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Anthocyanins. III. Disc Sensitivity Assay of Inhibition of Bacterial Growth by Pelargonidin 3-Monoglucoside and Its Degradation Products[a,b]

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(Manuscript received September 12, 1960)

SUMMARY

Pelargonidin 3-monoglucoside was isolated from frozen strawberries and subjected to heat degradation in 1*N* HCl. The six fractions isolated were characterized chemically and physically. The unheated and heated pigment inhibited the growth of *Escherichia coli* and *Staphylococcus aureus*, and exerted both a stimulatory and inhibitory effect on *Lactobacillus casei* culture. The stimulation may be due to a decrease in the oxidation-reduction potential of the media affected by the pigment, and/or the ability of the organism to split the β-glycosyl bond and use the glucose moiety. The influence on growth of the test organisms of the various fractions resulting from heating the pigment was reported.

Markakis *et al.* (1957), Tinsley and Bockian (1960), and others (Lamort, 1958a,b); Livingston and Markakis, 1956; Lukton *et al.*, 1956; Mackinney *et al.*, 1955; Meschter, 1953; Pratt *et al.*, 1954) reported that P3-MG (pelargonidin 3-monoglucoside) is unstable to heat and that several factors (temperature, pH, oxygen, ascorbic acid, riboflavin, 5-hydroxymethyl-2-furaldehyde, and carbohydrates) can affect the rate at which this pigment degrades. Kinetic studies on this degradation (Markakis *et al.*, 1957) revealed a first-order reaction rate at 45°C only when air in the reaction vessel was replaced by nitrogen. Some of the natural pigments such as P3-MG, cyanidin 3-monoglucoside, and delphinidin 3-monoglucoside were reported (Pratt *et al.*, 1960) to suppress the maximum growth of *Lactobacillus acidophilus* and *Clostridium* sp. P.A. 3679, though the lag phase of growth was shortened for P.A. 3679 and *Escherichia coli.* Powers *et al.* (1960) found that the growth of *Staphylococcus aureus* was inhibited by the synthetic compounds 5-desoxy-apigenidin-chloride-4′-methyl ether, apigenidin chloride, and 5-desoxy-apigenidin-chloride-4′-methyl ether. Portis and Fishbein (1941) showed that the percentage of Gram-positive microorganisms in the intestines increased following oral administration of grape juice, a rich source of anthocyanins.

The experiments reported herein were designed to examine further the effect of P3-MG and its heat-degraded products *per se*, as well as the chromatographically isolated fractions, on the activities of certain species of microorganisms that may be found in the intestines and foods. Attempts were also made to elucidate the mode of action of this pigment against *Lactobacillus casei.*

EXPERIMENTAL

Isolation of P3-MG. The anthocyanins were extracted from commercially frozen strawberries as previously described (Powers *et al.*, 1960). The P3-MG was separated from other anthocyanins and contaminating materials by cellulose-column chromatography with acidified methanol (pH 3.5)

[a] Journal Paper No. 138 of the College Experiment Station of the University of Georgia College of Agriculture Experiment Stations.

[b] Portions of this study were supported in part by U. S. Public Health Service Grant No. E-3156 and by the California Wine Advisory Board Contract M-40.

as a solvent. The cellulose column was prepared with Whatman standard-grade cellulose powder (200-mesh) packed to give a column of 80 × 2.2 cm. The absorption characteristics of the various eluted fractions were determined with a Beckman Model DU spectrophotometer to help establish their identity and purity. The purified P3-MG fraction was then crystallized. The absorption curves of an aqueous solution of this pigment seemed identical to those reported by Pratt *et al.* (1960) and Livingston and Markakis (1956).

Degradation of P3-MG. Degradation was accomplished by heating the pigment at 250°F in 1*N* HCl for 30 min in an Erlenmeyer flask or for 2.0 hr in an autoclave, followed by immediate cooling and filtration. The degraded pigment was condensed *in vacuo* and quantitatively fractionated by chromatography using the upper phase of a 1-butanol–acetic acid–water mixture (4:1:5 v/v).

Cultures. The test organisms, *E. coli*, *L. casei*, and *S. aureus*, were maintained by several passages through a tryptone-glucose-yeast extract (TGY) medium before the inhibition tests. The organisms were incubated at 37°C.

Media and assay procedure. The disc sensitivity method was used to demonstrate the effect of the pigment or its degradation products on growth of the test organisms. TGY agar (Difco) was seeded with a 1% inoculum of an 18-hr culture of the test organism. The aqueous solutions (0.05 ml) containing the desired concentrations of the test substances were added to sterile discs and allowed to dry. The discs were placed on the surface of the hardened agar, incubated at 37°C, and examined at 24 and 48 hours for inhibition or stimulation of growth.

RESULTS

Effect of P3-MG on growth. Both *E. coli* and *S. aureus* cultures were inhibited by the nondegraded pigment, as evidenced by a clear zone surrounding the discs. The pigment markedly stimulated *L. casei*. as well as inhibiting it (Fig. 1). Inhibition appeared to be linearly dependent at levels of 3–15 mg/disc. In each case the diameter of the zone of inhibition was highly significantly correlated with pigment concentration. Neither *L. casei* nor *S. aureus* was measurably affected by concentrations below 3 mg/disc.

Several experiments were conducted to investigate the mode of action of P3-MG on *L. casei*. The assumption was made that these lactic acid bacteria, being microaerophilic, require a decrease in the oxidation-reduction potential of the medium before growth is stimulated or initiated. Various compounds known to reduce the oxidation-reduction potential were tested, and it was found that 2–4 μM of L–(–)–cystine or 5–10 μM of sodium

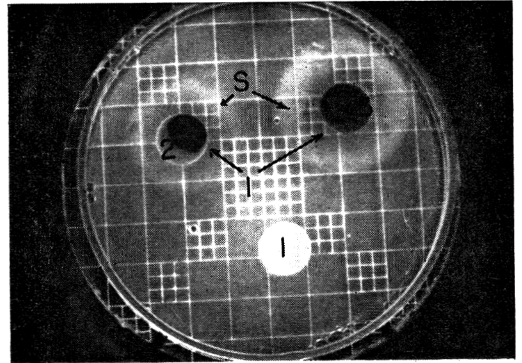


Fig. 1. The stimulatory and inhibitory effects of pelargonidin 3-monoglucoside on the growth of *L. casei* on glucose yeast extract agar media. Disc No. 1 (control) contained distilled water, whereas 2 and 3 had 8 and 15 mg of unheated pigment. I = inhibition, S = stimulation.

2-mercaptoethanoate (thioglycolate) per disc stimulated the growth of *L. casei* as much as 6–12 mg of P3-MG. Experiments with media devoid of added glucose showed that growth was initiated and stimulated around discs containing 2–4 μM of glucose.

Heat degradation of P3-MG and its effect on the test organisms. Heating P3-MG in 1*N* HCl caused a rapid degradation as detected by a sharp decrease in the absorption characteristic of the pigment at 500 mμ. During degradation, the pigment changed from brilliant red to brown, and a dark-brown precipitate increased with extensive heating.

When P3-MG was heated at 250°F in 1*N* HCl for either 30 min or 2 hr, followed by neutralization with NaOH, the precipitate that formed had no effect on the growth of the test organisms in the concentration ranges of 40 μg to 8 mg/disc. The neutral filtrate of the degraded pigment, however, inhibited the growth of all the test organisms at levels of 3–15 mg/disc based on anthocyanin weight (Fig. 2, 3). The 30-min filtrate at 1–5 mg/disc (Fig. 2) inhibited *S. aureus* markedly more than *E. coli* or *L. casei*. The 2-hr filtrate, in contrast, inhibited *E. coli* the most (Fig. 3). In the 2-hr filtrate the curves for *E. coli* and *S. aureus* deviated significantly from linearity when subjected to analysis of variance. Neither filtrate at lower concentrations (1–3 mg/disc) had a detectable effect on the growth of *L. casei*. At 3–15 mg/disc, however, both filtrates gave to the growth of *L. casei* culture a definite stimulation that seemed to be concentration-dependent.

Several control experiments were conducted to investigate the effect on the test organisms of sodium chloride resulting from neutralization of the degraded P3-MG. The results showed no

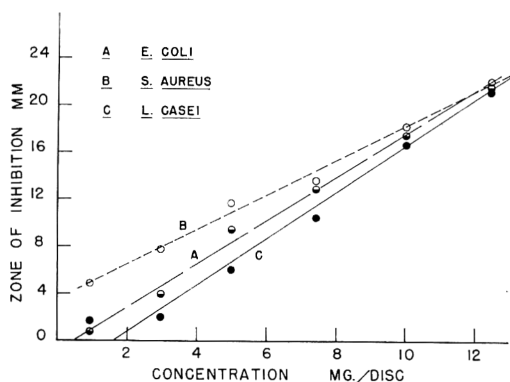


Fig. 2. The effect on the test organisms of various concentrations of neutral filtrate produced by heating pelargonidin 3-monoglucoside 30 min at 250°C in 1N HCl.

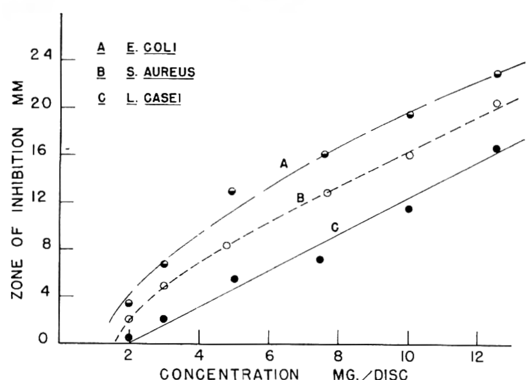


Fig. 3. The effect on the test organisms of various concentrations of neutral filtrate produced by heating pelargonidin 3-monoglucoside 2 hr at 250°C in 1N HCl.

effect from 0.05 ml of 4N NaCl solution per disc (11.7 mg).

Characterization of the degradation products in P3-MG. Efforts were directed toward learning the nature of the substances affecting growth. Five fractions were separated from the neutralized filtrate. Table 1 shows their physical and chemical characteristics. Only 75.5% of the initial pigment could be accounted for as isolated degradation products. This might indicate that some fraction or fractions could not be separated with the butanol–acetic acid–water solvent or detected by the indicators used. Fractions No. 2, 3, 4, 5, and 6, under spectral analysis, absorbed strongly in the ultraviolet range with a maximum at 280 mμ indicating the presence of an aromatic ring. Fraction No. 1 was identified as glucose, fraction No. 4 as hydroxymethylfurfural, and fraction No. 5 as phloroglucinol. Fraction No. 6, the brown precipitate consistently reported as an end-product of anthocyanin degradation (Hartman, 1959; Livingston and Markakis, 1956; Markakis *et al.*, 1957; Pederson *et al.*, 1947; Pratt *et al.*, 1960), was insoluble in water, ether, acetone, concentrated HCl, or H_2SO_4; slightly soluble in ethyl or methyl alcohol; and completely soluble in dilute NaOH, yielding a yellow color. Its properties are very similar to those reported by Pederson *et al.* (1947) and Markakis *et al.* (1957).

Effect of degradation products of P3-MG on the test organisms. The various soluble fractions resulting from heating the pigment 2 hr in 1N HCl at 250°F in an autoclave were studied for their effect on the test organisms using TGY medium. The results (Table 2) showed that fractions No. 1, 2, 3, and 5 stimulated the growth of *L. casei* whereas fraction No. 4 had no effect on it. Fraction No. 1, also tested on tryptone-yeast extract

Table 1. R_f values, percentage yields, and characterization of fractions isolated after pelargonidin 3-monoglucoside was heated 2.5 hr at 250°F in 1N HCl.

Fraction no.	R_f [a]	% yield [b]	Physical and chemical characterization [c] A	B	C	Identification
1	0.24	34.3	–	+	–	Glucose
2	0.62	7.2	Yellow	–	–	Aromatic compound
3	0.71	9.2	White	+	+	Phenolic compound with reducing group(s)
4	0.90	9.8	Yellow	+	+	(Hydroxymethylfurfural) and other furfural derivatives
5	0.95	5:1	White	–	+	Phloroglucinol
6		9.4	–	–	–	Polyphenolic [d]

[a] Solvent system: upper phase of 1-butanol–acetic acid–water mixture (4:1:5 v/v).
[b] Percentage yields based on the weight of the pigment before heat treatment.
[c] A, UV light (3600 A); B, ammoniacal silver nitrate; C, diazotized sulfanilic acid; – = no reaction; + = present.
[d] This fraction represents the dark-brown precipitate.

agar without glucose, stimulated the growth of *L. casei*. Fractions No. 3 and 4 inhibited *E. coli* and *S. aureus* with fraction 4 (hydroxymethylfurfural) being the most active. The inhibitory effect of these fractions is not explained at present except that hydroxymethylfurfural, 20–30 mg/disc, prepared by the method of Haworth and Jones (1944) inhibited the growth of both *E. coli* and *S. aureus* but had no effect on *L. casei*.

DISCUSSION

Stimulation of the growth of *L. casei* by P3-MG, detected by the disc sensitivity method, is in line with the previous observation (Pratt *et al.*, 1960; Powers *et al.*, 1960) that anthocyanin reduced the lag phase in liquid culture media as measured turbidimetrically. This pigment may poise the oxidation-reduction within favorable limits for growth stimulation. It was found that 2–4 μM cystine or 5–10 μM of sodium thioglycolate stimulated the growth of the *L. casei* to an extent similar to that of 2–6 μM P3-MG. It is also possible that this stimulation may be due to the ability of the organism to split the β-glycosyl bond in the P3-MG molecule and use the glucose moiety, but until it has been determined that *L. casei* can split the β-glycosyl bond of the P3-MG, no conclusion can be drawn as to the constitutive or inducible nature of the enzyme. It has been reported, however, that a β-glucosidase was induced in *Streptococcus lactis* by the microbial inhibitor 5-amino-2,4-bis (2-thenylamino) pyrimidine acetic acid (Kunkee, 1960).

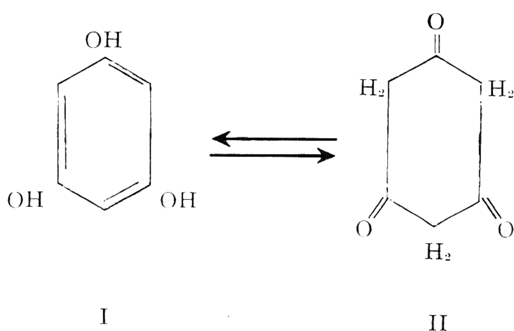
No explanation can be offered at present for the inhibitory effect of the pigment on the growth of *E. coli* and *S. aureus*, but it is speculated that the anthocyanin may poise the oxidation-reduction potential at levels unfavorable for growth initiation of these organisms as evidenced by growth after 2–3 days in the clear zone around the disc. Studies are now being conducted to examine the effect of P3-MG on the metabolic activities of the test organisms as well as on the Eh of the medium.

Results obtained in degradation of the pigment in 1*N* HCl revealed that P3-MG was apparently hydrolyzed, yielding pelargonidin and glucose (fraction 1). The former may undergo direct condensation and polymerization to form polyphenolic compounds (fraction 6) or another type of hydrolysis involving opening of the pyrilium ring at position 1–2, with formation of open-chain unsaturated ketones (chalcones), as suggested by Markakis *et al.* (1957). Further disruption may lead to formation of phloroglucinol (fraction 5), which is a tautomeric compound, reacting not only in the phenolic form (I) but also as a hexamethylene triketone (II) (possible fraction 2 or 3), reduction of which is an admissible as-

Table 2. Effect of the various fractions of heat-degraded pelargonidin 3-monoglucoside on the activities of the test organisms.

Fraction no.	Conc. (mg/disc)	Stimulation or inhibition[a]		
		E. coli	*L. casei*	*S. aureus*
1	3.5	0	0	0
	7.0	0	0	0
	14.0	0	+	0
	28.0	0	+	0
2	2.3	0	0	0
	4.6	0	0	0
	9.2	0	+	0
	18.4	0	+	+
3	2.3	0	0	0
	4.6	−2.0	+	−3.0
	9.2	−6.2	+	−5.2
	18.4	−8.2	+	−8.1
4	2.4	0	0	0
	4.8	−1.0	0	−1.0
	9.6	−8.0	0	−7.2
	19.2	−12.0	0	−12.0
5	1.7	0	0	0
	3.4	0	0	0
	6.8	0	+	0
	13.4	0	+	0

[a] 0 = no measurable effect; + = stimulation; − = inhibition, diameter (mm) of the zone of inhibition minus that of the disc.



sumption, and this could lead to the formation of resorcinol.

Hartman (1959) established that *p*-hydroxybenzoic acid was formed upon heating the pigment 1.5 hr at 180°F in citrate buffer (pH 3.4), but he failed to detect this acid when P3-MG was subjected to more extensive heat treatment.

Glucose (fraction 1) dehydration seems to be catalyzed by HCl, during the heat degradation process of the pigment, with the loss of three molecules of water, yielding 5-hydroxymethyl-2-furaldehyde (fraction 4). This intermediate compound was experimentally found in a model system, to undergo hydration, cleavage, and dismutation, forming levulinic and formic acid. This confirmed the results of Sowden (1949), who stated that the aldehyde carbon of the glucose eventually becomes the carbon of the resultant formic acid. The presence of 5-hydroxymethyl-2-furaldehyde (fraction 4), and possibly formate or formaldehyde, may also explain, in part, the inhibitory effect of the degraded pigment on the test organisms. Dood and Stillman (1944) reported that both non-nitrated furan and nitrofuran are bactericidal in low concentration when tested against *S. aureus, E. coli.* and *Salmonella typhosa.*

ACKNOWLEDGMENT

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Free Sugars and Sugar Phosphates in Muscle of Chill-Stored Aquarium Cod *(Gadus callarias)*

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(Manuscript received March 7, 1961)

SUMMARY

The concentrations of ribose, glucose, ribose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate, fructose monophosphate and fructose 1,6-diphosphate in muscle extracts of aquarium-kept cod were determined and their changes followed throughout a period of chill-storage. A comparison is drawn between these results and those obtained previously from a similar study on trawled cod, and the probable enzymic processes responsible for the changes are discussed. It is observed that on the whole, rested cod muscle contains considerably more free sugars and sugar phosphates than trawled cod muscle and it is concluded that muscle from rested fish is more liable to "brown" than is that from exhausted fish.

An earlier study (Burt and Jones, 1961) on changes in the concentration of sugar phosphates in cod muscle during chill-storage was carried out on fish that had been trawl-caught. Under trawling conditions, individual fish struggle to different extents, depending principally on the length of time spent in the trawl. These differences would lead to quite considerable variations in the concentrations of carbohydrate metabolites in the muscle (Amano *et al.,* 1953) overlying natural variations due to different nutritional states and different stages of growth. It was hoped that keeping cod alive for a number of weeks before killing them would minimize differences due to differences in struggling and food. The earlier results also indicated that the processes going on in the muscle would be better understood if sampling were more frequent during the period 1–4 days after death.

The concentrations of free sugars in fresh, spoiling, and sterile trawled cod muscle have already been reported (Jones, 1958a,b, 1959), and this knowledge is now extended to cover the amounts of ribose and glucose present in a free state in fresh and spoiling aquarium cod.

The conditions of chill storage were chosen for the following reasons: a) ice-melt water washing over the skin surface of the fish tends to keep down bacterial growth and accumulation there, b) muscle enzymes are less likely to be denatured at 0°C than at higher temperatures, and c) chemical interactions are slower at lower temperatures. It is known that cod muscle is sterile under these conditions until about the sixth day, and that the bacterial count within the muscle itself is comparatively low at eight days (Shewan).

Were great differences to be found between trawled and aquarium cod in sugar and sugar phosphate content, this study might have some bearing on commercial use of cod in respect of keeping quality and tendency to Maillard browning of dehydrated muscle. Line-caught fish do not struggle as much as trawl-caught ones, and consequently would lie somewhere between trawled and aquarium fish with regard to carbohydrate constitution.

This paper describes the concentrations of free sugars and sugar phosphates in aquarium-kept cod, and the changes in concentration during storage in ice.

EXPERIMENTAL

Cod 18–24 in. long were maintained in an aquarium for 8 weeks after their capture by trawl in the North Sea off Aberdeen. They were fed squid muscle so that they might replenish their lost glycogen reserves and return to a "rested" condition. At the start of the experiment the cod were caught and killed, by a blow on the head, with a minimum of struggling. They were then eviscer-

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ated, packed in ice, and stored for various periods in a cold room maintained at an ambient 2.5°C. Batches of 2–4 fish were removed from the ice for perchloric acid extraction of the muscle at each of the following times: 6½, 20, 22½, 29½, 46, 53, 71, 77, 94½, 101, 168, and 240 hours after death. As controls, whole fish were stunned by a blow on the head, dropped alive into liquid air and kept in solid carbon dioxide until the muscle was excised for extraction.

Preparation of extracts. Perchloric acid extracts of muscle were prepared by the method of Burt and Jones (1961). Aliquots of extracts from individual fish, stored for the same length of time, were pooled prior to analysis for glucose and the sugar phosphates.

Estimation of free sugars and sugar phosphates. Glucose was estimated by the method of Huggett and Nixon (1957) as modified by Matheson and Penny (unpublished). Aliquots of the pooled perchloric acid extracts were adjusted to pH 6.5 and diluted 1 : 1 in water. Extract (3 ml) and 5 ml glucose oxidase reagent (5.0 mg glucose oxidase and 0.5 mg horseradish peroxidase, both enzymes from the Sigma Chemical Company, dissolved in 0.2*M* pH 6.5 phosphate buffer, 2 ml of a 1% acetone solution of *o*-dianisidine added and volume made to 100 ml) were incubated 30 min. at 37°C. Reaction was stopped by the addition of 2 ml 0.4*N* sulphuric acid, and the optical density at 400 mμ was read 5 min later. A range of glucose standards (10–200 μg) was processed along with the extracts.

Ribose was determined chromatographically by the method of Jones (1958a) after treatment of individual extracts (10 ml) with Amberlite IR-120 (H^+) (1 g) and Amberlite IR-4B (OH^-) (1 g) resins. After concentration by lyophilization, solution equivalent to ½ g of tissue was applied to Whatman No. 4 paper. Chromatograms were developed (descending, 18 hours) in butanol–acetic acid–water (3 : 1 : 1) at 20°C. The two spots found were identified as ribose and glucose. Maltose was not detected.

Sugar phosphates were determined colorimetrically after ion-exchange separation of the pooled extracts (Burt and Jones, 1961; Jones and Burt, 1960).

RESULTS

The concentration of free glucose in "rested" cod muscle at death was 210–220 μmoles/100 g (Fig. 1). There was a noticeable fall during the first three days of chill-storage, and then a sharp rise on the fourth day to about 340 μmoles/100 g. Then glucose declined, and by the tenth day had mostly disappeared.

Fig. 2 shows the pattern of changes in free ribose concentration. Free ribose is not present at death, and during the period 1–4 days in ice there is a steady increase in the concentration of this

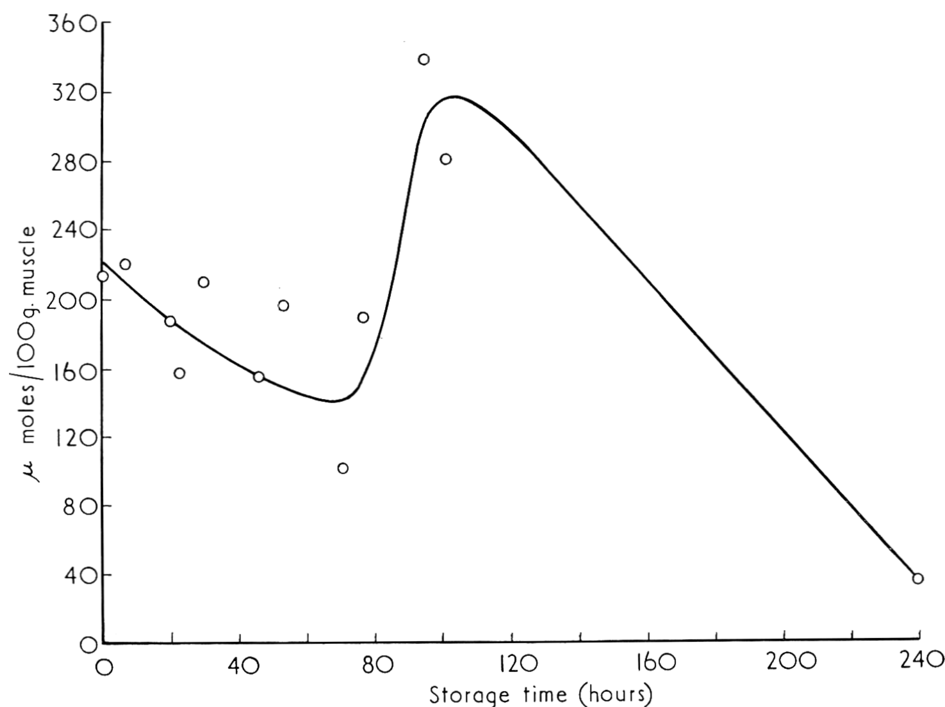

FIG. 1. Free glucose in muscle of chill-stored aquarium cod.

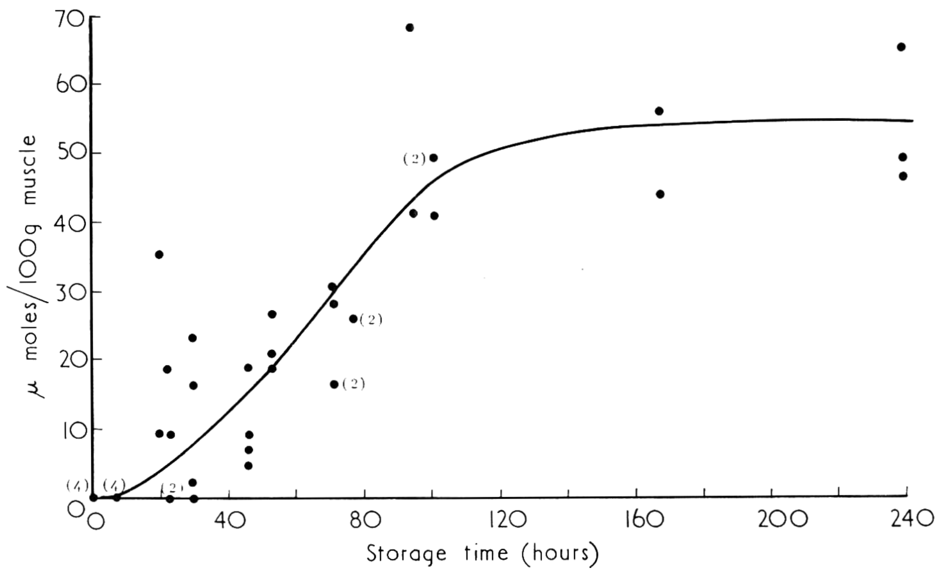

FIG. 2. Free ribose in muscle of chill-stored aquarium cod. (Figures in parentheses indicate the number of samples with a common value.)

sugar to 40–50 μmoles/100 g. From 4 to 10 days, ribose values are fairly constant, around the level of 50–60 μmoles/100 g.

Figs. 3, 4, and 5 show the concentrations at different times of various hexose phosphates and of ribose 1-phosphate. Separations of fructose 1-phos-

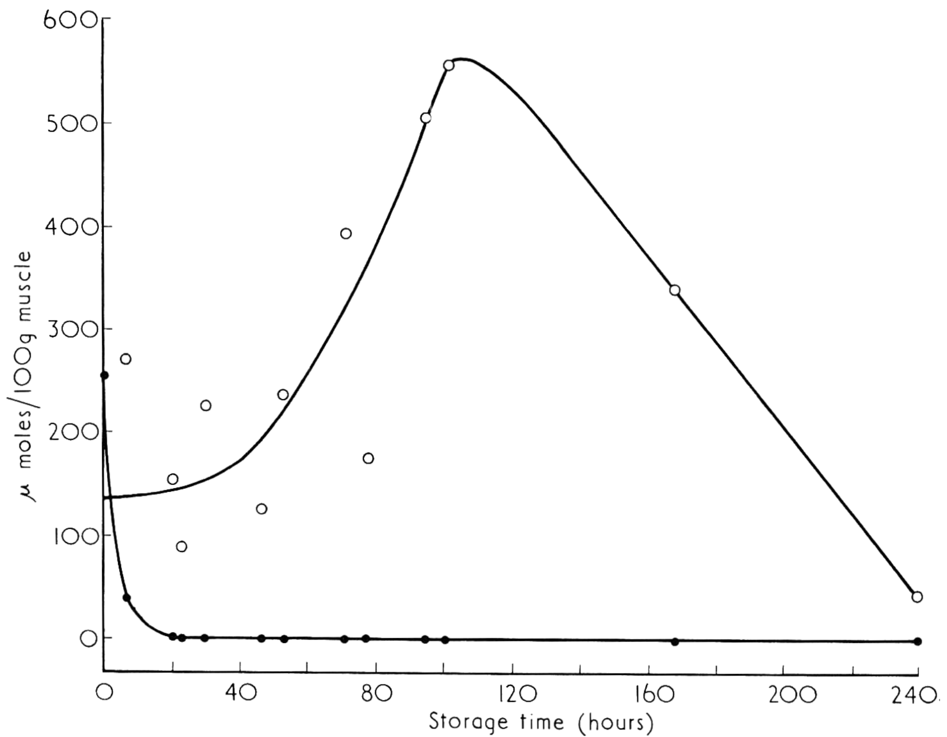

FIG. 3. Glucose 6-phosphate and fructose 1,6-diphosphate in muscle of chill-stored aquarium cod. Glucose 6-phosphate, ○—○; fructose 1,6-diphosphate, ●—●.

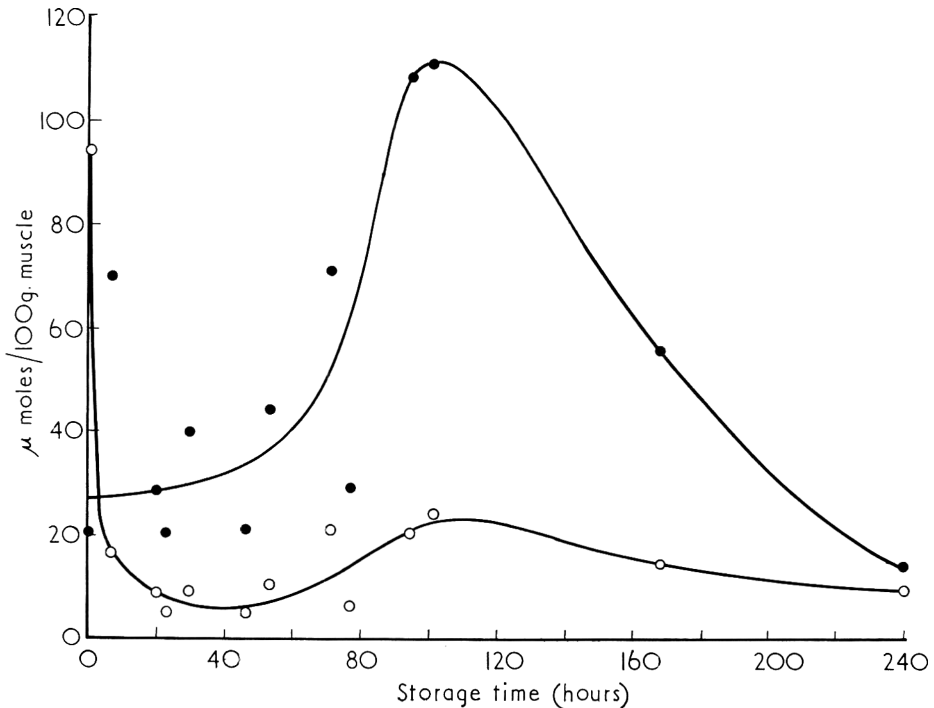

FIG. 4. Glucose 1-phosphate and fructose monophosphate in muscle of chill-stored aquarium cod. Glucose 1-phosphate, ○—○; fructose monophosphate, ●—●.

phate and fructose 6-phosphate were unsatisfactory in this material owing to the very high glucose phosphate levels relative to those in exhausted cod muscle (Jones and Burt, 1960). A combined value (fructose monophosphate) has been plotted in each case. For similar reasons, the unidentified ribose phosphate and ribose 5-phosphate fractions reported for trawled fish are not reported here.

Glucose 6-phosphate increased from 60–70 μmoles/100 g at death to a peak of 540–560 μmoles/100 g at four days, and then dropped to 40–50 μmoles/100 g at ten days (Fig. 3), whereas glucose 1-phosphate fell rapidly within the first day, from 90–100 μmoles/100 g to about one-tenth this value, then rose slowly to 20 μmoles/100 g at four days, and tailed off eventually to around 10 μmoles/100 g at ten days (Fig. 4). The concentration of fructose monophosphate at death was about 20 μmoles/100 g (Fig. 4). This rose during the first four days of chill-storage to a maximum of 110 μmoles/100 g, and then fell to 10–15 μmoles/100 g. Fructose 1,6-diphosphate concentrations fell from around 250 μmoles/100 g to zero in the first day after death (Fig. 3).

Fig. 5 shows the changes in concentration of ribose 1-phosphate. From zero concentration at death this compound increased to a maximum of 5–6 μmoles/100 g in 1–2 days, fell to less than 1 μmole/100 g at 4 days, rose again steeply to 9–10 μmoles/100 g and tailed off to around 7.0 μmoles/100 g at 10 days.

Rigor mortis set in 6–7 hr after death, and resolution of rigor followed about 40 hr later.

DISCUSSION

The muscle of sculpin *(Cottidae)* and tomcod *(Microgadus proximus)* frozen live in liquid nitrogen does not contain any free glucose, nor does lingcod *(Ophiodon elongatus)* muscle in *rigor mortis* (Tarr, 1954). In contrast with this, free glucose is present at death in the muscle of cod (40–180 μmoles/100 g) (Jones, 1958a,b, 1959) and of herring *(Clupea harengus)* (44–200 μmoles/100 g) (Hughes). Rested cod muscle contains about twice the concentration of free glucose as does that of exhausted cod (quoted above), and the changes in free glucose concentration during chill-storage follow different patterns in the two types of cod muscle since in trawled cod, the initial glucose level is maintained for 5–6 days, when it starts to decrease, reaching approx 70% of the initial value at 10 days (Jones, 1958b).

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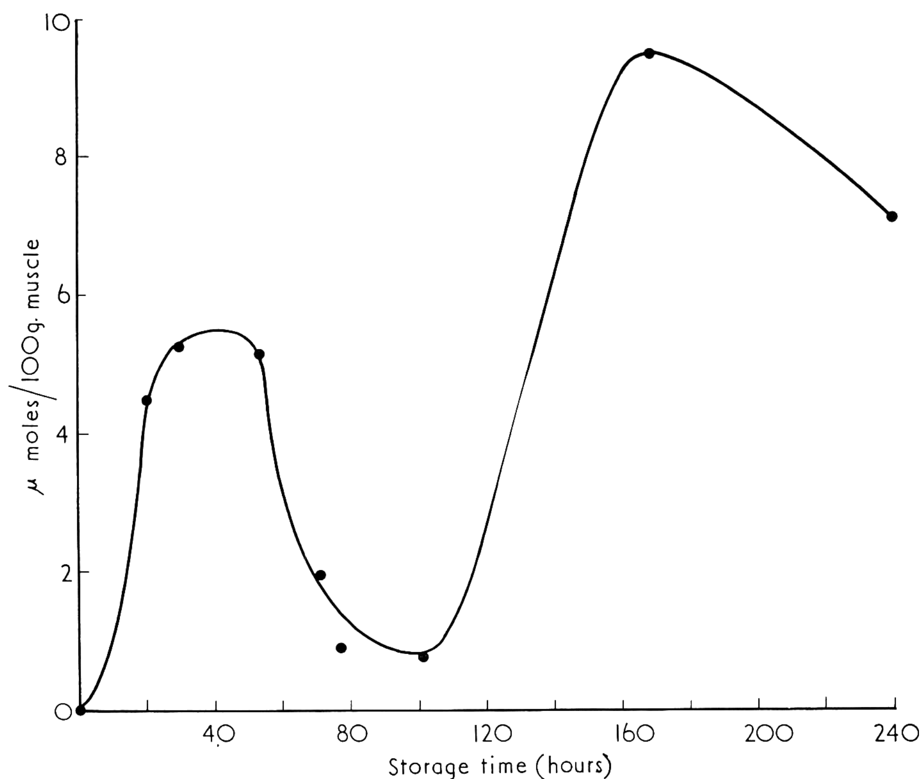

Fig. 5. Ribose 1-phosphate in muscle of chill-stored aquarium cod.

Other workers, investigating the following fish, have found that ribose is invariably absent from muscle at death, and that it appears there to varying degrees during chill-storage: lingcod (Tomlinson and Creelman, 1960), 4 species of Pacific salmon (genus *Oncorhynchus)* (Creelman and Tomlinson, 1960), herring (Hughes), sculpin (Tarr, 1954) and cod (Jones, 1958a,b, 1959). The changes in free ribose concentration (Fig. 2) resemble those obtained for exhausted cod (Jones, 1958b) in that free ribose is absent at death but increases steadily during 1–4 days in ice though the increase is much faster in the rested samples. A big difference is that, in rested cod, ribose levels are fairly constant, around 55 μmoles/100 g during the period 4–10 days, whereas the maximum for exhausted fish (23 μmoles/100 g) is not reached till 12 days after death.

A striking feature of the results obtained for sugar phosphates is that concentrations of glucose 1-phosphate and fructose 1,6-diphosphate at death are considerably higher in aquarium cod (90–100 and 240–260 μmoles/100 g respectively) than in trawled cod (20–25 and 80–90 μmoles/100 g, respectively) (Burt and Jones, 1961). A similar study by Fujimaki and Kojo (1953) on *Auxis tapeinosoma* (Bleeker) shows that glucose 1-phosphate in muscle is depleted in struggling, whereas such struggling increases the concentrations of glucose 6-phosphate and fructose monophosphate slightly, or has no effect on them. This agrees with the results presented here and earlier (Burt and Jones, 1961). Except for fructose 1,6-diphosphate, the pattern of changes in the sugar phosphates is markedly different in rested and exhausted cod muscle, but this difference could be ascribed in part to more frequent sampling in the rested case. In trawled cod muscle at 4 days glucose 1-phosphate, glucose 6-phosphate and fructose monophosphate concentrations have all dropped to a final level of less than 10 μmoles/100 g whereas in rested cod muscle, by this time, glucose 6-phosphate and fruc-

tose monophosphate have risen to the maximum concentrations shown in Figs. 3 and 4.

Brown (1960), using radioactively labeled glucose, showed that the Embden-Meyerhof pathway is primarily responsible for the oxidation of glucose in live carp and that the pentose cycle participates little. However, his methods do not make possible any differentiation between metabolism in muscle and that in the organs. Our results confirm that the Embden-Meyerhof pathway is of major importance in cod muscle also (Burt and Jones, 1961). Glycogen has been shown (Amano *et al.,* 1953) to be removed from fish muscle during struggling before death, and consequently the quantities of potential metabolites remaining to be supplied at the start of this pathway are greater in rested than in exhausted cod. This would account for the higher general levels of sugar phosphates found in aquarium cod muscle during storage. Glycogen present in fish muscle is broken down under the action of amylase (Andreev, 1958) and phosphorylase (Ono *et al.,* 1957) to dextrins (and eventually to maltose and glucose) and to glucose 1-phosphate, respectively. From the fact that maltose was not found in muscle extracts, it is to be expected either that the degradation did not proceed far enough during storage, or that any maltose formed was rapidly acted on further to form, possibly, free glucose. The latter course could account for the steep rise in glucose concentration in the third to fourth day after death. On the other hand, the similarity between the curves obtained for glucose (Fig. 1) and the hexose phosphates (Figs. 3 and 4), particularly in the period 3–10 days, might presage some connection whereby one is the precursor of the others.

