considerable decrease for the semimembranosus and semitendinosus muscles. Panel scores for tenderness tended to substantiate these findings. Connective tissue softens on heating (Winegarden *et al.*, 1952), but the muscle fibers become firmer, even tougher (Deatherage and Harsham, 1947; Satorius and Child, 1938), so the relative proportion of these constituents in a muscle would tend to determine whether or not muscle would become more tender when heated. A great deal of connective tissue could result in an increase in tenderness if conditions permitted the connective tissue to soften, whereas with very little connective

Table 3. Shear values and tenderness and residue scores.

Muscle	No. of muscles	Shear force (lb)			Tenderness panel scores [a]			Residue panel scores		
		140°F	158°F	176°F	140°F	158°F	176°F	140°F	158°F	176°F
Longissimus dorsi	4	3.5	3.5	3.7	18.6	19.2	18.4	17.8	19.0	18.8
Semimembranosus	2	6.2	5.7	4.7	16.6	15.7	17.2	14.1	16.1	17.3
Semitendinosus	2	6.7	5.5	4.1	17.4	18.0	18.5	17.0	18.0	18.0

[a] Maximum score possible for each roast was 20 (4 judges, each having a possible score of 5). The higher the score under "tenderness," the more tender; the higher the score under "residue," the less residue.

tissue, the effect on the meat fibers would predominate and the muscle might become less tender.

Strandine *et al.* (1949) stated that the semitendinosus has a large amount of connective tissue, both collagenous and elastic, the longissimus dorsi has only a small amount of each type, and the semimembranosus comes in between. The relatively low shear value for the longissimus dorsi at 140°F meat temperature substantiates the likelihood that there is very little connective tissue present. In this muscle, the increase in firmness of the fibers seemed to balance any change in connective tissue. In the semimembranosus and the semitendinosus the softening of the connective tissue appeared to have overshadowed the effect of heating on the muscle fibers, and decreases in shear values were found.

Comments of the judges tended to reinforce this assumption. Samples at 140°F were very soft when bitten into, and the first impression was one of great tenderness even though connective tissue was present. The judges became aware of the connective tissue in the 140°F samples when it failed to disintegrate readily with chewing. At 158°F the samples were firmer but the connective tissue was less evident. At 176°F internal temperature, the connective tissue gave no difficulty but the mouthfeel of the meat was dry, and frequently the meat was very friable or, as one judge noted, powdery though easy to bite through. The conflict between desirable and undesirable sensations made scoring the meat for tenderness a complex and difficult task. This recognition of different characteristics of tenderness supports, at least in part, the work of Cover (1959). In the present study the judges indicated a need for an evaluation of tenderness other than a simple numerical rating. However, no attempt was made to use the more detailed score card of Cover and Hostetler (1960).

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A Method for Determining Press Fluid in Cooked Beef

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SUMMARY

Methods are presented for cooking a small sample of meat in a Pyrex tube, termed tube-cooking, and for determining press fluid in a small sample (½ g) of cooked beef, referred to as the 1-minute method.

The 1-minute method was compared with the 15-minute method since both of these methods use the Carver Press. When the results were averaged and compared, the correlation was close. Besides being faster than the 15-minute method, the 1-minute method has the advantages that a small sample of meat is used, no grinding of the meat is necessary, and a greater percent of press fluid is obtained.

INTRODUCTION

Juiciness in cooked beef is a highly rated characteristic. Early workers tested the juice pressed out of raw meat. To obtain the juice from raw meat, Grindley and Emmett (1905) placed ground meat in a screw press under as much pressure as possible. As much as 700 ml of juice (30%) was obtained from 4½ lb of meat. Botazzi, as reported by Child and Fogarty (1935), obtained 40% and 63% of fluid from smooth and striated muscle, respectively, by applying a pressure of 350 atmospheres.

In another study (Empey, 1933) a pressure of 70 g per sq cm was exerted for 24 hr on raw meat in rubber bags. Child and Baldelli (1934) devised a "pressometer" whereby 2 or 3 g of meat were subjected to 250 lb pressure for 10 min.

Shortly after this, the hydraulic press began to be used. Vail and Hall (1937) reported using the Carver Laboratory Press and obtained 18–25 ml of juice per 100 g of cooked beef. This method was modified, and, as reported by Harrison *et al.* (1953) employed a gradually increasing pressure for 15 min. By this method, the juice pressed out was readily available for analysis.

More recently, the water-binding capacity of raw meat has been associated with quality and has been emphasized in some of the research, notably that of Hamm (1959), Wierbicki and Deatherage (1958), and Wierbicki *et al.* (1957).

The present report concerns a method for determining press fluid from cooked lean beef which employs filter paper and applied pressure similar to that of Wierbicki and Deatherage (1958), but uses the difference in weight of the meat sample before and after pressing to determine the amount of press fluid released.

EXPERIMENTAL PROCEDURE

Preliminary work tested the methods reported by Wierbicki and Deatherage (1958) and Wierbicki *et al.* (1957) for determining the water-holding capacity of fresh meat and the shrinkage of cooked meat. In the latter, 25 g of meat were cooked in a special Pyrex tube. This method was so simple and quick that in later studies a modification of this was used and is referred to as "tube-cooking."

Tube-cooking. Raw meat approximately ½ × ¾ in. in cross section and 2–2½ in. long, freshly cut from the meat under test and with a thermometer inserted 1/3 of the length, was wrapped in foil and fitted into a Pyrex tube designed for the centrifuge method (Wierbicki *et al.*, 1957). A foil cover, through which the thermometer extended, covered the tube. This tube was placed in a rack in hot water, which was held at 158°F until the meat reached 140°F, and then increased to 176°F for an internal temperature of 158°F or to 200°F for an internal temperature of 176°F.

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Based on a study carried out by Mabel Sanderson for the Ph.D. degree at Purdue University. Present address is Macdonald Institute, Guelph, Ontario, Canada.

When the required temperature was reached, the tube was taken from the heat; the meat remained in the foil wrapping until the temperature dropped to 104°F, and was then unwrapped and rolled once on a paper towel to remove excess surface moisture.

Determination of press fluid (1-minute method). Samples of cooked muscle weighing approximately 0.5 g (0.4–0.7 g) were used. These were cut from the inner portion of the cooked meat, where the internal temperature was taken, in such a way that the samples could be expected to have about the same temperature. Four samples were cut quickly, wrapped in aluminum foil, carried to the balance, and removed individually from the foil with tweezers, and each sample was placed between two numbered foils of known weight. The sample and the foils were weighed. Next, the sample, still between the foils, was transferred to a tray, and an inverted beaker was placed on top to anchor the top foil and prevent undue evaporation.

When the four samples had been weighed, each sample was transferred to a Plexiglas plate (6 × 6 × ¼ in.), the top foil was removed, and a piece of Whatman filter paper No. 42, 9 cm in diameter, was slipped between the lower foil and the meat sample, and a Plexiglas plate was placed on top. A second sample was placed on this plate in a similar way, and the process was repeated with the third and fourth samples. The whole pile of 4 samples and 5 Plexiglas plates was then placed in the Carver press with paper cushions above and below the pile. A pressure of 2000 lb (hydraulic pressure lb per sq in.) was exerted for 1 min.

After pressing, the samples were peeled off the filter paper, replaced between the same two foils, as before pressing, and weighed again. The loss in weight from the original weighing was termed "press fluid."

To check the reproducibility of the 1-min period, 4 pieces of meat each from the semimembranosus muscle from 4 different steaks purchased in the market were tested. Each piece was tube-cooked for 30 min at 158°F to an internal temperature of 140°F. Four samples were pressed from each piece.

Comparison of two methods for press fluid determination. The 1-min press-fluid method described above was compared in two experiments with the method used by Harrison *et al.* (1953), here termed the 15-min method.

In the first experiment to compare the 1-min and the 15-min methods, a pair of semimembranosus muscles from a Good-grade carcass were cut into 4 roasts each. An oven setting of 220°F was used for internal temperatures of 140 and 158°F. For 176°F the oven temperature was increased to 250°F after the meat reached 158°F. As each desired internal temperature was reached, the meat was removed from the oven, samples were taken as quickly as possible and wrapped in foil, and the roast was returned to the oven. The roasts were sampled for pressing at two of the following internal temperatures: 140, 158, and 176°F.

In the second experiment, muscles from right and left sides of the same Good-grade carcass were used for further comparison of the two methods. Internal meat temperatures of 122, 131, 140, and 149°F were used. Half of each biceps femoris served to test at four temperatures, while a whole adductor or semitendinosus muscle was used for two temperatures.

RESULTS AND DISCUSSION

When the 1-min method for pressing was used on tube-cooked steak samples in 4 experiments, the results were reasonably consistent (Table 1).

The tube-cooking method used in this study had a number of advantages. It was faster than oven-cooking, used less meat, and gave more even cooking. Meat cooked in this manner was desirable for testing purposes. This method is reported (Sanderson and Vail, 1963) to give results comparable to oven-cooking at 220°F.

The 1-min method for press fluid proved to be easy, and had the advantage of using a very small sample.

Tables 2 and 3 compare the 1-min method with the 15-min method used by Harrison *et al.* (1953), which requires a larger amount of meat. There was greater consistency in values determined by the 1-min method for both the semimembranosus and biceps femoris for meat cooked to internal temperatures up to 140°F. The two methods show similar variation at 140, 149, and 158°F. At 176°F, the 15-min method has a narrower range of values. The amounts of press fluid from meats cooked to 131 and 122°F were

Table 1. Percent press fluid using 2000 lb pressure for 1 min.

Sample	Steak 1	Steak 2	Steak 3	Steak 4
1	49.4	47.7	48.4	46.2
2	48.2	48.2	46.2	46.8
3	51.2	48.0	45.8	47.8
4	51.0	47.8	51.4	43.9
Av.	49.9	47.9	48.0	46.2

Table 2. Comparison of two methods for percent press fluid determination of the semimembranosus muscle.

Side of carcass	1-min method			15-min method		
	140°F	158°F	176°F	140°F	158°F	176°F
Left (top)	.....	48.4	35.9	.....	33.6	19.6
Left	56.5	41.7	.....	47.2	31.2	.....
Left	56.7	.....	37.6	42.0	.....	20.4
Left (bottom)	55.9	47.0	.....	38.1	29.1	.....
Right (top)	55.8	44.3	.....	38.4	26.8	.....
Right	56.1	.....	33.2	39.2	.....	19.6
Right	.....	41.5	32.7	.....	26.0	20.0
Right (bottom)	.....	48.5	31.6	.....	29.2	21.2
Av.	56.2	45.2	34.2	41.0	29.3	20.2

similar. This would be expected since the effect of these degrees of heat on the amount of press fluid would be small. In both cases the meat appeared to be practically raw.

