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CONTENTS

Predominance of Fructose Accumulation in Cold-Stored Immature Potato Tubers

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(Manuscript received August 14, 1961)

SUMMARY

Freshly harvested immature potato tubers contain appreciable amounts of free sugar, mainly as sucrose. As the tuber matures, the percentage of sucrose present decreases and that of starch increases. When stored at 25°C, immature tubers lose sucrose but starch does not accumulate. At 0°C storage, starch content decreases, immature tubers losing starch faster than mature tubers. Free sugars accumulate at 0°C, the major one being fructose in immature tubers, whereas sucrose accumulation predominates in the mature tubers.

As is well known, reducing sugars in cold-stored potatoes are a major factor in the nonenzymatic browning that occurs in many forms of processed potatoes (Schwimmer and Burr, 1959; Schwimmer *et al.*, 1957). Sugar content is quite low in freshly harvested mature tubers (Schwimmer *et al.*, 1954), whereas immature tubers contain appreciable amounts of free sugars, mainly sucrose (Appleman and Miller, 1926; Samotus, 1958). Samotus found that the sugar content of tubers harvested at the end of the bloom stage of development was as high as 1.5–2.0% for different varieties. Reducing sugars constituted 10–35% of the total sugars, and thus could possibly cause nonenzymatic browning during subsequent processing. The effect of storage on the levels of the individual sugars has not been investigated hitherto.

The present paper confirms previous observations on the presence of higher percentages of free sugars and lower percentage of starch in immature tubers as compared with mature tubers. In addition, it is shown that the characteristic responses of immature tubers to post-harvest storage is loss of sucrose and increase in reducing sugars at 25°C and the predominant accumulation of fructose, rather than sucrose, at 0°C.

MATERIALS AND METHODS

Source of tubers. White Rose and Kennebec varieties, used in the present study, were planted on March 4, 1960, in Hesperia sandy loam near Shafter (Kern County), California. Table 1 summarizes pertinent horticultural and harvest data.

Preparation of tubers for storage and analysis. Only tubers of medium size were used. After being carefully washed under tap water and dried, the tubers were divided into three lots of 2 kg each. One lot was analyzed immediately (2 days maxi-

Table 1. Potato cultural and harvest data.

Harvest number	I	II	III
Date harvested	May 3	May 23	June 14
Days of growth after planting	60	80	102
Stage of development[a]	Bud (pre-bloom)	After bloom	Near maturity
Weight of tubers/plant, g[b]	160	279	428
Soil temperatures (°C)	14–19	14–18	22–26

[a] The pre-bloom stage as a criterion for development is not commonly used in Kern County. High temperatures affect flowering, and tops do not often bloom although flower buds are evident. The state of maturity, expressed as days of growth after planting, are close to bud (pre-bloom), bloom, and after-bloom in more-northern growing areas. Normal harvest dates in this area are 105–115 days after planting.

[b] Data for White Rose variety.

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mum between harvest and analysis). The other two lots were stored, one at 25°C and the other at 0°C.

For analysis, each tuber was sliced longitudinally into halves. One set of half-tubers was used for extraction of starch and the other for chemical analyses and determination of dry weight.

Analytical methods. The glucose, fructose, and sucrose contents of deionized aqueous solution derived from alcoholic extraction of 100 g of potatoes were determined according to the procedures of Potter *et al.* (1959). The amounts of each of these three sugars are calculated from reducing sugar values before and after treatment of separate portions of the solution with glucose oxidase and invertase. (The various procedures for extraction, clarification and analysis are set forth in detail in the following sections of the Ninth Edition, Official Method of Association of Official Agricultural Chemist (1960): 6002b; 6074b; 6076b; 6076; 6078; 6079; 6083(2); 20:055; 29:056.) Dry weight was determined by loss of weight from drying for 1 hr at 130°C. Starch was determined in the residue from extraction of 100 g of freshly diced tubers with 5% trichloroacetic acid according to the method of Grossfeld (1930). A Rudolf Polarimeter was used.

RESULTS

Composition of freshly harvested potatoes. Carbohydrate analyses of fresh tubers are summarized in Table 2. Accumulation of solids during tuber growth is largely accounted for by the increase in starch content. The rate of accumulation is greater in the early stage, and is more pronounced in the White Rose than in the Kennebec variety. Total sugar concentration in tubers from the first harvest amounted to 1.5% for the White Rose variety and 0.8% for the Kennebec variety, but progressively decreased with increasing maturity. The main sugar component at all stages of development was sucrose. However, the percentages of sucrose and glucose both decreased during maturation to a greater extent than did that of fructose. The presence of appreciable amounts of sugar cannot be attributed to low soil temperature (Table 1).

Table 2. Carbohydrate content (percent fresh-weight basis)[b] of freshly harvested potato tubers.

Component or measurement	Variety[a]	Days after planting 60	80	102
Dry weight	W	16.2	21.2	23.2
	K	15.9	22.0	22.8
Starch	W	11.4	15.8	18.5
	K	11.3	16.7	18.3
Total sugars	W	1.53	0.84	0.58
	K	0.85	0.46	0.44
Sucrose	W	1.03	0.38	0.56
	K	0.56	0.39	0.32
Fructose	W	0.12	0.08	0.08
	K	0.08	0.05	0.05
Glucose	W	0.38	0.12	0.12
	K	0.21	0.02	0.07

[a] W = White Rose variety; K = Kennebec variety.
[b] Calculated on freshly-harvested-weight basis.

Table 3. Changes in tuber composition during storage at 25°C.

Changes in:	Variety[a]	Days after planting 60 / Storage time, weeks 5	80 / 6	102 / 5
		Percent fresh-weight basis[b]		
Dry weight	W	−0.3	−0.2	−1.5
	K	+0.3	−1.5	−0.6
Starch	W	−0.2	−0.0	−1.1
	K	−0.1	−1.7	−1.8
Total sugars	W	−0.43	−0.46	−0.24
	K	−0.15	−0.15	−0.15
Sucrose	W	−0.58	−0.43	−0.19
	K	−0.23	−0.16	−0.10
Fructose	W	+0.32	+0.01	+0.03
	K	+0.10	+0.02	+0.03
Glucose	W	−0.17	−0.04	−0.08
	K	−0.02	−0.02	−0.02

[a] W = White Rose variety; K = Kennebec variety.
[b] Calculated on freshly-harvested-weight basis.

Effect of post-harvest storage at 25°C. Table 3 summarizes data on the change in composition of tubers after storage for 5 or 6 weeks at 25°C. The percentage loss of total weight of immature tubers was greater than that of mature tubers. The values for total solids (% dry weight) and for starch are corrected for the total weight losses. The small observed changes in these values probably reflect sampling errors. The mean value of the percent change of percent dry weight for both varieties amounted to -1 ± 4. The mean value of the percent change of percent total sugars amounts to -35 ± 9. Sugar losses thus appear to be significant. The loss in sugar is primarily a loss in sucrose and, to a lesser extent, a decrease in glucose. In contrast, fructose increased, especially in the immature tubers.

Effect of post-harvest storage at 0°C. The principal sugar that accumulated in immature potatoes during storage at 0° was fructose (Table 4); the amount of fructose accumulated was more than

Table 4. Changes in tuber composition during storage at 0°C.

Days after planting / Storage time, weeks		60 / 6	80 / 7	102 / 6
Changes in:	Variety [a]	Percent fresh-weight basis [b]		
Dry weight	W	+0.2	−1.2	−0.6
	K	+1.0	−2.0	+0.2
Starch	W	−5.1	−5.2	−4.2
	K	−5.6	−5.9	−3.4
Total sugars	W	+4.87	+3.91	+3.86
	K	+5.81	+3.88	+3.05
Sucrose	W	+0.73	+1.25	+2.09
	K	+1.53	+2.09	+1.99
Fructose	W	+3.94	+1.90	+1.30
	K	+3.81	+1.32	+1.01
Glucose	W	+0.20	+0.76	+0.47
	K	+0.47	+0.47	+0.05

[a] W = White Rose variety; K = Kennebec variety.
[b] Calculated on freshly-harvested-weight basis.

three times that of sucrose. In mature potatoes sucrose accumulation was predominant, as has been frequently reported (Schwimmer *et al.*, 1954). The mature potatoes accumulated almost twice as much sucrose as fructose. That this represents a progressive shift toward predominance of sucrose accumulation in 0° storage with increasing maturity of the tubers is borne out by data from the second harvest, in which the ratio of accumulated fructose to sucrose was intermediate to values for the first and last harvest.

DISCUSSION

The appearance of significant quantities of sugars in freshly harvested immature potatoes is, on first sight, rather surprising in view of the heavy demands for rapid, continuous starch synthesis and heightened respiration rates. Apparently the rate of translocation of sugars from the leaves into the tubers exceeds the combined rates of those two processes. When these immature tubers are harvested and stored at 25° for 5 or 6 weeks, neither starch decomposition nor sugar accumulation occurs. On the contrary there is some indication of a disappearance of sucrose, which would indicate that sucrose is utilized preferentially in the respiratory process. The observation that the fructose content increases suggests that this sugar is not as directly involved in respiration as glucose and that the glucose moiety of the sucrose molecule is utilized at greater rate than the fructose moiety. Thus, White Rose and Kennebec tubers from the first harvest respectively increased 0.32% and 0.10% in fructose, and decreased 0.29% and 0.11% in the glucose moiety of sucrose.

The hypothesis that the glucose moiety of sucrose is preferentially utilized for respiration in the post-harvest state is corroborated by the sugar accumulation pattern at 0° storage. In this case the two pertinent overall reactions are presumed to be the rapid conversion of starch to sucrose followed by respiration. If respiration does indeed proceed *via* a mechanism in which the glucose moiety of sucrose is preferentially utilized, then fructose would accumulate provided that the respiration rate of the immature tuber is large in comparison to the rate at which sucrose is formed from starch. On the other hand, the fact that mature tubers respire at a slower rate than immature tubers (Appleman and Miller, 1926; Shaw *et al.*, 1958) suggests that the competition between sucrose accumulation and respiration favors the sucrose accumulation so that storage of mature tubers would result in a predominant increase in sucrose.

The hypothesis herein presented is in partial agreement with the conclusion of Barker (1936) that sucrose is the immediate respirable substrate, but is at variance with the picture presented by Barker in that the fructose rather than the glucose moiety of sucrose is considered to be utilized preferentially in respiration. In this connection, recent reports on the central role of uridine diphosphate glucose in the metabolism of both starch (Fekete *et al.*, 1960) and sucrose (Schwimmer and Rorem, 1960) in potatoes is suggestive of the dynamic role played by glucose.

The low starch content and the presence of appreciable reducing sugars in immature tubers, even after storage at room temperature, renders such tubers unsuitable for most processing purposes. It is of interest to note that the total sugar content of immature potatoes stored at 0° exceeded the starch content and accounted for 40% of the total solids. These levels of sugar and sugar-to-starch ratio are quite comparable to those of sweet potatoes (Sistrunk *et al.*, 1954; Scott and Matthews, 1957).

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Reference to a company and/or product name does not imply approval or recommendation to the exclusion of other products that may also be suitable.

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Amino Acid Composition of Normal and Degenerated Pig Muscle

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(Manuscript received July 22, 1961)

SUMMARY

The distribution of nineteen amino acids was found to be almost identical in *Longissimus dorsi* from a normal pig and a pig affected with total muscle degeneration.

INTRODUCTION

There might be no disagreement regarding the manifestations of muscle degeneration referred to in this paper. However, there is no uniform designation of this disorder, which is characterized by a pale, exudative (watery) appearance of the musculature, structural changes, a rapidly elapsing post-mortem glycolysis with consequent rapid lowering of pH, and low water-holding capacity. The condition has been studied during the last 10 years, primarily in England, Denmark, France, USA, Sweden, Holland, and Poland. In Scandinavia the disease is called "muskeldegeneration," in France "la myopathie exudative dépigmentaire du porc," in English literature the Scandinavian term appears—sometimes in English spelling—along with "muscular dystrophy," "waxy muscle degeneration," "myopathy," and "white muscle disease."

Causes of muscle degeneration. Many causes of muscle degeneration in pigs have been recorded. No exhaustive enumeration of these causes or a comprehensive survey of the literature in this large field of research is made here. Ludvigsen (1955, 1957, 1958, 1959) emphasized the relation between muscle degeneration and breed, and pointed to the fact that pigs selected for a high rate of growth and increased body length produce an enhanced amount of growth-stimulating hormone and reduced amounts of ACTH, thyroid-stimulating hormone, and adrenocortical hormones. Deficiency of the last three types of hormones reduces the adaptive mechanism of the body. Consequently, stress of any kind may cause development of muscle degeneration. Henry (1957), Henry and Billon (1959), and Bugard *et al.* (1959) reached similar conclusions. In addition, these investigators particularly refer to the hyperkalaemia in muscle degeneration, owing to excretion of potassium ions from the cells and indicating a disturbance in the production of adrenocortical hormones.

The impairing effect of domestication and stress on the quality of pork muscles can be reduced or eliminated by exercising the pigs for some days prior to slaughter, according to Briskey (1959) and Briskey *et al.* (1959a,b).

In agreement with what was found by Ludvigsen (1955, 1957, 1958, 1959), *viz.*, the importance of the heredity factor, certain breeds, e.g., Poland China, Piétrain, and Danish Landrace, are particularly liable to muscle degeneration. In addition, Wilson *et al.* (1959) showed that the concentration of myoglobin and the degree of "two-toning" of hams are heritable characteristics.

The influence of the intensity of feeding and the composition of the foodstuff, especially its content of protein and vitamins, on the development of muscle degeneration has been studied by several investigators. Cereal grown and harvested under unfavorable weather conditions, rancid fats as well as polyunsaturated fats deficient in tocopherol may cause muscle degeneration in pigs (Orstadius and Åberg, 1961; Orstadius, 1961). The disorder can be successfully treated prophylactically with tocopherol and sodium selenite and therapeutically with Na-selenite alone. Muth *et al.* (1959) reported

that Na-selenite exerts a strongly protective effect against white muscle disease in lambs and calves.

Background of the present investigation. We know that in the first place the most expensive parts of the carcass—the loin and the ham—are concerned with the disorder. Shall the veterinarian condemn pork with muscle degeneration as unfit for human consumption? It is evident that the inspecting veterinarians would need some basis for a decision. The practice in Sweden is to condemn the parts of the carcass that have total muscle degeneration, whereas those with partial muscle degeneration are approved for sausage manufacture.

The different appearance of normal and degenerated pig muscle might by itself not be a reason for assuming a possibly different amino acid composition of the respective proteins. Regarding the well established different biochemical behavior of normal and degenerated muscle from a general point of view, however, it cannot be taken for granted that protein synthesis and metabolism proceed in the same way in the two cases.

No consistent results exist with regard to color score in relation to content of pigment. Thus, Wismer-Pedersen (1959) found no systematic difference in pigment concentration between loins of differing color. He concluded that meat with normal water-holding capacity has a desirable color even at low contents of pigment. On the other hand, Lawrie (1960) reported a virtual absence of pigment in pale muscles. The myoglobin content, however, is so small a fraction of the meat protein that a varying content will not substantially influence the amino acid composition of the meat protein as a whole. Another, and more decisive, reason for the present investigation was the findings of Spangenberg (1957), which reveal great differences in the composition of essential amino acids in protein from well-fed and emaciated cattle. For instance, the content of phenylalanine in the *Musculus gracilis* from 20 well-fed animals was on an average 3.9%, whereas that of the same muscle from 20 emaciated animals was 10.1%. In contrast, Greenwood *et al.* (1951) found virtually the same percentages of eighteen amino acids in the protein of six different cuts of both choice and utility grades of beef. In agreement with these results are those of Schweigert *et al.* (1949) on pork and lamb cuts. At the Veterinary College, Hannover, where Spangenberg (1957) made the investigations just mentioned, Salobir (1958) also studied protein chemistry. By paper electrophoresis he recorded significantly different protein composition of meat from pigs fed a nutritionally complete diet and pigs fed a protein-deficient ration. Although this finding does not necessarily mean differences with regard to the amino acid composition, it is an indication of changes that may be of importance in consideration of the problem as a whole.

EXPERIMENTAL

About 0.5 g dry, acetone-treated meat (*Longissimus dorsi*) from a normal sow (dressed weight 57 kg) and from a barrow (dressed weight 66 kg) with total muscle degeneration was hydrolyzed by boiling 16 hr in 50 ml 6*N* HCl using reflux. The hydrolysate was diluted to 500 ml, and pH was adjusted to 2.0.

It may be noted that 23 kg of the loins and the hams in the carcass with muscle degeneration were condemned. Samples were taken of the loin at the lumbar region, where the disease is most pronounced and, according to Lawrie (1960), the ultimate pH of the meat is very much lower than that in the thoracic region.

Two ml of the diluted hydrolysate was run in a continuously operating amino acid analyzer for determining the amino acids, using ninhydrin as a reagent. The acidic and neutral amino acids were initially eluted from the column (Amberlite IR 120) with a 0.20*N* citrate buffer of pH 3.25. After proline the buffer solution was shifted to pH 4.25. The basic amino acids were determined in a separate column, using a 0.35*N* citrate buffer of pH 5.28 for elution.

Since tryptophan will be completely destroyed by acid hydrolysis, this amino acid was determined separately on the original material, using the method of Graham *et al.* (1947), slightly modified. In addition, hydroxyproline, for which ninhydrin cannot be used as a reagent, was determined separately as well, using the method of Stegemann (1958). Finally, the content of water-soluble guanidino compounds (essentially creatine and very little free arginine) was determined according to Rosenberg *et al.* (1956). It was calculated as percent creatine of total (crude) protein.

RESULTS

Table 1 lists the amino acid composition expressed as percent by weight of the sum of amino acids present in the hydrolysate of acetone-dried *Longissimus dorsi* from the normal pig, and from the pig with total muscle degeneration. In addition, the content of water-soluble guanidino compounds is given. The amino acids are listed in the order they appear in the effluent. The methionine-sulfoxides, which precede aspartic acid, are for practical reasons placed after methionine. Tryptophan and hydroxyproline, which were determined separately, are placed at the end of the table.

The yield of amino acids + ammonia, reduced by the water bound during hydrolysis, was found to be 98.5% (normal pig) and 100.9% (degenerated pig) of the amount of protein used for analysis. The content of protein in the acetone-dried meat was determined in two ways: 1) by multiplying the nitrogen content by 6.25, 2) by subtracting the content of water (7.4–7.0%), ash (3.6–3.4%), and lipids (0.8–0.7%) from 100. The respective results were 88.4 and 88.2% protein (normal pig) and 88.6 and 88.9% (degenerated pig). Thus, there was good agreement between the methods. For calculation the means were used.

Table 1. Relative percentage of the various amino acids found in hydrolysates from acetone-dried *Longissimus dorsi* of pigs.

Amino acid	Normal pig	Pig affected with total muscle degeneration
Aspartic acid	9.89	9.88
Threonine	4.75	4.75
Serine	4.19	4.15
Glutamic acid	15.65	15.66
Proline	3.94	4.04
Glycine	4.34	4.19
Alanine	5.80	5.74
Cystine	~0.3	~0.3
Valine	5.10	5.27
Methionine	2.93	3.25
Methionine-sulfoxides	~0.3	0.00
Isoleucine	4.76	4.94
Leucine	8.28	8.32
Tyrosine	3.64	3.72
Phenylalanine	4.27	4.14
Lysine	9.50	9.61
Histidine	4.45	4.25
Arginine	6.45	6.40
Tryptophan	1.29	1.26
Hydroxyproline	0.17	0.13
Total	100.00%	100.00%
Water-soluble guanidino compounds (essentially creatine), calculated as percent of total protein	2.64	2.60

From Table 1 it is evident that, within the limits of analytical error, the amino acid composition of the protein in normal and degenerated pig muscle is the same. That is also true for the water-soluble guanidino compounds. In the case of methionine a significantly lower content was found in the meat of the normal pig, but the difference could be accounted for by methionine-sulfoxides present in this sample. It might be assumed that these oxides are artifacts formed during the hydrolysis process or the manipulations. Among the other differences, the one for histidine is the largest. The histidine value for the protein in the degenerated pig muscle is hardly 5% (relative) lower than that for the protein in the normal pig muscle. However, this difference is nevertheless too small to be significant.

In view of these results it would be of interest to know whether there might also be nutritional equality between normal and degenerated muscles with respect to other constituents, e.g. thiamine.

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Spectral Examination of Cured-Meat Pigments During Frankfurter Processing [a]

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SUMMARY

Spectrophotometric analyses of aqueous-acetone extracts of frankfurters were useful for characterizing the porphyrin pigments that formed during smokehouse treatment or laboratory heating. The absorption curves showed differences among samples in spectral structure, rates of pigment development, and pigment stability. They also showed promise of differentiating among samples on the basis of storage stability.

Frankfurters are an important and popular food in the armed forces of the United States. Providing a high-quality product in remote overseas areas requires a minimum freezer-storage life of six months. Even though all frankfurters are produced in accordance with the requirements of the Federal Specification (1954) and appear identical when freshly prepared, their shelf life varies. Experience indicates that some lots exceed minimum stability needs while others become rancid within two months. It would therefore be desirable to develop objective methods capable of differentiating among lots on the basis of their predisposition to become rancid. For this purpose, our attention was directed to a study of the cured-meat pigments of frankfurters.

The heme pigments of uncured meat catalyze the oxidation of lipids (Watts, 1954; Younathan and Watts, 1959). Cured meats are more stable (Younathan and Watts, 1959), but evidence on the relation of cured-meat pigments to lipid oxidation is less definite. Bauernfiend *et al.* (1954) and Wiesman and Ziemba (1946) showed that flavor stability paralleled pigment stability during freezer storage of such comminuted cured meat products as wieners and sausages.

Heme pigments are present in cured meat products principally as nitrosohemochrome (the reduced or ferrous form), as brown denatured metmyoglobin (the oxidized or hemichrome form), or as a mixture of the two. Greenish discoloration can result from advanced oxidation, which ruptures the porphyrin ring to form verdohemochromes (Am. Meat Inst. Foundation, 1960; Brown and Tappel, 1958). The globin portion of the pigment may be present in either the undenatured or denatured form, depending on the extent of heat processing.

Younathan and Watts (1959) hypothesized that the oxidized form of the pigment is the active catalyst in the oxidation of the fat. However, this cause-and-effect relation may at times be reversed because other changes accompanying the development of the cured-meat pigments during processing and their role have not been fully assessed. Brown and Tappel (1958) reported that nitrosohemochrome forms in many reducing media but that it is most stable when formed in the presence of sulfhydryl groups released

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by heat denaturation of the protein. Metmyoglobin may therefore be formed in the presence of oxidized fat when there is a deficiency of sulfhydryls. Insolubilization of the pigment protein by heat may also retard interactions with the fat in the meat emulsion. Thus the physical state of the emulsion as well as its chemical composition may be a controlling factor in stability.

This paper reports the changes in the absorption spectra of the pigments as they develop under smokehouse and laboratory conditions. Some observations were also made on the relation of spectral characteristics to stability, but a more intensive study will be the subject of future work.

EXPERIMENTAL

Materials. Two local processors of frankfurters furnished samples withdrawn from the smokehouse at approximately one-hour intervals during the smoking treatment. They also provided unsmoked samples. The smokehouse temperature and the product internal temperature were recorded at each withdrawal (Table 1).

Table 1. Processing temperatures (°F) of Company A and Company B frankfurter samples.

Processing time (min)	Company A		Company B	
	Smokehouse temp.	Product temp.	Smokehouse temp.	Product temp.
0	.....	60	.....	.....
60	160	122	130	130
90	.....	.....	140	135
120	160	152	150	140
180	180	155	150	146

The product of company A contained equal portions of beef and pork and was part of a military procurement under the Federal Specification (1954). The product of company B was 60% beef and 40% pork and was processed for the commercial market. The samples contained no added color or ascorbic acid. The samples were delivered to the Institute laboratories on the day prepared, stored overnight at 4.4°C, and tested the following day.

Methods. *Sample preparation.* After the casings were removed the frankfurters were ground through a food chopper and further triturated with a mortar and pestle. Spectral analyses and protein solubility tests were conducted on portions of the prepared material, directly, and also after laboratory heating.

Laboratory heating. Twenty-gram samples of prepared frankfurters were wrapped and sealed in flat aluminum-foil packets of approx 3 × 5 × 3/16 in. The packets were immersed in a 60°C water bath for periods up to 3 hr. Above 60°C, the color developed too rapidly for study. Individual packets were withdrawn at intervals and the entire contents were tested.

Absorption spectra. Hornsey (1956) and others (Siedler and Schweighert, 1959; Tappel, 1957) used acetone-water solutions (40:3) to extract cured-meat pigments for spectral analysis. The extracted pigment, no longer attached to the globin moiety, was shown to be a nitrosoheme-acetone complex (Hornsey, 1956). It has also been hypothesized that the heme component probably exists in the meat unattached to the globin (Am. Meat Inst. Foundation, 1960).

In our work, the sample size was increased over that used by Hornsey and others in order to reveal the spectral structure more clearly. All extractions were conducted in subdued light to avoid light-catalyzed oxidation of the acetone complex (Tappel, 1957). Twenty grams of the prepared sample in a mortar were covered with 43 ml of the aqueous acetone. The sample was squeezed and pressed with a pestle for 5 min, care being taken to avoid undue aeration. The extract was filtered through Whatman No. 1 filter paper. Absorption spectra from 450 to 700 mμ were then obtained with a Model 14 M Cary recording spectrophotometer (mention of commercial products does not imply endorsement by the Department of Defense over other similar products not mentioned).

Soluble protein. A modification of Coretti's (1957) method was used to judge the extent of protein denaturation as an index of heat treatment during processing. Five-gram samples were extracted by intermittent shaking for 30 min in a flask with 25 ml of distilled water and filtered through Whatman No. 42 filter paper. Ten-ml portions of the filtrates were heated 15 min at 65°C to coagulate the soluble protein. The absorbancy of the turbid solution was measured with an Evelyn colorimeter with the 515 filter.

Keeping quality. Frankfurters in their cellulose casings were wrapped, 6 to a package, in aluminum foil and stored at −18°C in cardboard cartons. After 4–12 months of freezer storage, a frankfurter from each processing treatment was cooked 10 min in boiling water in a covered beaker. The intensity and character of the odor of the hot frankfurter samples were judged by a panel of laboratory personnel.

RESULTS AND DISCUSSION

Heretofore, aqueous-acetone extraction has been used largely for quantitative determination of cured-meat pigments. The purpose here was to observe qualitative and quantitative differences in spectra as they developed during processing or laboratory treatment.

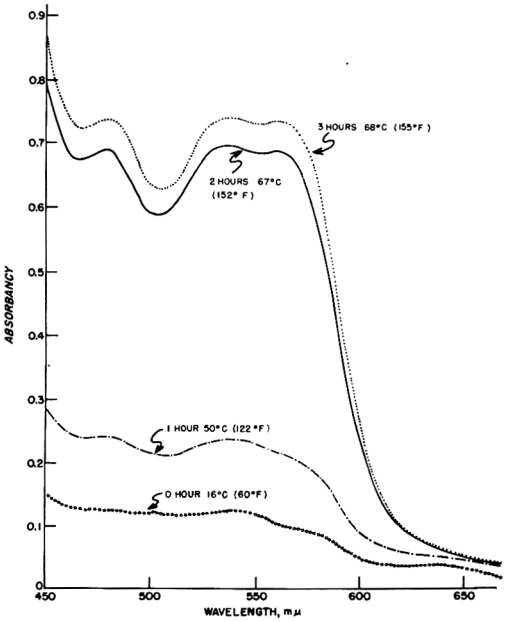

Fig. 1. Development of cured-meat pigment in Company A samples during smoking.

Pigment spectra of company A samples. Fig. 1 shows the pigment spectra of samples removed from the smokehouse at hourly intervals. The indicated temperatures are internal sample temperatures. The absorption maxima at 480, 540, and 565 mμ, characteristic of nitrosohemochrome (Hornsey, 1956; Tappel, 1957), were fully developed in approx 2 hr when the internal temperature reached 67°C. Most of the color developed during the second hour of smoking.

Fig. 2 shows that the same characteristic spectrum developed during laboratory heating of the unsmoked frankfurters at 60°C. The small dimensions of the aluminum-foil packet permitted rapid heat penetration and faster development of color.

Pigment spectra of company B samples. The pigment in samples from company B developed in a different manner from that described above. Pigment concentration was near a maximum after only 1 hr of smokehouse treatment when the internal temperature was 54°C. The curves in Fig. 3 gave evidence of instability upon heating in the smokehouse beyond the first hour. The absorption maximum at 565 mμ became progressively less well-defined until it disappeared, at 3 hr. Concurrently, a peak developed at 635 mμ, suggestive of a hemichrome form of the pigment.

Fig. 4 shows the results of laboratory heating of the unsmoked frankfurters at 60°C. Under these conditions, as in the smokehouse, the pigment developed more rapidly than it did in company A samples. In other respects, however, the pigment characteristics were more like those of Figs. 1 and 2 than those of Fig. 3. The spectral structure was that of nitrosohemochrome, and stability was good upon continued heating for 3 hr. The results in Figs. 3 and 4 indicated that the meat emulsion and the curing ingredients were inherently capable of developing the stable nitrosohemochrome, but that a smokehouse or other processing factor led to instability.

The fact that the same procedure was used for obtaining the data in Figs. 3 and 4 indi-

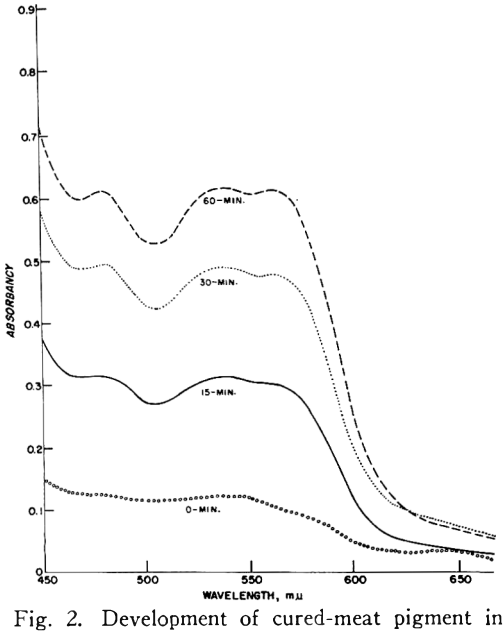

Fig. 2. Development of cured-meat pigment in unsmoked Company A samples during laboratory heating at 60°C.

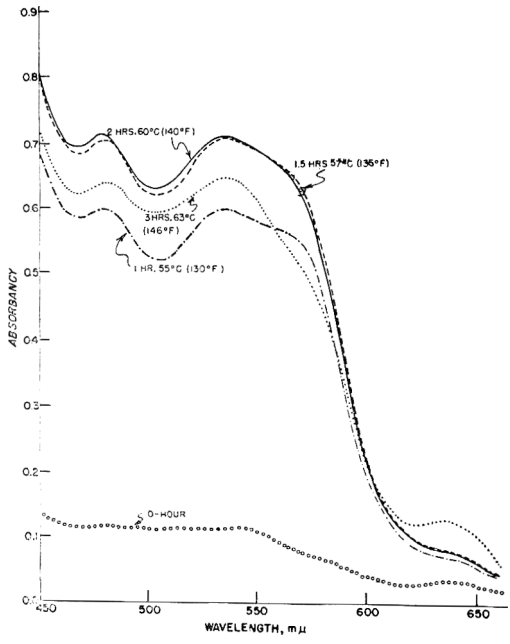

Fig. 3. Development of cured-meat pigment in Company B samples during smoking.

cated that the 635-mμ peak of Fig. 3 was not the result of light-catalyzed oxidation of the extracts. Extracts of the smoked samples did not develop the 635-mμ peak when exposed to subdued light for 2 hr. Furthermore, Hornsey (1956) showed that the light-catalyzed oxidation product had a single absorption maximum, at 566 mμ.

Samples of the material smoked for 3 hr were subjected to laboratory heating at 60°C. The absorption curves in Fig. 5 show that the spectral shift from 565 to 635 mμ that occurred during smoking could be reversed by the laboratory heating. Total exclusion of air under these conditions suggested that the smokehouse may not have had a sufficiently reducing atmosphere to prevent oxidation of the nitrosohemochrome (Fox, 1959).

Absorbancy index of frankfurter pigment. The sharpness of the α-band in the 560-mμ region is characteristic of the hemochrome structure (Lemberg and Legge, 1949). An absorbancy index employing the measurements at 505, 540, and 565 mμ may therefore be useful for characterizing the extracted pigment. Table 2 shows the values

Table 2. Absorbancy index of frankfurter pigment.

$$\frac{A_s\ 565\ m\mu - A_s\ 505\ m\mu}{A_s\ 540\ m\mu - A_s\ 505\ m\mu}$$

Smoking time, or lab. heating time (min)	Company A samples		Company B samples		
	Smoked	Unsmoked, lab. heated	Smoked	Unsmoked, lab. heated	Smoked 3 hr, lab. heated
15	.....	0.67	.....	0.94	−1.90
30	.....	0.80	.....	0.94	0.00
60	−0.18	0.94	0.59	0.94	0.53
90	.....	.....	0.47	.....	.....
120	0.91	.....	0.32	1.00	.....
180	0.97	.....	−1.20	1.00	.....

of this index $\frac{A_s\ 565\ m\mu - A_s\ 505\ m\mu}{A_s\ 540\ m\mu - A_s\ 505\ m\mu}$ for the spectra in Figs. 1 to 5. They ranged from 1.00, for nitrosohemochrome, to −1.90, for oxidized pigment.

Relation of temperature during smoking to pigment development. The effect of temperatures on pigment development, independent of other factors, could not be ascertained from these experiments. The greatest pigment concentration (Fig. 1) and highest product temperature (68°C, 155°F, Table 1)

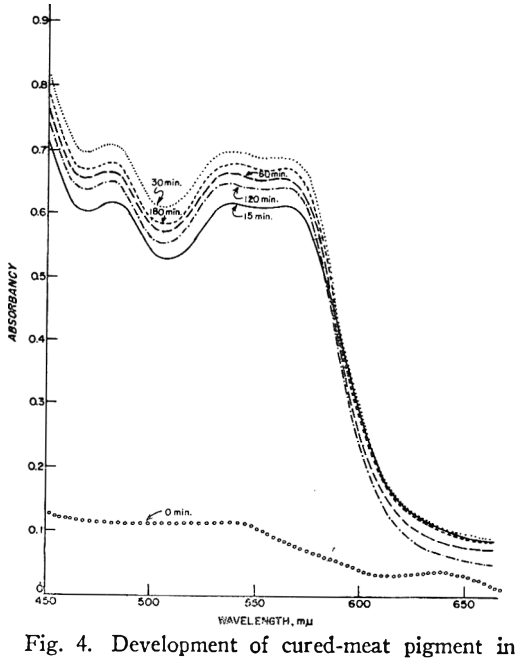

Fig. 4. Development of cured-meat pigment in unsmoked Company B samples during laboratory heating at 60°C.

were attained in the company A smokehouse. The concentration could not be equaled by 60 min of laboratory heating at 60°C (Fig. 2). The highest product temperature reached in the company B smokehouse was 63°C. The unstable pigment that developed could be equaled in concentration by 30 min of laboratory heating (Figs. 3 and 4). The greater stability of the pigment developed in the company A product appeared to be related to the higher product temperatures reached at the end of 120 and 180 minutes of smoking. The final product temperature prescribed by the Federal Specification (1954) for frankfurters is 66–71°C (150–160°F). A second source (Am. Meat Inst. Foundation, 1960) considers 66°C to be a minimum end-point temperature but recommends a temperature of 68°C to provide a safety margin for any variation that may arise from position in the smokehouse or errors in thermometer placement. This higher processing temperature is said to provide better keeping quality in the product.

The processing temperatures in Table 1 and the pigment spectra in Figs. 1 and 2 suggest that a product temperature of 68°C is not a required minimum for pigment development but is important for conferring stability on the pigment that develops at lower temperatures.

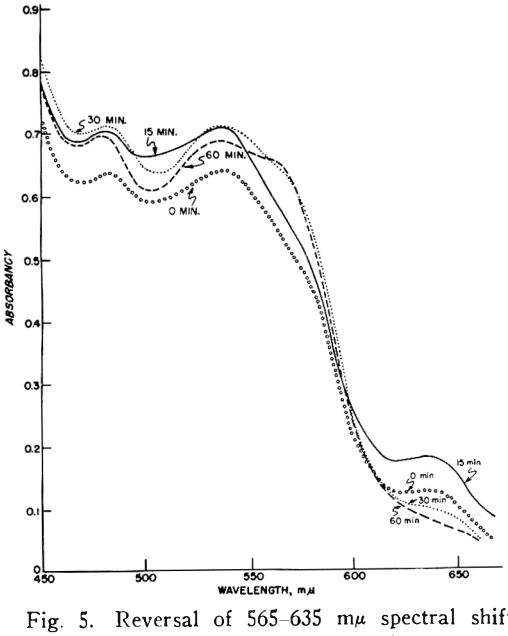

Fig. 5. Reversal of 565–635 mμ spectral shift (3-hr sample, Fig. 3) by additional laboratory heating at 60°C.

Relation of protein solubility to pigment formation and stability. A close relation between pigment formation and stability and the extent of protein denaturation has been reported (Am. Meat Inst. Foundation, 1960; Fox, 1959; Siedler and Schweigert, 1959). The protein-solubility results on the company A samples (Table 3) showed a consistent pattern with time of smoking. Complete protein denaturation as defined by loss of solubility occurred sometime between the first and second hours of smoking. At the same time a definite increase in pigment concentration occurred (Fig. 1) and the absorbancy index reached 0.91 (Table 2).

The smoking time for minimum solubility in the company B samples also coincided with the interval of rapid pigment development—approx 1 hr. However, results of the solubility test on the smoked samples were not entirely consistent with processing time. Although sample variation may be partly responsible, there may be a relation between the erratic behavior of this product and the instability of the pigment.

Relation of pigment development to keeping quality. Sensory evaluations were made after storage at −18°C. A rank order of stability was assigned that represented degree of retention of normal frankfurter odor in the cooked samples. Throughout 12 months of storage the stability of company A samples was directly related to processing time, pigment concentration, absorbancy index, and

Table 3. Soluble protein test on frankfurters (absorbancy, A_s 515 filter).

Smoking time, or lab. heating time (min)	Company A samples	Company B samples	
	Smoked	Smoked	Lab. heated
0	0.429[a]	0.301[a]	0.301[a]
15	.....	.....	0.143
30	.....	.....	0.137
60	0.296[a]	0.027	0.056
90	.....	0.375	.....
120	0.080	0.029	0.061
180	0.017	0.127	.....

Absorbancy of 0.080 or lower represents negligible turbidity.

[a] Test conducted on 1-g rather than 5-g sample.

insolubility of the protein. From the sixth to the twelfth months, only the samples smoked for 2 or 3 hr had a normal frankfurter odor. All of the smoked samples retained good color for 12 months. Among company B samples, which were more erratic in their pattern of pigment development, only the sample smoked 2 hours retained an odor resembling that of normal frankfurters after 4 months of storage. Off-odors were detectable in this sample after 6 months, and severe greenish discoloration occurred in all samples after 10 months. Storage stability of the company B product was definitely inferior to that of the company A product.

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Spectrophotometric Determination of Sorbic Acid in Apple Cider[a]

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SUMMARY

An improved method for measuring sorbic acid in cider is based on dilution of the sample and direct ultraviolet readings at 262 mμ. This method was compared with the distillation method for sorbic acid usually applied to cider. The latter method produced ultraviolet-absorbant materials that interfered with sorbic acid determinations and were shown to be affected by the sugar content of the cider and the distillation techniques. The ultraviolet-interfering materials in cider distillates have absorption peaks and chemical properties similar to those of 5-hydroxymethylfurfural.

In this paper, "cider" refers to unpasteurized unfermented juice from apples, not the heat-processed canned (or bottled) product. Freshly pressed cider has distinctive consumer appeal. Freezing, chemical preservation, and pasteurization have been studied (Marshall, 1947; Robinson *et al.*, 1958; Smock and Neubert, 1958; Tressler and Joslyn, 1954) as means of maintaining cider in good condition.

Smaller cider producers are particularly interested in the addition of chemical preservatives to extend the shelf life of cider during warm fall weather. Weaver *et al.* (1957), Robinson and Hills (1959), and Dryden and Hills (1959) found sorbic acid and its salts to be good preservatives, because their fungistatic properties increased storage life with the least flavor change. Therefore, it appeared desirable to be able to follow concentration changes in sorbic acid during the storage life of cider.

Distillation and spectrophotometric procedures have been developed and used by Melnick and co-workers (Gooding *et al.*, 1955; Luckmann and Melnick, 1955; Melnick and Luckmann, 1954) for sorbic acid determination in dairy products and other foodstuffs. Other workers (Böhme and Bertling, 1959; Spanyár and Sánder, 1958; Hardon and Visser, 1958; Alterton and Lewis, 1958) have used alterations of these procedures for their studies on sorbic acid. This paper presents a simple and accurate method of determining sorbic acid in cider based on the ultraviolet absorption of diluted cider rather than cider distillates.