The enzyme phosphoglucomutase catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate, but the kinetics of this reaction are very much in favor of formation of the 6-phosphate. In mammalian skeletal muscle (Najjar, 1955), the equilibrium mixture contains 94.5% glucose 6-phosphate. In aquarium cod muscle, glucose 6-phosphate constitutes 94.0–96.6% (mean 95%) of total glucose phosphate (Table 1) from 6 hours to 7 days after death. In mammalian muscle, glucose 6-phosphate can be metabolized further by phosphoglucose isomerase (Slein, 1955), yielding an equilibrium mixture of glucose 6-phosphate (68%) and fructose 6-phosphate. In aquarium cod muscle, glucose 6-phosphate constitutes 81.8–88.6% (mean 84.7%) of total glucose 6-phosphate plus fructose monophosphate (Table 1) from 20 hours to 7 days after death. The constancy of these two percentages over a wide range of actual concentrations supports the theory that the enzymes phosphoglucomutase and phosphoglucose isomerase are present in cod muscle. The discrepancies immediately after death and after 7 days in ice are probably due to equilibrium not being established in the first instance and to some other factor (? bacterial) interfering in the second. The effects of high aldolase activities and low ATP concentrations have already been discussed (Burt and Jones, 1961) in the case of trawled cod during chill-storage, and the phenomena encountered in this respect in the case of aquarium cod substantiate those explanations.

Table 1. Amount of glucose 6-phosphate relative to glucose 1-phosphate and fructose monophosphate in muscle of chill-stored aquarium cod.

Time in ice (hr)	No. of samples in pooled extract	Glucose 6-phosphate	
		% [a]	% [b]
0	4	44.1	76.5
6½	4	94.0	79.3
20	2	94.3	88.6
22½	4	94.7	81.8
29½	4	96.1	85.0
46	4	96.2	85.8
53	3	95.8	83.8
71	4	94.9	84.7
77	3	96.6	85.9
94½	3	96.0	82.4
101	3	95.9	83.3
168	3	95.8	86.0
240	3	82.4	76.3

[a] Per cent of total glucose phosphates.
[b] Per cent of total glucose 6-phosphate plus fructose monophosphate.

The changes taking place in ribose 1-phosphate concentration are very different from those occurring in trawled cod muscle, where there is a sharp rise to a maximum of

10 μmoles/100 g between 1 and 4 days followed by a gradual tailing off to zero at 19 days (Burt and Jones, 1961). This compound is presumably formed from purine ribonucleosides by the action of a phosphorylase (Tarr, 1956), but the reasons for the fluctuations (Fig. 5) are unknown.

The fact that sugar phosphates can contribute appreciably to "browning" in dehydrated or dry-salted cod has been discussed (Burt and Jones, 1961). From the known reactivities of glucose 6-phosphate and fructose 1,6-diphosphate (Schwimmer and Olcott, 1953), and the higher concentrations of these compounds and of glucose and ribose in rested than in exhausted cod, it is to be expected that line-caught cod would "brown" more than trawled cod.

ACKNOWLEDGMENTS

Dr. N. R. Jones supplied the extracts of cod muscle that were used in this work. Drs. N. A. Matheson and I. F. Penny of the Ministry of Agriculture, Fisheries and Food, Experimental Factory, Aberdeen, supplied details of their unpublished glucose oxidase procedure. The work described was carried out as part of the program of the Torry Research Station of the Department of Scientific and Industrial Research.

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The Autoxidation of Crystallized Beef Myoglobin [a]

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SUMMARY

The autoxidation rate in air for crystallized beef muscle myoglobin and the temperature dependence of the autoxidation were studied. Results show that autoxidation of beef muscle myoglobin is essentially the same as that reported by others for horse heart myoglobin. The rate constant for autoxidation was found to be dependent upon the amount of sodium hydrosulfite used to reduce metmyoglobin. It was shown that increased autoxidation, resulting from relatively high concentrations of sodium hydrosulfite, was not due to hydrogen peroxide. Control of the amount of sodium hydrosulfite used to 0.005% or less substantially reduced fluctuations observed in rate constants for autoxidation. For crystallized beef muscle myoglobin at pH 5.7 and 30°C, the rate constant is 0.21 ± 0.02 hr^{-1}. The abnormally high temperature dependence of myoglobin autoxidation and the relative importance of bacteria and autoxidation as causes of meat discoloration were discussed.

Deteriorative color changes take place in fresh red meats packaged for self-service sale. The changes may be considered to be of two types: 1) a darkening of the surface of the meat due to desiccation, and 2) a brown discoloration resulting from exposure of the meat to oxygen.

The darkening of the surface of meat has been attributed to the concentration of pigments as a result of the loss of water (Urbain, 1952). This type of discoloration can be controlled by selection of packaging films with low permeability to water vapor (Kraft and Ayres, 1954) and by increasing the relative humidity of the surrounding air (Penrod and Baker, 1954).

The second type of discoloration is less amenable to control. The cause is generally agreed to be oxidation of myoglobin or oxymyoglobin (Mb or MbO_2) to metmyoglobin (Mb^+). Such an oxidation occurs when purified myoglobin or hemoglobin solutions are exposed to air. With meat the situation is complicated by the fact that bacteria are inevitably present on the surface of the meat, and appearance of the brown color can be accelerated by inoculating meat with bacteria (Butler *et al.*, 1953).

Brooks (1929) observed that metmyoglobin in meats first forms beneath the surface, where the partial pressure of oxygen is less than at the surface. This observation led Brooks to investigate the autoxidation of hemoglobin at different partial pressures of oxygen (Brooks, 1935). He found that the rate of autoxidation of hemoglobin was first-order with respect to reduced hemoglobin ($Hb + HbO_2$) and was greatest when the partial pressure of oxygen was such (*ca.* 20 mm Hg) that hemoglobin was half oxygenated.

George and Stratmann (1952a, b) studied the autoxidation of crystallized horse heart myoglobin and found that the reaction was first-order with respect to reduced myoglobin ($Mb + MbO_2$) and that the rate of autoxidation was greatest when myoglobin was half saturated with oxygen. In addition, the temperature and pH dependence of the autoxidation was studied. With regard to temperature (George and Stratmann, 1952b), it was found that the activation energy for autoxidation of myoglobin in air was 25,000 calories. The rate of autoxidation in air was found to increase approxi-

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mately fourfold for a decrease in pH from 6.44 to 5.35 (George and Stratmann, 1954).

Watts (1954) has noted the lack of studies on myoglobin and its oxidation with regard to meat color, whereas hemoglobin is frequently used for such a purpose due to its ready availability.

In addition to applicability to meat color problems, the autoxidation of myoglobin is of considerable general interest. The mechanism of the oxidation is not clearly understood, particularly in view of the finding that a tenfold excess of oxygen is utilized per mole of myoglobin oxidized (George and Stratmann, 1952a). Furthermore, the function of myoglobin in muscle and other tissues of living animals and the susceptibility of myoglobin to autoxidation *in vivo* are areas in which data on the autoxidation of crystallized myoglobin may apply.

The present study was undertaken to learn more about the autoxidation of myoglobin from beef skeletal muscle, with the purpose of relating these findings to meat color problems and more general aspects of myoglobin oxidation. Since George and Stratmann (1952a, b, 1954) have made a thorough study of autoxidation of crystallized horse heart myoglobin, the immediate objective of this study was to determine if George and Stratmann's results applied to crystallized myoglobin from beef skeletal muscle.

METHODS

A. Preparation of crystalline myoglobin. The starting material for extraction and purification of myoglobin was lean beef muscle from animals grading U. S. Standard or U. S. Utility. Muscles from the shoulder and the round were used. The meat was trimmed of fat, passed twice through a food chopper, and then hemogenized for one min in a Waring blender with an equal weight of water (or sufficient water that the homogenate could be easily transferred for centrifugation). The homogenate was centrifuged for 15 min at 2,000 rpm and the supernatant extract decanted. The residue was re-extracted by mixing with an equal weight of water and allowing to stand overnight at 5°C. After a second centrifugation the two extracts were combined and concentrated to approximately 1/10 of the original volume by pervaporation from dialysis tubing at room temperature.

The concentrated extract was then heated 5 min at 55°C and the precipitated protein removed by centrifugation. Myoglobin is not precipitated by the heat treatment, but is usually oxidized to Mb^+ at this stage of purification. The partially purified Mb^+ was further concentrated by pervaporation from dialysis tubing at room temperature. The original volume of extract from 10 lb of meat was approximately 8 L. At this stage of concentration and purification, the volume was reduced to 100–200 ml.

To crystallize myoglobin, it is necessary to saturate the solution with $(NH_4)_2SO_4$. This may be done by dialyzing against saturated $(NH_4)_2SO_4$ as used by Theorell (1932), or by adding solid $(NH_4)_2SO_4$ until the solution is saturated (Lewis and Schweigert, 1955). With either procedure it is important to maintain the pH at about 6. Lower pH values may result in protein denaturation. As the concentrated myoglobin solution became saturated with $(NH_4)_2SO_4$, colorless protein precipitates were removed by centrifugation until crystals of myoglobin formed. The crystals were washed with saturated $(NH_4)_2SO_4$, dissolved in H_2O, and recrystallized by saturation with $(NH_4)_2SO_4$. Finally the crystals were washed with saturated $(NH_4)_2SO_4$, dissolved in 0.6*M* phosphate buffer pH 5.7, and dialyzed against the same buffer until no precipitate formed when tested with 10% $Ba(OH)_2$. The purified myoglobin solutions were stored at 5°C.

B. Measurement of autoxidation rates. The autoxidation of myoglobin was followed by measuring the decrease in light absorbancy at 580 mμ for an MbO_2 solution using a Beckman DU spectrophotometer and 1-cm cuvettes. A quantity of the purified Mb^+ solution sufficient to give an absorbancy of 0.6–0.8 when oxygenated and diluted was pipetted into a 50-ml Erlenmeyer flask. A small quantity of sodium hydrosulfite ($Na_2S_2O_4$) was added to reduce the Mb^+, and the solution was shaken in air until the reduced myoglobin became oxygenated. The solution was made up to 10 ml with 0.6*M* phosphate buffer at pH 5.7, and incubated at the desired temperature in a water bath. Samples were withdrawn periodically for determination of light absorbancy, and returned to the flask. After about 2 hr (at 30°C) the myoglobin solution was oxidized by adding a small amount of potassium ferricyanide. Taking the initial absorbancy reading as 100% MbO_2 and the final reading as 100% Mb^+, the per cent MbO_2 remaining after various intervals can be calculated.

RESULTS AND DISCUSSION

A typical plot for autoxidation of crystallized beef myoglobin at 30°C is shown in Fig. 1. When log per cent MbO_2 is plotted against time, a straight line results, indicating that the autoxidation is first order with respect to unoxidized myoglobin. The rate

constant in this particular experiment is 0.22 hr^{-1}. For the same set of conditions (0.6*M* phosphate buffer at pH 5.7 and 30°C), George and Stratmann (1952a) found that autoxidation rate constants for

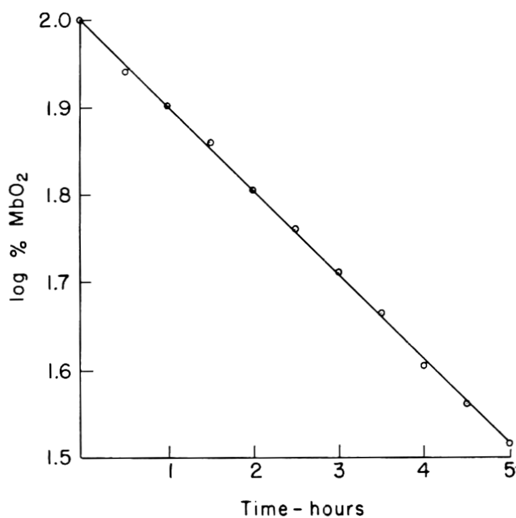

Fig. 1. The autoxidation of myoglobin showing the first-order dependence on MbO_2 concentration. Rate constant = 0.22 hr^{-1}.

crystallized horse heart myoglobin ranged between 0.38 and 0.24 hr^{-1}. They reported that rates for any one preparation were reproducible and that the observed differences in rates were attributable to different myoglobin preparations. In the present study, however, considerable variation in rate constants, 0.30 to 0.18 hr^{-1}, was observed with the same preparation of crystallized myoglobin.

In an attempt to minimize the fluctuation in rate constants, a study was made of the effect of varying the amount of $Na_2S_2O_4$ added to reduce Mb^+. The resulting rate constants for different amounts of $Na_2S_2O_4$ are shown in Fig. 2. It is apparent from these results that the rate of autoxidation of myoglobin is dependent upon the amount of $Na_2S_2O_4$ used to reduce Mb^+.

With the larger amounts of $Na_2S_2O_4$ (0.1 to 0.2%), the rate curves for autoxidation showed two distinct phases, an initial rapid rate followed by a slower rate. An example of this type of curve is shown in Fig. 3. The data in Fig. 2 were derived in each instance from the slower, straight-line portion of the rate-constant curves. It is known that some H_2O_2 is produced when $Na_2S_2O_4$ reacts with heme pigments (Dalziel and O'Brien, 1957). Consequently, it was of interest to determine the effect of added H_2O_2 and of added catalase on the autoxidation rates.

When catalase was added to MbO_2 solutions that had been reduced with a minimal amount of $Na_2S_2O_4$, no effect was observed on subsequent autoxidation rates. When catalase was added to MbO_2 solutions reduced with 0.1–0.2% $Na_2S_2O_4$, the initial rapid rate of pigment oxidation was not observed, but the rate constants obtained were comparable to those prevailing in the absence of catalase. If H_2O_2 (0.0004% final concentration) was added to MbO_2 solutions reduced with a minimal amount of $Na_2S_2O_4$, the initial oxidation was rapid, but the subsequent slower rate was no different from that in the absence of added H_2O_2. From these experiments the conclusion was drawn that some H_2O_2 was being produced when 0.1 to 0.2% $Na_2S_2O_4$ was added to reduce Mb^+. However, the effect of the H_2O_2 was to cause an initial rapid oxidation of myoglobin, and the results shown in Fig. 2 of increased autoxidation with increasing amounts of $Na_2S_2O_4$ could not be attributed

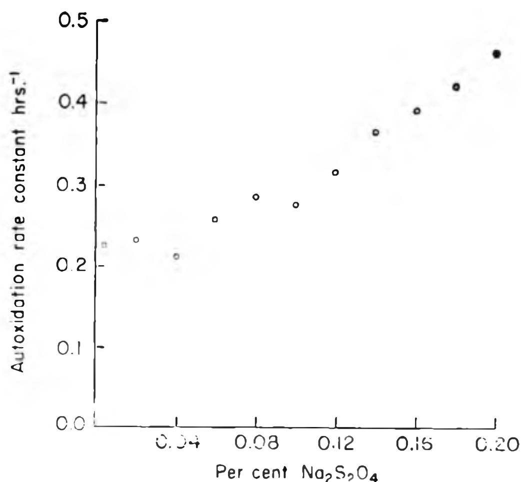

Fig. 2. The variation in autoxidation rate constants obtained with increasing amounts of sodium hydrosulfite.

to H_2O_2. The exact cause of the increased autoxidation rates has not been determined.

From Fig. 2 it is not readily apparent if a further decrease in the amount of $Na_2S_2O_4$ used to reduce Mb^+ would result in decreased rates of autoxidation. Since $Na_2S_2O_4$ rapidly reacts with O_2 in solution, and since $Na_2S_2O_4$ had to be put into solution for accurate measurement of the fractions of a milligram used to reduce Mb^+ solutions, there is a limit below which one cannot go in decreasing the amount of $Na_2S_2O_4$ used. Consequently, to determine if a further decrease in $Na_2S_2O_4$ would decrease the autoxidation rate, $Na_2S_2O_4$ was put into solution under N_2 and added to Mb^+ solutions that were made anaerobic by N_2 gassing. This technique allowed a tenfold decrease in the amount of $Na_2S_2O_4$ used (0.0005%, compared with the minimal amount of 0.005% shown in Fig. 2). The autoxidation rate under these conditions remained approximately 0.2 hr^{-1}, showing that further decrease in $Na_2S_2O_4$ did not decrease the rate of autoxidation.

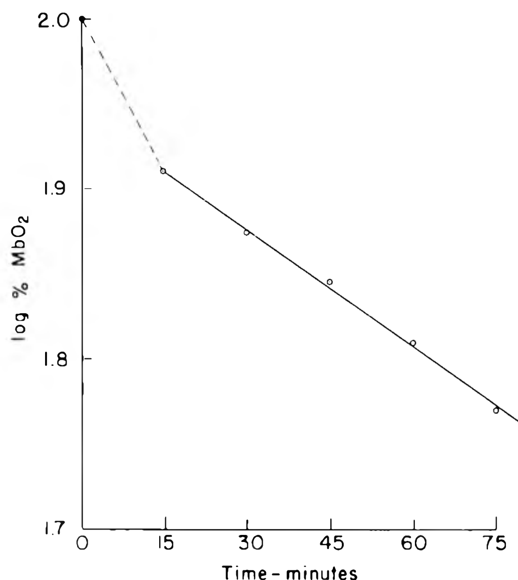

Fig. 3. Form of the rate-constant curve obtained with 0.12% sodium hydrosulfite.

By controlling the amount of $Na_2S_2O_4$ used to 0.005% or less it was possible to reduce considerably the amount of fluctuation found in rate constants for autoxidation of myoglobin. For example, in 18 separate experiments using several different crystallized myoglobin preparations, the autoxidation rate constants all fell within the range of 0.19 to 0.23 hr^{-1}. These results indicate that the autoxidation in air of beef skeletal muscle myoglobin has essentially the same rate as for horse heart myoglobin. The findings that autoxidation rates are increased by relatively high concentrations of $Na_2S_2O_4$ and that this effect can be eliminated by lowering the $Na_2S_2O_4$ concentration are pertinent to the study of autoxidation of any heme pigment systems in which the use of a reducing agent is necessary.

Since George and Stratmann (1952b) found an unusually high activation energy for the autoxidation of horse heart myoglobin, it was of interest to compare the beef muscle myoglobin in this respect, also. Autoxidation rates were determined at 40, 36, 32, 28, 20, 10, and 0°C for crystallized beef myoglobin (Fig. 4). The activation energy was found to be 23,700 calories, a value that checks closely with the 25,000 calories found by George and Stratmann for

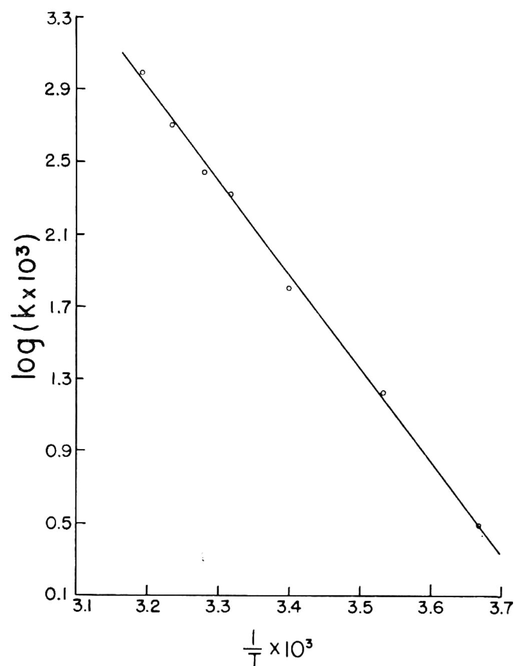

Fig. 4. Relationship between rate constants for autoxidation of myoglobin and temperature.

horse heart myoglobin. Of interest with respect to meat color is the high Q_{10} of 4.8 found for 0 to 10°C. This means that the rate of autoxidation of myoglobin would approximately double for a change in temperature from 0 to 4°C. The general observation has often been made that the color of meat is highly dependent upon the storage temperature. Results shown in Fig. 4 may provide a basis for the high degree of temperature dependence.

To control effectively the brown discoloration in fresh red meats packaged for self-service sale, it is necessary to know more about the oxidative reaction causing metmyoglobin formation. If, as postulated by Butler *et al.* (1953), the oxidation is due primarily to the presence of bacteria, then control measures directed at reducing the initial contamination and inhibiting the growth of bacteria would be most effective. The data presented in this study show that myoglobin is subject to an autoxidation not caused by bacteria. The MbO_2 solutions used to determine autoxidation were free of gross microbial contamination, and the short time used for determining rates, the relatively low pH and high buffer strength, and the constancy of rates with different myoglobin preparations preclude involvement of bacteria. Apparently, meat that has not been intentionally inoculated with bacteria will undergo a color deterioration that is independent of the bacterial contamination. Since Brooks (1929) has shown that the initial discoloration takes place 2–3 mm below the surface of meat, it is difficult to understand how bacteria growing on the surface cause the oxidation of myoglobin at some distance removed but not in immediately adjacent areas.

At present, information is lacking for determining if bacterial contamination, autoxidation, some unknown mechanism, or a combination of these factors is responsible for meat discoloration. If techniques were available for precise measurement of the rate of discoloration in a piece of meat, experiments could be designed to elucidate the causative mechanism. For example, the rate of discoloration by autoxidation in which a constant per cent of myoglobin is oxidized per unit of time would be quite different from the rate due to a constantly increasing bacterial population. Furthermore, precise rate studies could be used to differentiate between the temperature dependence of biological oxidation (characterized by an activation energy of 10–15 kcal and a Q_{10} of 2–3) and the unusually high temperature dependence of autoxidation of myoglobin.

With regard to maintaining the bright red color of fresh meats, the best procedure at present would be to keep storage temperatures as low as possible without freezing the meat. Such a procedure should effectively retard the discoloration, regardless of the causative mechanism.

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The Use of Natural Gamma Activity to Measure the Composition of Pork and Lamb Samples [a]

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SUMMARY

The gamma activity of 20 pork and 15 lamb samples, varying over a wide range in composition and each weighing 38 lb, was used to predict their chemical composition in terms of percentage of water, fat, and protein. All correlations between the estimated potassium content of the samples and the sample components were highly significant. However, the standard errors of the regression equations indicated that none of the relationships between the chemical components of the lamb samples and their potassium content were close enough to be of practical significance. The results on the pork samples seemed more promising. The standard errors suggest that chemical analysis should be used when accurate results are required or when the range in composition is small. However, advances in instrumentation may increase the accuracy of the K-40 method. Some of the difficulties in applying the method to the measurement of meat composition are discussed.

The possibilities of using the natural gamma activity of an isotope of potassium to measure the composition of meat has been discussed from a theoretical viewpoint by Anderson (1959). The isotope, K-40, is a constant proportion of naturally occurring potassium, which is a normal component of meat. Anderson has given the rationale for the relationship between potassium and the mass of lean tissue.

The gamma activity of K-40 has been used to measure the composition of humans (Woodward *et al.*, 1956), live pigs (Zobrisky *et al.*, 1959), pork hams (Kulwich *et al.*, 1958; Kulwich *et al.*, 1960a), and live sheep (Kirton *et al.*, 1961). The beta activity of K-40 has been used to predict the chemical composition of pork hams with reasonable accuracy (Kulwich *et al.*, 1960b). The only studies based on a reasonable number of observations, however, are the studies of Kulwich *et al.* (1960b), which suggest that the K-40 method of measuring meat composition may be useful.

The present experiment was designed to check the usefulness of the K-40 method with comminuted pork and lamb samples.

EXPERIMENTAL

Pork and lamb samples. Both the pork and lamb samples were obtained from several carcasses which were boned out and prepared at the Michigan State University Meat Laboratory. No attempt was made to keep separate the meat from different carcasses within each species. The range in chemical composition of the meat samples approximated that commonly found in sausage emulsions. The meat was ground into a homogeneous mixture in a silent cutter, which is used for making sausage emulsions. Exactly 19.0 lb of the meat emulsion were packed into separate waterproof cardboard cartons about 10 in. high and 9.5 in. in diameter. At the same time a sample was taken for chemical analysis. Each carton of meat was capped to prevent evaporation and frozen for ease of handling and to prevent deterioration. Twenty pork samples and seventeen lamb samples were prepared.

Chemical analyses. Water and protein were determined according to the procedure of Benne *et al.* (1956). Ether-extract was determined by a modification of the method of Benne *et al.* as described by Orme *et al.* (1958).

Scintillation counter. Radioactivity was measured with the Radiation Counter Laboratory Model 55400 Ratio Computer (Regas *et al.*, 1959). It is

[a] Journal Article 2796, Michigan Agricultural Experiment Station, East Lansing.

a large scintillation detector with a centrally positioned cylindrical counting well 12 inches in diameter and 24 inches deep. Pulse heights were counted on the range of 1.2–1.6 Mev., which includes the K-40 peak (1.45 Mev.). Limiting the range reduced the possibility of counting radioactivity from other elements. Only five of the eight photomultiplier tubes were functional, which probably reduced counting efficiency.

Counting methods. Two samples were always counted at the same time; thus, the gamma activity of the meat was determined on 38-lb batches. Each of the 19-lb samples was counted in two different combinations, giving a total of 20 observations for pork and 15 for lamb. Samples were measured in pairs to increase the precision of counting. All samples and backgrounds were counted for two consecutive 5-min periods. With the first 10 pork combinations, background counts were taken after each sample counting period. Since the background remained fairly constant for the remaining pork, two pairs of samples were counted between each background determination. With lamb, background counts were made only after counting every third pair of samples (40-min intervals).

Potassium standards. Four 19-lb lots of sugar, which were shown to have no gamma activity, were put in cardboard containers identical to those used for the meat samples. Three lots of chemically pure KCl containing 33, 66, and 132 g of potassium were added to three of the sugar cartons to act as potassium standards as outlined by Anderson (1959). Then each standard was counted with the pure sugar sample, giving 38-lb standards. The potassium standards were counted for two 5-min periods twice during the experiment.

RESULTS AND DISCUSSION

Potassium standards. The counts per second (cps) on the potassium standards were related to the grams of potassium in the standards by the following linear equation:

$$K\ (g) = 2.6554\ \text{cps} + 0.52$$

Since the potassium standards fulfilled the requirements discussed by Anderson (1959), the above equation was used to predict the potassium content of the meat samples. The predicted potassium content was then expressed as a percentage of the fresh meat sample. The scintillation detector recorded an average of 0.3716 cps per gram of potassium. Since the natural mixture of potassium isotopes emits 2.96 gamma rays per second per gram (Anderson, 1959), the efficiency of the detector was estimated to be 12.6%. It is probable that a higher counting efficiency would have been achieved if all eight photomultiplier tubes had been functioning. Conceivably, any lowered efficiency could have reduced the accuracy of all determinations.

Meat samples. The composition of the meat samples is presented in Table 1. There was quite a wide range in composition, with the variation for pork and lamb being about the same.

Table 2 shows the correlation and regression equations used in predicting the composition of the meat samples from the estimated potassium percentages. In pork, the composition could be predicted quite accurately. The standard errors of the equations based on the gamma activity of the pork are almost exactly the same as those given by Kulwich *et al.* (1960b) from the beta activity of pork ham samples. In lamb, in contrast, the error in estimating composition was quite high. The magnitude was similar to that observed in the most accurate regression equations for predicting the composition of lamb in a previous study (Kirton *et al.*, 1961). With both pork and lamb, the counting error (S.D.) was approximately 3.6, when expressed as a percentage of total number of counts. Since the background was

Table 1. Chemical composition of the counted meat samples.

	Mean	S.D.	Range
Pork (20 observations)[a]			
Water %	51.1	7.4	37.7 – 63.1
Fat %	33.5	9.5	17.5 – 50.7
Protein %	14.5	2.2	10.8 – 18.3
Potassium[b] %	0.244	0.050	0.153–0.323
Counts per second	15.7	3.22	9.7 – 20.8
Lamb (15 observations)[a]			
Water %	53.7	7.7	36.6 – 63.1
Fat %	30.0	10.0	18.4 – 52.5
Protein %	15.6	1.8	11.5 – 18.0
Potassium[b] %	0.256	0.046	0.171–0.323
Counts per second	16.5	3.00	10.9 – 20.8

[a] Each observation was made on a pair of 19-lb samples. The mean and range are therefore based on the 38-lb samples counted.

[b] Estimated from cps.

Table 2. Correlation coefficients and regression equations between % potassium and the chemical components of the meat samples.

Dependent variate	Correlation [a]	Regression equation [b]	$S_{y.x}$
Pork (20 observations) [c]			
Water %	0.977	Y = 144.89X + 15.70	1.61%
Fat %	−0.975	Y = 79.36 − 187.60X	2.20%
Protein %	0.962	Y = 42.14X + 4.20	0.61%
Lamb (15 observations) [c]			
Water %	0.917	Y = 153.13X + 14.39	3.20%
Fat %	−0.908	Y = 80.21 − 196.15X	4.35%
Protein %	0.883	Y = 34.93X + 6.62	0.89%

[a] All correlations significant at the 1% level.
[b] X = % potassium as estimated from cps.
[c] Each observation was made on a pair of 19-lb samples. The composition of these 38-lb samples has been related to their potassium content.

determined more frequently with pork, the over-all accuracy may have been greater than with lamb.

The work of Mounib and Evans (1960) and others provides a possible explanation for the lower relationships observed for lamb. It suggests that the concentration of potassium in the blood and skeletal muscle can vary between individual sheep and between breeds of sheep. Little information was available on the lamb used in this experiment, and it is possible that the different carcasses may have included lambs with different potassium levels, which could be responsible for at least part of the variation. Individual variation is unlikely to be the complete answer, however, since Mounib and Evans (1960) found no significant difference in potassium content of the lean tissue from lambs having different levels of blood potassium.

Applying the K-40 method to a continuous industrial process would not be easy, because of the difficulty of ensuring that a constant mass of material was being counted (Anderson, 1959). Similarly, when this method is used as a non-destructive experimental procedure, it is unlikely that an experimenter would want (or be able) to count large samples of exactly the same weight. Samples of different mass would complicate the calibration procedure and might reduce the accuracy of the results.

Present indications are that the K-40 method would not be accurate enough for estimations of lamb composition. For pork, the degree of accuracy appears more promising. It should be remembered that the statistical precision of the counting method (Anderson, 1959) applies only to the accuracy with which potassium can be estimated and takes no account of the errors of estimating meat composition from its known potassium content. Also, the variability of potassium in the bone and fat of carcasses and cuts will tend to lower the accuracy of the K-40 method. When Anderson (1959) stated that "there is no potassium in fat and very little in bone," he probably referred to pure fat and crystalline bone. Kirton *et al.* (1961) recently showed that there is about half as much gamma activity in fresh bone and a quarter as much in fatty tissues as in muscular tissue.

Another disadvantage of the K-40 method was the counting time required before estimates of the amount of potassium could be accurately obtained. For example, in the present experiment the lamb samples were counted for 10 minutes per pair to produce the relatively inaccurate estimates of sample composition. On the other hand, it may be noted that the more accurate estimates of the composition of pork samples were obtained when the background was determined more frequently than for the lamb. If the more frequent background determinations are partly responsible for this greater accuracy, then this would increase the difficulty of fitting the K-40 technique to a continuous system, as suggested by Anderson (1959).

ACKNOWLEDGMENTS

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Cellulose Crystallinity and the Reconstitution of Dehydrated Carrots

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SUMMARY

Blanched and unblanched dice of carrot phloem were dehydrated at two different rates and stored for periods up to 6 months. The rehydrated volume was always greater for blanched dice than for unblanched. In all samples, the rehydrated volume decreased as a result of storage. No consistent differences among samples could be attributed to differences in rate of dehydration. Cellulose, extracted from the dice, was examined for crystalline content by water-vapor sorption, X-ray diffraction, iodine adsorption, enzymatic digestibility, and density methods. All methods agreed in showing a higher crystallinity in unblanched than in blanched samples, a continual increase in crystallinity in all samples as storage time increased, and no notable differences in crystallinity as a result of different dehydration rates.

When carrots are dehydrated without being blanched (scalded), they not only lose much of their color during storage but also are tough when rehydrated and cooked (Morris, 1947). Their cellular reconstitution was considered briefly by Gane and Wager (1958), who suggested that cell walls absorb water and soften when a dried vegetable is put into water. Then, owing to natural elasticity, the walls tend to return to their original shape, and in so doing suck water into the cavity of the cell.

Thus, Gane and Wager (1958) concluded that any treatment that affects the natural elasticity of cell wall materials should also affect the over-all volume of the rehydrated tissue. They claimed that high temperatures during drying reduce that elasticity. Kaloyereas (1950) also found that reabsorption of water was greater for carrots dried at lower temperatures than for carrots dried at higher temperatures.

Earlier work by the present authors (Shimazu and Sterling, 1961) has shown that crystallinity increases in gels of cellulose and of calcium pectinate during dehydration, and even during storage. (Cellulose is taken in the physiological sense, i.e., including the more resistant hemicelluloses.) Those results were not related to the volumetric properties of rehydrated plant tissues, however, nor were the investigated polysaccharides derived from a dehydrated tissue. In the present study, blocks of carrot tissue were used to examine the roles of blanching, drying rate, and storage time in volumetric reconstitution. In addition, cellulose *(sensu lato)* was extracted from each sample and examined for its crystalline/amorphous composition.

MATERIALS AND METHODS

Experimental. About 23 kg of carrots (Imperator variety) were washed and peeled. The slender tips of the taproots and the green portions of the hypocotyls were removed, and the secondary xylem was trimmed away. The remaining secondary phloem segments were diced (7-10 mm on a side), yielding about 14 kg of sample. After the dice were mixed well, they were divided into four groups. Two groups remained unblanched, and two groups were blanched in steam (7 min at 98–102°C) and cooled. One blanched and one unblanched group were dehydrated rapidly, and the other blanched and unblanched group were dehydrated slowly: dehydration conditions were as described previously (Shimazu and Sterling, 1961). The samples were dehydrated until moisture content was about 4%. Final weight of material was about 2 kg.

Four storage periods at 30°C were used: 0, 2, 4, and 6 months, with a separate container for

every sample. Each sample was placed in a lacquered can, which was sealed under 15 in. of vacuum by a mechanical double seamer.

Analytical. When a can was opened, all except 30 g of carrot dice were ground and then extracted to remove the non-cellulosic components. The extraction procedure followed the earlier method (Shimazu and Sterling, 1961): washing in ethanol, extracting with ethanol-benzene mixture, refluxing with dilute HCl, washing, refluxing with dilute NaOH, and washing again. For crystallinity determinations the cellulose samples were dried in air at room temperature and then ground to pass an 80-mesh screen. The 30 g of untreated dice were used to determine moisture content and rehydration volume.

Moisture content. Ten g of carrot dice were ground to pass a 30-mesh screen and then weighed in duplicate in tared aluminum moisture dishes. The carrots were dried 10 hr at 70°C under 27 in. of vacuum.

Rehydration volume. Twenty g of dice were placed in a 250-ml volumetric flask nearly filled with distilled water, and held at room temperature during the swelling period. At 1, 2, 4, 6, 8, and 24 hours, distilled water was added to the flask until the total volume became exactly 250 ml. Then the flask was inverted and allowed to drain 2 min, while the unabsorbed water was received in a graduated cylinder. The volume of the carrots was then the difference between the volume of unabsorbed water and 250 ml.

Crystallinity determinations. The methods of the earlier study (Shimazu and Sterling, 1961) were followed exactly for water vapor sorption, X-ray diffraction, iodine adsorption, enzymatic digestibility, and density measurements. The rationale of this choice of methods is given in that study. Suffice it to say that there is adequate evidence that the relative accessibility of cellulose molecules is related directly to the reactivity of the cellulose product and inversely to its density (Hermans, 1949; Nickerson and Haberle, 1946; Shimazu and Sterling, 1961; Urquhart and Williams, 1925).

After 24 hours of swelling, some samples were prepared for histological observation by the usual techniques (Johnsen, 1940). Sections were cut at 10 μ and stained with methylene blue, particularly to delineate the cell walls.

RESULTS

During storage, there is little change in moisture content among the specimens (Table 1). The moisture content is virtually the same at 6 months as immediately following dehydration. Slowly dehydrated carrots appear to retain a higher moisture content than rapidly dehydrated ones, and the moisture content of the blanched carrots is somewhat lower than that of unblanched ones. Unlike results with model systems (Shimazu and Sterling, 1961), the values of the moisture content here are not consistently related with the other analytical findings.

Volume changes during a 24-hour rehydration period are also shown in Table 1. The rate of rehydration was somewhat erratic among the samples, posibly as a function of the relatively low temperature used. Nevertheless, on the basis of the 24-hour results, blanched carrots reconstitute to larger volumes than unblanched ones, and the reconstituted volume tends to decrease during storage. Fig. 1 shows a cross section of phloem tissue of a rehydrated unblanched carrot at 6 months, and Fig. 2 shows a cross section of phloem tissue of a rehydrated blanched carrot at 6 months. Note the more wrinkled cell walls and smaller cell size in the former, which indicate poorer reconstitution. Because the slowly dehydrated blanched sample has the largest rehydrated volume and the slowly dehydrated unblanched one has the smallest, little can be said about the effect of dehydration rate. No sample attained the original fresh volume (160.8 ml).

Uptake of water vapor by the extracted cellulose (Table 2) showed similar effects. Again, blanched samples adsorb more water vapor than unblanched, and adsorption capacity decreases continuously during storage. Likewise, the comparison of rapid versus slow dehydration leads to no consistent results.

When the sorption ratio (Hermans, 1949) is computed, i.e., when the desorption isotherm is related to that for native cellulose of cotton, crystallinity values for the samples may be estimated (Table 2). These are based on an amorphous content of 30% in cotton cellulose (Hermans and Weidinger, 1949). It will be seen that the crystallinity values reflect the differences that are apparent in the water vapor uptake: the higher the crystallinity values, the less water vapor adsorbed. Crystallinity values, as determined from X-ray diffraction measurements, are likewise presented here. They corroborate the other results in showing: 1) lower crystallinity in blanched than in unblanched samples; 2) an increase in crystallinity in all samples during storage; and 3) no notable differences between rapidly and slowly dehydrated samples.

Crystallinity values yielded by the iodine-adsorption method are extremely high (*cf.* Nickerson and Haberle, 1946), so high that few differences can be detected among the samples. The data do show that crystalline content is lowest in slowly dehydrated blanched carrots. In addition, crystal-

Table 1. Analytical results for dehydrated carrots.

Rate of dehydration	Blanching conditions	Storage (months)	Moisture content [a] (%)	Rehydration volume (ml) for six swelling periods (hr)					
				1	2	4	6	8	24
Rapid	Unblanched	0	3.92	67.5	87.9	104.5	112.5	116.5	117.0
		2	......	69.0	80.5	88.0	90.5	91.0	102.5
		4	......	70.0	80.0	86.0	98.0	99.0	107.0
		6	3.90	71.0	81.0	87.0	91.5	94.5	101.0
	Blanched	0	3.56	56.1	71.4	94.5	112.5	128.5	138.0
		2	......	61.5	69.0	75.0	77.0	78.5	104.5
		4	......	59.5	65.0	69.0	86.5	92.5	124.0
		6	3.58	57.5	65.0	66.5	79.5	90.5	103.5
Slow	Unblanched	0	4.68	47.3	68.7	91.1	104.0	110.0	111.5
		2	......	53.5	76.0	88.5	90.5	93.0	96.0
		4	......	53.5	62.5	70.5	87.5	91.5	101.0
		6	4.69	54.5	55.0	69.5	79.0	86.5	93.0
	Blanched	0	3.99	53.5	76.4	107.0	126.3	139.0	145.0
		2	......	57.0	82.5	99.5	106.5	120.0	124.5
		4	......	56.5	72.0	81.0	102.0	110.5	136.0
		6	3.95	59.0	70.5	78.5	97.5	110.0	126.0

[a] Per cent of dry weight.