The relation between the two methods seems to be fairly good, but not regular. For each increase in internal temperature from 131 to 140°F and 140 to 149°F, the amount of press fluid decreased by both methods. The 1-min method released a greater percentage. Sanderson and Vail (1963) demonstrated that the amount of press fluid decreased in a highly significant manner as internal temperature rose from 140 to 158°F and from 158 to 176°F.

Meat is not homogeneous, so the smaller sample should reflect the spread of values more accurately, and where fat or connective tissue is visible at least part of any difference can be accounted for by observation of such tissues. Where a small strip of meat can be cut so that a representative portion of lean, intramuscular fat, and connective tissue can be accounted for as well as any variation in degree of doneness, then the average of four ½-g samples, suitably spaced, should give the over-all value of the press fluid.

The 15-min method affords greater opportunity for loss of press fluid by evaporation during grinding and by adherence to the press parts or absorption by the cheesecloth and filter paper. An additional hazard was the possibility that the protein in the fluid would be coagulated by the high pressure and then exuded in a relatively solid state. The 1-min method avoided these difficulties, although some fat was pressed out and remained on the Plexiglas plate. With lean meat this was a relatively small amount.

The 1-min method gave higher values for the percent of press fluid released, but the correlation between the methods is good, r being 0.89 when computed from wide ranges of correlated values. These ranges are from 19.6 to 47.2 for the 15-min method, and from 31.6 to 56.7 for the 1-min method. As is usual, the value is less when the ranges of correlated values are less, the average within-temperature r being 0.39.

Table 3. Comparison of two methods for percent press fluid determination of the biceps femoris, adductor, and semitendinosus muscles.

Muscle	1-min method				15-min method			
	122°F	131°F	140°F	149°F	122°F	131°F	140°F	149°F
Biceps femoris	55.0	55.1	53.2	44.7	35.0	33.0	29.0	27.5
	55.6	56.4	44.6	39.3	38.8	37.5	24.2	19.3
	55.7	56.4	49.6	43.5	44.7	40.4	34.0	21.8
	55.6	57.3	49.4	48.4	40.8	33.6	29.6	24.8
Av.	55.5	56.3	49.2	44.0	39.8	36.1	29.2	23.4
Adductor	53.3	49.3	47.9	46.5	40.8	35.0	32.8	27.0
Semitendinosus	56.4	54.5	49.7	42.4	42.2	39.8	34.0	26.4

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Nutrient Composition of Cuban Foods [a, b] III. Foods of Vegetable Origin

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SUMMARY

One hundred and six samples representing 57 species and varieties of edible plants were collected (Mar. 4–Oct. 27, 1955) in Cuba. Eighty of the collections were made in the field, 19 in market places, and 7 in factories. In most instances, the food samples were trimmed to remove the portions not commonly eaten by the Cuban people. The edible portions were analyzed for content of moisture, lipid (ether extract), crude fiber, nitrogen, ash, calcium, phosphorus, iron, carotene, ascorbic acid, thiamine, riboflavin, niacin, tryptophan, lysine, and methionine. Results are expressed on the basis of grams or milligrams per 100 grams of edible portion, and are compared with data obtained from samples of similar food collected previously in Cuba.

INTRODUCTION

Initial steps in the development of a food and nutrition program for Cuba were taken several years ago when 360 samples of 137 representative edible plants were collected and analyzed for nutrient content. The results have been published (Lopez *et al.*, 1956; Navia *et al.*, 1954, 1955a, b, 1957).

The present report is concerned with the nutrient content of 106 additional samples representing 57 species and botanical varieties. The geographic origins of these samples were as follows: Oriente Province, 3; Camaguey Province, 4; Las Villas Province, 67; Matanzas Province, 15; Havana Province, 15; undetermined, 2. Eighty collections were made in the field, 19 in markets, and 7 in factories. Many of the foods were sampled because they were commonly consumed by the people; others were taken for analysis even though seldom eaten, because they represented plant species reputed to have high nutritional value and therefore potentially of greater use in the diets of the people.

METHODS

The techniques used in collection, preparation, shipment, and analysis of both fresh and dry samples were as reported for the earlier studies (Navia *et al.*, 1955a, 1957), and by Munsell *et al.* (1949, 1950a,b,c,d,e,f,g) in their study of the composition of Central American foods. Thus, a direct comparison may be made between the nu-

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tritive values of Central American and Cuban foods. The following nutrients were determined: moisture, ether extract, crude fiber, nitrogen, ash, calcium, iron, phosphorus, carotene, ascorbic acid, thiamine, riboflavin, niacin, tryptophan, lysine, and methionine.

DESCRIPTION AND USES OF FOODS STUDIED

Earth vegetables. *Asiatic lotus (Nelumbo nucifera).* The Asiatic or sacred lotus *(Yuca china),* is closely related to the water chinkapin and is grown in the New World principally for the beauty of its large pink and white flowers. In Asia, it is a sacred symbol of Buddhism and is widely grown for its edible seeds and starchy rhizomes. The rhizomes, the parts used in this sample, are usually cooked by boiling or steaming. The locally grown sample was purchased at a Chinese vegetable stall in the Cienfuegos market and was identified with the plant grown as an ornamental in the Atkins Garden. In preparing the sample, the roots were thoroughly washed and the outer part scraped off.

Garlic (Allium sativum). Garlic *(ajo)* is a basic flavoring material among peoples of Mediterranean origin, but the plant has been grown since remotest times for its underground bulbs and bulb offsets, or "cloves." The finely chopped or mashed "cloves" of garlic are used to add piquancy to other foods, such as lime juice, peppers, and cherry tomato, and to make a "sofrito" or seasoning for soups, meats and fish. The papery outer skin of the "cloves" was removed in preparing the present sample.

Turnip (Brassica rapa). The turnip *(nabo)* is an ancient crop plant of the Old World that produces flattened or globular white-fleshed roots. It is not grown as widely in Cuba as in cooler countries, but is occasionally found in markets, as was this sample.

Fruit vegetables. *Akee (Blighia sapida).* A tree native of West Africa, the akee was introduced in the West Indies many years ago. The fleshy gray arils that cap each of the three seeds of the fruit look like pieces of brain tissue (hence the name "seso vegetal," vegetable brain) and are considered safe as food only when the scarlet apple-sized fruits have opened naturally to expose the shiny black seeds and the arils. It has not found much acceptance in Cuba, although it is immensely popular in Jamaica, where the national dish is of the arils cooked with salt cod and seasoning.

Balsam pear (Momordica charantia). Native of Old World tropics, the balsam pear *(cundeamor* or *cundiamor chino)* is a highly variable climbing herb that has escaped from cultivation throughout the warmer parts of the world. The small, warty, yellow fruits are attractive when fully ripe, at which time they open to show the bright red arils. At this stage they are violently purgative and it has been reported that they are used as an abortifacient. In tropical Asia, they are soaked in salt water while still unripe and cooked as a vegetable or as an ingredient of curries. In Cuba, the plant is prepared in the same way as a vegetable, but it is used far more commonly in country medicine, with a host of virtues and special uses attributed to it. The sample was prepared from the whole green fruits purchased in a market.

Maya (Bromelia pinguin). A relative of the pineapple and similar to it in general appearance and in manner of fruiting, the maya *(piña de raton)* bears a fruit that is used primarily as an antihelminthic in country medicine. The white, mealy pulp of fully ripe fruits, which are rather like acid pineapples in taste, is occasionally eaten as fresh fruit. The plant is native in the Caribbean region and was formerly much used as an impenetrable cattle fence, before barbed wire became popular. The pulp alone was used for the three samples, after the hard outer rim had been discarded.

Herbage vegetables and flavorings. *Allspice (Pimenta dioica).* Commercial allspice *(pimienta de Jamaica)* is made from the dried, unripe berries of this small tree, which is native to the West Indies, Mexico, and Central America. The spice is recorded as one of the many ingredients used in the preparation of beverage chocolate in precolonial Mexico, and the plant caught the attention of the spice-and-gold-seeking Spaniards in the earliest days of exploration and settlement. In its native region the leaves are used for flavoring since they contain the same aromatic oils, although in lesser quantity. The sample was of the fresh leaves.

Amaranth, fruiting spikes (Amaranthus dubius). This is one of several species of amaranth *(bledo)* that are common weeds of wastelands and cultivated areas throughout the Americas, and are often used as pot-herbs. Other species, of South America and Eastern Asia, are cultivated for their seeds, which are used as pseudo-cereal grains. For these samples, the fruiting spikes containing the chaffy bracts and numerous small seeds in all stages of maturity were collected from the weedy plants.

Amaranth greens (Amaranthus hybridus). This amaranth *(bledo blanco)* is found fairly often in the markets, in the form of tender young plants, where it is sold as "espinacas," in substitution for European and Malabar spinaches. Sample (a) was of this nature and the other (b) of mature plants in flower.

Cauliflower (Brassica oleracea var. *botrytis).* The cauliflower *(coliflor)* is one of the many cultivated races of a wild species of the European coasts. In this race, a monstrous overgrowth and condensation of the flowering parts forms a structure, which when wrapped by the grower in the outer leaves, forms the tender, white edible mass. Although not common in Cuba, the vegetable was gaining increasing acceptance. It is easily grown as a winter (dry-season) crop with irrigation.

Justicia (Justicia pectoralis). Justicia *(tila* or *tilo)* is a low herb native throughout much of tropical America. In Cuba it is widely cultivated in dooryard gardens or in pots as a home medicine. From it is made a syrup for coughs and a mildly sedative tea for treatment of "nervousness." It is widely accepted in Cuba as a substitute for the true "tilo" of Spain, made of the flowers of European linden *(Tilia europea),* and is similarly prepared from the flower heads or, when these are not available, from the leaves. Freshly-collected 2–4-inch shoots were washed to remove soil and used for the sample.

Manioc shoots (Manihot esculenta). The actively growing leafy stem tips of the manioc or cassava plant *(yuca)* were collected because other laboratories (Munsell *et al.,* 1949) had reported it to have a high nutritional value. Only the starchy roots, previously reported on, are used for food in Cuba.