MATERIALS AND METHODS

Sorbic acid. Commercial samples of sorbic acid and potassium sorbate from Union Carbide Chemicals Company were used (mention of companies or products does not imply endorsement by the United States Department of Agriculture over others not named). Ultraviolet absorption curves for carefully prepared solutions of known concentrations agreed with the published spectra of Alterton and Lewis (1958), Luckmann and Melnick (1955), and Melnick and Luckmann (1954).

Cider. Freshly pressed cider was obtained from a local producer. Fresh cider was compared with pectinol clarified, heated and unheated, fermenting and fermented samples, with and without added sorbic acid. Commercially bottled cider was also studied.

Instrument. Adsorption spectra in the 210–320-mμ region were recorded on a Cary Model 14 spectrophotometer in a 1-cm cell versus acidified distilled water.

Sorbic acid distillation procedure. Cider samples of 5–10 ml, with and without added sorbic acid, were placed in a 500-ml distilling flask containing 50 g of $MgSO_4 \cdot 7H_2O$, 50 ml of distilled water, and 1 g of citric acid. The electrically

[a] Presented at the Institute of Food Technologists, San Francisco, California, May 15–19, 1960.

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heated flask was connected to a Friedrichs condenser. When 45 ml of distillate had been collected in a graduated cylinder cooled in an ice bath, 50 ml of distilled water was added through the side opening of the flask. The distillation was stopped after an additional 45 ml was collected, and the condenser rinsed with 90 ml of hot water. The distillate and rinse water were diluted to 1000 ml with acidified distilled water (pH 3.2–3.5) to prepare the sample for ultraviolet absorption spectrum measurements.

Malic acid may be substituted for citric acid, or 50 ml of 0.1*N* H_2SO_4 for the distilled water and citric acid, without affecting the results. An excess of acid (10 ml of conc. H_2SO_4) added to the distilling flask greatly increased the amount of interfering materials in the distillate. Variations in distilling techniques will affect the distillate of the test samples and their blanks by incomplete distillation or excessive pyrolysis.

Dilution method. Recovery errors are eliminated by this method, in which 1 ml of cider, with or without sorbic acid, was diluted to 100 or 200 ml by acidified distilled water (1 ml of 1*N* HCl per 100 ml of distilled water). The cider solution may be filtered through No. 50 Whatman paper, before or after dilution, to ensure clarity. The optimum sorbic acid concentration for measurement in a 1-cm cell is 1–3 ppm, which can be determined with an error no greater than ± 1%.

Calculation of sorbic acid concentration. For both the distillation and dilution procedures:

$$\text{ppm sorbic acid} = \frac{A_s - A_b}{.23} \times \text{dilution factor}$$

Where A_s = absorbance of cider + sorbic acid at 262 mμ

A_b = absorbance of cider blank at 262 mμ

.23 = constant (absorbance of 1 ppm sorbic acid at 262 mμ in 1-cm cell)

$$\text{Dilution factor} = \frac{\text{diluted volume}}{\text{original volume}} \text{ of sample}$$

RESULTS AND DISCUSSION

Spectrophotometric determination of sorbic acid in apple cider is based on the degree of ultraviolet absorbance of this substance at the wave length of maximum absorption. This maximum occurs at 262 mμ, and the absorbance increases proportionally with concentration, in agreement with Beer's Law.

Fig. 1 illustrates application of the dilution method for sorbic acid determination at various levels in cider. Curve A is for the 1% cider blank. Similarly curves B, C, D, and E respectively represent cider samples containing 23, 46, 91, and 182 ppm of sorbic acid. Because no heat is used, this method avoids the generation of irrelevant ultraviolet absorbant materials influenced by the cider's quality (clarified, pasteurized, fresh and/or fermenting) and no recovery losses are encountered.

However, many sorbic acid determination procedures are based on its recovery by distillation. These methods were applied to cider; and under some conditions, irrelevant materials interfere with the absorbance readings.

Fig. 2 and Table 1 are included in this paper to demonstrate problems in the application of the distillation procedure for sorbic acid recovery and determination from cider.

Fig. 2, curve A, indicates that fresh cider distillates contain materials that have a maximum absorption peak at 282 mμ. When sorbic acid is distilled from cider (B and C curves), peak absorption (between 262 and 282 mμ) is shifted relative to the sorbic

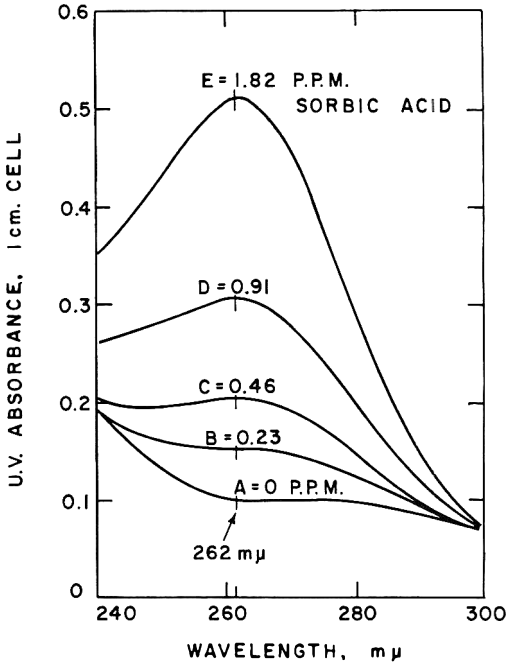

Fig. 1. Sorbic acid determinations in cider by dilution; dilution factor = 100. A) cider blank; B) cider + 23 ppm sorbic acid; C) cider + 46 ppm sorbic acid; D) cider + 91 ppm sorbic acid; E) cider + 182 ppm sorbic acid.

Table 1. Effect of microbiological activity on sorbic acid determinations in cider.

Storage time at room temperature (days)	By dilution: Ultraviolet absorbance at 262 mμ		By dilution: Calculated sorbic acid content[b] (ppm)	By distillation: Ultraviolet absorbance at 262 mμ		By distillation: Calculated sorbic acid content[b] (ppm)
	Sorbated-cider[a]	Blank-cider[a]		Sorbated-cider[a]	Blank-cider[a]	
0	0.428	0.068	156	0.440	0.100	148
4[c]	0.422	0.065	155 (154)[d]	0.414	0.050	158 (137)[d]
8[c]	0.420	0.058	157 (153)[d]	0.410	0.021	169 (135)[d]

[a] The sorbated-cider and its blank (same lot) were inoculated with spoilage organism from fermenting cider, then similarly sampled and analyzed by above methods. The cider was of late season and had a pH 3.95.

[b] $\frac{(\text{sorbated-cider}) - (\text{blank-cider})}{0.23} \times 100 = \text{ppm sorbic acid.}$

[c] There was considerable microbiological activity indicated by gas pressure and rising bubbles in cider.

[d] Recalculated using the zero or initial blank values, the differences show that the distillation method is considerably affected by microbial quality changes in the cider (sorbated or blank), altering the value of its calculated sorbic acid content. The dilution method is not similarly affected.

acid-cider ratios. The effect is especially large at low sorbic acid levels because of superimposition of the spectra of the interfering cider distillate materials on that of sorbic acid. The quantity of interfering materials in the distillate is affected by the amount of cider distilled, the distillation procedure, and the quality of cider, but the amount of interfering materials in the distillation procedure can be kept constant by using a standardized procedure and cider of constant quality (prevention of microbial growth). Then the sorbic acid content can be calculated by the absorbance difference of a sorbated-cider minus its blank measured at 262 mμ. Thus, the sorbic acid contents of cider used for B and C curves of Fig. 2 respectively calculate to 174 and 103 ppm for cider containing 180 and 96 ppm.

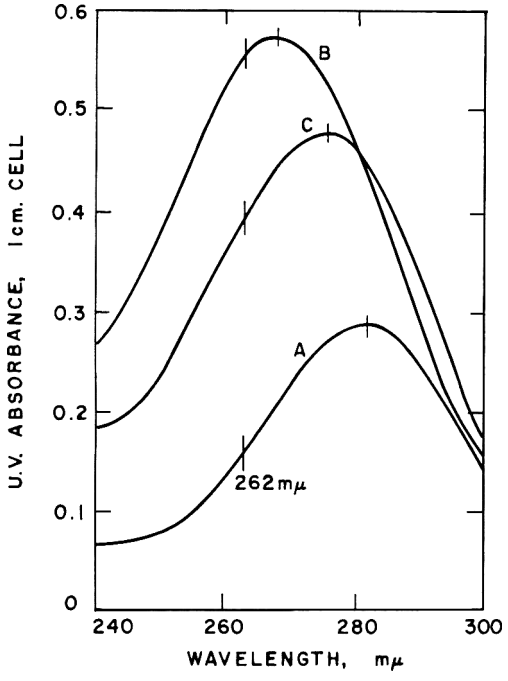

Fig. 2. Ultraviolet absorption spectra of sorbate-cider distillate; dilution factor = 100. A) cider blank; B) cider + 180 ppm sorbic acid; C) cider + 96 ppm sorbic acid.

However, when microbial growth alters cider quality, then varying amounts of interfering absorbing materials are distilled and we have no accurate basis for calculating the amount of recovered sorbic acid. This is demonstrated by Table 1.

From Table 1 it may be seen that microbial spoilage had less quantitative effect on the sorbic acid determined by dilution than by the distillation procedure, whether calculated using the original blank or one that had equal storage and microbial growth. Since the effect of microbial spoilage on fresh or sorbate-cider is evidently complex, accurate calculations for determination of sorbic acid levels cannot be made on dis-

tillates of cider in which microbial growth has occurred. This uncertainty is due to the variation in amounts of the interfering materials distilled from either blank or sorbated-cider which are changing in quality.

Sugars are known to produce ultraviolet-absorbing materials upon heating (Wolfrom *et al.*, 1948; Haas *et al.*, 1948); spectra of heated sugar solutions were compared with the interfering substances observed in cider distillates. A 1% solution of D-fructose, the predominant and least heat-stable sugar in apples (Smock and Neubert, 1958), was acidified as outlined in the dilution process. Spectra were obtained before and after 2 min of boiling. The unheated sample showed little absorption; the heated sample had at 282 mμ a strong maximum similar to that observed for cider distillates and boiled cider. Distillates of acidified fructose also exhibited maxima at 282 mμ, as did heated sucrose solutions. Increased absorption at 282 mμ in cider distillates can therefore result partly from sugar decomposition, which is accentuated by longer heating periods, higher temperatures, and addition of acid, but diminish if fermentation occurs.

Singh *et al.* (1948) and Wahhab (1948) have established that the principal decomposition product of sugars heated under acid conditions is 5-hydroxymethyl-2-furfural, especially when hexoses are present. The similarity in the spectra of a commercial sample of hydroxymethylfurfural and a fresh cider distillate is shown in Fig. 3. Cider distillates gave a positive Molisch test and had similar paper chromatographic R_f values compared to commercial 5-hydroxymethylfurfural, thus suggesting that the principal interfering material is 5-hydroxymethylfurfural.

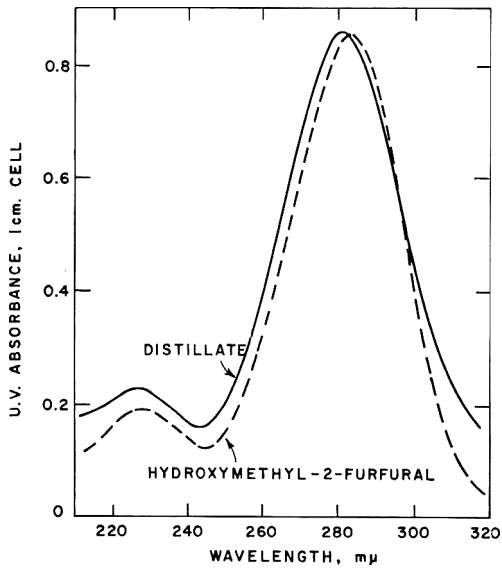

Fig. 3. Comparison of ultraviolet absorption curves for cider distillate and hydroxymethylfurfural.

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An Ion-Exchange Study of the Free Amino Acids in the Juices of Six Varieties of Citrus[a]

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SUMMARY

Free amino acids were determined in the juices of mature Valencia and Washington navel oranges, Eureka and Lisbon lemons, Dancy tangerines, and Marsh grapefruit. Twelve amino acids and ammonia were identified and measured quantitatively. Eleven unidentified ninhydrin-positive compounds were also detected. Alanine, asparagine, aspartic acid, glutamic acid, proline, serine, γ-aminobutyric acid, and arginine occurred in substantial amounts in all fruits. Proline was the most prominent amino acid in every fruit except the grapefruit, but appeared in especially high concentrations in the Valencia orange. Aspartic acid predominated in the grapefruit. Nitrogen calculated from the amino acids accounted for approximately 70% of the Kjeldahl nitrogen in each of the juices.

Recent developments in chromatographic and microbiological methods have stimulated interest in the free amino acids in plant materials. The results of such studies with the citrus fruits have been summarized in several reviews (Agr. Research Service, 1956; Kefford, 1959; Rockland, 1959, 1961; Underwood and Rockland, 1953). In general, data regarding the orange, grapefruit, lemon, mandarin, lime, and several hybrid varieties indicate a qualitative similarity in the amino acid compositions of the citrus varieties studied. A number of the amino acids appear to be common to most of the varieties. These include alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine. In addition, β-alanine, α-aminobutyric acid, citrulline, cystine, hydroxyproline, methionine, and ornithine have been reported in certain varieties.

Most of the studies pertaining to the amino acids in the citrus fruits have been qualitative or semi-quantitative. Quantitative investigations, in general, have been limited to the measurement of specific compounds or groups. Because of the importance of the amino acids in any consideration of fruit biochemistry and such processes as growth, maturation, and senescence, a quantitative study of the amino acids and related compounds in the citrus fruits has been initiated. The application of established ion-exchange techniques permits an accurate analysis of amino acids in biological materials. Moreover, such methods require a minimum of preparation of the material prior to assay, and thus avoid or reduce many of the errors that are inherent in other methods. For this purpose, the recently developed procedure of Moore *et al.* (1958) has been adapted. This procedure, and its antecedents, have found wide application in biochemistry, but have been used in only a few fruit studies (Carangal *et al.*, 1954; Saravacos *et al.*, 1958; Tinsley and Bockian, 1959). The following data represent a preliminary survey of amino acids in some important citrus varieties. Intensive studies are now being made of the individual varieties to determine the effects of seasonal changes and various environmental factors.

[a] Presented in part at the 20th Annual Meeting of the Institute of Food Technologists, San Francisco, May, 1960.

EXPERIMENTAL

Sample preparation. Valencia oranges, Eureka lemons, and Lisbon lemons were picked from trees in Citrus Experiment Station plots at Riverside, California. Fruits attained color on the tree, and conformed to commercial maturity standards. Washington navel oranges, Dancy tangerines, and Marsh grapefruit (Coachella Valley) were purchased on the local market. For analysis, two lots of each variety, six fruits each, were selected for duplicate determinations. The fruits were peeled, albedo was removed, and the pulps were macerated (with removal of seeds) and squeezed through cheesecloth. The juice was frozen, thawed, and filtered through Celite to give a clear serum. Kjeldahl nitrogen, soluble solids, total acidity, and pH were determined on this fraction.

Amino acid separations. The amino acids in the serum were determined by the procedure of Moore *et al.* (1958), with minor modifications. Separation of the more acidic compounds (aspartic acid through proline) required operation of the 150-cm column at 30°C in this region. After elution of proline, the temperature could be increased to 50° C for the remainder of the run. However, application of this procedure to citrus juices proved to be impractical. Some of the more acidic amino acids are present in the juices in relatively high concentrations, and application of a sample sufficient for measurement of the slower-moving acids resulted in overlapping of the early peaks. Conversely, when the sample size was reduced sufficiently for complete resolution of the early peaks, many of the later peaks appeared only as traces. Therefore, two 0.9 × 150-cm columns were used. One column *(Column A)* was operated at 50°C throughout, with elution at pH 3.25 followed by pH 4.25 (after collection of approx 260 ml of effluent). Fractions containing the unresolved acidic compounds (fractions 100 through 200) were discarded. The second column *(Column B)* was operated at 30°C throughout, at pH 3.25, with application of a smaller load. This separation was terminated with the elution of proline. In both cases, the columns were regenerated with 0.2*N* sodium hydroxide and equilibrated with pH 3.25 buffer after each run. The basic amino acids were separated on a 0.9 × 50-cm column *(Column C)* with elution at pH 4.30. This column was operated at 30°C until only arginine remained on the column, whereupon the temperature was elevated to 50°C. After elution of arginine, the column was reused without regeneration.

The serum was added to the columns as follows: *Column A* (150 cm at 50°C): pH of the serum (3 ml) was adjusted to pH 2 and the mixture was added to the column. *Column B* (150 cm at 30°C): The serum (5 ml) was diluted (to 25 ml) with pH 2.2 citrate buffer, and an aliquot (1 ml, containing 0.2 ml serum) was added

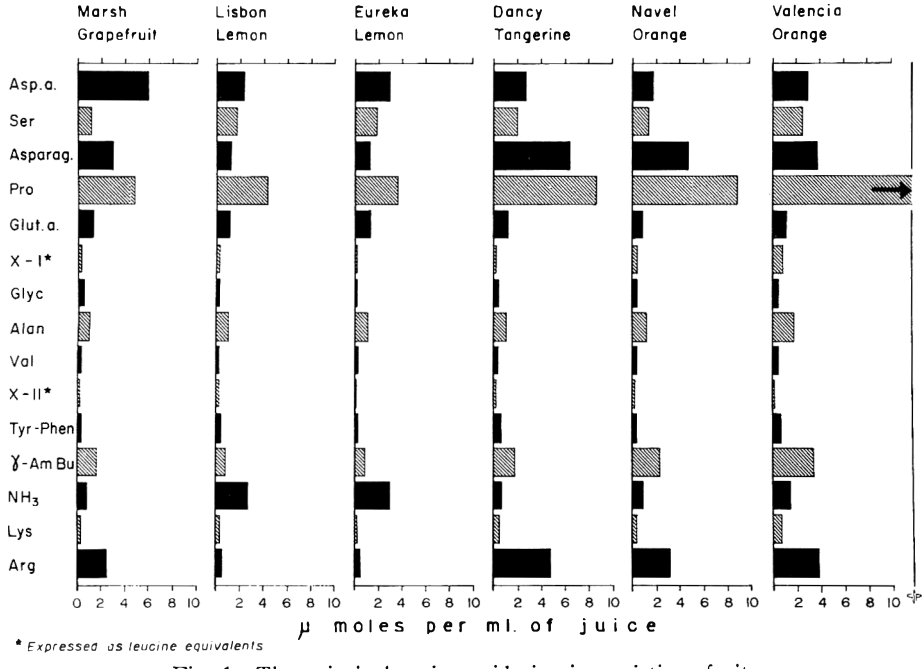

Fig. 1. The principal amino acids in six varieties of citrus.

Table 1. Compositions of some citrus juices.

	Navel orange			Valencia orange			Dancy tangerine		
	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids
Alanine	1.3	12	0.12	1.5	13	0.10	0.8	7	0.06
γ-Aminobutyric acid	2.3	24	0.24	3.1	32	0.26	1.7	18	0.17
Ammonia	0.6	1	0.01	1.0	2	0.02	0.4	1	0.01
Arginine	3.1	54	0.53	3.3	57	0.46	4.8	84	0.77
Asparagine	5.1	67	0.66	3.8	50	0.40	6.4	85	0.78
Aspartic acid	2.0	27	0.27	2.5	33	0.27	2.7	36	0.33
Glutamic acid	0.8	12	0.12	1.2	18	0.15	1.1	16	0.15
Glycine	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02
Lysine	0.2	3	0.03	0.3	4	0.03	0.3	4	0.04
Phenylalanine (+ tyrosine)	0.2	3	0.03	0.3	5	0.04	0.3	5	0.05
Proline	9.3	107	1.06	20.8	239	1.93	8.7	100	0.92
Serine	1.7	18	0.18	2.1	22	0.18	1.8	19	0.17
Valine	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02
Soluble solids (%)		10.1			12.4			10.9	
Acid (%, as citric)		0.84			1.29			0.89	
Solids-acid ratio		12.0			9.6			12.2	
Total μmoles amino acids per ml		27.8			41.3			30.4	
Total μ equivalents N per ml (from amino acids)		42.3			55.3			51.5	
Total μ equivalents N per ml (Kjeldahl)		61.5			77.9			74.0	
Mg N per ml (Kjeldahl)		0.86			1.09			1.04	
Percent N accounted for		69			71			70	

to the column. *Column C* (50 cm): The serum (1 ml) was added directly to the column.

Buffers were delivered by gravity, and fractions (2 ml) were collected with drop-counting fraction collectors (Research Specialties Co.). The flow rates were the maximum obtainable under the conditions with gravity feed. These were approximately 8 ml/hr for the 150-cm column at 30°C, 12 ml/hr for the 150-cm column at 50°C, and 25 ml/hr for the 50-cm column.

Quantitative and qualitative analysis. The effluents were analyzed photometrically (Coleman Universal Spectrophotometer) according to the method of Moore and Stein (1954), with absorption measurements at the appropriate wavelength (570 or 440 mμ). A standard leucine curve was prepared, and the color values of Moore and Stein (1954) were used. Peak positions and recoveries were verified by application of standard mixtures of amino acids (Spackman *et al.*, 1958).

The amino acids were identified by their elution thresholds and by paper chromatography. For the latter procedure, samples were separated on the columns and alternate fractions were analyzed to determine peak positions. The remaining fractions were combined into individual peaks, and desalted on a short column of Amberlite IR-120 resin. The resulting amino acids were chromatographed simultaneously with known compounds by two different one-dimensional systems (phenol-ammonia and butanol-acetic acid-water) (Clements and Deatherage, 1957). Some compounds remain unidentified, either because of the low levels present or because of a lack of known reference compounds with corresponding characteristics. Pending identification, these compounds are expressed in leucine equivalents.

RESULTS AND DISCUSSION

The results (averages of single analyses of duplicate samples) are tabulated in Tables 1–3, and shown graphically in Fig. 1. The graphs are drawn to a single scale to permit a direct comparison of the profiles. Since this is a preliminary study involving limited sampling, the data do not necessarily represent typical profiles of the varieties studied. However, the results are accurate quantitative measurements of what may be assumed to be typical specimens of the particular varieties grown under typical conditions. The data and techniques are to be employed

as a basis for future determinations of "normal" ranges, and for studies of factors contributing to variations.

The citrus fruits appear to be similar to other fruits in that the free amino acid content is subject to the influence of physiological and environmental factors. The amino acids that are characteristic of plants in general are present in varying amounts, and any specific differences appear to be quantitative rather than qualitative. Although some citrus varieties appear to be characterized by high levels of certain amino acids, the composition of a given sample may be a reflection of the particular environmental and physiological conditions, rather than of variety. The significance of such apparent varietal differences must await a complete quantitative study of seasonal changes, nutrition and rootstock effects, and perhaps climatic and water relations.

The navel orange, Valencia orange, and tangerine were characterized by a definite preponderance of proline. This is most striking in the Valencia orange (239 mg per 100 ml) with a concentration almost identical to the maximum reported by Rockland and Underwood (1956) for this variety. These authors report proline in even higher concentrations in the navel orange, but the samples analyzed in the present study contained less than half as much proline (107 mg per 100 ml) as the Valencia variety. Proline was also the most prominent amino acid in the lemons, but was present at much lower levels (41–47 mg per 100 ml). The grapefruit contained somewhat more proline (59 mg per 100 ml), but aspartic acid (81 mg per 100 ml) predominated in this fruit. In general, alanine, asparagine, aspartic acid, glutamic acid, proline, and serine were the most prominent acidic and neutral amino acids in all varieties. The dominant basic compounds were γ-aminobutyric acid and arginine, with substantial amounts of ammonia. Glycine, valine, tyrosine, phenylalanine, and lysine appeared in all varieties in relatively low concentrations.

Eleven unidentified components were detected, most of them present in traces (Table

Table 2. Compositions of some citrus juices.

	Eureka lemon			Lisbon lemon			Marsh grapefruit		
	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids	μmoles per ml juice	mg per 100 ml juice	mg per 100 mg solids
Alanine	1.0	9	0.12	1.1	10	0.12	1.0	9	0.09
γ-Aminobutyric acid	0.7	7	0.09	0.7	7	0.08	1.8	19	0.18
Ammonia	3.0	5	0.06	2.5	4	0.05	1.0	2	0.02
Arginine	0.2	3	0.04	0.2	3	0.04	2.7	47	0.45
Asparagine	1.2	16	0.21	1.3	17	0.20	3.2	42	0.40
Aspartic acid	2.7	36	0.46	2.4	32	0.39	6.1	81	0.77
Glutamic acid	1.3	19	0.24	1.2	18	0.22	1.5	22	0.21
Glycine	0.1	1	0.01	0.1	1	0.01	0.3	2	0.02
Lysine	0.1	1	0.01	Tr	1	0.01	0.2	3	0.03
Phenylalanine (+ tyrosine)	0.1	2	0.03	0.2	3	0.04	0.2	3	0.03
Proline	3.6	41	0.53	4.1	47	0.57	5.1	59	0.56
Serine	1.6	17	0.22	1.8	19	0.23	1.4	15	0.14
Valine	0.1	1	0.01	0.1	1	0.01	0.2	2	0.02
Soluble solids (%)		7.7			8.1			10.4	
Acid (%, as citric)		5.17			5.30			1.75	
Solids-acid ratio		1.5			1.5			5.9	
Total μmoles amino acids per ml		15.6			16.0			25.3	
Total μ equivalents N per ml (from amino acids)		17.4			17.9			36.8	
Total μ equivalents N per ml (Kjeldahl)		25.7			27.5			56.2	
Mg N per ml (Kjeldahl)		0.36			0.39			0.79	
Percent N accounted for		68			65			66	

Table 3. Unidentified ninhydrin-positive substances in some citrus juices.

Compound	Unidentified compounds: Leucine equivalents (μmoles per ml juice)						Peak position (fraction no.)		Notes
	Navel orange	Valencia orange	Dancy Tangerine	Eureka lemon	Lisbon lemon	Marsh grapefruit	150-cm column (50°)	50-cm column (30°)	
I	0.2	0.4	0.1	0.1	0.1	0.2	29–35		Measured at 440 mμ
II	.1	.1	.1	Tr	.1	.1	197–203		Leucine + ?
III	.2	.1	.1	Tr	Tr	.2		125–135	Histidine (?) + ?
IV	....	.1	.3	Tr	Tr	....	93–96		Column at 30°
V	.1	.1	.1	Tr	Tr	.1	45–48		Methionine sulfoxide (?)
VI	Tr	.1	Tr	Tr	Tr	Tr	54–59		Methionine sulfoxide (?)
VII	.1	.1	.1	Tr	.1	.1	187–192		Isoleucine (?)
VIII	.2	.1	.3	....	....	....		90–95	
IX	Tr	Tr	Tr	Tr	Tr	Tr	178–184		Methionine (?)

3). One compound (I) appeared at significant levels in all varieties, but was especially prominent in the Valencia orange. This compound reacted with ninhydrin to give a yellow color, and absorption was consequently measured at 440 mμ. The color and early elution threshold suggest an imino acid, probably more acidic than proline. Paper chromatography of the effluent did not produce a spot detectable with ninhydrin or isatin. In view of the low ninhydrin color values for the imino acids, the concentrations of this compound could be much greater than the leucine equivalents indicate (perhaps approaching 1 μ-mole per ml of juice in the Valencia orange). Tentative identifications are given for some of the other unidentified compounds. Methionine is probably present (also accounting for traces of compounds corresponding to methionine oxidation products, since thiodiglycol was not added to the buffers). Other ninhydrin-positive compounds are undoubtedly present, but probably in traces. Under the conditions employed, glutamine is only partially recovered; glutathione would be obscured by glutamic acid (Spackman *et al.*, 1958).

Total amino nitrogen in each juice was calculated from the individual amino acid concentrations, and this value was compared with the total nitrogen as determined by the Kjeldahl method (Tables 1 and 2). It may be noted that the Kjeldahl nitrogen ranged from 0.36 mg per ml of juice, in the Eureka lemon, to 1.09 mg per ml, in the Valencia orange, but in each case the amino acids accounted for approximately 70% of the total nitrogen in the clarified juice.

Insofar as the individual amino acids are concerned, the results are qualitatively similar to those of previous workers. However, extreme quantitative variations are to be noted in many instances. It has been shown that the total nitrogen content of citrus fruits is influenced by position on the tree (Koo and Sies, 1956), mineral nutrition (Jones and Parker, 1947), and rootstock (Marsh, 1953). It has also been established that total nitrogen, as well as concentrations of certain amino acids in certain varieties, are related to maturity (Bain, 1958; Cameron *et al.*, 1935; Safina and Sara, 1955; Wedding and Horspool, 1955; Zidan and Wallace, 1954). The technique involved in extracting the juice may also be important, since it has been shown that increased pressure during extraction results in a juice with higher nitrogen content (Solarino, 1938).

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The Concentration of Myoglobin and Hemoglobin in Tuna Flesh

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SUMMARY

Amounts of myoglobin and hemoglobin were determined in light and dark muscle of yellowfin tuna, *Neothunnus macropterus*. Myoglobin concentration ranged from 0.37 to 1.28 mg/g wet weight in light muscle and from 5.3 to 24.4 mg/g in dark muscle; hemoglobin concentrations ranged from 0.12 to 0.58 mg/g in light muscle and from 0.5 to 3.8 mg/g in dark muscle. The fish were divided into two groups on the basis of means of capture, i.e., taken on hooks from a bait boat, or seined. Average percentage of hemoglobin was slightly, but not significantly, higher in the seined fish. Of the total heme pigments, myoglobin constituted 69–85% in light muscle and 81–95% in dark muscle from bait-boat fish, and 47–81% in light muscle and 84–98% in dark muscle of seined fish.

INTRODUCTION

A recently developed technique utilizing DEAE-cellulose chromatography (Brown, 1961) has made possible a simple and accurate determination of the amounts of myoglobin and hemoglobin in tissue extracts. This paper presents results of a study in which such chromatography was employed to determine the concentration of these two heme pigments in light and dark muscle of yellowfin tuna.

The heme proteins myoglobin and hemoglobin, and various of their derivatives, are responsible for normal and off colors of canned tuna (Brown and Tappel, 1957; Brown *et al.*, 1958) as well as most meat and other fish products.

EXPERIMENTAL

Materials. Frozen sections of yellowfin tuna muscle approx 2×4×6 in., each containing both light and dark muscle, were supplied by Roland Finch and Louis Canedo, Westgate-California Corporation, San Diego. The samples were divided into two lots on the basis of means of capture: taken on hooks from a bait boat, or taken in a seine. All fish within a group were similarly handled. The bait-boat group ranged from 21 to 38 in. long and had been held frozen in the well of the boat for 16–31 days. The seined fish were 20–31 in. long and had been held frozen in the well for 14 days.

Methods. Samples of 10 g each of light muscle and 2 g each of dark muscle were taken from the interior of the sections of the fish. These were blended for 1 min with 35 ml of cold ion-free water in a Waring blender micro-cup. The cup was rinsed with an additional 5 ml of water, and the combined mixture was stored overnight in a refrigerator (6°C) and then centrifuged 20 min at 10,000 G in a Servall refrigerated centrifuge (2°C). Aliquots of the resulting supernatant were placed onto DEAE-cellulose columns (described below), and others were suitably diluted for absorbancy measurements in the Soret region. The heme pigments were in the met (oxidized) form, as determined spectrophotometrically. A second extract of the residue was made and treated in similar fashion. The second extraction yielded less than 10% of the total pigments. Preliminary studies indicated that a third extraction yielded no additional pigment. Final figures in the data are summaries of results from the two extracts.

The columns (2 × 6 cm) were prepared from DEAE-cellulose (Bio-Rad Laboratories) equilibrated with 0.05*M* Tris buffer, pH 8.6; they were packed under 1 lb air pressure. Details of these procedures have been given (Brown, 1961). Usually 2 ml of the supernatant was placed on the column and eluted with the same buffer. Under these conditions, myoglobin is not retained on the column, whereas hemoglobin is retained on the top portion of the packing and does not migrate. The color of the myoglobin makes it possible to collect a single fraction of eluate by

visual inspection. The absorbancy of this fraction in the Soret region reflects only the heme proteins present. Absorbancy of the original extract diluted with the same buffer was compared to absorbancy of the eluate to obtain the percentage of myoglobin. Absolute concentrations were determined from extinction coefficient measurements on highly purified yellowfin tuna myoglobin (Brown *et al.*, 1961, 1962); yellowfin tuna metmyoglobin in this buffer has its Soret peak at 410 mμ and $A_{1\%} = 67$.

RESULTS AND DISCUSSION

The measurements are presented in Table 1. Duplicate determinations are shown for most of the samples. The variations in duplicate values reflect actual variation in myoglobin (or hemoglobin) content of the extract; losses of myoglobin on the column are negligible under these conditions, numerous control studies have shown (Brown, 1961). This variation was considerably more pronounced in the light muscle tissue than in the dark. Variation of myoglobin concentration within pork muscle has been reported (Ginger *et al.*, 1954; Hornsey, 1959). The average values for myoglobin concentration in tuna light muscle are about the same as those reported for light pork muscle (Ginger *et al.*, 1954); they are also approximately equal to the levels in rat and guinea pig muscle, but lower than levels in horse, dog, rabbit, and human muscle (Perkoff and Tyler, 1958). Matsuura and Hashimoto (1959) have reported higher values for myoglobin concentration in *Thunnus orientalis*.

Most of the pigment extractable from light muscle and essentially all of that from dark muscle is myoglobin. Matsuura and Hashimoto (1959) obtained similar results on extracts from *Thunnus orientalis* except that they found all of the pigment from light muscle to be myoglobin. Naughton *et al.* (1958) suggested that 95% of the pigment in tuna muscle is hemoglobin.

If the amount of bruising undergone by fish handled by seine is significantly different from that of fish caught on hooks, it might be expected to be reflected in a relatively higher percentage of hemoglobin than myoglobin in muscle tissue. There did appear to be some small difference in the

Table 1. Concentration (mg/g wet weight) of heme proteins in tuna muscle.

Means of capture and sample	Light muscle				Dark muscle			
	Total	Myoglobin	Hemoglobin	% myoglobin	Total	Myoglobin	Hemoglobin	% myoglobin
Bait boat								
A	0.49	0.37	0.12	76	16.5	15.5	1.0	94
	0.88	0.68	0.20	77	15.6	14.8	0.8	95
B	0.78	0.64	0.14	82	16.5	15.5	1.0	94
	0.88	0.75	0.13	85	16.5	15.2	1.3	92
C	1.42	1.05	0.37	74	22.6	20.8	1.8	92
	1.68	1.28	0.40	76	.....	.....	....	....
D	0.81	0.59	0.22	73	8.0	7.2	0.8	90
	0.84	0.59	0.25	70	6.6	5.3	1.3	81
E	0.75	0.61	0.14	82	9.3	7.7	1.6	83
	0.68	0.47	0.21	69	10.6	9.4	1.2	89
Av.	0.92	0.70	0.22	76	13.6	12.4	1.2	90
Seined								
G	1.09	0.69	0.40	63	21.5	21.0	0.5	98
	1.27	0.93	0.34	73	19.2	18.0	1.2	94
H	1.23	1.03	0.20	81	17.3	14.5	2.8	84
	1.35	1.06	0.29	79	17.8	14.9	2.9	84
I	0.82	0.41	0.41	50	28.2	24.4	3.8	87
	1.12	0.53	0.58	47	.....	.....	....	....
J	1.16	0.88	0.28	76	20.6	17.5	3.1	85
	1.15	0.88	0.27	76	.....	.....	....	....
Av.	1.15	0.80	0.35	68	20.8	18.4	2.4	89

relative amounts of the pigments between the two groups, with the seined fish having slightly more hemoglobin. One fish, Sample I, is the most noticeable in this regard, having about 50% hemoglobin in the muscle extract. However, the limited numbers of samples in this study and the smallness of the differences do not permit any generalization about bruising.

The major limitation to application of the techniques described here is in the extraction procedure. Tuna myoglobins in aqueous solution are remarkably stable proteins; tuna hemoglobins are not (Brown *et al.,* 1961). There was no difficulty with loss of solubility of these proteins in the *extracts* used in this study, and since the differentiation of myoglobin and hemoglobin depends on elution of myoglobin and retention of hemoglobin, it is of no consequence if hemoglobin should become insoluble on the column. However, there was some pigment that was not extracted, for the tissue residues were colored. In the case of light muscle, the residual color was not great; the dark muscle residues had considerably more color. Based on experience with these proteins it would be expected that hemoglobin would more likely be denatured (and therefore not extractable) than would myoglobin. Thus the absolute values for myoglobin concentration are likely correct; the values for hemoglobin, particularly in dark muscle, may be in error on the low side. However, observation of the original and the extracted muscle clearly indicates that most of the total pigments have been removed, and therefore any correction factor should not be large.

There has been a tendency in the past to assume that the properties of myoglobin and hemoglobin were sufficiently similar to make it possible to apply any results obtained with either of these proteins to any color problem or question concerning pigments of fish and meats. It can be seen that such assumptions might be misleading. The methods used in this study should be applicable to other muscle tissue.

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Fractionation of the Component(s) Responsible for Sex Odor/Flavor in Pork[a, b, c]

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SUMMARY

Sex odor/flavor in pork was produced when fat, lean (with fat), and most organs from a boar were heated in a skillet or in boiling water. Odor seemed not to be entirely absent in boar fat at body temperature, although volatilization appeared to occur to a much greater extent at approx. 100–108°C. Sex odor was found to be water-insoluble, ether-soluble, and definitely associated with the fatty tissues of boars. It was absent in rehydrated moisture-free, fat-free lean. Distillation methods proved unsatisfactory for collecting the sex odor components in recognizable form, since no consistent and reproducible differences could be detected between boar and barrow fat when collected volatile compounds were analyzed by heat test and gas chromatography. Cold saponification of boar fat yielded a small quantity of unsaponifiable matter that produced a concentrated, permeating sex odor on exposure to heat. Thus, the agent(s) responsible for sex odor in pork are located in the unsaponifiable material. Cholesterol and squalene were found in this fraction in both boar and barrow fat, but sex odor was not produced when these compounds were heated.

Heating the flesh of certain swine causes the production of an objectionable odor or flavor that was described by Lerche (1936) as being "onionlike" or unpleasantly perspirative in nature. It is commonly referred to as sex odor or boar odor, although Self (1957) indicated that it occurred in the flesh of both sexes, being about as prevalent in female hogs as in males.

Dutt *et al.* (1959) concluded that the preputial glands produced a fat-diffusible material responsible for sex odor in boar carcasses. Christian and Turk (1958) noted in taste-panel studies that sex odor or flavor was less prevalent in boar tissues frozen and stored 5 months at freezer temperatures than in fresh, unfrozen samples from the same animal.

Sex odor in pork undoubtedly causes some adverse consumer-processor relations and has been the reason for condemnation of a large number of boars and stags annually under Federal Meat Inspection. Nevertheless, little is known about the nature of the odor or the responsible component(s).

Thus, this study was undertaken to ascertain if sex odor was located in the fat or lean or both; to study its solubility properties; and to attempt to collect, isolate, and identify the responsible component(s).

MATERIALS AND METHODS

Use of controls. Unless otherwise indicated, sex-odor-free barrow tissues were used as controls throughout the study.

Production of sex odor by heat. Tissues were examined for sex odor by heating small cubes in a skillet or by boiling them in an Erlenmeyer flask in a small amount of distilled water. At least three individuals verified the presence or absence of the odor. The temperature required for odor production was ascertained by heating cubed tissue in Erlenmeyer flasks fitted with stoppers containing a short length of glass tubing. The flasks were im-

[a] Journal article 2835, Michigan Agricultural Experiment Station, East Lansing.

[b] This paper presents a portion of research reported in the Ph.D. thesis of the senior author at Michigan State University.

[c] Presented at the 21st Annual Meeting of the Institute of Food Technologists, New York, May 10, 1961.

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mersed in a lard bath, and the temperature was increased gradually until sex odor emerged from the tubes.

Determination of solubility properties and tissue location of sex odor component(s). A number of organs, as well as fat and lean tissues, were obtained from a young Yorkshire boar (18 mo. old). Solubility in water of the component(s) responsible for sex odor in pork was determined on both fat and lean tissues. The twice-ground material was blended for 3 min with 5 parts of distilled water. The resulting slurry was centrifuged, and the supernatant was decanted and strained through cheesecloth; and both it and the residue were heat-tested for sex odor.