Table 2. Analytical results for cellulose from dehydrated carrots.

Rate of dehydration	Blanching conditions	Storage (months)	Water-vapor sorption relative humidity (%)			Crystallinity (%)			Cellulose digestibility (%)		Density (g/ml)
			30	50	70	Sorption ratio	X-ray	Iodine adsorption	6 hr	24 hr	
Rapid	Unblanched	0	6.98	8.04	11.00	52	56	98	4.5	18.0	1.51
		2	6.43	7.61	10.35	55	59	98	4.0	16.5	1.51
		4	6.01	7.80	10.05	56	62	98	3.2	14.9	1.61
		6	5.50	7.52	9.34	59	63	98	2.9	11.8	1.61
	Blanched	0	7.19	9.41	12.61	46	52	97	5.4	19.0	1.54
		2	6.82	9.19	11.12	50	57	98	5.0	18.2	1.57
		4	6.60	8.63	10.85	52	58	98	4.7	18.0	1.59
		6	6.30	8.00	10.20	54	58	98	4.7	17.3	1.61
Slow	Unblanched	0	6.42	7.98	10.84	54	57	99	4.8	18.0	1.52
		2	6.00	7.84	10.21	56	59	98	3.0	15.0	1.53
		4	5.86	7.72	9.86	57	60	98	2.9	12.6	1.61
		6	5.45	7.20	9.30	60	63	98	2.6	11.5	1.65
	Blanched	0	7.23	9.74	12.94	45	52	93	5.0	18.0	1.55
		2	6.30	7.96	10.42	54	58	95	4.7	17.0	1.54
		4	6.22	7.80	10.10	55	58	95	4.6	16.8	1.63
		6	5.81	7.72	9.92	57	58	96	4.6	16.9	1.71

linity values tend to increase in such carrots during storage.

Digestibility of cellulose, like water-vapor adsorption, reflects the accessibility of cellulose molecules—in this case, to enzymatic attack. Hence, the higher the digestibility, the greater the amorphous content. Immediately after dehydration, the blanched samples show slightly greater digestibility than the unblanched, during a 6-hour digestion period. (For a 24-hour digestion period, no real differences are obvious). During storage, however, digestibility declines for all samples, but much more in unblanched samples than blanched samples. Seemingly, the cellulose of the rapidly dehydrated samples has a slightly greater digestibility than the cellulose of slowly dehydrated ones.

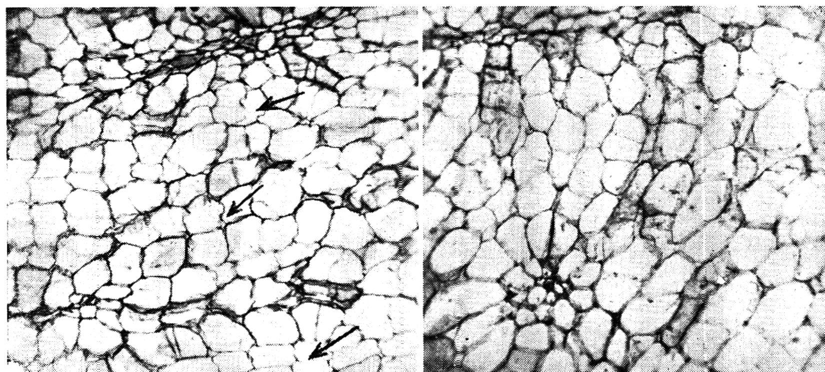
The principal consistent relationship in density determinations is that the density of the cellulose increases continuously in storage in all cases. It might also be suggested that cellulose has a higher density in slowly dehydrated carrots than in rapidly dehydrated ones. Density is not consistently related to the blanching history of the material. Incidentally, the 6-month sample of cellulose from slowly dehydrated blanched carrots has a spuriously high value, which should very likely be discarded. It must be noted that occasional density determinations can be quite erratic and that, in general, their duplicability is not very good.

DISCUSSION

In agreement with the results found earlier, most measures of extracted carrot cellulose indicate that a continued increase in crystallinity, at the expense of amorphous cellulose, occurs during storage. It is most interesting to note that this agreement was found despite the fact that the carrots were extracted *after dehydration*, with manipulations involving heating and immersion in hot water. Perhaps, because of this treatment, the difference in crystallinity between rapid dehydration and slow dehydration noted earlier (Shimazu and Sterling, 1961) has not been apparent in the present experiment.

Although there is an erratic increase in rehydration volume of carrot dice at the 4 months' testing period, this apparent change in the material is not reflected in the other measurements that were made. These latter show a continuous decrease in amorphous content with time. Gane and Wager (1958) also reported a slow increase in toughness and a decrease in rehydration capacity in stored dehydrated vegetables. In general, the rehydrated volume is quite significant. Twenty g of dried dice expand to a minimum volume of 93 ml and a maximum volume of 145 ml after 24 hours of soaking at room temperature.

From previous results (Shimazu and Sterling, 1961), it is known that the maxi-



Figs. 1–2. 1) Cross section through secondary phloem of rehydrated unblanched carrot after 6 months of storage (slow rate of dehydration). Arrowheads are located near regions of marked cell-wall wrinkling. Note that the cell walls in the whole section all show some degree of wrinkling or folding. X102. 2) Cross section through secondary phloem of rehydrated blanched carrot after 6 months of storage (slow rate of dehydration). Little evidence exists of any wrinkling in the cell walls. The cells are somewhat larger than those of Fig. 1 (samples from the same carrot root). The slightly lighter degree of cell-wall staining in this section is perhaps a consequence of the loss of cell-wall constituents (probably pectin) and the more swollen state of the cell wall, due to blanching. X102.

mum water uptake in rehydrated calcium pectinate gels is sufficient to give a volume of 48 ml per 20 g of dried gel. Because of its higher crystallinity, the cellulosic component of the cell wall will take up considerably less water during swelling. Even if this mitigating effect of the cellulose is ignored, it is apparent that cellular reconstitution is not simply a question of gel swelling but that it also involves a re-establishment of cell wall configurations. The folded, wrinkled walls tend to straighten out during rehydration. This effect is confirmed by microscopic sections made after 24-hour rehydration of the tissues.

The data presented here can be used to relate the crystallinity of cell-wall cellulose with the relative capacity of the walls to assume their original shapes. It is at once obvious that, contrary to the opinion of Gane and Wager (1958), the assumption of shape is not a question of elasticity of the wall. [The property of elasticity is a function of the crystalline content of the wall (Alfrey, 1948; Hermans, 1949; Meredith, 1956; Press, 1943.)] In fact, the more highly crystalline (and elastic) the cell wall, the poorer the volume of reconstitution. Volume recovery is promoted instead by a higher amorphous content of the cell wall components, permitting these to swell more. The highly crystalline substance swells poorly.

The regular increase of crystallinity during storage is a marked feature in all samples. Note that this change occurs without perceptible loss of moisture. It seems not unreasonable to suggest that crystallization in cellulose occurs in the same way as it does in retrograding starch: an increase in polysaccharide-polysaccharide bonding at the expense of polysaccharide-water bonding (Caesar, 1950). It is much easier for this process to take place in cellulose than in starch because most of the molecules in the cellulose microfibrils are already lined up in parallel fashion (Roelofsen, 1959). In starch, in contrast, the microfibrillar structure (Sterling and Pangborn, 1960; Sterling and Spit, 1958) is readily dispersed during gelatinization, and the molecular arrangement is more random. [Possibly, crystallization can also explain the increased toughness of dehydrated fish in storage (Connell, 1958) and the poor reconstitution of dehydrated meat (Wang *et al.*, 1953)].

The present study shows that blanching helps maintain the amorphous phase, apparently hindering the crystallization process during storage. Perhaps this preventive effect is related to further molecular separation in the amorphous regions with an increase in the extent of molecular disorder. Perhaps the blanching process also may cause some hydrogen-bond rupture at the margins of crystalline micelles. It is of interest that the blanching effect is still readily perceptible after six months. Without presenting evidence, Crafts (1944) declared that "prolonged" blanching tends to reduce the ability of cell walls to absorb water, swell, and reassume their original form. Further investigation of this point would certainly be highly desirable.

The method of enzymatic digestibility has not been used previously in the estimation of cellulose crystallinity. It appears to be an extremely sensitive technique, particularly at shorter reaction times. Moreover, even with longer reaction times, there is always a difference between cotton and carrot celluloses (Shimazu and Sterling, 1961), and changes in amorphous content due to storage are apparent. Possibly, the sensitivity of this technique is also related to the parallel orientation of cellulose molecules in native microfibrils: the formation of new crystallites will retard swelling in water and thus limit the physical access of the enzyme; it will also dramatically reduce the length of the molecular segments available for attack.

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A Study of the Free Amino Acids in Bovine Muscles

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SUMMARY

Paper chromatography was used in studying variations in the free amino acid content of beef muscle. Analysis of eleven cuts representing nine muscles from a cow shows a variation in the content of certain free amino acids. In general, the variation of the peak of the curve obtained from the chromatogram by a photoelectric densitometer is greatest in the leucine-isoleucine spot. The more tender cuts (*Longissimus dorsi* and *Psoas major*) contain more leucine-isoleucine than the less tender *Semitendinosus*. This was further evidenced in a comparative study of these same muscles from seven beef animals. In each of these seven animals the amounts of these free amino acids increased from the less tender to the more tender muscles. Since the results agree in general with those found by other methods, this study is now being extended to many more animals.

It was thought that a study of the free amino acids from muscle to muscle and animal to animal might show some properties or constituents of meat that would reflect their quality. It was not thought that certain free amino acids in themselves rendered meat tender or tough, but that more of one than another would be required in the process of growth of different muscles. Thus, it would be present in larger quantity in the meat juice as a source of supply for the formation of certain types of muscular tissue. It was hoped that a relationship could be found between the relative amounts of certain free amino acids and meat quality as represented by tenderness.

Paper chromatography was chosen as the best method of establishing the differences in free amino acids. Previous investigators (Bianchi, 1955; Colombo and Gervasini, 1955a, b, 1956; Grau and Böhm, 1958a, b; Leinati, 1957; Niewiarowicz, 1956; Walker, 1952) have used this method, but not in a systematic study of the variability of free amino acids from muscle to muscle or in the same muscles of different meat animals.

PREPARATION OF EXTRACTS

Five grams of meat were cut into fine pieces and boiled 1 hr in 100 ml of distilled water. The material was then filtered through a folded filter paper and the residue washed 3 times with hot distilled water. The combined filtrate and washings were concentrated to a small volume and made up to 2.5 ml. This means that 1 ml of the extract was equivalent to 2 g of the meat.

CHROMATOGRAPHY

These meat extracts were spotted on Whatman no. 1 filter paper cut to 28.5×23.0 cm for one-dimensional chromatography and 28.5×28.5 cm for two-dimensional chromatography. In one-dimensional chromatography five samples were chromatographed at one time by placing 5 lambda on five evenly spaced spots on the lower edge of the paper. In two-dimensional chromatography 20 lambda were spotted in the lower right-hand corner of the paper. The moist spots were dried in a current of warm air and the paper was rolled into stainless-steel coils as described by Ma and Fontaine (1949). The coils with the paper were inserted into 1000-ml graduated cylinders to which had been added 60–65 ml of the developing solvent system. The most satisfactory solvent found for the one-dimensional chromatogram and the first dimension of the two-dimensional chromatogram was *n*-butanol, acetic acid, and water (60 : 15 : 25, v/v). The stoppered cylinders containing the solvents and coiled paper were placed in an oven at 60°C for development until the solvent ascended to the upper edge of the paper. This took 6–6.5 hr. For two-dimensional chromatography, the paper

was removed from the cylinder, dried at 60°C in the oven, and rolled again into coils in the proper direction and placed in the cylinder containing the second solvent system: phenol, water, and ammonium hydroxide (100:20:0.1, v/v). Development was carried out in an oven at 60°C. This phase took 5.5–6.0 hr. The papers were dried, as before, in a chromatographic oven at 60°C.

For color development the papers were sprayed uniformly with ninhydrin reagent (0.5 g ninhydrin, 100.0 ml 70% ethanol, and 0.5 ml 1*N* sodium hydroxide). Approx 5 ml of reagent was needed for each 28.5×23.0-cm sheet. The sprayed chromatograms were allowed to develop at room temperature. The time required for the spots to appear may be used as a guide to the identity of the amino acids. Isatin (0.2% in 70% ethanol plus 1% glacial acetic acid) was used to confirm the identification.

After spraying and color development, the one-dimensional chromatograms were cut into strips, one for each unknown sample. The relative densities of the spots were measured on a "Spinco Analytrol," using a B-1 cam and 550-mμ filters. (Made by Spinco Division, Beckman Instruments, Inc. In listing the name of a manufacturer no discrimination is intended and no guarantee of reliability is implied.) A map (Fig. 1) showing the locations of thirty known amino acids was prepared with the same solvent systems and conditions as used in preparing the two-dimensional chromatograms. Identities were established by com-

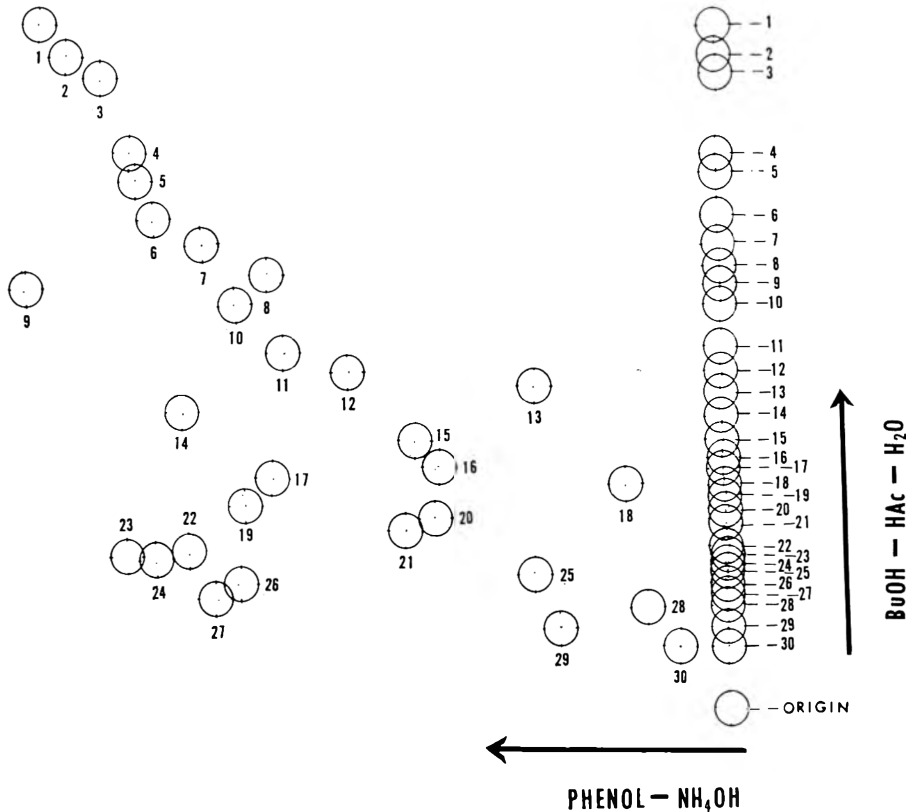

Fig. 1. Map of 30 known amino acids. This is a composite map from three two-dimensional chromatograms and averaged R_f values of six sets of one-dimensional chromatograms developed by using solvent systems: BuOH-HAc-H_2O (60:15:25) and phenol-H_2O-NH_4OH (100:20:0.1). The numbers represent: 1) leucine, 2) isoleucine, 3) phenylalanine, 4) valine, 5) methionine, 6) tryptophan, 7) α-aminoisobutyric acid, 8) tyrosine, 9) proline, 10) β-alanine, 11) alanine, 12) threonine, 13) glutamic acid, 14) hydroxyproline, 15) glycine, 16) serine, 17) glutamine, 18) aspartic acid, 19) citrulline, 20) taurine, 21) asparagine, 22) arginine, 23) carnosine, 24) anserine, 25) ornithine, 26) lysine, 27) histidine, 28) cysteine, 29) cystine, 30) cysteic acid.

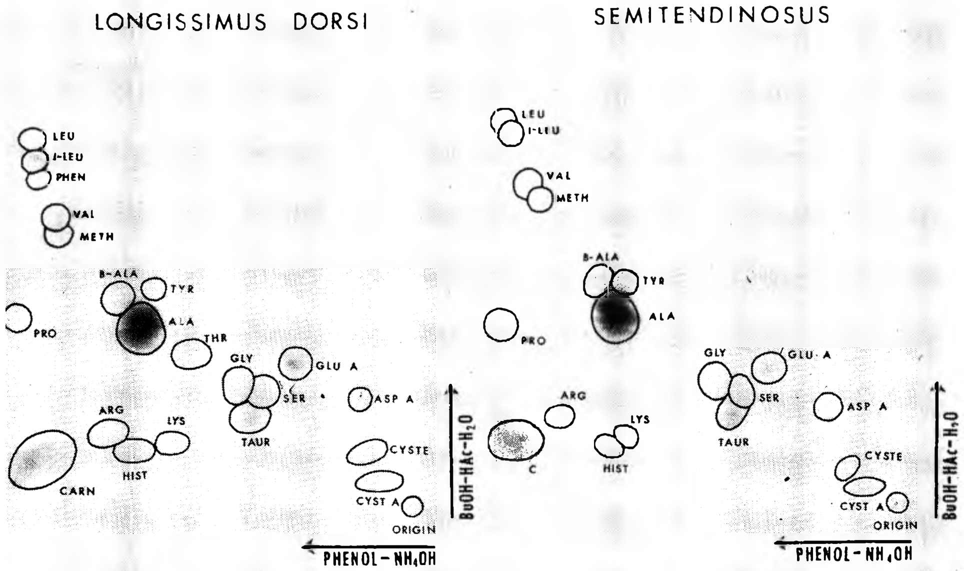

Fig. 2. Two-dimensional chromatograms showing spots of the individual amino acids present in the *Longissimus dorsi* and in the *Semitendinosus* muscles of the same animal.

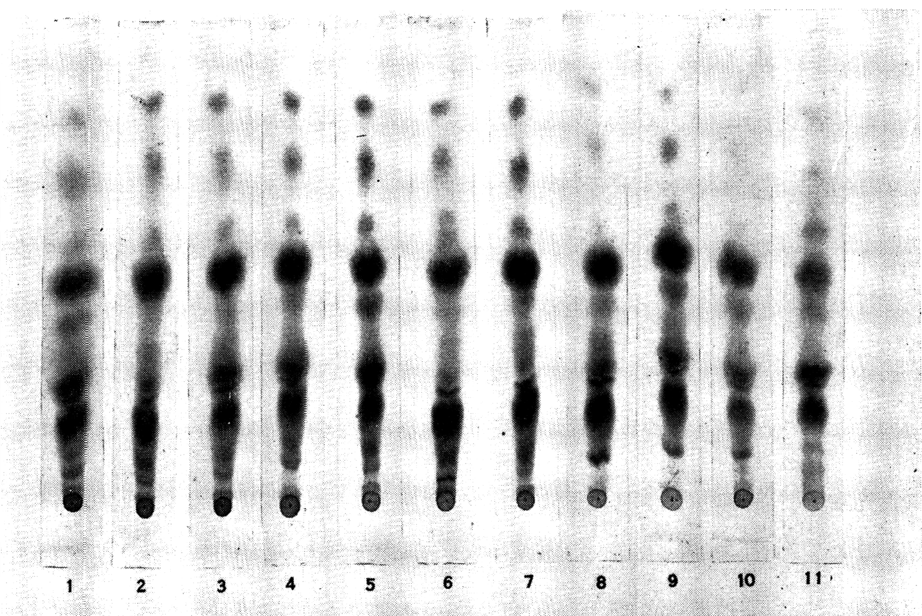

Fig. 3. One-dimensional chromatograms showing 11 muscles from a 4-year-old cow: 1) *Semitendinosus*, 2) *Semimembranosus*, 3) *Biceps femoris*, 4) *Gluteus medius*, 5) *Psoas major*, 6, 7, 8) *Longissimus dorsi*, respectively, shortloin, 8th rib, and 3rd rib, 9) *Triceps brachii*, 10) *Digital flexor*, and 11) *Serratus ventralis*.

paring the locations of the spots from muscle extracts with those on the map of the known amino acids.

The two-dimensional method has shown the following amino acids and peptides to occur free in bovine muscles: leucine, isoleucine, phenylalanine, valine, methionine, β-alanine, alanine, tyrosine, proline, carnosine, arginine, lysine, histidine, threonine, glycine, serine, taurine, glutamic acid, aspartic acid, cysteine, and cysteic acid (Fig. 2). Specific tests were made for certain amino acids: glycine, lysine, and phenylalanine by the Peronnet and Truhaut reaction (1933); tyrosine by the method of Schwartz and Pallansch (1957); and serine and threonine by the method suggested by Schwartz (1955).

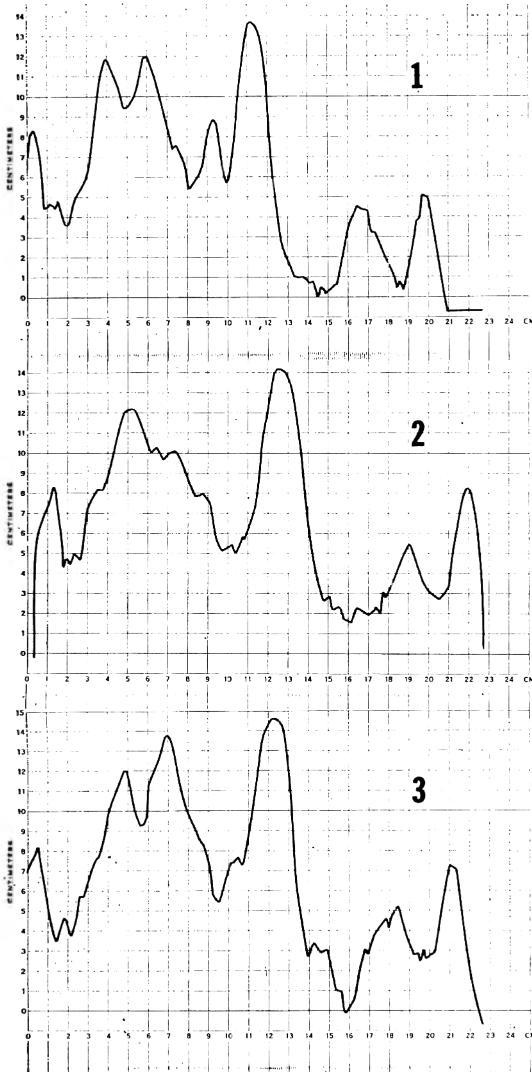

Fig. 4. Spinco analytrol curves of one dimensional chromatograms of free amino acids of beef muscle: 1) *Semitendinosus*, 2) *Semimembranosus*, 3) *Biceps femoris*.

EXPERIMENT ON MUSCLES

The meat samples used came from beef animals grown for the breeding, feeding, and management programs of the Animal Husbandry Research Division, USDA, Beltsville. The samples were stored at 35°F until the experiments began. Eleven samples, representing cuts from nine muscles, were selected for the study: 1) 1½ in. from cut surface of the round-*Semitendinosus*; 2) same round, but the *Semimembranosus* muscle; 3) same round, but the *Biceps femoris* muscle; 4) loin end or sirloin, the *Gluteus medius* muscle; 5) tenderloin, *Psoas major*; 6) short loin, *Longissimus dorsi*; 7) eighth rib, *Longissimus dorsi*; 8) third rib, *Longissimus dorsi*; 9) chuck, *Triceps brachii*; 10) foreshank, deep *Digital flexor*; 11) neck, *Serratus ventralis*.

Fig. 3 shows one-dimensional chromatograms of these eleven samples. Figs. 4, 5, and 6 show the curves obtained from these chromatograms made on a Spinco Analytrol. As expected, curves 6, 7, and 8 from the three samples of *Longissimus dorsi* are very similar.

Since the spot on the one-dimensional chromatogram (top spot, Fig. 3) that showed the greatest variability from muscle to muscle was leucine, isoleucine, and small amounts of phenylalanine (when present), the variability of intensity of this spot was used as a measure of these free amino acids in the *Semitendinosus*, *Psoas major* and *Longissimus dorsi* muscles. The muscles were from seven beef animals: one cow, 4 years old; four twins about two years old (2 steers and two heifers); and two yearling bulls. Fig. 7 shows that the content of these amino acids varies among the muscles of the seven animals used.

Fig. 8 shows the relative amounts of 21 free amino acids obtained from the *Longissimus dorsi* and *Semitendinosus* muscles. The greatest variation between *Longissimus dorsi* and *Semitendinosus* muscles was in free threonine content.

DISCUSSION

In general, the variation of the leucine-isoleucine peak on the curves follows a pattern similar to that found by Hiner and Hankins (1950) by other methods. The more tender cuts (*Longissimus dorsi* and *Psoas major*) contain more leucine-isoleucine than the less tender *Semitendinosus*. This is shown by the density of the spots produced by equivalent quantities of extract from the different muscles (Fig. 7).

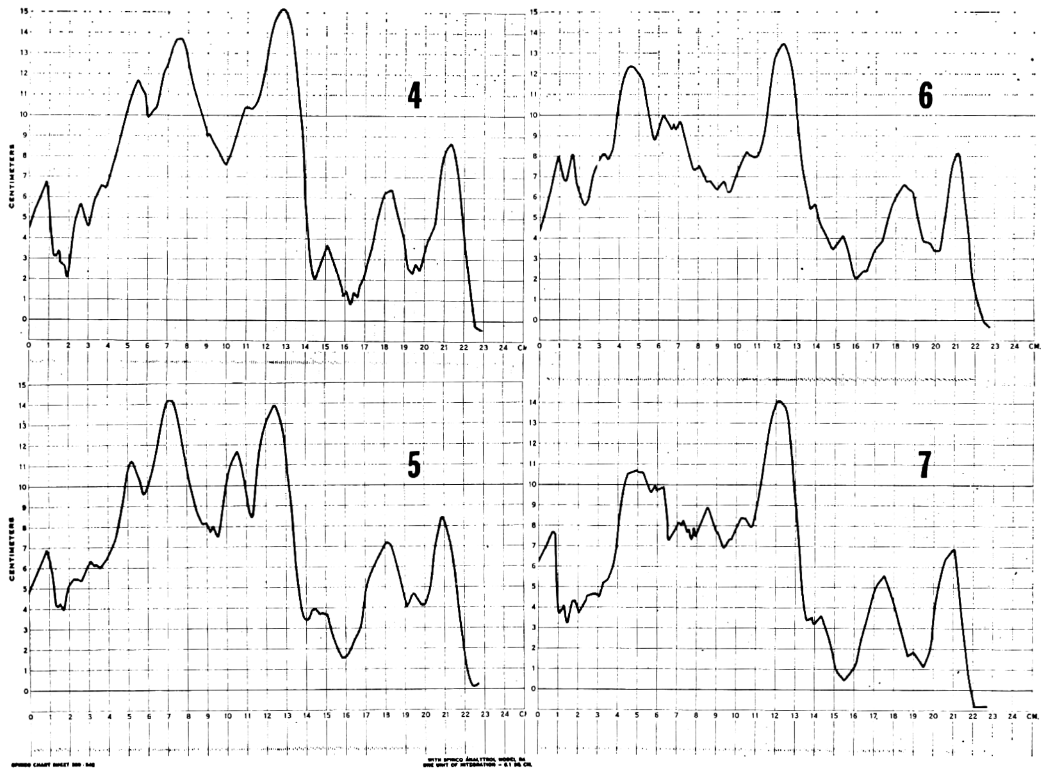

Fig. 5. Spinco analytrol curves of one-dimensional chromatograms of free amino acids of beef muscles: 4) *Gluteus medius*, 5) *Psoas major*, 6) *Longissimus dorsi* (shortloin), 7) *Longissimus dorsi* (8th rib).

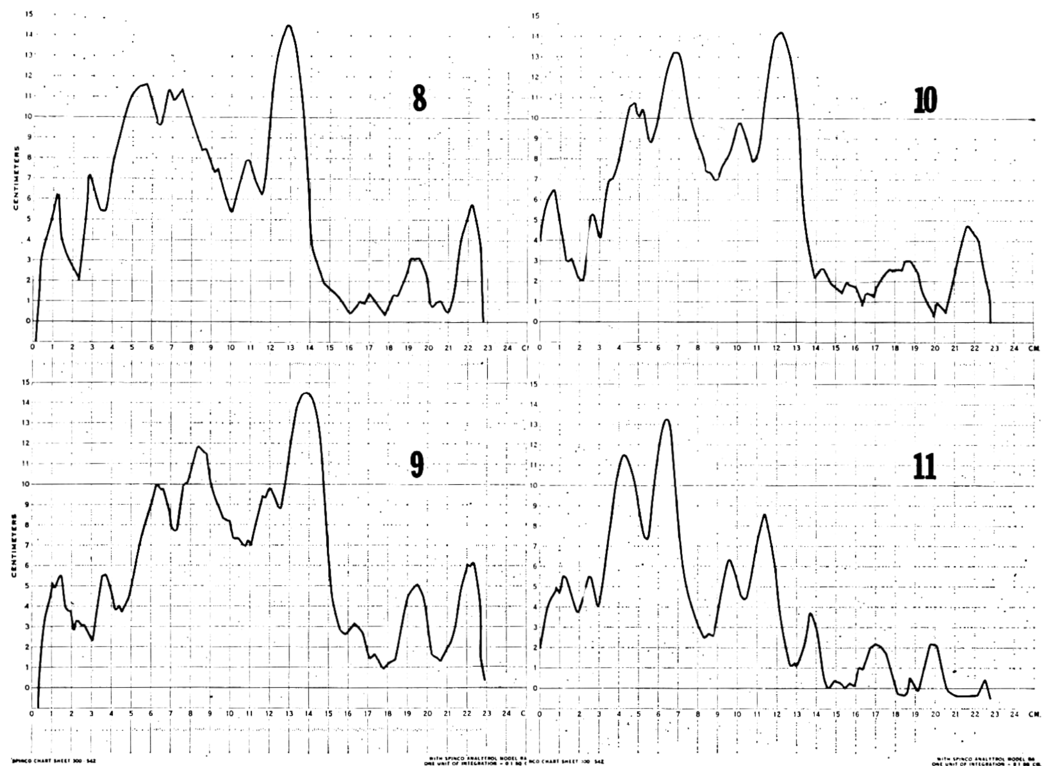

Fig. 6. Spinco analytrol curves of one-dimensional chromatograms of free amino acids of beef muscles: 8) *Longissimus dorsi* (3rd rib), 9) *Triceps brachii*, 10) *Digital flexor*, 11) *Serratus ventralis*.

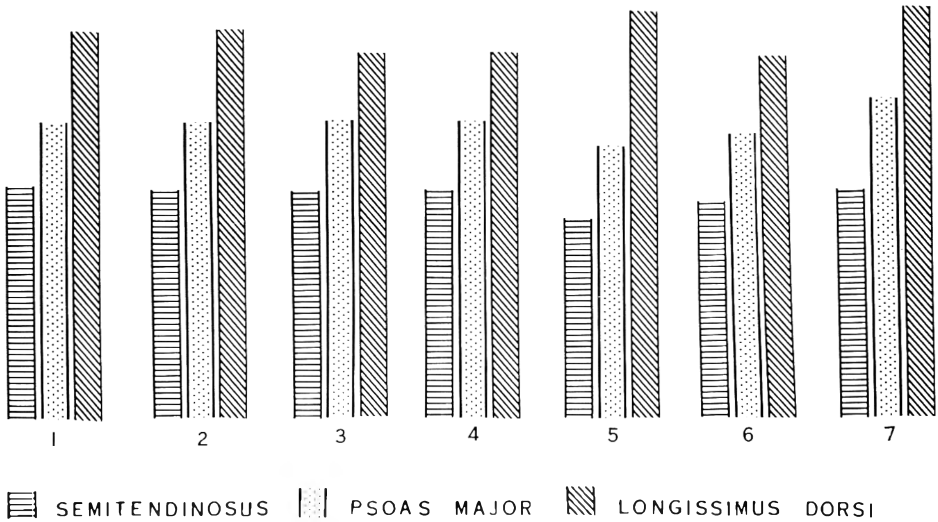

Fig. 7. Variations of leucine-isoleucine peaks obtained from one-dimensional chromatograms by use of a Spinco Analytrol. Three muscles were studied from seven animals, as follows: 1 and 2, twin steers; 3 and 4, twin heifers; 5 and 6, yearling bulls; 7, a cow.

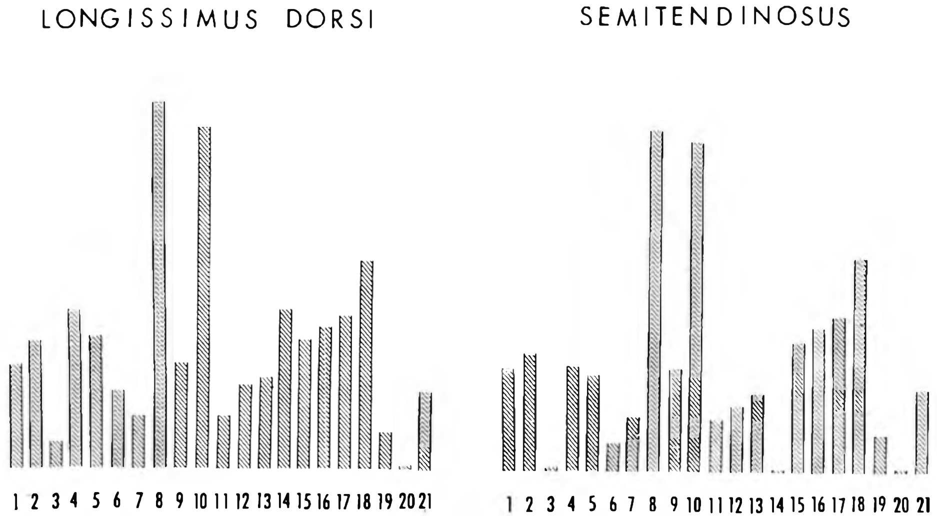

Fig. 8. The comparative values of amino acids present in *Longissimus dorsi* and *Semitendinosus* muscles of beef animals shown here is a summary of data from six different chromatograms for each muscle. 1) leucine, 2) isoleucine, 3) phenylalanine, 4) valine, 5) methionine, 6) β-alanine, 7) tyrosine, 8) alanine, 9) proline, 10) carnosine, 11) arginine, 12) lysine, 13) histidine, 14) threonine, 15) glycine, 16) serine, 17) taurine, 18) glutamic acid, 19) aspartic acid, 20) cysteine, 21) cysteic acid.

The relative amounts of leucine-isoleucine were measured in the *Semitendinosus, Psoas major,* and *Longissimus dorsi* of seven animals (Fig. 7). In each animal the amounts of these amino acids increased from the less tender to the more tender muscles. Threonine was found in the *Longissimus dorsi,* but was not identifiable in the *Semitendinosus* in the sample sizes used.

The results suggest that the method used is suitable for a survey to determine the relative tenderness of the same muscles in different beef animals. Such a study is in progress.

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The Distribution of Thiamine and Ascorbic Acid in the Potato Tuber [a]

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SUMMARY

Two zones that together approximate the cortical layer of two varieties of stored potatoes were compared in thiamine and total ascorbic acid content with the center region. In both varieties the cortical layer contained a slightly higher concentration of thiamine than the center. In Sebago but not in Russet Burbank, total ascorbic acid was higher in the cortical region. The components of total ascorbic acid were determined separately in the Russet Burhank variety. The biologically active fraction (reduced and dehydroascorbic acid together) constituted 90–97% of the total ascorbic acid.

Potatoes are known to vary in starch, mineral, and tannin content in various areas or zones of the same tuber. It was of interest to learn the distribution of thiamine, of reduced ascorbic and dehydroascorbic acid in the various zones or depths of tuber tissue. The effect of surface treatments or of depth of peeling could then be related to losses of these vitamins. This paper reports the distribution of these vitamins.

METHODS

Two varieties, Russet Burbank and Sebago, both grown in Wisconsin, were obtained in 100-lb lots from the retail market and stored at 40°F. A fourth of each of 10 tubers in each lot was taken by cutting the tuber from stem to bud-end. These quarters were peeled with a stainless-steel peeler, and the peel put aside. The peeled quarters were separated into three zones. The first zone consisted of a layer just beneath the peel, about 0.2 cm thick, which was obtained by again removing a layer with the hand peeler. The second zone consisted of the next two peelings taken together. These two zones together approximately comprised the cortical layers of the peeled tuber. The rest of the tuber quarter, treated as the third (central) zone, constituted the bulk of the potato tuber.

The samples were weighed, blended 2 min with extracting acid at maximum speed in an Osterizer, and aliquots were taken for analysis. Thiamine was determined as described by the Association of Vitamin Chemists (1951). Clarase was used to liberate bound forms of thiamine. The acid extracts were purified by adsorption on activated Decalso columns, and the thiamine was then estimated as thiochrome.

Ascorbic acid was determined by the methods of Roe and Oesterling (1944) and Roe *et al.* (1948) as combined into a general procedure by the Association of Vitamin Chemists (1951). Dehydroascorbic and diketogulonic acid were determined in the thiourea-metaphosphoric acid extracts, and total ascorbic acid in metaphosphoric acid extracts. Data were analyzed by methods of analysis of variance and significance determined between means by use of Duncan's multiple-range technique, (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Preliminary work showed that the thiamine content of the potato peel set aside was less than 2% of the total thiamine in the whole tuber. Table 1 shows the thiamine content of three zones in both varieties. The statistical analysis of the thiamine values in the three zones of both varieties revealed that the F values for zones were significantly different. Duncan's multiple-range test indicated that the first zone was not significantly different from the second in thiamine content, but the first and second zones were significantly different from the third zone in thiamine content. The variation due to lots was significant at the 5% level in

[a] Publication approved by the Director of the Wisconsin Agricultural Experiment Station, University of Wisconsin.

Table 1. Distribution of thiamine, total and reduced ascorbic acid, dehydroascrobic acid, and diketogulonic acid in Russet Burbank, and thiamine and total ascorbic acid in Sebago variety of potatoes.