Mint (Mentha nemorosa). The samples of mint *(yerba buena)* were of the kind commonly grown in Cuba as a digestive stimulant and, less commonly, as a flavoring herb. The specific identification given here is tentative; it may be no more than a variety of the European horse mint *(Mentha longifolia).* The two samples were of freshly-picked upper stems and leaves.

Purslane (Portulaca oleracea). Purslane *(verdolaga)* is a common, succulent weed found throughout the warmer parts of the world. It is an ancient vegetable, and a number of selected races are grown in Europe as pot-herbs. Although it is widely used throughout its range, in either the weedy or the improved forms, it is not popular in Cuba except in the eastern province. The sample was of terminal shoots about 4 inches long.

Royal palm cabbage (Roystonea regia). The "cabbage" of the Cuban royal palm *(palmito de palma real)* is a delicacy, since this stately single-trunked palm is protected by law. It is composed of the tightly rolled creamy-white immature leaves in the center of the long, apical leaf bud of the palm, which is primarily a texture food, of a mild slightly nutty flavor, which may be eaten fresh as a salad or cooked like cabbage.

Spring onion (Allium fistulosum) The spring or welsh onion *(cebolla de la tierra)* was incorrectly identified as the chive *(Allium schoenoprasum)* in the second paper of the series (Navia *et al.,* 1957). It is similar to the chive in appearance and in use.

Squash (Cucurbita moschata). The flowers, vine tips, and shoots of the squash *(calabaza),* were reported by other laboratories to have high nutrient content (Munsell *et al.,* 1950a) These parts of the plant are not known to be used as food in Cuba.

Sweet potato (Ipomoea batatas). Terminal shoots of the sweet potato vine *(boniato)* were sampled, although there is no evidence that they are used for food in Cuba. Sample (a) was of 3–5-inch growing tips of the "Alquizar" variety; sample (b) of the same kind of material from a mixed planting.

Tea weed (Sida rhombifolia). The tea weed *(malva de cochino)* is a vigorous herbaceous or semi-woody weed found along roadsides and in disturbed soils throughout the warmer parts of the world. It is widely used in Malaya as a medicinal herb and is reported to be browsed by pigs in many parts of its range. The leaves and some immature fruits were stripped by hand from the strong fibrous stems of freshly-collected plants. In Cuba, the species appears to have only a limited human use—as a medicinal.

Legumes and oil seeds. *Adzuki bean (Phaseolus angularis).* The Adzuki bean *(frijol diablito)* is a twining annual from eastern Asia. It produces small, flattened, rounded, oblong, dark-red beans that have a white crest around the hilum. In Cuba it is grown primarily in the eastern province, where it is used like other dried beans, particularly with rice as a variant of the dish called "congri." Its history of cultivation in Cuba is obscure, and even its identification, until now, has been in doubt. The sample was purchased in a market near the place of origin.

Cashew nut (Anacardium occidentale). The cashew nut *(semilla de marañon)* is the seed contained in the small, green, kidney-shaped fruit borne at the end of the swollen peduncle, which is called the cashew apple, also reported here. The shell contains a violently caustic oil, which is driven off or detoxified in the roasting, after which the nut meats are extracted. The sample was of freshly roasted nuts harvested locally the year before.

Corojo (Acrocomia armentalis). The corojo *(corojo)* is a tall palm whose single, rather carrot-shaped stem is heavily covered with long spines when young. Native throughout Cuba, the palm is especially common in Camaguey province, where there was a small local industry for the preparation of an edible fat, "manteca de corojo,"

from the oily meat of the fruits. The fruits are yellow and about an inch in diameter, and are borne in large clusters. The hard, inner shell was cracked by hand, and the fresh meats extracted for the sample.

Macadamia nut (Macadamia ternifolia). The macadamia nut *(nuez de Macadamia)*, the product of a tree native to northeastern Australia, is now increasingly planted in the tropics and subtropics as a tree crop. The nut itself is very hard-shelled except in a few improved varieties, and is round and about an inch in diameter. It is rarely planted in Cuba. Freshly extracted meats were taken from a thick and hard-shelled seedling variety.

Pigeon pea (Cajanus cajan). The pigeon pea, or cajan pea *(frijol gandul)*, is a small erect shrub native of the Old World, now grown widely in the tropics and warm temperature zones either as a forage crop, or for its seeds, which are used either green (sample *a*) or dry (sample *b*) for both human and animal food. It is frequently seen in Cuba in farmyard and dooryard plantings, where it provides food for poultry, and is grown less commonly for human food.

Royal palm fruit (Roystonea regia). The large clusters of small, round fruits of the Cuban royal palm *(palmiche de palma real)* are cut while still on the palm and stored under cover until the outer skin and pulp are dry and fall off. The seeds are then used as pig food and are reputed to give the meat a fine texture and flavor. An edible fat for human consumption, once prepared from the oily endosperm of the seed, is apparently made no longer. The two samples were of fully ripe fruit, one green and one dried.

Squash seed (Cucurbita moschata). This sample was of whole seeds *(semillas de calabaza)* taken from a locally grown squash, washed free of adherent pulp, and dried. Little use seems to be made of cucurbit seeds in Cuba except in country medicine, where a few entire seeds are swallowed as a remedy for intestinal parasites. In other countries, such as Mexico, pumpkin seeds are a popular nibble.

Fruits. *Apple banana (Musa paradisiaca* var. *sapientum).* The small apple banana *(platano manzano)* is the sweetest and most aromatic of the varieties used in Cuba as dessert fruits. Unfortunately, it is highly susceptible to the serious Panama disease. The sample was prepared from ripe fruits purchased in a market.

Barbados cherry (Malpighia glabra). This small fruit *(acerola* or *cereza del pais)*, produced by a small shrubby tree native from Texas to northern South America, is eaten raw or cooked. Most wild plants bear rather small and acid fruits, and in Cuba are seldom eaten by humans. With the discovery of the extremely high ascorbic acid content of fruits from some plants of this species (Asenjo and Guzman, 1946) there has developed a selection and breeding program to improve the quality of the fruit while maintaining its high vitamin content.

Bignay (Antidesma bunius). The bignay *(bignay)* is a much more commonly grown relative of the Antidesma discussed in the second paper of this series (Navia *et al.*, 1957). It is a small tree bearing long, many-fruited clusters of small, dark-red to black berries, and is found wild from India through Malaya to northern Australia. Widely cultivated in the tropics, although sparingly in Cuba, it makes excellent jelly and jam but is of little importance as a fresh fruit. The juice of ripe fruits pressed through a jelly cloth was used for the sample.

Governor's plum (Flacourtia indica). The Governor's plum, or Ramontchi *(ciruela gobernadora)*, is a species from southern Asia closely related to the Paniala reported on previously (Navia *et al.*, 1957) and is also called "ciruela gobernadora" in Cuba. The small, spiny bush or tree bears round, plum-like but many-seeded fruits, which may be eaten out of hand or made into preserves. The plant is not well known in Cuba. The sample was of the soft, tan pulp of fresh fruit, which was separated from the skin and seeds by pressing through a jelly cloth.

Litchi (Litchi chinensis). The litchi *(mamoncillo chino)* is a small tree of subtropical and tropical China whose fruit is highly prized either fresh or sun-dried, the latter being the "litchi-nuts" of Chinese restaurants. It is now cultivated widely throughout the warmer parts of the world and exists in a number of varieties. The strawberry-red, ovoid fruits, borne in clusters, are about one to two inches in diameter and are covered with blunt angular tubercles. The outer leathery skin is peeled off, exposing the white parchment-like covering of the subtly-flavored, translucent, white juicy pulp. There is a single stone in the pulp, quite large in ordinary varieties and small in the best. The sample was prepared from the pulp of fresh fruits of the "Brewster" variety.

Mombin (Spondias purpurea). The purple mombin *(ciruela campechana o morada)* and yellow mombin *(ciruela criolla o amarilla)* are now considered to be two races of a single species. The plants are small trees native to the American tropics that are now widely planted for their fruits. They were given the Spanish name for plum *(ciruela)*, presumably because they resemble plums in having a single large stone, a slightly acid but agreeably flavored pulp, and a smooth, firm skin. These varieties are propagated easily

by planting branch cuttings, and this has led to dual use of the plant as a live fence post and fruit tree.

Miscellaneous. *Invert molasses (Saccharum hybrids).* Invert molasses *(miel rica invertida)* is produced by sugar mills when they have cane in excess of that needed to produce granular sugar. The processing method is the same as that used in the manufacture of granular sugar up to the point of partial concentration. Then about one half of the total sucrose is "inverted" to fructose and glucose by treatment with acid or, more commonly, by enzymatic hydrolysis. Since the resulting syrup cannot be crystallized it is not included in the granular sugar quota. After further evaporation to reduce the water content, the invert molasses is sold mostly to distilleries, and minor amounts are used in cattle feed. This sample was collected during the grinding season at a commercial sugar mill that produced invert molasses by use of a commercial preparation of invertase.

Sugar cane juice (Saccharum hybrids). These samples of sugar cane juice *(guarapo de caña)* were taken in order to determine differences in composition of the juice taken from the first crusher rolls *(guarapo del primer molino)* and the last, or fifth, rolls *(guarapo del quinto molino).* POJ2878 was the main cane variety being ground. A third sample of cane juice was purchased from one of the many small retail establishments that sell fresh juice extracted by small hand or power crushers. The sample was from the cane variety "Pepe Cuca," a rather tender-rinded variety that crushes easily in such machines.

RESULTS AND DISCUSSION

Table 1 presents the data obtained. It must be remembered that these data relate to raw foods and that the content of certain nutrients may be somewhat lower after preparation for human consumption. It would be ideal if each of these foods could be evaluated and compared in terms of the contribution that one serving would make toward supplying the dietary requirement of each nutrient. This is not possible, because no data are available relating to the size of food serving in Cuba. It is not satisfactory to compare these foods on the basis of the amount of each nutrient per 100 grams or per 100 calories, since certain foods (such as parsley) are consumed in small amounts whereas others (such as potatoes or rice) are eaten in large quantities daily. In the following discussion, therefore, the comments are made in terms of similar types of foods consumed by people in other areas of the world.

In the following paragraphs the figures quoted refer to "mg per 100 g wet weight" unless otherwise noted.