Samples of boar backfat weighing 200 g were extracted with several organic solvents. Solvents and fat were blended and strained through cheesecloth, and the relative amounts of fat extracted by each solvent were noted. Samples of fat thus extracted were freed of solvent and heated to determine sex odor. The following solvents were tested: acetone, carbon tetrachloride, chloroform, diethyl ether, petroleum ether (bp 30–60°C), dioxane, and ethyl alcohol.

A 100-g sample of lean from the ham of a boar was freeze-dried for 26 hr. The moisture and a sample of dry lean were heat-tested. The remaining dry lean was extracted for 3 hr. in a Goldfisch fat extractor. The fat and the moisture-free, fat-free lean were heated to check for sex odor.

High-temperature, atmospheric-pressure distillation. Various modifications of high-temperature, atmospheric-pressure distillation were attempted. The first apparatus employed was similar to that described by Kramlich and Pearson (1960) for fractionation of volatile flavor compounds from cooked beef. Their system was modified by removing the liquid air traps and the water-cooled condenser (replaced by a short air-cooled condenser) and replacing the nitrogen stream with a stream of air. In addition, the sample flask was heated in a hot lard bath, thus preventing localized burning of the sample and allowing more even heat distribution. The flask was charged with 2500 g of cubed fat or liquid fat and heated for 5 hr at 140–150°C. The liquid fat was obtained by grinding intact boar fat, heating slightly, and straining through cheesecloth. Volatile compounds were collected from both the cubed fat and strained (liquid) fat. The compounds collected in the dry ice-ethanol trap were vaporized by heating the trap in hot oil (145°C) and injecting them into a Barber-Coleman model 20 ionization detection system gas chromatograph by means of a 6-way gas sampling valve. The gas chromatograph was equipped with a 10-ft × ¼-in. copper column packed with diethylene glycol succinate (DEGS) on Chromasorb. The contents of the trap immersed in ice were extracted with ethyl ether, and the ether extract was analyzed by gas chromatography.

A second type of high-temperature atmospheric-pressure distillation apparatus was assembled so as to allow short-time heating of a smaller quantity of fat. Skinless boar fat was cut into small cubes, and 615 g were placed in a 1-L Pyrex suction flask. The top of the flask was closed with a cork stopper containing an L-shaped glass tube. This tube was connected to a trap immersed in ice (100-ml suction flask). The sample flask was heated for 30 min on an electric stove at medium heat. Volatiles produced were drawn through the traps by a slight vacuum. The procedure was repeated ten times. The total aqueous distillate obtained was 300 ml. This liquid was saturated with sodium chloride and extracted with four 50-ml portions of diethyl ether. The ether extract was dried over anhydrous sodium sulfate, reduced to a volume of 0.2 ml, and analyzed by gas chromatography using 10-ft × ¼-in. copper columns packed with DEGS, flexol plasticizer, mannitol, or silicone 200. All liquid phases were coated on Chromasorb "W".

Tests for ammonia, carbonyls, and sulfur compounds in boar fat. Ammonia test paper was used to test for ammonia in the volatile stream coming from the distilling flask containing boar fat with connective tissue. The paper was prepared by mixing 10 ml of 20% silver nitrate solution with 5 drops of 40% formalin and a few drops of dilute sodium hydroxide. This mixture was filtered, and the filtrate was immediately absorbed on strips of Whatman No. 1 filter paper.

Carbonyl compounds distilled from intact and rendered boar fat were detected by passing the volatile stream through a solution of 2,4-dinitrophenylhydrazine (2 g per L in 2*N* HCl) and by mixing the contents of the dry ice-ethanol trap with the reagent. A yellow precipitate indicated the presence of carbonyl compounds.

Examination of preputial glands and contents of preputial diverticulum. The yellowish-brown preputial grands were removed from a number of boars at slaughter and analyzed by heat test and gas chromatography. Further, since the contents of the preputial diverticulum are claimed by some to contain sex odor, this material was collected from several boars, combined, and filtered, and the filtrate was extracted with an equal quantity of ether. This extraction was repeated, and the combined extracts were washed with distilled water. After evaporation of the ether, some of the residue was smelled before and after heating; and some was analyzed by gas chromatography using a 100-ft capillary column coated with Apiezon "L".

Saponification. Both boar and barrow fat was saponified as an aid in fractionation. One hundred g each of boar and barrow fat was extracted by blending 1½ min with 200 ml of diethyl ether. The resulting slurry was filtered through 4 layers of cheesecloth. The liquid portion was placed in a 2-L flask, and 400 ml of ether was added. Sodium ethylate, prepared by dissolving 16 g of kerosene-free metallic sodium in 200 ml of 95% ethyl alcohol, was then stirred into the ether-fat mixture. The flask was stoppered, shaken vigorously, and allowed to remain at room temperature for 24 hr. Soap was removed from the liquid by suction filtration using Whatman No. 41 filter paper. The soap was extracted once with diethyl ether, refiltered, and allowed to dry at room temperature. The combined filtrate was washed repeatedly with distilled water, dried over anhydrous sodium sulfate, and reduced under vacuum to a volume of 1 ml.

Gas chromatography and heat test of unsaponifiable matter from fat. The unsaponifiable matter from boar and barrow fat was heat-tested and analyzed by gas chromatography using a 10-ft × ¼-in. copper column packed with DEGS on Chromasorb or silicone-SE 30 on Chromasorb "W." Several compounds suspected of being present in unsaponifiable matter were obtained in pure form and subjected to gas chromatographic analysis. Their retention times were compared with those of peaks produced by similar analysis of the unsaponifiable matter.

Column chromatography of unsaponifiable matter from boar fat. A 161-mg sample of unsaponifiable matter from boar fat was subjected to silicic acid chromatography by the method of Hirsch and Ahrens (1958). The column was 253 mm long and 23 mm in inside diameter. It was packed to a depth of 150 mm with 325-mesh silicic acid. The type and volume of eluants were the same as those recommended by Hirsch and Ahrens (1958) for the stepwise elution of complex lipid mixtures.

RESULTS AND DISCUSSION

Figure 1 illustrates the lean tissue fractionation procedure and indicates the fractions where sex odor was detected. Sex odor was evident in heated lean tissue and in the residue from water extraction but could not be detected in the water extract of ground lean either while cold or on exposure to heat. Thus, the component(s) responsible for sex odor are not readily soluble in water.

It was possible to fractionate lean boar tissue into lean, fat and moisture fractions by freeze-drying and ether-extraction of the freeze-dried lean (Fig. 1). Sex odor was not evident when heat was applied to the water fraction; however, a faint ammonia-like smell was noted. Sex odor was evident in freeze-dried lean (with fat) when this tissue was heated, but no odor could be detected in this fraction after removal of the fat; only the "brothy" smell of cooked pork was evident. The fat fraction was present in rather small amounts, and was burned slightly on heating. This precluded definite detection of the odor in this fraction. However, since sex odor could be detected in lean tissue before, but not after, ether extraction, this would appear to be sufficient proof of its presence in the fat.

Examination of some of the major organs of a boar indicated that sex odor was present in all except the liver and lung. The lung,

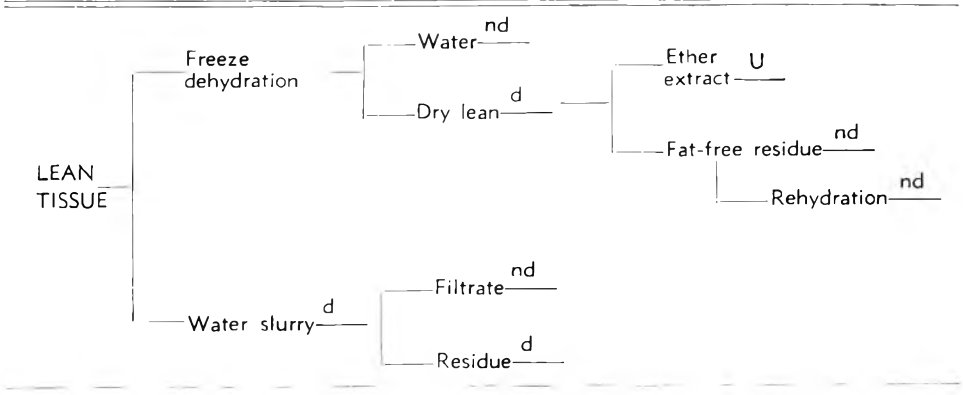

Fig. 1. Lean tissue fractionation procedure. [d] sex odor detectable. [nd] sex odor not detectable. [U] questionable.

being low in odor-containing fat, would not be expected to produce any appreciable odor on heating. Liver, when exposed to heat, produced a characteristic "liver-like" smell, which probably masked any sex odor present.

The procedure used for the fractionation of boar fatty tissue is presented in Fig. 2. Sex odor was detected in the residue from a water extract of fat but was absent in the filtrate, thus indicating further its lipophilic character and lack of solubility in water. When boar fat was heated in a skillet, the characteristic sex odor was produced quickly and profusely. The odor was less pronounced when fat was heated with water in an Erlenmeyer flask. Although sex odor was not entirely absent from fatty tissues at body temperature, volatilization appeared to be maximum at approximately 100–108°C. This indicates that the responsible component(s) are not volatile to any great extent until about 100°C is reached, or that they are present as precursors. When boar fatty tissues were heated in a skillet, the odor continued to be produced even when the temperature was much higher than 108°C. In addition, if a sample of fat was heated, cooled, and reheated, the odor seemed to be as intense as that produced initially.

Gas chromatographic analysis of volatile compounds obtained by distillation. Having established that the sex odor component(s) were present in the fat, attempts were made to trap the odor component(s) by heat (Fig. 2).

When fat, with its adhering connective tissue, was heated in the sample flask, a number of familiar odors were produced but sex odor was difficult to detect. Fumes emerging from the flask were "meaty" and ammoniacal in nature—somewhat resembling cooking bacon or rendering lard. These fumes rapidly turned ammonia test paper black, indicating that ammonia was present.

The contents of the trap immersed in ice were mostly water. Other volatile compounds were also collected in this trap, and they possessed an odor very reminiscent of cooked fat. No sex odor was evident when the trap contents were exposed to heat; however, a positive test for sulfur was obtained.

Analysis of the contents of the dry ice–ethanol trap was accomplished with a 5-ft. × ¼-in. copper column packed with DEGS. No apparent differences were noted between the volatile constituents from boar and barrow fat. Several additional peaks were obtained by increasing column length and decreasing gas flow rate but still no distinct differences were observed.

Fat, free of connective tissue (Fig. 2), still produced sex odor when heated; but tests for ammonia and sulfur compounds were negative. Volatile compounds produced by distillation of this fat were captured in a trap immersed in a dry ice–ethanol mixture. No ice trap was used since it was possible that the sex odor component(s) were being retained in this trap. As a further precaution against premature condensation of the volatile components, the air-cooled condenser was maintained at 100°C with an electric mantle. Analysis of the cold trap contents by gas chromatography yielded a number of peaks, but still no distinct and reproducible differences were noted between boar and barrow fat. When the trap was heated, no sex odor was detected; only the aroma of cooked fat was evident. Reaction of the trap contents with 2,4-dinitrophenylhydrazine yielded a yellow precipitate, indicating the presence of carbonyl compounds. Gas chromatographic analysis of the volatile components after removal of the carbonyl compounds showed that several peaks had disappeared.

Short-time heating of boar fat minimized the volatile material produced by excessive heat. The distillate obtained in the ice trap consisted mostly of water and had a cooked fat smell. Gas chromatographic analysis of the dry ice–ethanol trap contents indicated that the volatile compounds obtained from boar and barrow fat were essentially identical. One one occasion, the contents of the ice trap seemed to evolve a faint sex odor when heated; however, this could not be reproduced in other trials.

Mannitol on Chromasorb "W" proved to be the most suitable column packing material for separation of the ether extract of the aqueous distillate obtained in the ice trap. Thus, this column was used for all future gas chromatographic analysis of volatile compounds collected by distillation using

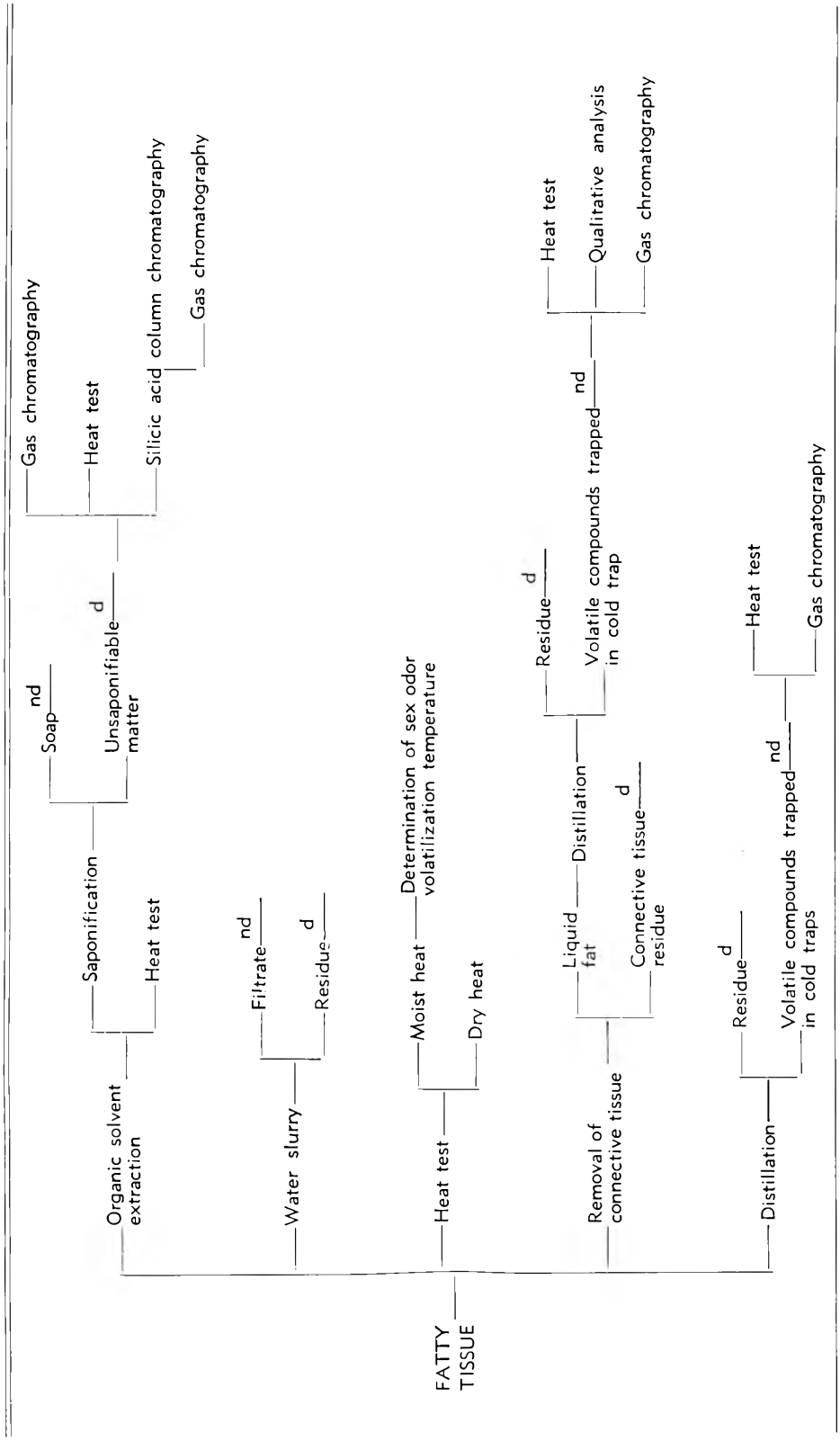

Fig. 2. Fat tissue fractionation procedure. [d] sex odor detectable. [nd] sex odor not detectable.

short-time heating. In most cases 14 peaks were obtained from both boar and barrow fat. Minor differences were observed occasionally, but they were inconsistent and could not be reproduced. No attempts were made to identify any of the peaks. Similar analysis of the ether extract of the steam distillate from boar and barrow fat with a 10-ft DEGS column yielded no differences. Only one peak other than the solvent peak was obtained from each type of fat. The steam distillate was devoid of any sex odor.

A number of determinations were made with the several types of distillation methods mentioned. In all cases the results were similar; that is, no distinct and reproducible differences could be detected between volatile compounds obtained from boar and barrow fat. Thus, under the conditions of this study, the agent(s) responsible for sex odor in pork were not retained in recognizable form in any of the traps used. The fact that the odor was produced in heated fat but was not detected in the traps, indicated that the component(s) might have been altered by being decomposed, recombined, or destroyed during heating of the fat. Residues of sex odor component(s) might well have been retained in the traps but did not produce sex odor on exposure to heat. The roles played by the solvent and the heat in alteration of sex odor are, at this time, poorly defined.

Heating excised preputial glands failed to produce a more intense sexual odor than heating boar fat alone. If these glands are the site of production of the odor (Dutt *et al.*, 1959) then it must be distributed to the fatty tissues as rapidly as it is produced. Gas chromatographic analysis of an aqueous distillate of these glands as well as an ether extract of the preputial diverticulum contents showed no major differences between boar and barrow fat. A urine-like smell was present in the ether extracts, but no other odor was noted.

Table 1 shows the results of the solvent extraction of fat from fatty tissue. Fat extracted with carbon tetrachloride, dioxane, or ethanol did not seem to possess sex odor; but these solvents were difficult to remove from the fat, and solvent odor was predominant when heat was applied to the samples, perhaps masking any sex odor present. Ethyl ether was the solvent of choice since it was very effective in removal of the fat, containing the sex odor, from associated connective tissue.

Table 1. Solubility of fat and sex odor components in various organic solvents.

Solvent	Fat yield	Sex odor [a]
Acetone	fair	d
Carbon tetrachloride	good	nd
Chloroform	good	d
Dioxane	fair	nd
Ethyl ether	excellent	d
Petroleum ether	good	d
Ethanol	fair	nd

[a] d, detectable; nd, not detectable.

Saponification of boar and barrow fat (Fig. 1) yielded a small quantity of unsaponifiable matter. Application of heat to a small drop of unsaponifiable matter from boar fat produced a very potent sex odor that rapidly permeated the surrounding atmosphere. No such odor was noted when the corresponding fraction from barrow fat was exposed to heat. Likewise, the soap from both the boar and barrow fat was devoid of sex odor. Thus, sex odor component(s) are lipophilic and, further, are located in the unsaponifiable fraction of the fat.

The silicone–SE 30 column was superior to the DEGS column for gas chromatographic analysis of the unsaponifiable matter. High temperatures are required for analysis of this material, and the latter column packing bleeds excessively at temperatures above 250°C. Peaks from unsaponifiable matter were similar for boar and barrow fat although the boar sample was known to contain sex odor. The last compound that emerged from the gas chromatograph produced a large peak and seemed to be the major component present. This was assumed to be cholesterol since this compound is known to be a component of the unsaponifiable fraction of some fats. Analysis of pure cholesterol by gas chromatography produced a peak that compared in shape and retention time to the unknown peak observed in boar and barrow fat. Further evidence for the presence of cholesterol was shown by the fact that a positive test was obtained for

Δ-5 sterols (Liebermann-Burchard reaction). Since cholesterol was found in both boar and barrow fat, it was not expected to be one of the agent(s) responsible for sex odor in boars. When this compound was heated, no odor reminiscent of sex odor was observed. Squalene was similarly analyzed by gas chromatography and tentatively identified as being a component of the unsaponifiable fraction of boar and barrow fat. On heating a sample of pure squalene, a rather odd, sweetish odor was detected; but it in no way resembled sex odor. It is still possible, however, that cholesterol and squalene may contribute to the development of sex odor by combination with other component(s) present in the unsaponifiable fraction.

Column chromatographic analysis of a small sample of the unsaponifiable fraction of boar fat yielded three fractions. The first fraction was eluted with 200 ml of 25% ethyl ether in petroleum ether. It was yellow and present in small amount. Pure ethyl ether eluted the largest fraction. This fraction was also yellow, and amounted to about 85–90% of the total charge placed on the column. The third fraction, eluted with pure redistilled methanol, possessed a yellowish-brown color. When the three fractions were exposed to a heat test, it was found that the fraction eluted with ethyl ether contained the sex odor component(s). The first fraction also contained a trace of the odor, but it was absent in the third fraction. Gas chromatographic analysis of the three fractions indicated that separation by column chromatography was not complete. Peaks obtained from the second (largest) fraction appeared to be very similar to the unfractionated unsaponifiable matter. Thus, the separation of this material was not complete when undertaken with the eluants employed. It is possible that a different series of eluants could be used to achieve a more efficient separation of the unsaponifiable fraction into its component(s).

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Some Relations Between the Chemical and Physical Characteristics of Bovine Muscles

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SUMMARY

Variations in the distribution of water in bovine muscles were investigated with histological and chemical analyses. A histological method has been developed to measure the extracellular space. This property, along with water and protein content and pH values, was determined in four muscles from each of four animals. The results indicate that extracellular space varied among the muscles of the animals according to a definite pattern. Among the relations found, extracellular space was positively correlated with the water-protein ratio, whereas intracellular water content was negatively correlated. The evidence indicates that, in the muscles that characteristically contain a relatively high proportion of water to protein, the additional water is located in extracellular spaces.

Recent investigations in this laboratory have been concerned with the properties of bovine muscle tissues related to their capacity for holding water (Swift and Berman, 1959; Swift *et al.*, 1960; Berman, 1961; Gibbs and Fryar, 1961). Patterns established for the composition and certain properties of the muscles have been found to repeat in the muscles of each different animal studied. Among the components conforming to pattern and having a direct statistically significant relation to holding water are the chloride content and water-protein ratio.

From the chloride content is calculated "chloride space," a measure of the extracellular space in which aqueous fluid is found. The variation in chloride content therefore suggested that differences may exist in the distribution of water within the muscles. The work reported here deals with an investigation of this possibility.

The measurement of differences in extracellular areas is a direct approach to obtaining information on variations in chloride space and water-protein ratios. A satisfactory histochemical method was needed as an independent and direct approach to this measurement. To accomplish this, histological techniques that minimize distortion were investigated and devised.

The newly developed method and complementary chemical methods were then applied to a group of four muscles from each of four bovine animals. The muscles selected were those that, in our earlier work, had been found to vary markedly in composition and properties. The present paper describes our new histochemical method, presents the histochemical and chemical data obtained, and discusses the significance of the knowledge obtained on the distribution of water among the muscles.

EXPERIMENTAL PROCEDURE AND RESULTS

Animals and muscles used. The carcasses used were of three Holstein animals (a cow 34 months old, a bull 15 months old, and a bull 6 months old) and a Brown Swiss cow (39 months old). Within one hour of slaughter the muscles listed in Table 1 were removed from each carcass. Centrally located cubes (2 cm^3) were cut from each muscle for histological analysis, and adjacent cubes for chemical analysis.

Histological method. The cubes to be used for histological analysis were immediately frozen by immersion in liquid nitrogen (−195°C) contained in DeWar flasks. The cubes were then transferred to solid CO_2 (−78°C) and stored at dry-ice tem-

Table 1. Bovine muscle studied

Number	Muscle	Location	
		Forequarter	Hindquarter
1	*Longissimus dorsi*	Rib, chuck	Sirloin, porterhouse
2	*Semimembranosus*		Round
3	*Serratus ventralis*	Rib, chuck	
4	*Rectus abdominus*	Plate	Flank

perature until sectioned (two or more hours later).

The sample (stored at −78°C) was removed from storage and allowed to warm until microtome cutting temperature was reached. The 2-cm^3 sample was quickly cut to 1-cm^3 size and attached to the stage of a Spencer sliding microtome (trade names are mentioned for identification, implying no endorsement) with a drop of water that was then frozen in a stream of CO_2 gas. Cross sections of the muscle were cut 15 μ thick and mounted with glycerol directly on alcohol-cleaned slides. The sections were stained lightly according to Lillie's (1942) modification of Mayer's hematoxylin method and blotted with filter paper. A drop of glycerol was placed on the slide, and the section was covered with a 2-mm No. 0 micro cover glass.

By using liquid nitrogen and solid CO_2, this method avoided fixation procedures. Flotation was also eliminated by direct mounting with glycerol. This treatment resulted in minimal distortion of the muscle sections.

The mounted sections were photographed with a microscope and camera, using 35-mm high-contrast copy film. To measure the magnification factor, a stage micrometer slide marked in .01-mm units was photographed with each group of slides, using the same setting of microscope and camera. Enlarged 5 × 8-in. prints were made of muscle cross sections and of the stage micrometer with the same setting of the enlarger. In this way, accurate measurements of the original cell dimensions were possible.

The cellular and extracellular areas were cut out of the photograph, the respective areas of the paper were weighed, and the percentage of extracellular space was calculated from the weights. Calculation of space, a volume, from area, a plane surface, was possible because of the geometry of muscle tissue and the technique of sectioning tissue. Muscles are composed of bundles of fibers that may be assumed to be parallel over comparatively short distances. A transverse cross section of striated muscle would thus be approximately equivalent to a number of cylindrical fibers imbedded in a bath of extracellular material. The volumes of the fiber and the non-fiber materials can be calculated by multiplying these cross-sectional areas by this length. The lengths of the fiber and non-fiber material are equal when a sectional cut is made perpendicular to the axis of the fibers. Consequently, the volumes of the fiber and non-fiber materials are directly proportional to their areas. The average cellular diameter was calculated by dividing the total cellular area in a photograph by the number of cells, assuming the area of each cell to be a circle. The effect of post-mortem age was determined on samples from the other half of the carcass after 24 hr at *ca.* 3–4°C. In all instances histological measurements were made on 5–8 samples of tissue, and the data given are averages of these values.

Analytical methods. The cubes cut from the muscles for chemical analyses were ground through a Hamilton-Beach grinder equipped with a plate having approx 5-mm^2 openings. The analytical methods were applied to duplicate samples of ground tissue. Total nitrogen was measured by the macro-Kjeldahl method, and moisture by the oven drying method of the AOAC (1960). To determine pH, 20 g of each sample were mixed with 20 ml of water and stored at 5°C in airtight containers. After 24 hours, the samples were allowed to warm to room temperature and the pH was determined with a Beckman Model GS pH meter. This value has become known as the ultimate pH and corresponds to the low pH attained in tissue after rigor. Chloride was determined by the modified Volhard method described by Wilson and Ball (1929). Chloride space was calculated from the chloride content of the tissue and of blood, using the equation:

$$\text{Chloride space, \%} = \frac{\text{tissue chloride, mg \%}}{\text{blood chloride, mg \%}} \times 100.$$

Results and calculations. Table 2 shows average values obtained in the chemical analyses and histological measurements, along with the results of calculations of moisture-to-protein ratios. Fig. 1 shows averaged data for each of the four muscles of the four animals.

The correlation coefficients of relationships between components are given in Table 3. One property shown, intracellular water percent, was calculated as the difference between the percentages of total water content and of extracellular space. This calculation is based on the assumption

Table 2. Analytical data from histochemical and chemical analysis of four beef muscles from four different animals.

Animal	Muscle no.	Moisture	Protein	m/p Ratio [a]	Extracellular space	pH [b]	Chloride space [c]
A (cow,		%	%		%		%
34 mo)	1	75.81	23.13	3.28	11.35 (18.70) [d]	5.50	
	2	75.72	22.15	3.42	14.40 (22.90)	5.39	
	3	76.10	20.41	3.73	18.39 (28.43)	5.63	
	4	75.79	22.01	3.44	15.02 (28.35)	5.51	
B (bull,							
15 mo)	1	73.71	22.38	3.29	12.50	5.54	15.99
	2	73.40	22.06	3.33	12.01	5.42	18.67
	3	74.20	21.28	3.48	16.79	5.81	19.92
	4	74.45	21.31	3.49	13.62	5.69	20.76
C (bull,							
6 mo)	1	75.00	22.82	3.29	15.54	5.45	
	2	75.34	22.99	3.28	14.48	5.39	
	3	75.89	19.53	3.89	19.48	5.69	
	4	75.57	20.42	3.70	18.11	5.59	
D (cow,							
39 mo)	1	71.86	21.25	3.38	15.19	5.45	8.28
	2	74.25	22.10	3.36	14.27	5.40	10.85
	3	67.24	18.47	3.64	19.80	4.70	14.25
	4	72.45	20.82	3.48	16.33	5.47	12.74

[a] Correlation coefficients of relation of extracellular to the water-protein ratios for individual animals are: Animal A, $0.980 \pm .019$, $P < .01$; B, $0.745 \pm .212$, not sig.; C, $0.985 \pm .013$, $P < .01$; D, $0.992 \pm .007$, $P < .01$.

[b] Correlation coefficients of relation of extracellular spaces to the pH values for individual animals are: Animal A, $0.604 \pm .284$, not significant; B, $0.898 \pm .087$, $P < 0.10$; C, $0.998 \pm .002$, $P < .01$; D, $0.998 \pm .011$, $P < .01$.

[c] Calculated as $\frac{\text{chloride tissue, mg \%}}{\text{chloride blood, mg \%}} \times 100$.

[d] Determined 24 hr after slaughter.

Table 3. Statistical analyses of data from analyses on four muscles from each of four beef animals.

	Water-protein ratio		pH [a]		Protein, %	
	Averaged [b] df 3	Not averaged [c] df 15	Averaged [b] df 3	Not averaged [c] df 15	Averaged [b] df 3	Not averaged [c] df 15
Extracellular space, %	$0.988 \pm .011$ $P < .01$	$0.846 \pm .051$ $P < .001$	$0.958 \pm .037$ $P < .01$	$0.557 \pm .124$ $P < .05$	$-0.996 \pm .004$ $P < .001$	$-0.850 \pm .050$ $P < .001$
Intracellular water, [d] %	$-0.950 \pm .044$ $P < .02$	$-0.575 \pm .121$ $P < .01$	$-0.962 \pm .033$ $P < .01$	$-0.476 \pm .139$ $P < 0.10$	$0.972 \pm .025$ $P < .01$	$0.876 \pm .042$ $P < .001$

[a] pH 24 hr after slaughter.
[b] Based on averages of data for each of the four muscles.
[c] Data treated as determinations of 16 unrelated muscles.
[d] Calculated as the difference between content of water and extracellular space.

that extracellular space consists primarily of water and that the rest of the water is intracellular.

The statistical calculations were made on two bases. One involved averaged data for each muscle and emphasizes variations between the properties of muscles within animals, excluding between-animal differences; the other was based on individual measurements, independent of the muscles or animals from which the samples were obtained.

DISCUSSION

The histological method, developed after many attempts with alternative techniques, produced excellent slides for photography that were not detectably distorted (Fig. 2 and 3). In addition, the operations were carried out rapidly so that slides were immediately available for microscopic inspection and assessment. This opportunity to screen tissue sections before photomicrography eliminated lengthy operations on unsatisfactory sections.

Another method was also successful. This likewise involved initial freezing with liquid nitrogen at about −195°C. The frozen cube was then dehydrated in solutions of 1% $HgCl_2$ in absolute ethanol at temperatures ranging from −40°C to room temperature, treated successively with 4% collodion in alcohol, chloroform-alcohol, and paraffin in chloroform, and then finally embedded in paraffin, cut, and stained in the conventional manner. However, elapsed time was almost a month, in contrast with the rapid preferred method, which only took a few hours.

Since the measurement of extracellular space has frequently been approached through the measurement of the chloride content of tissue and the calculation of *chloride-space,* as discussed by Davson (1952), analyses by this method were made on two animals (Table 2, animals B and D). This afforded an opportunity for a limited comparison of the two methods. Statistical analyses of the results show that estimates of extracellular space based on the chloride method approximated those based on the histological method. However, estimates of extracellular space based on the chloride method varied more widely than those based on the histochemical method, and statistical analyses showed that data obtained by the two methods were not closely related.

As shown by the data in Table 2 (animal A), a drastic increase, amounting to an average of 66%, occurred in the extracellular spaces of the four muscles during the 23-hr period between samples taken at 1 hr and samples taken at 24 hr after slaughter. The correlation coefficient showing the relation between the measurements made at 1 hr and those made at 24 hr ($r = 0.874 \pm .113, p < .05$) indicates that the increases were moderately uniform. Therefore, any changes in the dimensions of the extracellular spaces in the hour that normally elapsed from time of slaughter until initial freezing would probably affect all muscles proportionally, and consequently can be eliminated as a factor significantly affecting the results reported here.

The changes in space after slaughter cannot be studied by determining chloride, sucrose, or inulin space. On the other hand, as shown above, the histological method can be used to measure changes occurring during the first 24 hr. Also, measurements (not reported here) on muscles aged 8 days at 3°C showed that structural deformation was marked during this period.

Analytical and statistical results are shown in Table 2 and the principal statistical calculations in Table 3. The data were subjected to calculations and statistical analyses in the manner previously followed in these investigations (Swift and Berman, 1959). The statistical analyses show that average extracellular spaces were significantly and positively correlated with average water-protein ratios ($r = 0.988 \pm .011, P < .01$) and average pH values ($r = 0.958 \pm .037, P < .01$) and negatively with average protein content ($r = -0.996 \pm .004, P < .001$). When based on analyses of individual animals (Table 2) the relations of extracellular spaces to water-protein ratios and pH values were also significant in three of four cases. The results indicate that variation of extracellular space occurs among the muscles of bovine animals according to definite patterns. As mentioned above, earlier work has shown that variation of pH, protein content, and other components and properties among muscles similarly occurs according to definite patterns, i.e., muscles of each animal, when listed in order of increasing

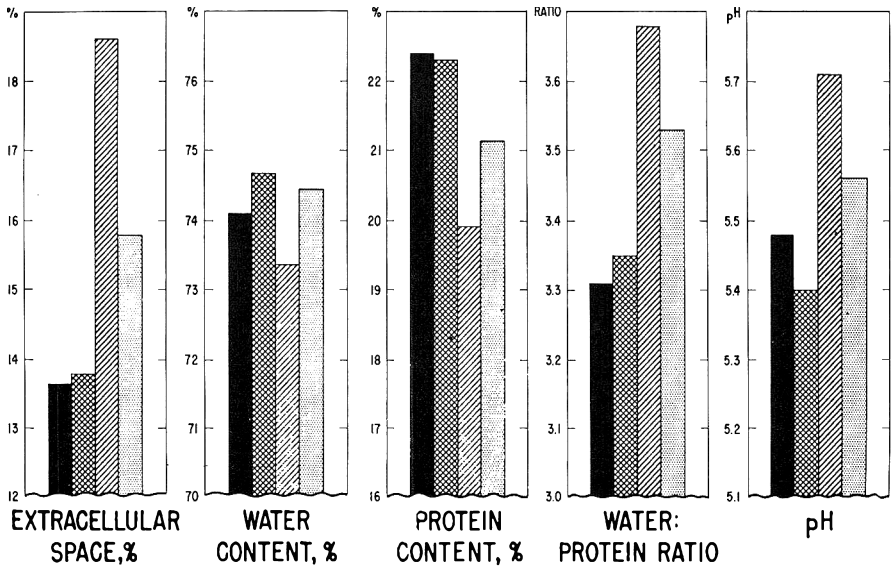
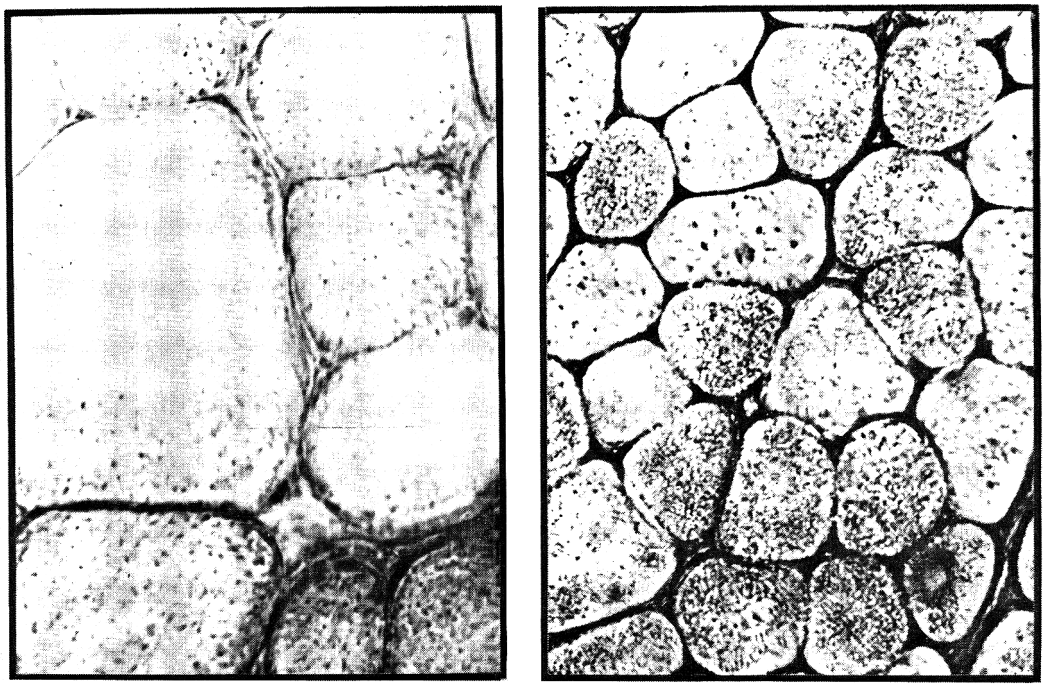


Fig. 1. Averaged data, for each of the four muscles of the four animals.



LONGISSIMUS DORSI RECTUS ABDOMINUS

Fig. 2. Frozen sections 1 hour after slaughter, enlarged 317×.

amounts of extracellular space, tend to fall in the pattern $2 < 1 < 4 < 3$.

The statistical analyses in Table 3, calculated from the data on 16 muscles but not averaged or grouped according to animal or muscle sources, also show that significant correlations exist between extracellular spaces and water-protein ratios ($r = 0.846 \pm .051, P < .001$), pH values ($r = 0.557 \pm .124, P < .05$), and protein contents ($r = -0.850 \pm .050, P < .001$). Coefficients were lower than those obtained with averaged data, as was to be expected since calculation with the unaveraged data was influenced by between-animal differences. These arise from differences in leanness, age, sex, or conditions, which may raise or lower the levels over which the properties of all tissues may range, but do not influence patterns of between-muscle variation. The differences noted between correlation coefficients obtained in calculations based on averaged and unaveraged data derive from this fact, namely, that variations of properties and composition of muscles of animals occur in patterns that are similar but not necessarily superimposable.

The significant and direct relation of extracellular spaces to water:protein ratios shown in Table 3 indicates that the increment of water in the extracellular spaces is higher in muscles that contain a relatively high proportion of water-to-protein than in muscles that contain a lower content of water relative to protein. The *serratus ventralis* and the *rectus abdominus* muscles are those containing the high proportion of water to protein, and the *longissimus dorsi* and the *semimembranosus* the low. The validity of the interpretation depends on water being the principal component filling extracellular spaces. That the space contains fluid that is 90% water or more can be readily calculated from data on the composition of interstitial fluid published by Davson (1952).

Assuming that extracellular spaces are occupied by water allows intracellular water to be calculated. To do this, the difference between total water content and extracellular space (as water) is used as an estimate of intracellular water. This assumption was the basis for the calculations in Table 3 of the statistical relations involving intracellular water. The significant negative relation of average percentages of intracellular water to average water-protein ratios ($r = -0.950 \pm .044, P < .02$) indicates that the water in the extracellular areas and not that in the cellular areas accounts for the relatively high proportion of water relative to protein in the *serratus ventralis* and *rectus abdominus* muscles.

In support of the thesis that the high water-protein ratio in the *serratus ventralis* and *rectus abdominus* muscles is entirely explained by water in their larger extracellular spaces rather than in their cellular areas, the proteins, which are principally located in the intracellular areas, appear to be hydrated at a relatively uniform level, as shown by the positive relation of percentages of intracellular water to protein content ($r = 0.972 \pm .025, P < .01$).

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A Simplified Method for Determination of Some Aspects of the Carotenoid Distribution in Natural and Carotene-Fortified Orange Juice

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SUMMARY

The unique and complex mixtures of carotenoids in oranges present difficulties in analysis and interpretation of results. The situation is at times further complicated by the addition of β-carotene to orange juice. Existing analytical procedures, in combination, have been adapted to a rapid quantitative method for both total carotenoids and carotenes. The method involves extraction with a foaming solvent mixture, saponification of esterified oxygenated carotenoids, separation of carotenes by column chromatography, and measurement with a spectrophotometer. An increase in carotenes from the normal level of less than 10% of the total carotenoids to approximately 20% is readily detected, reflecting a sensitivity adequate to the interpretive needs in examining commercial products.