	Zone 1 [a]	Zone 2 [a]	Zone 3 [a]
Russet Burbank			
Weight of tissue in zone (g)	35.5 (28.3–43.6) [b]	70.1 (60.8–79.3) [b]	197.3 (167.6–237.3) [b]
% weight in each zone	11.7	23.1	65.2
Thiamine (μg/g)	1.15 (1.12–1.17)	1.22 (1.18–1.24)	1.02 (0.91–1.11)
% thiamine in each zone	12.6	26.2	61.2
Total ascorbic acid (mg/100g)	6.2 (5.5–6.7)	6.5 (6.3–6.6)	8.0 (7.1–8.6)
% total ascorbic acid in each zone	8.7	18.4	72.9
Ascorbic acid (reduced form) (mg/100g)	4.4 (3.8–4.9)	4.9 (4.8–5.0)	7.2 (6.4–7.7)
% ascorbic acid in each zone	6.2	13.8	66.3
Dehydroascorbic acid (mg/100g)	0.8 (0.5–1.0)	0.8 (0.4–1.3)	0.4 (0.1–0.9)
% dehydroascorbic acid in zone	1.1	2.2	3.0
Diketogulonic acid (mg/100g)	1.0 (0.8–1.2)	0.8 (0.5–1.1)	0.4 (trace–0.5)
% diketogulonic acid in zone	1.4	2.4	3.6
Sebago			
Weight of tissue in zone (g)	38.5 (36.0–40.8)	79.1 (76.7–81.8)	210.7 (197.8–223.4)
% weight in each zone	11.7	24.1	64.2
Thiamine (μg/g)	1.21 (1.13–1.28)	1.24 (1.23–1.06)	1.00 (0.93–1.07)
% thiamine in each zone	13.1	27.7	59.2
Total ascorbic acid (mg/100g)	7.0 (6.8–7.1)	7.1 (6.8–7.3)	5.8 (5.6–5.9)
% total ascorbic acid in each zone	12.8	25.8	61.4

[a] Zones 1 and 2 together roughly represent the cortical layer left on a peeled potato, whereas Zone 3 is the center region.
[b] Average with range given in parentheses.

the Sebago, but not in the Russet Burbank variety. Since the first and second zones together correspond approximately to the cortical layers of the tuber, it is clear that these layers have a slightly higher concentration of thiamine than the center of the tuber. The thiamine content of Russet Burbank, when the peeled whole tuber is considered, varies from 1.00 to 1.13 μg/g. In Sebago the overall thiamine content varies from 1.03 to 1.14 μg/g of the tuber. These results agree with those reported by Cover and Smith (1948).

Table 1 shows the concentration of ascorbic acid, dehydroascorbic acid, and diketogulonic acid in the three zones of Russet Burbank, the amount of each of these expressed as per cent of total ascorbic acid in the tuber, and the total ascorbic acid of the Sebago variety. The total ascorbic acid content of both varieties and all zones varies from 5.5 to 8.6 mg/100 g of potato. On the basis of the whole peeled tuber, total ascorbic acid content varies from 6.9 to 8.0 mg/100 g in Russet Burbank, and from 6.0 to 6.3 mg/100 g in Sebago.

It must be pointed out that the ascorbic acid content of these varieties is lower than values for potatoes reported by Watts and Merril (1950). One reason may be the length of storage at 40°F, 6–8 weeks after purchase in October. The pattern of reduced ascorbic acid in the three zones of Russet Burbank is similar to that of total ascorbic acid. Thus, total ascorbic acid may give an approximation of reduced ascorbic acid in the raw sample. The statistical analysis indicated that, in both varieties, zones one and two were not significantly different in total ascorbic acid. In Russet Burbank there was no significant difference between zones one and two for reduced ascorbic acid, dehydroascorbic acid, or diketogulonic acid. In zone three, however, the concentration of total ascorbic acid was higher in Russet Burbank, and lower in the Sebago.

Data have been presented to show that the concentration of thiamine is slightly less in the center than in the cortical layers in both varieties. With total ascorbic acid, this holds good for Sebago but not for Russet Burbank. Since the center zone constitutes the bulk of the potato tuber, it contains the

major portion of the thiamine and ascorbic acid found in the tuber.

In Russet Burbank, the reduced ascorbic acid content of each zone was found to follow the same pattern as total ascorbic acid content. Ascorbic acid and dehydroascorbic acid together, which form the biologically active fraction of total ascorbic acid, form 90–97% of the total ascorbic acid content. Diketogulonic acid composed 3–9% of total ascorbic acid content of potatoes.

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Proximate Composition and Sodium and Potassium Contents of Four Species of Commercial Bottom Fish

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SUMMARY

The proximate composition and sodium and potassium contents of four species of bottom fish from Pacific Coast waters were investigated. Pacific Ocean perch, Pacific cod, and lingcod were quite uniform in composition regardless of species, size, season and area of capture, and method of preservation on board the fishing vessel. The averages were 79–81% moisture, 18–19% protein, 0.5–1.5% oil, 1.1–1.3% ash, 60–70 mg% sodium, and 390–440 mg% potassium. Sablefish varied greatly in oil content, with a range of 3–23% and an average of 15%. Variations in the other constituents were rather large; averages were 71% moisture, 13% protein, 1% ash, 56 mg% sodium, and 348 mg% potassium.

INTRODUCTION

This is the fourth and final report in a series on studies of the proximate and mineral composition of Pacific Coast bottom fish. Previous papers include data on halibut (Thurston and MacMaster, 1960), rockfish, and sole (Thurston, 1961a, b).

Pacific Ocean perch *(Sebastodes alutus)*, Pacific cod *(Gadus macrocephalus)*, lingcod *(Ophiodon elongatus)*, and sablefish *(Anoplopoma fimbria)* are fished extensively in Pacific Coast waters from Alaska to southern California (except that Pacific cod is seldom taken in California waters). In 1958, landings of these fish in California, Oregon, and Washington amounted to more than 30 million pounds. The 1958 catch of other important species was: salmon (all species) 66, halibut 21, and herring 11 million pounds (Power, 1958).

This paper compares the variation in composition of fillets of Pacific Ocean perch, Pacific cod, lingcod, and sablefish with regard to species; notes the effect that such factors as size of fish, season of capture, area of capture, and method of preservation may have on composition; and compares the variation in composition of the inedible parts.

EXPERIMENTAL

Collection of specimens. The fish from which samples were prepared for analysis were collected in 1958–60, from February to October, in an area extending from Hecate Strait south to Cape Lockhart, Oregon (Table 1). Most of the fishing was within 50 miles of shore and at 30–100 fathoms. The specimens were either frozen or stored in ice on board the fishing vessel, and were similarly held at the laboratory until the filleting was begun.

Preparation of samples. The fish were processed and the fillets removed as described (Thurston *et al.*, 1959). After removal of skin and bones, the raw fillet was ground to a homogeneous state, and a portion was hermetically sealed in a ½-lb can and held at −18°C until analyzed.

Methods of analysis. The prepared samples were removed from the cans and analyzed by standard methods as described (Thurston *et al.*, 1959) for the following constituents: moisture, protein (Kjeldahl N × 6.25), oil (mixed-ether extraction following acid hydrolysis), ash, and sodium and potassium (by flame photometer).

RESULTS AND DISCUSSION

Variations in physical data. In increasing order of weight, the species are Pacific Ocean perch, Pacific cod, sablefish, and lingcod (Table 1). Fishermen remove viscera and heads from sablefish immediately on capture, and drain the blood to prevent darkening of the flesh. Thus, for this species, data are based on the dressed fish; for the other species, the whole fish is used, since they are delivered "in the round" to processors.

Table 1. Source and physical data for four species of bottom fish.

Series No.	No. Fish	Cond. [a]	Place of capture	Date Mo.	Date Yr.	Length (cm) Av.	Length (cm) Range	Weight (kg) Av.	Weight (kg) Range	Fillet yield (%) Av.	Fillet yield (%) Range
Lingcod											
1	12	F	W. Cape Lockhart, Ore.	4	59	72	55–87	4.28	1.56–7.21	25.6	15.1–31.4
2	6	F	S.W. Cape Flattery, Wash.	4	60	63	53–73	2.06	1.40–3.24	41.2	40.5–42.1
3	10	F	S.W. Cape Flattery, Wash.	5	60	71	57–90	3.50	1.58–6.23	33.6	29.0–39.7
4	7	F	S.W. Cape Flattery, Wash.	6	60	82	69–96	5.20	2.96–7.35	32.6	28.0–35.8
5	12	I	W. Cape Flattery, Wash.	6	60	64	58–71	2.41	1.83–2.75	33.4	29.6–37.5
6	14	I	Hecate Strait, B. C.	8	60	69	64–81	3.03	1.58–5.51	34.1	31.9–36.0
7	14	F	N.W. Vancouver Is., B. C.	8	60	69	60–76	3.02	1.83–4.46	34.4	27.4–40.5
	75					70	53–96	3.36	1.40–7.35	33.6	15.1–42.1
Sablefish [b]											
1	14	F	S.W. Cape Flattery, Wash.	10	58	49	42–64	2.01	1.30–4.53	34.1	26.4–39.0
2	13	F	S.W. Cape Flattery, Wash.	5	60	56	45–72	3.16	1.26–6.28	43.1	37.0–49.1
3	15	I	Hecate Strait, B. C.	8	60	60	51–70	2.48	1.69–3.86	48.3	36.9–53.9
4	14	F	N.W. Vancouver Is., B. C.	8	60	56	49–74	3.28	1.76–6.67	40.5	30.4–47.1
	56					55	42–74	2.73	1.26–6.67	41.5	26.4–53.9
Pacific Ocean perch											
1	11	F	Cape Flattery, Wash.	10	58	40	34–43	1.00	0.61–1.33	24.2	21.4–29.4
2	12	I	W. Cape Flattery, Wash.	7	59	39	32–46	1.04	0.52–1.71	26.1	23.5–29.9
3	12	I	N.W. Vancouver Is., B. C.	10	59	40	34–44	0.90	0.55–1.16	26.4	24.5–30.1
4	14	I	W. Cape Flattery, Wash.	2	60	40	38–45	1.07	0.74–1.66	27.8	23.0–31.8
5	14	F	S.W. Cape Flattery, Wash.	3	60	39	31–52	0.99	0.51–2.42	26.2	21.0–29.9
6	14	I	W. Vancouver Is., B. C.	4	60	38	32–44	0.78	0.47–1.10	27.6	24.7–29.7
7	14	I	S.W. Cape Flattery, Wash.	6	60	37	31–42	0.75	0.49–1.25	27.7	23.8–31.6
8	14	F	N.W. Cape Flattery, Wash.	6	60	41	37–43	1.05	0.81–1.27	27.9	22.0–31.7
9	14	I	Hecate Strait, B. C.	8	60	40	33–43	0.89	0.53–1.20	27.7	24.8–32.4
10	14	F	N.W.Vancouver Is., B. C.	8	60	41	37–44	1.05	0.81–1.27	37.9	22.0–31.7
11	14	I	Cape Scott, B. C.	10	60	39	33–44	0.86	0.57–1.29	29.3	26.0–32.6
	147					39	31–52	0.94	0.49–2.42	27.1	21.0–32.6
Pacific cod											
1	14	F	Washington	2	58	59	50–66	2.65	1.45–3.42	21.7	15.0–26.1
2	13	F	S.W. Cape Flattery, Wash.	4	60	57	53–65	2.11	1.72–3.32	28.7	26.9–33.8
3	13	I	Straits, Wash.	4	60	55	49–60	1.56	1.02–3.21	29.5	25.3–34.3
4	14	I	S.W. Cape Flattery, Wash.	6	60	57	52–60	2.00	1.50–2.45	29.2	26.0–31.0
5	13	F	N.W. Cape Flattery, Wash.	6	60	57	51–62	1.98	1.38–2.83	30.6	24.8–33.4
6	13	I	Estaban Point, B. C.	8	60	60	54–67	2.20	1.35–3.15	29.9	27.8–35.0
7	14	F	N.W. Vancouver Is., B. C.	8	60	55	49–58	1.85	1.37–2.45	31.3	27.8–35.8
8	14	I	Cape Scott, B. C.	10	60	57	49–62	2.19	1.13–2.96	29.5	26.4–31.2
	108					57	49–62	2.07	1.02–3.42	28.8	15.0–35.8

[a] F = frozen; I = iced.

[b] Heads and viscera removed.

Table 2. Composition of fillets and inedible parts.

Species	No. fish	Moisture (%)		Protein (%)		Oil (%)		Ash (%)		Sodium (mg%)		Potassium (mg%)	
		Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range
A) Fillets													
P.O. perch	147	79.0	76.1–81.9	19.2	17.4–20.4	1.54	0.36–4.03	1.15	1.04–1.26	62	46–96	393	327–440
Pac. cod	108	81.5	79.7–83.2	17.9	15.8–19.1	0.62	0.31–0.78	1.20	1.06–1.30	71	53–120	405	326–441
Lingcod	75	81.1	79.1–85.1	17.7	14.4–19.4	1.01	0.57–2.31	1.22	1.09–1.31	60	43–93	438	371–492
Sablefish	56	71.5	63.9–82.4	12.9	11.8–14.1	15.2	2.8–22.6	1.00	0.86–1.15	56	39–82	358	305–396
B. Inedible parts													
P.O. perch	147	70.4	69.1–73.8	16.3	15.7–17.3	7.77	4.54–10.4	6.47	4.84–8.07	164	128–206	247	215–271
Pac. cod	108	78.6	77.3–79.7	16.5	15.0–17.7	1.92	0.94–5.17	3.54	2.17–5.85	160	136–207	259	200–312
Lingcod	58	75.0	73.8–75.7	15.6	15.1–16.1	5.7	4.4–6.2	4.2	3.3–4.8	147	132–170	281	257–308
Sablefish	41	61.7	55.5–65.4	10.8	10.3–11.4	25.0	22.0–29.3	2.4	1.8–2.9	131	120–138	207	200–218

In length, weight, and fillet yield, all four species showed large variations from the averages shown in the table. A comparison between length and weight can be misleading, since the latter varies considerably with stomach contents. For example, one 80-lb lingcod dressed 40 lb, and filleted 16 lb, whereas a 40-lb specimen had in its stomach three rocks totalling 11 lb. The fillet yield for the various species is of interest because of the large variation (15–42% for the whole fish) and also because, on the average, only about ⅓ of the whole fish is edible flesh.

Variation in the composition of fillets. *Species.* Pacific Ocean perch, Pacific cod, and lingcod are high in protein and low in oil and sodium, whereas sablefish is very high in oil and correspondingly low in protein (Table 2).

Pacific Ocean perch has the highest average protein content of the group and is higher in this constituent than are all other bottom fish except halibut. The low average oil content (1.5%) was quite uniform; although one specimen analyzed 4%, very few exceeded 3%. The 62 mg% average for sodium is low for salt-water fish and not much higher than values for many fresh-water species (Thurston *et al.*, 1959).

Pacific cod has a very uniform composition, averaging: moisture 81.5%, protein 17.9%, oil 0.6%, ash 1.2%, sodium 71 mg%, and potassium 405 mg%. This species offers a good source of protein for low-fat diets and, also, is not high in sodium.

Lingcod compares favorably with Pacific cod. The averages are moisture 81.1%, protein 17.7%, oil 1.0%, ash 1.2%, sodium 60 mg%, and potassium 438 mg%.

Sablefish is in definite contrast to the other three species. Oil content is very high (15.2% average), and the other constituents are correspondingly lower. The averages are moisture 71.5%, protein 12.9%, ash 1.0%, sodium 56 mg%, and potassium 348 mg%. Variations from these averages were large, owing to the great fluctuation in the oil content: 2.8–22.6%.

Size. In all of the four species studied, the changes in composition with increasing size of fish were slight except in a few instances. The Pacific Ocean perch specimens showed little variation in any of the con-

stituents. In Pacific cod and lingcod, some of the constituents varied slightly with size. Sablefish, in contrast, showed a large increase in oil content as fish size increased, with a corresponding drop in the amount of moisture.

Season. The season of capture did not have any uniform effect on the composition. Some fluctuation was noted between spring and fall specimens of Pacific Ocean perch, but these fluctuations were minor compared to the variations found in the different series. All of the constituents were relatively constant for Pacific cod. The summer specimens of lingcod showed some variation compared to the spring and fall series, but the variation was not large. Although somewhat lower values for protein, oil, and potassium were found for sablefish caught in the summer, none of the differences were large except in the sodium and potassium values, which varied as much as 10–20%.

Area. No great differences were noted for specimens taken from different areas. Thus, fish captured in the coastal waters of British Columbia and Washington had essentially the same composition. The differences in oil and sodium content for Pacific Ocean perch were not significant.

Preservation. The method of preservation did not appear to affect composition greatly. Only minor differences were noted in the oil and sodium contents of the two groups. It has been reported (Thurston and MacMaster, 1960) that halibut undergo extensive leaching of ash and mineral content during storage in ice. These four species, however, are held in ice for relatively short periods compared to the holding time for halibut. Thus, leaching would not be appreciable.

Characteristics. The low sodium and oil content of Pacific Ocean perch, Pacific cod, and lingcod recommend them as excellent sources of protein for all diets, including those restricted in oil and sodium content.

Variations in the composition of inedible parts. The inedible parts—carcass, skin, and viscera—provide a by-product of value for animal or fish food, either in the freshly ground state or as a processed meal. Pacific Ocean perch, Pacific cod, and lingcod are high in protein and mineral content, and the variations are not great (Table 2b). Oil content, however, shows considerable variation. The lowest values were found in Pacific cod (1.9%), and the highest in sablefish (25%). Because of this high oil content, sablefish is correspondingly lower in the other constituents.

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Volatile Constituents of Black Pepper[a]

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SUMMARY

Black pepper oil, separated by gas chromatographic techniques, had at least 23 volatile components. On the basis of relative retention volumes and infrared spectroscopy, these include α-pinene, β-pinene, D-limonene, and β-caryophyllene. α-phellandrene, reported by others, was not detected. Four samples of pepper oil differed in the relative amounts of various constituents. The methods described permit rapid screening of samples, and selection for greater flavor uniformity from batch to batch.

Much effort has been directed toward characterizing the constituents of some spices and condiments, but the early work was generally handicapped by the fact that many of the components are closely related and sometimes isomeric. Isolation of individual components by conventional means was frequently difficult and sometimes not possible. Recent technological advances such as gas chromatography have vastly improved the possibilities of obtaining separations of volatile mixtures. The amount of a purified fraction ultimately obtained is frequently quite small, but techniques such as micro-infrared spectroscopy and mass spectrometry greatly enhance the possibility of characterizing these isolated materials. With a view to developing methods permitting characterization of the volatiles in spice and condiment oils, it was decided to apply these techniques to a substance that had been relatively well characterized by conventional techniques —oil of black pepper.

Early studies of the composition of black pepper oil have been well reviewed (Guenther, 1952; Hasselstrom *et al.*, 1957). The work of this latter group is a particularly good example of resolving a complex mixture and identifying its components with conventional means of analysis. Most of our present knowledge of pepper oil composition comes from this work. Beginning with the steam-distilled oil from 1000 lb of freshly ground Malabar peppercorns, they reported their sample to consist of approximately 25% DL-limonene, 23% β-pinene, 19% β-caryophyllene, 14% α-pinene, 7% l-α-phellandrene, and 2% dihydrocarveol. They also reported small amounts (0.1-0.5%) of piperonal, phenylacetic acid, epoxydihydrocaryophyllene, cryptone, piperidine, 3 high-boiling alcohols, and other unidentified trace components.

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METHODS AND PROCEDURE

Pepper oil. Commercial samples of steam-distilled pepper oil of unknown origin were obtained from three sources, and a steam distillate of Ceylon black peppercorns was prepared especially for this study by the research department of Wm. J. Stange Co., Chicago. These oils were subjected to gas chromatography without further separation.

Gas chromatography. Commercial gas chromatographs with four-filament thermal conductivity cells were used. Analytical columns were ¼-in. O.D. × 10-ft. stainless-steel packed columns, except as otherwise noted. Used in some preliminary fractionation was a stainless-steel preparatory column, ½-in. O.D. × 5 ft.

Separated fractions were recovered by passing the effluent gas stream through a 5–6-cm length of 2-mm glass tubing, with a right-angle bend 1½ cm from one end. The long end was inserted in a 2-ml conical test tube, and the mouth of the tube was protected with a loose plug of Pyrex wool. The trap assemblies were supported in an ice bath, and collected samples were stored at −20°C until needed.

Infrared spectroscopy. Infrared spectra were determined on a Beckman Model IR-5 spectrometer. When material was sufficient and sample volatility was not a severe problem, the thin-film technique was used, with the sample layered between two KBr discs. When sample size was severely re-

stricted (*ca.* 1 μl), micro-cavity cells were used to determine the spectra in spectro-grade CCl_4.

Enrichment procedures. Oxygenated compounds were separated on silicic acid, using essentially the technique of Kirscher and Miller (1952), and the hydrocarbon-enriched fraction and oxygenated-compound-enriched fraction examined on the gas chromatograph.

The method described by Stanley *et al.* (1960) was used to concentrate carbonyl materials, and the carbonyl-rich and carbonyl-poor fractions were examined by gas chromatography.

Knowns. Known compounds were obtained from commercial chemical supply houses. Because they invariably consisted of more than one component, they were chromatographed, and the major fraction collected and its structure verified by infrared spectroscopy.

RESULTS AND DISCUSSION

A large number of stationary phases were investigated to determine which did the best job of separating black pepper oil into its component fractions. Included were LAC 446, diethylene glycol succinate, Apiezon L, silicone grease, carbowax 1500, Ucon Polar, Ucon Non-polar, sucrose-acetate-isobutyrate, and polyamine #6. Ucon Polar was selected as giving the best over-all separations resulting in the largest number of components.

Figs. 1-4 show chromatograms typical of black pepper oil from four sources. It is apparent that these four oils differ in their relative concentrations of individual fractions, particularly fractions 2, 4, 8, 15, 19, and 23. Subsequent work (reported below) has shown that fraction 5-6 is really two components about equal in amounts. This fraction gave an infrared spectrum that, though exhibiting absorption bands characteristic of terpene-like materials, was not in close agreement with that of any single known compound. When a recovered mixture that the ¼-in. packed column separated into 4 major fractions was separated on a capillary column coated with LAC 446, it was resolved into five major and several minor fractions. This improved resolution is to be expected, since the capillary column is generally conceded to be capable of much higher resolution that are packed columns. Too, the flame ionization detector used with this column has a sensitivity perhaps 10^3 times that of the thermal conductivity cell, so it is not surprising that what formerly

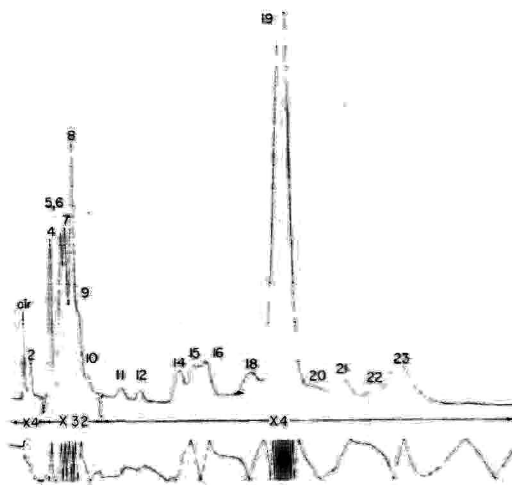


Fig. 1. Chromatogram of a commercial sample of black pepper oil. Ucon Polar on firebrick at 175°C. Flow, 75 cc/minute.

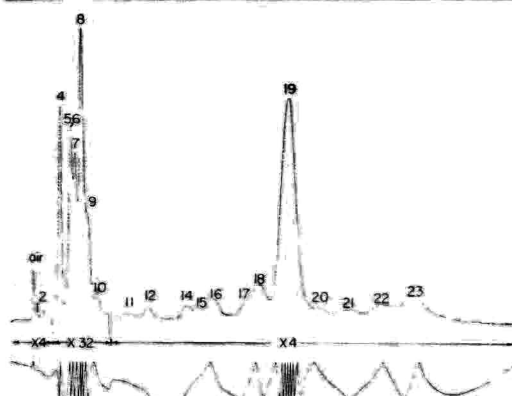


Fig. 2. Chromatogram of a commercial sample of black pepper oil. Ucon Polar on firebrick at 175°C. Flow, 75 cc/minute.

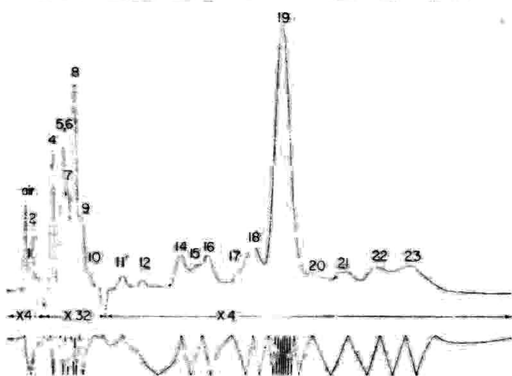


Fig. 3. Chromatogram of a commercial sample of black pepper oil. Ucon Polar on firebrick at 175°C. Flow, 75 cc/minute.

appeared to total 6–7 fractions now shows 10 components. The relationship between peak area and the amount of a given fraction is less direct and straightforward with the flame ionization detector than with the thermal conductivity cell. From study of Figs. 1–4, it would seem logical that fractions 2, 4, 5-6, and possibly 1 would give sizable peaks on a capillary column and flame ionization detector. The fact that five such peaks occur indicates that at least one of the early peaks from the packed columns is really two or more components whose retention values are not sufficiently different for separation on Ucon Polar (Bernhard, 1957; James, 1956). Changing the temperature of separation to 120 or 200°C did not affect the resolution of fractions 1–6.

Samples of fraction 5-6 from the preparatory column were rechromatographed at very low flow rates on a ¼-in. × 10-ft packed column coated with LAC 446. Fraction 5-6 then appeared as a doublet, apparent as a slight dimple at the peak. Attempts were made to collect isolated portions of the two sides of this doublet by limiting the collections to the first and last portions of the peak, avoiding the center cut. The two fractions so collected were re-examined by gas chromatography, and re-collected for infrared analysis. The chromatograms indicated that a relatively pure sample of fraction 5 was achieved; its infrared spectrum is identical with that of β-pinene (Fig. 6). Because fraction 7 follows closely on the peak in question, preparations of fraction 6 (the last portion of the peak) resulted in a mixture of fractions 6 and 7. Comparison of the infrared spectrum of a pure specimen of fraction 7 (Fig. 7) with that of this mixture indicated that certain of the spectral features of the mixture were due to fraction 6. Later attempts to purify fraction 6 yielded a material whose infrared spectrum was significantly different from either fraction 5 or fraction 7 (Fig. 8). The infrared absorption exhibited by fraction 6, particularly in the region 6.8-7.1 and 7.25-7.45, suggested a structure similar to that of terpinolene. When a commercial sample of terpinolene was subjected to gas chromatography, it was found that one of its four major fractions had on Ucon Polar a retention volume agreeing with that of fraction 6. The infrared spectrum of this isolated fraction, however, differed significantly from that of fraction 6. Similarly, the infrared absorption spectrum of fraction 7 shows strong similarity to D-limonene in the region 7.25-7.45 μ (Figs. 7, 10). This suggests that fraction 7 might be

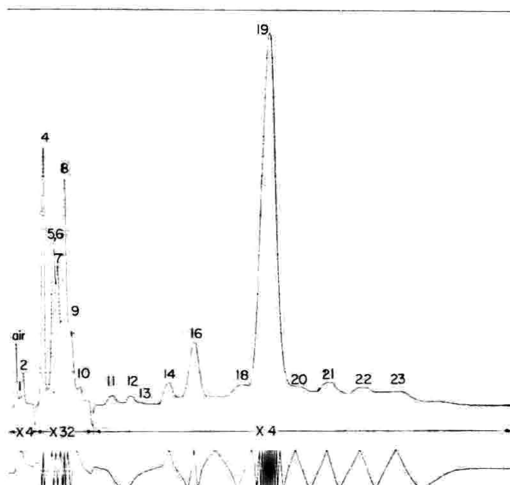


Fig. 4. Chromatogram of a specially prepared steam distillate of Ceylon black peppercorns. Ucon Polar on firebrick at 175°C. Flow, 75 cc/minute.

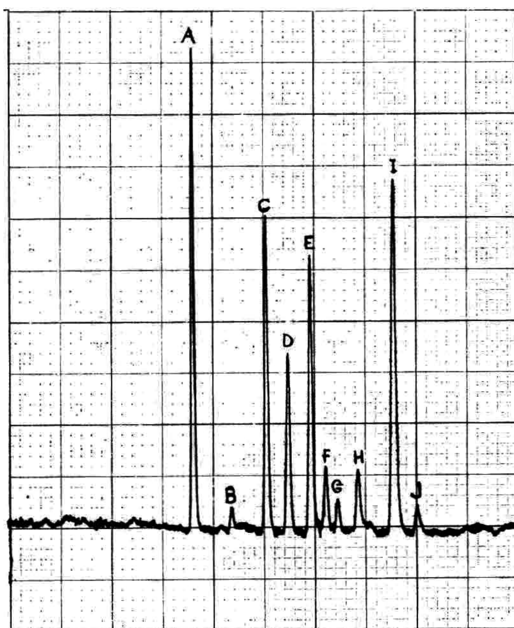
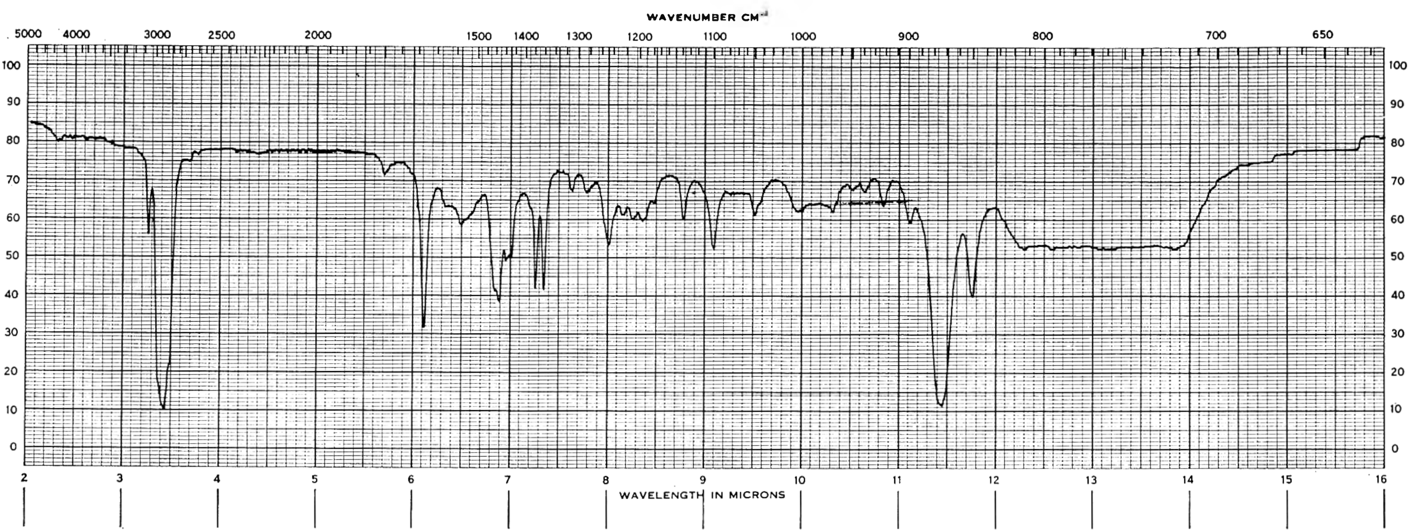


Fig. 5. Chromatogram of fractions 1 through 6 reseparated on a capillary column using flame ionization detector.



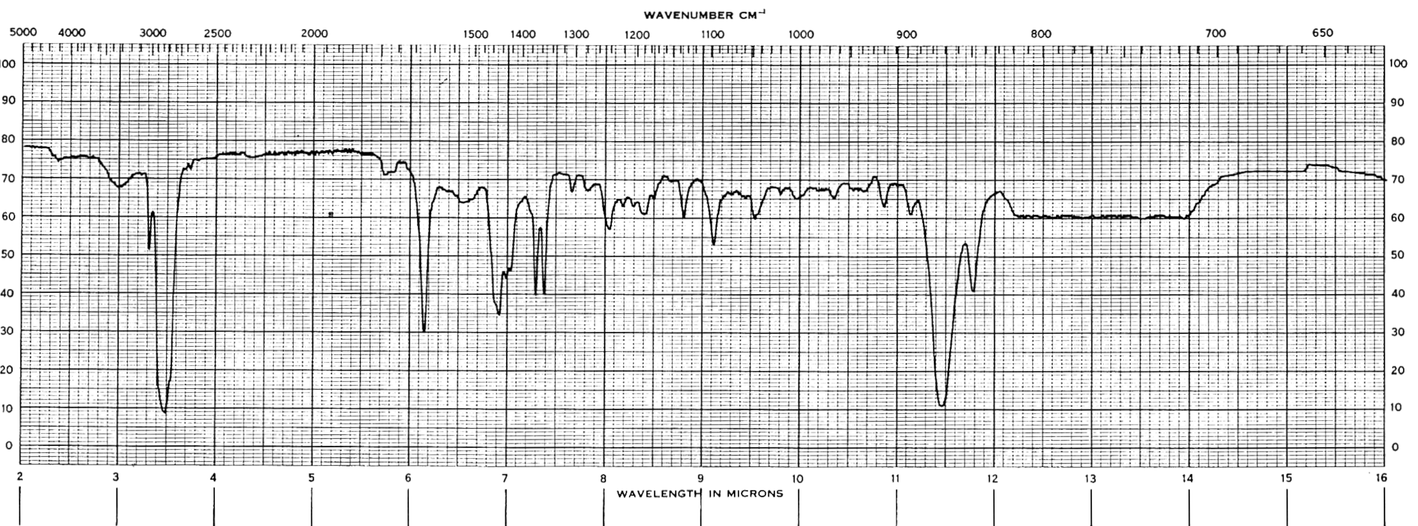


Fig. 6. Comparison of the infrared spectra of β-pinene and fraction 5. About 2% CCl_4 solution in micro-cavity cell.

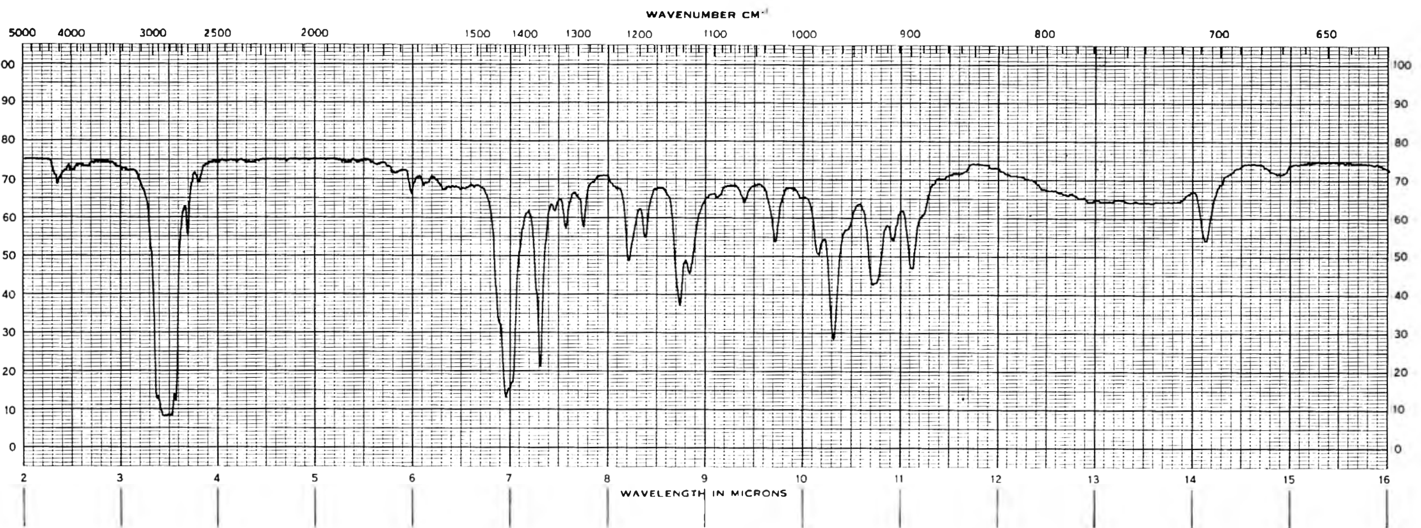


Fig. 7. Infrared spectrum of fraction 7. Thin film technique.

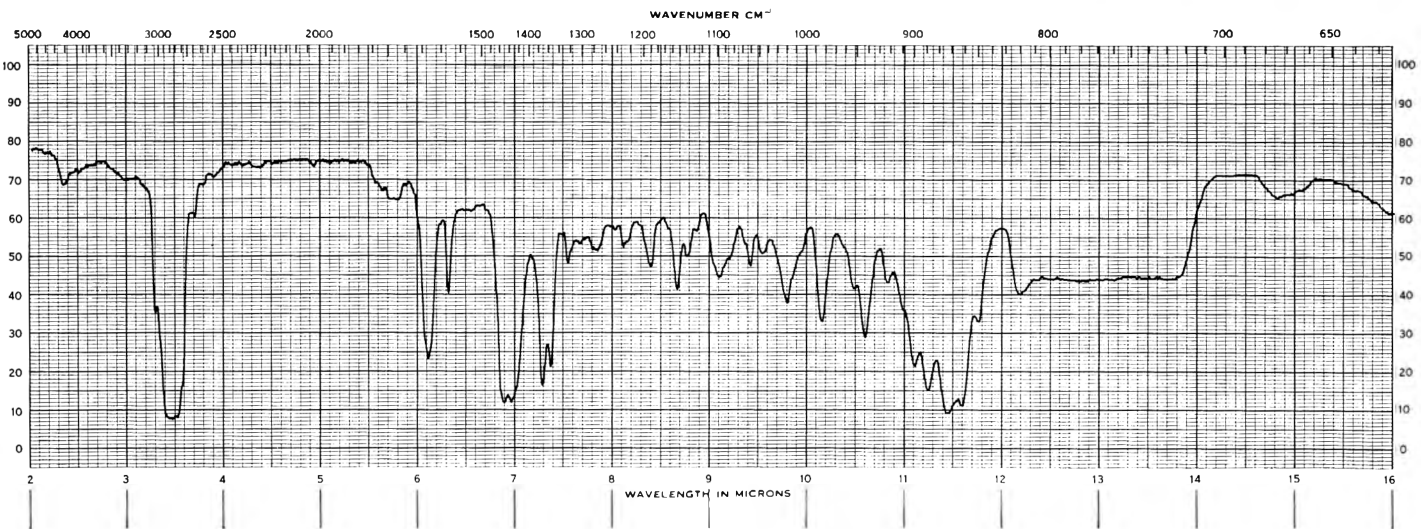


Fig. 8. Infrared spectrum of fraction 6. About 2% CCl_4 solution in micro-cavity cell.