Cereals. *Corn.* The whole corn meal contained more iron (6.51 mg) but less thiamine (.26 mg) than usual. The green corn contained normal amounts for carotene, iron, and thiamine.

Earth vegetables. *Asiatic lotus.* The Asiatic lotus contained the most ascorbic acid (65.9 mg) among all the earth vegetables studied.

Carrots. Carrots are usually rich in carotene (Navia *et al.,* 1955a, 1957). Of the three samples analyzed, *a* was highest (7.05 mg) in total carotene, even higher than the value reported in a previous publication (Navia *et al.,* 1957).

Garlic. This sample contained significant amounts of phosphorus, iron, and thiamine, but its limited use as a seasoning prevents it from making a significant contribution to the diet.

Malanga. Though consumed abundantly, this root does not have outstanding nutritional value and its use as food should be discouraged. The white variety contained much less carotene than the yellow variety.

Potato. Besides starch, the principal nutrient found in the potato sample was ascorbic acid (32 to 41 mg), an amount equivalent to an equal weight of citrus juice. Of the two varieties, sample *a* was higher in ascorbic acid, in niacin, and in iron content.

Fruit vegetables. *Akee.* Akee is richer in lipid content (15%) than any other fruit vegetable in Cuba except for avocado (Navia *et al.,* 1955a, 1957). It contains moderate amounts of nitrogen (1.12%) and is a good source of iron and carotene. It was reported recently (Fox and Miller, 1960) that akee contains an antimetabolite of riboflavin that may have significant effect on its nutritive value.

Balsam pear. The fruit of the balsam pear is commonly used in infusions. It was found to contain a significant amount of ascorbic acid (75.0 mg).

Maya. This fruit is a relative of the pineapple, but is seldom consumed except

for medicinal purposes. It contained very significant amounts of ascorbic acid (93.8, 121.3 mg).

Pepper. The six samples of peppers studied represented three pairs of green and ripe fruit. In agreement with previous reports (Navia *et al.*, 1955a, 1957) the carotene, riboflavin, and ascorbic acid values were higher in the ripe than in the green fruit.

Plantain. Even though the plantain is usually a good source of carotene, the three samples analyzed were only fair (.50, .71 mg). The green samples (*a, b*) contained less carotene than the ripe sample (*c*).

Squash. The carotene and niacin contents of both samples of squash were nearly the same as previously reported (Navia *et al.*, 1957).

Tomato, cherry. This type of tomato, which is used to season foods, was unusually high in carotene.

Herbage vegetables. *Allspice, leaves.* The allspice leaves contained very significant amounts of carotene (4.73 mg), calcium (209.0 mg), and ascorbic acid (80.9 mg).

Amaranth, fruiting spikes. The fruiting spikes of amaranth were higher in nitrogen and lysine content than the amaranth greens, but lower in carotene and ascorbic acid.

Amaranth, greens. The two amaranth samples were found to be excellent sources of iron (4.16, 6.30 mg), calcium (361.5, 238.0 mg), carotene (3.57, 3.29 mg), ascorbic acid (113.0, 67.5 mg), and lysine (244, 120 mg). The nitrogen content was only fair (.73, .38).

Cabbage. The two samples of cabbage were moderately rich in ascorbic acid.

Cauliflower. The one sample gave a high value for ascorbic acid.

Chard. The chard was lower in calcium, carotene, and ascorbic acid than previously reported (Navia *et al.*, 1957).

Justicia. These fragrant leaves were significantly high in calcium (663.0 mg) and iron (7.40 mg), and were rich sources of carotene (3.05 mg) and niacin (2.51 mg). However, only the infusion is used by the people.

Leek. The leek is usually a fairly good source of carotene (Navia *et al.*, 1957) but the samples analyzed proved to be somewhat low in this nutrient (.24, .41 mg).

Lettuce. Previous studies (Navia *et al.*, 1955a, 1957) demonstrating that this vegetable is a good source of carotene were confirmed by the results obtained for the sample analyzed here. It contained relatively large amounts (1.27 mg).

Manioc, shoots. In contrast to the results obtained in the roots of the manioc, which were nearly devoid of all nutrients except starch (Navia *et al.*, 1955a, 1957) the shoots were found to be high in carotene (3.39 mg) and ascorbic acid (218.0 mg) content. Lysine (207 mg) and riboflavin (.27 mg) were moderate.

Mint. The two samples of mint were high in carotene (2.13, 2.89 mg) and iron (2.23, 5.89 mg), but only moderate in riboflavin (.15, .20 mg). This food is used in limited amounts to flavor dishes.

Parsley. In accord with previous reports (Navia *et al.*, 1955a, 1957), this vegetable proved to be rich in iron (3.49 mg), carotene (3.33 mg), and ascorbic acid (145.3 mg). Its value is limited, however, because only small quantities can be consumed in the diet due to its strong flavor and aroma.

Purslane. The sample analyzed was high in carotene (2.14 mg) and low in ascorbic acid (41.9 mg).

Squash, shoots. The squash shoots contained less nutrients than the manioc shoots.

Squash, vine-tips. The two samples of squash vine-tips were lower in carotene than the squash shoots or manioc shoots.

Squash, flowers. The three samples of squash flowers were fairly rich in iron content (4.23 mg) and moderate in carotene and niacin content.

Sweet potato, shoots. The two samples were moderately high in ascorbic acid, carotene, and riboflavin content.

Tea weed. This sample contained remarkable amounts of carotene (6.91 mg), riboflavin (.47 mg), calcium (466.0 mg), and iron (4.96 mg), and moderate amounts of thiamine (.22 mg) and ascorbic acid (89.5 mg). This vegetable appears to be one of the best of all the foods studied.

Watercress. The cress sample was moderately rich in calcium (123.1 mg), iron (3.51 mg), and ascorbic acid (66.3 mg).

Table 1. Composition of

Name of food sampled					
English	Scientific	Spanish	Moisture (g)	Ether extract (g)	Crude fiber (g)
CEREALS:					
Corn meal	*Zea mays* L.	Maíz, harina de	13.1	2.63	1.01
Corn, green	*Zea mays* L.	Maíz tierno	55.7	2.15	.81
EARTH VEGETABLES:					
Asiatic lotus	*Nelumbo nucifera* Gaertn	Yuca china	81.6	.25	.74
Carrot (a)	*Daucus carota* L.	Zanahoria	89.8	.13	.87
Carrot (b)	*Daucus carota* L.	Zanahoria	86.4	.10	1.05
Carrot (c)	*Daucus carota* L.	Zanahoria	88.5	.10	.84
Garlic	*Allium sativum* L.	Ajo	65.7	.01	.59
Malanga (a), white	*Xanthosoma sagittifolium* Schott	Malanga blanca	67.9	.19	.50
Malanga (b), yellow	*Xanthosoma sagittifolium* Schott	Malanga amarilla	59.4	.15	.79
Potato (a)	*Solanum tuberosum* L.	Papa	83.3	.02	.38
Potato (b)	*Solanum tuberosum* L.	Papa	83.1	.02	.33
Turnip	*Brassica rapa* L.	Nabo	90.8	.10	.87
FRUIT VEGETABLES:					
Akee	*Blighia sapia* Koen.	Seco vegetal	71.9	15.12	1.46
Balsam pear	*Momordica charantia* L.	Cundiamor chino	93.3	.26	1.08
Maya (a)	*Bromelia pinguin* L.	Piña de ratón	89.8	.10	.25
Maya (b)	*Bromelia pinguin* L.	Piña de ratón	86.0	.48	.29
Maya (c)	*Bromelia pinguin* L.	Piña de ratón	86.4	.62	.24
Okra	*Hibiscus esculentus* Moench	Quimbombó	90.0	.08	.86
Pepper (a), green	*Capsicum frutescens* L.	Ají cachucha, verde	91.2	.22	1.15
Pepper (b), ripe	*Capsicum frutescens* L.	Ají cachucha, rojo	90.0	.33	1.11
Pepper (c), green	*Capsicum frutescens* L.	Ají, verde	92.1	.28	.67
Pepper (d), ripe	*Capsicum frutescens* L.	Ají, rojo	88.9	.48	.78
Pepper (e), green	*Capsicum frutescens* L.	Ají pimiénto, verde	92.6	.15	1.09
Pepper (f), ripe	*Capsicum frutescens* L.	Ají pimiénto, rojo	91.1	.18	.63
Plaintain (a)	*Musa paradisiaca* L.	Plátano macho	62.0	.10	.36
Plantain (b)	*Musa paradisiaca* L.	Plátano macho	62.4	.22	.35
Plantain (c)	*Musa paradisiaca* L.	Plátano macho	60.8	.16	.36
Squash (a)	*Cucurbita moschata* Poir.	Calabaza Camagüey	86.3	.10	.73
Squash (b)	*Cucurbita moschata* Poir.	Calabaza Camagüey	87.0	.22	.62
Tomato, cherry (a)	*Lycopersicum esculentum* var. *cerasiforme* Alef.	Tomate placero	93.0	.33	1.15
Tomato, cherry (b)	*Lycopersicum esculentum* var. *cerasiforme* Alef.	Tomate placero	93.6	.38	.59
HERBAGE VEGETABLES:					
Allspice leaves	*Pimienta dioica* Merr.	Pimienta de Jamaica, hojas	62.3	1.32	6.67
Amaranthus greens (a), shoots	*Amaranthus hybridus*	Bledo blanco, hojas y tallos	84.0	.59	1.39
Amaranthus greens (b), shoots	*Amaranthus hybridus* L.	Bledo blanco, hojas y tallos	89.6	.35	.77
Amaranthus, fruiting spikes (a)	*Amaranthus dubius* Mart.	Bledo, espiga	71.1	1.12	5.41
Amaranthus, fruiting spikes (b)	*Amaranthus dubius* Mart.	Bledo, espiga	65.2	.99	4.69
Cabbage (a)	*Brassica oleracea* var. *capitata* L.	Col	92.2	.13	.74
Cabbage (b)	*Brassica oleracea* var. *capitata* L.	Col	93.0	.06	.67
Cauliflower	*Brassica oleracea* var. *botrytis* L.	Coliflor	89.7	.13	1.09
Chard	*Beta vulgaris* var. *cicla* L.	Acelga blanca	92.3	.30	.62
Justicia	*Justicia pectoralis* Jack	Tilo, hojas	85.0	.59	2.84
Leek (a)	*Allium porrum* L.	Ajo puerro	82.4	.15	1.09
Leek (b)	*Allium porrum* L.	Ajo puerro	93.4	.31	1.17
Lettuce	*Lactuca sativa* L.	Lechuga	95.1	.18	.54
Manioc shoots	*Manihot esculentus* Crantz	Yuca, puntas y hojas	82.5	.85	1.71
Mint (a)	*Mentha nemorosa* Willd.	Yerba buena	90.6	.77	1.41
Mint (b)	*Mentha nemorosa* Willd.	Yerba buena	89.0	.50	1.83

food samples from Cuba.