INTRODUCTION

A variety of analytical methods have been used for determination of carotenoids in citrus fruit and juice. However, some of these procedures do not make the necessary separation of oxygenated carotenoid compounds from the hydrocarbon carotenes, and other methods are too cumbersome for routine use. Because of this situation and the general practice of reporting all carotenoids in terms of colorimetric equivalent amounts of β-carotene, much confusion and conflicting analytical data have resulted in different laboratories. The problem has been further accentuated by the advent of synthetic β-carotene and its resulting widespread use to color citrus juices and beverages. The work described here was done to develop a simple yet valid quantitative method for the determination of total carotenoids and carotenes in orange juice. These two groups of compounds are of interest because the total carotenoids constitute the visible pigmentation of the juice, and the carotenes are commonly used as a measure of the pro-vitamin A content. Addition of β-carotene is reflected by a change in the relative proportions of the two groups of compounds, the altered ratio being in some ways a better indication than the absolute level of carotenes.

REVIEW OF LITERATURE

Early work by Zechmeister and Tuzson (1933, 1936a, b) confirmed the presence of carotenes in oranges but showed that most of the carotenoid pigments are oxygenated compounds, a majority of which are esterified. Methods of analysis involved saponification of the esters and separation on a chromatographic column.

More recently Curl (1953) and Curl and Bailey (1955, 1956, 1957, 1959, 1961) separated and identified the carotenoid pigments of Valencia and navel oranges using the techniques of countercurrent distribution and column chromatography. They found that the carotenoids of orange juice comprise an unusualy complex mixture, of which less than 10% is made up of carotenes. The latter are distributed as zeta-carotene, which predominates, beta-carotene, and still less alpha-carotene.

To extract and separate the pigments into fractions for final separation and identification, Curl and Bailey used an elaborate pro-

cedure. The juice was mixed with an equal volume of methanol, allowed to stand, then filtered. The filter cake was washed with 50% methanol, then acetone. The acetone and methanol were evaporated *in vacuo*, and the carotenoids were removed from the aqueous residue by exhaustive extraction with ether. Subsequently the ether extract was separated and evaporated. Before and after overnight saponification with 20% potassium hydroxide in methanol, the compounds of interest were separated by counter-current distribution and column chromatography. By this procedure the carotenoids arranged themselves according to the number of unesterified hydroxyls, regardless of the combined total of esterified and free hydroxyl groups. Thus, without saponification, the completely esterified oxygenated compounds cannot be separated from the hydrocarbons.

The A.O.A.C. (1960) lists several methods for estimation of carotenes, and for some materials specifies a saponification step. In the procedure for fresh plant materials, however, this step is not included. Briefly, the separation involves blending with acetone-hexane, filtration, solvent treatment of the filtrate, and chromatographic separation. For the latter, a packed 2×10-cm column composed of equal parts activated magnesia and diatomaceous earth is used with 50 ml of acetone-hexane (1 + 9) for the eluant. Measurement is made with a spectrophotometer or other suitable instrument.

A method of extraction that avoids the large volumes of initial filtrate was developed by Moore and Ely (1941). They discovered that certain mixtures of ethyl alcohol, water, and petroleum ether form semistable foams. Thus, a sample included as the aqueous portion of this mixture can be extracted in a blender without losses due to splashing. Not mentioned by the authors, though also a possibility, is that low surface tension as indicated by foaming will make for better extraction.

Of the reported values for carotenes (reported as β-carotene) in oranges, the data of Taylor and Witte (1938) are the most comprehensive. They used the procedure of Guilbert (1934), which employs saponification but separates the oxygenated compounds from carotenes by their methanol extraction from a petroleum ether solution rather than by column chromatography. Many samples were analyzed, and extreme values for carotenes ranged from 0.005 mg/100 ml, for Florida pineapple oranges, to 0.297 mg/100 ml, for California navel oranges.

Pilcher (1945), using the foaming-solvent method, without saponification and adsorbing extraneous pigments on Ca_2HPO_4, found 0.01 to 0.12 mg as β-carotene per 100 g. Hinman *et al.* (1947), using foaming solvent plus the A.O.A.C. chromatographic procedure, obtained 0.02–0.22 mg per 100 g, and Burger *et al.* (1956), using essentially the same technique, found 0.003–0.014 mg per 100 g. Recently Joseph *et al.* (1961), with the above method, found 1.35–3.20 mg per 100 g total carotenoids, and 0.02–0.17 mg per 100 g carotenes. Mackinney (1961) gives values for total carotenoids ranging from 0.44 to 3.4 mg per 100 g in several species and varieties of oranges. He states that his results with extracts of Biondo, Sanguigno, Sanguinella, and Tarocco varieties permit the assertion that the carotenoid mixtures are qualitatively similar to those of California Valencias and Washington navels, with the hydrocarbon (carotene) fraction varying from 3 to 10% of the total carotenoids.

The confusion in methodology was mentioned by Calvarano (1960), who gave values ranging from 0.98 to 2.32 mg per 100 ml for total carotenoids in the filtered juice of commercially significant Italian varieties. Calvarano also determined unesterified xanthophylls by solvent extraction of the total carotenoids without saponification.

Rowell (1960) published a method based on centrifugation for detecting the addition of β-carotene beadlets to orange juice. This is a qualitative procedure, however, and would not necessarily detect β-carotene added in a form other than beadlets, nor does it reveal information on the distribution of carotenoids.

It appears, then, that much of the published data on carotenes in orange juice may be of questionable value for comparative

purposes because of the analytical procedures used. Where adequate procedures have been used to eliminate the esterified oxygenated carotenoids, the methods are too cumbersome to be directly applicable to routine analysis of many samples.

EXPERIMENTAL

The problem was approached by starting with Curl's thorough but lengthy extraction procedure. Various simplifications, suggested either in the literature or by observation of results, were tried. Many variations were rejected; only the method as developed is presented.

Materials. Unless otherwise specified, chemicals should meet A.C.S. reagent specifications: *hexane,* commercial, b.p. 60–75°; *isopropyl alcohol,* colorless, 99.6%; *alkaline methanol,* 20 g KOH dissolved in 100 ml methanol; *sodium sulfate,* powdered, anhydrous; *column adsorbent,* equal parts by weight of Hyflo Super-Cel (Johns-Manville) and activated magnesia (Micron brand, No. 2642, Westvaco Chlorine Products Company, Newark, California) or Sea Sorb 43; *acetone,* dry, alcohol-free.

Principles of method. Using separate and different isopropanol-hexane-juice combinations for total carotenoids and for carotenes, the first steps in either analysis are: blend, add water, and separate the washed hexane solution. For total carotenoids this solution is dried and the absorbance measured. Carotenes are determined after saponification, followed by column chromatography of the hexane solution. Elution is accomplished with acetone-hexane and absorbance of the eluate measured.

Total carotenoids. In a semi-micro Waring blender bowl, mix 10 ml single-strength orange juice, 30 ml isopropyl alcohol, and 10 ml hexane for one minute. Empty the blended solution into a 125-ml separatory funnel containing some water, rinse the blender bowl three times with water, and add the rinse water to the contents of the separatory funnel. Add water to the separatory funnel to give a total volume of 80–100 ml. The solution will separate into an aqueous layer and a hexane layer, the latter containing the carotenoids. Moisten the stopper and insert it in the separatory funnel, then mix the contents by swirling. Wetting the stopper helps to prevent leakage of the hexane layer, but, during mixing, contact of the solution with the stopper should be kept to a minimum.

Allow the solution to stand for 30 minutes, then discard the uncolored (unemulsified) portion of the aqueous layer. This will appear milky but will not contain a significant acount of carotenoid material. Separation can be hastened by centrifugation using special separatory funnels. After draining off the aqueous phase, wash down the funnel several times with water, separating and discarding the uncolored aqueous phase after each washing. This will transfer all but an insignificant amount of the colored material to the hexane layer. Finally, drain the hexane layer into a 50-ml volumetric flask through a small quantity of anhydrous sodium sulfate powder supported in a funnel by a piece of cotton. Wash the separatory funnel twice with 3 or 4 ml of hexane, discharging the hexane through the funnel. If necessary, wash any color from the sodium sulfate with more hexane. The powdered sodium sulfate as used here and throughout this work is for the purpose of producing an anhydrous system. Add 5 ml acetone to the volumetric flask and fill to the mark with hexane. A small amount of sodium sulfate added to the flask will ensure dryness. This solution is now ready to measure, although in some cases further dilution with acetone-hexane may be necessary to bring the absorbance of the sample into the proper range for measurement with the particular colorimeter or spectrophotometer to be used. If necessary, this solution can be stored for up to 24 hr at 0°C or lower.

Carotenes. Using 20 ml. of single-strength orange juice, 50 ml isopropyl alcohol, and 10 ml hexane, follow the above procedure for total carotenoids through the step of washing the separated hexane layer with water. After that step, drain the hexane into a 125-ml ground-glass-jointed flask. Rinse the separatory funnel first with 10 ml of alkaline methanol, then with 10 ml of isopropyl alcohol, and drain both of these into the flask with the hexane. Place 2 or 3 glass beads in the flask to prevent bumping, and attach the flask to a reflux condenser. Heating gently on a water or steam bath, reflux for 10 min. Pour the solution into a previously moistened 125-ml separatory funnel and rinse the flask three times with water, pouring the rinse water into the funnel. Bring the volume in the separatory funnel to 80–100 ml, moisten and insert the stopper, and then, as before, mix by swirling.

Allow the solution to stand for 30 minutes, then drain off the aqueous lower layer, but retain any emulsified colored material. The aqueous layer will be colored by carotenoid compounds made water-soluble by the saponification step. Wash the hexane layer 3–4 times with water. This will transfer emulsified carotenes to the hexane and remove all alcohol from that layer. Drain the washed hexane layer through a funnel containing sodium sulfate powder supported on cotton into a previously prepared chromatographic column. Wash the separatory funnel and sodium sulfate

with several 3–4-ml portions of hexane to remove colored material.

A suitable chromatographic tube can be prepared by joining a 1.8-cm O.D. tube 20 cm long to a smaller tube (*ca.* 1.0 cm O.D.) about 10 cm long. The column of adsorbent should be about 2.5 cm deep. It is supported with a cotton plug, and packed by applying vacuum while tamping the surface with a cork on a stirring rod, or with some similar flat object. Top the adsorbent with a 0.5-cm layer of sodium sulfate powder. It is convenient to insert the discharge tube from the column in a rubber stopper and then insert the stopper into a filter flask. A test tube for sample collection can be placed in the filter flask so the discharge tube fits into the test tube. By applying vacuum, draw the hexane layer through the column. The carotenes and other carotenoids will be retained at the top of the column. As the surface of the hexane sinks below the sodium sulfate in the column, wash the color through the sodium sulfate with small amounts of a 25-ml portion of the acetone-hexane solution, then add the remainder of the acetone-hexane. If bands of color separate before addition of the acetone-hexane, the hexane solution was not washed free of alcohol. Either alcohol or incomplete saponification will prevent a clean separation of carotenes by the acetone-hexane. Break the vacuum, place a test tube under the discharge from the column, and reapply vacuum. With pure juice the first 10–15 ml of acetone-hexane effluent will contain all of the carotenes, and subsequent column effluent will be colorless. This can be checked by breaking the vacuum and inserting another test tube, then collecting two or three more ml of effluent. In the case of β-carotene addition, somewhat more effluent may need to be collected.

After all colored effluent fractions are combined, in either a glass-stoppered graduated cylinder or in a volumetric flask, this solution is ready to measure. Working with pure juice, the final volume should be kept to a minimum to ensure that the absorbance will be measurable. Extracts from β-carotene-fortified juice may need considerable dilution with acetone-hexane to bring the absorbance into a suitable range for measurement.

RESULTS

To test the method, a 24% semisolid suspension of β-carotene in vegetable oil was obtained from Hoffmann-La Roche, Inc. (lot No. A.N. 174–3). A dilution was made of 0.416 g (0.100 g β-carotene) of this suspension in 50 ml cold-pressed citrus oil. The β-carotene preparation was not completely soluble at that concentration, but, by agitating, a uniform mixture suitable for further dilution could be obtained. Dilutions of this solution were measured with a Beckman Model DU spectrophotometer—as is, after various treatments duplicating different stages of the separation procedure, and after addition to and separation from orange juice. In calculating concentrations, an absorptivity of 250 L/g-cm was used. Various other values are reported for beta- and other carotenes of citrus, as shown in Table 1. The authors from whom the data in the table are taken, also report the presence of various cis-trans isomers of the carotenes, some occurring naturally and some formed during extraction, but all differing somewhat in absorbance characteristics. The maximum for β-carotene observed here occurred from 450 to 454 mμ, but the absorptivity of 250 was applied in each case. Some values obtained for the β-carotene stock solution are given in Table 2. All dilutions were made with 1+9 acetone-hexane solvent.

The hydrolysis and chromatography steps of the method were checked as shown in Table 3 for possible loss of β-carotene; using dilutions from the β-carotene in citrus oil. The theoretical amounts are based on the original β-carotene sus-

Table 1. Absorbance characteristics of some carotenes as reported in the literature.

Carotene isomer	Source	Absorptivity						Reference	
		mμ	a	mμ	a	mμ	a		
beta	purified AOAC			451	250			Lime *et al.*	(1957)
beta	pure			450	256			Beadle and Zscheile	(1942)
beta	from spinach			450	245			Beadle and Zscheile	(1942)
beta	purified, heat-isomerized			451	244	478	212	Zechmeister and Polgar	(1943)
beta	tomatoes	417	179	452	250	479	229	Zscheile and Porter	(1947)
alpha	tomatoes	421	180	447	270			Zscheile and Porter	(1947)
alpha	purified, heat-isomerized			444	257	474	233	Zechmeister and Polgar	(1947)
zeta	tomatoes			401	220	426	227	Zscheile and Porter	(1947)
zeta	tomatoes	378	130	400	221	425	226	Nash and Zscheile	(1945)

Table 2. Absorbance of various dilutions of 24% β-carotene suspension.

Dilution of 0.416 g/50 ml solution	Absorbance at 454 mμ	Indicated β-carotene in stock solution (g/100 ml)	Calculated β-carotene in stock solution (g/100 ml)	% recovery
1:1250	0.387	0.193	0.200	96.5
	0.398	0.199	0.200	99.5
1:1000	0.490	0.196	0.200	98.0
1:500	0.96	0.192	0.200	96.0
	0.99	0.198	0.200	99.8
			Average recovery	97.8

Table 3. Effect of hydrolysis and chromatography procedures on recovery of β-carotene.

Solution	β-carotene (mg/ml)	
	Before	After
Solution with 0.00156 mg β-carotene per ml hydrolyzed 1 hr	0.00156	0.00162
Solution with 0.0391 mg β-carotene per ml chromatographed, then diluted to 0.00196 mg/ml for measurement	0.00188	0.00192

pension containing 97.8% of the declared 24% β-carotene, or 23.5% β-carotene. It is evident that there is no significant effect on recovery of β-carotene that can be attributed to these two steps.

The adequacy of the saponification was established by extracting Valencia juice and estimating carotenes after saponification treatments of increasing severity. Results in terms of absorbance at 427 mμ, where absorption was maximum, are given in Table 4. From these data it is evident that 10 min of reflux is adequate for complete hydrolysis of esterified carotenoids and that reflux temperatures at atmospheric pressure are required. Since the amount found after 30 min reflux is virtually the same as after 10 min, there is no problem of degradation. The relatively small loss indicated after 60 min is probably within the limits of normal variation.

Samples of Valencia and navel juice, reconstituted from concentrates, were carried through the complete analysis, and the extracts were examined with a Beckman Model DK2 Recording Spectrophotometer, giving the curves in Fig. 1. Assuming the major carotene components of Valencia orange juice to be zeta-, beta-, and alpha-carotene plus the colorless compound phytofluene, the various peaks of the curve for Valencia carotenes in Fig. 1 probably result from these compounds as follows: 348 mμ, phytofluene; 377 mμ, phytofluene and zeta-carotene; 402 mμ, zeta-carotene; 426 mμ, zeta-carotene; 450 mμ, alpha- and beta-carotene; 477 mμ, alpha- and beta-carotene. Using the data of Zscheile and Porter (1947), it would be possible to calculate the concentrations of each component. For a simplified method such as this, however, it suffices to estimate the concentration by applying an arbitrary absorptivity of 250 L/g-cm to the highest peak observed between 390 mμ and 490 mμ. When data are made with a properly calibrated spectrophotometer, calculate the concentration of carotenes from the formula below:

$$C = \frac{A \times 100}{250 \times L \times W},$$

where C = concentration of carotenes as mg/100 ml in original sample; A = absorbance; L = cell length in cm; and W = ml of original sample per ml of the final dilution measured.

From a qualitative standpoint, Fig. 1 shows that any significant addition of beta-carotene to either California Valencia or navel juice would greatly change the curves for the carotene fraction at 450 mμ and 477 mμ, making the addition very evident.

Table 4. Effect of saponification treatment on separation of carotene from orange juice.

Saponification treatment	Volume of extract (ml)	Absorbance at 427 mμ
1 min cold	no separation on column	.....
10 min cold	no separation on column	.....
10 min reflux	15	0.400
30 min reflux	15	0.397
60 min reflux	15	0.379

Table 5. Ratio of total carotenoids to carotenes in several samples of orange juice.

Variety	Total		Carotenes		Ratio
	λ max.	mg/100 ml	λ max.	mg/100 ml	
Valencia	425	1.31	425	0.092	14:1
	425	1.31	426	0.070	19:1
	442	0.87	427	0.062	14:1
Navel	450	0.85	425	0.050	17:1
	427	0.644	426	0.024	27:1
Hamlin	445	0.32	.....	nil	.....
Mandarin	440	3.46	427	0.258	13:1

Samples of orange juice were analyzed for total carotenoids and carotenes so that the ratio between the two quantities could be calculated. These data are shown in Table 5.

For the method of analysis to be of value in regulating coloring of orange juice, it is necessary to show that added β-carotene can be detected. Brief tests showed that, to produce a significant increase in color of single-strength orange juice, β-carotene must be added in an amount approximately equivalent to the total carotenoids naturally present, or about 1 mg/100 ml. Some of the previously mentioned dispersion of β-carotene in citrus oil was homogenized into a weighed quantity of single-strength orange juice and the juice concentrated to approx 70° Brix to remove the oil. Analysis of this sample following dilution to single strength is shown in Table 6. Recovery of the added β-carotene was very good, and the percentage reported would have been even better if the control sample had been calculated at 454 mμ rather than at 425 mμ. The very marked effect on the ratio of carotenoids to carotenes should be noted.

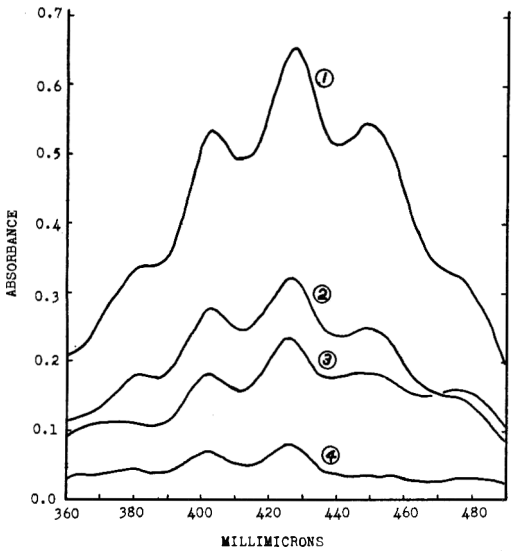

Fig. 1. Absorbance curves for total carotenoids and carotenes in orange juice: 1) Valencia, total, 0.2 ml juice/ml; 2) Navel, total 0.2 ml juice/ml; 3) Valencia, carotenes, 1.33 ml juice/ml; 4) Navel, carotene, 1.33 ml juice/ml. Solvent and reference 1+9 v/v acetone-hexane.

DISCUSSION

There may be some question regarding the use of separate samples for total carotenoids and for carotenes. Using a single extraction, an aliquot of the hexane layer could be removed for measurement of total carotenoids. To do this, however, would require diluting the first extraction to known volume so an aliquot could be removed. For this the solution would either have to be dried or a solvent such as isopropyl alcohol added to achieve a single phase. The added manipulation and change in the sample size represented by the final solution measured would tend to make error more likely. Two samplings thus present a more simple approach.

Table 6. Analysis of navel orange juice with added β-carotene.

β-carotene added	Total carotenoids		Carotenes		Recovery of added carotene (%)	Ratio
	λ max.	mg/100 ml	λ max.	mg/100 ml		
No addition	450	0.70	425	0.029	.....	24:1
0.978 mg/100 ml	450	2.12	454	0.990	98.9	2.1:1

The foaming-solvent technique is probably the most time-saving feature of the method. There was concern that the milky aqueous layer discarded after the initial extraction might contain some carotenoids. Additional extractions and centrifuging showed that this is not the case if enough time is allowed for separation of the phases. In this step, leakage of the hexane past the ground-glass stopper of the separatory funnel can cause losses. Isopropyl alcohol was used rather than ethyl alcohol because of the tax complexities concerning the latter.

No investigation was made of column material. The particular type of magnesia used has been employed in most of the work reported in the literature and is specified by the A.O.A.C. method, which suggests that not all magnesia is equally suitable. The extract applied to the column must be dry and alcohol-free, or oxygenated carotenoids will come through with the carotenes, When the solvent is dry there is a very clean separation and no difficulty in ascertaining when all carotenes have been eluted.

Three or four hours are required to complete the analysis of one sample, but, in only slightly more time, several samples could be run simultaneously.

If many samples are to be run routinely and qualitative aspects of the carotene fraction ignored, a more simple instrument than the spectrophotometer can be used, provided that it is equipped with a suitable filter and is properly calibrated. One such instrument is the Klett-Summerson photoelectric colorimeter with a K.S.44 (410–480-mμ) filter.

With this method the concentrations of carotene and total carotenoids in orange juice and orange beverages can be measured quickly and reliably. This measurement is of interest in the regulation of β-carotene added to orange juice and in the estimation of pro-vitamin A activity.

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Studies on the Fate of Staphylococci During the Processing of Hams

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SUMMARY

Staphylococci are destroyed during the heat processing of both *smoked* and *fully cooked* hams. Although these organisms are capable of growth in the presence of sodium chloride, sodium nitrate, and sodium nitrite in the concentrations used in cured meats, they are readily destroyed in the presence, as well as the absence, of these agents at a temperature of 137°F. Twenty-two of 53 strains of *Staphylococcus aureus* were killed in excess of 99% after 10 min exposure at this temperature. Thirty-one strains exhibited more resistance, but were destroyed in excess of 99.9% after 60 min at 137°F. The significance of these results in terms of previous studies is discussed.

Growth of staphylococci with attendant production of enterotoxin is virtually unknown in unprocessed meats, but cured meats, particularly ham, are frequently implicated in outbreaks of staphylococcus food poisoning.

In the production of hams, the uncured meat is pumped via the arterial system with a pickle that is a solution containing sodium chloride, sodium nitrate, sodium nitrite, sugar, and disodium phosphate. The pumped hams are maintained at 36–38°F and may be held for a while before being heat processed. In the smoke house the temperature of the product is gradually raised to a minimum of 137°F for *smoked hams* and 148°F for *fully cooked hams*. After heat processing, the hams are chilled and held under refrigeration until they leave the processing plant.

Lechowich *et al.* (1956) reported that food-poisoning staphylococci die rapidly in the presence of curing pickle. Further, those workers found that the organisms do not grow in pumped hams during the so-called curing period (36–38°F). Earlier findings of Gross and Vinton (1947) were similar.

Stritar (1941) reported that temperatures as high as 148°F are not sufficient to destroy enterotoxogenic staphylococci in ham. Gross and Vinton (1947), working with a heat-resistant staphylococcus (No. 184) obtained from Stritar, conducted extensive studies in broth as well as in cured and uncured meats. They found thermal death times of over 2 hr at 139°F and over 5 hr at 136°F. These results suggest that the 137°F process given to *smoked hams* is insufficient for the destruction of staphylococci. Gross and Vinton's data indicated that 148°F is sufficient for staphylococcus destruction in 10–17 min. Lechowich *et al.* (1956) reported destruction of staphylococci in the interior of hams at a temperature below that realized in the production of *smoked hams* (less than 137°F). Dack (1946) has stated that hams, as they leave the packers, are safe; he would thus imply that the heat processing given to hams is sufficient to destroy staphylococci.

The present study was undertaken in an effort to reconcile these apparent inconsistencies with regard to the fate of staphylococci during the heat processing of hams.

EXPERIMENTAL

Bacteriological methods. The determination of staphylococci in hams was accomplished by surface

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plating on tellurite glycine agar (Difco). The potassium tellurite solution was sterilized by Seitz filtration and added to the melted agar immediately before the plates were poured. Quantitative surface plating was done according to the method outlined by Snyder (1947). Plates were incubated 24 hr at 98.6°F.

In experiments involving inoculation of sterilized culture media with pure and mixed cultures of staphylococci, conventional total plate counts were determined using tryptone glucose yeast extract agar (Difco), with incubation for 24 hr at 98.6°F.

The staphylococci used were all coagulase-positive strains isolated from clinical materials and foods implicated in food poisoning.

Survival of staphylococci during normal processing of hams. Ham pumping pickle containing conventional quantities of sodium nitrite, sodium nitrate, brown sugar, and sodium chloride was inoculated with a mixture of eight different strains of staphylococci, each isolated from ham implicated in food poisoning. Four of the cultures had been authenticated by monkey feeding tests for ability to produce enterotoxin. The pickle was made up in water at 140–150°F, and after all ingredients were in solution, disodium phosphate was added. The staphylococci were added after the pickle had been cooled below 100°F. Bacteriological analyses of the pickle were done before and after pumping. Hams in the 14–16-lb weight range were pumped with the pickle to 112% of their original weight.

To establish the initial level of staphylococci, duplicate whole hams were sampled immediately after pumping. At various stages of processing, duplicate whole hams were sampled, i.e., each ham was taken from the smoke house, and the meat was trimmed from the bone and placed in a polyethylene bag. Since the hams could not be analyzed immediately, the bags were placed in an ice bath for approx 1 hr and then placed in a −10°F freezer. When the entire set of samples had been accumulated, they were partially thawed and the entire meat mass was ground aseptically and subjected to bacteriological analysis. This method of sampling was chosen since it was felt to yield a more representative sample than could be obtained by the more conventional core-sampling procedure. An 8–10°F differential is normal between the center and a point midway between the center and the surface of 14–16-lb hams (Schack, 1961). Thus, when the minimum internal temperature reaches 100°F, a major portion of the ham is still in a temperature range that allows growth of staphylococci. Hams were sampled when the minimum internal temperature reached 100°F (optimum growth temperature for staphylococci), 113°F (minimum temperature at which *smoked hams* are removed from processing), and 148°F (minimum temperature for *fully cooked* hams).

Minimum internal temperatures of the hams were obtained with an automatic recording potentiometer. Experiments were performed using various levels of staphylococci.

Heat processing was carried out in a Julian oven. The oven temperature increased from approx 130 to 160°F during 22 hr, by which time the minimum internal temperature of the hams had reached 148°F.

Simulated processing. Studies were conducted in a medium consisting of nutrient broth (Difco) containing 0.405% brown sugar. This medium was tested at various salt levels (0, 2.5, 5.0, and 10.0%), in both the presence and the absence of sodium nitrate and sodium nitrite. A Seitz-filtered solution of sodium nitrite and sodium nitrate was added to autoclaved nutrient broth so as to give final concentrations of 0.0135% and 0.0075% respectively. These studies were carried out at pH 7.0 and pH 6.0, the acidity being adjusted with lactic acid. The medium was dispensed in 100-ml amounts in bottles for use. The inoculum consisted of a mixture of the same eight strains of coagulase-positive staphylococci used in the ham inoculation experiments described above. The organisms were grown separately in trypticase soy broth (B.B.L.) incubated 18 hr at 98.6°F. After incubation, equal volumes of each of the cultures were mixed, and a 1:1000 water dilution prepared. Each test bottle was then inoculated with 1 ml of the diluted mixture so as to receive approx 1,000,000 staphylococci.

The medium was at a temperature of 40°F at the time of inoculation. The test bottles were placed in a 98.6°F incubator and held 60 hr. It required 4 hr for the medium to reach the temperature of the incubator. At the end of the incubation period, the bottles were placed in a water bath with an initial temperature of 106°F. During a 4.5-hr period, the water-bath temperature was gradually raised to 139°F and the temperature of the medium to 137°F (see Table 2 for intermediate temperatures). When the bottles had reached 137°F they were held for 5 min, shaken, and then held for an additional 5 min. The bottles were then cooled in ice water. Total bacteria counts were determined on the broth after inoculation, after incubation, and after raising the temperature to 137°F.

Destruction of various strains of Staphylococci at 137°F. Pure cultures of each of the 53 strains were grown in trypticase soy broth and incubated 24 hr at 98.6°F. For survival studies, 0.1 ml of the incubated culture was inoculated into 10 ml of trypticase soy broth. The inoculated tubes were then completely immersed in a water bath set at

137°F. After the tubes had reached the temperature of the bath, they were held for a period as indicated, and then immersed in an ice bath. The chilled tubes were analyzed immediately for surviving organisms by the methods described under Bacteriological Methods.

RESULTS

Survival of staphylococci during normal processing of hams. Table 1 summarizes the results of three separate experiments wherein ham pumping pickle was inoculated with various levels of staphylococci before injection. In Experiments 1 and 2 there is indication of a slight increase in numbers of staphylococci during the 6 hr required for the minimum internal temperature of the hams to reach 100°F. Such an increase is not apparent in the third experiment, in which a larger number of organisms was inoculated into the pickle. In all three experiments there was a reduction in the number of organisms by the time the product reached 113°F. This is of interest since coagulase-positive staphylococci are known to be capable of growth at temperatures as high as 113°F (Evans and Niven, 1950). The inoculated organisms were not recovered from the product by the time it reached 137°F, and likewise, the analyses of hams that had reached 148°F yielded negative results.

Simulated processing. Table 2 presents a compilation of results obtained in experiments designed to simulate in a culture medium a ham-processing schedule. The inoculated staphylococci were able to grow in all cases. Minor differences were observed in the final numbers of organisms, with the least growth occurring in bottles containing 10% sodium chloride. No explanation is apparent for the small number of staphylococci recovered from the pH 7.0 variable containing sodium nitrate, sodium nitrite, and 5% sodium chloride. When the individual cultures were heated to 137°F, a 99+% reduction in viable staphylococci occurred in all variables.

Destruction of various strains of staphylococcus aureus in broths at 137°F. Table 3 shows that 22 of the 53 strains tested were more than 99% destroyed during 10 min at 137°F. The remaining strains showed greater than 1% survival after this heat treatment, and these are referred to as "resistant" strains. Table 4 summarizes the data in Table 3.

The resistant strains were further tested by 60 min at 137°F. Under these conditions, all 31 strains exhibited greater than 99.9% reduction in numbers. One of the test organisms, *Staphylococcus aureus* No. 184, obtained from the American Meat Institute Foundation, had originally been obtained from Dr. G. M. Dack, who had found it to be the most heat-resistant of 16 enterotoxin-producing strains in his collection. It was subsequently used by Stritar (1941) and by Gross and Vinton (1947) in work on heat resistance. In our studies, this organism fell into the resistant group, showing only 90% reduction in numbers after 10 min at 137°F. After 60 min at 137°F, however, the broth culture showed less than 1 organism per ml, the level before heat treatment being 11,000,000 per ml.

DISCUSSION

The present results provide additional evidence that food-poisoning staphylococci do not survive the processing temperatures used in the production of either *smoked* or *fully cooked* hams. These results confirm those of Lechowich *et al.* (1956), who found that inoculated hams showed a progressive decrease in numbers of staphylococci during 48 hr at 120°F, even though the maximum internal temperature of the product reached only 118°F.

The previously cited work of Stritar (1941) and of Gross and Vinton (1947) would suggest the necessity for much higher temperatures than are employed in ham processing, particularly *smoked* hams. The present studies are not, however, completely at variance with either of these groups. In those earlier studies, complete destruction of large numbers of staphylococci rather

Table 1. Survival of mixed cultures of staphylococci in hams during curing and heat processing.

Sample	Time in smoke house	Staphylococcus count per g		
		I	II	III
Pickle (100°F)	.....	12,000	32,000	290,000
Ham, after pump	.....	900	2,400	52,000
Ham, 100°F[a]	6 hours	3,800	5,700	16,000
Ham, 113°F	8 hours	300	1,200	7,200
Ham, 137°F	12 hours	<100	<100	<100
Ham, 148°F	20 hours	<100	<100	<100

[a] Temperatures shown refer to minimum internal temperature.

Table 2. The effects of curing salts and pH on the growth and heat resistance of staphylococci.

Experimental conditions		Staphylococci per ml: After 98°F incubation	Staphylococci per ml: After raising temperature to 137°F	% destruction
pH 7.0, NO_3^-–NO_2^- absent				
%NaCl	0	100,000,000	11,000	99.9+
	2.5	140,000,000	560,000	99.6
	5.0	120,000,000	180,000	99.85
	10.0	43,000,000	2	99.9+
pH 7.0, NO_3^-–NO_2^- present				
% NaCl	0	280,000,000	0	99.9+
	2.5	120,000,000	80	99.9+
	5.0	70,000	0	99.9+
	10.0	100,000,000	0	99.9+
pH 6.0, NO_3^-–NO_2^- absent				
% NaCl	0	600,000,000	11,000	99.9+
	2.5	660,000,000	120,000	99.9+
	5.0	470,000,000	44,000	99.9+
	10.0	57,000,000	160,000	99.82
pH 6.0, NO_3^-–NO_2^- present				
% NaCl	0	400,000,000	50,000	99.9+
	2.5	400,000,000	1,900,000	99.53
	5.0	280,000,000	72	99.9+
	10.0	18,000,000	28	99.9+

Inoculum: 13,000 staphylococci per ml trypticase soy broth, consisting of mixture of eight different cultures of *Staphylococcus aureus*.

Temperature schedule: Initial temperature of broth −40°F. Temperature raised to 98.6°F in air incubator within 4 hr. Held additional 12 hr at 98.6°F before "heat processing."

Processing schedule:

Time	Bath	Medium	Time	Bath	Medium
0	106°F	98.6°F	3 hr	124°F	121°F
1 hr	112°F	190°F	4 hr	137°F	134°F
2 hr	116°F	112°F	4½ hr	139°F	137°F

NO_3^-–NO_2^-: Final concentrations in medium where indicated:
$NaNO_2$: 0.0135%
$NaNO_3$: 0.0075%

than percent destruction in numbers was used as a criterion of heat lethality. On this basis, one surviving cell out of a billion would be interpreted as a positive result, although no information would be gained in terms of the efficiency of the heat process. Since ham pumping pickle does not support the growth of staphylococci (Lechowich *et al.*, 1956) and since fresh meat seldom contains more than a few thousand staphylococci per g (Jay, 1961; Silliker, 1959), it is unnecessary to consider a heat process sufficient to kill millions of these organisms per g of meat.

The extensive experiments of Gross and Vinton (1947) indicate a thermal death time for staphylococcus No. 184 of between 1 and 10 min at 150°F. Those workers reported much higher orders of thermal resistance when the test organism had been allowed to grow in meat. They acknowledged the difficulty of determining staphylococci in a mixed environment. A critical analysis of their data suggests that the higher order of resistance reported for staphylococci that had grown in meat may be an artifact attributable to the growth of other organisms (micrococci and/or enterococci) in the inoculated and incubated meat samples. Those organisms have a higher order of heat resistance than staphylococci, and yet they would have been determined as staphylococci with the analytical techniques used, i.e., counts on phenol red mannitol agar contain-

Table 3. The destruction of various strains of staphylococci at 137°F.

Culture number	Staphylococci per ml broth			
	10-min treatment		60-min treatment	
	Before	After	Before	After
161 [a]	21,000,000	16,000 (>99%)		
264 [a]	26,000,000	11,000 (>99%)		
269	29,000,000	840 (>99%)		
K-57 [a]	22,000,000	220 (>99%)		
S-6 [a]	20,000,000	2,400 (>99%)		
NB-1	33,000,000	12,000 (>99%)		
NB-4	13,000,000	88,000 (>99%)		
2	25,000,000	4,500 (>99%)		
4	32,000,000	<1 (>99%)		
29	9,200,000	1,000 (>99%)		
52A/79	34,000,000	2 (>99%)		
3B	10,000	2 (>99%)		
3C	19,000,000	16 (>99%)		
55	11,000,000	17 (>99%)		
7	19,000,000	1 (>99%)		
42E	31,000,000	5 (>99%)		
47	16,000,000	77,000 (>99%)		
70	24,000,000	84,000 (>99%)		
42B	21,000,000	95,000 (>99%)		
42D	14,000,000	14,000 (>99%)		
81	23,000,000	116,000 (>99%)		
80	19,000,000	12,000 (>99%)		
184	18,000,000	1,800,000 (90%)	11,000,000	<1 (>99%)
254 [a]	19,000,000	1,000,000 (94.2%)	12, 000,000	<1 (>99%)
258 [a]	19,000,000	650,000 (96.6%)	15,300,000	5 (>99%)
267 [a]	25,000,000	390,000 (98.2%)	4,000,000	<1 (>99%)
273 [a]	28,000,000	1,900,000 (93.2%)	19,000,000	<1 (>99%)
276 [a]	27,000,000	1,300,000 (97.9%)	8,400,000	<1 (>99%)
NB-6	12,000,000	1,000,000 (91.7%)	21,000,000	9 (>99%)
OB-1	17,000,000	242,000 (93.0%)	12,000,000	1 (>99%)
OB-2	29,000,000	1,100,000 (96.2%)	2,100,000	18 (>99%)
OB-9	11,000,000	350,000 (96.8%)	23,000,000	450 (>99%)
OB-11	15,000,000	247,000 (93.9%)	11,000,000	<1 (>99%)
OB-23	26,000,000	570,000 (98.0%)	26,000,000	<1 (>99%)
9 [a]	120,000,000	2,200,000 (98.2%)	2,100,000	1 (>99%)
3A	17,000,000	1,000,000 (94.1%)	13,000,000	<1 (>99%)
6	11,000,000	1,100,000 (90.0%)	12,000,000	<1 (>99%)
54	14,000,000	230,000 (98.4%)	8,400,000	< (>99%)
75	15,000,000	650,000 (95.7%)	24,000,000	<1 (>99%)
VA-4	5,000,000	72,000 (98.6%)	4,100,000	<1 (>99%)
210 [a]	25,000,000	2,900,000 (88.5%)	11,000,000	6 (>99%)
247 [a]	22,000,000	4,000,000 (81.8%)	13,000,000	1 (>99%)
260 [a]	18,000,000	12,000,000 (33.0%)	5,000,000	1 (>99%)
279 [a]	19,000,000	3,300,000 (82.7%)	13,000,000	<1 (>99%)
307 [a]	17,000,000	4,700,000 (72.3%)	7,400,000	<1 (>99%)
NB-12	36,000,000	30,000,000 (16.0%)	21,000,000	<1 (>99%)
NB-13	23,000,000	4,800,000 (79.1%)	26,000,000	<1 (>99%)
OB-8	24,000,000	18,000,000 (75.0%)	4,800,000	900 (>99%)
OB-19	27,000,000	7,000,000 (74.0%)	3,900,000	<1 (>99%)
11 [a]	6,600,000	1,000,000 (84.9%)	11,000,000	<1 (>99%)
16 [a]	17,000,000	2,800,000 (83.5%)	5,600,000	1 (>99%)
21 [a]	9,000,000	840,000 (90.7%)	22,000,000	<1 (>99%)
53	7,500,000	1,170,000 (84.4%)	150,000	<1 (>99%)

[a] Known to be enterotoxin-producing *Staphylococcus*.

Source of coagulase-positive staphylococci:

1, 2, 3, 4, 9, 11, 16, 21: Swift & Company strains isolated from foods implicated in poisoning.

161, 184, 210, 307, K-57, S-6, NB-13: American Meat Institute Foundation strains isolated from foods implicated in poisoning.

247, 254, 258, 260, 264, 267, 269, 273, 276, 279, NB-1, NB-2, NB-6, NB-12, OB-1, OB-2, OB-8, OB-9, OB-11, OB-19, OB-23, OB-27: American Meat Institute Foundation strains isolated from clinical materials.

29, 52, 52A, 79, 3A, 3B, 3C, 55, 6, 7, 42E, 47, 53, 54, 70, 73, 75, 77, 42D: Strains used in *Staphylococcus* bacteriophage typing, supplied by Dr. J. E. Blair.

ing 7.5% salt. In their work, growth in heat-sterilized meat did not appear to increase the thermal resistance of staphylococci, whereas pasteurized and fresh meat samples appeared to promote a tenfold increase in resistance.

If the findings of Gross and Vinton (1947) on the heat resistance of staphylococci in meat is discounted (except for their data on sterilized cured meats), their reported resistances in a variety of systems do not appear to be inconsistent with the results of the present study. The inordinately high level of cells dealt with in thermal resistance studies, combined with the "all-or-nothing" analytical aspects of such a procedure, frequently lead to exaggerated concepts of heat resistance. Studies on the heat resistance of staphylococci in other foods suggest that these organisms are relatively heat sensitive.