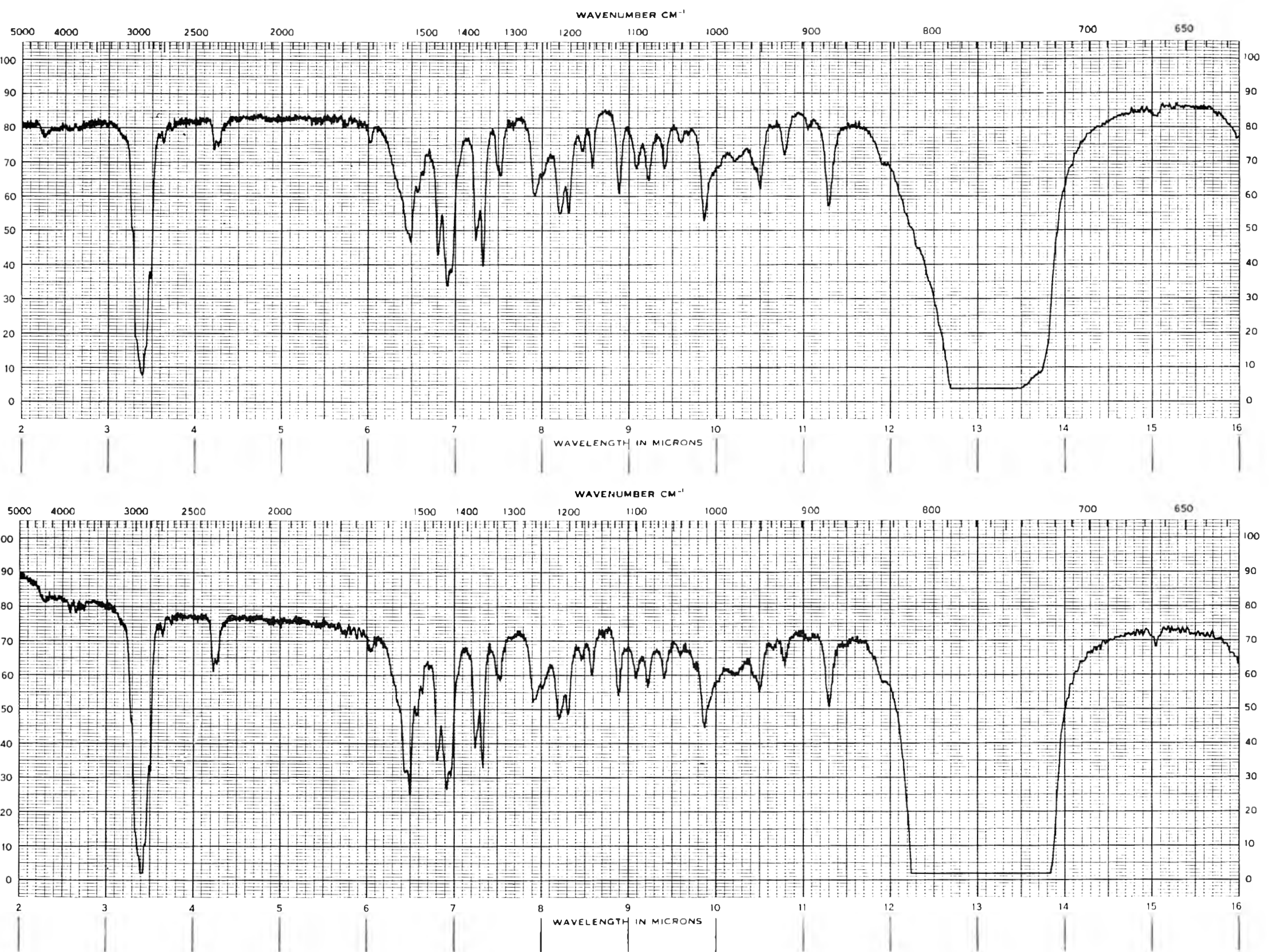


Fig. 9. Comparison of the infrared spectra of α-pinene and of fraction 4. About 2% CCl_4 solution in micro-cavity cell.

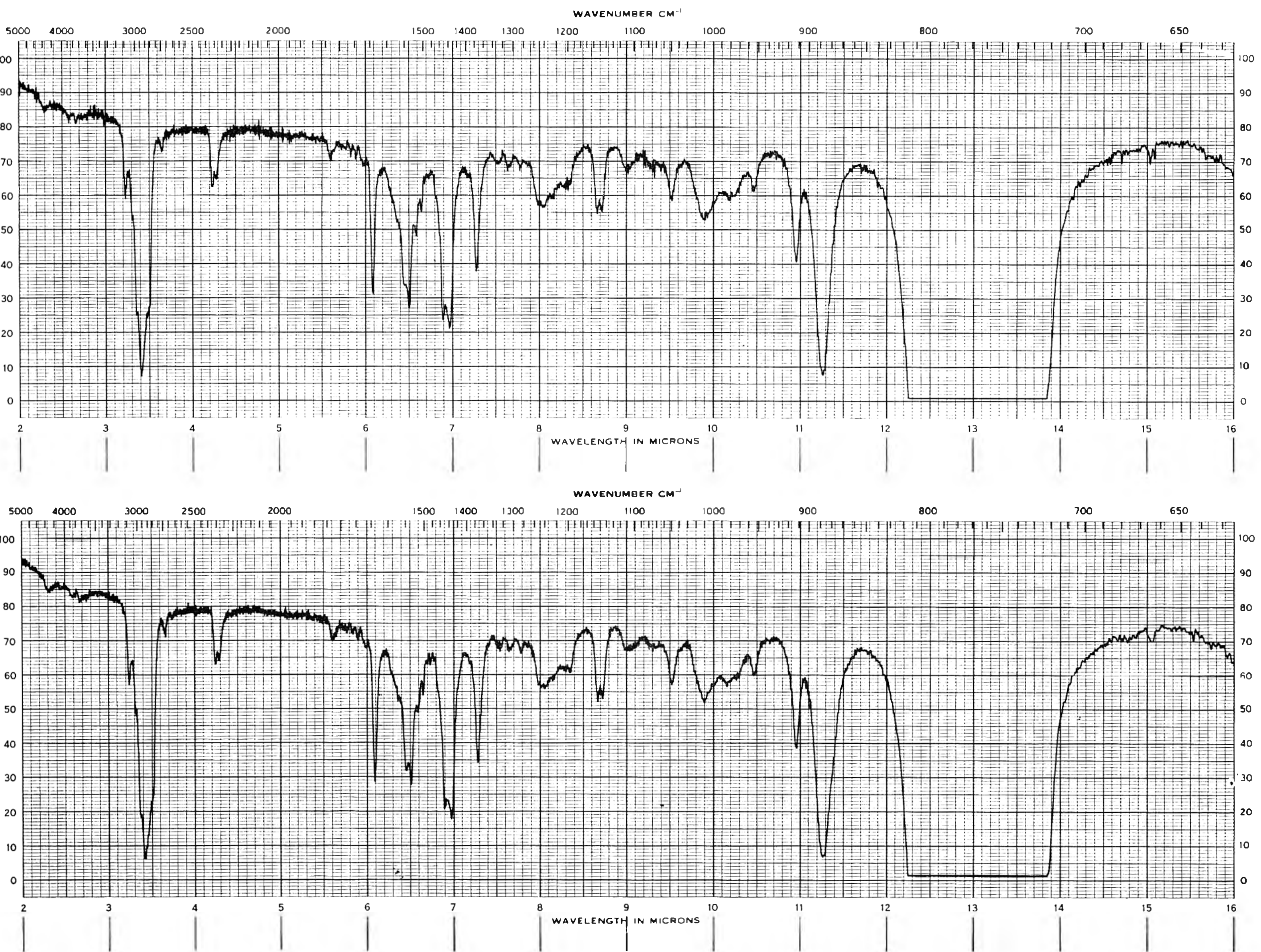


Fig. 10. Comparison of the infrared spectra of D-limonene and fraction 8. About 2% CCl_4 solution in micro-cavity cell.

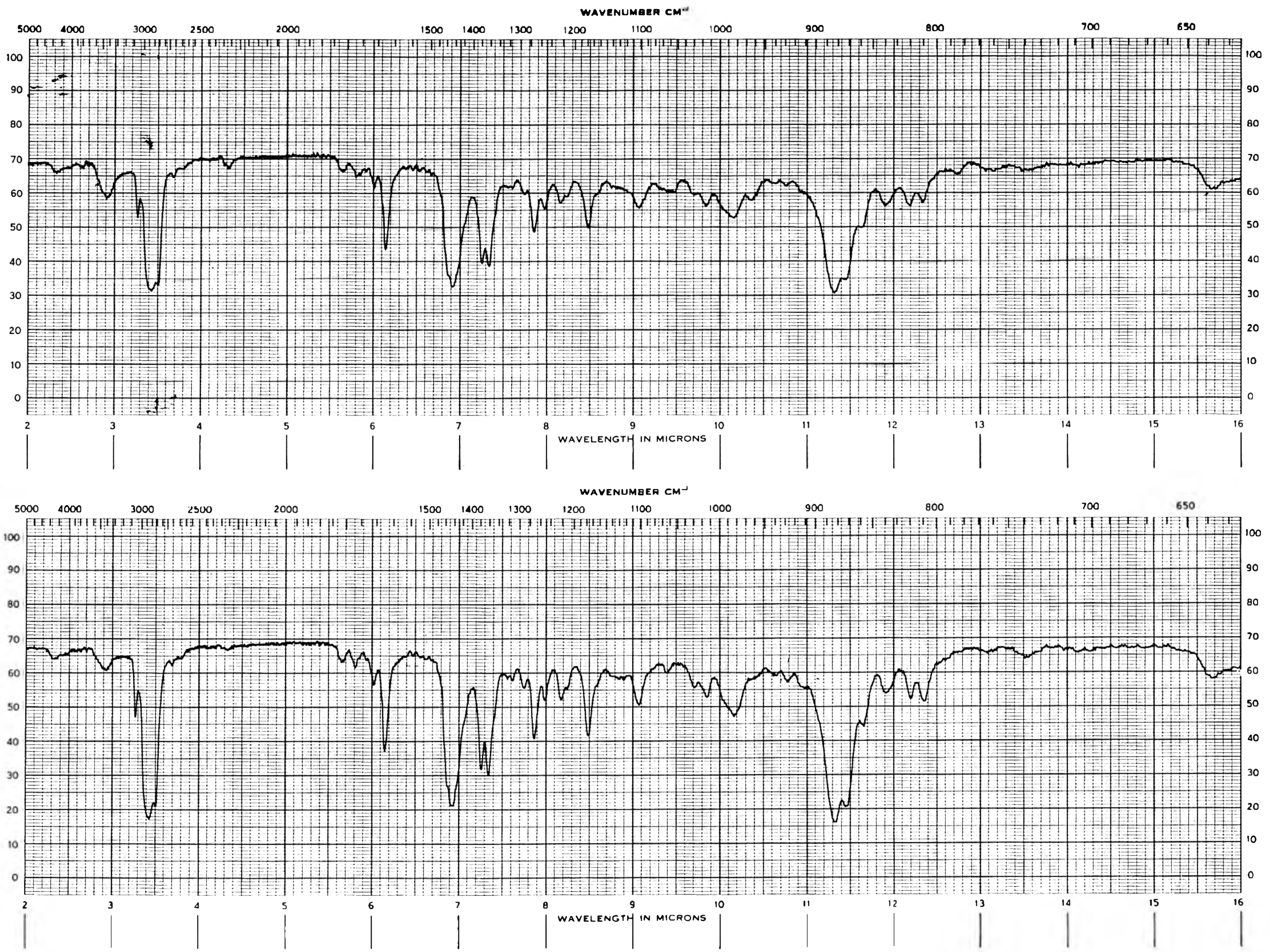


Fig. 11. Comparison of the infrared spectra of β-caryophyllene and fraction 19. Thin-film technique.

iso-limonene. Because attempts failed to obtain a sample of iso-limonene, this suggestion could not be confirmed.

Procedures have been suggested for isolation of carbonyls (Stanley *et al.,* 1960), and hydrocarbons and oxygenated compounds (Kirscher and Miller, 1952) in essential oils and related materials and their subsequent examination by gas chromatography. These procedures are not always definitive, in that solubility relationships in these complex mixtures, or other factors, may act to prevent clear-cut separations. It would, however, seem reasonable to consider the enrichment of a given fraction through the use of these techniques as at least indicative of structure or functional group.

Gas chromatographic analysis of the carbonyl-enriched fraction (Stanley *et al.,* 1960) showed a several-fold increase in fractions 11, 12, and 15. Investigation of the oxygenated fraction, isolated by adsorption on silicic acid (Kirscher and Miller, 1952), indicated that fractions 11, 12, 15, 16, 21, 22, 23, and 24 were oxygenated. Similarly, by investigating the unadsorbed fraction, it was demonstrated that fractions 1, 4, 5, and 7 were probably hydrocarbons. Because the functional group tests applicable to this study (Dubois and Monkman, 1959) require good chromatographic resolution (limiting the use of the preparative column) and appreciable quantities (10–20 μl), attempts to apply them to the minor components of pepper oil were restricted. The use of these techniques indicated that fractions 12, 16, 19, 21, and 22 were unsaturated compounds, and that fractions 14, 16, 19, 21, and 22 were aromatic.

From comparison of the relative retention values (Scholly and Brenner, 1959) of the pepper oil components with compounds reported by Hasselstrom *et al.* (1957) and with related compounds, tentative identification was made of several components (Table 1). A ½-in. × 5-ft preparatory column was used to obtain fractions of pepper oil comparatively richer in selected components, and these fractions were chromatographed on a ¼-in × 10-ft column to test their purity and obtain relative retention values.

From the relative retention values on Ucon Polar (and in some cases, LAC 446) (Table 1) and, where obtainable, the infrared spectra of the isolated component (Figs. 6, 9, 10, 11), fraction 2 agrees with piperidine, fraction 4 appears to be α-pinene, fraction 5 β-pinene, fraction 8 D-limonene, and fraction 19 β-carophyllene. Hasselstrom *et al.* reported about 7% α-phellandrene, based on boiling point, carbon-hydrogen balance, and melting point of the maleic anhydride adduct. The retention values for fraction 7 are quite close to those for α-phellandrene on both Ucon Polar and LAC 446 (Table 1). Separations on LAC 446 at low flow rates and low temperature (107°C) show that, though the retention values are close, the difference is significant. When α-phellandrene was added to black pepper oil and the mixture chromatographed under these conditions, the α-phellandrene appeared as a distinct peak following fraction 7. In addition, there are notable differences between the infrared spectra of α-phellandrene and of fraction 7. These data indicate that α-phellandrene was not present in significant amount in the oils investigated. The comparatively large retention values exhibited by piperonal and phenylacetic acid indicate they are not among the components investigated in this study (Figs. 1-4).

ACKNOWLEDGMENTS

The authors are grateful to the Norda Essential Oil and Chemical Company, the William J. Stange Company, F. Ritter and Company, and Fritzche Brothers, Inc., for samples of pepper oil. The authors acknowledge the encouragement and suggestions of Mr. Dan Ridenour, and the cooperation of R. A. Bernhard, of this department, particularly in the capillary column gas chromatographic separations.

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Table 1. Comparison of pepper oil fractions and synthetic knowns.

Pepper oil fraction of total oil	Approx %	RV Rel [a] Ucon Polar	RV Rel [b] LAC 446	Remarks
1	<0.1	.02		
2	0.4	.03		[c] piperidine
3	0.1	.04		
4	11.6	.11	.42	[c, d] α-pinene
5	13.9	.15	.69	[c, d] β-pinene
6			.71	
7	10.1	.165	.78	
8	26.3	.19	1.00	[c, d] D-limonene
9	10.1	.21		
10	2.3	.27		[e, f]
11	0.5	.37		
12	0.3	.43		[e, f, g]
13	<0.1	.49		
14	0.1	.60		[g]
15	0.5	.63		[e, f]
16	1.3	.71		[e, f, g]
17	0.5	.84		
18	2.0	.88		
19	13.3	1.00		[c, d] β-caryophyllene
20	0.8	1.11		
21	1.1	1.23		[e, f, g]
22	1.5	1.38		[e, f, g]
23	2.3	1.49		[f]
24	0.1	1.56		[f]
Synthetic knowns				
α-pinene		.11	.42	[c, d] fraction 4
β-pinene		.15	.69	[c, d] fraction 5
α-phellandrene		.14, .17	.81	
D-limonene		.19	1.00	[c, d] fraction 8
citronellal		.44, .6 [h]		
citral		.95		
citronellol		.37, .72, .89, 1 [h]		
linalool		.51		
terpinolene		.11, .13, .17, .20 [h]		
piperonal		4		
phenylacetic acid		8–9		extremely broad, diffuse peak
piperidine		.03		[c] fraction 2

[a] ¼-in. packed column at 175°C, β-caryophyllene = 1.00.
[b] ¼-in. packed column at 175°C, D-limonene = 1.00.
[c] Retention value in close agreement with.
[d] Infrared spectrum in close agreement with.
[e] Carbonyl test positive.
[f] Oxygenated test positive.
[g] Aromatic test positive.
[h] Commercial sample containing more than one major fraction.

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Comparison of Four Methods of Isolating Salmonellae from Foods, and Elaboration of a Preferred Procedure

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(Manuscript received November 18, 1960)

SUMMARY

From comparative study of four methods of isolation of *Salmonella* from egg products, of six enrichment broths and five selective media, and of several secondary technical operations, a recommended scheme for isolation of salmonellae from foods was devised and shown to be effective for diverse foods containing as few as 0.15 salmonellae per g, even in the presence of extreme coliform and other bacterial contamination.

When salmonellae in foods are few, attempts to isolate them often fail. One reason for this is that salmonellae are easily overgrown by members of the coliform and paracolon groups when these are more numerous. Various enrichment broths and selective agar media, and different methods of preparing the test specimen have been used in attempts to overcome this difficulty.

This paper evaluates 4 methods of isolating salmonellae, 6 enrichment broths, 5 selective media, and several modifications of specimen manipulation. The methods studied were in use, at the start of this project, at laboratories experienced in isolating salmonellae from foods. The criteria were: 1) numbers of *Salmonella* developing on specifically recommended differential media; 2) inhibition of coliforms; 3) differentiation from "late" lactose-fermenting or lactose-negative bacteria that were not *Salmonella;* 4) recovery of *Salmonella* from heavily contaminated specimens containing few salmonellae.

The specific media and the manipulations most conducive to a high degree of attainment within these criteria were assembled as a specific procedure and applied to the isolation of *Salmonella* from market specimens of several different foods.

MATERIALS AND METHODS

The four "methods" of *Salmonella* isolation are those parts of the respective total procedures leading to establishment of *Salmonella*-suspect colonies on differential agar media. After that, an identical determinative procedure was used. All media used were Difco products unless otherwise specified. The sources of the methods were: I) The Institute of the American Poultry Industries (IAPI) (1957), a method that seems to be based upon that of Ayres (1949) and similar to that described by the American Public Health Association (1958); II) the Food Hygiene Laboratory, Colindale, England, through the courtesy of Dr. Betty Hobbs (1960); III) personal communication (1960), from Dr. G. Slocum, Division of Microbiology, Food and Drug Administration, Washington (originally used for isolation of *Salmonella* from dried egg white); IV) after Silliker and Taylor (1958). The test food was frozen whole-egg melange containing known populations of *Salmonella* of two serotypes (*S. gallinarum* and *S. oranienburg*) in numbers ranging from 0.15 to 2.0 cells per gram, and in the presence of diverse contaminants including up to 40,000 coliforms per gram. To allow equilibration of the added bacteria, aliquots of the inoculated specimens were held 2 weeks at −12°C before use.

Method I. Specimens in triplicate of the thawed egg preparation were added at the respective rates of 10, 1, and 0.1 g to 90.0, 9.0, and 9.0 ml of selenite-F (SF) enrichment broth (Baltimore Biological Laboratories), each bottle containing one teaspoonful of glass beads. Each preparation was thoroughly shaken by hand and incubated 18–24 hr at 37°C, and one loopful of the resultant culture streaked on Kauffman brilliant green (KBG) and bismuth sulfite (BiS) agars, which were respectively incubated 18–24 hr and 48 hr at 37°C. The "most probable number" (MPN) of salmonellae present in each specimen was estimated after confirmatory tests for *Salmonella* were completed for each tube showing growth. The authors have since learned that a new pro-

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cedure for cooperative study has been prepared by the IAPI.

Method II. Two specimens of 25 g were placed in sterile jars containing 25 ml of quarter-strength Ringer's solution. The mixtures were shaken by hand and incubated 1–2 hr at 37°C before 50 ml double-strength selenite broth (S) was added to one preparation and 50 ml double-strength tetrathionate (T) broth to the other. After incubation at 37°C for 24 and 72 hr these enrichment cultures were used to inoculate plates of both desoxycholate citrate agar (DC) and BiS agar.

Method III. The significant modification in this method is pre-enrichment of the food preparation in lactose-broth. The details were: specimens weighing 10 g were added to 90 ml of lactose broth and incubated overnight at 37°C. One ml of this culture was transferred to 9 ml of selenite-F plus cystine (SFC) broth which was incubated 18-24 hr at 37°C, streaked upon KBG and Salmonella-Shigella (SS) agars, and incubated 18–24 hr at 37°C. SFC was prepared by addition of l-cystine (BBL) at the rate of 10 mg/L to SF (BBL) (North and Bartram, 1953).

Method IV. Specimens weighing 11 g were each added to 99 ml of standard phosphate buffer at pH 7.2 (American Public Health Association, 1953) contained in stoppered glass bottles, shaken by hand, transferred to plastic centrifuge bottles, and centrifuged 10 min at 4500 rpm in an angle-head centrifuge. The supernatant was decanted and the sediment resuspended in 99 ml of S-FC broth and incubated 18–24 hr at 37°C before streaking upon KBG agar.

Determination of Salmonella. Colonies resembling *Salmonella* were streaked on MacConkey agar for purification, and representative lactose-negative colonies were transferred to triple-sugar iron agar (TSI) and to the two tubed media of Gillies (1956), the latter being prepared in the laboratory. They are now available from Baltimore Biological Laboratories.

Gillies' media are the determinative media used routinely in this laboratory. Their use obviates the need for the many diagnostic media enumerated by Edwards and Ewing (1955) and avoids the defect of Taylor and Silliker's (1958) dulcitol-lactose-iron agar, which does not detect *S. gallinarum (pullorum)*. The use of one tube of each of the Gillies media allows the observation of fermentation of glucose, mannitol, sucrose, and salicin, of motility, and of the production of urea, indole, and H_2S. Cultures with positive reactions to urea, indole, lactose, sucrose, and the mannitol and glucose-negative strains are discarded. The remaining cultures are tested serologically with "pooled" 'O' group-antisera and then by standard typing procedures, the latter by the National Enteric Typing Center of the Department of National Health and Welfare through the courtesy of Dr. E. T. Bynoe. Gillies' media were adopted for determinative purposes after extensive trials prompted by the knowledge that they were in routine use at the Colindale laboratory. Previously, other abbreviated determinative procedures such as those described by Ayres (1949) and Byrne *et al.* (1955) had allowed the isolation from diverse foods of cultures that satisfied commonly used presumptive tests (including agglutination with polyvalent-O sera) but failed to be confirmed as *Salmonella*. This occurred often enough to lead us to use the much more numerous biochemical tests of Edwards and Ewing (1955) or a compromise between these and shorter procedures such as described by Ross and Thatcher (1958) and Thatcher and Loit (1961). Since adoption of Gillies' media, every one of 185 cultures tentatively considered *Salmonella* has been confirmed by the Typing Center.

Comparison of enrichment media. For comparison of enrichment broths, 25-g portions of the test specimen were manipulated as described in method II, but with substitution of standard phosphate buffer at pH 7.2 instead of Ringer solution. The two diluents seemed interchangeable, but the phosphate buffer was chosen because it is a standard bacterial diluent in use at this laboratory. The selective plating media used were KBG, KBG plus sulfapyridine (KBGS) and SS agars. The enrichment broths compared were: SF, SFC, selenite brilliant green (SBG), selenite brilliant green plus sulfapyridine (SBGS), tetrathionate (T), and modified tetrathionate (MT), the last advocated for use in the isolation of salmonellae from feces (Hajna and Damon, 1956). Dilutions of the enriched cultures were also prepared, with phosphate buffer the diluent, before inoculation of selective media. The ability of salmonellae to multiply in an enrichment broth in the presence of large coliform inocula and the relative degree of coliform inhibition are described as "good," "fair," and "poor."

Comparison of selective agar media. The preparatory procedure described in the foregoing paragraph was also applied to the comparison of selective agar media. SFC was used as the enrichment broth. Dilutions of 1:100 and 1:1000 were prepared from the enriched culture after incubation at 37°C for 18–24 hr. The original and the diluted cultures were each used to inoculate the following agar media: SS, KBG, KBGS, BiS, and DC. All were incubated at 37°C: BiS for 48 hr, and the others for 18–24 hr. The media were rated in accord with their ability to inhibit coliform organisms, ease of differentiation of salmonella from coliforms, the number of lactose-

negative colonies appearing on the plates, and the proportion of colonies appearing to be *Salmonella*, that could be confirmed as *Salmonella*.

Because finite counts cannot be made from "streaked" plates, an approach was made toward comparative appraisals by expressing the respective number of coliform and lactose-negative colonies appearing on a selective medium after inoculation with 0.1 ml of enriched culture in the arbitrary categories: very many, many, moderate, few, and none. In these experiments the first four categories roughly corresponded, respectively, to the four quartiles of the percentage values in descending order of the total number of colonies on a plate.

Secondary technical operations. After selection of a general method, and of enrichment and selective media, the following technical modifications were applied at the appropriate points in the overall procedure: manual shaking of the food specimen in pre-enrichment or enrichment broths versus comminution in a Waring blender for 3 min, centrifugation and no centrifugation of the food suspension, the application of various dilutions of the enriched inocula to the selective media, and the use of a loop or glass "spreader bar" to spread the diluted inocula.

Colonies not typical of coliforms present on plates too "crowded" with coliforms to develop characteristic properties of salmonellae, were restreaked on KBG agar after the method of Taylor (1958) to allow better indication of *Salmonella* properties.

Application of a preferred procedure. After selection of a preferred procedure based on the results of all foregoing tests, the method was applied to 136 samples of frozen whole egg, to powdered egg white, to commercial "cake mix" preparations containing egg white, to "cut-up" chicken, and to cheese, the two last foods having been inoculated with known numbers of *Salmonella*. For comparative purposes Method IV and the MPN procedure using SFC were also applied to the inoculated specimens.

Chicken meat was inoculated by spreading with a Pasteur pipette droplets of a dilute suspension of *S. oranienburg* over the surface of 500-g portions of chicken. The added inoculum provided 110 organisms per 500 g of chicken as estimated from plate counts of an aliquot of the inoculum suspension. Inoculated specimens were refrigerated in glass jars for 2 hr, by which time the inoculum film was not visible, then shaken with 500 ml of phosphate buffer on a mechanical shaker for 15 min. The wash water was then used in 25-ml portions as the test specimen. Control chicken specimens were treated similarly except for omission of the added salmonellae.

Cheddar cheese was inoculated by injecting into 200-g specimens, 100 1/100-ml portions of a suspension of *Salmonella* of known cell content by the use of a syringe with a fine needle. For each injection a hole was first made in the cheese with a slightly larger needle to avoid blocking the injecting needle. The cheese was refrigerated for 2 days, and aliquots were prepared by comminution in enrichment broth in a Waring blender, and with and without the addition of 5 ml of a 10% solution of the detergent "Tergitol" per 100 ml of broth as recommended by Galton *et al.* (1954) for fatty foods. Control cheese was uninoculated.

For the examination of market specimens of powdered egg white, the "preferred" method was applied both with and without pre-enrichment of the specimen in lactose broth, and comparison was made with the method of Slocum (unpublished). The pre-enrichment was accomplished as follows: 25-g specimens of egg white were mixed with 25 ml buffer before 50 ml lactose broth was added. The whole suspension was "blended" for 3 min, and incubated at 37°C for 5 hr, this period having been shown preferable during preliminary tests. Portions of 10 ml and 1 ml were each transferred to 90 ml SFC broth and incubated 24 hr at 37°C.

RESULTS

The four methods used without modification on each of three tests with frozen whole egg each allowed recovery of *S. oranienburg*, which had been added at the rate of 1.5/g, but Method III and the tetrathionate-treated replicate in Method II failed to isolate *S. gallinarum*, which was present at the rate of 0.4 cells/g. Method I (MPN), with SF broth as enrichment medium, as originally described, effected only partial recovery of each serotype. The method was lengthy and the most laborious, requiring 9 separate weighings and presumptive determination of *Salmonella* from each tube showing growth. Method II, with BiS and DC, provided sparse numbers of salmonellae and very large numbers of coliforms. Continued incubation of the enrichment broths (S and TT) for up to three days did not once, in our experience with the test egg specimens, provide an increase in the number of *Salmonella* colonies, but instead tended to promote a heavy growth of coliforms, *Proteus*, *Pseudomonas*, and other organisms, and fewer recognizable *Salmonella* colonies. Method III, which was not designed for the present food but for powdered egg white, was of little use for frozen whole egg because of pronounced overgrowth of coliforms. Method IV gave more numerous *Salmonella* colonies than either Method II or III when compared without modification. This preliminary order of appraisal became subject to change after introduction of the

Table 1. The relative development of coliform and lactose-negative bacteria from specific *Salmonella* enrichment broths.[a]

Enrichment broth	Enrichment dilution	Colony development	
		Coliforms	Lactose-negative
TT	none	very many	none
	1:1000	many	none
MT	none	very many	none
	1:1000	very many	none
SF	none	many	few
	1:1000	many	few
SFC	none	moderate	many
	1:1000	few	many
SBG	none	very many	none
	1:1000	very many	none
SBGS	none	very many	none
	1:1000	very many	none

[a] Test specimen: frozen whole-egg melange containing: SPC, 11 x 10[illegible]; coliforms, 39,000; *Salmonella*, 0.15/g (MPN). (MPN based on use of SFC and KBGS). Plating media for each specimen: KBG, KBGS, and SS agar.

media and manipulations shown to be of advantage during the rest of this study.

Table 1 summarizes the comparative effect of different enrichment broths upon the numbers of coliforms and the lactose-negative organisms developing on specific selective media. The appraisals in Table 1 show that SFC is the medium most favorable to multiplication of lactose-negative organisms and inhibition of coliforms. Without dilution, this medium provided many salmonellae and a moderate development of coliforms. At a dilution of 1:1000 the proportion of salmonellae was increased. (It should be recalled that the test specimen for the findings expressed in this table contained 0.15 *Salmonella* (100,000 total count) and 39,000 coliforms per g.) SF yielded few salmonellae among many coliforms. All other broths yielded very many coliforms but failed to provide a single colony of *Salmonella* from the test egg specimens.

Similar evidence for the comparative values of the different enrichment broths is provided by the data in Table 2, which are based upon application of the broths to the MPN procedure, and using two frozen egg specimens: one containing *S. gallinarum* only, at the rate of 0.4/g, the other containing the same numbers of *S. gallinarum* but having in addition *S. oranienburg* at the rate of 1.5/g. From the first specimen, both selenite broths provided full recovery of *S. gallinarum*; T and MT failed to yield a single *Salmonella* colony. From the second specimen, both serotypes were recovered by use of each of the selenite preparations, the SFC providing complete recovery as shown by an MPN of 2.10/g for the two serotypes together. The comparable MPN value for SF was 0.44. The other test broths yielded *S. oranienburg* only, and at MPN values of 0.03 for T and 0.094 for MT.

These results led to selection of SFC as the preferred enrichment medium.

Table 3 offers similar appraisal estimates for the different selective media based on use of the preferred enrichment broth (SF plus cystine). The test specimen contained 0.53/g (MPN) salmonellae. The largest numbers of lactose-negative colonies on the plates were provided by SS and KBGS, respectively providing moderate numbers and large numbers from the undiluted and diluted enrichments. However, many of the apparent *Salmonella* colonies present on SS agar were frequently found to be other than salmonellae. A high degree of dependability was provided by KBGS. "Poor" in this respect were SS, BiS, and DC. High numbers of coliforms were provided by KBG, BiS, and DC. Coliform inhibition was greatest with KBGS and SS. KBG alone was "poor," though still allowing colony distinction between coliforms and salmonellae. KBG and KBGS each provided more consistent and easily recognized differentiation in colony characters be-

Table 2. The influence of specific enrichment media on an MPN method for recovery of Salmonellae from frozen whole egg.

Enrichment broth	No. of salmonellae/g recovered (MPN)			
	Control specimen[a]		Inoculated specimen[b]	
	No./g	Serotypes recovered	No./g	Serotypes recovered
SF	0.44	S. gallinarum	0.44	both
SFC	0.42	S. gallinarum	2.10	both
T	0	none	0.03	S. oranienburg
MT	0	none	0.094	S. oranienburg

[a] Containing *S. gallinarum*, 0.4/g (MPN).
[b] Containing *S. oranienburg*, 1.5/g (MPN), and *S. gallinarum*, 0.4/g (MPN). Plating

Table 3. The relative development of coliform and lactose-negative bacteria from specific *Salmonella*-selective agar media.[a]

Selective medium	Enrichment dilution	Colony development: Coliforms	Colony development: Lactose-negative colonies	Colony differentiation	Dependability of Salmonella indication
SS	none	moderate	moderate	fair	poor
	1:100	few	many	fair	poor
KBG	none	very many	none	good	fair
	1:100	many	few	good	fair
KBGS	none	moderate	moderate	good	good
	1:100	few	many	good	good
BiS	none	very many	none	poor	poor
	1:100	many	few	poor	poor
DC	none	many	few	fair	poor
	1:100	many	few	fair	poor

[a] Using frozen whole egg containing: standard plate count, 11 x 10^5/g; coliforms 39,000/g; Salmonella 0.53/g (MPN).

tween salmonellae and coliforms than any of the other media. Thus the medium with advantage seemed to be KBGS, particularly when inoculated with a 1:100 dilution of the enriched culture.

Further evidence of the superiority of KBGS over the other media, in dependability of apparent lactose-negative colonies proving to be *Salmonella*, is shown by the data in table 4, which tabulates the proportion of *Salmonella*-suspect colonies that were fished from plates during examination of egg products and confirmed as *Salmonella*.

Table 4. The proportion of *Salmonella*-suspect colonies confirmable as *Salmonella* developing on specific selective agar media.

Selective agar medium	Lactose-negative colonies resembling salmonellae[a]: No. tested	No. *Salmonella*	% *Salmonella*[b]
SS	94	39	41
KBG	123	86	70
KBGS	84	64	76
BiS	38	7	18
DC	92	36	39

[a] Source: commercial egg products.
[b] Positive for Gillies' reactions and with polyvalent-O sera.

Methods II and IV, having been shown to be the more effective plating procedures, were modified by substitution of the preferred enrichment broth (SFC) and of the more effective selective agars, KBGS and KBG. The modified methods were then used to determine the effect upon *Salmonella* recovery of: a) dispersing the food specimen in the preparatory diluent by shaking by hand or by using a Waring blender; and b) the effect of centrifuging the suspended specimen. The results are appraised in Table 5.

In the modified Method II the use of a blender gave advantage, with the plates from specimens so treated giving a much higher yield of *Salmonella* colonies. Centrifuging of the blended specimen provided no additional advantage. In Method IV the use of a blender and centrifuging gave additive advantage. The combined effect, provided KBGS was used, was equivalent to the blended specimens treated by the modified Method II.

Table 5. The effect of different treatments of the specimen suspension on recovery of salmonellae by two methods.[a]

Methods[b]	Specimen preparation	Colony development on KBGS agar: Coliforms	Lactose-negative organisms
II	Shaken	many	few
II	Blended	few	many
II	Blended and centrifuged	few	many
IV	Shaken	many	few
IV	Blended (*not* centrifuged)	moderate	moderate
IV	Blended and centrifuged	few	many

[a] Using frozen whole egg containing: SPC 620,000/g, coliforms 38,000/g, *Salmonella* 0.93/g. (MPN, using blended specimen, SFC and KBGS).
[b] Modified as described in text.

These observations were confirmed by multiple tests. Use of the spreader-bar as described by Silliker and Taylor (1958) was advantageous under circumstances where plates were overcrowded with coliforms, a condition found to be influenced by the nature of the specimen, the ratio of coliforms to salmonellae within the specimen, and the length of the incubation period of the enrichment culture.

Below is the preferred procedure for routine food analysis and for survey purposes, where sensitivity is desirable but quantitation is not essential, selected on the basis of the total experience gained in these experiments. The procedure is based on Method II since this uses the larger amount of food specimen and, with the introduction of the blender for suspending the specimen and use of the preferred enrichment and selective media, provided a high yield of *Salmonella* colonies, easily differentiated from coliforms and most paracolon organisms. It also obviates the need for centrifuging of the specimen, the nine separate weighings, and the multiple tube inoculations needed for the MPN procedure. However, the MPN procedure (Method I) permits a useful quantitative recovery after substitution of SFC as the enrichment broth and of KBGS as the plating medium.

The preferred method. Weigh 25 g of thawed egg melange (or other food). Add to 25 ml standard phosphate buffer at pH 7.2, contained in a sterile Waring blender, equipped with a screw-threaded lid, and "blend" for 3 min (an automatic timing device is used in this laboratory). Transfer to a sterile glass jar and incubate 1–2 hr at 37°C. Add 50 ml of double-strength SFC and incubate 24 hr. Transfer one loopful to one plate of each of KBG and SS agars and distribute the inoculum with a loop by the streaking technique. Also transfer 3 loopfuls to KBGS and apply the spreader-bar. The use of both KBG and KBGS is recommended because on rare occasions a specific *Salmonella* strain may be inhibited by the sulfapyridine. If no interest in *Shigella* is involved the SS agar may be omitted.

If large populations of coliforms are expected, prepare dilutions of the enriched culture at 1:100 and 1:1000 with standard phosphate buffer and inoculate one plate of KBG agar from the original culture and from each dilution, using 3 loopfuls or 0.1 ml of culture, and disperse with a spreader-bar. Incubate 24 hr at 37°C. (If coliforms number less than 10,000/g, dilution of the enriched culture is not necessary.) Select colonies resembling *Salmonella* from the plates showing the more discrete colonies, and subculture to MacConkey agar for preliminary purification. Use lactose-negative colonies from MacConkey agar to inoculate with each a slope of TSI, and one tube of each of the two Gillies media. Colonies with the biochemical characteristics of salmonellae are then tested for agglutination by polyvalent 'O' antisera, using the cultures from the TSI slope. Positive cultures are "typed" serologically.

Table 6. The recovery of salmonellae from commercial frozen-egg preparations of varying degrees of coliform contamination. Montford-Thatcher method.

SPC No./g	Coliforms No./g	Escherichia coli No./g	Salmonellae isolated (serotypes)
67,000	60,000	30,000	S. gallinarum
9,300	3,500	2,600	S. gallinarum
280,000	7,500	5,600	S. gallinarum
520,000	170,000	174,000	S. gallinarum
380,000	150,000	120,000	S. gallinarum
280,000	80,000	60,000	S. gallinarum
400,000	7,000	5,300	S. gallinarum
150,000	4,000	1,000	S. gallinarum
380	100	51	S. gallinarum
73,000	9,000	4,500	S. thompson
97,000	13,000	3,300	S. gallinarum
130,000	33,000	33,000	S. gallinarum
11,000,000	2,800,000	2,800,000	S. gallinarum
3,700	450	220	S. gallinarum
190,000	5,600	0	S. gallinarum
5,200	200	100	S. gallinarum
1,200,000	32,000	16,000	S. gallinarum
2,000,000	150,000	150,000	S. bareilly, S. oranienburg
1,100,000	50,000	50,000	S. oranienburg, S. gallinarum, S. bareilly
1,200,000	20,000	20,000	S. oranienburg
130,000	13,000	13,000	S. gallinarum
130,000	26,000	26,000	S. gallinarum
870,000	54,000	0	S. manhattan, S. oranienburg
5,500,000	360,000	360,000	S. thompson
3,400,000	400,000	200,000	S. oranienburg
1,080,000	130,000	130,000	S. manhattan

Applications of the preferred method. The method as outlined above was applied to 136 specimens of commercial frozen egg products, including some of extremely poor quality as judged by standard plate count and coliform content. Salmonellae distributed among five different serotypes were found in 27 specimens. Table 6 lists the values for standard plate count and coliform content for each specimen from which *Salmonella* was isolated. It is clear that the method is able to recover salmonellae from egg specimens containing extreme levels of coliform and other bacterial contamination.

The method is not limited in application to egg products. *Salmonella* was readily recovered from poultry meat to which *S. oranienburg* has been added at the rate of 0.22 cells/g. The control poultry preparations were free from salmonellae. Cheddar cheese (48% butter fat) containing *S. oranienburg* at the rate of 0.5 cells/g yielded a predominance of *Salmonella* on both KBG and KBGS agars. Tergitol was without effect. No salmonellae were found in the control specimens.