Constituents measured and yields per 100 g of edible portion												
Nitrogen (g)	Ash (g)	Calcium (mg)	Phosphorus (mg)	Iron (mg)	Carotene (mg)	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)	Ascorbic acid total (mg)	Tryptophane (mg)	Methionine (mg)	Lysine (mg)
1.81	.98	19.8	116.8	6.51	.31	.25	.08	1.12		37	154	188
1.00	.73	10.9	151.5	2.00	.16	.20	.09	1.36	6.7	27	78	147
.27	.71	39.4	66.2	.50	.00	.10	.04	.45	65.9	15	12	50
.12	.83	36.3	39.9	1.45	7.05	.04	.04	.65	11.5	5	3	36
.09	.69	35.8	33.9	1.77	5.37	.04	.04	.70	9.1	5	5	39
.21	1.06	34.5	30.3	.71	5.91	.04	04	1.16	10.7	9	7	41
.10	1.41	28.2	202.5	1.06	.01	.26	.07	.23	7.4	58	40	233
.36	1.21	21.7	100.8	1.04	.02	.12	.04	.58	17.2	24	20	109
.77	1.06	36.0	72.5	1.37	.64	.14	.05	1.41	13.3	55	34	65
.29	.66	12.2	41.2	1.15	.00	.04	.02	1.31	41.4	12	20	102
.25	.60	11.3	36.7	1.05	.00	.06	.02	1.21	31.8	12	19	92
.18	.70	64.9	40.1	.09	.01	.05	.04	.94	36.9	11	2	55
1.12	1.28	37.7	62.3	1.78	.36	.07	.18	1.33	46.1	39	26	276
.50	.54	18.5	31.0	.68	.13	.05	.04	.44	23.0	5	6	51
.40	.90	98.4	16.9	2.21	.00	.14	.03	.27	121.2	59	18	75
.43	.76	142.9	17.3	.98	.00	.18	.05	.53	97.9	58	21	68
.55	.77	113.4	16.7	1.14	.01	.13	.04	.42	93.8	63	26	77
.49	.78	58.2	55.8	1.34	.17	.11	.08	.71	13.0	27	10	68
.26	.56	19.9	27.0	1.18	.23	.10	.05	.83	108.6	10	5	63
.29	.62	20.7	26.7	1.38	1.17	.08	.16	1.37	153.9	15	9	49
.18	.41	13.4	21.5	.67	.48	.05	.02	1.04	72.7	10	5	53
.22	.51	14.0	28.0	.90	1.56	.08	.12	2.17	132.8	15	7	57
.11	.34	9.8	18.1	.79	.20	.02	.03	.67	255.4	5	5	39
.11	.46	10.0	21.2	1.33	.50	.04	.10	.85	231.5	7	6	35
.19	.80	11.0	40.2	.95	.54	.05	.06	.65	31.4	12	10	58
.36	.93	13.0	37.8	.61	.50	.06	.04	.47	21.6	11	7	46
.21	.75	10.2	45.5	1.61	.71	.04	.04	.56	34.0	10	10	62
.24	.64	17.8	33.1	1.25	1.01	.05	.06	1.05	35.2	11	10	60
.52	.89	17.8	67.0	.86	.76	.07	.05	.81	20.6	11	8	49
.18	.56	11.4	29.8	.94	.84	.07	.12	.59	48.4	9	12	69
.24	.41	14.4	18.5	1.11	.61	.07	.06	.76	47.4	14	8	51
.82	2.56	209.0	44.8	2.27	4.73	.06	.14	.55	80.9	46	28	144
.73	2.00	361.5	82.7	4.16	3.57	.01	.14	1.59	113.0	54	42	244
.38	1.68	238.0	50.7	6.30	3.29	.04	.09	.91	67.5	36	23	120
1.56	2.16	206.8	135.3	6.30	1.94	.02	.27	1.43	72.9	77	67	330
1.06	3.26	394.5	124.6	17.76	1.67	.01	.21	2.06	65.6	83	41	317
.18	.56	65.0	25.1	.75	.04	.04	.03	.44	58.9	7	2	63
.13	.50	65.8	21.8	.52	.03	.03	.03	.36	51.9	5	2	45
.20	.69	31.3	44.7	.78	.01	.07	.05	.37	114.3	17	12	100
.40	1.46	105.5	29.9	2.44	1.33	.04	.17	.95	48.0	34	13	87
.63	2.29	663.0	34.6	7.40	3.05	.04	.20	2.51	27.5	29	19	109
.30	.54	58.7	27.2	1.02	.24	.14	.06	.39	15.1	15	9	91
.41	.65	59.5	31.3	1.52	.41	.06	.10	.44	18.0	12	8	75
.16	.59	44.9	24.0	1.30	1.27	.07	.10	.38	20.3	17	12	73
.61	1.11	170.8	71.1	1.80	3.39	.08	.27	.92	218.0	50	38	207
.21	.82	138.3	42.9	2.23	2.13	.05	.15	.40	32.5	15	13	78
.56	1.29	193.0	38.4	5.89	2.89	.05	.20	.61	40.0	26	21	160

Parsley	*Petroselinum crispum* Mansf.	Perejil	88.6	.42	.96
Purslane	*Portulaca oleracea* L.	Verdolaga, hojas y ramas	88.8	.44	.94
Royal palm cabbage	*Roystonea regia* O. F. Cook	Palmito de palma real	91.6	.44	.60
Spring onion	*Allium fistulosum L.*	Cebolla de la tierra	89.4	.21	1.60
Squash shoots	*Cucurbita moschata* Duch.	Calabaza, tallos y puntas	91.1	.46	1.01
Squash vine-tips (a)	*Cucurbita moschata* Duch.	Calabaza, puntas	92.0	.50	1.02
Squash vine-tips (b)	*Cucurbita moschata* Duch.	Calabaza, puntas	94.1	.27	.76
Squash flowers (a)	*Cucurbita moschata* Duch.	Calabaza, flores	93.3	.42	.55
Squash flowers (b)	*Cucurbita moschata* Duch.	Calabaza, flores	94.8	.39	.55
Squash flowers (c)	*Cucurbita moschata* Duch.	Calabaza, flores	96.0	.23	.40
Sweet potato shoots (a)	*Ipomoea batatas* L.	Boniato, puntas y hojas	87.1	.78	1.27
Sweet potato shoots (b)	*Ipomoea batatas* L.	Boniato, puntas y hojas	86.6	1.37	1.36
Tea weed	*Sida rhombifolia* L.	Malva de cochino	80.2	1.42	3.32
Watercress	*Nasturtium officinale* R. Br.	Berro	94.3	.40	.62
LEGUMES AND OIL SEEDS:					
Adzuki bean	*Phaseolus angularis* Wight	Frijol diablito	13.1	.57	5.70
Asparagus bean (a)	*Vigna sesquipedalis* Fruw.	Habichuela china	89.9	.23	1.21
Asparagus bean (b)	*Vigna sesquipedalis* Fruw.	Habichuela china	89.1	.25	1.21
Bean, black	*Phaseolus vulgaris* L.	Frijol negro	11.3	1.38	3.83
Bean, spotted red	*Phaseolus vulgaris* L.	Frijol chino	11.7	1.16	3.63
Cashew nut (roasted)	*Anacardium occidentale* L.	Marañón, semilla tostada	9.1	45.90	1.43
Corojo	*Acrocomia armentalis* Bailey	Corojo	3.2	74.10	6.60
Hindu cowpea	*Vigna cylindrica* Skeels	Frijol precioso	12.5	1.69	4.43
Macadamia nut	*Macadamia ternifolia* T. Muell	Macadamia, nuez de	4.8	72.55	6.53
Peanut (a), raw	*Arachis hypogaea* L.	Maní, crudo	9.2	42.50	4.16
Peanut (b), roasted	*Arachis hypogaea* L.	Maní, tostado	2.2	48.10	2.41
Pigeon pea (a)	*Cajanus cajan* Millsp.	Frijol gandul	70.5	.50	2.57
Pigeon pea (b)	*Cajanus cajan* Millsp.	Frijol gandul	16.4	.92	4.19
Royal palm fruit (a)	*Roystonea regia* O. F. Cook	Palmiche	24.8	17.54	23.52
Royal palm fruit (b)	*Roystonea regia* O. F. Cook	Palmiche	56.7	7.79	12.25
Royal palm fruit (c)	*Roystonea regia* O. F. Cook	Palmiche	49.5	12.84	13.49
Royal palm fruit (d)	*Roystonea regia* O. F. Cook	Palmiche	50.3	15.00	12.37
Squash seeds	*Cucurbita moschata* Duch.	Calabaza, semillas	16.8	30.07	24.76
FRUIT: CITRUS					
Grape fruit	*Citrus paradisi* Macf.	Toronja	90.7	.03	.07
Sweet lime	*Citrus aurantifolia*, Swingle	Lima	88.7	.08	.14
FRUIT: OTHER					
Banana, apple	*Musa paradisiaca* var. *sapientum* Ktze.	Plátano manzano	73.1	.10	.34
Barbados cherry	*Malpighia glabra* L.	Acerola (cereza del país)	93.5	.46	.16
Bignay	*Antidesma bunius* Spreng	Bignay	88.2	.28	.14
Cashew apple, juice	*Anacardium occidentale* L.	Marañón, rojo, jugo	88.7	.09	.89
Coconut meat	*Cocos nucifera* L.	Coco, masa de	56.2	32.60	13.17
Coconut milk	*Cocos nucifera* L.	Coco, agua de	95.1	.02	.02
Guava (a), ripe	*Psidium guajava* L.	Guayaba, madura	85.7	.99	4.90
Guava (b), semi-ripe	*Psidium guajava* L.	Guayaba, pintona	81.7	1.31	8.13
Guava (c), peruvian	*Psidium guajava* L.	Guayaba del Perú	87.5	.35	4.07
Litchi	*Litchi sinensis* Sonn.	Mamoncillo chino	83.1	.06	.11
Mango	*Mangifera indica* L.	Mango mamey	84.8	.08	.81
Mombin (a)	*Spondias purpurea.* L.	Ciruela criolla	86.0	.13	.74
Mombin (b)	*Spondias purpurea.* L.	Ciruela campechana	86.9	.11	.24
Myrobalan	*Phyllanthus emblica* L.	Mirobalanos	78.3	.09	2.47
Papaya	*Carica papaya* L.	Fruta bomba	90.8	.30	.97
Plum, Governor's	*Flacourtia indica* Merr.	Ciruela gobernadora	81.7	.23	1.69
Sapodilla	*Achras zapota* L.	Níspero (zapote)	78.0	.30	1.51
Sweetsop (a)	*Annona squamosa* L.	Anón	78.6	.13	1.03
Sweetsop (b)	*Annona squamosa* L.	Anón	75.2	.66	1.42
MISCELLANEOUS:					
Molasses, blackstrap	*Saccharum* hybrids	Caña, miel final	22.1	.90	.19
Molasses, invert	*Saccharum* hybrids	Caña, miel rica	17.9	.04	.21
Sugar cane juice (a)	*Saccharum* hybrids	Caña, guarapo	76.8	.02	.07
Sugar cane juice (b)	*Saccharum* hybrids	Caña, guarapo	96.2	.02	.17
Sugar cane juice (c)	*Saccharum* hybrids	Caña, guarapo	74.1	.08	3.89
Sugar cane syrup	*Saccharum* hybrids	Caña, melado de	31.1	.20	9.45