Husseman and Tanner (1947) studied one strain of staphylococcus in pastry-cream fillings. Their study indicated an 83% destruction within 8 min at 130°F in product containing an initial level of approx 1×10^8 cells per ml. Destruction in excess of 99% takes place at 148°F in 4 min. Castellani *et al.* (1953), working with what they selected as heat-resistant staphylococci, showed that no strain was capable of surviving 149°F for 5 min, and that two heat-resistant strains could survive 140°F for 15 min but not 30 min. These same workers demonstrated that inoculated turkey stuffing removed at 140°F during roasting showed destruction of staphylococci in excess of 99.9%. Angelotti *et al.* (1961) conducted studies on the heat resistance of enterotoxigenic strains of *Staphylococcus aureus* in custards and chicken à la king. Their results indicated that 140°F for 40–59 min was sufficient for complete destruction of 10,000,000 organisms. Similar studies on ham salad gave lower end points of survival than were observed in either custard or chicken à la king. A computation of the exposure at 140°F necessary to effect a 90% reduction in the numbers of inoculated organisms indicated that the most heat-resistant staphylococcus was 90% destroyed in 7.68 min in custard and 5.17 min in chicken à la king.

The present results, combined with the others cited, indicate that the heat processing given to *smoked* and *fully cooked* hams is sufficient to eliminate any reasonable number of staphylococci. It becomes obvious that the most critical time for the introduction and multiplication of food-poisoning staphylococci in hams occurs after heat processing. Both retailer and consumer require thorough education on the handling of cured meat products. Because of the size of the product, the housewife may be inclined to store ham at room temperature either before or after preparation for the table. Dack (1946) states, ". . . . a food may be perfectly safe when it leaves the processor, but if it is not kept under refrigeration, staphylococcus food poisoning may occur."

Unfortunately, the average consumer regards this type of product as highly stable. This concept of stability is erroneous in the

Table 4. Destruction of *Staphylococcus aureus* in trypticase soy broth at 137°F.

	Staphylococci per ml broth				% destruction	
	Before heating		After heating			
Test series	Log av.	Antilog	Log av.	Antilog	Av.	Range
Sensitive group						
10-min exposure						
22 stains	7.166	14,700,000	2.926	843	99.994 [a]	99.497–100.000
Resistant group						
10-min exposure						
31 strains	7.257	18,100,000	6.149	1,410,000	93.60	16.7 – 98.56
Resistant group						
60-min exposure						
31 strains	6.943	8,770,000	0.300	2.00	99.999+ [b]	99.981–100.000

[a] One of twenty-two (1/22) strains showed complete destruction.
[b] Twenty of thirty-one (20/31) strains showed complete destruction.

light of the milder cures becoming more prevalent in the meat industry. Furthermore, a product that has been mishandled to the point of becoming toxic may not change in taste or appearance. The salt tolerance of staphylococci is well documented in the literature (Evans and Niven, 1950). Because of this tolerance, handling of the product can be equivalent to the inoculation of staphylococci into a selective medium. The most obvious solution for control of staphylococcus food poisoning is refrigeration at temperatures that will inhibit rapid growth of staphylococci.

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Iron and the Bacterial Spoilage of Shell Eggs

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SUMMARY

The finding that iron has a profound effect in increasing both the rate and extent of *Pseudomonas* spoilage of experimentally infected shell eggs was extended to other egg spoilage bacteria. All bacteria tested were so affected. These gram-negative bacteria included five strains of *Proteus*, three of *Paracolobactrum*, two of *Alcaligenes*, two of *Aerobacter*, two of *Achromobacter*, and two of *Salmonella*.

Iron salts have a striking effect on increasing the rate and extent of *Pseudomonas* spoilage in experimentally inoculated shell eggs (Garibaldi and Bayne, 1960). The ease of visual detection and demonstration with ultraviolet light focused attention on fluorescent rots, but other types of bacterial spoilage also have great economic significance. At least 16 different species of bacteria have been isolated from spoiled eggs (Florian and Trussell, 1957). The importance of organisms other than *Pseudomonas* in shell egg spoilage was emphasized by the many studies reviewed by Romanoff and Romanoff (1949). Garibaldi (1960) showed that conalbumin is the most important antibacterial factor controlling the growth of gram-negative egg spoilage bacteria in egg white. That this inhibition of growth of various bacteria is reversed by iron is well known (Schade and Caroline, 1944; Feeney and Nagy, 1952). The present study was made to determine if iron also affects the extent of spoilage of shell eggs experimentally inoculated with egg spoilage bacteria other than *Pseudomonas* species.

MATERIALS AND METHODS

The bacteria used were mainly those isolated from spoiled eggs (Florian and Trussell, 1957). Two strains of *Pseudomonas* used in the previous study (Garibaldi and Bayne, 1960) were included for comparison. Because of the importance of *Salmonella* in egg products, the effect of iron on spoilage by two species of this pathogen was also determined.

The eggs laid by hens 9–12 months old were nest-clean and unwashed. They were collected on day of lay and subjected to experimental contamination after 18 hr at 35°C. The eggs were inoculated as previously described (Garibaldi and Bayne, 1960). Briefly, one dozen warm (35°C) eggs were immersed for 15 min in 2 L of a cold (5°C) suspension containing approx 10^7 bacteria per ml. Actual bacterial numbers were determined by plating appropriate dilutions of this distilled water suspension. Eggs were inoculated in both the presence and absence of iron; iron was added to the bacterial suspension, as $FeSO_4$, immediately before immersion of the eggs. Changes in the iron concentration of the inoculating suspension during treatment of the eggs were determined by the 1,10-phenanthroline method (Sandell, 1959). Controls were similarly treated in sterile water with and without added iron. Eggs were air dried at room temperature, and stored 21–25 days at 20°C.

Bacterial populations of the egg meats were determined as follows: The eggs were broken under aseptic conditions and the egg meats dropped into a sterile blender containing 120 ml of sterile 0.1% tryptone solution. After homogenization of the mixture, 0.1 ml of the appropriate dilution was spread onto trypticase soy agar plates, and incubated 1 or 2 days at 28°C. Colonies were then counted. Penetration and spoilage were considered to have occurred only when counts were at least several hundred cells per ml of egg material. This method eliminated positive results from chance contamination of the egg contents by a few surviving cells in the shell-membrane system.

RESULTS AND DISCUSSION

Iron in the bacterial inoculum increased the extent of spoilage of shell eggs by all

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Table 1. The effect of soluble iron on the bacterial spoilage of experimentally inoculated eggs.

Bacterium used to inoculate eggs	Eggs spoiled (of 12)[a]	
	Without added iron	With 10 ppm iron
Proteus vulgaris (5 strains)	0	12
Paracolobactrum intermedium (b) (2 strains)	0	12
Achromobacter liquifaciens (2 strains)	0	9
Salmonella typhimurium Tm-1	0	12
Salmonella pullorum 1431	0	10
Pseudomonas fluorescens 7	6	12
Pseudomonas ovalis 1	6	12
Aerobacter cloacae 24a	4	12
Alcaligenes bookeri (a) 29d	6	11
Paracolobactrum intermedium (a) 56a	9	12
Controls	0	2

[a] Eggs were classified as spoiled when the egg meats contained more than 200 bacteria per ml.

species tested (Table 1). In many instances, no eggs spoiled when inoculation was done without added iron. When inoculation included 10 ppm iron, at least 75% of the treated eggs spoiled. Immersion of control eggs in sterile water containing 10 ppm iron allowed the natural inoculum on these nest-clean eggs to penetrate and spoil two of the twelve eggs tested. The pattern of spoilage remains the same if 100,000 bacteria per ml of egg meat is set as the spoilage criterion. In all instances of spoilage following inoculation, the organism isolated on the plates was that used to inoculate the egg.

The inoculating suspensions contained approx 10^7 bacteria per ml in the absence of iron. In its presence, however, the population dropped to 10^5 bacteria per ml. This drop in population is not surprising, since the toxicity of minute amounts of some metallic ions is well documented (Salle, 1948).

Iron in sufficient quantities reverses the bacteriostatic action of raw egg white that is due to conalbumin (Schade and Caroline, 1944). The amount of iron necessary for complete saturation of the conalbumin in the white of an average large egg is approx 0.7 mg. The change in iron concentration in the bacterial suspension showed that less than 0.06 mg of iron may enter the egg contents during inoculation. Therefore the iron effect observed in this study does not result from saturation of the conalbumin.

As reported earlier (Garibaldi and Bayne, 1960), iron may influence the spoilage of experimentally infected shell eggs in the following manner. Both the infecting bacteria and the iron are pulled through the shell to the egg membranes as the warm egg (35°C) contracts when immersed in the cold (5°C) suspension. The bacteria now find sufficient amounts of this essential nutrient to multiply readily at this site. As a consequence, the immediate environment may be altered by diffusable metabolic products so that bacterial growth in the egg white can occur. Among these metabolic products may be the iron-transport compounds (Garibaldi and Neilands, 1956) and/or acidic products, which would lower the pH of the surrounding white. The excretion of either or both of these products would allow the bacteria to satisfy their iron requirements even in the presence of the large excess of conalbumin in egg white.

Some of the organisms used in this study have been characterized previously as either primary or secondary invaders of shell eggs (Florian and Trussell, 1957). Primary invaders are able to infect the egg contents when inoculated in pure culture in the air sac, whereas secondary invaders are not. Our observations, in the absence of added iron, agree with those of the above authors with one exception; they listed *Aerobacter cloacae* 24a as a secondary invader. Note that all the bacteria listed in our Table 1 would be classified as primary invaders if they had been tested in the presence of added iron. Therefore, whether an organism is a primary or secondary invader depends greatly on the environmental conditions used in making the tests.

Comparing these results with those of a previous study (Garibaldi, 1960), we note that most bacteria able to spoil eggs in the absence of added iron, are also those able to grow in egg white whose pH has been adjusted to 7.9 or whose conalbumin has been partially saturated by iron. To grow under these conditions of iron deprivation, these

organisms must have a very efficient mechanism to satisfy their requirement for iron.

The data emphasize the need to control iron contamination of shell eggs; particular attention should be given to the elimination of iron in waters used to wash shell eggs.

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The Effect of Tylosin on Coagulase-Positive Staphylococci in Food Products

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SUMMARY

The ability of Tylosin to prevent the growth of coagulase-positive staphylococci was examined in four food substrates. Staphylococcal development was prevented during the organoleptically acceptable shelf life of ice cream mix by 20 ppm of the antibiotic. Staphylococci were controlled in processed cheddar cheese spread, ham, and domestic sausage by 2.5, 3.0, and 5 ppm Tylosin, respectively. The possible use of Tylosin as a preventive of staphylococcal food poisoning in foods is discussed.

The destruction of spore-forming bacteria by Tylosin has been reported by Denny *et al.* (1961) and by Greenberg and Silliker (1961). Both papers suggest the possibility that the antibiotic could prove useful in reducing the thermal processing requirements of certain shelf-stable canned foods.

Coagulase-positive staphylococci were found to be highly susceptible to Tylosin *in vitro* (Greenberg, 1958; McGuire *et al.*, 1961). Foods that are heat processed but subject to subsequent handling (e.g., custards and cream-filled pastries; semiperishable meats; fish, meat or egg salads; sandwich fillers) are frequently implicated in food-poisoning outbreaks. Investigation by public authorities often reveals that foods were inadvertently contaminated with enterotoxigenic staphylococci after preparation, and mishandled sufficiently to permit toxin formation (Dack, 1956; Dewberry, 1959). This paper describes the effect of Tylosin on the microflora of four foods inoculated with enterotoxigenic staphylococci and incubated at various temperatures.

MATERIALS AND METHODS

Tylosin was supplied by the manufacturer, Eli Lilly and Company, Indianapolis, Indiana. Water solutions of the antibiotic were prepared fresh, as needed, and added to meat emulsion, ham pickle, cheese sauce formulation, and ice cream mix without sterilization. When utilized as an additive in pure-culture determinations, Tylosin solutions were filtered through Seitz filters before use.

Five strains of coagulase-positive *Staphylococcus aureus* were used. Nos. 247, 254, and 258, from the American Meat Institute, had been isolated from enteritis patients. Nos. 9 and 16 were isolated from ham implicated in food-poisoning cases. Trypticase soy-broth (Baltimore Biological Laboratories) cultures were incubated 24 hr at 98°F, after which equal volumes of the cultures were pooled and added to the food substrate by appropriate means. Staphylococcus counts were made by spreading measured amounts of diluted samples on tellurite glycine agar plates (Difco) with a sterile glass rod, incubating the plates 24 hr at 98°F, and counting the resultant colonies.

Product formulation. *Ice cream mix.* Butterfat, 12%; non-fat milk solids, 11%; sucrose, 12%; corn syrup solids, 6%; Gelox stabilizer, 0.4%; and water, 58.6%. The product was divided into three portions, and Tylosin was added at 0, 5, and 20 ppm. The products were pasteurized by steam injection to 210°F. Holding time at 210°F was 8 seconds. After being cooled the products were inoculated with staphylococci, placed in sterile screw-cap jars, and incubated at 98 and 68°F. Samples were removed at various intervals and plated on tellurite glycine agar.

Processed cheddar cheese spread. Cheddar cheese, 21%; smoked cheese, 6%; rework or curd, 23%; whey powder, 6%; butter, 1%; 32% cream, 10%; spices, 2%; disodium phosphate, 2%; sodium citrate, 1%; sodium chloride, 0.5%; lactic acid, 1%; skim milk powder, 0.3%; steam condensate, 8%; and water, 18.2%. The product was heated to 175°F in an open steam-jacketed kettle and divided into three portions, and sufficient Tylosin was added to give aliquots containing 0, 2.5, and 10 ppm. After being cooled to about 100°F, the products were inoculated with

the staphylococcus mixture and spooned into sterile screw-cap jars. Samples were incubated at 98°F.

Ham. A ham pickle was prepared containing 11% sodium chloride, 2% sucrose, and 0.15% sodium nitrite. Tylosin was added to portions of the pickle so as to give final concentrations of 0, 5, and 20 ppm of the antibiotic. Hams were pumped with sufficient amounts of these curing solutions to result in 0, 0.75, and 3.0 ppm Tylosin in the tissue. The hams were processed at 148°F, sprayed with a mixture of staphylococci, and incubated at 70 and 98°F.

Domestic sausage. Tylosin in concentrations of 0, 5, and 20 ppm was added to a domestic sausage emulsion of the following composition: pork, 46%; beef, 30%; water, 19.2%; commercial curing mix, 0.2%; commercial bologna flavor, 0.4%; sodium chloride, 3.0%; and sucrose, 1.2%. The product was brought to an internal temperature of 155°F in a smoke house, sprayed with the staphylococcus culture mixture, and incubated at 70°F.

Brain heart infusion broth. Sufficient Tylosin was added to screw-cap bottles containing 100 ml brain heart infusion broth to give final concentrations of 0, 5, and 20 ppm. Each bottle was inoculated with 0.1 ml of a mixture of the 5 staphylococcus cultures grown 24 hr in brain heart infusion broth (Difco). The reaction bottles were incubated at 78°F, sampled periodically, and plated on tryptone yeast extract agar (Difco).

RESULTS

The data in Table 1 demonstrate that no growth of coagulase-positive staphylococci occurred during the shelf life of ice cream mix containing 20 ppm Tylosin. In the absence of Tylosin, the staphylococcus population increased from an initial 4,600 per g to 130 million per g during 24 hr at 98°F. Approximately 18 generations of staphylococcus growth occurred during the first day at 98°F, and about 15 generations were observed within 48 hr at 68°F. The presence of 5 ppm Tylosin delayed, but did not prevent, the development of large numbers of staphylococci during the period in which the product was acceptable organoleptically at 98°F.

The staphylococcus population of cheddar cheese sauce increased a thousandfold during the initial 24 hr of incubation. In product containing 2.5 and 10 ppm Tylosin, no growth of staphylococci was observed. Indeed, the data in Table 2 suggest that destruction of the staphylococcus inoculum occurred in the Tylosin-treated sauce during the first few days of incubation.

Hams pumped with pickle containing 0 and 5 ppm Tylosin and sprayed with staphylococci developed high surface staphylococcus populations within 3 days at 70 and 98°F (Table 3). However, hams pumped with 20 ppm Tylosin pickle (con-

Table 1. Effect of Tylosin on staphylococci in ice cream mix.

Days at 98°F	Staphylococci per g, ppm Tylosin 0	5	20
0	4,600	10,000	11,000
1	130,000,000	8,400	5,000
2		50,000	10,000
3		7,200,000	20,000
4			10,000 [a]
Days at 68°F			
0	4,600	10,000	11,000
2	20,000,000	20,000	5,500
4		30,000	2,000
7		920,000	3,400 [a]

[a] Experiment terminated, product organoleptically unacceptable.

Table 2. Effect of Tylosin on staphylococci in processed cheddar cheese spread.

Days at 98°F	Staphylococci per g, ppm Tylosin 0	2.5	10
0	2,700	4,500	1,200
1	2,600,000	<100	1,700
4	55,000,000	<100	<100
6		<100	<100
8		<100	<100
10		<100	<100
14		<100	<100
18		<100 [a]	<100 [a]

[a] Experiment terminated, product moldy.

Table 3. Effect of Tylosin on staphylococci in ham.

Days at 98°F	Staphylococci per g, ppm Tylosin 0	0.75	3.0
0	6,000	3,800	5,400
3	260,000,000	360,000,000	100
7			220,000,000 [a]
Days at 70°F			
0	2,000	4,800	5,200
3	26,000,000	26,000,000	600
7			280,000,000 [a]

[a] Product organoleptically unacceptable.

taining approximately 3 ppm in the tissue) showed a decrease in staphylococcus level after 3 days at both temperatures. Within 7 days, the coagulase-positive staphylococcus counts of both hams were in the hundreds of millions.

Table 4. Effect of Tylosin on staphylococci in domestic sausage.

Days at 70°F	Staphylococci per g		
	ppm Tylosin		
	0	5	20
0	100	<100	100
1	10,000	100	<100
2	100,000	2,000	1,000
7	600,000	2,000 [a]	1,000 [a]

[a] Experiment terminated, product organoleptically unacceptable.

Data summarized in Table 4 show that domestic sausage containing 5 and 20 ppm of Tylosin resisted the development of staphylococci during the shelf life of the product at 70°F. Control product containing no Tylosin experienced a hundredfold increase of staphylococci during the initial 24 hr of incubation. By the second day, the control product contained 100,000 staphylococci per g. At the time the product became organoleptically unacceptable (7 days) the control product contained 600,000 staphylococci per g whereas product fortified with 5 and 20 ppm Tylosin contained 2,000 and 1,000 staphylococci per g, respectively.

Table 5 lists results obtained in the experiment with brain heart infusion broth. The populations of both of the Tylosin cultures declined during the initial 4.5 hr of incubation. At this point, the control culture apparently began exponential growth. The 5 ppm culture population remained approximately the same from the 4.5-hr sampling through 72 hr, whereas the 20 ppm culture contained fewer than 10 organisms per ml at 72 hr.

Table 5. Effect of Tylosin on staphylococci in brain heart infusion broth.

Hours at 78°F	Staphylococci per ml		
	ppm Tylosin		
	0	5	20
0	760,000	1,010,000	240,000
2.5	850,000	334,000	201,000
4.5	1,240,000	185,000	109,000
5.5	2,600,000	199,000	42,550
6.5	3,125,000	144,000	41,000
7.25	5,630,000	131,000	24,500
72	280,000,000	150,000	<10

DISCUSSION

Staphylococcus food poisoning is a major public health problem. Coagulase-positive staphylococci grow best in foods in which competing microogranisms are present in low numbers or absent (cooked foods) or inhibited by some ingredient (sodium chloride). Lack of a reliable easy method for detecting staphylococcus enterotoxin has slowed research on the mechanism of toxinogenesis in foods. However, it is generally accepted that susceptible foods, inoculated with virulent staphylococci and held at 50–110°F for 4 or more hr, are potentially hazardous (Dack, 1956). As Dewberry (1959) pointed out, "The prevention and control of staphylococcal food poisoning are fraught with considerable difficulty owing to the abundance and widespread nature of various staphylococcus strains and to the fact that the infected or intoxicated food is not altered in appearance, taste or smell. Consequently, there are no physical signs to indicate whether or not the food is contaminated." Control of staphylococcal growth would be highly desirable in mishandled or accidentally inoculated product until such time that it becomes organoleptically repulsive.

Our results show that Tylosin is particularly suitable as an anti-staphylococcal agent in foods. It is essential that a material used for this purpose possess as narrow an antibacterial spectrum as possible, so that it cannot be substituted for good sanitation. Also, if put into general use as a food additive, it must not have an effect on the normal intestinal flora of man. Tylosin is ineffective at concentrations of at least 100 ppm against organisms such as *Aerobacter aerogenes, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa,* and various yeasts and molds (McGuire *et al.,* 1961).

Tylosin at 20 ppm prevented the proliferation of staphylococci in ice cream mix through the time at which the product became organoleptically unacceptable (4 days at 98°F, 7 days at 68°F). Processed cheddar cheese spread containing 2.5 ppm Tylosin and 10 ppm Tylosin developed visible mold in 18 days at 98°F. No development of staphylococci was observed during the 18-day incubation period, although the staphylo-

coccus population of control product increased from its original 2700 per g to 2,600,000 per g within 24 hr. Hams containing 3.0 ppm Tylosin resisted the development of staphylococci for at least 3 days at 70 and 98°F. Domestic sausage containing 5 and 20 ppm Tylosin became organoleptically unacceptable within 7 days at 70°F. Fewer than 4 generations of staphylococcus growth occurred during the incubation period, although a six-thousand-fold increase was observed in control product.

Previously reported work with spore-forming bacteria (Greenberg and Silliker, 1961) showed no evidence that germination was inhibited in Tylosin-fortified brain heart infusion broth. Spore germination occurred at the same rate both in the presence and in the absence of Tylosin. Once heat resistance was lost, the cells either followed a typical growth curve (Tylosin-free controls) or, in the presence of Tylosin, were destroyed exponentially. The data in Table 5 (and in portions of Tables 1 and 4) suggest that moderate levels of the antibiotic may be bacteriostatic rather than truly bactericidal for enterotoxigenic staphylococci in certain substrates.

The results suggest that Tylosin may be useful in delaying or preventing staphylococcal enterotoxin formation in a variety of food products. It is apparent from the food substrate data that the concentration of Tylosin required will differ from product to product. For example, 2.5 ppm effectively controlled the staphylococci in cheddar cheese sauce, whereas 5 ppm delayed staphylococcus development only temporarily in ice cream mix. These results can be explained, at least in part, on the basis that the rate of Tylosin deterioration in one food environment is unlike that in another. The beneficial effects of Tylosin can thus be assessed only in terms of specific systems. It would be improper to generalize as to what constitutes a safe or effective level of the antibiotic in foods on the basis of experience with a few products. Other studies, specifically designed to test the effectiveness of Tylosin as a food additive in widely differing food categories, have clearly shown that the anti-spore-former activity of the material varies greatly from one environment to another (Greenberg and Silliker, 1960).

Regardless of these limitations, the fact remains that, with economically practical levels of Tylosin, control over the growth of staphylococci has been demonstrated during the normal shelf-life of 4 perishable food products.

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The Action of Tylosin on Spore-Forming Bacteria

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SUMMARY

Tylosin at levels up to 100 ppm did not inhibit the germination of spores of *Bacillus cereus* and *Clostridium* species PA 3679 in brain heart infusion broth. Death of *B. cereus* in the presence of Tylosin was correlated chronologically with the start of exponential growth in Tylosin-free control cultures. With PA 3679, the stage most susceptible to Tylosin occurred after germination but prior to exponential growth.

The possibility of using antibiotics to extend the shelf life of perishable foods or as an adjunct to heat processing in shelf-stable products has long intrigued the food scientist. Although chlortetracycline was approved in 1955 for use in and on uncooked poultry in the United States, no antibiotic has been applied on a commercial basis to canned foods. Lack of heat stability and of specific activity against spore-forming bacteria eliminates most antibiotics from consideration as additives to heat-processed foods.

Nisin and Tylosin have recently been given attention as potential additives in canned foods. Tylosin has been observed as the more effective of the two against a variety of spore-forming bacteria (Greenberg and Silliker, 1958; Denny *et al.*, 1961b). Denny *et al.* indicated that their results with Tylosin are explicable on the basis of an acceleration in the rate of spore destruction by heat or, alternatively, an inhibition of spore germination in the heat-treated growth menstruum. Subsequently, Denny *et al.* (1961a) demonstrated that Tylosin has no effect on spore destruction rates. This report presents evidence that Tylosin has no effect against spores *per se*, but, in the manner of most antibiotics, attacks cells at that stage of their life cycle when they are capable of outgrowth.

MATERIALS AND METHODS

Tylosin, as Tylosin lactate, was supplied by the manufacturer, Eli Lilly and Company, Indianapolis, Indiana. Equivalent activity: 1.1 g Tylosin lactate = 1 g Tylosin. Water solutions of the antibiotic were prepared fresh, as needed, and sterilized by passing through Seitz filters. Aliquots of these solutions were added to previously sterilized brain heart infusion broth (Difco) to give the desired concentration of Tylosin.

Bacillus cereus, type T, and *Clostridium* species PA 3679-29 were utilized in the study. Spore stocks of *B. cereus* were prepared from 48-hr shake cultures in a modification of G medium first described by Stewart and Halvorson (1953). Spores were harvested by centrifugation and suspended in 0.0025*M* phosphate buffer (pH 7.2). The spore stocks were heat-shocked 20 min at 176°F before introduction into test media. Vegetative cell suspensions were obtained by introducing spores into brain heart infusion broth and incubating without shaking for the desired time at 98°F.

Spore stocks of PA 3679 were prepared according to the method of Zoha and Sadoff (1958), washed twice in 0.0025*M* phosphate buffer, and stored in the refrigerator. Before use they were heat-shocked 10 min at 210°F.

The reaction vessels were screw-cap bottles containing 100 or 200 ml of brain heart infusion broth. All of the work was carried out at 78±2°F. After inoculation, each bottle was sampled periodically and dilutions were made in 0.0025*M* phosphate buffer. In experiments where both spore and total viable cell concentrations were determined, a single sample was withdrawn from the reaction bottle and diluted in buffer for subsequent handling. Spores were defined as those organisms capable of surviving 20 min at 170–172°F. Pasteurization was done by placing in a 173°F water bath the dilution bottles from which the corresponding total count samples were taken. A similar bottle, fitted with a thermometer, was used in following the temperature of these suspensions during heating.

B. cereus counts were made by plating on tryptone dextrose yeast extract agar (Difco) and

incubating 24 hr at 98°F. PA 3679 was quantified by 3-tube or 5-tube most-probable-number determinations (Hoskins, 1934) in brain heart infusion broth incubated 72 hr at 98°F. Development of both turbidity and putrefaction constituted a positive result. Recovery tubes contained 25 ml of broth.

RESULTS

To ensure that plate count differences could not be attributable to carryover of inhibitory levels of Tylosin, a test was conducted in which *B. cereus* spores were plated in TY agar containing graded levels of the antibiotic. The results (Table 1) showed that the organism could be recovered quantitatively in TY agar containing 0.4 ppm Tylosin. A limit of 0.1 ppm was imposed in recovery media for *B. cereus*.

Table 1. Restrictive levels of Tylosin for *Bacillus cereus* and PA 3679 in recovery media.

ppm Tylosin	*B. cereus* per ml in TY agar[a]	ppm Tylosin	PA 3679 per ml in BHI broth[b]
0	132.0	0	38
0.1	130.3	0.01	32
0.2	133.1	0.03	32
0.4	127.0	0.05	6.1
0.8	<1	0.07	<1.8
1.2	<1	0.09	<1.8

[a] Mean of 10 replicate platings.
[b] 5-tube most probable number.

Tylosin at 0.05 ppm was found to restrict recovery of PA 3679 spores in brain heart infusion broth (Table 1). Consequently, bottles containing 100 or 200 ml of broth were used as recovery units for samples containing relatively high concentrations of Tylosin so that the antibiotic would be diluted sufficiently to permit outgrowth of viable bacteria.

Tables 2 and 3 and Fig. 1 show results with *B. cereus*. In the first of these tests, *B. cereus* spores were suspended in brain heart infusion broth containing 0, 10, and 100 ppm Tylosin. At least 99.5% of the populations of all three test systems were incapable of surviving pasteurization 1.5 hr after the start of incubation, demonstrating that germination had not been retarded by Tylosin at either 10 or 100 ppm. Cell viability in the Tylosin systems was apparently lost after germination, but the pattern was not altogether clear.

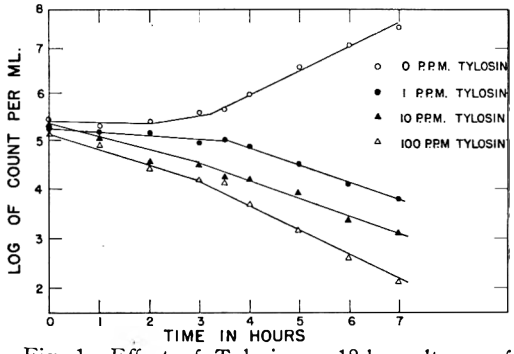

Fig. 1. Effect of Tylosin on 18-hr cultures of *B. cereus* in brain heart infusion broth.

Table 3 shows that death in the presence of Tylosin was correlated chronologically with the start of exponential growth in Tylosin-free control cultures. Forty-eight-hour vegetative cells were suspended in 0, 10, and 100 ppm Tylosin in brain heart infusion broth. The viable population of each suspension remained approximately the same until the population of the control began to increase exponentially (between the 4th and 6th hr of incubation). Approximately 5 generations of growth occurred during the 90-min period between the 4.5th and 6th hr of incubation. During the same incubation period, approximately 99% of the cells in the Tylosin-containing cultures were destroyed.

Table 2. Effect of Tylosin on spores of *Bacillus cereus* in brain heart infusion broth.

		Log number viable bacteria per ml broth					
		Hours at 78°F					
ppm Tylosin		0	1.5	3.5	5.0	7.0	24
100	Total	5.36	5.34	5.28	5.10	4.83	1.3
	Pasteurized	5.39	<3.00	2.00	<2.00		
10	Total	5.61	4.78	4.62	4.92	4.40	1.76
	Pasteurized	5.34	3.00	2.30	<2.00		
0	Total	5.38	5.15	5.60	6.01	6.89	7.30
	Pasteurized	5.00	<3.00	2.00	2.00		

Table 3. Effect of Tylosin on vegetative cells of *Bacillus cereus* in brain heart infusion broth.

	Log number viable bacteria per ml broth					
	Hours at 78°F					
ppm Tylosin	0	1	3	4.5	6	23
100	3.80	3.92	3.87	3.76	1.80	<1.3
10	3.74	3.92	3.73	3.61	1.80	<0.3
0	3.79	3.80	3.88	4.48	6.00	7.38

Fig. 1 shows the kinetics of Tylosin-induced death of *B. cereus* obtained in one experiment. Young (16-hr) cells were suspended in bottles of brain heart infusion broth containing 0, 1, 10, and 100 ppm Tylosin. Exponential growth began in the control bottle at approx 3.5 hr. The populations of the Tylosin-containing suspensions decreased during the 0–3.5-hr period at a rate dependent on Tylosin concentration. Beginning with the 3.5-hr samples, however, exponential death ensued in all 3 Tylosin cultures—and at a rate independent of Tylosin concentration.

A preliminary experiment in which PA 3679 spores were suspended in brain heart infusion broth containing 0, 10, and 100 ppm Tylosin and sampled periodically, suggested that the germination of mesophilic anaerobic spores is also independent of Tylosin. The data (Table 4) consisted of 3-tube most-probable-number determinations. The hypothesis was confirmed by an additional 5-tube MPN test (Table 5) with 5 ppm Tylosin in the test system. Again, Tylosin had no demonstrable effect on germination.

Some insight into the role of Tylosin in killing PA 3679 was obtained by incorporating 10% NaCl into brain heart infusion broth in the presence and absence of Tylosin. Outgrowth of germinated PA 3679 spores was prevented by the 10% NaCl. As can be seen in Fig. 2, approx 98% of the initial inoculum was destroyed during the first 2.5 hr of incubation in brain heart infusion broth containing 5 ppm Tylosin. During the same period the population of a similar culture containing 5 ppm Tylosin plus 10% NaCl was reduced approx 50%.

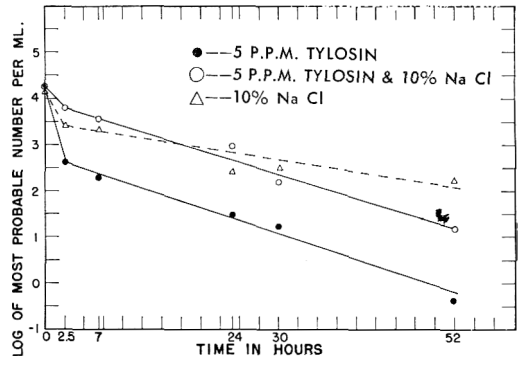

Fig. 2. Effect of Tylosin on spores of PA 3679 suspended in brain heart infusion broth. (Unpasteurized count)

The rate of death in the Tylosin and Tylosin-NaCl cultures appears to have been parallel from the 2.5-hr point throughout the experiment (52 hr). The survivor levels at 2.5 hr were considerably higher in the 10% NaCl cultures, in both the presence and absence of Tylosin, than in the salt-free Tylosin system. Thus, blocking outgrowth,

Table 4. Effect of Tylosin on spores of *Clostridium* species PA 3679 in brain heart infusion broth.

		Log most probable number cells per ml broth					
		Hours at 78°F					
ppm Tylosin		0	1.5	3.25	5	6.5	25
100	Total	3.36	3.18	0.48	0.48	0.80	0[a]
	Pasteurized	3.63	1.48	1.48	1.71	1.48	
10	Total	3.45	2.48	1.81	1.64	1.56	0[a]
	Pasteurized	3.97	1.48	2.97	1.48	2.36	
0	Total	3.18	3.36	4.36	4.18	4.36	7.18
	Pasteurized	3.36	1.96	2.36	2.36	1.96	

[a] Fewer than 3 cells per ml.

Table 5. Effect of Tylosin on spores of *Clostridium* species PA 3679 in brain heart infusion broth.

		Log most probable number cells per ml broth							
		Hours at 78°F							
ppm Tylosin	MPN	0 [a]	0.5	1.0	4.0	5.5	7.0	24	30
5	Total	4.30	3.18	2.97	1.91	1.65	2.36	1.65	1.23
	Pasteurized	4.30	3.14	1.30	1.89	2.36	2.23	<1.26	<1.26
0	Total	4.30	4.66	3.08	2.30	3.89	4.96	8.23	....
	Pasteurized	4.30	3.60	3.08	1.65	2.69	2.67	<1.26	....

[a] Initial concentration of heat-shocked spores calculated: 0.2 ml of stock spore suspension containing 20,000,000 spores per ml added to 200 ml brain heart infusion broth = 20,000/ml.

even by a system somewhat toxic in its own right, interfered with Tylosin's antibiotic activity. The data in Tables 4 and 5 and in Fig. 2 demonstrate, however, that, unlike *B. cereus,* PA 3679 cells were attacked successfully before the time when exponential outgrowth occurred in Tylosin-free media.

DISCUSSION

The usefulness of any canned-foods antibiotic must be measured by its ability to maintain a given container of product free of post-processing microbiological growth. Since bacterial spores are the surviving flora in canned foods receiving nonsterilizing thermal treatment, it is essential that the mode of anti-spore-former activity of the agent be understood clearly. Should single-event contact with a spore prevent germination, effective levels of the antibiotic need not remain in the product after processing. If the antibiotic retards spore germination, it must persist in the product at effective levels throughout the expected shelf life of the product.

Our data support the hypothesis that spore germination is not affected by Tylosin in environments where germination and outgrowth can take place. Tylosin seems to function in the manner of other antibiotics tested by various investigators as possible canned-food antibiotics. Wynne and Harrell (1951) showed that penicillin had no effect on the germination of spores of *Cl. botulinum 62A* or PA 3679. Wynne *et al.* (1952) demonstrated that streptomycin was incapable of delaying germination of *Cl. chauvei, Cl. perfringens,* or PA 3679 in Brewer's thioglycollate medium. Andersen (1952) found, in studies with *Cl. botulinum 62A,* that subtilin was very effective in a medium capable of supporting rapid germination, but was relatively ineffective under conditions where germination was delayed. Michener (1955) concluded that subtilin affects *B. subtilis* spores only in an environment suitable for germination. Campbell and Sniff (1959) demonstrated that neither subtilin nor nisin prevented spore germination or reduced heat resistance of spores of *B. coagulans.*

As with the antibiotics listed above, Tylosin destroys cells after they have undergone the metabolic events that result in loss of thermal resistance. In the case of the aerobic spore-forming organism tested in this study, Tylosin is of greatest effect at the point where exponential growth would begin in Tylosin's absence. With PA 3679 cells, the stage most susceptible to Tylosin occurs after the loss of thermal resistance but before exponential growth.

If Tylosin is to be used as a canning antibiotic, levels of the material in a container must be effective at all times when germination can take place. The use of Tylosin may make it possible to reduce materially the thermal processing requirements of canned-food products in which the antibiotic remains effective for long periods or in which spore germination occurs readily.

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Effects of Tetracycline Antibiotics on the Products of Anserinase Action in Chill Stored Haddock (*Gadus aeglefinus*) Muscle

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SUMMARY

Anserine (β-alanyl-*L*-1-methylhistidine) is cleaved hydrolytically by a haddock muscle enzyme. The inclusion of chlortetracycline and oxytetracycline in ice preserving eviscerated haddock increases the free β-alanine and *L*-1-methylhistidine recoverable from the muscle during chill storage. This results from their lower utilization by the microfloras selected by tetracyclines. Technological implications are discussed briefly.

Recent evidence (de Silva and Hughes, 1960) has indicated that proteolysis in the flesh of iced herring *(Clupea harengus)* proceeds independently of bacterial action. The pattern of amino acid accumulation in the chill stored fish is governed by rates of utilization that vary with the type of microflora that develops. Since herring are subject, in commercial practice, to the activities of the gut enzymes (Baalsrud, 1951), and since dark flesh usually possesses a higher inherent proteolytic activity (Jones, 1961), the conclusions of de Silva and Hughes are not readily applicable to considerations of the deterioration of eviscerated "white" fish.

During an investigation into biochemical changes in the flesh of iced haddock, material that had been stored in the presence of antibiotics was made available to us through the kindness of Dr. J. M. Shewan. The flesh of the species contains an enzyme, anserinase, that cleaves anserine (β-alanyl-*L*-1-methylhistidine) hydrolytically at the peptide bond. This gave us an opportunity to provide an illustration of the effects of modifications of the microflora on its interrelations with a fairly simple and well-characterized autolytic system.

The anserinase of haddock muscle closely resembles that of the related species cod *(Gadus callarias)* (Jones, 1955, 1956a). In life it forms part of a simple cycle in which the liberated β-alanine reacts enzymically with adenosine 5′-triphosphate to form β-alanyladenylate (Jones, 1960). A combination of this compound with *L*-1-methylhistidine completes the cycle to anserine. A rapid fall in adenosine 5′-triphosphate concentration after the death of the fish (Jones and Sanz Perez, unpublished data) allows the accumulation of the free amino acids in the stored fish.

EXPERIMENTAL

Materials

Haddock were caught by commercial trawler 45 miles S-E of Aberdeen in October, 1959. They were eviscerated, packed in ice and landed within a day of death. DL-1-Methylhistidine was prepared from anserine isolated from pike (*Esox lucius*) (Jones, 1955). The amino acid was separated on a Dowex 1 (OH^-) column and crystallized as the monohydrate, m.p. 251.5°C (Jones, 1959a). β-Alanine was purchased (Light and Co., Colnbrook, Bucks, England). Chlortetracycline (Acronize B1: American Cyanimid Co., New York, N.Y.) was kindly donated by the maker. Oxytetracycline (Biostat: Pfizer Ltd., Folkestone, England) was kindly donated by the maker.

These antibiotic preparations contained dispersing agent.

Methods

Chill storage. The fish were divided into 3 groups ashore. They were packed in aluminum boxes containing a) commercial ice (CI), b) ice containing $5 \times 10^{-4}\%$ (w/w) chlortetracycline (CIC), c) ice containing $5 \times 10^{-4}\%$ (w/w) oxytetracycline (CIO). The refrigerated temperature was 2.5°C, simulating that in a trawler's hold. Fish were removed periodically, and fillets were prepared for analysis.

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Analyses for L-1-methylhistidine and β-alanine. Muscle (20 g) from the anterio-dorsal portion of fillet was extracted initially into ethanol (54 ml) and then repeatedly with 75% (v/v) ethanol-water to a total 250 ml of extract. Aliquots (25 ml) from the combined extracts of 6 fish were shaken with chloroform (75 ml). Of the upper, aqueous layer that separated, containing the free amino acid fraction (Awapara, 1948), 4 ml were lyophilized and stored at −2°C to await analysis.