Where powdered egg white was the test food, pre-enrichment with lactose broth, as described, was of decided advantage. Without pre-enrichment, neither the "preferred" method nor the MPN method, with SFC used as enrichment, yielded any colonies resembling *Salmonella* on plates of KBG agar, but small numbers were recovered from plates of KBGS. With pre-enrichment, salmonellae were obtained (in small numbers) from only the most dilute enrichment preparation when KBG agar plates were used, but with KBGS as the selective agar medium, plates from all dilutions of the enrichment broth were crowded with salmonellae. Comparative MPN values obtained after pre-enrichment with lactose broth and the use of plates of KBG and KBGS to indicate presumptive tubes were respectively 0.06/g and greater than 11.0/g. Thus, recovery of salmonellae from egg white is good when the "preferred" method is adapted by suspending the specimen in buffer, mixing and "blending" with lactose broth, and incubating the whole preparation 5 hr at 37°C. As judged from present results, using KBGS rather than KBG makes the method more sensitive than that of Slocum (unpublished). This method allowed the isolation of salmonellae from 11 of 14 randomly purchased packages of "angel-food cake mix." With pre-enrichment, SFC, and KBGS, the products of five manufacturers were shown to contain *Salmonella*, and at MPN values ranging from 15 to 280/g.

CONCLUSIONS

The advantages of the over-all scheme are: 1) Relatively large amounts of food specimen may be used. This is important when salmonellae are present in fractional numbers per gram. 2) No centrifuging is required. 3) Salmonellae develop readily from specimens containing large numbers of coliforms. 4) Distinction between salmonellae and atypical coliforms and members of the paracolon group is positive. (Not one of our isolates submitted for *Salmonella* typing after use of this method has yet failed to be *Salmonella*. This occurred occasionally with other methods.) 5) Only three determinative media are required between isolation and serological typing, thanks to Gillies' media. 6) KBG and KBGS each provide clear color distinction between colonies of salmonellae and other bacteria. The inclusion of sulfapyridine, as in the latter medium, greatly improves inhibition of coliforms and allows the use of larger volumes of inocula from the enrichment broth. This provides a larger number of salmonellae on the agar media.

Pre-enrichment of the specimen blended in the specified mixture of buffer and lactose broth for 5 hr at 37°C is recommended for application of the method to powdered egg white.

About 30 specimens of food can be prepared and cultured by one technician in a 7-hr day if the number of blender jars available is sufficient. Time required for completion of a positive determination is 5 days. A negative indication is provided in two days.

This empirical study makes no attempt to explain why the recovery of salmonellae is substantially increased by using a Waring blender to disperse the food specimen in buffer; nor does it explain why, after "blending" and the use of the preferred media, centrifuging the suspended specimen (Silliker and Taylor, 1958) is without advantage. The method is the most sensitive we have used, and its application to surveys of specific market foods, to be described elsewhere, has decidedly changed our opinion of the frequency of distribution of *Salmonella* in these foods.

Since this manuscript was completed, Sugiyama *et al.* (1960) have reported the value of the addition of *Salmonella* agglutinating antisera to a pre-enrichment culture medium as a means of concentrating the salmonellae present in a food specimen, a principle used effectively by one of us in isolating brucellae from cheese (Thatcher *et al.*, 1956). The authors point out, however, that availability of polyvalent flagella antiserum precludes, for the present, its general use in routine isolation of salmonellae from food. Hence, the practical value of the method we have advocated is retained.

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Lipolytic Activity of Microorganisms at Low and Intermediate Temperatures. III. Activity of Microbial Lipases at Temperatures Below 0°C

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SUMMARY

The lipolytic activity at −7, −18, and −29°C of strains of *Pseudomonas fragi*, *Staphylococcus aureus*, *Geotrichum candidum*, *Candida lipolytica*, *Penicillium roqueforti*, and an unidentified *Penicillium* sp. in emulsions of corn oil, coconut oil, and lard was determined. The action was measured by titratable acidity and by quantitative determination of the fatty acids by chromatography. The lipases from these microorganisms showed considerable activity within 2–4 days at −7°C and within a week at −18°C. Activity at −29°C was evident within 3 weeks by some of the cultures, particularly on corn oil. The rate of lipolysis in frozen substrates was directly related to their degree of unsaturation. However, there also were differences among genera. The lipase of *G. candidum* had considerably more specificity for oleic and linoleic acids than any of the others. *P. fragi* and *C. lipolytica* were least able to attack the β-esterified palmitic acid of lard. The ability of *S. aureus* to attack this position readily at 35° C was nullified by lowering the temperature to −18° C. A similar effect was observed on the mold lipase.

It is generally agreed that microbial counts of frozen foods decline slowly during storage even though a few bacteria and molds can grow at temperatures down to −5 to −10°C (Borgstrom, 1955). Nevertheless, changes in quality occur in frozen foods and are often quite pronounced after relatively short storage times in the range of −18 to −5°C. This deterioration is generally attributed to enzymes native to the product.

Balls and Tucker (1938) observed that pancreatic lipase was active at temperatures as low as −30°C, and Balls and Lineweaver (1938) found that chymotrypsin and pepsin were capable of bringing about proteolysis at similar temperatures. Sizer and Josephson (1942) studied lipase, trypsin, and invertase and found them active as low as −18°C. Wagenknecht *et al.* (1952) concluded that the loss of color in unblanched peas stored at −18°C was caused at least in part by an increased acid number (lipolysis), which favored the conversion of chlorophyll to pheophytin. Kuhl (1940) reported that wheat-germ lipase and the oxidase and catalase activity of wheat bran were inhibited below 0°C. Peterson and Gunderson (1960a, b) reported that proteinases from *Pseudomonas fluorescens* were elaborated and active at 0–5°C in defrosted chicken pies; activity in frozen substances was not reported. Studies on the blanching process for vegetables (Lee, 1958) have been concerned primarily with native enzymes, although high initial bacterial counts on peas and beans have some relationship to flavor and color deterioration in storage (Hucker *et al.*, 1952).

Previous reports from this laboratory (Alford and Elliott, 1960; Alford *et al.*, 1961) have shown that the lipases produced by different species of *Pseudomonas* are similar in their activity at temperatures ranging from 0 to 30°C. The present work was undertaken to include a study of lipases from selected bacteria, yeast, and molds, with particular emphasis on the effect of sub-freezing temperatures on their activity and specificity.

Table 1. Titratable acidities produced by lipases from different microorganisms at 35°C.

	Corn oil			Lard			Coconut oil		
	45 min	3 hr	24 hr	45 min	3 hr	24 hr	45 min	3 hr	24 hr
P. fragi	5.4[a]	9.6	14.8	5.8	11.8	19.2	7.6	14.8	18.7
S. aureus	0.7	2.1	10.1	0.6	2.0	10.6	1.9	5.0	21.5
G. candidum	3.8	9.1	20.0	2.4	5.0	14.6	1.8	3.0	5.5
C. lipolytica	1.9	4.0	7.8	1.9	4.3	10.7	3.1	6.7	10.3
P. roqueforti	3.7	6.0	7.6	2.3	3.0	2.0	5.3	9.0	11.3
Penicillium sp.	5.5	12.4	11.6	5.6	12.8	33.2	7.2	12.8	26.3

[a] ml of 0.02*N* acid produced.

EXPERIMENTAL METHODS

Sources and cultivation of microorganisms. The species used were obtained from the following sources: *Pseudomonas fragi,* NRRL B-27, from Dr. W. C. Haynes, NURDD, USDA, Peoria, Illinois; *Staphylococcus aureus,* D-87, from Food and Drug Administration, Washington, D. C.; *Geotrichum candidum,* from Dr. A. R. Colmer, Louisiana State University, Baton Rouge, Louisiana; *Candida (Mycotorula) lipolytica,* NRRL Y-1094, from Dr. C. W. Hesseltine, NURDD, USDA, Peoria, Illinois; *Penicillium roqueforti,* NRRL 849, from Dr. Hesseltine; and the *Penicillium sp.,* isolated from the surface of aged ham.

Cultures of *P. fragi, S. aureus, G. candidum,* and the unidentified *Penicillium* were grown on 1% peptone broth buffered at pH 7.0 with 0.05*M* phosphate. *C. lipolytica* was grown on the medium recommended by Peters and Nelson (1948), and the *P. roqueforti* on the medium suggested by Morris and Jezeski (1953) except that the butterfat was omitted. All cultures, except *S. aureus,* were incubated at 20°C; the *S. aureus* cultures were incubated at 32°C. Incubation periods were: *P. fragi* and *S. aureus,* 3–4 days; *G. candidum* and *C. lipolytica,* 4–6 days; and the molds, 6–10 days.

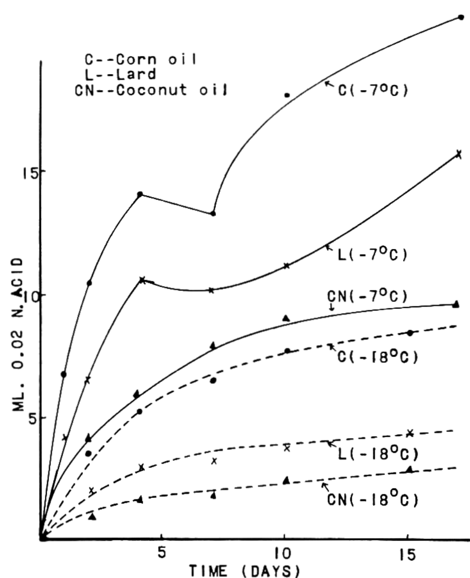

Fig. 1. Activity of *Pseudomonas fragi* on emulsified fats at low temperatures.

Enzyme preparation. The cells or mycelia were removed by filtration or centrifugation for 10 min at 2500 × G, and the filtrates and supernates were used as the enzyme source.

Substrate preparation. The lard, corn oil, and coconut oil emulsions were prepared as previously described (Alford and Elliott, 1960) except that homogenization was carried out at 4000–4500 lb with a recycling period of 15 min, and the emulsions were not heated after preparation. Phosphate buffer at pH 7.0 was added to the emulsions to be used for the lipases from *P. fluorescens, S. aureus,* and the two molds so that the final concentration after addition of enzyme would be 0.05*M* buffer and 2% fat. A similar procedure was used for *G. candidum* and *C. lipolytica* except that citrate buffer at pH 5.0 was used. Previous work (Alford and Elliott, 1960: Morris and Jezeski, 1953; Peters and Nelson, 1948) as well as additional observations in our laboratory indicated that assays at these pH values would give near-optimum activity.

Assay. To tubes containing 2 ml of enzyme at 3–5°C were added 8 ml of buffered substrate (also at 3–5°C), and the contents were immediately frozen in an alcohol-dry ice bath. The tube contents were below −30°C within two minutes of

Table 2. Activity of lipases from different microorganisms at −29°C.

	Lard		Corn		Coconut	
	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk
P. fragi	0.8[a]	1.3	3.0	3.8	0.7	1.0
S. aureus		0.5		0.9		0.4
C. lipolytica	1.2	1.3	1.0	1.5		0.9
Penicillium sp.		0.9	3.1	4.2		1.7

[a] ml of 0.02*N* NaOH produced.

Table 3. Effect of temperature on percentages of fatty acids released from corn oil by different microorganisms.

Temperature (°C)	C_{16}	C_{18}	C_{18}—	C_{18}=	C_{16}	C_{18}	C_{18}—	C_{18}=
	Composition of original fat							
	13	2	28	56				
	Pseudomonas fragi				*Staphylococcus aureus*			
35°	14	1	27	57	28	6	20	45
− 7°	12	tr	27	60	19	4	24	52
−18°	7	tr	26	66	14	tr	25	59
−29°	tr	tr	35	63				
	Geotrichum candidum				*Candida lipolytica*			
35°	3	0	40	57	14	1	31	53
− 3°					20	2	27	50
− 7°	tr	tr	4	95	9	tr	32	57
−18°	tr	tr	35	64	8	2	34	54
	Penicillium roqueforti				*Penicillium* sp.			
35°	19	tr	26	55	20	tr	24	55
− 7°	15	1	25	58	16	2	28	52
−18°	8	1	28	63	9	2	29	59

being mixed. Replicate samples were stored at −7°C, −18°C, and −29°C. Samples were removed periodically for determination of titratable acidity by titration with 0.02*N* NaOH, and fatty acid composition by gas chromatography. Samples for chromatography were usually taken when 15–35 mg of free fatty acids were present. The procedures were as reported previously (Alford and Elliott, 1960; Alford *et al.*, 1961; Hornstein *et al.*, 1960). Although activity in the control flasks was essentially negative, the zero-time blank values ranged from 1.0 to 1.6 ml of 0.02*N* NaOH. All values shown in the data are net values.

RESULTS AND DISCUSSION

Table 1 shows the titratable acidities produced by the different microorganisms at 35°C. Coconut oil is the most readily attacked of the three substrates by the lipases from all cultures except *G. candidum*. The low activity of *P. roqueforti* on lard is not readily explained.

Fig. 1 and Table 2 show the rate and amount of acid production by *P. fragi* lipase below 0°C on corn oil, lard, and coconut oil. Unlike the results at 35°C, the rate of lipolysis in frozen substrates was directly related to their degree of unsaturation. If one considered the average molecular weights of the fatty acids in the fats, these differences in rate would be even greater since there are more molecules of fatty acid available in the 200 mg of coconut oil than in the others. This might also be a factor in the greater coconut oil activity at 35°C.

The apparent lag in lipolysis between the 4th and 7th days on corn oil and lard may be related to substrate utilization. As was

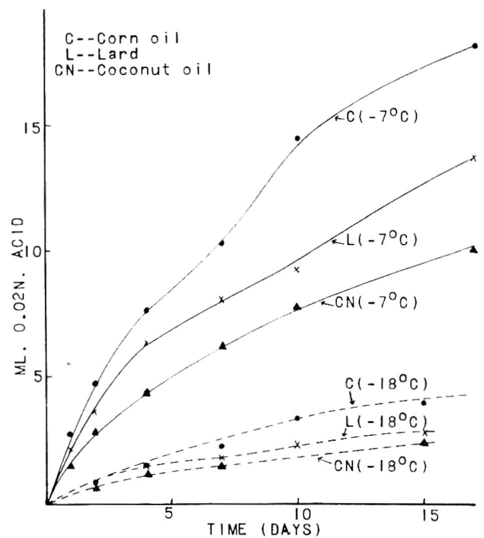

Fig. 2. Activity of *Staphylococcus aureus* on emulsified fats at low temperatures.

postulated previously (Alford *et al.*, 1961), the lipase may have a position specificity, or at least affinity, for the α-position on the triglyceride. If one assumes such a specificity, then most of the fatty acid available in the α-position will have been released when a titratable acidity of 10-15 ml of 0.02*N* acid is obtained. The subsequent lag may be related to the time required for the shifting of fatty acid molecules from the β-position to the α-position (isomerization). This is supported by the increase in palmitic acid released by *P. fragi* after several days at 35°C (Alford *et al.*, 1961).

Similar curves are presented in Figs. 2–6 for the other microorganisms. Essentially the same ratio of activity on the three substrates is observed for all cultures. The relatively high titratable acidity produced by *P. roqueforti* lipase on coconut oil at 35°C, coupled with the similarity of rate of hydrolysis at −7°C of coconut oil to that of lard and corn oil (Fig. 5), suggests an affinity of this lipase for the lower fatty acids. This might be expected when one considers the known activity of *P. roqueforti* on the lower fatty acids of butterfat in blue cheese manufacture (Morris and Jezeski, 1953).

Balls *et al.* (1937) reported that pancreatic lipase had very little activity on the higher triglycerides (above C_8) at 0°C. The data presented here indicate a similar relationship of rate of attack to degree of unsaturation for microbial lipases. There is a definite shift toward increasing percentages of the unsaturated acids as the temperature decreases. More conclusive evidence is shown in Tables 3, 4, and 5, which show the types of fatty acids released from the natural triglycerides. This apparent increase in af-

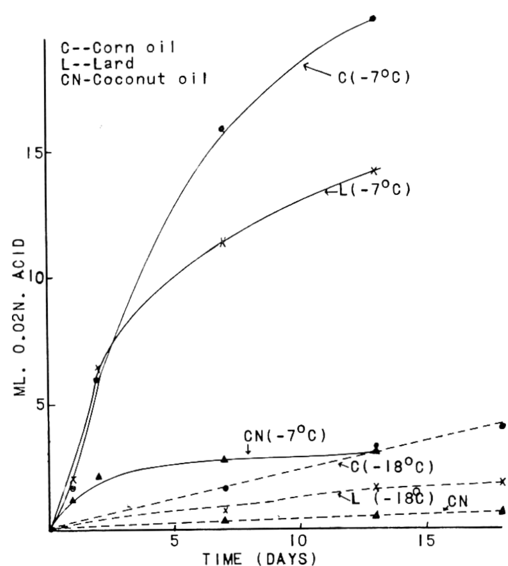

Fig. 3. Activity of *Geotrichum candidum* on emulsified fats at low temperatures.

Table 4. Effect of temperature on percentages of fatty acids released from lard by different microorganisms.

Temperature (°C)	C_{16}	C_{18}	$C_{16}-$	$C_{18}-$	$C_{18}=$	C_{16}	C_{18}	$C_{16}-$	$C_{18}-$	$C_{18}=$
	Composition of original fat									
	26	14	5	47	8					
	Pseudomonas fragi					*Staphylococcus aureus*				
35°	6	9	1	68	16	26	12	5	41	14
− 7°	2	2	1	73	21	10	3	4	63	20
−18°	tr	1	tr	76	20	tr	3	1	67	26
	Geotrichum candidum					*Candida lipolytica*				
35°	5	tr	2	73	19	3	7	tr	83	6
− 7°	tr	tr	3	72	25	1	2	tr	76	18
−18°	tr	tr	4	66	28	1	3	tr	72	21
	Penicillium roqueforti					*Penicillium* sp.				
35°	20	17	1	56	6	14	20	1	50	13
− 7°	10	2	5	67	14	6	3	2	71	16
−18°	5	3	6	57	28	1	1	3	68	26

Table 5. Effect of temperature on the percentages of fatty acids released from coconut oil by different microorganisms.

Temperature (°C)	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{18}—	C_{18}=	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{18}—	C_{18}=
	Composition of original fat															
	8	8	43	17	11	2	8	2								
	Pseudomonas fragi								*Staphylococcus aureus*							
35°	7	12	46	19	8	2	5	3	6	8	45	16	11	3	4	6
− 7°	1	4	27	12	13	1	24	18	1	2	33	10	18	6	20	10
−18°	tr	4	36	14	12	6	22	6	tr	4	34	12	20	7	18	6
	Geotrichum candidum								*Candida lipolytica*							
35°	tr	3	39	19	11	3	14	11	12	7	36	16	12	3	13	1
− 7°	tr	tr	4	tr	14	0	51	29	2	4	34	15	18	4	18	4
−18°									1	5	37	15	10	8	14	11
	Penicillium roqueforti								*Penicillium* sp.							
35°	tr	5	45	26	14	3	5	tr	tr	4	40	26	15	4	2	8
− 7°	2	4	34	8	12	1	22	14	2	2	36	15	12	2	19	14
−18°									1	4	35	15	15	7	18	6

finity for unsaturated fatty acids as the temperature decreases may actually be an effect on the physical structure or degree of crystallinity of the fat. In addition to the effect of temperature on the activity of the enzymes, fundamental differences in specificity among the different genera of microorganisms are evident. This is in contrast to the slight variations in activity among different psychrophilic strains of *Pseudomonas* reported previously (Alford *et al.*, 1961).

Factors other than generic differences enter into this specificity, however. Decreasing temperature generally caused a decrease in percentage of saturated fatty acids released, but the effect was not the same on individual fatty acids by the same enzyme from different substrates. For example, the extensive activity of *S. aureus* on palmitic acid in lard is almost nullified by lowering the temperature. Its activity on palmitic acid in corn oil is reduced only about half, while in coconut oil there is an actual increase in percentage of this acid liberated as the temperature decreased. Mattson and Beck (1956) reported that pancreatic lipase is specific for the α-position of triglycerides. Employing this specificity, Mattson and Lutton (1958) gave further evidence that the palmitic acid of lard is primarily esterified at the β-position. Alford and Blankenship (1961) indicated that the lipase from *S. aureus* has an affinity for palmitic acid at 35°C and that it could attack the β-position of triglycerides to a limited extent. Table 3 indicates that this ability to attack the β-position in lard is seriously retarded by lowering the temperature.

As indicated above, *G. candidum* was more

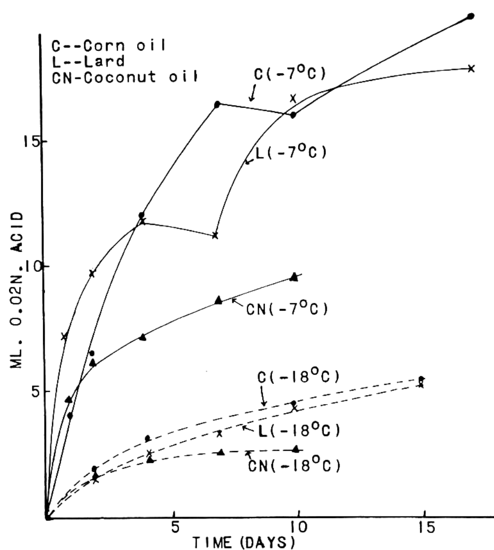

Fig. 4. Activity of *Candida lipolytica* on emulsified fats at low temperatures.

active on corn oil and lard than on coconut oil. This was particularly apparent at the lower temperatures (Fig. 3). When the types of fatty acids released were determined, a decided preference for the unsaturated acids was observed. In Table 3, the oleic and linoleic acids compose 99% of the acids hydrolyzed by *G. candidum* from corn oil at the lower temperatures. These acids account for over 90% of the acids from lard (Table 4) and 80% from the coconut oil (Table 5) even though these unsaturated acids are only 10% of the total acids in this oil.

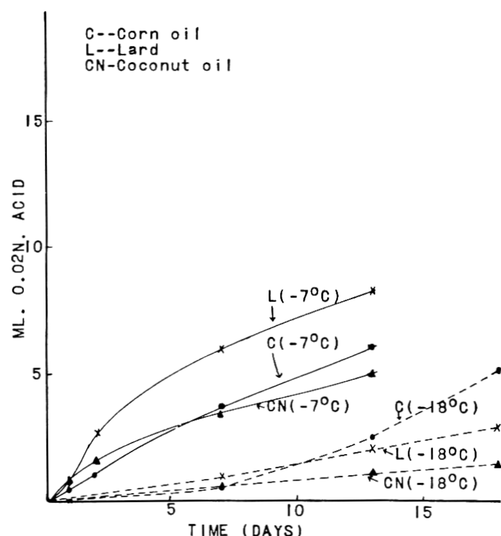

Fig. 5. Activity of *Penicillium roqueforti* on emulsifed fats at low temperatures.

The other yeast-like fungus, *C. lipolytica,* showed a similar affinity for unsaturated acids from lard, but to a much less extent when corn or coconut oil was the substrate. This indicates a degree of position specificity similar to that suggested for *P. fragi.*

The unidentified *Penicillium* produces the most active lipase, but it is similar to *P. roqueforti* in the pattern of fatty acids released from the various substrates at different temperatures.

Other less pronounced differences in the activity of these lipases might be inferred from a closer examination of these data. However, factors which may affect the quantitative if not the qualitative aspects of their activity must be considered. Different lots of culture supernatant from the same microorganisms may vary in the concentration of lipase present; the average size of the globules of the emulsified substrate may vary slightly between batches as well as among the fats; finally, the method of extraction and esterification may cause discrepancies of 5–10% between duplicates. These factors combine to make exact quantitative comparisons between different experiments difficult.

Nevertheless, these data show basic differences in microbial lipases, and that these enzymes are active in frozen substrates. They could bring about changes in quality if present in sufficient concentration. It is apparent that the type of fatty acid, as well as its position on the triglyceride, is important in determining the activity of microbial lipases. The delineations between enzymes from different sources are not sharp, and specificities overlap considerably. To the enzyme chemist interested primarily in pure systems exhibiting a high degree of specificity, the study of such systems has little to offer. However, to the food microbiologist or technologist interested in the subtle differences in the flavor of certain foods or the incipient off-flavors associated with the beginning of spoilage, these enzymes offer intriguing possibilities for further study.

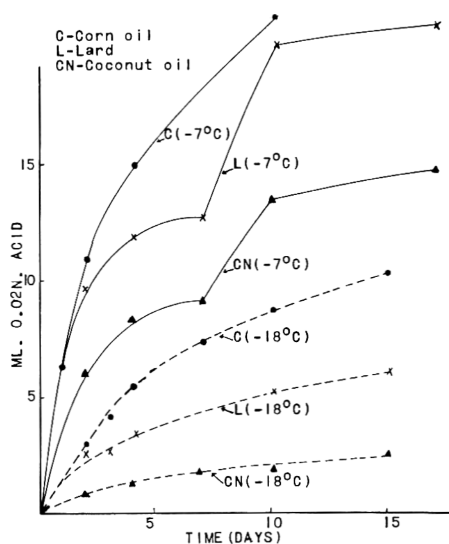

Fig. 6. Activity of *Pencillium* sp. on emulsified fats at low temperatures.

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Nutritive Value of Central American Beans. IV. The Essential Amino Acid Content of Samples of Black Beans, Red Beans, Rice Beans, and Cowpeas of Guatemala [a]

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(Manuscript received June 1, 1961)

SUMMARY

The rice bean *(Phaseolus calcaratus)*, cowpea *(Vigna sinensis)*, red bean, and three black bean *(Phaseolus vulgaris)* samples grown in Guatemala, were found to be fairly similar in chemical composition. Average percentage values were: moisture 14.5, protein 22.3, ether extract 1.3, ash 3.5, crude fiber 6.0, and carbohydrate 52.4. The range in amino acid content, expressed as mg of amino acid per gram of nitrogen, was: arginine 356–528, histidine 159–232, isoleucine 143–339, leucine 189–260, lysine 322–544, total sulfur-containing amino acids 80–94, phenylalanine plus tyrosine 362–471, tryptophan 52–73, threonine 215–348, and valine 191–383. The cowpea sample was higher in lysine; the rice bean and red bean samples were higher in tryptophan. The isoleucine and valine contents were lower, and the leucine higher, in the red beans than in the other samples. When compared with the amino acid pattern of the FAO Reference Protein, methionine plus cystine was the most-limiting amino acid, and leucine and tryptophan respectively the second- and third-most limiting. All the samples contained high amounts of lysine, making beans a good source of this amino acid.

Among vegetable foods, legume seeds represent a rich source of protein, although research on their use and dietary value has been somewhat neglected. They not only contain a relatively large amount of protein but also have an essential amino acid pattern that complements that of corn and other cereal grains (FAO, 1954). Since, in most of the Central-American countries, beans of the *Phaseolus vulgaris* variety provide 20–30% of the protein in the rural diet (Tandon *et al.*, 1957), the quality of their protein is of considerable practical importance. The chemical composition and the lysine, methionine, and tryptophan content of some varieties of beans and other leguminous seeds (Bressani *et al.*, 1954, 1960; Jelliffe *et al.*, 1956) have been studied previously. This paper reports on the essential amino acid content of additional samples.

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EXPERIMENTAL METHODS

The leguminous seeds studied were rice beans *(Phaseolus calcaratus)* grown in the highlands of Guatemala and obtained from the local market in Guatemala City, cowpeas *(Vigna sinensis)* and red beans *(Phaseolus vulgaris)* also from a market in Guatemala City, and three black bean samples *(Phaseolus vulgaris)* respectively from markets in the highland towns of San Pedro Ayampuc, Tecpán, and Parramos.

Five-pound samples were brought to the laboratory, ground to pass 60 mesh, and stored at 4°C pending analysis. The moisture, nitrogen, ether extract and ash content were determined by A.O.A.C. (1945) methods. The essential amino acid content was measured microbiologically by hydrolizing 1-g samples 8 hr in the autoclave with 6N HCl or 4N NaOH. The lysine, methionine, leucine, isoleucine, arginine, cystine, phenylalanine, and tyrosine contents were determined with Difco media (Difco Laboratories, Detroit, Michigan)

Table 1. Proximate chemical composition of the six bean samples (per cent).

Sample	Moisture	Protein	Ether extract	Ash	Crude fiber	Carbo-hydrates [a]
Cowpea	14.2	24.2	1.2	3.2	6.2	51.0
Rice bean	13.5	20.7	0.7	3.8	7.2	54.1
Red bean	14.1	22.2	1.0	4.0	6.9	51.8
Black bean (Parramos)	15.7	22.8	1.8	3.4	5.3	51.0
Black bean (San Pedro Ayampuc)	15.5	22.6	1.5	3.3	4.8	52.3
Black bean (Tecpán)	14.5	21.4	1.8	3.2	5.4	53.7
Average	14.5	22.3	1.3	3.5	6.0	52.4

[a] Values obtained by difference (100 − % H_2O − % protein − % ether extract − % ash − % crude fiber).

and *Leuconostoc mesenteroides* P-60. The valine and histidine were determined using the organism and the media recommended by Steele *et al.* (1949). The threonine assay also employed the medium of Steele *et al.* (1949) with *Streptococcus faecalis* 8043 as the microorganism. Tryptophan was determined with Difco media and *Lactobacillus arabinosus* 17-5.

RESULTS

Table 1 shows the proximate chemical composition of the six samples. The four *Phaseolus vulgaris* samples contained a similar quantity of protein, the cowpea sample a slightly higher amount, and the rice beans the least. All samples had a similar ash content, whereas crude fiber was higher in the red bean, rice bean, and cowpea samples than in the black beans; the reverse was found for the ether extract content.

When the essential amino acid composition of the six legume seeds is expressed on a percentage basis, the common black beans and the cowpea showed a higher percentage of tryptophan than the rice bean. The lysine content of the rice bean samples was slightly higher than that of the other samples, particularly that of the red bean, which had the lowest amount. The total sulfur-containing amino acids were higher in the cowpea, red bean, and rice bean samples. In all samples but the red bean, the isoleucine content was higher than that of leucine.

Differences in essential amino acid content among the samples became more evident when expressed on the basis of mg of amino acid/g of nitrogen (Table 2). The rice bean sample was highest in lysine, and the red beans and Parramos beans contained the most tryptophan. Methionine and cystine contents were similar in all the samples. The red bean samples had the lowest isoleucine and valine content. Also included in the table is the amino acid pattern of the FAO Reference Protein for comparison with that of the amino acid composition of the samples studied.

DISCUSSION

Leguminous seeds, particularly beans, have often been mentioned as good foods to correct the amino acid deficiencies of cereals. For most of them, however, relatively little of the biological work essential for their

Table 2. Essential amino acid content in the six bean samples (mg A.A./g nitrogen).

Amino Acid	Cowpea	Rice bean	Red bean	Black bean (Parramos)	Black bean (San Pedro Ayampuc)	Black bean (Tecpán)	FAO Protein Reference
Arginine	398	470	528	370	356	511	
Histidine	193	215	232	185	159	176	
Isoleucine	291	339	143	331	317	309	270
Leucine	211	260	219	202	189	223	306
Lysine	461	545	322	486	490	523	270
Methionine	71	77	70	64	66	71	270 (Methionine + Cystine)
Cystine	15	17	17	16	17	14	
Phenylalanine	302	365	390	320	320	330	360 (Phenylalanine + Tyrosine)
Tyrosine	60	52	81	67	61	58	
Tryptophan	59	52	73	71	66	67	90
Threonine	215	287	343	348	277	304	180
Valine	328	371	191	383	354	342	270

efficient use in human feeding has been done. The leguminous seeds in this study had a relatively high essential amino acid content, but, compared with the FAO Reference Protein (FAO, 1957), they were deficient in methionine, cystine, leucine, and tryptophan.

The sulfur amino acids were the first-limiting, with a protein score of around 32%. Other investigators have also reported the marked deficiency of methionine in the leguminous seeds (Baptist, 1954; Cerighelli *et al.*, 1960; Jaffe, 1950). The second-limiting amino acid was leucine, with an average score of 72% for the samples. These results are important since the average ratio of leucine to isoleucine in all the samples was 1.0 : 1.33, compared to a leucine to isoleucine ratio in the FAO Reference Protein of 1.0 : 0.68; red beans were an exception, with a ratio similar to that in the FAO Reference Protein. The samples also contained a relatively high proportion of valine, which could cause an imbalance of the type reported by Harper and Kumta (1959) between isoleucine, valine, and leucine. In addition, it would be useful to know whether leucine is, in fact, the second-limiting amino acid, and the effect of correcting the disproportion of this amino acid in relation to isoleucine and valine. From a practical point of view, however, the low leucine content of beans may be an advantage for populations that consume tortillas, since the leucine content of corn is relatively high (Bressani and Mertz, 1958).

By comparison with the amino acid pattern of the FAO Reference Protein (FAO, 1957), tryptophan is the third-limiting amino acid, averaging 72% for all samples, but further study is necessary to determine if a true deficiency exists. At least, the tryptophan deficiency is not important as a niacin precursor in beans, since Bressani *et al.* (1954) and Cravioto *et al.* (1945) reported that, like most leguminous seeds, they are good sources of niacin.

The lysine content was relatively high in all the samples studied, about twice that of the amino acid pattern of the FAO Reference Protein (FAO, 1957). Therefore, beans should complement the lysine deficiency in cereal protein. Further study is needed to determine the optimum combination of beans and cereal grains, and the degree of improvement in the protein quality obtained in the final combination (Bressani *et al.*, 1961).

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Studies on Domestic Dates. III. Effect of Temperature on Some Chemical Changes Associated with Deterioration [a, b]

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(Manuscript received March 23, 1961)

SUMMARY

The rates of various reactions associated with deterioration of whole dates such as darkening, pH decrease, sucrose hydrolysis, and oxygen and carbon dioxide gas exchange were studied at 28.4, 38.0, and 49.0°C and their apparent activation energies calculated. Also, changes in the activities of phenolase and peroxidase were followed in dates stored at 38.0°C. It was shown that dates darken by both oxidative and nonoxidative browning pathways, which respectively have apparent activation energies of 23.4 and 34.5 kcal/mole. Demonstration of enzymic browning in ground date tissue suggested that oxidative browning of whole dates is enzyme-catalyzed. Oxygen absorption and carbon dioxide production gave respective apparent activation energies of 17.6 and 25.3 kcal/mole in unheated dates, and 19.0 and 27.4 kcal/mole in heated dates, indicating the existence of enzymic and nonenzymic sources. The nonenzymic nonoxidative decrease in pH and invertase-catalyzed sucrose hydrolysis had apparent activation energies of 22.8 and 26.0 kcal/mole, respectively. Apparent phenolase activity increased during storage of dates, whereas apparent peroxidase activity decreased.

Paper II of this series (Maier and Schiller, 1961) reported the rates of several chemical and physical changes in dates during accelerated deterioration at 49°C. Evidence of both oxidative and nonoxidative browning reactions were found in the darkening process, while enzymic and nonenzymic reactions were responsible for the oxygen and carbon-dioxide gas exchange. Sucrose hydrolysis was found to be catalyzed by invertase. The reactions responsible for the decrease in pH were primarily nonenzymic and nonoxidative.

This paper reports similar studies on dates held at 28.4 and 38.0°C. The results show more clearly the effects of the various treatments on the deteriorative reactions. In addition, the combined rate data for the three temperatures allow the apparent activation energies of the various reactions to be calculated and compared.

EXPERIMENTAL

All procedures were as already described (Maier and Schiller, 1961). High-quality, non-hydrated Deglet Noor dates of 19.1% moisture content were used from the same randomized lot used in the previous study. The dates were stored in jars at 28.4 and 38.0°C. Enzyme activities, rates of reflectance decrease, pH decrease, sucrose hydrolysis, oxygen absorption, and carbon dioxide production were determined by methods already reported. In the previous study the soluble dark pigment increase in 600 hr at 49°C was used as a measure of the rate of darkening; this amounted to 26.0 mg/g for the control. At 38°C a 26.0 mg/g increase occurred in 120 days for the control; therefore, pigment increase in 120 days was used as a measure of the rate of darkening at this temperature. At 28.4°C the data gave linear plots that permitted the rates to be calculated from the slopes. All rates of darkening as measured by soluble dark pigment production are given in terms of mg/g/hr. Ap-

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[b] This investigation was supported in part by the Date Administrative Committee, Indio, California.

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Table 1. Effect of treatments and temperature on the rates of reactions associated with deterioration of dates.

Temp. (°C)	Air [a]		Nitrogen [a]		Air-nitrogen [b]		Heated [c]	
	Meas.	Calc. [d]	Meas.	Calc. [d]	Meas.	Calc. [d]	Meas.	Calc. [d]
Rate of reflectance decrease ($-\Delta\%$ reflect/hr $\times 10^4$)								
28.4	6.55	6.55	3.79	3.25	2.76	2.80	22.0	23.8
38.0	29.0	30.0	15.5	18.0	13.5	10.1	52.0	57.5
49.0	186	186	149	145	37.0	43.5	197	167
Rate of soluble pigment production (mg/g/hr $\times 10^4$)								
28.4	14.8	15.5	9.15	9.66	5.65	6.19	8.36	8.81
38.0	89.5	75.0	69.5	55.2	20.0	18.3	49.3	48.7
49.0	443	486	376	455	67.0	67.5	380	374
Rate of pH decrease ($-\Delta$ pH/hr $\times 10^4$)								
28.4	1.43	1.43	1.28	1.23	......	......	1.53	1.36
38.0	4.39	4.49	3.48	3.60	......	......	4.25	4.60
49.0	17.7	17.7	13.1	13.1	......	......	19.2	19.7
Rate of oxygen absorption (μg/g/hr)								
28.4	.128	.127	......	......	......	......	.0720	.0710
38.0	.322	.305	......	......	......	......	.162	.183
49.0	.867	.890	......	......	......	......	.555	.574
Rate of carbon dioxide production (μg/g/hr)								
28.4	.0845	.0670	......	......	......	......	.0475	.0390
38.0	.175	.240	......	......	......	......	.113	.153
49.0	1.23	1.13	......	......	......	......	.864	.820
Rate of sucrose hydrolysis ($hr^{-1} \times 10^4$)								
28.4	1.15	1.15	1.00	1.03	......	......	.000	......
38.0	4.06	4.22	3.70	3.86	......	......	.207	......
49.0	20.5	20.5	20.5	18.9	......	......	.943	......

[a] Averages of duplicate determinations.
[b] Difference between the rate in air and that in nitrogen.
[d] Long heat treatment of Maier and Schiller, 1961; both peroxidase and phenolase were inactivated.
[d] Calculated rates read from Arrhenius plots of the experimentally measured rates.

parent activation energies were calculated from Arrhenius plots of logarithm rate versus reciprocal absolute temperature. The lines of best fit for the Arrhenius plots were calculated by the method of least squares. Measured reaction rates are listed in Table 1; theoretical rates read from the regression lines of the Arrhenius plots are given for comparison.

Date paste was prepared by grinding 30 g of date tissue and 40 ml of water with a mortar and pestle until a paste of smooth consistency was obtained. A portion of the paste was heated 4 min over a steam bath at 86–90°C, the water of evaporation was replaced and the paste cooled to room temperature. The rate of darkening of the unheated control and the heated sample was determined by reflectance measurements over 26 hr at 25°C. The samples were poured into 3.2 cm O.D. cuvettes with optically flat bottoms and their reflectances measured with a photoelectric reflection meter equipped with a green tristimulus filter (Maier and Schiller, 1960).

RESULTS AND DISCUSSION

Darkening. The rate of darkening as measured by reflectance decrease or soluble dark pigment increase (Table 1) is retarded in the absence of oxygen. In general the oxygen effect becomes more pronounced as the temperature decreases. With the exception of the 38°C data the two methods give closely similar results for percentage retardation of darkening rate in the absence of oxygen. With heated dates, however, the

reflectance data show an increased rate and the soluble pigment data show a decreased rate when compared with unheated dates. It was previously shown (Maier and Schiller, 1960) that the results of the reflectance method are closely related to the visually observed darkness, whereas results of the soluble pigment method are not. It is apparent, therefore, that heated dates darken considerably more rapidly than unheated dates, but produce less soluble dark pigment (more insoluble dark pigment) in the process of darkening. Thus, the heat treatment not only increased the initial darkness of the dates from 16.8 to 8.04% reflectance (Maier and Schiller, 1961), but also resulted in a vastly increased susceptibility to darkening. This is probably caused by the production of brown pigment precursors by the heat, in addition to an increased lability of the polyphenolic constituents to autoxidation as suggested by Nielsen *et al.* (1950). Because of the pronounced effect of heat on the darkness of the dates, the original purpose of this treatment, to serve as a nonenzymic darkening control, was not achieved.