.32	1.31	157.4	34.2	3.49	3.33	.00	.22	.92	145.3	38	21	146
.37	1.61	62.1	26.5	.59	2.14	.01	.13	.99	41.9	33	17	108
.52	.96	72.3	28.7	.61	.01	.03	.05	.67	15.9	33	19	121
.26	.61	75.3	67.5	1.45	.87	.05	.12	.54	40.2	16	15	85
.49	.96	67.1	64.1	2.83	1.18	.09	.14	1.19	38.7	26	25	181
.67	1.05	55.0	67.3	4.58	.73	.08	.12	.83	23.1	19	20	182
.63	.74	42.9	60.6	1.47	.69	.08	.12	.73	12.7	21	22	121
.26	.69	53.6	23.3	4.23	.54	.05	.12	1.33	30.5	21	13	71
.43	.62	49.8	37.7	1.21	.36	.04	.12	.86	24.9	16	9	62
.47	.46	26.5	35.4	1.72	.45	.06	.08	.84	17.6	14	9	45
.38	.83	87.5	46.6	2.97	2.15	.09	.25	.92	60.9	41	34	179
.50	1.12	56.2	49.7	.99	1.47	.12	.28	1.44	39.1	42	33	224
1.19	1.56	466.0	58.4	4.96	6.91	.22	.17	2.10	89.5	79	49	215
.37	.92	123.1	26.7	3.51	.70	.06	.10	.84	66.3	24	7	88
4.04	3.88	252.0	316.8	7.55	.02	.57	.18	3.16	....	179	196	1382
.52	.62	39.4	56.1	2.05	.44	.13	.08	1.05	29.8	26	22	136
.88	.64	41.6	53.6	1.11	.33	.13	.09	1.26	26.4	24	18	129
3.67	3.43	143.5	315.5	8.30	.01	.42	.22	2.95	....	212	163	1225
4.42	3.72	121.2	381.0	7.65	.01	.55	.19	3.76	....	222	169	1337
3.30	2.12	66.7	383.0	6.04	.01	.31	.29	2.58	1.8	478	305	1025
1.96	1.48	211.5	155.9	3.19	.03	.06	.07	2.64	.9	239	169	474
3.99	3.26	128.5	585.5	6.35	.02	.68	.19	3.38	....	221	213	1223
1.69	1.48	104.3	102.0	3.01	.00	.35	.11	2.14	....	80	66	273
5.15	1.92	56.3	294.3	2.48	....	.76	.28	9.75	....	530	279	1310
6.19	2.09	53.9	319.4	2.12	....	.09	.21	9.59	....	531	304	1180
1.03	1.19	36.4	72.4	1.55	.20	.25	.17	2.50	45.6	28	58	283
3.11	3.30	75.1	316.5	4.14	....	.65	.20	5.35	....	113	165	1065
1.31	2.19	197.3	161.5	5.86	.05	.12	.03	.77	1.2	48	42	236
.57	1.86	217.9	75.9	2.66	.28	.07	.09	.68	28.1	39	20	122
.57	1.68	170.3	50.6	2.68	.50	.24	.07	1.39	13.6	65	23	120
.75	2.35	234.8	95.6	7.68	.43	.14	.08	.67	8.0	45	21	139
5.40	3.77	81.7	873.5	16.47	.31	.40	.38	2.98	2.5	360	409	1383
.05	.15	13.9	12.5	.29	.01	.04	.01	.13	45.9	1	1	10
.10	.35	15.1	18.9	.77	.00	.01	.02	.23	48.1	3	2	17
.18	.68	8.8	31.3	1.09	.04	.04	.05	.62	21.1	11	8	55
.05	.31	16.3	6.9	.73	.25	.02	.03	.55	109.8	2	9	8
.08	.90	23.0	27.3	.84	.05	.01	.03	.36	3.8	4	2	12
.09	.23	6.6	17.0	.73	.06	.05	.05	.48	253.1	7	4	19
.61	.82	12.2	82.8	1.33	....	.02	.02	.78	6.8	21	47	117
.00	.25	28.8	15.6	.38	....	.00	.01	.02	1.8	....	....	....
.41	.60	18.8	27.4	.87	.15	.07	.07	1.14	225.0	11	8	42
.60	.79	19.5	33.2	.90	.01	.06	.06	1.04	175.0	13	11	42
.39	.52	11.4	10.3	1.26	.01	.05	.05	1.04	33.3	11	4	26
.13	.30	5.4	30.6	1.11	.00	.02	.03	.87	71.9	7	9	40
.09	.29	5.5	11.3	.95	2.32	.04	.05	.51	15.0	6	5	24
.09	.44	13.6	28.4	.75	.16	.08	.05	.32	37.7	6	2	19
.14	.37	19.1	28.1	.44	.10	.06	.03	.65	31.1	7	2	18
.33	.42	15.6	24.4	.63	.03	.01	.05	.28	212.0	6	4	26
.30	.47	12.2	10.1	2.16	.23	.04	.02	.39	102.7	7	3	19
.28	.45	16.8	19.0	.91	.02	.03	.05	.31	9.9	3	2	17
.05	.29	13.1	14.1	.86	.02	.00	.02	.21	21.9	4	3	33
.42	.60	25.0	17.6	1.10	.00	.07	.08	.72	55.0	12	6	41
.60	.62	25.4	28.1	3.11	.01	.08	.10	.90	41.5	10	8	52
.40	5.61	470.5	35.8	10.73	....	.08	....	2.47	....	8	4	14
.09	1.24	187.0	19.6	2.33	.01	.04	....	.55	12.1	3	2	14
.08	.25	31.1	6.1	.67	.01	.02	.03	.09	1.1	0	....	....
.05	.11	30.5	3.6	.54	.04	.02	.02	.08	.9	1	....	....
.04	.26	29.1	2.8	.53	.03	.02	.03	.19	....	3	....	....
.05	.97	139.0	8.7	1.77	....	.03	....	.51	0	3	....	....

but the carotene (.70 mg) and riboflavin (.10 mg) values were below those reported previously (Navia *et al.*, 1957).

Legumes and oil seeds. *Adzuki bean.* This bean sample is noted for its high nitrogen content (4.04 mg), which makes it valuable as a source of protein. The lysine content was extremely high (1382 mg), although the tryptophan content (179 mg) was not as high as in the two *Phaseolus vulgaris* samples reported in this paper (222, 212 mg). The iron (7.55 mg), calcium (252 mg), thiamine (0.57 mg), and niacin (3.16 mg) values were high, and the lipid content was low (0.57%). The high nutrient content of this bean indicates that it should be consumed more extensively.

Asparagus bean. The results on the two samples were quite similar and revealed significant amounts of carotene and iron.

Black and spotted red bean. These bean samples, like the adzuki, were extremely rich in nitrogen value (3.67, 4.42%), lysine (1225, 1337 mg), and also in iron (8.50, 7.65 mg), rich in thiamine (.42, .55 mg), riboflavin (.22, .19 mg), and niacin (2.95, 3.76 mg). Black beans are commonly included in the diet of the people, and therefore its high values in nutrients make it an important factor in their nutrition.

Cashew nut. This nut sample was found to be rich in lipids (45.9%) and nitrogen (3.30%), and moderately good as a source of iron (6.04 mg), thiamine (.31 mg), riboflavin (.29 mg), and niacin (2.58 mg). The amino acid content was quite high, especially in tryptophan (478 mg) and methionine (305 mg).

Corojo. Exceptionally high in lipids (74.10%) and also in crude fiber (8.60%), the corojo is low in nitrogen (1.96%) compared to other nuts analyzed (Navia *et al.*, 1955a, 1957). While the thiamine and riboflavin contents were low, niacin content was high.

Hindu cowpea. The cowpea was characterized by a high content of nitrogen (3.97%), crude fiber (4.43%), phosphorus (585.5 mg), iron (6.36 mg), thiamine (.68 mg), and niacin (3.38 mg). This sample contained less thiamine than those reported previously (Navia *et al.*, 1955a).

Macadamia nut. This nut was analyzed to be nearly as rich as the corojo in lipid content (72.55%). It was high in crude fiber (6.53%), iron (3.01 mg), thiamine (.35 mg), and niacin (2.14 mg), and low in nitrogen (1.69%) content.

Peanut. Of the two samples of peanut studied, *a* was a raw peanut and *b* was roasted. Corrected to the same moisture content, there were no important differences in the ether extract, nitrogen, and iron contents. The niacin content of the roasted sample was slightly lower, and there were significant losses in thiamine (.76 vs. .09) and riboflavin (.28 vs. .21). The peanut is an excellent source of lysine, tryptophan and methionine. The amino acid content of these samples of peanuts was higher, but the niacin content was lower, than previously reported (Navia *et al.*, 1955a).

Pigeon pea. Sample *a* was green fresh, whereas *b* was dried. The nitrogen and lysine contents of both samples were high, and the tryptophan and methionine contents were low.