Water (400 μl) was added, and aliquots containing 3–12 μg of β-alanine or *L*-1-methylhistidine (as predetermined by rough experiment) were chromatographed 2-dimensionally on 8-in.-sq Whatman No. 1 paper in phenol-ammonia and collidine-lutidine (Datta *et al.*, 1950; Dent, 1948). The papers were dried in air and sprayed on both sides with 1% (w/v) ninhydrin in butan-1-ol. Color was developed at 20° for 48 hr in darkness. Spots in the positions of β-alanine and *L*-1-methylhistidine were eluted quantitatively into 50% (v/v) acetone-water and evaluated against reference standards at 570 mμ. The accuracy of the analyses was of the order of ±5%.

RESULTS

Fig. 1 indicates that, after an initial lag period during the first day of storage, concentrations of *L*-1-methylhistidine rose rapidly, with relatively little difference between the groups at 3 days' storage. Thereafter, levels were higher in CIO fish than in CIC fish and CI fish. After 7 days, concentrations in CI fish fell rapidly. A less rapid fall was found in the CIC fish after 10 days.

Fig. 2 illustrates changes in concentrations of β-alanine. After initial lags similar to those found for *L*-1-methylhistidine, the levels increased rapidly between 1 and 3 days' storage. Thereafter, levels were consistently higher in the presence of the tetracyclines than in their absence

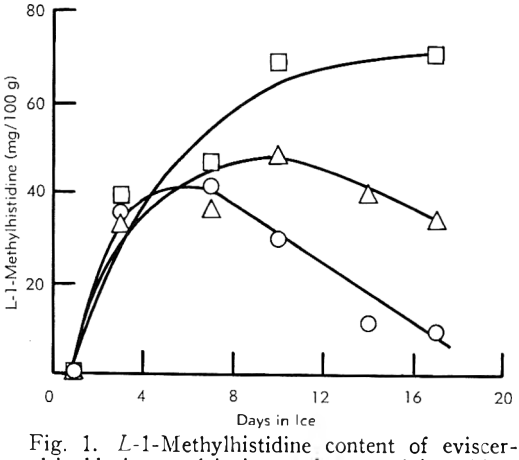

Fig. 1. *L*-1-Methylhistidine content of eviscerated haddock stored in ice ○, ice containing chlortetracycline △, and oxytetracycline □. (Points represent average values for 6 fish. Extracts of individual fish of a group were combined for amino acid analysis. Analyses were performed in triplicate, and averaged.)

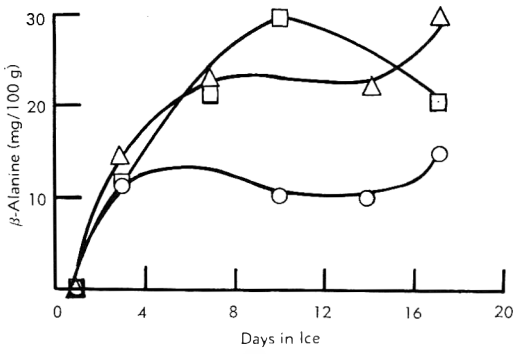

Fig. 2. β-Alanine content of eviscerated haddock stored in ice ○, ice containing chlortetracycline △, and oxytetracycline □. (Points, mean values as in Fig. 1.)

DISCUSSION

Neither β-alanine nor *L*-1-methylhistidine occur to any appreciable extent in fish muscle proteins (Connell and Howgate, 1959). The appearance of *L*-1-methylhistidine in the flesh presumably derives solely from the hydrolysis of anserine since it precedes bacterial invasion of the tissue (see below) and it has not been implicated in the intermediary imidazole metabolism of microorganisms. β-Alanine must also derive predominantly from anserine during the early stages of chill storage. Alternative direct sources of the amino acid, such as pantothenate, are present in relatively very small concentration. A small proportion (~ 4%) may possibly derive from the presence of carnosine (β-alanyl-*L*-histidine) in the tissue. Although most workers have failed to detect this compound in gadoid muscle (Yudaev, 1950; Shewan *et al.*, 1952; N. R. Jones, unpublished data), Lukton and Olcott (1958) found 4.5 mg/100 g in the closely related species cod (*Gadus callarias*) using a refined ion-exchange chromatographic technique. Haddock contains some 132–170 mg of anserine per 100 g of fresh muscle (N. R. Jones, unpublished data).

With the development of a spoilage microflora on the fish an alternative pathway to β-alanine may be postulated. Aspartate could conceivably be subject to bacterial

decarboxylation (e.g., David and Lichstein, 1950). The concentration of aspartate remained below 2 mg/100 g throughout the course of these experiments but this in itself would not preclude this substance as a precursor of β-alanine. It could be an intermediary with a very high rate of turnover in the spoiling tissue. The possible precursors of aspartate itself include active proteolysis, the amination of fumarate produced by the operation of the citric acid cycle and transamination reactions involving oxaloacetic acid produced in the cycle. Data collected in this laboratory indicate that these would not be seriously operative until towards the end of the experiment on CI fish. Therefore, de-carboxylation of aspartate was not an important consideration during most of the experiment but it could account, perhaps, for the terminal rise observable in β-alanine concentration.

It is commonly accepted that a spoilage microflora does not invade the muscle of gadoids from the epidermal layers during approximately the first 6 days of chill storage (Stewart, 1932; J. M. Shewan, personal communication). Therefore, a marked lack of stoichiometry [calculated *L*-1-methylhistidine: β-alanine, 1:1 (moles); observed 1.6:1] in the concentrations of the amino acids after 3 days probably reflects a partial removal of β-alanine along an unknown pathway of muscle catabolism. In this respect our results differ from those in earlier experiments (Jones, 1955; Shewan and Jones, 1957) on cod in which stoichiometry was good and there was no observable lag phase in the disappearance of anserine. Antibiotics did not interfere with the action of the muscle enzymes, apparently, for the 3 groups of haddock behaved similarly during the preliminary autolytic phase.

The evidence indicates, then, that we have measured in these studies the combined effects of (a) muscle anserinase, (b) the probable partial removal of one of the products of anserinase action by other catabolic systems, (c) the utilization of the products of anserinase action by the spoilage microflora, coupled, perhaps, with the effects of bacterial dipeptidases on anserine and late bacterial synthesis of β-alanine via aspartate, (d) the effects of tetracycline antibiotics on (c). The information presently available on the comparative bacteriology of fish chill-stored in the presence and absence of antibiotics, and on the biochemistry of the species selected by such treatments, does not permit the precise evaluation of these factors. But we do have evidence from earlier experiments (Shewan and Jones, 1957) that the *Pseudomonad* group of the spoilage microflora can utilize β-alanine rapidly. The higher levels of β-alanine in tetracycline-treated fish after a period of chill storage may well reflect the effects of retarding the development of such highly active spoilage species (G. Hobbs, personal communication).

No data are available on the fate of *L*-1-methylhistidine in comparable model systems. From Fig. 1 it seems likely that there is a considerable utilization of the amino acid by the spoilage microflora under conventional conditions of chill storage. It appears that the type and intensity of the microflora selected by the presence of oxytetracycline in the preservative ice results in a lower requirement for the amino acid than that selected by chlortetracycline.

These experiments provide an illustration of interaction between an essentially autolytic activity and bacterial agencies in a stored foodstuff. They also indicate that the introduction of antibiotic practices can modify the chemical composition of flesh foodstuffs in a manner that may be significant technologically. *L*-1-Methylhistidine is known to have a high capacity for both sugar-activated (Jones 1959b) and spontaneous (Jones, 1956b) non-enzymic "browning" reactions. β-Alanine is also one of the more "browning" reactive amino acids (Jones, 1959b).

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The Composition of Commercially Important Fish Taken From New England Waters. II. Proximate Analyses of Butterfish, Flounder, Pollock, and Hake, and Their Seasonal Variation

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SUMMARY

The protein, oil, ash, moisture, sodium, and potassium content of samples of butterfish, flounder, pollock, and hake was determined. In addition, length, weight, and fillet yield have been recorded. Fillet yield differed markedly both with species and with the size of the fish. Analysis showed a wide variation in the percentage of oil, not only in butterfish (considered an oily fish) but also in lean fish such as silver hake and flounder. Protein content proved to be consistently higher in pollock than in the other fish. Mineral and oil components showed a much higher percentage in offal than in corresponding fillets. Analysis of variance indicated that significant differences in results were related to the season in which the fish were caught.

INTRODUCTION

Because existing information on the composition of many species of fish in New England waters is inadequate in many respects, representative data are being collected at this laboratory. Factors under consideration in relation to composition include species and size, and season and area in which caught.

This report is concerned with quantitative differences in the components of the fillets and offal of butterfish, flounder, pollock, and hake. (Offal is the entire body of the fish, less the fillet. It includes head, bone, skin, and viscera. The offal of many species is used in animal feeds after being frozen, canned, or converted to meal.) The sodium and potassium content is aso under consideration. Thurston (1958) reported that the sodium content of the flesh of most Pacific ocean fish he has studied was less than 100 mg per 100 g. The importance of this element in certain special diets is well known, and its concentration in any major food is of significance. The sodium-potassium balance is also of interest from the standpoint of its influence on human metabolism.

EXPERIMENTAL

Collection of samples. The fish studied were selected from boats of the in-shore fishing fleet. They had been carefully iced after catching and were never more than 48 hr old when received at the laboratory. With the exception of butterfish, which were caught south of Cape Cod (off Block Island), all the fish were taken in areas north of Cape Cod (in the Gulf of Maine).

Preparation of samples. Immediately on arrival at the laboratory, the fish were individually measured for weight and length, and the fillet was removed from each side of the fish. The two fillets from each fish were weighed and blended into a homogeneous mass for analysis. The offal of each fish was weighed separately, ground in a meat chopper, and finally homogenized in a blender.

Methods of analysis. The analytical methods are described by Sohn *et al.* (1961). Sodium and potassium analyses were made on acidified solutions of the ash from aliquots of the homogenized fillets. The determinations were carried out using the flame attachment to the Beckman model DK spectrophotometer. All analyses were carried out in duplicate.

RESULTS AND DISCUSSION

Table 1 shows the physical data for butterfish, flounder, pollock, and hake. The data for pollock indicate that the fillet yield,

Table 1. Physical data for butterfish, flounder, pollock, and hake.

	No.	Origin	Length (cm)		Weight (g)		Fillet yield (%)	
			Av.	Range	Av.	Range	Av.	Range
Butterfish *(Poronotus triacanthus)*	12	Block Island	24.0	21.5–25.9	198.0	138–248	33.1	28.9–37.4
	18	Point Judith	21.4	19.0–23.0	157.0	112–176	32.2	28.5–39.2
Flounder								
Greyback *(Glyptocephalus cynoglossus)*	9	Boothbay area	40.0	38.0–42.7	408.0	318–537	26.0	22.6–29.7
Yellowtail *(Limanda ferruginea)*	7	Jeffrey's Bank	35.1	30.0–42.7	333.0	297–409	26.5	18.9–34.9
Blackback *(Pseudopleuronectes americanus)*	7	The Cultivator	33.4	28.5–36.5	459.0	292–640	31.9	27.7–40.9
Dabs *(Hippoglossoides platessoides)*	12	Jeffrey's Bank	36.2	34.0–40.0	397.0	292–570	32.3	29.5–36.0
Dabs *(Hippoglossoides platessoides)*	12	Cape Cod	36.7	31.0–41.0	407.0	259–553	31.5	25.0–35.5
Pollock *(pollachius virens)*								
Large	6	Jeffrey's Bank	64.5	62.0–71.0	2365.0	2151–2800	35.1	31.0–36.2
Small	6	Gloucester	31.3	28.6–34.3	312.5	270–487	27.5	20.3–30.8
Medium	12	Jeffrey's Bank	43.6	37.0–49.0	804.0	482–1115	33.4	26.6–47.7
Hake								
Squirrel *(Urophycis chuss)*	6	Jeffrey's Bank	107.0	102.0–110.0	2728.0	2106–3818	24.7	23.8–28.9
Silver *(Merluccius bilinearis)*	6	Cape Cod	36.0	26.0–45.0	374.0	172–574	33.1	30.0–39.5
	12	Middle Bank	36.6	31.3–41.3	389.0	206–870	32.1	24.3–39.1
	12	Narraganset Bay	32.3	29.5–35.0	218.0	171–276	35.6	33.6–40.0

Table 2. Composition data for fillets of butterfish, flounder, pollock, and hake.

Description	Season caught	No.	Protein (%)	Oil (%)	Ash (%)	Moisture (%)	Sodium (mg/100 g)	Potassium (mg/100 g)
Butterfish	Fall	12[a]	18.1±0.5	5.06±1.5	1.3±.05	76.3±1.9	96±13	411±21
	Spring	12[a]	16.7±0.5	8.92±4.1	1.2±.06	74.0±4.9	81±15	338±30
Flounder								
Greyback	Fall	9	16.2±1.2	0.05±.03	1.1±.06	82.2± .8	97±24	.....
Yellowtail	Fall	7	17.5±1.0	0.13±.07	1.3±.10	80.8± .8	79±19	.....
Blackback	Fall	7	18.8±1.5	0.15±.09	1.3±.14	79.9±1.5	.....	.....
Dabs	Fall	12[a]	17.6± .5	0.23±.01	1.2±.09	80.6± .7	83±17	333±35
Dabs	Spring	12[a]	18.1± .8	0.12±.06	1.2±.05	79.6±1.3	98±13	325±39
Pollock								
Large	Fall	6[a]	18.9± .3	0.38±.20	1.4±.06	77.4± .3	100±43	360±72
Small	Fall	6[a]	19.2± .4	0.27±.04	1.3±.16	78.0±1.1	72±16	312±31
Medium	Spring	12[a]	18.6± .5	0.33±.17	1.4±.06	77.7± .9	86±20	371±48
Hake								
Squirrel	Fall	6	16.7± .4	0.56±.16	1.2±.05	82.0± .9	92±32	.....
Silver	Fall	6	15.2± .9	0.43±.06	1.2±.10	82.4±1.5	104±11	.....
Silver	Fall	12[a]	16.9± .5	2.01±.61	1.2±.09	79.3±1.5	77±18	341±36
Silver	Spring	12[a]	16.4± .4	1.99±.80	1.1±.07	80.1±1.1	105±27	257±32

[a] These groups were used to determine the significance of seasonal variation in composition. See Table 4.

expressed as percent of the entire pollock, is greater from large and medium fish than from small ones.

The findings are quite different for the two species of hake. Silver hake (200–400 g) provide fillet yields of about 33%. Much larger squirrel hake (*ca.* 2700 g) provide fillet yields of about 25%.

The data for flounder suggest that blackbacks and dabs have a significantly higher fillet yield than do greybacks and yellowtails. If this relation holds true in further studies on fish caught in widely different areas, it will be of considerable importance to fish processors and fishermen.

Table 2 presents values for the protein (nitrogen × 6.25), oil, ash, sodium, potassium, and moisture content of the fillets. The seasons in which the fish were caught, and the number of samples subsequently used for analyses of variance are also shown.

Butterfish, which is considered to be an oily fish, shows a wide variation in oil content. The lean fish, however, such as dabs and particularly silver hake, exhibit remarkably large variations in oil content on a percentage basis. The protein content of pollock appears unaffected by size but is consistently higher than in butterfish, flounder, or hake. This higher content of protein is reflected in the moisture content of pollock fillets, which is lower than that of the other lean fish.

Except with hake and large pollock, the mean values of sodium and potassium fall below 100 mg/100 g. Pollock is of particular interest in that the sodium percentage increases with the size of the fish.

Table 3 lists the values for the proximate components of the offal. The difference in composition between fillets and offal is immediately apparent in all the species. The skeletal structure and scales are responsible for the high ash and the liver contributes greatly to the oil content. The high ash and oil contents appear to affect moisture more than protein—a fact that is of importance to manufacturers who utilize flounder and pollock offal in the preparation of fish meal. The value of this product is largely determined by its protein content. Its manufacturing cost is largely determined by the cost of water removal.

Table 4 shows the levels of significance of the variation with season of the proximate components of the fillets and offal of butterfish, dabs (flounder), pollock, and silver hake. Two series of analyses were se-

Table 3. Composition data for offal of butterfish, flounder, pollock, and hake.

Description	Season caught		Protein (%)	Oil (%)	Ash (%)	Moisture (%)
Butterfish	Fall	12[a]	15.4±0.6	8.4±3.0	3.1±0.3	71.9±2.8
	Spring	12[a]	14.7±0.4	13.0±3.5	3.1±0.4	68.7±3.6
Flounder						
Greyback	Fall	9	14.1±0.3	1.2±0.3	3.8±0.1	80.9±0.62
Yellowtail	Fall	7	16.2±0.2	0.8±0.2	5.8±0.6	75.3±2.2
Blackback	Fall	7	16.9±1.6	3.2±1.2	5.1±0.1	74.2±1.8
Dabs	Fall	12[a]	17.2±0.7	2.2±0.6	5.5±1.5	74.7±1.7
Dabs	Spring	12[a]	15.7±0.9	2.8±1.2	4.7±0.4	76.3±1.8
Pollock						
Large	Fall	6[a]	17.0±1.4	6.6±1.4	3.4±0.4	71.3±1.3
Small	Fall	6[a]	16.1±0.7	9.6±2.6	4.0±0.3	70.1±1.0
Medium	Spring	12[a]	16.5±1.1	8.1±2.5	3.7±0.5	70.7±1.7
Hake						
Squirrel	Fall	6	14.1±0.6	5.3±0.5	3.0±0.7	71.6±6.0
Silver	Fall	6	13.3±0.6	0.3±0.0	5.9±1.0	79.7±1.3
Silver	Fall	12[a]	15.5±0.7	4.7±1.7	3.6±0.6	75.4±1.6
Silver	Spring	12[a]	14.8±0.4	3.2±1.4	3.5±0.4	78.0±1.3

[a] These groups were used to determine the significance of seasonal variation in composition. See Table 4.

lected so that data from fish caught in the fall of 1960 could be compared with those from fish caught in the spring of 1961. Analysis of variance (F-test) was used to determine the relation of the variation to season.

In those cases where variation in composition is related to the season, the components showing variation usually exhibit an increase in the fall. Exceptions to this are: the oil of the fillets and offal of butterfish, protein of the fillets and moisture of the offal of dabs, oil and ash of the fillets and moisture of the offal of pollock, and moisture of the offal of hake. These components are present in higher concentration in the spring than in the fall.

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Table 4. Significance of seasonal variation in the composition of fillets and offal.

	Fillet				Offal			
	Protein	Oil	Ash	Moisture	Protein	Oil	Ash	Moisture
Butterfish	***	**	*	(−)[a]	***	***	(−)	*
Flounder	(−)	*	(−)	*	***	(−)	(−)	*
Pollock	*	*	***	***	(−)	***	(−)	***
Silver Hake	**	(−)	*	(−)	**	*	(−)	***

*** Variation related to the season at a level of significance *higher* than 1%.
** Variation related to the season at a level of significance of 1%.
* Variation related to the season at a level of significance of 5%.
[a] (−) Variation not related to season.

Radiation Sterilization of Food. II. Some Aspects of the Growth, Sporulation, and Radiation Resistance of Spores of Clostridium Botulinum, Type E [a, b]

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SUMMARY

The radiation resistance of spores of 6 strains of Type E *Clostridium botulinum* was determined in a beef stew substrate. The results are evaluated in terms of the minimum dose showing no spoilage for a 2 billion total inoculum level (20 cans each with 100 million spores per can) and in terms of a calculated radiation D value. The mean radiation D value for 6 strains is 0.132 megarad (range 0.125–0.138). A comparison with results for 6 strains of Type A and 5 strains of Type B shows that Type E strains possess about 45–55% of the resistance to ionizing radiations of Types A and B. It is concluded that any radiation dose for food sterilization based upon the maximum resistance of Type A or B strains would provide ample protection against Type E strains. The unswelled cans at the lowest radiation level showing no spoilage were free of toxin and recoverable Type E spores.

INTRODUCTION

Since Morgan and Bohrer (1953) and Morgan and Reed (1954) first presented evidence that spores of *Clostridium botulinum* possessed radiation resistance as high as or higher than spores of the usual types of nontoxic food-spoilage organisms, it has been evident that the minimum dose requirement for radiation sterilization of food must be based on the radiation resistance of *Cl. botulinum*. Data on the radiation resistance of spores of *Cl. botulinum* Types A and B in various substrates have been published by a number of workers: Kempe *et al.* (1954), Kempe (1955), Wagenaar and Dack (1956), Denny *et al.* (1959), Pratt *et al.* (1959), Ingram *et al.* (1959), Schmidt and Nank (1960). Only the spores of Types A and B *Cl. botulinum* are considered to have significant thermal resistance in relation to the thermal processing of food products. However, since there appears to be no essential relation between thermal and radiation resistance of bacterial spores (Morgan and Reed, 1954), it was deemed necessary to study the radiation resistance of spores of Types A, B, C, and E of *Cl. botulinum* under comparable conditions. In 1958 a program was initiated to study the comparative radiation resistance of spores of 6 strains each of Types A, B, C, and E *Cl. botulinum* in a single stubstrate using procedures of evaluation previously developed in this laboratory (Schmidt and Nank, 1960). The data on the resistance of Types A and B spores will be reported by Schmidt *et al.* (1961), and data on Type C spores will be reported later. This paper presents and discusses data on the radiation resistance of spores of 6 strains of Type E *Cl. botulinum*.

[a] Presented at the Twentieth Annual Meeting of the Institute of Food Technologists, May 18, 1960.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 2144 in the series of papers approved for publication. The views or conclusions are those of the author(s). They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

EXPERIMENTAL METHODS

Substrate. The substrate used was a Quartermaster Food and Container Institute specification beef stew, modified by replacing carrots with peas. The product consisted of parboiled beef (33%), blanched white potatoes (21%), blanched peas (12%), and gravy (33%). The meat was parboiled in a minimum quantity of water, and the broth from this operation used in preparing the gravy. The gravy was prepared by mixing a paste of 3 lb of wheat flour and 6 lb of water with approx 41 lb of broth. The meat was cut into approx ⅜-in. cubes, and the potatoes were cut to ¼-in. cubes to provide more uniform distribution when the product was repacked in 202 × 202 cans. The product was prepared commercially under supervision of Quartermaster Food and Container Institute personnel, packed in No. 10 cans, and frozen without heat processing.

Preparation and standardization of suspensions. The following cultures were used: strains VH, Beluga, 8E and 1304E received from the Western Laboratory of the National Canners Association, Berkeley, California, and strains Iwanai and Alaska received from Dr. C. E. Dolman, University of British Columbia, Vancouver, B. C.[c] Stock cultures of all strains were carried in PE2 medium (Folinazzo and Troy, 1954), in which sporulation was moderately good. The trypticase-peptone medium used for the preparation of spore suspensions of Types A and B *Cl. botulinum* (Schmidt and Nank, 1960; Schmidt *et al.*, 1961; Schmidt, 1961) yielded only slight growth and poor sporulation with the Type E strains. This was not unexpected, however, since the Type E strains are predominantly saccharolytic whereas the Type A and B strains are proteolytic. The addition of 0.4% glucose to the trypticase-peptone medium provided a quite satisfactory growth and sporulation medium for all strains used.

The medium (5% trypticase, 0.5% peptone adjusted to pH 7.0) was distributed in 150-ml quantities in 8-oz screw-cap bottles or in 10-ml quantities in 16 × 125-mm screw cap tubes and sterilized 15 min at 250°F. Sterile concentrated sodium thioglycollate (10%) and glucose (20%) solutions were added to the medium just prior to inoculation, giving respective final concentrations of 0.2 and 0.4%. For the preparation of spore suspensions, tubes of the trypticase-peptone-glucose medium were inoculated from PE2 stock cultures, heat-shocked 13 min at 140°F, and incubated at 85°F for 40–48 hr, at which time maximum sporulation had occurred. The tubes were heat shocked for 13 min at 140°F, and 1-ml quantities were inoculated into 8-oz screw-cap bottles containing 150 ml of the same medium. The bottles were incubated at 85°F and mixed by inversion on an 8- and 16-hr schedule as described by Schmidt (1961) for the production of Types A and B spore crops. Growth and sporulation was followed at 16, 24, 40, and 48 hr by direct counts with a Petroff-Hauser counting chamber and dark-phase microscopy. The incubation time at which the refractile spore population was at peak depended on the level of the inoculum and the peculiarities of the strain. In general, the peak refractile spore count occurred after 24–40 hr of incubation. Shortly thereafter many spores germinate and become nonrefractile. Therefore, the cultures were centrifuged at the time it was estimated that the peak refractile spore population was reached, or the cultures were refrigerated for not longer than 24 hr before centrifuging. After centrifugation for one hour at 2000 rpm in 250-ml bottles, the supernatant was siphoned off and the spore sediment resuspended in a quantity of sterile water to give the desired stock concentration and held in refrigerated storage. Usually 8 bottles were used for the production of each spore crop. At the time of centrifuging, a total count of refractile spores was made on each of 4 bottles. The mean count of the 4 bottles was used to calculate the total volume of sterile water to be added to resuspend the spores to a final concentration of approx 1×10^9 per ml.

Preparation and standardization of dilute suspensions for inoculation. Dilution of the stock suspension to contain 100 million viable spores per ml was based on previous standardization of the stock suspensions. Both a total refractile and viable spore count were made upon the dilution. With total count used as a guide to the proper dilution, viable counts were made on aliquots of a dilution using liver infusion agar plus 0.14% sodium bicarbonate in 16 × 150-mm screw-cap tubes. Since gassing was excessive at 85°F, the viable spore counts were conducted at an incubation temperature of 70°F. Usually the maximum colony count appeared after 40 hr of incubation, with excessive gassing occurring by 48 hr of incubation. Since preheating even as mildly as 13 min at 140°F markedly reduced the spore count, the inoculum was based upon an unheated spore count in order to measure the resistance of the maximum portion of the population.

Packing and inoculation. The beef stew substrate in No. 10 cans was thawed and filled into

[c] The strains received from the National Canners Association were originally sent them by Dr. C. E. Dolman. The VH, Beluga, and Alaska strains were isolated by Dr. Dolman, 8E is one of the prototype strains of Gunnison *et al.* (1936), and strains Iwanai and 1304E were sent to Dr. Dolman by Dr. Iida of the Hokkaido Institute of Public Health, Hokkaido, Japan.

202 × 202 cans to give a fill-in weight of 75 grams. Since the product contained little free liquid, 10 ml of sterile water was added. The cans were inoculated with 1 ml of suspension containing 100 million spores and vacuum-closed as described by Schmidt and Nank (1960). The closed cans were immediately chilled in cracked ice and held at 38°F or lower until shortly before irradiation.

Irradiation. The cans were irradiated at ambient temperatures in the gamma radiation facility of the Argonne National Laboratory by procedures described by Schmidt and Nank (1960). The irradiated cans were held at room temperature for not longer than 1–2 days, and returned to the laboratory for incubation at 85°F.

Incubation and observation for swells. The inoculated irradiated cans were incubated at 85°F and observed for swells every 3 or 4 days (Monday and Friday of each week) for at least 6 months. Then the lowest radiation level showing no spoilage was examined for toxin and recoverable viable spores.

Toxin assay of swelled cans. The swelled cans were chilled in the refrigerator and opened aseptically, and the contents of each can were transferred to a sterile aluminum Waring blender with a threaded screw cap. An equal volume of sterile water was added, giving a 1:2 dilution. After being blended, a 20-ml retained sample was transferred to a 20 × 150-mm screw-cap tube and stored at 38°F or below. One ml of the blend was transferred to a 16 × 125-mm screw-cap tube and mixed with 1 ml of a 2% solution of trypsin (Difco 1-250) in *M*/15 phosphate buffer at pH 6.2. Trypsin digestion was carried out for 1 hr at 98°F. For the toxin assay, either 0.5 ml of the digest or 0.5 ml of dilutions of the digest in gelatine-phosphate buffer (Duff *et al.*, 1956) was injected intraperitoneally into 15–20-g Swiss Webster mice with and without Type E antitoxin protection. The antitoxin used was a lot of Porton antiserum received from Dr. Gail Dack, University of Chicago, or Mr. M. A. Cardella, Fort Detrick, Maryland. Trypsin digestion was used to assure detection of any toxin in the prototoxin form (Duff *et al.*, 1956; Dolman, 1957; Nakamura *et al.*, 1956), and in some cases dilution was necessary to prevent overwhelming of the antitoxin.

Toxin assay on unswelled cans. After at least 6 months of incubation the 20 cans representing the lowest radiation level showing no swells were examined for detectable toxin. The contents of each can were blended as described above, and toxin was assayed after trypsin digestion.

Spore recovery on unswelled cans. The 20 cans at the lowest radiation level showing no swells were also examined for dormant Type E spores. Two procedures have been used for the spore recovery tests.

In the first procedure, 20 ml of the blended sample was transferred to each of three 1 × 8-in. screw-cap tubes, and subculturing was conducted by pouring into each tube approx 40 ml of semi-solid liver infusion agar containing 0.14% sodium bicarbonate and 0.1% sodium thioglycollate added from concentrated sterile solutions. Since only a small headspace was left in the tubes, a vaspar seal was omitted. The tubes were incubated for at least 4 weeks at 85°F for the detection of survivors. This procedure did recover survivors, but the semi-solid agar gave rise to problems in determining the toxicity of the outgrowth because of the restricted nature of the growth in this medium.

Further recovery tests were therefore conducted by subculturing the 20-ml quantities of blend in liver infusion broth with 0.14% sodium bicarbonate and 0.1% sodium thioglycollate and using a vaspar seal. All recovery cultures were tested for Type E toxin after trypsin digestion as previously described.

Calculation of results. In addition to recording the results as survival and destruction dose for the 100 million initial population per can, the partial spoilage data were evaluated in terms of a radiation D value as suggested by Schmidt and Nank (1960). Assuming an approximately logarithmic rate of destruction over the major portion of the 9–10-log cycle reduction which occurs to reduce the inoculum to the level of partial spoilage, the radiation D value (dose for 90% destruction) was calculated as follows:

$$D = \frac{\text{radiation dose (megarads)}}{\log M - \log S}$$

where M = the total inoculum, i.e., the spores per container times the number of containers; and S = the number of swelled containers, assuming one survivor per container

RESULTS AND DISCUSSION

Table 1 summarizes the average spore yields for the 6 strains of Type E *Cl. botulinum*. The degree of sporulation was somewhat higher than would be calculated from these figures since only the count of refractile spores is indicated in this table. The different cultures contained different percentages of nonrefractile or germinated spores. The data show that the procedure suggested for the preparation of spore crops of Type E strains appears to give satisfactory yields of refractile spores when reason-

Table 1. Spore yields of Type E *Cl. botulinum* cultures obtained in the preparation of suspensions.

Strain	Incubation time (hr)	Population (millions/ml)	
		Total	Spores[a]
VH	24	185	54
Beluga	72	172	88
8	24	165	129
1304	24	165	110
Iwanai	24	89	66
Alaska	24	198	61

[a] Refractile spores.

ably large quantities of suspension are desired.

Table 2 shows the refractile and viable spore counts on dilutions of some stock suspensions. Preheating, when used, was carried out in dilute aqueous suspension for 13 min at 140°F. It is apparent that only a fraction, about half, of the refractile spores appear to be viable under the conditions of the counting procedure even without preheating. However, the short incubation period (40 hr) imposed by the excessive gassing that takes place a few hours later may result in a lower count than would be obtained if a longer incubation period were possible. Preheating reduced spore viability markedly in 3 of the suspensions, and reduced it moderately in the fourth. Evidence was obtained with some of the suspensions that survival of preheating is better in liver infusion agar than in dilute aqueous suspension. However, other strains showed no difference due to the suspending medium.

Tables 3 and 4 show the complete data on spoilage and toxicity for each of the 6 strains. To verify toxicity, when 10 or more cans of 20 swelled, at least 10 cans were assayed, and when fewer than 10 cans swelled all of the swelled cans were assayed. In most instances Type E toxin was demonstrated in the swells. However, 2 cans of putrefactive nontoxic spoilage were encountered, and in 3 instances putrefactive spoilage yielding Type A toxin was found. The significance of these findings is discussed later herein. In calculations of the D values at each spoilage level for each strain, the total spoilage at that level was corrected for the presence of either Type A or nontoxic spoilage. In general, the D values calculated from 2 or more partial spoilage levels for any given strain are in very good agreement. In 5 of the 6 strains, two successive dose levels following the highest level showing spoilage show no spoilage, which demonstrates the absence of "skips." In the other strain, only one dose level showing no spoilage was obtained.

Table 2. Total and viable spore count of suspensions of Type E *Clostridium botulinum*.

Strain	Total count (millions/ml)	Viable count (millions/ml)			
		No preheat	%[a]	Preheat	%[a]
VH	137	65	47	8	6
Beluga	119	54	45	18	12
8E	226	155	68	112	50
1304E	166	66	40	18	11

[a] Percent of total spore count.

Table 5 summarizes the radiation resistance of the 6 strains in terms of the spoilage-no spoilage end point and the mean radiation D value for each strain. The range of variation in radiation resistance, whether judged by the no-spoilage end point or the D value, is surprisingly narrow. The maximum difference between the D values of strains 8E and Iwanai is about 10%, and the overall maximum variation of strains from the mean value is ±5%. The fact that the 8E and Alaska strains are indistinguishable in either end point or D value is especially interesting since, according to Dolman (1960), 8E is one of the prototype strains sent by the Russian workers to Dr. K. F. Meyer in 1935, whereas the Alaska strain was isolated by Dr. Dolman a few months before being sent to us.

Table 6 shows the comparative radiation resistance in terms of D values for the 6 Type E strains, 6 Type A strains, and 5 Type B strains. The data for the Type A and B strains are taken from Schmidt *et al.* (1961). It is evident that in respect to the range of D values, the Type E strains represent a much more homogeneous group than do either the Type A or B strains. In terms of the ratio of either the mean D values or the highest D value of each type, the Type E strains have approx 45–55% of the resistance of the Type A and B strains. The most important conclusion to be de-

Table 3. Spoilage and toxicity in 202 × 202 cans of beef stew inoculated with spores of strains VH, Beluga, and 8E of Type E *Cl. botulinum* and irradiated to various dose levels.

Strain	Dose (megarad)	Swells[a]	D value (megarad)	Toxicity of swelled cans		
				Type E	Type A	Non-toxic
VH	0.90	20/20	.....	....	....	....
	1.00	14/20	0.122	9	0	1
	1.10	10/20	0.133	10	0	0
	1.20	0/20	.....	....	....	....
	1.30	0/20	.....	....	....	....
Beluga	1.00	10/10	.....	....	....	....
	1.10	11/20	0.133	10	0	0
	1.20	2/10	0.138	2	0	0
	1.20	1/20	0.129	1	0	0
	1.30	2/20	0.144	2	0	0
	1.40	0/20	.....	....	....	....
8E	1.00	10/10	.....	....	....	....
	1.10	14/20	0.135	10	0	0
	1.20	8/20	0.142	7	1	0
	1.20	3/20	0.136	3	0	0
	1.30	0/20	.....	....	....	....
	1.40	0/20	.....	....	....	....

[a] Number swelled/number incubated.

Table 4. Spoilage and toxicity in 202 × 202 cans of beef stew inoculated with spores of strains 1340E, Iwanai, and Alaska of Type E *Cl. botulism* and irradiated to various dose levels.

Strain	Dose (megarad)	Swells[a]	D value (megarad)	Toxicity of swelled cans		
				Type E	Type A	Non-toxic
1304E	1.00	15/20	0.123	10	0	0
	1.10	10/20	0.133	10	0	0
	1.20	3/20	0.136	3	0	0
	1.30	0/20	.....	....	....	....
	1.40	0/20	.....	....	....	....
Iwanai	1.00	14/20	0.122	9	1	0
	1.10	6/20	0.128	5	1	0
	1.20	1/20	.....	0	0	1
	1.30	0/20	.....	....	....	....
	1.40	0/20	.....	....	....	....
Alaska	1.00	20/20	.....	....	....	....
	1.10	12/20	0.134	12	0	0
	1.20	5/20	0.140	5	0	0
	1.30	0/20	.....	....	....	....
	1.40	0/20	.....	....	....	....

[a] Number swelled/number incubated.

Table 5. Summary of radiation resistance of six strains of Type E *Cl. botulinum* in beef stew.

Strain	Radiation dose (megarad)		Mean radiation D value (megarad)
	Spoilage	No spoilage	
VH	1.10	1.20	0.128
Beluga	1.30	1.40	0.136
8E	1.20	1.30	0.138
1304E	1.20	1.30	0.131
Iwanai	1.10	1.20	0.125
Alaska	1.20	1.30	0.137
Mean (6 strains)			0.132

Table 6. Comparative radiation resistance of strains of *Cl. botulinum* Types A, B, and E in beef stew.

Type	Number of strains	Radiation D value (megarad)	
		Range	Mean
E	6	0.125–0.138	0.132
A	6	0.251–0.308	0.279
B	5	0.180–0.284	0.238

rived from this data is that, with the limitation of the representativeness of the number of strains tested, there is no evidence that the radiation resistance of Type E strains could exceed that of Type A and B strains. Therefore, any radiation dose for the sterilization of food based upon the maximum resistance of Types A and B strains should provide ample protection against the survival of Type E spores.

Table 7 shows the toxin assay and spore recovery on 20 cans of each strain at the lowest radiation dose level showing no swells. Toxin assays were conducted on blended samples with trypsin digestion. The dilution level tested varied in the different experiments, being gradually reduced as completely negative results were obtained. As may be seen, there was no evidence of toxin in cans at the lowest radiation level showing no spoilage. As mentioned in the procedure, 3 samples of 20 ml each of the 1:2 blend were subcultured from each can. This corresponds to the subculture of approx 33% of the contents of each can. The first three strains were subcultured into a semi-solid liver agar. Where growth occurred, it was restricted to one or more colonies that were difficult to assay for toxin or to subculture further from the agar medium. Therefore, the last 3 strains were subcultured in liver broth with a vaspar seal. With one exception, the recovery cultures were not toxic. One recovery culture from the series inoculated with the Iwanai strain produced Type A toxin. This was the same inoculum that yielded 2 swelled cans of putrefactive spoilage containing Type A toxin. Three nontoxic putrefactive clostridia were found in the subcultures; the remaining positive subcultures were either cocci or non-spore-forming rods. No culture examination was made of the agar subcultures of the first three strains, since the restricted growth was removed and digested with trypsin for toxin assay. The restricted nature of the growth in agar, however, suggests that these cultures were not clostridia but rather non-spore-forming types. When the nontoxic cultures in liver broth were digested with an equal volume of 2% trypsin, and 0.5 ml of digest was inoculated into

Table 7. Toxin assay and spore recovery from 20 cans of each strain at the lowest radiation level showing no spoilage.

Strain	Radiation dose (megarad)	Toxin assay		Number of positive subcultures[a]	Type of culture	
		Result	Dilution tested		Clostridia	Non-spore-forming
VH	1.20	NT	1–800	3	....[c]	...
Beluga	1.40	NT	1–80	8	....	...
8E	1.30	NT	1–80	4	....	...
1304E	1.30	NT	1–16	7	1	6
Iwanai	1.30	NT	1–32	10	2[b]	8
Alaska	1.30	NT	1–8	4	1	3

[a] Number of positive subcultures in 60 (3 subcultures from each of 20 cans).
[b] One toxic, Type A.
[c] Cultures not characterized.

mice, a nonspecific toxicity was encountered. Tests were conducted with trypsin-digested sterile medium, and then with the trypsin solution in pH 6.2 phosphate buffer. It was found that the injection of 0.5 ml of a 1% or 1 ml of a 0.5% trypsin solution was lethal to mice in most cases. No deaths resulted from the injection of 0.5 ml of 0.5% trypsin. Also it was found that 0.5 ml of the blended stew digested with an equal volume of 2% trypsin was not toxic. Therefore, when using trypsin to release Type E toxin, it seems necessary to determine that the conditions of trypsin digestion used in the toxin assay do not result in nonspecific toxicity.

An attempt was made to determine the source of the Type A spoilage encountered on 3 occasions in the examination of swells for Type E toxin. The Type A contamination either could be a natural contamination of the product, or could have occurred during the packing operation despite efforts to decontaminate all equipment thoroughly between experiments. Using equipment in another area of the laboratory where spores of *Cl. botulinum* had not been used, 500 cans of stew were packed and closed in the regular manner without inoculation. The 500 cans were irradiated in the usual manner with a dose of one megarad, and incubated at 85°F. A total of 112 cans swelled during incubation. All spoilage was toxin-assayed, but no toxic samples were found. Twenty-five retained samples selected as representative were subcultured into PE2 medium, and all yielded cultures of putrefactive clostridia, which were not toxic. This result appears to exclude naturally occurring contamination as the source of the Type A spoilage encountered. The demonstration of natural contamination with clostridial spores at a level sufficient to produce 20% spoilage at a dose level of one megarad would seem to explain the occurrence of one can of putrefactive nontoxic spoilage at one megarad with the VH strain and at 1.2 megarad with the Iwanai strain. These results, however, would emphasize the necessity for the verification both of the presence of toxin and its type when experimental work is conducted in one laboratory using different toxin types of *Cl. botulinum* and when the substrate may contain a normal nontoxic and potentially resistant flora.