Another approach, using date paste rather than whole dates, was therefore used to help clarify this point. The semi-liquid properties of the paste permitted rapid thermal enzyme inactivation without concomitant darkening. In fact, the heated sample was slightly lighter initially. In addition, the high moisture content of the paste accelerated and favored enzymic darkening (as opposed to nonenzymic darkening), allowing the study to be completed in 24 hours. Fig. 1 shows the rates of darkening of heated and unheated date paste as studied by the reflectance method. It is clear that darkening under these conditions is primarily enzymic. Thus, it can be concluded that a functional enzymic browning system is present in date tissue. It appears probable, therefore, that the oxidative darkening of intact unheated dates is an enzyme-catalyzed reaction.

The effect of the treatments on the apparent activation energy of darkening is shown in columns A and B of Table 2. The rate of oxidative darkening was obtained by subtracting the rate in nitrogen from the rate in air. The apparent activation energy for oxidative darkening was calculated from these rates. The respective average activation energies of oxidative and nonoxidative darkening were found to be 23.4 and 34.5 kcal/mole. With unheated dates the good agreement of the activation energies obtained by the reflectance and soluble pigment methods suggests that similar reactions are measured in each case. With heated dates the large difference in activation energies obtained by the reflectance

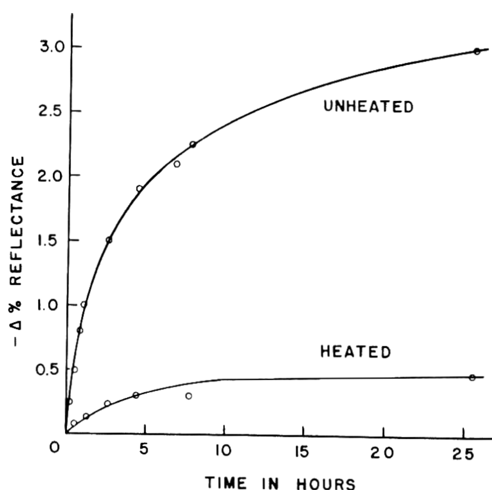

Fig. 1. Darkening of heated and unheated date paste as measured by the decrease in reflectance at 25°C.

Table 2. Apparent activation energies of reactions associated with deterioration of dates.

	Activation energies in kcal/mole					
Treatment	A Reflectance decrease	B Soluble pigment production	C pH decrease	D Oxygen absorption	E Carbon dioxide production	F Sucrose hydrolysis
Air	29.9	31.3	22.6	17.6	25.3	26.0
Nitrogen	34.5	34.5	21.5	......	......	26.0
(Air-nitrogen)[a]	25.3	21.5	......	......	......	......
Heated	17.6	33.6	24.2	19.0	27.4	......

[a] See text.

method, 17.6 kcal/mole, and the soluble pigment method, 33.6 kcal/mole, indicates a fundamental difference in the reactions measured. The high activation energy and slow rate of darkening observed with the soluble pigment method suggest that in heated dates soluble dark pigment is produced primarily by the nonoxidative darkening pathway. Conversely, the low activation energy and rapid rate observed by reflectance measurements suggest that nonenzymic oxidative darkening or a low energy phase of nonoxidative darkening caused by accumulation of labile dark pigment precursors during heat treatment are primarily responsible for the over-all darkening of heated dates. Nielsen *et al.* (1950) reported that darkening of pasteurized dates is primarily an oxidative reaction.

The activation energy of nonoxidative darkening in dates is similar to that reported for nonenzymic browning of dehydrated white potatoes, i.e., 32 and 37 kcal/mole, respectively, for moisture contents of 8.8 and 4.7% (Hendel *et al.*, 1955). A considerably lower activation energy, 26 kcal/mole, has been reported for the nonenzymic browning of sulfured, dried (23.5% moisture) apricots (Stadtman *et al.*, 1946).

Ingraham (1954) reported activation energies, determined in dilute aqueous solution, of 3.9 and 11.6 kcal/mole, respectively, for polyphenolases from apple and mushroom tissue. The higher activation energy observed in this work for oxidative darkening of unheated dates, 23.4 kcal/mole, does not, however, rule out the possibility that this is an enzyme-catalyzed reaction. The activity of date phenolase is known to be reduced by high sucrose and glucose concentrations (Rashid, 1950). It is possible that the very high solute concentration of date tissue (primarily sucrose, glucose, and fructose) in addition to reducing the reaction rate also results in a high apparent activation energy. Invertase-catalyzed sucrose hydrolysis is an example of an unequivocal enzyme-catalyzed reaction in date tissue with a high apparent activation energy (see section below on sucrose hydrolysis).

Enzyme activities. Peroxidase and phenolase activities were assayed at several storage periods for the fruit held at 38.0°C.

Table 3. Changes in apparent enzyme activities (Δ absorbance/min) during storage of dates at 38.0°C in air and in nitrogen.

Time (days)	Air		Nitrogen	
	Phenolase	Peroxidase	Phenolase	Peroxidase
0	0.021	0.090	0.021	0.090
5.5	0.033	0.072		
55	0.141	0.040	0.181	0.042
128	0.140	0.008	0.210	0.009

The results (Table 3) show an increase in apparent phenolase activity and a decrease in apparent peroxidase activity, which is in agreement with previous findings at 49°C. Heat-treated dates gave negative tests for both enzymes at all times, indicating no regeneration of enzyme activity. In general, phenolase activity was somewhat higher in dates stored in nitrogen than in dates stored in air. This may have been the result of less reaction-inactivation of the enzyme. Phenolase appears to approach a maximum activity after 128 days' storage, whereas peroxidase activity continues to decrease. This suggests that phenolase is most probably the enzyme responsible for enzymic browning of dates.

Decrease in pH. The results in Table 1 show that heating dates to inactivate enzymes has very little effect on their subsequent rate of decrease in pH. The reaction responsible for this change is therefore primarily nonenzymic. In the absence of oxygen the rate of pH decrease is somewhat slower than that of the control in air, varying from 74.0% of the control rate at 49.0°C to 89.5% at 28.4°C. Thus, pH decrease is caused primarily by a nonenzymic, nonoxidative reaction. It is not known whether the small acceleration in the rate caused by ogxyen is the result of an indirect effect on the nonoxidative reaction or the result of a separate nonenzymic, oxidative reaction.

The apparent activation energies for pH decrease listed in Column C of Table 2 give an average value of 22.8 kcal/mole. This is considerably lower than the activation energy of nonoxidative darkening. We previously proposed that pH decrease might be closely associated with the darkening system (Maier and Schiller, 1961). Unfortunately, these results do not prove or disprove this hypothesis. If pH decrease is a

result of the nonoxidative darkening system, it must occur in a reaction that precedes the formation of dark pigments since it has a much lower activation energy. Such a reaction could be the condensation of amino acids with reducing sugars, the lowering of pH resulting from loss of basic amino groups. It is interesting in this regard that a decrease in concentration of total amino acids has been reported to occur during darkening of dates (Rinderknecht, 1959).

Gas exchange. Absorption of oxygen and production of carbon dioxide by dates have been reported to involve complex reaction systems (Maier and Schiller, 1961). The results in Table 1 bear this out. The heat treatment reduces the rate of gas exchange more pronouncedly at lower storage temperatures. This indicates that enzymic reactions contribute to the over-all gas exchange. Reactions such as enzymic browning and tissue respiration are probably involved.

The nonenzymic reactions (heated dates), which account for about 60% of the over-all gas exchange rates at 28.4°C, have apparent activation energies of 19.0 and 27.4 kcal/mole, respectively, for oxygen absorption and carbon dioxide production. The respective energies obtained for oxygen absorption and carbon dioxide production are lower with unheated dates, 17.6 and 25.3 kcal/mole, than with heated dates. Lower energies agree with previous findings in showing that gas exchange of unheated dates is partially enzymic and partially nonenzymic. It has been shown that carbon dioxide production is largely dependent on the presence of molecular oxygen (Maier and Schiller, 1961), indicating that the two gases are involved in closely similar reaction systems. The activation energies indicate that oxygen is converted into carbon dioxide in multistep reaction systems. It is interesting that the activation energy for oxygen absorption in heated dates is about the same as that for over-all darkening (by reflectance) of heated dates.

Sucrose hydrolysis. The virtual complete absence of sucrose hydrolysis in heat-treated dates (Table 1) leads to the obvious conclusion that sucrose hydrolysis in unheated dates is the result of invertase action. As would be expected, the presence or absence of oxygen has little effect on the rate of hydrolysis. The apparent activation energy of the hydrolysis shown in column F of Table 2, 26.0 kcal/mole, is much higher than the energies reported for yeast (Sizer and Josephson, 1942) and malt (Sizer, 1937) invertase in dilute aqueous solutions, respectively 11 and 13 kcal/mole. Sucrose hydrolysis in dates follows first-order kinetics over the major portion of the reaction at all three temperatures studied. In addition, the rate data fit the straight-line Arrhenius plot extremely closely, and the energies of the two separate determinations agree perfectly. These facts argue against partial heat inactivation of the enzyme or changes in reaction mechanism as causes for the high activation energy observed. It is probable that the very high solute concentration of date tissue is responsible for this high energy, possibly through its effect on the catalytic properties of the enzyme or on the diffusional properties of the reaction system.

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Relationship of Extensibility of Muscle Fibers to Tenderness of Beef

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SUMMARY

Greater muscle fiber extensibility in 1-in. steaks from 24 steers was closely related to higher shear force for both *Longissimus dorsi* and *Biceps femoris* muscles cooked to 100°C. At 61°C only *L. dorsi* showed this close relationship. Greater extensibility for both muscles was associated with lower scores (tougher meat) for softness to tooth pressure, ease of fragmentation and mealiness of muscle fibers at 100°C. At 61°C these relationships were not as marked for either muscle.

The role of muscle fibers in meat tenderness is not yet fully understood. It is becoming more and more apparent, however, that not all individual muscle fibers are alike and that differences between them contribute to variations in meat tenderness. Cover and Smith (1956) found that fibers of some braised *Longissimus dorsi* steaks fell apart readily into strings and were tough, whereas those from some *Biceps femoris* steaks prepared in the same way broke easily and were mealy. Work by Wang *et al.* (1956) indicated, but did not establish, a relationship between muscle fiber extensibility and tenderness. This study was conducted to compare the extensibility of the fibers of two muscles with shear-force values and judges' scores for six components of tenderness.

EXPERIMENTAL

The samples of meat came from 24 yearling steers produced in 1958–59 at Substation No. 23 of the Texas Agricultural Experiment Station. The freshly slaughtered carcasses were stored at about 36°F for 7 days before the experimental steaks were removed. Four 1-in steaks were obtained from each animal, including two steaks from each of two muscles (*L. dorsi* from loin and *B. femoris* from bottom round). These steaks were wrapped individually, frozen at −20°F, and stored at 0°F.

About 15 hr before being cooked the four steaks from one carcass were placed in a conditioning refrigerator. Just before cooking, when the internal temperature of the steaks was about −2°C (28.4°F), the bulb of an L-shaped thermometer was inserted to the center of each steak from the outside edge. Cooking was begun when the internal temperature reached +1°C. One of the steaks from each muscle was cooked at 175°C by dry heat to an internal temperature of 61°C; the other was cooked in 100°C steam to an internal temperature of 100°C and held 25 min at that temperature. Cover and Hostetler (1960) have described the cooking procedures, and the details of scoring by the taste panel and of obtaining and shearing the 4 cores from each steak. The samples for the panel were taken from the center of the steak, and a ½-in core for shear tests was taken from each quarter of the steak (Fig. 1). The core halves resulting from shearing were saved. The halves closest to the judges' samples were used for extensibility determinations.

Extensibility was determined in essentially the manner described by Wang *et al.* (1956). A piece of meat of about 15 x 6 x 3 mm, with the fibers running the long way, was removed from a core. This piece and enough water to cover the somewhat dulled blades were placed in the glass jar of a Waring Blendor. The blender was then run for about 15 sec. A rheostat in the line set at 40 V reduced the speed of the blender.

Equipment used in gathering extensibility data included a pair of watchmaker's forceps with fine points and a Petri dish with a transparent millimeter ruler placed on the bottom. Water was kept in the dish to keep the fibers from drying while being stretched. A binocular microscope with 15x eye pieces and 0.7x objectives was used in examining the fibers for injuries incurred during blending and in observing them while they were being stretched.

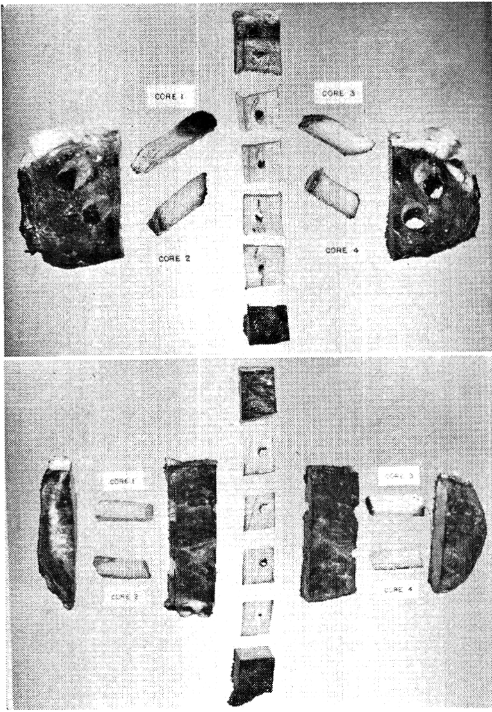

Fig. 1. *Longissimus dorsi* (top) and *Biceps femoris* (bottom) steaks with samples for taste panel cut from center of steak and a ½-in core cut from each quarter of the steak. The browned edges were removed from the samples for the taste panel.

Extensibility determinations were made by grasping a single fiber at two points 5 mm apart with two forceps. The forceps in the left hand was kept stationary, and that in the right hand moved slowly to the right until the fiber broke. The amount of stretch beyond the original 5 mm was recorded as the extensibility. Values were measured to the nearest 0.25 mm. The averages reported are to the nearest 0.1 mm.

Six extensibility determinations were made on each core from which fibers were available. Fig. 2 shows the contrasting appearance of the blended samples from which fibers were and were not available within one steak. When it was not possible to obtain six fibers for extensibility determinations from a sample, it was assumed that blending was sufficient to break the majority of them, and zero values were assigned to each of the six possible measurements of that core. The 24 determinations, including the six zeros for those cores from which fibers were not obtainable, were averaged. These averages were used in calculating analyses of variance and coefficients of correlation.

In a preliminary study, extensibility had been determined for fibers from only one area of a steak. The area chosen for *B. femoris* steaks had been between cores three and four (Fig. 1). For *L. dorsi* steaks the chosen area had been between cores one and two. If no fibers had been found in these areas, other areas were tried until fibers were obtained. Twenty extensibility determinations had been made for each steak. It was then discovered that certain areas, particularly in *B. femoris* steaks, yielded single fibers more easily than others. Since there was the possibility that the extensibility of muscle fibers, like shears, might vary from area to area within a steak, it was decided to try to get fibers from several areas of the steaks. For this reason samples for extensibility determinations were taken from the four cores of a steak in this study.

RESULTS AND DISCUSSION

Muscle fiber extensibility. The availability or successful isolation of single muscle fibers for extensibility determinations differed for the two muscles and from one position to another within the same steak. It was possible, with only a few exceptions, to get single fibers from each of the four cores of *L. dorsi* steaks. With *B. femoris* steaks, however, single fibers were seldom obtained from all of the cores. There is little evidence that cooking method had any effect on the availability of fibers from steaks in either muscle.

Extensibility values were higher for steaks cooked to 100°C than for steaks cooked to 61°C (Table 1). Fibers from *L. dorsi* muscles showed the greatest difference in extensibility between cooking methods. Wang *et al.* (1956) reported that cooked fibers from *Semitendinosus* and *L. dorsi* muscles were more extensible than raw fibers from the same muscles. Smith (1957) found that cooking increased the tensile strength of muscle fibers. Bramblett *et al.* (1959) reported, in contrast, that cooking meat for unusually long periods at low temperatures (18 hr at 68°C and 30 hr at 63°C) generally decreased the tensile strength of muscle fibers from five muscles of the bottom round (*Biceps femoris, Semitendinosus, Semimembranosus, Adductor,* and *Gracilis*).

Extensibility values were higher for *L. dorsi* than for *B. femoris* steaks within each of the cooking methods (Table 1). This had also been true in preliminary work at

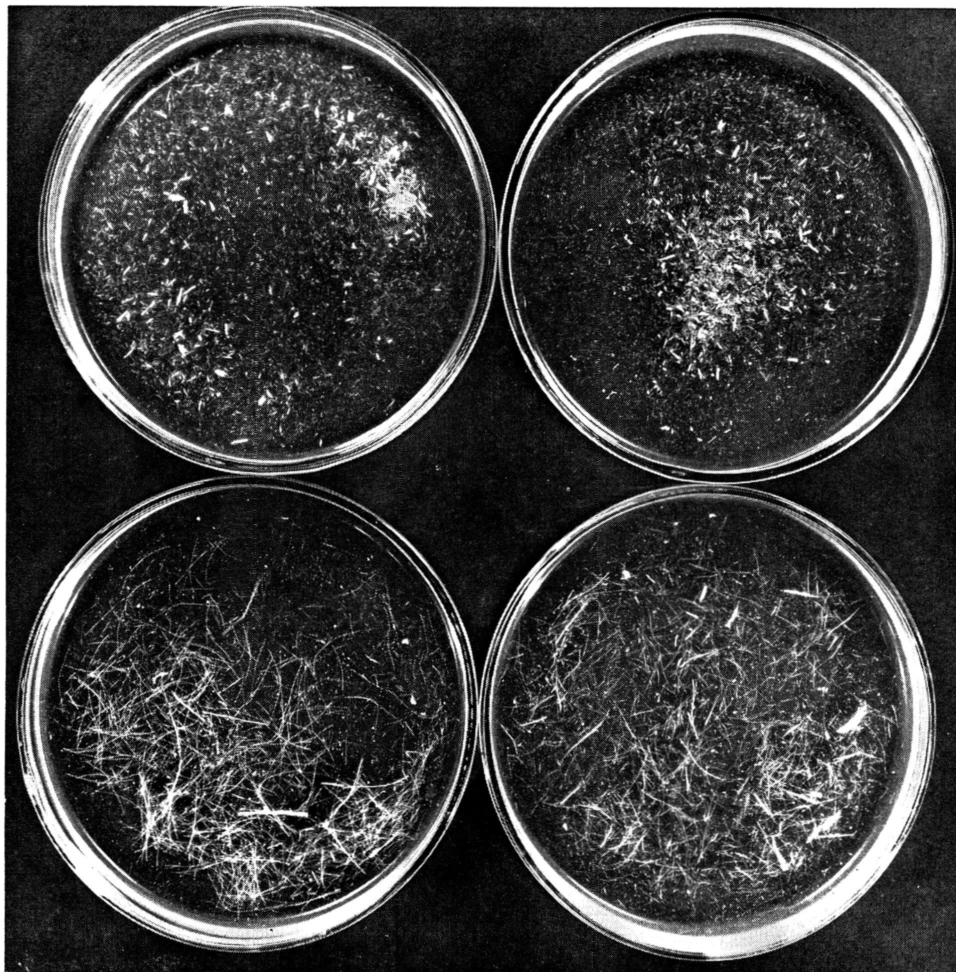

Fig. 2. Variation in availability of single muscle fibers from the four cores of a steak from *Biceps femoris*. Upper left, core 1 unsuccessful; lower left, core 2 successful; upper right, core 3 unsuccessful; lower right, core 4 successful.

100°C but not at 61°C. The fibers that had been readily available from *B. femoris* at 61°C had not differed in extensibility from those of *L. dorsi* at the same temperature, but at 100°C, fibers that had been readily available from *B. femoris* had not been as extensible as those from *L. dorsi* muscles.

A wide variation in extensibility values within each muscle-method combination indicated differences among the same muscles from different animals (Fig. 3). The ranges of extensibility values for fibers from *L. dorsi* steaks were about the same for both methods of cooking, though their minimum and maximum values were quite different. The range of extensibility values for fibers from *B. femoris* was slightly greater for steaks cooked to 100°C than for steaks cooked to 61°C.

Each muscle had two extensibility values (mean of 24 fibers) associated with it: one for 61°C, and one for 100°C. Correlation coefficients were used to measure the degree of association between these two extensibility values for the 24 animals in this test. Coefficients of 0.76 for *L. dorsi* and 0.88 for *B. femoris* steaks indicated positive relationships (sig. at 0.1% level) for both muscles. In preliminary work, when fibers had been taken from only one area of a steak, this relationship had not been sig-

Table 1. Mean of extensibility and shear-force values (24 animals).

Muscles	Steak temp. (°C)	Mean extensibility (mm)	Mean shear (lb)
Variation between steak temperatures			
L. dorsi	61	3.9	9.7
	100	6.8	15.3
	Sig. of dif.	***	***
B. femoris	61	2.2	8.1
	100	3.2	10.3
	Sig. of dif.	*	*
Variation between muscles			
L. dorsi	61	3.9	9.7
B. femoris	61	2.2	8.1
Sig. of dif.		***	n.s.
L. dorsi	100	6.8	15.3
B. femoris	100	3.2	10.3
Sig. of dif.		***	***

n.s., *, ***, respectively, indicate significance above the 5% level, and at the 5% and 0.1% levels.

nificant. The coefficients of correlation for 32 animals had been 0.22 for *L. dorsi* and 0.33 for *B. femoris*. The increase in coefficients was possibly an indication that the sampling techniques had been improved.

Shear-force values. When shear-force values were compared by steak temperatures within muscles, it was found that steaks cooked to 100°C sheared higher than steaks cooked to 61°C (Table 1). The difference was greater in the *L. dorsi* muscle. At 61°C the *L. dorsi* did not shear significantly higher than the *B. femoris* muscle, but at 100°C it sheared considerably higher.

Shear-force values varied widely for each group of 24 steaks within a muscle-method combination (Fig. 4). This is another indication of differences between animals in the same muscles. For both *L. dorsi* and *B. femoris* steaks the range of values was larger at 100°C than at 61°C. Within a cooking method the ranges were larger for *L. dorsi* than for *B. femoris* steaks.

Each muscle had two shear values (mean of 4 cores) associated with it: one for 61°C and the other for 100°C. Correlation coefficients were used to measure the degree of association between these two shear values for 24 animals. Coefficients of 0.86 for *L. dorsi* and 0.51 for *B. femoris* steaks indicated positive relationships that were significant at the 0.1% level for *L. dorsi*, and at the 1.0% level for the *B. femoris* muscle.

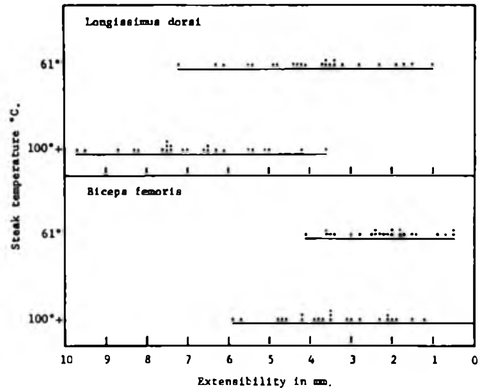

Fig. 3. Distribution of extensibility values for 24 steaks within each cut and condition of cooking. Each dot represents the mean value of 24 fibers from one steak.

Relationship between extensibility and shear. Both shear and extensibility values varied from one position to another within many of the steaks. In general, cores with high shear values also had high extensibility values, and those with low shear values had low extensibility values. The relationship between extensibility (mean of 24 fibers) and shear-force values (mean of 4 cores) can perhaps best be shown by the use of

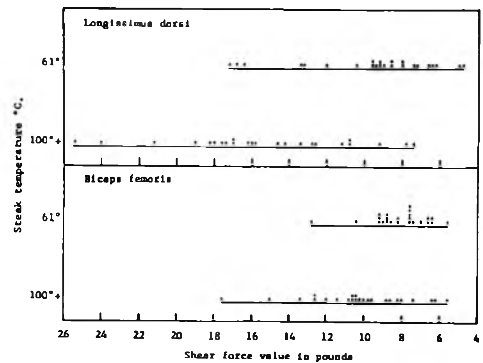

Fig. 4. Distribution of shear force values for 24 steaks within each cut and condition of cooking. Each dot represents the mean value of 4 cores from one steak.

Table 2. Coefficients of correlation of extensibility value with panel score for 24 steers.

Steak temp. (°C)	Muscle	Coefficients of correlation for extensibility versus panel scores for: Softness: To tongue and cheek	Softness: To tooth pressure	Muscle fibers: Ease of fragmentation	Muscle fibers: Mealiness	Muscle fibers: Apparent adhesion	Connective tissue
61	*L. dorsi*	−.51*	−.60**	−.41*	0	−.49*	.38 n.s.
	B. femoris	−.62**	−.02 n.s.	−.31 n.s.	−.32 n.s.	−.54**	.25 n.s.
100+	*L. dorsi*	−.49*	−.80***	−.80***	−.72***	−.77***	−.30 n.s.
	B. femoris	−.64***	−.81***	−.85***	−.86***	−.73***	−.10 n.s.

n.s., *, **, *** respectively, indicate significance above the 5% level, and at the 5%, 1%, and 0.1% levels.

correlation coefficients. The coefficients were higher within *L. dorsi* than within *B. femoris*, being 0.92 for 61°C and 0.88 for 100°C within *L. dorsi*, and 0.24 for 61°C and 0.79 for 100°C within *B. femoris* steaks. The coefficients were significant at the 0.1% level for both methods of cooking for *L. dorsi* steaks and for *B. femoris* steaks cooked to 100°C, but were not significant for *B. femoris* steaks cooked to 61°C.

In preliminary work, when fibers for extensibility determinations had been taken from only one area of a steak, the correlation coefficient for shear versus extensibility had been 0.73 for 61°C and 0.49 for 100°C within *L. dorsi*, and 0.28 for 61°C and 0.18 for 100°C within *B. femoris*. Thus, getting fibers from the same areas as the cores markedly increased the correlations between extensibility and shear values for all muscle-cooking method combinations except for *B. femoris* at 61°C.

The improved sampling technique seems to associate extensibility with shear-force values for *L. dorsi* steaks cooked to 61°C and 100°C, and for *B. femoris* steaks cooked to 100°C. *B. femoris* cooked to 61°C, in contrast, must have factors not measured by extensibility that influence shear-force values.

Relationship between extensibility and panel scores. When steaks from either *L. dorsi* or *B. femoris* were cooked to 100°C, correlation coefficients indicated that 4 of 6 recorded components of tenderness were rather closely related to extensibility values (Table 2). The four closely related components were: softness to tooth pressure, ease of fragmentation, mealiness, and apparent adhesion between muscle fibers. Somewhat less closely related was softness to tongue and cheek, especially in *L. dorsi*. Tenderness of connective tissue gave no indication of any relationship to extensibility values. A negative coefficient indicated that high extensibility values were associated with low scores for the four components and that such meat was not soft to tooth pressure, the muscle fibers were not easily fragmented or very mealy, and there was apparent adhesion between the muscle fibers.

The correlations were lower for 61°C than for 100°C. The only two of the six components that showed a significant relationship to extensibility values for both muscles were softness to tongue and cheek, and apparent adhesion of muscle fibers. Softness to tooth pressure and ease of fragmentation were related significantly to extensibility for only *L. dorsi*. For *B. femoris* at 61°C, the relationship was very low between extensibility and softness to tooth pressure. Something other than muscle fibers must have influenced scores for softness to tooth pressure. Softness to tooth pressure was correlated more highly with tenderness of connective tissue (coefficient 0.71, significant at 0.1% level) than with any other component of tenderness or with shear. Perhaps the impression of softness to tooth pressure in *B. femoris* was influenced more by muscle fibers at 100°C, and more by connective tissue at 61°C.

Muscle fiber extensibility and tenderness. This study seems to bear out the suggestion of Wang *et al.* (1956) of an inverse relationship between muscle fiber extensibility and tenderness. Both studies compared muscle fiber extensibility with shear and

taste panel tests, but the present study extended the tests to include two conditions of cooking, and taste-panel scores to include six components of tenderness.

The relationship between shear and extensibility values was closer for some muscle-cooking method combinations than for others. The softness and muscle fiber components of the taste-panel scores were rather closely related to extensibility values at 100°C, but not at 61°C. Scores for connective tissue did not show any relationship to extensibility values for either muscle cooked by either method. The physical or chemical changes responsible for increasing the extensibility of muscle fibers as cooking takes place are not yet clear.

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Effect of Some Proteins on the Binding Quality of an Experimental Sausage [a]

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Muscle materials were prepared that answered the requirements of the present experiments, as judged by results on the quantities of extracted protein estimated by chemical analysis and the appearance of structure observed by electron microscope. Nevertheless, in the results obtained from the determination of ATPase activity and from superprecipitation, it was clear that the protein involved had not been completely removed from the fibrils. Water-soluble protein did not appreciably affect the binding quality of sausage. There was a shade of differences between the binding quality of sausage made from intact fibrils, and that of sausage made from actin- and tropomyosin-poor fibrils. In the experimental sausages, binding quality was low when myosin-poor fibrils were used, and was negligible when "ghost" fibrils were used.

The changes in the structural proteins of muscle in the sausage manufacturing process have not been sufficiently clarified, since the material used for experimental sausages has usually been whole muscles. Between the cell level and the molecular level seem to be many factors that should be taken into consideration.

On the basis of this idea, the present study deals with the preparation of subcellular muscle preparations from the muscle fibrils free of water-soluble protein to the so-called "ghost" fibrils.

It was found that sausage made from myosin-poor muscle fibrils showed a considerable decrease in binding quality, whereas sausage from water-soluble protein-free muscle fibrils showed little change in binding quality.

EXPERIMENTAL

Materials. Beef *M. semitendinosus* was used within 2 hr of slaughter. Adenosine triphosphate (ATP) was isolated from fresh rabbit muscle by the method of Kerr (1941) and used as the potassium salt.

Preparation of myofibrils. As shown in Fig. 1, various muscle models were prepared. The method of preparing myofibrils (Fig. 1) was similar to one reported by Perry (Corsi and Perry, 1958; Perry, 1951; Perry and Corsi, 1958; Perry and Grey, 1956). Chilled, freshly minced muscle was homogenized 1 min with 5 vol of 0.1*M* KCl containing 5m*M* ethylenediaminetetraacetate (EDTA), pH 7.0, and centrifuged 15 min at 2500 rpm. The residue was resuspended with 5 vol of the same solution and homogenized 2 min. After centrifugation for 15 min at 2500 rpm, the supernatant was discarded and the light-colored upper layer of the sediment, mainly myofibrils, was removed with the aid of a little KCl-borate buffer (0.1–0.039*M*), pH 7.1. The myofibril paste was diluted with KCl-borate to 1 L/200 g of muscle taken, then centrifuged 3 min at 1500 rpm. The precipitate was discarded, and the suspension was centrifuged again

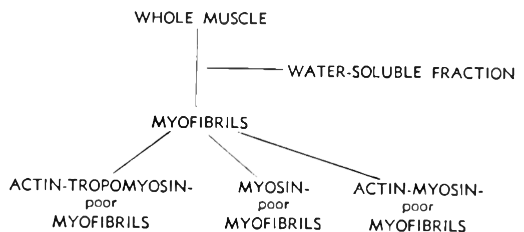

Fig. 1. Classification of various muscle models from fresh beef muscle.

[a] In this paper, purified actin-free myosin and actin-combined myosin prepared by extracting muscle with the 0.6*M* NaCl solution for 24 hours are respectively called myosin A and myosin B (natural actomyosin). The term "myosin" is also conventionally used to indicate both proteins.

for 15 min at 2500 rpm to precipitate the myofibrils. After two further resuspensions and centrifugation to remove sarcoplasmic proteins and granules, the suspension was centrifuged 20 min at 8000 rpm at 0°C to obtain a concentrated myofibril paste.

Preparation of actin-poor myofibrils. Extraction by procedures designed mainly to remove actin from isolated myofibrils was carried out with 5m*M* tris(hydroxymethyl)aminomethane buffer, pH 7.7, as suggested by Perry and Corsi (1958). Immediately after the myofibrils were prepared, 5 volumes of 5m*M* tris-buffer were added, and a suspension was made. The suspension was transferred to a stoppered flask in the presence of a trace of toluene, and 24 hours later the myofibril suspension was centrifuged 15 min at 3000 rpm. The supernatant was discarded except when the concentration of extracted protein was to be determined, and the sediment was again suspended with 5 vol of 5m*M* tris-buffer. To achieve extraction, the suspension and centrifuging were repeated every day for 7 days, instead of dialysis against 40-50 vol of the medium as reported by Perry and Grey (1956). According to Perry and Corsi (1958), this method of extraction simultaneously removed tropomyosin from the myofibrils. The prepared actin- and tropomyosin-poor myofibrils were washed thrice further with 10 vol of distilled water and KCl-borate buffer.

Preparation of myosin-poor myofibrils. Immediately after the myofibrils were prepared, 6 vol of Straub solution (0.3*M* KCl, 0.09*M* KH_2PO_4, 0.06*M* K_2HPO_4, pH 6.4) were added, and a suspension was made. Fifteen minutes later the myofibril suspension was centrifuged 15 min at 3000 rpm. The prepared myosin-poor myofibrils were washed thrice further with 10 vol of distilled water and KCl-borate buffer.

Preparation of "ghost" myofibrils. Immediately after the myofibrils were prepared, 10 vol of Hasselbach-Schneider solution (0.47*M* KCl, 0.01*M* $K_4P_2O_7$, 0.01 *M* phosphate buffer) were added, and a myofibril suspension was made. Twenty minutes later, the myofibril suspension was centrifuged 15 min at 3000 rpm. The precipitate was resuspended with 4 vol of the same solution, and 60 min later the suspension was centrifuged 15 min at 3000 rpm. Then, the precipitate was resuspended with 4 vol of the same solution, and 120 min later the suspension was centrifuged 15 min at 3000 rpm. Next, the precipitate was suspended with 6 vol of 0.6*M* KCl solution, and 18 hr later the pH of the suspension was adjusted to 7.0 by adding 0.05*M* $NaHCO_3$ solution. The suspension was centrifuged 15 min at 3000 rpm. The precipitate was washed thrice further with 10 vol of distilled water and KCl-borate buffer.

The four models of myofibrils mentioned above were centrifuged 20 min at 12000-13000 rpm to obtain a constant moisture content. All manipulations were carried out at 0°C.

Procedures. *Microscopic technique.* All observations by electron microscope (JEM-4B style) were carried out as follows: The sample of myofibrils was suspended with 100, 1000, and 10000 volumes of 0.025*M* KCl solution; a falling drop of the suspension was placed on a mesh of synthetic resins treated with collodion; the drop was dried under low pressure; and the sample to be used was shadowed with chromium.

ATPase activity. Methods of estimating the adenosine triphosphatase (ATPase) activity of myofibrils have been detailed by Perry (1951) and Bendall (1958). Their methods were modified for use in this study, as follows: Nine ml of a 0.1*M* potassium chloride suspension of myofibrils, containing 0.05*M* tris-aminomethane maleate buffer (pH 6.9) and 0.01*M* EDTA, was kept for 5 min in a constant-temperature bath at 20°C, and 1 ml of 0.01*M* ATP was added to the above mixture, thus starting the reaction. At measured intervals of time, aliquots of the reaction mixture were pipetted into equal volumes of 10% perchloric acid. The protein precipitated was removed by filtration through a sheet of dry filter paper. The amount of phosphate liberated was determined colorimetrically by the method of Martin and Doty (1949).

Determination of protein concentration. The nitrogen extracted from the whole muscle and the myofibrils with various media was estimated colorimetrically by the Biuret method or by Folin's reagent according to the concentration of the protein determined. The micro-Kjeldahl method was used to obtain the standard curve of the protein concentration and to determine the protein content of the homogenates before extraction. That was calculated by multiplying the nitrogen content determined by a factor of 6 in myosin A and myosin B, and by a factor of 6.25 in water-soluble protein and the other proteins. Potassium chloride solution, normally used for extracting protein from muscle tissue, caused difficulty in these studies because of a white substance that clouded the solutions when the protein content was determined by Folin's reagent (Gellart *et al.*, 1959). Therefore, sodium chloride in dilute solution, having almost the same action as potassium chloride in the extraction of protein from muscle, was used as the medium. However, because there were no technical difficulties in using KCl with the Biuret method, the protein concentration was sometimes measured by this method.

Determination of pH. The pH value was determined with a glass-electrode pH meter.

Sausage manufacturing. The sample sausages of both whole muscle and myofibrils were manufac-

tured according to the standard method, but with the addition of 2.5% sodium chloride (W/W). Model sausage was well mixed, stuffed into casing of gum hydrochloride (31 mm in diam), and cooked for 30 min in a water-bath at 70°C.

Determination of tensile strength. The binding quality of the sausage was evaluated by estimating the tensile strength of the finished product (Swift and Rex, 1957). The tensile strength was measured with a tester (Fig. 2), and the quality of the product was judged on the basis of photographic evidence from the plane surface of the slice. The method of measuring tensile strength was as follows. The center section of finished products was cut into slices 10-12 mm thick. One slice was placed on the plate (c'), and the end of the rod (c) was lowered until it touched the surface of the slice. Then the pointer of the circular scale (b) was adjusted to zero by turning the plate (c') which can be made to go up and down by means of a screw. Next, increasing weights were added at (a), and the readings of the pointer were recorded one minute after the addition of each weight (reading A). Then the slice was removed from the plate without being turned, and the reading of the pointer when the end of the rod (c) reached to the plate (c') was recorded (reading B). The tensile strength values of the sample sausages were calculated from these data as follows:

$$\text{Tensile strength} = 100 \times \frac{\text{reading A}}{\text{reading B}}$$

RESULTS

Effect of extraction with 0.6M KCl solution and 0.6M NaCl solution. As mentioned, NaCl solution was used sometimes as the medium, according to the purpose of the extraction. As a preliminary examination, it was necessary to check the agreement between KCl and NaCl in the results of extraction from both whole muscle and myofibrils. As shown in Table 1, the result was that the action of NaCl solution showed almost no difference from that of KCl solution under the same condition and at the same ionic strength. Therefore, both of these media were used without any fear of giving trouble and without discrimination.

Table 1. Extracting effect of 0.6*M* KCl solution and 0.6*M* NaCl solution.

Sample	Solution	Extracted protein (%)
Whole muscle	KCl	33.05
	NaCl	36.64
Myofibrils	KCl	66.98
	NaCl	69.23

Table 2. Effect of homogenization time on amount of protein extracted from intact myofibrils with 0.6*M* NaCl solution at 2×10^4 rpm and pH 6.8.