Squash seeds. The squash seeds were rich in lipid (30.07%), crude fiber (24.76%), nitrogen (5.40%), iron (16.47 mg), thiamine (.40 mg), riboflavin (.38 mg), and niacin (2.98 mg). Values in amino acids were also high in tryptophan (360 mg), methionine (409 mg), and lysine (1383 mg). The hulls of squash seeds are generally removed preparatory to eating. Had these seeds been peeled before analysis, then all values except the crude fiber would have been increased by nearly 25%. Thus squash seeds are an excellent food.

Fruits (citrus). *Grapefruit.* The outstanding nutrient in this fruit was ascorbic acid (45.9 mg); the value reported here fell within the range of previous analysis (Navia *et al.*, 1955a, 1957).

Sweet lime. The value for ascorbic acid (48.1 mg) was not as high as that observed previously (Navia *et al.*, 1957), but its iron content was slightly higher (.77 mg).

Fruit (other). *Banana, apple.* The sample analyzed here was fairly rich in ascorbic acid content (21.1 mg), and its iron content was moderately high (1.09 mg).

Barbados cherry. The Barbados cherry is noted for its exceptionally high content of

ascorbic acid. Asenjo (1946) reported varieties that have 2000–3000 mg. The sample analyzed here contained 1093 mg of ascorbic acid.

Cashew apple. The juice of this fruit is consumed in preference to the whole fruit. The sample analyzed here was high in ascorbic acid (253.1 mg), but was lower than the value (372.0 mg) reported previously (Navia *et al.*, 1957).

Coconut, meat and milk. The coconut milk contained little of nutritional value but can serve as a safe source of water. The meat was high in lipids (32.6%) and crude fiber (13.17%) and quite low in nitrogen content.

Guava. Two of the three samples were of one variety, one was ripe *a* and the other semi-ripe *b*. Ripening tended to reduce the lipid, fiber, nitrogen, ash, and phosphorus content, and to increase the carotene and ascorbic acid content. The third sample *c*, representing a different variety (Peruvian), was generally less nutritious. Guavas are an excellent source of ascorbic acid, yet the values reported here (225.0, 175.0 mg) were lower than those (484.0 and 458.0 mg) reported previously (Navia *et al.*, 1955a).

Litchi. This fruit is not important nutritionally, even though it is a fairly good source (71.9 mg) of ascorbic acid.

Mango. The sample of mango studied was rich in carotene (2.32 mg), but extremely low in ascorbic acid (15.0 mg). Samples of other varieties reported previously (Navia *et al.*, 1957) were lower in carotene (1.07 mg) and higher in ascorbic acid (57.9 mg).

Mombin. The mombin is not important nutritionally.

Myrobalan. The sample analyzed had a high content of ascorbic acid (212.0 mg), but this was only one-third the amount (625.0 mg) previously recorded (Navia *et al.*, 1957) for this fruit.

Papaya. The sample proved to be a fairly good source of iron (2.16 mg) and ascorbic acid (102.7 mg), but only a fair source of carotene (.23 mg). Samples of this fruit previously studied (Navia *et al.*, 1957) contained less (23.1, 46.1 mg) ascorbic acid than that reported here.

Sweetsop. The sweetsop was found to contain moderate amounts of iron and ascorbic acid.

Miscellaneous. *Molasses, blackstrap.* The nutritional value of the sample analyzed was lower than in the sample previously studied (Navia *et al.*, 1957). The calcium values were high (470.5 mg).

Molasses, invert. This sample showed in general lower nutritional value than the blackstrap molasses.

Sugar cane juice. The three samples studied were taken from the first crusher rolls *a*, fifth crusher rolls *b*, and a retail store *c*. None of the samples were found to be important nutritionally, except as a source of calories.

Sugar cane syrup. The sample studied showed values similar to the previously reported value (Navia *et al.*, 1957).

CONCLUSION

The following foods are judged to have significant value as sources of *lipid*: akee, cashew nut, corojo, macadamia nut, peanut, royal palm fruit, squash seeds; *nitrogen*: adzuki bean, black bean, spotted red bean, cashew nut, Hindu cowpea, peanut, pigeon pea, squash seeds; *calcium*: adzuki bean, allspice, amaranthus greens and spikes, justicia leaves, manioc shoots, tea weed and molasses; *phosphorus*: beans (adzuki, black, spotted-red, asparagus), cashew nut, Hindu cowpea, peanut, squash seeds; *carotene*: allspice leaves, amaranthus greens and spikes, carrots, justicia, manioc shoots, parsley, teaweed; *iron*: amaranthus greens and spikes, adzuki beans, black beans, spotted beans, cashew, Hindu cowpea, justicia, mint, molasses, royal palm fruit, squash seeds; *thiamine*: adzuki bean, black bean, spotted red bean, cashew nut, macadamia nut, peanut, pigeon pea, squash seeds; *riboflavin*: squash seeds, tea weed; *niacin*: adzuki bean, spotted red bean, Hindu cowpea, peanut, pigeon pea; *ascorbic acid*: allspice leaves, amaranthus greens and spikes, Asiatic lotus, balsam pear, Barbados cherry, cabbage, cashew apple, cauliflower, chard, guava, manioc shoots, maya, myrobalan, papaya, parsley, peppers, sweet potato shoots, sweetsop, tea weed, watercress; *tryptophan*: cashew nut, peanut; *methionine*: cashew

nut; *lysine*: black and red bean, adzuki bean, cashew nut.

It will be noted that cashew nut, squash seed, adzuki bean, peanut, and black bean are mentioned most frequently, and therefore are rich in over-all nutritional value. The data indicate that mombin, litchi nuts, coconut meat, coconut milk, and sugar cane juice are so low in nutrient content that their use as food should perhaps be discouraged.

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Nitrogen Balance of Dogs Fed Lime-Treated Corn Supplemented with Proteins and Amino Acids[a, b]

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SUMMARY

The effect on the nitrogen balance of young dogs of supplementing lime-treated corn with small amounts of black bean flour, skim milk, fish flour, and torula yeast was investigated. Although the diets were kept isonitrogenous, all supplements increased nitrogen retention significantly, as did supplementing with lysine and tryptophan. The gain was most marked for skim milk, fish flour, and torula yeast plus lysine, and of sufficient magnitude to be of practical significance for human feeding. The better the protein quality of the supplement, the greater the decrease in nitrogen retention after its removal.

It is well recognized that lime-treated corn protein is most deficient in the essential amino acids lysine and tryptophan (Bressani, 1960; Bressani *et al.*, 1958; Scrimshaw *et al.*, 1958). It is also well documented that corn is the most important staple of the rural Central American diet (Flores, 1961). Therefore, efforts should be made to find ways of improving the quality of corn protein. Synthetic amino acids are still too costly to be practical for the enrichment of cereal foods. However, the nutritive value of the proteins of lime-treated corn can be improved by supplementing them with other proteins that are rich in its limiting amino acids. This method has the further advantage that the enriched product will contain higher amounts of protein, as well as be improved in protein quality. The present work determines the effect on nitrogen balance of supplementing lime-treated corn with small amounts of lysine and tryptophan, or proteins rich in these two amino acids.

MATERIAL AND METHODS

The lime-treated maize flour was prepared by methods described previously (Bressani *et al.*, 1958). The supplements tested were: skim milk powder (supplied by UNICEF), torula yeast (Lake States Yeast Corporation, Rhinelander, Wisc.), deodorized fish flour (VioBin Corp.) and cooked black bean powder prepared by cooking beans in the autoclave for 10 minutes (Bressani *et al.*, 1962). The effect of adding these foods to lime-treated maize flour was studied by the nitrogen balance method in young mongrel dogs 4–5 months old. The basal diet was: 78% lime-treated corn, 7% corn gluten, 2% mineral mixture (Hegsted *et al.*, 1941), 10% hydrogenated vegetable fat, 1% cod liver oil, and 2% cornstarch. Three ml of a complete vitamin solution (Manna and Hauge, 1953) were added per 100 g of diet. The average nitrogen content of the basal diet was 1.77% with a calculated calorie content of 425 cal/100 g.

The nitrogen content of the corn gluten was 7.60%, of the skim milk 4.48%, of torula yeast 8.00% and of the fish flour 12.14%. The protein and amino acid supplements, added to the basal diet, replaced part or all of the nitrogen from corn gluten (supplied by Dr. E. L. Powell, American Maize Products Co., Roby, Ind.), and the composition of the diet was adjusted to 100% with cornstarch. Thus, all diets were approximately isonitrogenous and isocaloric.

The amounts added were 5% skim milk, 3% torula yeast and 4% fish flour. The amounts of lysine and tryptophan added to the diet were equal to the quantities found in 5% skim milk or in 3% torula yeast. The cooked dehydrated black beans contained an average of 3.44% nitrogen and were fed in two periods in an amount equivalent to about 32% and 22% of the daily nitrogen intake of the dog respectively. This quantity of beans represents the range in

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daily family consumption in Guatemala (Flores and García, 1960; Flores, 1961). The diets were fed for 10 days, allowing two days for adaptation and the remaining eight for two balance periods of 4 days each.

Four series of studies were carried out. The two dogs used in the first weighed an average of 4.71 kg at the start and 7.32 kg at the end of the series. The diets fed were: basal plus 5% skim milk, basal plus 0.17% L-lysine plus 0.025% DL-tryptophan, basal plus black bean flour, basal plus 5% skim milk, and basal plus 5% skim milk plus black bean flour, with basal diet feedings in between each supplement feeding. Three dogs were used in the second series of experiments in which the sequence of diets tested was: basal plus skim milk, basal plus 3% torula yeast, basal plus 3% torula yeast plus 0.14% L-lysine HCl, with basal diet feeding in between supplement feedings. The average weight of the animals was 7.00 kg at the start and 8.01 kg at the end of the series.

In the third series of tests 3 dogs were also used, weighing 5.06 kg at the start and 7.28 kg at the end of the series. The order of diets fed was: basal plus 5% skim milk, basal plus 3% torula yeast, and basal plus 4% fish flour, with basal diet feedings in between the other treatments. In the fourth series of experiments, 4 animals were fed the basal diet plus 5% skim milk, basal and basal plus 4% fish flour. The average weight at the start was 6.36 kg and 6.79 kg at the end of the experiment.