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Radiation Resistance of Spores of Type E Clostridium As Related to Extension of the Refrigerated Storage Life of Foods [a, b]

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SUMMARY

Spores of three strains of *Clostridium botulinum* Type E were inoculated into 202 x 202 cans of beef stew and irradiated to permit the survival of a small number (50–250) of spores per container. The cans containing survivors were incubated at 43 and 49°F to determine whether germination and outgrowth of surviving spores could take place. With all three strains at least some of the survivors were capable of developing to the point of producing swell spoilage and toxin at both temperatures. The significance of these results in relation to extension of the refrigerated storage life of food by radiation "pasteurization" is discussed.

INTRODUCTION

Low doses of radiation for extending the refrigerated storage life of many food products has received considerable attention in recent years (Proctor *et al.*, 1955; Hannan, 1957; Niven, 1958; Coleby, 1959; Ingram, 1959a,b; Eukel and Huber, 1960). The preceding references are only representative since it is not our purpose to review the literature on this subject. Although many reports have appeared suggesting that the refrigerated storage life of a number of food products can be extended very considerably, different laboratories have varied considerably in the temperature range selected as representative of "refrigerated" storage. A large amount of the work has been conducted at storage temperatures between 35 and 41°F. In other cases, temperatures closer to 32°F have been used, while in some cases "refrigerated" storage has been conducted at temperatures somewhat higher than 41°F. Until recently the work in this area of investigation has proceeded with no consideration of the knowledge that spores of Type E *Clostridium botulinum* can germinate and develop over extended periods at temperatures that are within or closely border the temperature range that might be expected to prevail at times during the refrigerated storage and distribution of food products. Dolman *et al.* (1950) reported growth and toxin production by the VH strain at 43°F in herring. Ohye and Scott (1957), using a cooked meat medium, found growth initiation from spore inocula of four strains in 3–4 weeks at 41°F, with toxin detectable after 8 weeks of incubation. Six other strains showed growth, but the cultures did not kill mice though some appeared to produce transient symptoms. However, since that work was done before the recognition of the potentiation of Type E toxin by trypsin (Duff *et al.*, 1956), the complete absence of toxin in any of these cultures is uncertain.

Since spore suspensions of a number of Type E strains were available upon which radiation resistance had been determined (Schmidt *et al.*, 1961), the present experiments were carried out to determine whether a few spores permitted to survive irradiation

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[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 2145 in the series of papers approved for publication. The views or conclusions are those of the author(s), and are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

would be able to initiate growth at low incubation temperatures.

EXPERIMENTAL METHODS

Spore suspensions. The spore suspensions used as inocula were prepared and standardized by procedures to be described (Schmidt *et al.*, 1961). The inoculum levels used were 100 million or 25 thousand per can. Since the 100-million level required a dose level of 0.7–1.0 megarad for spore reduction, the 25-thousand level, requiring 0.25–0.40 megarad, was also used in order to bring the radiation treatment closer to the practical radiation pasteurization dose-level range.

Substrate and packing procedure. The substrate used was a modification of a Quartermaster Food and Container Institute specification beef stew described by Schmidt *et al.* (1961). The product was prepared commercially under Quartermaster Food and Container Institute supervision, packed in No. 10 cans, and frozen. For these experiments the product was thawed and repacked in 202 × 202 cans. A net fill-in weight of 75 g was used, together with 10 ml of sterile water since the product contained little free liquid. The cans were inoculated with 1 ml of suspension, vacuum-closed, and chilled in cracked ice according to procedures described by Schmidt and Nank (1960).

Irradiation and storage. At the gamma radiation facility of the Argonne National Laboratory, the inoculated cans were irradiated by the procedures described by Schmidt and Nank (1960). The radiation doses applied were designed to reduce the original inoculum level to 50–250 spores per can, based on the initial spore load per container and a radiation D value of 0.13–0.14 (Schmidt *et al.*, 1961) for the different suspensions. The irradiated cans were held below 38°F for 24–48 hr and then placed in incubators operating at 43±1°F or 49±1°F. The cans were observed regularly for the appearance of swells, with the longest time between observations being less than 72 hr.

Toxin assay. For toxin assay the contents of swelled cans were blended in a Waring blender with an equal volume of sterile water. One-ml samples of the blend were mixed with one ml of 2% trypsin (Difco 1-250) in *M*/15 phosphate buffer, pH 6.2, and incubated 1 hr at 98°F. After digestion, 0.5-ml portions of either the digest or further dilutions of the digest were injected intraperitoneally into white mice weighing 15-20 g. Control white mice protected with Type E antiserum were also injected to demonstrate the presence of specific Type E toxin. All mice were observed for at least 4 days and in most instances 7 days. Under the conditions used most deaths of mice occurred within 24 hr.

Experiments with heat-shocked spores. As a preliminary investigation, mildly heat-shocked spores (13 min at 140°F) were inoculated into tubes of either PE2 medium (Folinazzo and Troy, 1954) or tubes of heat-sterilized beef stew. The contents of the tubes were layered with vaspar, chilled, and incubated at either 43±1°F or 49±1°F. Outgrowth was judged by gas production. Toxin assays were conducted either on the liquid of the PE2 medium or a 1:2 blend of the stew. These experiments were conducted to provide assurance that at least some of the spores in the suspensions to be used were capable of outgrowth and toxin production at the incubation temperatures proposed in the radiation experiments.

RESULTS

The data in Table 1 show that mildly heat-shocked spores of these strains are able to initiate outgrowth at 43 and 49°F. Outgrowth at each temperature, as evidenced by gas production, appears somewhat more slowly in the beef stew medium. However, the maximum time for the appearance of outgrowth at 43°F in beef stew is not longer than 19–22 days. All samples showing growth contained Type E toxin.

Table 1. Outgrowth at low temperature from mildly heat-shocked[a] spores of *Cl. botulinum* Type E.[b]

	No. of days of incubation for visible gas production			
	PE2 medium		Beef stew medium	
Suspension	43°F	49°F	43°F	49°F
VH	16	5	19	12
Beluga	16	6	19	10
Iwanai	16	5	22	10

[a] 13 min at 140°F.
[b] Inoculum 6–12 million spores per tube.

Table 2 shows the results of experiments with the Beluga and VH strains when the inoculated beef stew was irradiated to dose levels leaving a small number of survivors and then incubated at low temperatures. The Beluga strain gave only one swelled can after 75 days at 43°F. At 49°F, however, 5 swelled cans appeared at 24 days of incubation. The results with the VH strain suggest some relation between the radiation dose level used to reduce the spore population and the ability of survivors to show outgrowth at low temperature. At 43°F, 3 cans swelled when a dose level of 0.70 megarad was used to reduce the 100-million-spore inoculum to a few survivors, whereas 16 cans swelled when a dose level of 0.25 megarad

Table 2. Development of spores of 3 strains of *Clostridium botulinum* surviving irradiation during storage at 43 and 49°F.

Strain	Inoculum level	Radiation dose (Mr)	Incubation temp. (°F)	Swells[a]	Days for swells		Toxicity test[b]	
					First	Last	Toxic	Non-toxic
Beluga	1×10^5	0.80	43	1/20	75	75	1	0
	1×10^5	0.80	49	5/20	24	24	5	0
VH	1×10^6	0.70	43	3/20	27	30	3	0
	1×10^6	0.70	49	6/20	11	67	6	0
	25×10^3	0.25	43	16/20	17	100	8	2
	25×10^3	0.25	49	19/20	10	11	10	0
Iwanai	1×10^8	1.0	43	0/20	None	120	....	....
	1×10^8	1.0	49	0/20	None	120	....	....
	25×10^3	0.40	43	8/24	28	44	6	2
	25×10^3	0.40	49	15/24	18	31	8	7
	25×10^3	0.40	43	8/20	21	30	1	7
	25×10^3	0.40	49	11/20	13	21	3	8

[a] Number swelled/number incubated.
[b] When fewer than 10 cans swelled, all cans were assayed. When more than 10 cans swelled, at least 10 cans were assayed.

was used to reduce a 25-thousand-spore inoculum. Also, the time of appearance of the first swell was reduced from 27 days to 17 days with the lower-inoculum lower-dose level. At the 49°F incubation temperature, the effect of inoculum-dose level is also apparent in the number of cans showing swells, though the time of appearance of the first swell was unchanged. However, an effect of the higher dose level may be noted in the much extended time required for the last swell to appear.

The results of toxin assay show that most, though not all, of the swells were toxic under the conditions of assay used. In some cases toxin levels of 800–8000 mld/g were found, which resulted in the overwhelming of antitoxin protection tests when low dilutions of the digested blend were tested. For this reason dilutions equivalent to 800 mld in some cases were the only levels tested for toxicity and may have been the cause of negative toxicity tests, because of the much lower level of toxin present in these samples. Also, it is possible that mixed growth occurred, resulting in toxin destruction, or that the radiation survivors produced nontoxic growth since nontoxic variants of Type E have been recorded by Dolman (1957a) and Nakamura *et al.* (1956).

Results with the Iwanai strain are also shown in Table 2. No spoilage resulted at either 43 or 49°F when a 100-million-spore load was reduced with a 1.0-megarad dose level. However, in two separate experiments when a spore inoculum level of 25 thousand was reduced with a 0.40-megarad dose level, outgrowth occurred in 21–28 days at 43°F and 13–18 days at 49°F. The results of the two separate experiments appear to be in reasonably good agreement with respect to percent swells and the time for the first and last swells to occur. The first experiment shows more toxic swells than the second, which may be due either to the dilution level selected for toxin assay or to the other causes assigned for the absence of detectable toxin with the VH strain.

These results show without doubt that a small number of spores surviving dose levels in the radiation pasteurization range are capable of germinating and producing toxic outgrowth at low temperatures within storage periods not excessively long in relation to some estimates of extended refrigerated storage life. Comparison of the results of high-inoculum high-dose level and low-inoculum low-dose level suggests that future work in this area should be based on low-inoculum low-dose experiments in order to avoid the effects of high-dose levels on the outgrowth of surviving spores at low temperature.

DISCUSSION

The ubiquity of spores of *Cl. botulinum* Types A and B is quite generally accepted (Meyer, 1956; Dolman, 1957a), and receives ample and adequate testimony each year in the number of cases of botulism arising in this country from home-processed foods. For several reasons the picture with regard to Type E spores is not quite so clear. Type E botulism has appeared pri-

marily as a clinical entity caused by non-thermally processed marine products (Nakamura *et al.*, 1956; Dolman, 1957a,b; Iida *et al.*, 1958). However, two outbreaks from food products thermally processed commercially—mushroom sauce (Geiger, 1941) and German canned sprats (Hazen, 1937)—demonstrate that unprocessed marine products are not the sole source of potential Type E botulism. Dolman and Kerr (1947) also found Type E toxin in underprocessed home-canned chicken.

The comparatively low thermal resistance of spores of Type E *Cl. botulinum* is the reason why they survive infrequently in thermally processed foods, even if grossly underprocessed, and is probably also the reason why it is so difficult to obtain any adequate estimate of their distribution in nature other than in marine products. However, Dolman (1957b,c), Nakamura *et al.* (1956), and Pederson (1955) have presented evidence that Type E spores are present in alluvial soils, terreaqueous deposits, and soil muds. Dolman and Kerr (1947) found Type E spores in the soil of a chicken run at Nainamo, B.C., where Type E botulogenic home-canned salmon caused 3 deaths and Type E toxin was found in underprocessed home-canned chicken. While some evidence suggests a special liability of marine life to serve as vehicles for the dissemination of Type E *Cl. botulinum* to widely separated parts of the world, Dolman (1957a,b) also argues strongly for the terrestrial origin of Type E spores that find a favorable habitat in the aqueous milieu. Furthermore, Meyer (1956) states: "that potentially botulogenic spores are carried in organs and tissues of healthy food animals is irrefutable, and in all probability these spores are not infrequently the source of the poison in meat or meat products responsible for human botulism when the anaerobes belong to Types A, B, and possibly E." In light of the evidence reviewed the authors would hesitate to accept the position that under any given set of circumstances Type E *Cl. botulinum* spores will not be present in any product. Any food product designed for human consumption and of such nature that it is capable of supporting the growth of *Cl. botulinum*, *must* be so processed and stored to assure public-health safety under the assumption that such contamination may be present.

The susceptibility of a food product to spoilage by Type E *Cl. botulinum* will depend on the presence of the organism, the nature of the product, the packaging conditions, the temperature of storage and handling, and the presence of other organisms that may grow more rapidly and produce obvious organoleptic defects.

The experimental data presented here show unequivocally that the radiation resistance of spores of Type E *Cl. botulinum* is of such an order that radiation dose levels applied to extend refrigerated storage life are insufficient to destroy even moderate levels of Type E spore contamination. Furthermore, it has been shown that small numbers of survivors of Type E spores can germinate and produce toxic outgrowth at 43 and 49°F. The time required is well within even not too optimistic estimates of the refrigerated storage life of some radiation-"pasteurized" food products. It must also be realized that the data presented here are based on the time required for obvious swelling of cans. The problem of toxicity prior to the appearance of a swell has not yet been investigated. This aspect of the problem may be of considerable importance since a particularly insidious type of spoilage is produced by the Type E strains. At the time when copious amounts of gas have been produced there is no marked organoleptic change in the product other than, occasionally, a mild sour odor. Gas formation, therefore, is the only major warning of a hazardous product.

It is probable that today the successful use of refrigeration in the limited prolongation of the storage life of perishable food products is primarily due to the fact that the normal indigenous flora, in great part psychrophilic in nature, produces very obvious organoleptic defects long before a public-health hazard can occur. The destruction of this flora by radiation or any other method for the purpose of extending refrigerated storage life removes an active agency in the assurance of public-health safety and suggests the necessity for the application of much more

stringent control of the temperatures of refrigerated storage and distribution of such products than has hitherto been required. Ohye and Scott (1957) found no growth initiation of ten Type E strains incubated 22 weeks at 36°F. However, it must be pointed out that these experiments consisted of duplicate tubes, each inoculated with 10 thousand spores of the strain being studied. An intensive study of this problem should be conducted in the near future to determine the absolute minimum temperature that permits outgrowth of spores of Type E strains using high-spore-level inocula. Since our results have shown a general parallelism of behavior at low temperatures by mildly heat-shocked spores and irradiation survivors, it should be possible to conduct the work first with heat-shocked spores to outline the areas in which studies with irradiation survivors must be conducted. Also, the time relationship for the development of swells and the formation of toxin at minimum growth temperatures must be determined. Such studies appear to be essential in establishing the necessary control of temperature and other variables that will be required to assure the public-health safety of foods receiving radiation "pasteurization" for the prolongation of refrigerated storage life.

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The Flavor of Cucumbers

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SUMMARY

Nona-2,6-dienal, non-2-enal, hex-2-enal, and three saturated aliphatic aldehydes were isolated from cucumbers. Quantitative and qualitative aspects of the flavor of nona-2,6-dienal and non-2-enal were examined.

The flavor of cucumbers was attributed by Takei and Ono (1939) to nona-2,6-dienol and nona-2,6-dienal. The alcohol was present in larger amounts, but they concluded that the aldehyde was the more important constituent of the flavor. The aldehyde was assigned the *2-trans,6-trans* configuration since it was identical with nona-2,6-dienal prepared from natural leaf alcohol, which Takei and Ono considered to be the *trans*-isomer of hex-3-enol. Since leaf alcohol is now known to be the *cis*-isomer (Crombie and Harper, 1950), the dienal isolated from cucumbers should be the *2-trans, 6-cis*-compound. Although it is now generally agreed (Sondheimer, 1952; Jutz, 1959) that the nona-2,6-dienal isolated from violet-leaf is the *2-trans,6-cis*-isomer, no further work on the aldehyde present in cucumbers has been reported.

During studies of oxidized flavor in dairy products (Forss, Dunstone and Stark, 1960), it was noticed that one compound, later identified as non-2-enal, had an odor reminiscent of cucumbers. The present work was undertaken to investigate the possibility that non-2-enal occurred in cucumbers and to check the identity and configuration of the nona-2,6-dienal reported by Takei and Ono. Non-2-enal, nona-*2-trans,6-trans*-dienal and nona-*2-trans,6-cis*-dienal were also examined organoleptically to compare their flavors and threshold concentrations.

MATERIALS AND METHODS

Three known varieties (Marketer, Supermarket, and Ashley) and several unknown varieties of cucumbers were used.

Samples of non-2-enal and nona-2,6-dienal were respectively obtained from Light and Co. Ltd., England, and from Haarmann and Reimer, Holzminden, Western Germany. Synthetic nona-*2-trans,6-trans*-dienal and nona-*2-trans,6-cis*-dienal were obtained from Dr. C. Jutz.

The cucumbers were diced, macerated, and distilled at 35°C under a pressure of 20 mm Hg, and the distillate examined by two different methods. In one, the distillate was passed directly into a solution of 2,4-dinitrophenylhydrazine in 50% sulphuric acid, and the 2,4-dinitrophenylhydrazones so formed were separated by column chromatography with the Celite-nitromethane system described by Day *et al.* (1960). Alternatively, the free compounds present in the distillate were extracted into light petroleum (b.p. < 40°C) and fractionated by gas-liquid chromatography using 20% silicone oil or "Carbowax 400" columns with the temperature programmed from 50 to 115°C. Fractions were collected for spectral examination both as the free compounds and after conversion to the corresponding 2,4-dinitrophenylhydrazones. In addition the 2,4-dinitrophenylhydrazones were characterized by paper chromatography (Lynn, Steele and Staple, 1956) and melting-point determination.

Infrared spectra were obtained with a Beckman IR 7.

Ultraviolet and visible spectra were obtained with a Far-ultra-violet Beckman DK 2.

Melting points were determined with a Kofler-Reichert microfusion apparatus.

For flavor evaluation, by an experienced laboratory panel, solutions were prepared from gas-liquid-chromatographically purified synthetic aldehydes, and distilled water. To determine threshold concentrations, eight graders were given a series of solutions and asked to select the one at which the flavor was just perceptible. Pairs of solutions were compared using the triangle test. In all cases, graders were asked to comment qualitatively on the flavors.

RESULTS AND DISCUSSION

Isolation and identification of aldehydes. Substantial quantities of three unsaturated aldehydes were isolated from cucumbers and identified. The data used in the characterization of the aldehydes are presented in Table 1. The identity of each was confirmed by comparison of its infrared spectrum with that of an authentic specimen. Although differences in variety and in ripeness had some effect on the quantities of aldehydes isolated, an average composition would be: ethanal 10%, propanal 10%, *n*-hexanal 15%, hex-2-enal 15%, non-2-enal 20%, and nona-2,6-dienal 30%. The yield of aldehydes was 0.0002–0.002% of the weight of fresh cucumbers.

Infrared spectra of the unsaturated aldehydes and their 2,4-dinitrophenylhydrazones respectively showed bands at 975 cm^{-1} and 980 cm^{-1}. These can be assigned to the C-H deformation of a *trans*-double bond conjugated to a carbonyl group (Bellamy, 1958). Thus all three unsaturated aldehydes had the *2-trans*-configuration. This is hardly surprising since no *2-cis*-enals have been reported in the literature, although attempts have been made to synthesize such compounds (e.g., Gamboni and Schinz, 1958). In addition the spectrum of the isolated nona-2,6-dienal showed a band at 718 cm^{-1} while that of its 2-4-dinitrophenylhydrazone showed no band at 965 cm^{-1}. This is the behavior to be expected of a compound containing a nonconjugated double bond with the *cis*-configuration (Bellamy, 1958). These facts, together with a comparison of the properties of the isolated nona-2,6-dienal with those of the two synthetic isomers, proved that the isolated nonadienal had the *2-trans,6-cis*-configuration. There was no evidence of the presence of nona-*2-trans,6-trans*-dienal among the aldehydes isolated from cucumbers.

Molecular structure and flavor. The threshold concentrations of non-2-enal, nona-*2-trans,6-trans*-dienal and nona-*2-trans,6-cis*-dienal in water were found to be 0.0005–

Table 1. Identification of aldehydes isolated from cucumbers.

		Free compound			2,4-Dinitrophenylhydrazone				
		Relative retention time in gas chromatography		λ max (mμ) (*n*-hexane)	λ max (mμ)		Relative R_f values on paper chromatogram	Melting point (°C)	Mixed melting point (°C)
		Silicone	"Carbowax" 400		EtOH	EtOH-NaOH			
Ethanal	isolated	.....	.....	.....	354	432	0.33	159[a]	
	synthetic	.....	.....	.....	355	432	0.33	Various[a]	
Propanal	isolated	.....	.....	.....	357	432	0.47	153–4[a]	
	synthetic	.....	.....	.....	356	433	0.48	155[a]	
n-Hexanal	isolated	0.13	0.19	.....	357	432	0.93	106–7	106–7
	synthetic	0.12	0.18	.....	357	432	0.93	106–7	
Hex-2-enal	isolated	0.16	0.22	214	373	456	0.81	144–5	144–6
	synthetic	0.15	0.22	214	373	456	0.81	145–6	
Non-2-enal	isolated	1.05	0.69	214	373	456	1.14	124–5	124–5
	synthetic	1.05	0.70	214	373	456	1.12	124–5	
Nona-2,6-dienal	isolated	1.01	1.10	214	373	456	0.93	114[b]	114
Nona-2-*trans*,6-*cis*-dienal	synthetic	1.02	1.10	214	373	456	0.93	113–4	
Nona-2-*trans*,6-*trans*-dienal	synthetic	1.00	1.00	214	373	456	1.00	136–7	

[a] Sublimes.
[b] A second crystalline form, m.p. 118°C, was also found.

0.001 ppm for the first two, but slightly lower (0.0001 ppm) for the *trans,cis*-isomer. These figures agree reasonably well with the value of 0.006 ppm obtained for non-2-enal by Lea and Swoboda (1958). At dilutions close to the threshold concentration, graders used the general description "oily" or "tallowy" for the flavors of non-2-enal and nona-2-*trans*,6-*trans*-dienal, but "green" or "like cucumbers" for that of the nona-2-*trans*,6-*cis*-dienal. As the concentration of the test solution increased, more graders used the description "like cucumbers" for each compound. However, at any particular concentration, it was clear that the order of increasing resemblance to cucumbers was non-2-enal, nona-2-*trans*,6-*trans*-dienal and nona-2-*trans*,6-*cis*-dienal.

In triangle tests at ten times the threshold concentration, most graders could differentiate easily between nona-2-*trans*,6-*cis*-dienal and either non-2-enal or nona-2-*trans*,6-*trans*-dienal, although not between the latter two. However, at 100 times the threshold concentration, graders were able to distinguish between non-2-enal and nona-2-*trans*,6-*trans*-dienal.

The flavor of non-2-enal is also characteristic of dec-2-enal although not of oct-2-enal. Thus, the "cucumber-like" flavor may be assigned to the molecular structure of a 2-*trans*-enal having a total chain length of 9 or 10 carbon atoms. Further unsaturation and conjugation to a 2,4-dienal produces a flavor unlike that of cucumbers—more like that of cardboard or linoleum.

Although introduction of a *trans*-nonconjugated double bond has little effect on the flavor, a nonconjugated *cis*-unsaturation increases the intensity of the flavor and adds a more pleasant, "green" note. Crombie and Shah (1955) found that the *trans,trans*- and *trans,cis*-isomers of deca-2,6-dienal had odors resembling those of the nona-2,6-dienals. We found that the odor of deca-2-*trans*,6-*cis*-dienal, regenerated by hydrolysis from its 2,4-dinitrophenylhydrazone, was typically "green," "plant-like," and "like cucumbers." Hoffmann (1961) found that the "green" reversion flavor of soybean oil was due to hex-3-*cis*-enal. Naves (1957) discussed the odors of several pairs of stereochemical isomers, and in most cases the *cis*-isomer was responsible for a pleasant, "green" note. From this, it appears that the *cis*-nonconjugated unsaturation is responsible for the production of a "green" or "plant-like" flavor.

Hex-2-enal has a strong odor of green leaves (Guenther and Althausen, 1949) whereas *n*-hexanal has a typically oily flavor. Therefore, in deciding the contribution each constituent makes to the flavor of cucumbers, it appears likely that the pleasant element is largely due to the nona-2-*trans*,6-*cis*-dienal, perhaps with some assistance from the hex-2-enal, and that the more unpleasant, astringent note is contributed by non-2-enal.

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Relation Between Olfactory Threshold Concentration and Pyruvic Acid Content of Onion Juice

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SUMMARY

A highly significant correlation (r = −0.97) exists between the amount of enzymatically developed pyruvic acid present in the juice of comminuted onion and the olfactory threshold concentration of the juice. The correlation indicates that determination of pyruvic acid in freshly prepared onion juice constitutes a fairly reliable, simple, and convenient method of estimating at least one aspect of onion flavor.

It is becoming increasingly clear from recent investigations that much of the flavor and odor of onions arises as the result of conversion of S-substituted cysteine sulfoxide derivatives (Carson and Wong, 1961; Virtanen and Matikkala, 1959a) to unstable alkyl sulfenic acid intermediates by an alliinase-type enzyme present in onions (Schwimmer *et al.*, 1960; Kupiecki and Virtanen, 1960). It has been suggested that this unstable intermediate can then be converted to the more stable sulfur-containing products reported to be present in the volatile fraction of comminuted onions (Schwimmer *et al.*, 1961). The reaction catalyzed by onion alliinase also results in the formation of ammonia and pyruvic acid as well as odoriferous sulfur volatiles (Carson and Wong, 1961; Schwimmer *et al.*, 1960; Niegisch and Stahl, 1956).

In a previous publication (Schwimmer and Weston, 1961) it was demonstrated that pyruvic acid develops in onion homogenates prepared by comminution with an equal volume of water. The amount of pyruvic acid produced from mature onion bulbs varied between 4 and 20 μmoles (0.35 to 1.16 mg) per gram of onion and appeared to depend on the pungency or strength of the onion, as evaluated informally and according to the generally accepted rating of the "lots" of commercial onion varieties used in that investigation.

A more rigorous demonstration of a highly significant correlation of enzymatically produced pyruvic acid with one aspect of onion flavor, viz., olfactory threshold concentration, constitutes the subject matter of the investigations reported herein. This correlation, together with further simplification of methods for preparation of onion juice for pyruvic acid analysis, offers a convenient, fairly reliable, and sensitive procedure for predicting the strength of onion odor.

MATERIALS AND METHODS

Preparation of extract. Twenty-one of the twenty-two lots of onion bulbs used in the present study were grown at the Greeley, Idaho, experimental acreage of the Plant Industry Station, U. S. Department of Agriculture. Table 1 shows the code number, pedigree, and source of inbred. The other lot, Number 100, consisted of Southport White Globe (strain "Sunspice") onions supplied by the Basic Vegetable Products Company, Vacaville, California.

Six to twelve onions, weighing 600–900 g, were peeled and sliced into wedges small enough to pass through the hopper (2 × 1.25 in.) of a fruit and vegetable juicer (Acme Supreme Model 5001, Acme Manufacturing Company, Sierra Madre, California). (Mention of commercial instruments and their manufacturers is for information only, and does not imply endorsement or recommendation over other instruments that may be available.) A strip of Whatman No. 1 filter paper covered the perforations in the side of the cylindrical basket. This apparatus made it possible to obtain 200 ml of clear onion juice within 3 min. After 30 min at room temperature, two 1-ml portions of this juice were removed for the determination of pyruvic acid, and a drop was used for the determination of

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Table 1. Genetic background of onions.

Greeley lot no.	Pedigree	Source of inbred [a]
569	B 4038 A Charteuse	(EYG × YGD)
607	B 9166 A	Ebenezer
609	B 12132 A	YSS
613	Colo 6 A	YSS
621	St G 36 A	Stockton Globe
627	B 1288 A	SWG
631	Red Globe A	SRG
787	p 52-474 A	YSS
819	RBW 101-A 3	Bronze Globe
854	B 2247 C	BYG
858	B 3954 C	(EYG × YGD)
867	B 12115-2 C	YSS
873	p 53-364-2 C	YSS
877	p 53-416 C	YSS
880	p 74-9-32 C	YSS
918	B 2215 C	BYG
1132	B 7-165 B (B 1288 B × L 303 B) F_3 iiccrr	(SWG × Eclipse)
1136	B 7-165 B (B 1288 B × L 303 B) F_3 iiccrr	(SWG × Eclipse)
1137	B 7-165 B (B 1288 B × L 303 B) F_3 iiccrr	(SWG × Eclipse)
1138	B 7-165 B (B 1288 B × L 303 B) F_3 iiccrr	(SWG × Eclipse)
1563	(Wh Creole × p 54-306) A	White Creole × YSS

[a] EYG, Early Yellow Globe; BYG, Brigham Yellow Globe; YSS, Yellow Sweet Spanish; WP, White Portugal; SWG, Southport White Globe; SRG, Southport Red Globe; YGD, Yellow Globe Danvers.

soluble solids (° Brix) with a Bausch and Lomb refractometer. Ten-ml portions of the juice were distributed among several vials and placed at −30°F until used for redetermination of pyruvic acid and for organoleptic evaluations.

Determination of pyruvic acid. For analysis of pyruvic acid content, 1-ml samples were diluted to 200 ml. A 2-ml sample of the diluted juice was added to 1 ml of 0.0125% of 3,4-dinitrophenylhydrazine in 2*N* HCl. After 10 min at 37°C, 5 ml of 0.6*N* NaOH was added and the absorbance of the solution measured with an Evelyn Colorimeter (420 mμ filter, set at zero absorbance with reagent blank). The method was calibrated with sodium pyruvate as standard, and the results are expressed as μmoles of pyruvic acid per ml of juice (Schwimmer and Weston, 1961).

Control onion samples were treated as above except that the onions were first heated 5 min in an electronic oven (Radarange, Raytheon Corp.) to destroy enzyme activity by radio frequency energy ($\lambda = 12$ cm), then extracted as previously described. There was no significant change in the pyruvic acid content after storage of the extract for 5 months at −30°F. The variation from the mean value of 4 replicates was ± 0.2 μoles per ml juice.

The difference between the pyruvic acid contents of the juice from unheated (P_T) and heated onion (P_C) is defined as μmoles of enzymatically produced pyruvic acid (P_E) per ml of juice.

Organoleptic evaluation. Because of the overpowering odor of undiluted onion juice, the best method of assessing the relative odor intensity of different samples is by determining the threshold concentration. Each sample of frozen juice was thawed rapidly and diluted with odor-free tap water to the approximate threshold range (1–30 ppm of onion juice in water). At least three concentrations of each onion juice sample were directly compared for odor with the water used in preparing the diluted samples. Ten-ml samples of water or diluted onion juice were placed in dark 2-oz glasses fitted with plate-glass covers. The

Table 2. Organoleptic and analytical data on onion juice.[a]

Code no.	ppm	P_E	P_T	P_C	° Brix
607	3	18.6	22.3	3.7	11.5
1137	3	18.2	21.4	3.2	12.3
627	5	17.5	21.4	3.9	11.8
100	5	16.1	19.6	3.5	12.9
918	5	15.7	20.2	4.5	7.0
1563	6	15.2	19.4	4.2	10.5
1132	7	13.2	16.3	3.1	10.7
1136	8	13.8	16.9	3.1	11.9
1138	9	13.0	16.8	3.8	12.6
854	10	13.0	17.4	4.4	11.0
569	10	13.3	18.6	5.3	6.9
858	10	13.5	18.1	4.6	10.1
819	10	13.6	18.3	4.7	12.6
877	12	12.0	16.5	4.5	7.7
613	12	11.8	15.2	3.4	6.3
631	13	8.9	12.2	3.3	9.1
621	14	8.2	12.6	4.4	7.0
787	15	10.0	12.8	2.8	10.3
880	16	8.1	12.1	4.0	8.1
867	16	9.1	12.5	3.4	8.7
609	22	5.5	10.8	5.3	7.0
873	23	5.3	9.9	4.6	8.2

[a] ppm, sensory threshold concentration; P_E, enzymatically produced pyruvic acid in μmoles/ml; P_T, total pyruvic acid; P_C, pyruvic acid of heated controls.

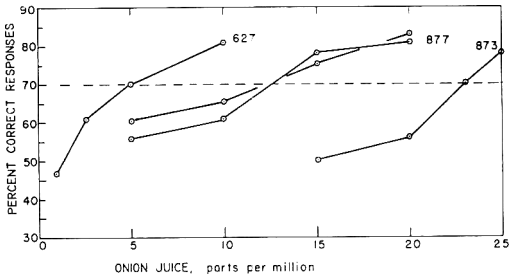

Fig. 1. Percent correct response vs. sensory threshold concentration (ppm) of diluted onion juice from representative samples of strong (627), medium (877), and weak (873) onions. The numbers refer to the code numbers of Table 1. Duplicate tests for sample 877 indicate reproducibility of method. Intersection of dotted line with each curve indicates threshold concentration for that sample.

samples were evaluated by a trained and selected panel of 12 judges, in the form of a duo-trio test (Peryam and Swartz, 1950). Evaluations were conducted in individual booths maintained at 70°F, and the order of presentation to the judges was randomized. Each concentration of onion juice was replicated three times, giving a total of 36 judgments.

The percentage of correct response was plotted against onion juice concentration in ppm. Typical examples of these plots are shown in Fig. 1. The threshold concentration is defined as the minimum concentration (ppm) of onion juice in water that can be detected by 70% of the judges. Examination of the results from all onion samples indicated that this degree of occurrence gave the most reproducible results. For the number of replications and panel size used, 70% correct responses corresponds to a probability level of about .01 according to Tables of the Binomial Probability Distribution (National Bureau of Standards, 1950). Repeated tests on a given onion sample showed that threshold concentration could be estimated within ± 1.5 ppm for the juice from either strong or weak onions (see Fig. 1).

RESULTS AND DISCUSSION

Table 2 shows the onion lots in order of increasing olfactory threshold concentration, with the corresponding amounts of enzymatically produced pyruvic acid, total pyruvic acid, pyruvic acid content of heated controls, and solids content. In general, the results substantiate previous findings that the amount of enzymatically produced pyruvic acid of comminuted onions varies between 5 and 20 μmoles per g (or ml). Also, note that strains considered mild, e.g., Yellow Sweet Spanish, produce less pyruvic acid than those generally considered strong, e.g., Ebenezer, Southport White Globe (Magruder *et al.*, 1941). The threshold concentration of the various juices ranged from 3 to 23 ppm.

Fig. 2 shows the relation between ppm of onion juice in water and P_E in μmoles/ml for all 22 samples. The correlation coefficient ($r = -0.97$) and computed standard error of estimate ($s = 1.38$) indicate a highly significant correlation between threshold concentration and pyruvic acid production. Total pyruvic acid (P_T) plotted against ppm also gives a highly significant correlation ($r = -0.94$, $s = 1.96$). Since the correlation coefficients are very similar and it is more convenient to perform P_T instead of P_E, it would probably be advantageous to use P_T as a measure of onion odor.

The results also indicate some relation between solids and odor, but the correlation coefficient ($r = 0.57$) is much lower than that obtained for P_E or P_T vs ppm. Platenius and Knott (1941) reported that total solids content reflects pungency to some extent.

The results tend to support the theory that the enzyme or enzyme system that produces volatile odor components when the cells of

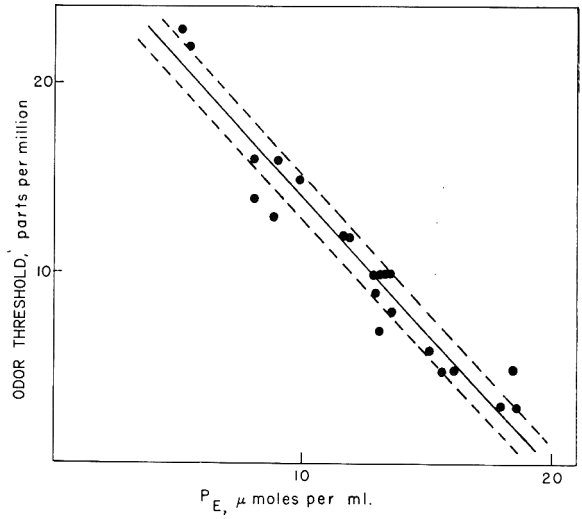

Fig. 2. Relationship of sensory threshold concentrations of diluted onion juice (ppm) and enzymatically produced pyruvic acid (P_E). Dotted lines indicate computed standard error of estimate, $S = 1.38$.

onion tissue are disrupted also gives rise to pyruvic acid (Schwimmer *et al.*, 1961). However, the amount of pyruvic acid produced is in excess of the preliminary estimates of the amount of the S-methylcysteine and S-propyl cysteine sulfoxides, the only two known substrates of onion alliinase so far detected in onion. This discrepancy can be eliminated if one assumes that a precursor to cycloalliin (Virtanen and Matikkala, 1959b) and the glutamyl peptides present in onion (Virtanen and Matikkala, 1960) can give rise by some yet unknown mechanism to both odoriferous volatile products and pyruvic acid.

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Quantitative Aspects of the Interaction of Carrageenan with Cationic Substances
II. Precipitation with Long-Chain Quaternary Ammonium Detergents

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SUMMARY

Using the interaction of carrageenan (Seakem type 5) with cetylpyridinium chloride as the type example, investigation was made of optimum conditions for the precipitation of carrageenan and its fractions by quaternary ammonium detergents. Found to be very critical factors for quantitative precipitation were the temperature and pH of the reaction medium. Comparatively, the amount of detergent cation bound by a particular sulfated hydrocolloid is indicative of its degree of sulfation.

Carrageenan is a strongly charged anionic polyelectrolyte that is extensively used in the food and pharmaceutical industries as an emulsifier and stabilizer. Its isolation and quantitative determination, especially in the presence of other food gums, is important from both the practical and theoretical viewpoints. Several qualitative tests for carrageenan are available. These are based largely on the reaction of the polyanionic electrolyte with methylene blue, a reaction that has been placed on a quantitative basis (Graham, 1960), and on reaction with Millon's reagent, ferric chloride, or thorium nitrate (Smith and Montgomery, 1959). Hansen and Whitney (1960) developed an ester sulfate method for the determination of carrageenan in milk, but rapid micro-methods for the quantitative separation and estimation of carrageenan are still sorely lacking.

Quaternary ammonium detergents have been used for the precipitation and purification of acidic polysaccharides, and, in conjunction with salt solutions of high molarity have been employed for the separation of certain neutral polysaccharides (Smith and Montgomery, 1959). However, they have not been extensively applied to the sulfated plant hydrocolloids. Slack (1958), while investigating the stimulation of connective tissue by carrageenan, observed that through the use of 1.2*M* KCl and an excess of cetylpyridinium chloride, this anionic polyelectrolyte could be selectively precipitated from mixtures containing other less sulfated mucopolysaccharides. Springer *et al.* (1957) noted that fucoidan, a polymer of L-fucose monosulfate, was precipitated and inactivated by cetyldimethylbenzylammonium chloride. The stringy or flocculent precipitate formed by Irish Moss (carrageenan) on reaction with alkyldimethylbenzylammonium chloride (Rodalon) is used as a test for detection and identification of the hydrocolloid (Smith and Montgomery, 1959). The interaction of sulfated plant polysaccharides in general, and of carrageenan in particular, thus becomes a matter of interest and importance.

Recently, the precipitation of sulfated mucopolysaccharides by cationic detergents has received much attention. Korn (1959a) described the quantitative isolation of heparin, a sulfated mucopolysaccharide, as the cetyltrimethylammonium complex, precipitated from 1*M* NaCl, and has used this procedure in studies on the synthesis of heparin by slices of mouse mast cell tumor. Cifonelli and Dorfman (1960) used cetylpyridinium chloride to precipitate heparin monosulfate in order to study its properties. Schiller *et al.* (1961) separated microquantities of hyaluronic acid, chondroitin sulfuric acids,

and heparin through the differential solubility of their cetylpyridinium chloride complexes. Urinary mucopolysaccharides have been purified and characterized through complexing with cetyltrimethylammonium bromide (Di Ferrante and Rich, 1956).

Much interest has been exhibited in the role of carrageenan in the stimulation of the synthesis of connective tissue (Slack, 1958; Williams, 1957). In addition, the mechanism of its interaction with proteins and other biological entities has not yet been fully explained. In view of the differential solubilities in simple salt solutions of detergent-polysaccharide complexes, it was deemed worthwhile to make a comprehensive study of the quantitative nature of the precipitation of carrageenan by quaternary ammonium detergents. If conditions for the quantitative precipitation of this hydrocolloid are known, then its isolation, purification, and assay from heterogeneous mixtures can be readily accomplished. Moreover, since there is a possibility that each detergent complex may display specific salt-solubility characteristics, information on the quantitative nature of the interaction of carrageenan with cationic detergents could greatly facilitate studies on the mechanism of reactions of this versatile phytocolloid with other compounds of biological interest.