Time (sec)	Extracted protein (%)
10	25.8
30	23.7
60	20.3
120	21.6
180	22.3

Observation of various conditions of extraction as a preliminary examination. It was considered, in determining the protein content that could be extracted from isolated myofibrils by some media,

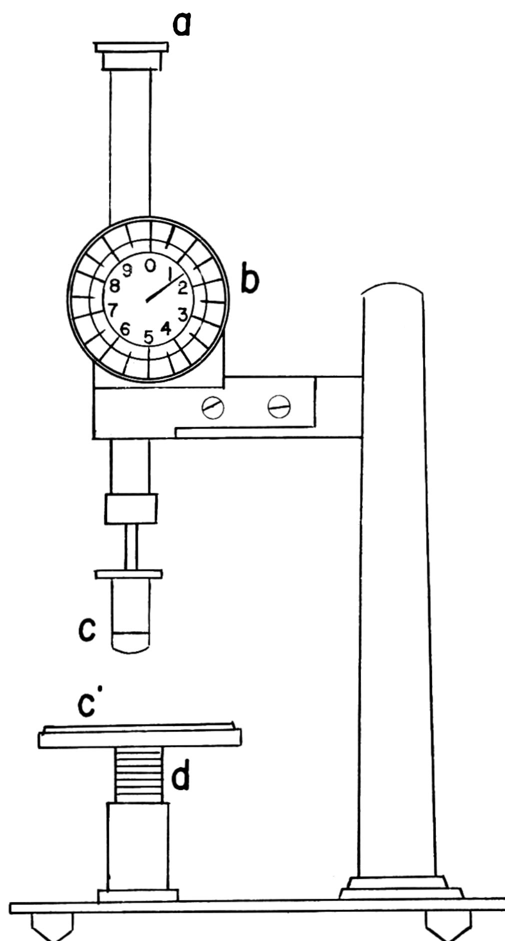

Fig. 2. Apparatus used to determine the tensile strength of a slice of sausage prepared from various muscle models.

that the concentration of protein that passed into solution might be influenced by the duration of homogenization and of extraction. Table 2 gives the results. In all these experiments the medium used was 0.6*M* NaCl solution, and the pH was controlled at 6.6 with dilute $NaHCO_3$ solution before centrifugation of the material. From this result it was found that homogenizing for 10-30 sec gave the best extraction. Extracting 12 hr or more after homogenizing increased the protein yield from myofibrils, but the amount extracted within the first 2 hr after homogenizing was constant. The results are indicated in Table 3.

Table 3. Effect of extraction time on amount of protein extracted from intact myofibrils homogenized 30 sec in 0.6*M* NaCl solution.

Extraction time	Extracted protein (%)
Immediately	21.4
15 min	21.3
30 min	21.4
60 min	22.7
120 min	21.4
12 hr	25.6
24 hr	25.0

The following investigation for the determination of the soluble protein content was carried out on the basis of the above results (Tables 2, 3).

Remaining protein content after extraction. Some soluble protein fractions were estimated; first, the protein extracted from fresh muscle by the 0.1*M* KCl solution and KCl-borate buffer (0.1-0.039*M*), and second, the protein extracted from myofibril paste by using various media. As shown in Table 4, the average remaining protein content of muscle extracted with 0.1*M* KCl solution and KCl-borate buffer was about 72% of the total protein. This quantity coincides almost exactly with that reported by Hanson and Huxley (1957). However, the yield of myosin, extracted mainly by Straub solution, was considerably less than their estimate of the percentage of myosin in total myofibrillar protein. This result may perhaps be due to the fact that actomyosin is formed during preparation after slaughter.

Table 4. Comparison of physicochemical properties in preparations of various muscle models.

	Remaining after extraction (%)		Super-precipitation	Binding quality
	Protein	ATPase		
Whole muscle	100	100	+	+
Isolated myofibrils	71.4	97.4	+	+
Actin- and tropomyosin-poor myofibrils	45.4	92.6	+	+
Myosin-poor myofibrils	55.2	25.9	+	±
"Ghost" myofibrils	24.8	9.3	+	−

Electron microscopic observations. Intact myofibrils isoalted from fresh muscle (Fig. 3) were similar in structure to those of rabbit muscle observed by other authors (Perry, 1951; Szent-Györgyi, 1951). As shown in Fig. 4, the features of fibrils with actin removed suggest that actin had been present as the I-substance; the I-bands and A-bands were shortened. Perry and Corsi (1958) and Corsi and Perry (1958) indicated that myosin does not pass into solution under the condition in the procedures described above. Therefore, it was thought that the I-band disappeared when actin was selectively extracted from fibrils with 5m*M* tris-buffer. When myosin was removed from fibrils, the electron micrographs showed that extraction was not complete. Even though the edges of both the I- and A-bands could not be seen clearly, some A-substance did remain (Fig. 5). After complete extraction of myofibrils with Hasselbach-Schneider solution, the only substance seen was in the region of the I-band as seen in Fig. 6. It was found that such images of "ghost" fibrils corresponded with the ones observed by interference microscopy (Corsi and Perry, 1958; Huxley, 1956; Huxley and Hanson, 1957). In the comparative measurements of the rabbit *psoas* muscle by interference microscopy and chemical analysis, Hanson and Huxley (1957) and Huxley and Hanson (1957) pointed out that at least four-fifths of the myosin in these fibrils is present as the A-substance. The present results could not be compared directly with that estimate, but the four preparations of myofibrils (Fig. 3, 4, 5, 6) can be helpful in this type of investigation because the relative amounts of actin and myosin, as estimated from the electron micrographs, agree with the quantities determined by chemical analysis (Table 4) (Figs. 3, 4, 5, 6).

Superprecipitation. The addition of ATP to a solution of myosin B containing a low concentration of KCl is known to result in a strong coagulation of the protein—"superprecipitation," after Szent-Györgyi (1951). This phenomenon was produced with every fibril preparation used in this study. In other words, even the "ghost" fibrils prepared with Hasselbach-Schneider solution still indicated myosin B. The results are in Table 4; the extracting procedure used for each preparation did not completely remove myosin B.

ATPase activity. It is clear from the results (Table 4) that the amount of ATPase remaining

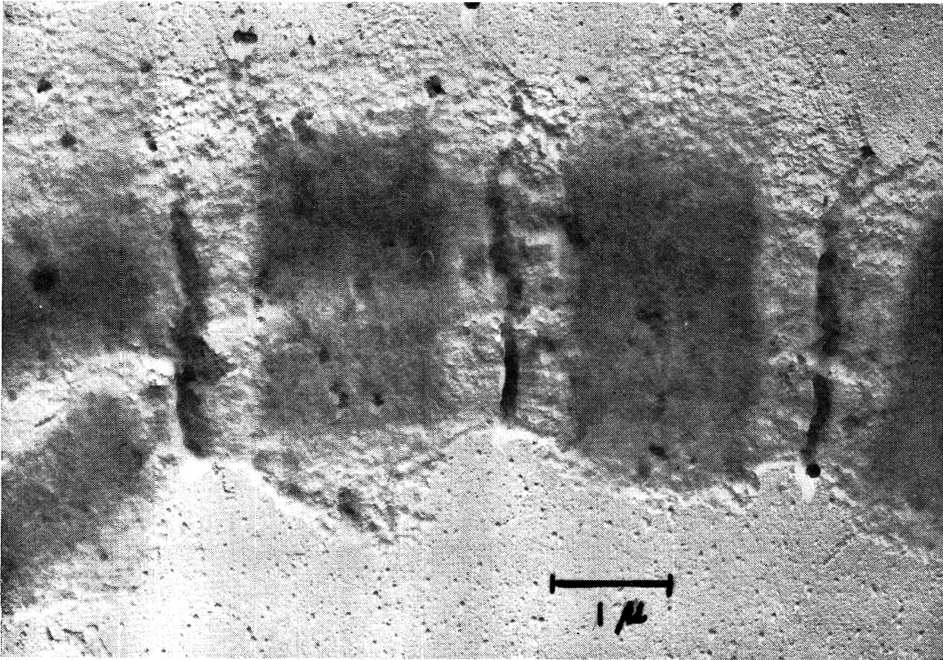

Fig. 3. An electron micrograph of a portion of the intact fibrils from fresh beef ***M. semitendinosus***. x 16,000.

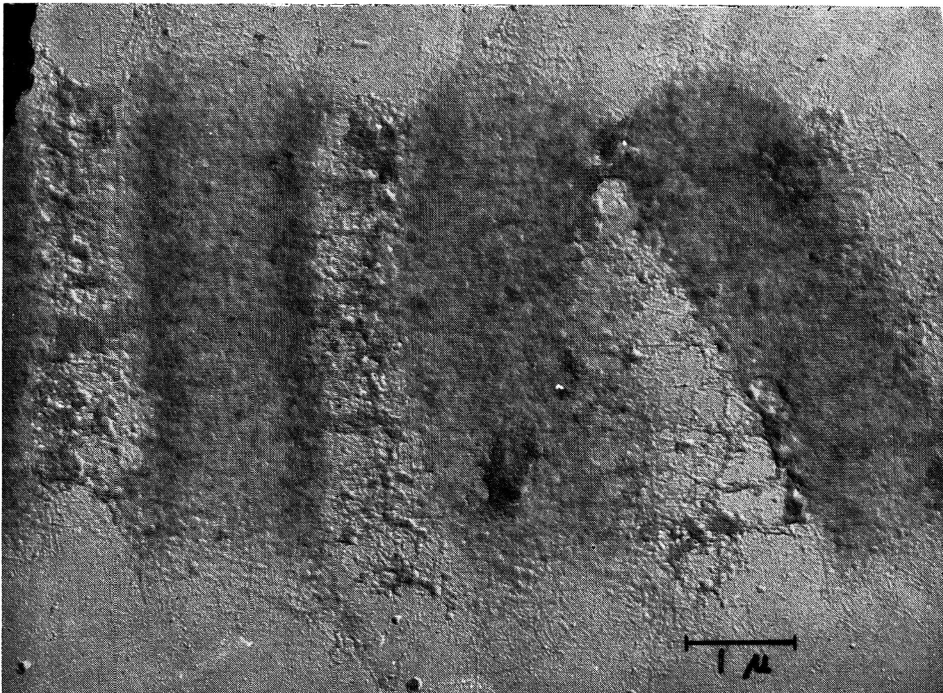

Fig. 4. An electron micrograph of a portion of the actin- and the tropomyosin-poor fibrils prepared from intact fibrils. Near the I-band, the fibrils were separated. x 16,000.

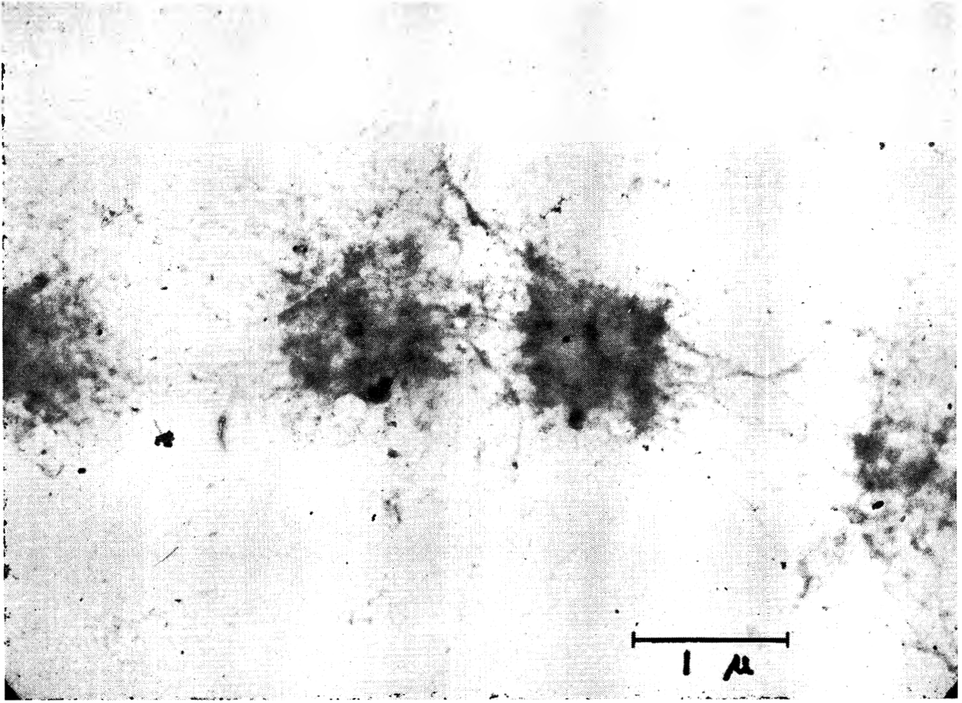

Fig. 5. An electron micrograph of a portion of the myosin-poor fibrils prepared from intact fibrils. It was notable that part of the A-band was removed. x 16,000.

Fig. 6. An electron micrograph of a portion of the "ghost" fibrils prepared from intact fibrils. The dense part seems to be near the I-band.

in each preparation depended on the extracting treatment. Because the presence of ATPase indicates the presence of myosin A too, it was apparent that myosin could not be removed completely from fibrils by the procedures employed. However, the degree of remaining ATPase activity in each preparation agreed well and qualitatively with the binding quality of the experimental sausage prepared from those materials. The results supported the writer's opinion that actomyosin, one of the structural proteins of muscle, might play an important role in meat quality; and that the nature and the quality of myosin and the ATPase activity have a close relationship with the binding quality of sausage.

Binding quality. It can be seen in Table 4 and Fig. 7 that the sausage prepared from myosin-poor fibrils decreased in binding quality, and that the "ghost" fibrils had no binding quality, expressed in tensile strength values. These facts suggest that myosin A is an essential protein in the binding quality of sausage. The sausages prepared from fibrils from which water-soluble protein had been removed, and from actin-poor fibrils were only slightly inferior in binding quality to sausage made from whole muscle. As Fig. 8 clearly shows, by photographic evidence of the plane surfaces of the slices described above, gross appearance varied with the remaining ATPase activity. In this case, the quantity of myosin A present in fibrils seemed to be an important factor in determining the quality of the sausage.

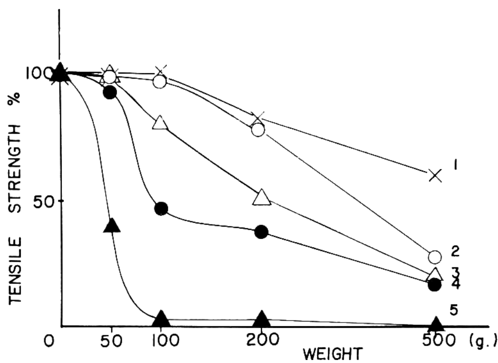

Fig. 7. Tensile strength of sausage from various muscle models: (1) whole muscle; (2) intact fibrils; (3) actin- and tropomyosin-poor fibrils; (4) myosin-poor fibrils; (5) "ghost" fibrils.

DISCUSSION

Many studies (Arnold *et al.,* 1956; Chili *et al.,* 1954; Grau and Fleischmann, 1957; Hamm, 1956, 1957; Suri, 1957; Swift and Hankins, 1954; Wierbicki *et al.,* 1957 a, b) on the quality of sausage have been based on the water-holding capacity. Other studies (Bendall, 1954; Swift and Rex, 1956) investigated the effects of various cations on the water-holding capacity of meat. On the other hand, it is reported that actomyosin in muscle proteins may play an important role in the binding quality (Bendall, 1954). The present authors reported in a previous paper (Hashimoto *et al.,* 1959) that since actomyosin is the chief constituent of muscle protein, it may be closely related to the binding quality of sausage, from investigations conducted on: A) the effect on nitrogen extractability of storing meat materials at 20°C, B) the pH value, C) the ATPase system of muscular tissue, and D) the quality of the sausage. From a study of the mechanism of the denaturation of myosin B (natural actomyosin) (Yasui *et al.,* 1958), it was suggested that the denaturation proceeds in two steps; a) a rapid first-order reaction that causes partial denaturation, and b) a pH-dependent reaction that proceeds slowly to complete denaturation. In recent research (Nihei and Tonomura, 1959), it was concluded that myosin B contains 10% of myosin A and that the denaturation in the rapid first-order reaction is due to the denaturation of the myosin A (Yasui *et al.,*

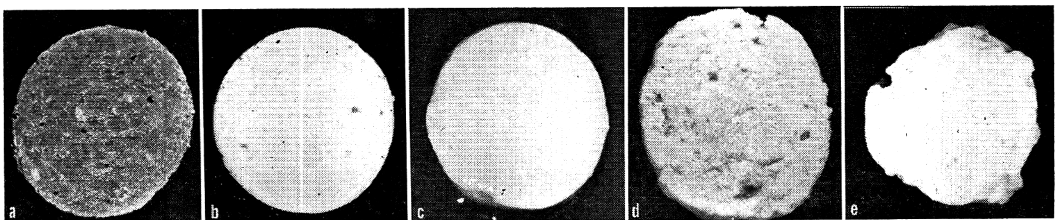

Fig. 8. Comparison of surface planes of sausage from various muscle models. (a) whole muscle; (b) intact fibrils; (c) actin- and tropomyosin-poor fibrils; (d) myosin-poor fibrils; (e) "ghost" fibrils.

1960). From these results, it was pointed out that myosin A, one of the muscle structural proteins, is the most important substance influencing the binding quality of sausage. Nevertheless, the relation of the muscle structural protein to the binding quality of sausage could not be clearly shown in a series of experiments (Hashimoto *et al.*, 1959; Yasui *et al.*, 1958), because the sample used was whole muscle in which various proteins were present as contaminants. In such a case, the matter of the proteins must be examined from all angles—including isolated protein both in whole muscle and in fibrils that are simplified as far as possible. This paper describes an investigation carried out at the level of the fibrils.

In excellent work of Huxley *et al.* (Hanson and Huxley, 1957; Huxley, 1956; Huxley and Hanson, 1957) and of Perry *et al.* (Corsi and Perry, 1958; Perry, 1951; Perry and Corsi, 1958; Perry and Grey, 1956), the investigation developed methods of comparative measurement of various protein fractions by chemical analysis and analysis of structure of fibrils by interference microscopy. Using these methods, the present writers prepared myofibrils simplified from beef *M. semitendinosus* and compared the binding quality of sausage manufactured from them in connection with physicochemical properties. The results indicated that myosin A plays an important role in the binding quality of sausage, because the binding of sausage prepared from fibrils of low ATPase activity is poorer than that of fibrils of high ATPase activity. Therefore, suitable quantities of remaining native myosin A in fibrils are necessary to good binding. It seemed that the binding quality of sausage, as shown by tensile strength, changed principally with the quantity of native myosin A in the fibrils; and in the present work, the water-soluble protein and the actin and tropomyosin had no immediate effects on that quality. Actin appears to be useful in maintaining stability in the form of actomyosin, being bound with myosin A. Although the various extracting procedures used were not fully suitable for making perfect preparations, electron microscopy clearly showed the features resulting from removal of most of the protein of the fibrils.

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The Relationship Between the Components of Myofibrillar Protein and the Effect of Various Phosphates that Influence the Binding Quality of Sausage[a, b]

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SUMMARY

Components of myofibrillar protein consist mainly of a "heavy" part of myosin B: the existence of small components was recognized in 43–50% of saturated ammonium sulfate from salting-out diagrams of native myofibrillar protein. Regarding the increase in the binding quality of sausage that the phosphate addition brought about, the facts are summarized: first, in the native state the effect has been correlated with the action of phosphate in promoting the extraction of protein from intact fibrils; and second, in the denatured state the effect has been correlated with the quantities of "light" components dissociated by ultracentrifugation as result of addition of phosphate. It was shown that the binding properties of sausage bear close relationship to the amount and the nature of myosin A contained and/or liberated in myosin B.

It is apparent, from results obtained heretofore (Fukazawa *et al.*, 1961 a, b; Hashimoto *et al.*, 1959), that myosin B is closely related to the binding quality of sausage, and that myosin A, which is contained in myosin B, plays an especially important role. On the other hand, that myofibrillar protein composes "heavy" components of myosin B seems to have been proved through the results on the inactivation of ATPase. In view of these observations, further studies on the components of myofibrillar protein were carried out.

On the other hand, with regard to various phosphates used in meat production, some reports (Bendall, 1954; Swift and Rex, 1956, 1957; Wierbicki and Deatherage, 1958) have already appeared on the increasing effect of the water-holding capacity of meat. Nevertheless, the cause of the increased binding from the addition of the phosphates has not been made clear.

MATERIALS AND PROCEDURES

Materials. Beef *M. semitendinosus* and rabbit skeletal muscle were used as materials. Myosin A solutions were prepared according to the method described by Perry (1955). The preparation of muscle fibrils was as reported in our paper (Fukazawa *et al.*, 1961a). Myosin B solution was prepared by extracting minced muscle with a 6-fold weight of Weber-Edsall solution for 24 hours. The resulting extracts were then purified by precipitating once or twice at an ionic strength of 0.06 μ and by resolubilizations at 0.6 μ. Then, 6 volumes of 0.6*M* NaCl Weber-Edsall solution were used for removing the myofibrillar protein from fibril paste. After standing for 20 minutes at 0°C the suspension was centrifiuged 15 min at 3000 rpm. The supernatant liquid was employed as a sample for estimating myofibrillar protein.

Procedures. *Salting-out analysis.* Stock solutions of phosphate buffer (0.5*M* KCl and 0.1*M* phosphate buffer, pH 7.0) saturated with ammonium sulfate were prepared at 5°C. From these, a series of salt solutions with varying concentrations of ammonium sulfate and a constant concentration of phosphate buffer was obtained. To 5 ml of these

[a] In this paper, purified actin-free myosin and actin-combined myosin prepared by extracting muscle with the 0.6*M* NaCl Weber-Edsall solution for 24 hours are called respectively myosin A and myosin B (natural actomyosin). The term "myosin" is also conventionally used to indicate both proteins.

[b] The following abbreviations are used: ATP = adenosin triphosphate, PP = pyrophosphate, TPP = tripolyphosphate, HMP = hexametaphosphate.

solutions was added 1 ml of the protein solution, and the mixture allowed to stand 20 hr at 1°C. Then, the precipitate was discarded by filtration (Snellmann and Tenow, 1954). The protein concentration in the filtrate was measured by colorimetry with Folin's phenol reagent, or directly by the method of Lowry *et al.* (1951).

Ultracentrifugal analysis. The ultracentrifugal sedimentation pattern was determined with a Spinco Model E ultracentrifuge at 28°C and 55,800 rpm.

Determination of extracted protein. Five grams of intact fibril paste, first buffered to pH 6.4 with 1/40M tris-maleate buffer, was weighed and suspended in 30 ml of 0.6M NaCl Weber-Edsall solution. Next, after standing 15 min at 0°C, the paste was centrifuged 15 min at 3000 rpm. The supernatant fluid was filtered through a cloth, and then, after the residue was washed several times with the same solution, total volume was made up to 50 ml. An aliquot of this solution was pipetted out, and the protein content was estimated colorimetrically with Folin's phenol reagent or the Biuret method. The effect of phosphate on the extractability of myofibrillar protein was examined by determining the extractable myofibrillar protein obtained when a 0.1M solution of various phosphate salts was added to fibrils, at the time of extracting, in such amounts that the final concentration was 0.01M. At the same time, control preparations were examined after addition of the same volume of distilled water. The pH of the suspension was adjusted to 6.6 by Br-thymol blue (BTB) indicator before centrifugation.

Ultracentrifugal separation. The protein solutions, which had a constant concentration of 0.6M NaCl, with and without phosphate salt, were ultracentrifuged 150 min at 37,000 rpm at 3°C in a Hitachi preparative ultracentrifuge (Nihei and Tonomura, 1959; Tonomura and Morita, 1959b). Then the protein solutions were separated into two or three parts: upper clear layer, turbid layer, and pellets (Yasui *et al.*, 1960). The protein was pipetted out from the upper layer in a polyethylene bottle, and the protein concentration was determined by the method mentioned above. The percentage of myofibrillar protein associated with the "light" component was calculated by dividing the protein concentration determined before the treatment.

Viscosity change. The viscosity of a 0.6M NaCl solution of the myosin-B-like protein extracted from fibrils was estimated by viscometer of the Ostwald type at 20°C and pH 6.7 in the presence of 1mM Mg (Cl salt), after removal of gross aggregates by centrifugation for 20 min at 12,000 rpm and 0°C. From the measured values the relative viscosity could be obtained by the following formula:

$$\text{relative viscosity} = \frac{\eta\ \text{rel} - 1}{c} \times 100$$

in which c = protein concentration.

Protein determination. Protein content of the extract was determined with either Folin's phenol reagent or the Biuret method, according to the concentration of protein to be extracted, as described in our paper (Fukazawa *et al.*, 1961a). The standard micro-Kjeldahl method was used to calculate the standard curve and to estimate the protein content of the fibrils after extraction.

Storage conditions. Incubation of fibrils and protein solution was for 8 hr at 35°C in the presence of a trace of toluene; but in the case of the viscosity test, storage of the protein solution was 12 hr at 30°C.

pH control. The pH of fibrils, in the way previously mentioned (Fukazawa *et al.*, 1961b), was controlled to 5.6, 6.4, or 7.0 by washing the fibrils with 50 volumes of various buffers.

RESULTS

Salting-out. The range of concentration of ammonium sulfate for the precipitation of the series of main muscle-structure protein was: actin, 9–20%; actomyosin, 28–32%; myosin 33–45%; "phosphate absorbing protein," 45–55%; and tropomyosin, 55–64% (Dubbison, 1954). As shown in Fig. 1, the salting-out pattern of myosin A extracted from beef muscle within 3 hours of slaughter showed some content of actomyosin. The pattern obtained did not show a single peak, such as seen in that of rabbit myosin A (Sasaki, 1958). The cause may be that the beef muscle was held longer after slaughter before use than was the rabbit muscle.

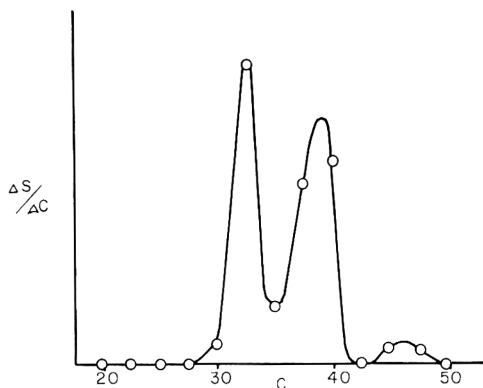

Fig. 1. The derived salting-out curve of the protein solution extracted from fresh beef muscle with Guba-Straub's solution. S = the extinction at 750 mμ, C = per cent (v/v) of saturated ammonium sulfate solution.

Fig. 2 has been given the salting-out curve of myosin B isolated by extraction for 24 hours from fresh beef muscle. The curve is considerably similar to the one (Fig. 3) for an extract obtained by extraction, with 0.6*M* NaCl Weber-Edsall solution, of intact fibrils stored 4 days at 4°C. It follows, from these salting-out curves, that the main component of intact myofibrillar protein is the heavy part of myosin B. Moreover, in the present study, the small component (43–50% saturated ammonium sulfate) shown in Fig. 3 cannot be positively identified, but is was probably "phosphate absorbing protein." More must be learned about this small component.

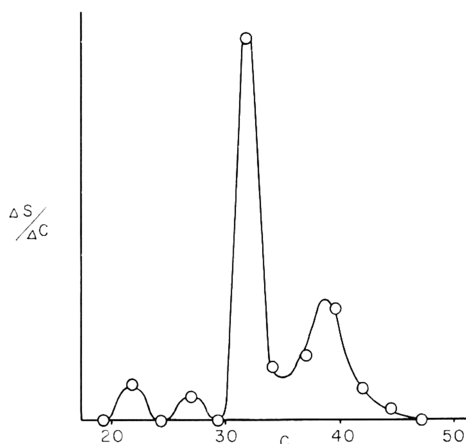

Fig. 2. The derived salting-out curve of the protein solution extracted from fresh beef muscle with 0.6*M* NaCl Weber-Edsall solution. S = the extinction at 750 mμ, C = per cent (v/v) of saturated ammonium sulfate solution.

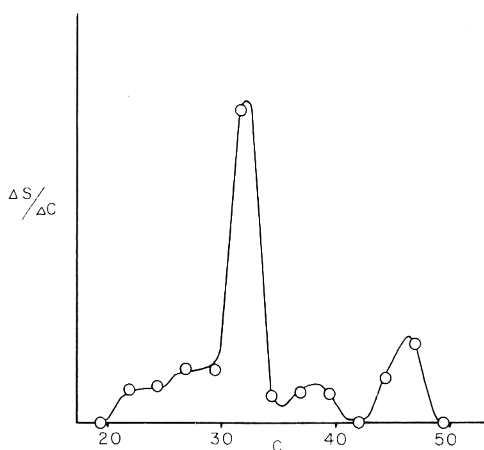

Fig. 3. The derived salting-out curve of the protein solution extracted from isolated intact muscle fibrils with 0.6*M* NaCl Weber-Edsall solution. S = the extinction at 750 mμ, C = per cent (v/v) of saturated ammonium sulfate solution.

Ultracentrifugal analysis. Fig 4 shows the results of an ultracentrifugal run made with the extract obtained from intact fibrils with 0.6*M* NaCl Weber-Edsall solution. It is pointed out that the extract consists of more than two components. Fig. 5 shows the results for myosin B isolated directly from fresh beef, and Fig. 6 shows those for myosin A extracted directly from fresh beef muscle. It was concluded that these results were in accordance with the results of salting-out analysis.

The effect of addition of phosphates on the amount of protein extracted from fibrils by 0.6M NaCl Weber-Edsall solution. As shown in Table 1, the effect of the extraction, in denatured fibrils, was notably promoted only by addition of PP. On the other hand, the phenomenon in native fibrils always developed in the order PP > TPP > HMP > control.

The effect of pH value, which affects the extractability of myofibrillar protein, is graphed in Fig. 7. The quantities of protein extracted increased at the neutral region, where the quantities extracted also depended on the kind of phosphate used, in the order PP > TPP > HMP > control, both before and after denaturation. That PP had a specific effect is clear from Fig. 7.

Ultracentrifugal separation. The protein concentration of the upper clear layer obtained by ultracentrifugal separation was estimated (Table 2).

Table 1. Effect of various phosphate-Na salts on the quantities of protein extracted with 0.6*M* NaCl Weber-Edsall solution before and after 8 hours at 35°C.

	Extracted protein % from fibrils	
	Native	Denatured
PP	57.16	55.11
TPP	50.39	47.12
HMP	50.88	42.04
Control	36.42	40.60

Table 2. Effect of various phosphate-Na salts on the protein content of the upper clear layer after ultracentrifugal separation.

	% of protein concentration of upper clear layer	
	Native	Denatured
PP	15.87	16.40
TPP	10.58	15.93
HMP	5.01	14.74
Control	13.78	6.56

It was concluded that PP has a specific effect differing from that of the other phosphates, even before and after storage, because the addition of PP resulted in the greatest amount of extracted protein. On the other hand, in the native state the protein concentration of the upper clear layer after addition of TPP or HMP (these were used additionally at the time of first extraction) was somewhat lower than that of the control. However, addition of TPP or HMP after denaturation resulted in a protein content in the upper clear layer that was higher than in the control. These facts, considered in connection with the results obtained on the denaturation of myosin A as reported in our work (Yasui *et al.*, 1960), suggested that the phosphate used has an important effect on the dissociable component of myosin B.

The main component of the separated upper clear layer probably has a direct and close relationship to the binding of sausage: it consists of myosin A and the "light" components of myosin B.

Viscosity change. Generally, it is believed that actomyosin, which is a complex substance composed of actin and myosin, is induced to dissociate by addition of PP. Though such a dissociation theory was not believed with certainty, viscosity always dropped upon addition of PP in this study, in spite of the degree of denaturation, as shown in Table 3.

Table 3. Changes in the decrease in viscosity of the protein solution extracted from intact fibrils with 0.6*M* NaCl Weber-Edsall solution.

	Native		Denatured	
	Before addition	After addition	Before addition	After addition
PP	2.19	1.57	1.57	1.14
TPP	2.19	1.99	1.57	1.55
HMP	2.19	1.99	1.57	1.55
Control	2.19	1.99	1.57	1.55

The TPP and the HMP caused no dissociation like that caused by the PP. Hence, it was apparent that only PP specifically acts upon the elongate part and the dissociable part in myosin B. Such behavior upon the addition of PP showed that it has a positive effect on the binding quality of sausage.

DISCUSSION

Many authors have studied the components of myofibrillar protein. Until now, most such investigations have used rabbit skeletal muscle, not beef muscle. The results of the research comparing myosin A and myosin B prepared from fresh beef muscle

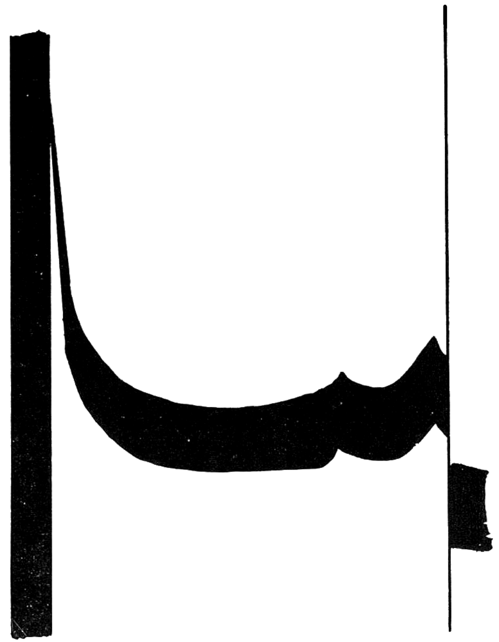

Fig. 4. Ultracentrifugal run of myosin B prepared from isolated intact muscle fibrils. Protein conc. 2.08 mg/ml, pH = 6.60, 0.5*M* NaCl, 8 min.

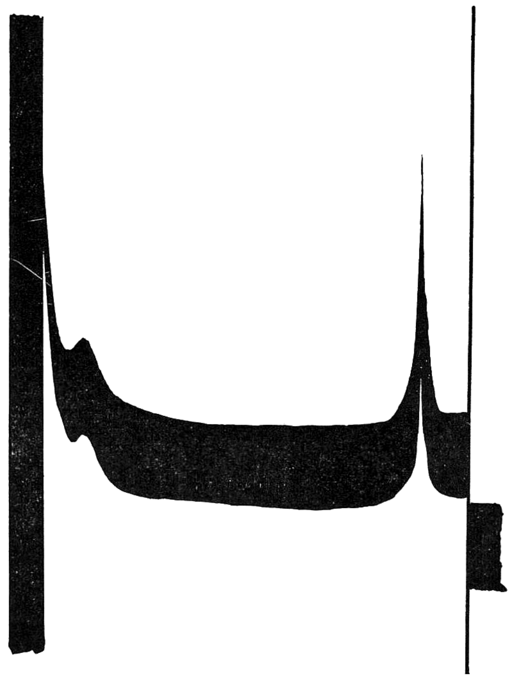

Fig. 5. Ultracentrifugal run of myosin B prepared from fresh beef muscle. Protein conc. 2.1 mg/ml, pH = 6.60, 0.5*M* NaCl, 8 min.

indicated a small difference between the two materials in myosin B, and a larger difference in myosin A. In other words, the myosin A obtained from beef muscle blended considerably with the "heavy" component of myosin B. This phenomenon might be dependent on the difference in processing time after death. From another angle, it is considered that such a difference may be due to the content of ATP in the fibrils, but this assumption has not been proved.

The components of myofibrillar protein extracted from intact fibrils with 0.6*M* NaCl Weber-Edsall solution were probably similar to those found by salting-out analysis. In the case of intact fibrils, however, a small component located in 43–50% saturated ammonium sulfate was observed. That point must be studied more definitely in the future.

From the study of the effect of various phosphates on the binding quality of sausage, the following conclusion was drawn. It is believed that the ionic strength of the cured meat maintains a condition such that the muscle structural protein is drawn to outside, through the sarcolemma of the muscle-cell, and that such action may be promoted by the use of phosphates.

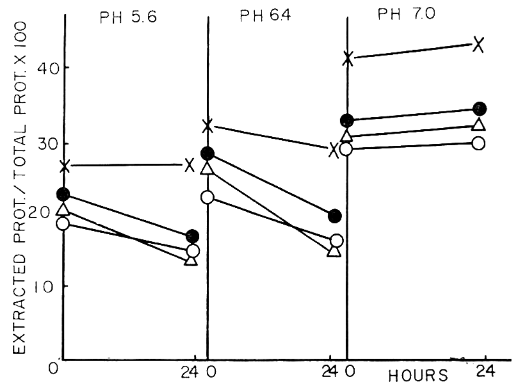

Fig. 7. The amount of protein extracted from intact muscle fibrils with 0.6*M* NaCl Weber-Edsall solution containing various sorts of phosphate-Na salts and at different pH values. Phosphate, $10^{-2}M$ to muscle fibrils; ×, pyrophosphate; ●, tripolyphosphate; △, hexametaphosphate; ○, control.

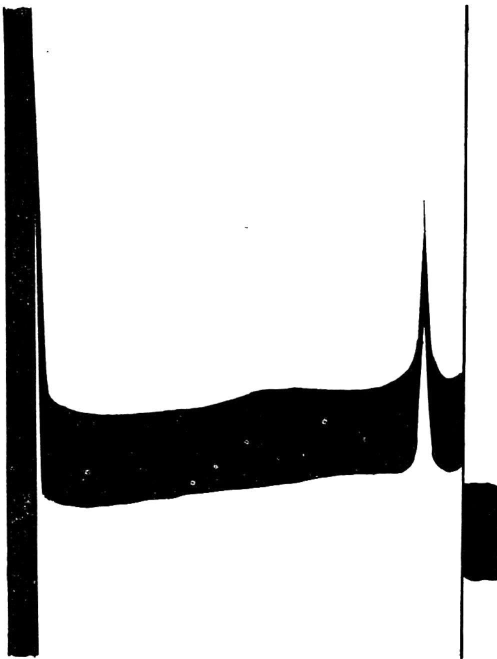

Fig. 6. Ultracentrifugal run of myosin A prepared from fresh beef muscle. Protein conc. 2.0 mg/ml, pH = 6.60, 0.5*M* NaCl, 16 min.

The authors have undertaken herein to clarify the role played by addition of various phosphates (PP, TPP, HMP) in the binding quality of sausage. Changes were in: 1) the quantity of protein extracted from intact fibrils by addition of phosphate; 2) the concentration of "light" components after ultracentrifugal separation; and 3) viscosity. From these results the following facts were presented, which satisfactorily explain the effect of phosphates on the binding quality of sausage. That is, the effect of phosphate in the native state is to increase the amount of protein extracted from fibrils by the use of 0.6*M* NaCl Weber-Edsall solution. In other words, if the myofibrillar protein is nondenatured, the phosphate used has an effect on the binding quality equivalent to the amount of myofibrillar protein extracted with 0.6*M* Weber-Edsall solution. In this case, the quantities of "light" component are not related to the binding quality of sausage. On the other hand, in the denatured state of myofibrillar protein (especially of myosin A) obtained after storage for 8 hours at 35°C, the effect of phosphate on the binding quality is due to an increasing concentration of "light" component rather than to the quantity of extracted protein.

When the fall in viscosity was observed, it was remarked that only PP had a specific

action. This indicates that the dissociable components have great importance in regard to the binding quality of sausage. It has been concluded, from these results, that the binding quality of sausage has a close relationship to the myosin A content and to the dissociable components of myosin B.

On the other hand, in the case of denatured myosin A, in which the ATPase activity had been completely inactivated after 8 hours at 35°C, only dissociable components proved to have an effect on the binding quality. So, in this case the phosphate used acts on the dissociable components, and the concentration of upper clear layer is increased, as compared with the sample to which no phosphate was added. These findings suggest important effects of phosphate on the binding quality of sausage.

When it was assumed that actomyosin itself was a dissociable component, it seemed that the addition of phosphate induced dissociation in the dissociable components. On the other hand, from the determination of the fall in viscosity, although the explanation has been presented as due to PP action only, the action of other phosphates could not be satisfactorily explained.

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