Protein and calorie intakes differed with each series of experiments but remained as constant as possible within each series. Feeding was carried out twice daily at 8:00 a.m. and 4:00 p.m., and water was available at all times. Feces and urine were collected twice a day and stored at 4°C until analysis was performed. Urine was collected with 1 cm^3 of concentrated acetic acid. The feces and urine were pooled every four days and after homogenizing they, as well as all diets fed, were analyzed for nitrogen by the Kjeldahl method.

RESULTS

Table 1 summarizes nitrogen-balance results of the first series of tests. The addition of 5% skim milk powder increased nitrogen retention significantly over the values obtained with the basal diet. The addition of 0.17% L-lysine plus 0.025% DL-tryptophan, the quantities of these two amino acids found in 5% skim milk, also improved nitrogen retention significantly; in fact, to the same level reached with skim milk. The addition of beans increased nitrogen retention, but supplementation with both skim milk and beans brought further improvement. The retentions of nitrogen decreased markedly and sometimes became negative with a return to the basal diet after feeding the basal plus any of the supplements.

Table 2 shows the results of individual periods as well as averages per treatment of the second series of experiments. The addition of skim milk again improved nitrogen retention significantly. The additions of torula yeast and torula yeast plus

Table 1. Nitrogen balance of dogs fed lime-treated corn supplemented with skim milk, lysine and tryptophan and black beans.[a]

Diet	Nitrogen						Average change in NR/NI[b] to basal diet	
	Intake	Fecal	Urine	Retained	Absorbed	Retained	Before	After
	mg/kg/day				% of intake			
Basal	578	165	398	15	71.4	2.6	....	....
B + 5% skim milk	643	179	327	137	72.2	21.3	.187	.283
Basal	560	194	405	— 39	65.3	— 7.0	....	....
B + L-lysine HCl + DL-tryptophan[c]	611	197	283	131	67.7	21.4	.284	.153
Basal	520	189	299	32	63.6	6.1	....	....
B + black beans[d]	635	254	250	131	60.0	20.6	.145	.302
Basal	394	143	289	— 38	63.7	— 9.6	....	....
B + 5% skim milk	457	133	215	109	70.9	23.8	.334	.189
Basal	370	124	228	18	66.5	4.9	....	....
B + 5% skim milk + black beans[e]	569	193	205	171	66.1	30.0	.251	....

[a] Average values from 2 dogs and 2 balance periods per dog per treatment.
[b] Nitrogen retention/nitrogen intake.
[c] Amino acid levels used: 0.17% L-lysine HCl and 0.025% DL-tryptophan.
[d] Amount of black bean flour fed equivalent to 32% of total nitrogen intake (weight/day 40–44 g).
[e] Amount of black bean flour fed equivalent to 22% of total nitrogen intake (weight/day 24–28 g).

Table 2. Nitrogen balance of dogs fed lime-treated corn supplemented with skim milk, torula yeast and torula yeast plus lysine.[a]

		Nitrogen						Average change in NR/NI[b] to basal diet	
		Intake	Fecal	Urine	Retained	Absorbed	Retained		
	Period	mg/kg/day				% of intake		Before	After
Basal	1	349	83	228	38	76.2	10.9		
B + 5% skim milk	1	420	104	204	112	75.2	26.7		
	2	416	103	172	141	75.2	33.9		
	$\bar{x}$	418	103	188	127	75.3	30.4	0.195	0.284
Basal	1	425	135	323	— 33	68.2	— 7.8		
	2	392	102	242	48	74.0	12.2		
	$\bar{x}$	408	118	282	8	71.1	2.0		
B + 3% torula yeast	1	447	131	219	97	70.7	21.7		
	2	469	139	213	117	70.4	24.9		
	$\bar{x}$	458	135	216	107	70.5	23.4	0.214	0.085
Basal	1	387	133	202	52	65.6	13.4		
	2	309	100	157	52	67.6	16.8		
	$\bar{x}$	348	117	179	52	66.7	14.9		
B + 3% torula yeast + 0.14% L-lysine HCl	1	344	128	131	85	62.8	24.7		
	2	341	117	134	90	65.7	26.4		
	$\bar{x}$	343	122	132	89	64.4	25.9	0.110	0.175
Basal	1	307	112	186	9	63.5	2.9		
	2	314	100	171	43	68.1	13.7		
	$\bar{x}$	310	106	178	26	65.8	8.4		

[a] Average per period of 3 dogs.
[b] Nitrogen retention/nitrogen intake.

0.14% L-lysine HCl as well as the amount of lysine found in 3% torula yeast, also increased nitrogen balance significantly, but the values were lower than that from skim milk addition. The torula-lysine addition was slightly more effective than torula alone in increasing nitrogen retention. Feeding of the basal diet after any supplemented period caused a decrease in nitrogen retention. The decrease was greatest after skim milk, followed by torula yeast plus lysine and torula yeast alone. This is indicated by the change in nitrogen retention/nitrogen intake upon adding and withdrawing the supplement.

Table 3 summarizes the average results obtained in the third series of experiments. The addition of skim milk and of torula yeast, increased nitrogen balance significantly, the increase being greater with skim milk, followed by the torula yeast. The fish flour supplement increased retention of nitrogen in the first period only, but the lower retention observed in the second was probably due to a decrease in nitrogen intake. Food intake with the basal diet was not maintained at a constant level, because the animals did not consume all that was offered, particularly at the end of the series. As before, nitrogen balance was decreased by feeding of the basal diet after any of the supplements and was lowest after skim milk.

Table 4 presents the results of the final series of studies. The addition of both skim milk and 4% fish flour significantly improved the nitrogen retention of dogs fed lime-treated maize flour. In this study, retention of nitrogen was superior for the fish flour supplement.

DISCUSSION

The results corroborate observations made with rats in which lime-treated corn flour was enriched with proteins of animal and vegetable origin (Bressani and Marenco, 1962; Bressani *et al.*, 1960). The improvement in nutritive value made by the protein added, as indicated by the increases in nitrogen retention, is probably due mainly to the contribution of lysine and tryptophan, the most limiting amino acids in lime-treated corn (Bressani, 1960; Bressani *et al.*, 1958; Scrimshaw *et al.*, 1958).

While it is true that nitrogen retention varies proportionally with nitrogen intake and that, in the experiments reported there was some variation in intake, this is not the primary cause of the effects noted. Nitrogen intake, although adjusted accord-

Table 3. Nitrogen balance of dogs fed lime-treated corn supplement with skim milk, torula yeast and fish flour.[a]

		Nitrogen						Average change in NR/NI[b] to basal diet	
		Intake	Fecal	Urine	Retained	Absorbed	Retained		
	Period	mg/kg/day				% of intake		Before	After
Basal	1	721	179	460	82	75.2	11.4	....	....
	2	738	188	437	113	74.5	15.3	....	....
	$\bar{x}$	729	183	448	98	74.9	13.4	....	....
B + 5% skim milk	1	813	179	350	284	78.0	34.9	....	....
	2	796	138	294	364	82.7	45.7	....	....
	$\bar{x}$	804	158	322	324	80.3	40.3	0.269	0.207
Basal	1	684	151	397	136	77.9	19.9	....	....
	2	665	159	378	128	76.1	19.2	....	....
	$\bar{x}$	674	155	387	132	77.0	19.6	....	....
B + 3% torula yeast	1	664	121	274	269	81.8	40.5	....	....
	2	684	159	307	218	76.7	31.9	....	....
	$\bar{x}$	674	140	290	244	79.2	36.2	0.166	0.117
Basal	1	552	135	308	109	75.5	19.7	....	....
	2	526	124	249	153	76.4	29.1	....	....
	$\bar{x}$	539	129	278	132	76.1	24.5	....	....
B + 4% fish flour	1	516	165	185	166	68.0	32.2	....	....
	2	393	142	180	71	63.9	18.1	....	....
	$\bar{x}$	454	153	183	118	66.3	26.0	0.015	....

[a] Average per period of 3 dogs.
[b] Nitrogen retention/nitrogen intake.

ing to the weight gain of the animals, could not be kept constant without force feeding, because the animals refused to consume all of the calculated unsupplemented diet. As suggested by Harper (1957–58), it may be harmful to the animal to consume large amounts of deficient proteins. Care should be taken in suggesting increased consumption of protein with amino acid deficiencies since the refusal of the unsupplemented diet is probably a defense mechanism.

Except for the bean supplement the decrease in nitrogen balance after removal of the supplement was greater the better the protein quality of the supplement. For example, the protein quality of skim milk is superior to that of torula yeast plus lysine and both are superior to torula yeast alone. Although torula yeast is a good source of lysine, only 80% is biologically available; this would account for the better quality when supplemented with lysine.

Table 4. Nitrogen balance of dogs fed lime-treated corn supplemented with skim milk and fish flour.[a]

		Nitrogen					
		Intake	Fecal	Urine	Retained	Absorbed	Retained
Diet	Period	mg/kg/day				% of intake	
Basal + 5% skim milk	1	472	103	275	94	78.2	19.9
	2	469	132	270	67	71.8	14.3
	3	497	132	269	96	73.4	19.3
	$\bar{x}$	479	122	271	86	74.5	17.9
Basal	1	491	125	323	43	74.5	8.7
	2	498	129	291	78	74.1	15.7
	$\bar{x}$	494	127	307	60	74.3	12.1
Basal + 4% fish flour	1	479	109	221	149	77.2	31.1
	2	481	110	216	155	77.1	32.2
	$\bar{x}$	480	109	218	153	77.3	31.9

[a] Each period represents the average of four dogs.

Although black beans are of a lower protein quality than skim milk or torula yeast, the findings with this supplement after its omission from the basal diet did not follow the pattern observed with skim milk, torula yeast with and without lysine. This was probably because of the larger increase in nitrogen intake when beans were fed and the significant decrease in intake when they were omitted. These results deserve further study because the rural populations in many areas in Latin America consume mainly corn and beans.

Nutritional surveys carried out in preschool children in Guatemala (Flores and García, 1960) have shown that some animal protein is consumed every two or three days, and in some cases every day. The effect of this sporadic supplement is not well known though it is assumed to be good. However, as indicated by the dog data, the change might be of little or no benefit to the extent that it reduces subsequent food consumption. The results are of practical importance for populations consuming diets which are poor on both quantity and quality of protein since lime-treated corn is the most important staple food in the rural diet of most Central American countries (Flores, 1961; Flores and García, 1960). Efforts should be made to find effective ways of improving its protein contribution.

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