Carrageenan can be precipitated in the presence of an excess of quaternary ammonium detergents for which rapid, simple colorimetric methods of determination are available. By determining the excess quaternary ammonium detergent in the supernatant, quantitative relationships for the interaction can be established. Alternately, cetylpyridinium chloride absorbs strongly in the ultraviolet region. Therefore, direct determination of the excess quaternary ammonium detergent in the supernatant can be made.

This study was undertaken to determine the effect of pH and temperature on the binding of detergent cations, the time required for maximum binding, the influence of buffer normality, and the amount of detergent that must be present for complete precipitation of a given quantity of hydrocolloid. Since several other sulfated hydrocolloids are gaining importance in the food industries, some of these were included in the studies.

EXPERIMENTAL

Reagents. Carrageenan (Seakem types 3, 4, 5, 7, and 402), lambda and kappa fractions of carrageenan, Gelcarin, and Viscarin, all from Marine Colloids Inc., New York; agar, from Meer Corporation, New York; furcellaran, from Duché Uni-Gum Corporation, New York; iridophycan, from Dr. W. Z. Hassid, University of California; hypnean, seaweed from Dr. Harold Humm, Duke University. Stock solutions of 0.25–1% (dry-weight basis) were prepared as previously described (Graham, 1960).

Cetylpyridinium chloride, from Wm. S. Merrell Company, Cincinnati, Ohio; cetyldimethylbenzylammonium bromide, from Fine Organics, Inc., New York; 0.1*M* H_3PO_4; 0.1*M* $(NH_4)_2HPO_4$; 0.1*M* NH_4OH. These were prepared as previously described (Graham, 1960).

Isopropyl alcohol, Fisher Certified Reagent, Fisher Scientific Company; Sodium Carbonate, Fisher Scientific Company; double-distilled water, twice distilled from glass; bromphenol blue, Fisher Scientific Company; toluene, Fisher Certified Reagent, Fisher Scientific Company; ethylene dichloride, purified, Fisher Scientific Company; dialysis membrane, *27/32*, Visking Company, Chicago, Illinois.

Equipment. Volumetric pipettes, Coleman Model DU spectrophotometer, 40-ml ground-glass-stoppered test tubes, 15-ml centrifuge tubes, Beckman pH meter, Model G; Burrell wrist-action shaker.

Precipitation studies. Zero to 100 mg of the particular hydrocolloid were added to a 100-ml glass-stoppered volumetric flask. Five ml of 0.1*M* $(NH_4)_2HPO_4$–NH_4OH buffer of pH 9.0 was added to each flask, and the volume was made up to 40 ml with distilled water. Finally, 10 ml of the detergent solution was added to give a final detergent concentration of 20μM/ml. The flasks were placed on a Burrell wrist-action shaker and incubated 12 hr at 27°C. Then the volume was made up to 100 ml with distilled water and the flasks shaken well. After standing for a short while, 1 ml of the supernatant was removed and added to a glass-stoppered centrifuge tube containing 9 ml of isopropyl alcohol. The mixture was centrifuged 10 min at 2500 rpm, and 1 ml of the supernatant was added to 9 ml of distilled water. Suitable aliquots of this last mixture were used to determine the amount of free quaternary ammonium detergent in the mixture. The amount of the quaternary ammonium detergent bound by the particular hydrocolloid was calculated as the

amount added minus the amount left in the system. In every case, duplicate controls containing all the ingredients except the hydrocolloids were included to account for any adsorption by the glass or losses occurring during manipulation.

Equilibrium dialysis. These experiments were carried out by placing into the dialysis sacks 20 ml containing amounts of the hydrocolloid ranging from 10 to 100 mg. The sacks were fashioned in the shape of U-tubes, both ends tied, and placed in 125-ml Erlenmeyer flasks. The ends of the sacks were tied to the outside of the neck of the flask by rubber bands. Five ml of the buffer solution and 10 ml of the particular detergent solution were added to the outside of the flask, and the final volume made up to 50 ml with distilled water. The flasks were stoppered with hard rubber stoppers and incubated, with shaking, for 72 hr at 27°C. Controls in duplicate were run by dialyzing the hydrocolloids against buffer only, and by including a system containing the dialysis sacks, buffer, and detergent only. Adsorption of the detergent by the dialysis sack was generally absent or negligible. At the end of the equilibration period, the amount of free detergent was determined both inside and outside of the dialysis sack.

Calculation of the amount of quaternary ammonium detergent bound at equilibrium was based on the difference between the amount added and the total amount found "free" in the solution on the outside of the sack and that on the inside. Both outside and inside solutions were made up to a volume of 100 ml with distilled water before final analyses were done. Experiments conducted by immersing the sacks completely in the detergent solution gave results that, within the range of experimental error, were the identical to those obtained by fashioning the sacks as U-tubes.

Methods used for determination of quaternary ammonium detergents. *Colorimetric.* The method used was the colorimetric method of Auerbach (1943), using a 9:1 mixture of toluene and ethylene dichloride. The intensity of the color developed was measured with a Beckman model DU spectrophotometer.

Direct spectrophotometry. Since cetylpyridinium chloride absorbs strongly in the ultraviolet region, direct determinations on gum-free supernatants were done in many instances. Thus it was possible to check the results based on the colorimetric method. In all instances agreement was good between the precipitation approach and the equilibrium dialysis approach (Table 2).

Influence of variables on the interaction. The influence of several factors on the completion of precipitation was studied at 27°C with 50 mg carrageenan (Seakem type 5). Variation of pH, interaction time, buffer molarity, or the amount of detergent present provided data for the establishment of optimum conditions.

Table 1. Influence of variables on the binding of cetylpyridinium by carrageenan (Seakem type 5) at 27°C.

pH of medium	Cetylpyridinium cation bound per mg of carrageenan (μM)
1.2	2.89
3.0	3.84
5.2	3.98
6.9	4.00
9.0	4.09
10.1	4.05
Buffer strength (M)	
0.01	4.08
0.05	4.07
0.10	4.08
0.20	3.99
0.50	4.00
1.00	3.89
Reaction time (hr)	
0.5	3.68
1.0	3.98
2.0	4.07
4.0	4.01
8.0	4.08
16.0	4.05
24.0	4.01
48.0	4.06
Detergent concentration (μM/ml)	
1.0	1.64
2.0	2.89
5.0	3.42
10.0	4.07
15.0	4.08
20.0	4.09

In the dialysis experiments, equilibrium time at 27°C was determined by dialyzing 50 mg of carrageenan (Seakem type 5) against a constant concentration of cetylpyridinium chloride in 0.01M buffer of pH 9.0 for periods of 4–96 hours. The

Table 2. Comparison of binding of cetylpyridinium cation by carrageenan at 27°C by the dye method and by the ultraviolet absorption method.

Carrageenan added (mg)	Cetylpyridinium cation bound (μM)/mg of carrageenan	
	Dye method	UV method
10	4.06	4.02
20	3.98	4.06
40	4.09	4.01
50	4.07	4.01

amount of cetylpyridinium chloride necessary to precipitate a given amount of the hydrocolloid completely was ascertained by dialyzing it for 72 hr against varying concentrations of the detergent.

The influence of pH was assessed by allowing 50 mg (dry-weight basis) of the hydrocolloid and an excess of cetylpyridinium chloride to react at 27°C at varying pH levels of 0.01*M* NH_4HPO_4–NH_4OH buffer. The amount of detergent cation-bound was determined by the general procedure. The final pH of each system was checked with a Beckman model G pH meter.

The concentration of detergent that must be present in order to allow maximum uptake of the detergent cation per milligram of the hydrocolloid was assessed at pH 9.0 in 0.01*M* buffer at 27°C by a procedure similar to that outlined above except that the amount of detergent added was varied as shown in Table 1

For buffering, Na_2HPO_4–NaH_2PO_4 or NH_4HPO_4–NH_4OH mixtures may be used. The ammonium phosphate buffer was selected since it offered less interference when alcohol was used in preparation of the supernatant. However, the ammonium ion can precipitate carrageenan (Stoloff, 1954). To assess any influence of such possible precipitation on the binding of the detergent cation, the influence of varying molarities of the $(NH_4)_2HPO_4$–NH_4OH buffer at pH 9.0 was determined. For this, 50 mg (dry-weight basis) of carrageenan (Seakem type 5), and an excess of cetylpyridinium chloride were allowed to react with buffers of varying molarities at 27°C, and the amount of cetylpyridinium cation bound determined according to the general procedure. The results are in Table 1.

The time necessary for maximum binding of the cetylpyridinium cation by carrageenan (Seakem type 5) at 27°C in 0.10*M* $(NH_4)_2HPO_4$–NH_4OH buffer was established in a similar manner and determining the amount of detergent cation bound over periods varying from 30 min to 72 hr.

Variations in the binding of the detergent cation by carrageenan (Seakem type 5) with temperature were assessed at 27, 37, and 45°C with a 0.01*M* buffer at pH 9.0 and a reaction time of 12 hr. Furcellaran and agar were included. The results are in Fig. 1.

Release and recovery of bound detergent cation. If the hydrocolloid-detergent complex can be dissociated, the amount of detergent cation released can be determined. If all experimental variables are adequately controlled, the amount of detergent cation released should be equivalent to that determined by the difference method in the binding studies. Wilson (1946) showed that cetylpyridinium chloride and other quaternary ammonium detergents were bound by orange juice, milk, and other food products. Such bound detergents could be released, almost quantitatively, by boiling the products in hydrochloric acid. Graham (1958) demonstrated that cetylpyridinium chloride bound to bacterial cells could be readily recovered by this procedure.

Table 3 summarizes the results of preliminary experiments conducted to establish the reported stability of cetylpyridinium chloride in acid.

Following this, varying amounts of carrageenan (Seakem type 5) were allowed to react for 12 hr with cetylpyridinium chloride at pH 9.0 in 0.01*M* $(NH_4)_2HPO_4$–NH_4OH buffer at 27°C in 50-ml centrifuge tubes. The precipitates formed were centrifuged down and washed five times with distilled water. Five ml of 0.2% bromphenol blue in absolute ethanol was added, and enough HCl was added to each tube to give a final normality of 0.5. Distilled water was added to make all final volumes up to 25 ml. At the end of the heating period 1*N* KOH was added to neutralize the acid, the hydrolysates were made up to volume 100 ml with 0.01*M* $(NH_4)_2HPO_4$–NH_4OH buffer, and suitable aliquots were used for determination by the colorimetric method. Table 4 shows the amount of cetylpyridinium cation bound per milligram of carrageenan (Seakem type 5) as determined by the difference method and the hydrolysis procedure.

Quantitative binding of detergent cations by various sulfated hydrocolloids. After the optimum conditions for binding were established, using the carrageenan (Seakem type 5)–cetylpyridinium chloride system as the example, determination was made of the uptake of the cetylpyridinium and cetyldimethylbenzylammonium cations by various sulfated hydrocolloids. Varying quantities of each hydrocolloid were mixed with an excess of each detergent, and after 12 hours the amount of the detergent cation bound determined according to the general procedure. The results are summarized in Table 5 and Fig. 2.

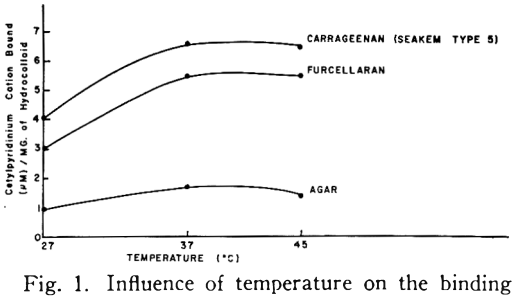

Fig. 1. Influence of temperature on the binding of cetylpyridinium cation by carrageenan, furcellaran, and agar.

Table 3. Stability of cetylpyridinium chloride to acid and heat.

Normality of HCl	Duration of heating (min)	Cetylpyridinium chloride (μM)		
		Added	Recovered	% recovery
		10	9.8	98.0
	15.0	20	19.7	98.5
		50	47.9	95.8
		10	9.8	98.0
0.1	30.0	20	19.8	99.0
		50	47.8	95.6
		10	9.7	97.0
	60	20	19.6	98.0
		50	47.6	95.2
		10	9.6	96.0
	15	20	19.8	99.0
		50	47.9	95.8
		10	9.7	97.0
0.5	30	20	19.9	99.5
		50	47.8	95.6
		10	9.6	96.0
	60	20	19.6	98.0
		50	47.8	95.6
		10	9.6	96.0
	15	20	19.5	99.0
		50	47.2	95.4
		10	9.5	95.0
1.0	30	20	19.5	97.5
		50	47.4	95.8
		10	9.4	94.0
	60	20	19.2	96.0
		50	46.2	92.4

RESULTS AND DISCUSSION

Table 1 records the influence of several variables on the detergent-hydrocolloid interaction. Between pH 3.0 and 10.1, the amount of detergent bound per milligram of carrageenan (Seakem type 5) does not vary drastically. This is readily explained by the fact that ionization of the strongly acidic sulfate group is not suppressed extensively at low pH levels and the polysaccharide is still highly charged at these low pH levels (Whistler, 1959). In addition, the polysaccharide is considered to be fairly stable even at such low pH levels (Whistler, 1959; Hansen and Whitney, 1960). Although only carrageenan (Seakem type 5) was used, furcellaran and the other sulfated polysaccharides listed in Table 5 would be expected to respond similarly. At the extremely low pH of 1.2, the binding of the detergent cation by the hydrocolloid was apparently lowered. Although a suppression of ionization could have subscribed to this effect, some hydrolysis of the hydrocolloid cannot be neglected, in view of the incubation period used.

Within the range investigated, buffer molarity had negligible effect on the binding of the detergent cation by the hydrocolloid. The ammonium ion is thus highly preferable to the sodium ion, which may interfere severely in the preparation of supernatants using alcohol as the precipitant.

The time necessary for completion of the

reaction is relatively short. Although the incubation period used in the direct precipitation procedure was 12 hr, a period as short as 2 hr will suffice, where time is a limiting factor or where a large number of determinations have to be done. If the interaction is studied by the method of equilibrium dialysis, a much longer incubation period is necessary and lower detergent concentrations must be employed to avoid complications due to micelle formation (Ehrenpreis and Fishman, 1960).

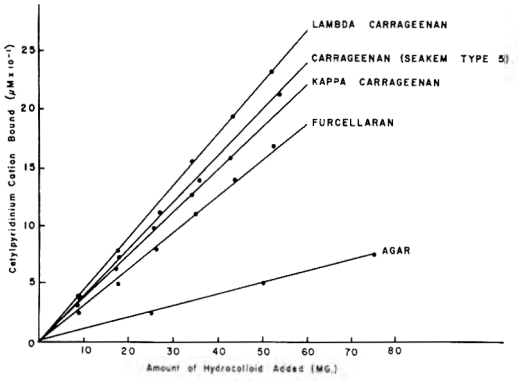

Figure 2. Quantitative binding of cetylpyridinium cation by carrageenan and other hydrocolloids.

When a maximum of 50 mg of carrageenan was used, maximum binding of the detergent cation was observed in the presence of a concentration of detergent of $10\mu M$/ml or more. A level of $20\mu M$/ml therefore assured an excess at all times.

From Table 3 it is seen that cetylpyridinium chloride is highly stable to heat and acid. This factor permitted hydrolysis of the precipitate and high recoveries of bound cetylpyridinium cation. Table 4 shows the amount of detergent cation bound as determined by the direct method and the recovery method. The close agreement indicates that the hydrolysis procedure provides a reliable check on the precipitation method. With detergents (such as cetylpyridinium chloride), that absorb strongly in the ultraviolet range, binding data can be correlated by three different methods, as shown by comparing Tables 2 and 4. Although the ultraviolet spectrophotometric method is more rapid and sensitive than the dye method, the latter method, because of its specificity, is less subject to interference from any precipitate that might have been carried over in the supernatant.

Table 4. Recovery of cetylpyridinium cation after hydrolysis of precipitate.

Carrageenan added (mg)	Cetylpyridinium cation bound (μM)/per mg of carrageenan	
	By difference method	After hydrolysis of precipitate
20	4.07	3.98
40	4.09	4.01
60	4.06	3.89

Fig. 1 shows the effect of temperature on the binding of the cetylpyridinium cation. Binding is much more pronounced at 37°C than at 27°C. However, the amount bound at 45°C by the three hydrocolloids tested is approximately the same as that bound at 37°C, indicating that ionization is complete at the latter temperature. Although low concentrations (0.01%) of agar were used, at 27°C possible errors due to gel formation of hydrocolloid cannot be overlooked. However, the results obtained at 45°C lend credence to the conclusion that its comparatively low binding capacity is real and is due to its supposedly small content of sulfate (Whistler, 1959).

The binding capacity of the hydrocolloids used reflects their degree of sulfation (Table

Table 5. Binding of detergent cations by carrageenan and other hydrocolloids at 27°C.

Hydrocolloid	μM per mg of hydrocolloid	
	Cetylpyridinium	Cetyldimethylbenzylammonium
Lambda-carrageenan	4.422	4.499
Carrageenan (Seakem type 5)	4.088	4.044
Kappa-carrageenan	3.814	3.820
Furcellaran	3.070	3.038
Agar	0.992	1.037
Hypnean	3.867	3.989
Iridophycan	3.745	3.894
Carrageenan (Seakem type 3)	4.180	4.329
Carrageenan (Seakem type 402)	4.040	4.023
Carrageenan (Seakem type 7)	3.944	3.807
Viscarin	4.048	4.107
Gelcarin	4.010	4.092

5). Lambda-carrageenan, which reportedly has a sulfate content of 24–33% (hydrochloric acid digestion) or 44% (nitric acid digestion), bound more detergent cation per milligram than kappa-carrageenan, with a sulfate content of 23–28% (hydrochloric acid digestion) or 33% (nitric acid digestion). Seakem type 5 showed an intermediate binding capacity, and furcellaran, whose sulfate content is known to be much less, exhibited a correspondingly lower binding capacity. The other carrageenans tested gave results closely similar to those obtained with Seakem type 5. Agar, a supposedly lightly sulfated polysaccharide, had the lowest binding capacity. Hypnean and iridophycan bound less detergent cation than any of the carrageenans used. Little is known about the chemical structure of hypnean, but it is considered to be somewhat similar to carrageenan. Some support for this is had from its detergent-binding capacity, if binding is conditioned mainly and perhaps exclusively by the sulfate content of the hydrocolloid. On a similar premise, the binding data indicate that iridophycan resembles hypnean more closely than it does the carrageenans. Close agreement in values was obtained with cetyldimethylbenzylammonium chloride.

These studies demonstrate that the precipitation of sulfated plant hydrocolloids can be exploited for their quantitative determination. Since the approach has been widely applied for their isolation (Scott, 1955; Springer *et al.*, 1957; Korn, 1959a, 1959b, 1959c; Palmstierna *et al.*, 1957; Schiller *et al.*, 1961), use of the techniques described affords simultaneous isolation and assay by simple, rapid, and reproducible means. Since the quaternary ammonium compounds can be readily assayed colorimetrically and cetylpyridinium chloride absorbs strongly in the ultraviolet region, two approaches for assay are available, each providing a ready check on the other.

The described procedure may find wide use in studying the interaction of carrageenan or other sulfated hydrocolloids with various biochemical entities and may be of value in monitoring the solubility characteristics of the detergent-hydrocolloid complex. These complexes have been widely studied (Scott, 1955) with animal and microbial polysaccharides. However, the plant-sulfated polysaccharides have been seriously neglected. Recently, they have been receiving attention in this laboratory and will be reported on in another communication. Detergent precipitation has been successfully applied to the isolation, purification, and quantitative assay of proteins (Jacox, 1953, 1959; Greendyke and Jacox, 1958), and could be of invaluable aid in studies on plant-sulfated hydrocolloids that are being used or are of potential use in the food and pharmaceutical industries.

ACKNOWLEDGMENTS

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Chew Count as a Measure of Tenderness of Pork Loins with Various Degrees of Marbling [a]

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SUMMARY

Some tasters were more repeatable than others in making chew counts, and were thereby able to discriminate more between loins of differing tenderness. Mean chew counts by six tasters for 36 loins ranged from 25.3 to 47.0, with one exceptional value at 60.0. These showed a high correlation with mean shear values based on 10 shears per loin. On average, an increase of 1 lb in shear value corresponded to an increase of 4 in the chew count in this experiment. Mean chew counts and shear values both showed significant correlations with two measures of marbling fat made on the 36 loins (visual scores and intramuscular fat contents), the less marbled loins being somewhat tougher. On average, an increase of 1% in intramuscular fat corresponded to a decrease of about 1.5 in chew count or 0.4 lb in shear value.

INTRODUCTION

Lowe (1949) suggested that, as far as the ordinary consumer is concerned, a major difference between tough and tender meat is the time and effort required to masticate a portion in the mouth. The number of chews needed to do this might, therefore, be a valuable way of measuring gross tenderness variations. Since this characteristic undoubtedly varies a good deal from person to person, care must be taken to use the same panel throughout an experiment, to supply them with samples of standard size, and to specify precisely the end point of the chewing process.

Various workers have used different definitions of this end point. Aldrich (1960) requires the sample to be "masticated so completely that nothing remains in the mouth," the taster being allowed to cut his own portions for chewing (about ½-in. squares) from slices of beef. Cobb (1960) examined several different end points for 1 × 1-in. samples from cold slices of beef roasts cut ⅜ in. thick, and prefers "chewing to the consistency at which the sample would normally be swallowed." This is similar to the definition used in studies of veal by Hanning *et al.* (1957) and in the present studies of pork.

The chew-count method is likely to be of value only if the meat under test contains no large deposits of tough connective tissue, which would render a sample impossible to chew to any of the above end points—a criterion that is usually satisfied by samples from the center of the Longissimus dorsi muscle of pigs, which were used in this study. However, it has also proved successful in an investigation of the effect of cold-storage and cooking methods on commercial-grade cow beef (Paul *et al.*, 1956). As far as the authors are aware, the work of Hanning *et al.* (1957) and Paul *et al.* (1956), and Wisconsin studies reviewed by Kauffman (1960), are the only published investigations in which chew counts have been used; in no case is detailed information

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given on within-judge repeatability of tenderness evaluations by this method.

Tenderness of pork has received much less attention from meat research workers than has that of beef. However, it is likely to gain importance in view of the increasing emphasis placed on the leanness of pigs over the last few years. It is often stated that flesh quality may deteriorate as quantity is increased, for a decrease in subcutaneous fat usually brings with it a decrease in the intramuscular or "marbling" fat frequently associated with superior eating qualities (Kauffman, 1960).

The experiment to be described had two objectives: (a) to study the "chew count" as a method of evaluating the tenderness of pork chops; and (b) to study the variation in tenderness among pork chops with various degrees of marbling.

METHODS

Repeatability trial. The main experiment was preceded by a small-scale repeatability trial involving 9 tasters. Since repeatability cannot be measured by giving tasters the same sample twice, the next best thing is to ask them to make independent assessments on immediately adjacent samples. This was achieved by taking 1-in. cores from cold pork chops, removing the browned surfaces and dividing the remainder into equal halves by cutting across the fibers. When this had been done for two chops, the four samples so produced were arranged on a plate in random positions. The panel member recorded his count on each sample, and his standard error was calculated from differences between values given to the two halves of the same core. Each of the nine tasters was given four plates set up in this way.

Main experiment. Thirty-six loins were chosen at the time of cutting from pigs slaughtered in the Michigan State University Meat Laboratory; 7 were from Yorkshires, 13 from Duroc × Hampshires, 9 from Chester White × Hampshires, and 7 from second and third crosses of these breeds, representing a range in lean cut yield from 47.8 to 58.4%. These were always chosen in pairs, or "blocks," the two loins of a block being kept together and treated alike throughout the experiment. One loin of the block had relatively abundant marbling fat whereas the other was considered deficient in this characteristic.

Seven chops 1½ in. thick were cut from each loin, the first being anterior to the 10th rib cut and the others in succession down the loin posteriorly. These were immediately wrapped and frozen at −20°C. Three chops from each loin were used for tasting, two for shearing, and two for chemical analysis, always from the same positions.

A subjective marbling assessment of a single chop from each loin was made on a black and white Polaroid photograph taken when the chop was still frozen. The pictures, projected about twice full size, were scored by six judges on a 5-point scale with the help of the photographic reference standards provided by Batcher and Dawson (1960). The values used here are the total scores of these six judges.

The chops for objective tenderness appraisal were thawed and cooked in the same way as those for tasting (see below). After being cooled to room temperature, five ½-in. cores were removed from each chop and sheared on the Warner-Bratzler apparatus. The value for each loin is therefore the average of 10 shears.

The two chops for chemical analysis were partially thawed to allow the eye muscles to be separated and ground. Duplicate values of intramuscular fat content (calculated on a fresh-weight basis) were obtained on each chop, so the values used here are the average of four determinations on each loin.

The three chops for tasting from each of the two loins of a block were thawed at room temperature, allocated at random to the six positions in the fryer, and cooked in deep fat (at 138°C) to an internal temperature of 80°C. They were cooled overnight and tasted cold the following morning. Two 1-in. cores were taken from each chop (six from each loin); the six tasters were allocated core positions at random for a particular comparison. The tasting was carried out in six sessions, three blocks being compared at each session. For the first three sessions, the allocation of cores to plates was the same as for the small-scale repeatability trial described above, the two chops compared on a particular plate being in this case the pair of chops forming a particular block. It is possible that the judges could have been able to pair off the duplicates on a plate by eye, because of slight differences in color and texture between the two loins. To test this possibility, the design was changed for the second three sessions of the experiment. At these, only two plates were given to each taster, the first having single samples from six different loins (from 3 blocks), whereas their duplicates were on the second plate in a different random order (see Fig. 1).

Analysis. All correlations were calculated on a within-block basis; these have 17 degrees of

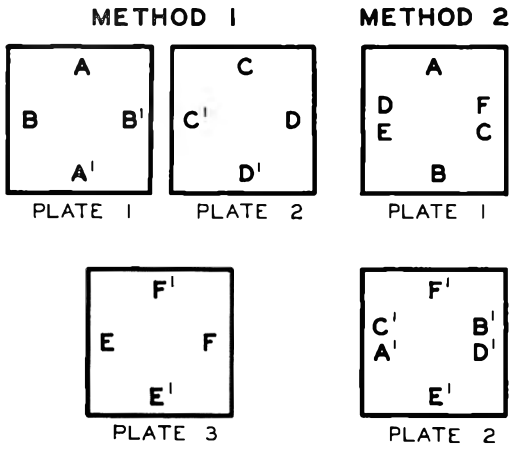

Fig. 1. Allocation of duplicate samples to plates according to Method 1, used for the first three sessions, and Method 2, used for the remaining sessions. A and B form the first block of two loins, and so on, three blocks being compared at each session.

freedom. Such correlations are not influenced by the effects of extraneous variations on the characteristics correlated, such as length of time kept in the frozen state, since the members of a block were kept together and treated alike from the time they were chosen. In the case of those correlations involving chew counts, the within-block correlations are not influenced by variations in the tasters' standards from session to session.

RESULTS

Variability of chew counts. The standard errors of repeated chew counts for the nine tasters in the small-scale preliminary study ranged from 2.3 to 12.8 chews, each being on 8 degrees of freedom. However, standard errors for six tasters lay in the range 2.3 to 3.4, and these were chosen for the main experiment; tasters with standard errors 5.2, 6.3, and 12.8 were rejected.

Table 1 shows the standard errors of repeated chew counts found in the two stages of the main experiment, together with measures of judging discrimination. Several of the tasters tended to be less repeatable in the experiment than they had been in the trial; in fact, the pooled standard error increased from 2.8 to 4.6. There was no evidence that repeatability was any better when the duplicate samples appeared on the same plate than when they appeared on different plates.

A taster could be repeatable in this sense merely by not allowing his scores to vary to any extent; if he did, his standard error would be small though discrimination between loins would be poor. It is for this reason that measures of each taster's discriminating ability (the F ratio of mean squares "between" and "within" loins in the same block, and the intraclass correlation) have been included in Table 1. These show that, although taster E had a large standard error, he proved to be the most discriminating, with C, who had the smallest standard error, the next-most discriminating. The low values of A for discriminating ability suggest that his small standard error was to some extent achieved by conscious or unconscious equalization of the counts for the samples in a particular session.

Mean chew count ranged from 25.3 to 47.0, with one exceptional loin having a value of 60.0; these are averages of duplicate assessments by the six judges. A standard error of 4 chews for a single observation shows that the standard error of the difference between two loin means in this experiment would be 1.6 chews (if there were no "taster × loin" interaction). Differences of less than 3½–4 chews between mean chew counts could therefore be regarded as showing no significant difference in tenderness.

Variability in shear values. The standard error of repeated shears on the same loin was 1.33 lb, although some of this variation was due to differences in tenderness between the 5 positions within the chops from which the cores were taken. The standard error of the difference between two loins was 0.59 lb, since each loin mean was based on 10 shear values; these means ranged from 5.1 to 9.1 lb, with the exceptionally tough loin having a shear value of 12.9 lb.

Shears from the more anterior of the two chops chosen for this objective measurement of tenderness were significantly greater than those from the posterior chop (7.3 vs. 6.8 lb), though this difference showed significant variation from loin to loin.

Table 1. Repeatability and discrimination in the main experiment.[a]

Taster	Method 1	Method 2	Overall	F	Intraclass correlation
A	3.9	2.7	3.4	3.3	54
B	7.5	5.3	6.5	4.8	66
C	2.6	3.7	3.2	6.2	73
D	4.1	2.5	3.4	4.6	65
E	5.3	6.4	5.9	8.4	79
F	4.3	4.3	4.3	5.8	70
Pooled	4.9	4.4	4.6	....	....

[a] Standard errors of repeated chew counts when tasting was by methods 1 and 2 (each on 18 degrees of freedom), and over the whole experiment (on 36), together with the F value (ratio of mean squares "between" and "within loins in the same block" on 18 and 36 degrees of freedom), and the intraclass correlation as a percentage.

Relation between objective and subjective tenderness. Table 2 shows the results of calculating the regression of chew count on the objective measure of tenderness (using loin mean values in both cases) for each taster and for the mean counts of the whole panel. These are calculated within blocks of loins.

Table 2. Subjective and objective tenderness measurements.[a]

Taster	Regression coefficient ± standard error (chews)	Correlation coefficient
A	2.6 ± 0.3	0.89***
B	5.0 ± 1.2	0.72***
C	3.0 ± 0.6	0.79***
D	2.5 ± 0.6	0.69**
E	7.4 ± 0.9	0.90***
F	4.2 ± 0.7	0.83***
Panel average	4.1 ± 0.4	0.92***

** $0.01 > p > 0.001$.
*** $0.001 > p$.
[a] The relation between mean chew counts and shear values for each taster and for the panel average. These have been calculated on a within-block basis.

The two tenderness measures were highly related, some 85% of the variation in chew counts averaged over the six tasters being explained by shear values. The correlations dropped to 0.85 (72% of the variation explained) when the block containing the extremely tough loin was omitted from the analysis. On average, an increase of 1 lb in shear value was equivalent to an increase of 4 in the chew count, although the regresion coefficient differed among the six tasters.

These results suggest that if increased toughness of pork results, at least as far as the average consumer is concerned, essentially in increasing the difficulty of chewing then the shearing apparatus gives a reasonably good prediction of toughness over a wide range, even though it simulates only one particular feature of the chewing process. It may not, however, be sufficiently sensitive to detect small differences in the difficulty of chewing.

Measurement of marbling fat. The within-block correlation between chemically determined intramuscular fat and the total subjective scores for marbling fat by a panel of six was 0.85. The total score had an average of 17.9, ranging from 6 to 27 for individual loins. Intramuscular fat averaged 3.45% on fresh-weight basis, ranging from 1.1% to 7.4%.

Although the loin chosen in a particular block as "marbled" always had a marbling score no lower than the "deficient" loin, the difference in the score among the 18 blocks ranged from 0 to 17. The mean difference between marbled and deficient loins was 7.4 on this 30-point scale. The mean difference in intramuscular fat was 2.83%, which was also highly significant.

Relation of tenderness to marbling. Shear values showed a significant difference of 1.3 lb between the two types of loin, the "marbled" loins being the more tender. Some 38% of the variation in shear values was explained by the amount of intramuscular fat ($r = -0.62$); an increase of 1% in intramuscular fat caused, on average, an improvement of 0.42 lb in shear value. The correlation of shear value with marbling score was −0.61 within blocks.

Table 3 shows, for each taster, the mean chew counts for the loins classified as relatively well marbled and relatively poorly marbled. The analysis showed that each taster was able to detect differences in tenderness between loins by this method, although the marbled loins were significantly more tender for only four of the six tasters; taster B failed to detect a significant difference on average between the two series. The extent of the difference varied from taster to taster and, of course, from block to block.

Table 3. Differences between loins within blocks.[a]

Taster	Mean of well-marbled loins	Mean of poorly marbled loins	Difference	F
A	36.4	41.3	4.9	28.2***
B	40.6	43.8	3.2	<1
C	34.5	38.1	3.6	4.3
D	20.8	24.8	4.0	7.2*
E	33.4	43.5	10.1	9.3**
F	36.0	42.0	6.0	8.7**

* $0.05 > p > 0.01$.
** $0.01 > p > 0.001$.
*** $p < 0.001$.
[a] The mean chew counts for the loins classified as relatively well marbled and relatively poorly marbled for each taster, together with the significance of the difference (F, based on 1 and 17 degrees of freedom).

Table 4 shows how the chew counts were related to two measures of marbling (regression of chew count on marbling score and intramuscular fat percent). Some 45% of the variation in the panel average chew count was explained by the marbling score (reduced to 35% when the extreme block was omitted), and some 33% by the percentage of intramuscular fat (reduced to 22%). Marbling score and intramuscular fat percentage

Table 4. Subjective tenderness measurements in relation to marbling fat.[a]

	Marbling score		Intramuscular %	
Taster	Regression coefficient (chews)	Correlation coefficient	Regression coefficient (chews)	Correlation coefficient
A	−0.47	−0.71***	−1.53	−0.77***
B	−0.56	−0.35	−1.06	−0.23
C	−0.51	−0.59**	−1.19	−0.46*
D	−0.60	−0.74***	−1.31	−0.55*
E	−1.19	−0.64**	−3.31	−0.60**
F	−0.71	−0.64**	−1.95	−0.58**
Panel average	−0.67	−0.67**	−1.73	−0.57*

* $0.05 > p > 0.01$.
** $0.01 > p > 0.001$.
*** $0.001 > p$.
[a] The relation between mean chew counts and measures of marbling fat for each taster and for the panel average. These have been calculated on a within-block basis.

together explained 44% of the variation in the panel average chew count within blocks of loins. An increase of 1% in the intramuscular fat corresponded to a decrease in chew count of about 1½ on average. Taster B was again exceptional in showing no significant relation between chew counts and marbling.

These results give support to the contention that pork loins with low levels of intramuscular fat tend to be somewhat tougher on average, when prepared and eaten in this manner, than those with more intramuscular fat, although the relation was not sufficiently close to be used for predictive purposes. Selection programs aimed at improving the lean meat content of pig carcasses should therefore keep a careful check on the changes that are occurring in the eating qualities of this lean meat as the level of fatness of the carcass is reduced. Shearing samples of cooked pork with apparatus of the Warner-Bratzler type seems the most practical way of making regular evaluations of tenderness in pigs from progeny testing stations, etc.; periodic comparisons with chew-count evaluations by a small trained panel would be desirable, however, to ensure that the shearing method continues to reflect differences in the difficulty of chewing as selection alters the chemical and physical composition of the meat.

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Lack of Correlation Between Egg Shell Thickness and Shell Permeability of Market Eggs[a]

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Interior physical quality and bacterial penetration of shell eggs have been reported as functions of egg shell permeability (Fromm, 1960; Kraft *et al.*, 1958). Romanoff (1943), using small sections of pretreated egg shells, found that shell thickness was not a factor contributing to the rate of air flow through the shell. Since an objective tool for measuring egg shell permeability *in vivo* was reported only recently (Fromm, 1959), the present study was conducted to employ the intact egg shell in its entirety to determine if a relationship exists between egg shell permeability and shell thickness.

The eggs used, which were obtained from a flock of Single Comb White Leghorns in their first year of production, were gathered 3 times daily. They were visibly clean and weighed 59–64 g. Immediately after the eggs were gathered, shell thickness was determined by placing the eggs in various aqueous NaCl solutions of known concentration and determining the specific gravity of each egg by flotation. The principle involved is that the contents of the freshly laid egg are relatively constant, so that any variation in thickness of shell will affect the specific gravity of the egg (Baker and Curtiss, 1958; Olsson, 1934; Phillips and Williams, 1944).

The eggs were divided into 5 specific-gravity groups (1.076 or less, 1.080, 1.084, 1.088, and 1.092 or more) and coded as groups 3 to 7. Respective mean shell thicknesses (inches $\times 10^{-3}$) for the specific-gravity groups were 10.86, 11.84, 12.77, 13.39, and 14.21, and statistical differences were detected between these means. The relation between shell thickness and permeability was determined by comparing the permeability of the various specific-gravity groups. Permeability was determined according to the method of Fromm (1959).

Twenty eggs in each of the 5 specific-gravity groups were analyzed for egg shell permeability (50 paired observations) on the day gathered. It has been demonstrated that permeability of the shell increases with age of the egg (Almquist and Holst, 1931; Fromm, 1960; Kraft *et al.*, 1958; Romanoff, 1943). To evaluate the relationship between shell thickness and permeability, 20 eggs within each of the 5 specific-gravity groups were analyzed at 3, 6, 9, and 12 days of age (held at 24°C and 55% RH), and correlation analyses were calculated. This experiment was repeated.

It has been reported that a simple method to increase permeability of the egg shell is to wash the eggs in warm water and rub the eggs thoroughly with a nonabrasive material (Fromm, 1960; Fromm and Monroe, 1960). To determine whether increased shell permeability of new-laid eggs affects the relation between shell thickness and shell permeability, the experiment described above was repeated except that eggs, on the day gathered, were held 3 min under agitation in 43.4°C water and thoroughly rubbed with cheesecloth. This experiment was repeated, and correlation analyses were made between shell thickness and permeability at the 5 different age levels.

Table 1 shows the correlation coefficients for specific gravity (shell thickness) of eggs and egg shell permeability according to age groups, and the influence of washing and rubbing the eggs on this relationship. Significant correlations were found among controls in Trial 1 at 3 days, in Trials 1 and 2 at 6 days, and in Trials 1 and 2 at 12 days of age. Among washed and rubbed groups, egg shell thickness and permeability were not related.

Among control trials, shell thickness and permeability were related in 5 of 10 age groups. At this level of probability one can readily assume that the relation is due to

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Table 1. Correlation coefficients for specific gravity (shell thickness) and egg shell permeability according to age of eggs and shell treatment.[a]

Shell treatment	Age (days)					Over-all mean OD of shell extractions[b]
	0	3	6	9	12	
Control						
Trial 1	0.02	−0.36*	−0.31**	0.22	−0.43*	0.37
Trial 2	0.12	−0.05	−0.32**	−0.23	0.33**	0.34
Washed and rubbed						
Trial 1	0.11	0	0.23	0.16	0.02	0.70
Trial 2	0.26	−0.07	−0.05	0.22	0.17	0.66

* $p = < 0.05$.
** $p = < 0.01$.
[a] Each coefficient represents 50 paired observations (100 eggs).
[b] Optical density is directly proportional to shell permeability.

chance. Evidence of this may be noted in that at 12 days of age a significant negative correlation existed in Trial 1 and a significant positive coefficient was calculated for Trial 2. This would indicate that thicker shells at 12 days of age were less permeable in Trial 1 and more permeable in Trial 2. Even with the highest correlation coefficient (controls, Trial 1), only 18% of the variation in egg shell permeability could be attributed to shell thickness. The remainder of the variability is probably associated with individual differences in the density or quantity of the cuticular layer on the shell and in the pores of the egg. Among washed and rubbed eggs, the complete lack of correlation could possibly be attributed to elimination of the cuticular layer or some other protective substance, increasing the permeability of the shell (Fromm, 1960; Fromm and Monroe, 1960). This phenomenon is evidenced in Table 1 by the higher level of permeability as shown by the greater mean optical density of shell extractions from washed and rubbed eggs than from control eggs. Standard errors between individual shell-permeability observations for control eggs and for washed-rubbed eggs were, respectively, 0.137 and 0.115, and the mean coefficients of variation were 38.59% and 16.91%. The lesser values of variability attributed to the washed and rubbed groups indicate that individual differences were negated by this treatment. Thus, under the conditions of this study, the factor that inhibited permeability of the egg shell was probably the cuticular layer on the shell and in the pores of the shell, and shell thickness was of minor importance.

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