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Alterations in the Contracture Band Patterns of Unfrozen and Prerigor Frozen Ovine Muscle Due to Variations in Post-Mortem Incubation Temperature

SUMMARY

Samples of unfrozen and prerigor frozen ovine semitendinosus muscle were incubated for 24 hr post-mortem at six temperature levels between 0 and 40°C. Examination of electron-micrographs showed that variations in temperature caused muscle fibers to be in various states of contraction. The variations in sarcomere length of unfrozen and prerigor muscle did not follow any specific course in relation to temperature. All the observed transverse striations were definable components of the sarcomere.

Prerigor frozen muscle incubated at 30 and 40°C showed severe disruption of the *Z* lines. However, *Z*-line material was observed to be present in a disoriented state. *H* zones and *M* lines were observed in all samples, and their boundaries were clearly defined. The lengths of the *H* zones and *M* lines were constant among all treatments.

INTRODUCTION

Numerous investigators (Bendall and Wismer-Pedersen, 1962; Cassens *et al.*, 1963a,b; Elliott, 1965) have reported the appearance of irregular transverse bands in post-rigor porcine musculature, usually associated with muscle that has passed through rigor at elevated temperatures. Ramsbottom and Strandine (1949) reported a similar phenomenon in beef muscle which had been cooked prerigor.

The processes involved in the formation of these bands have not been clearly elucidated. Bendall and Wismer-Pedersen (1962) have proposed the theory that the bands are formed by precipitation of the sarcoplasmic proteins, whereas Cassens *et al.* (1963c) have presented microscopic evidence suggesting that the bands are caused by severe disruption of the myofibrillar proteins during violent contraction.

The type of contracture band pattern found in thaw rigor musculature appears to vary among species. Thoennes (1940) and Cassens *et al.* (1963c) have noted the presence of irregular transverse striations in thaw-rigor frog and pig muscle, whereas Marsh and Thompson (1958) and Kaminez (1962) observed the complete disappearance of all transverse striations, and the disruption of the sarcomere in sheep and frog muscle.

Because of the conflicting nature of the evidence as to the effect of post-mortem treatment upon muscle ultrastructure, an experiment was conducted to investigate the effect of post-mortem incubation temperatures upon the contracture band patterns and the fiber ultrastructure of unfrozen and prerigor frozen ovine muscle.

EXPERIMENTAL

Materials. Both semitendinosus muscles were removed from two lambs immediately after slaughter. Electron-photomicrographs were prepared from specimens of unfrozen and prerigor frozen tissue which had been incubated for 24 hr post-mortem at 0, 5, 10, 15, 20, 30, and 40°C.

Methods. Within 10 min of slaughter, both semitendinosus muscles were removed and cut into 14 strips approximately 3 cm long and 0.5 cm^2 in cross-sectional area. Each strip was prepared so that the fibers were parallel to the long axis. Seven strips, which had previously been attached to a thin rod at either end, were placed in sealed plastic bags and frozen in a dry ice-ethanol mixture for 40 min. Six strips of frozen muscle, together with 6 strips of unfrozen muscle, were incubated at 0, 5, 10, 15, 20, 30, and 40°C for 24 hr in a moist atmosphere. The unfrozen strips were placed in the incubation chambers within 10 min post-mortem, while the frozen samples were incubated upon removal from the freezing mixture. The remaining unfrozen and frozen samples were prepared for immediate histological examination.

After 24 hr of incubation, a specimen, approximately 2 fibers thick and 0.5 cm long, was obtained from the center of each muscle strip. The samples were fixed in 0.2*M* 6.5% redistilled gluteraldehyde in pH 7.2 cacodylate buffer for 4 hr at 4°C, after which they were rinsed in 0.2*M* cacodylate buffer

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Table 1. The mean lengths of the contracture bands in frozen and unfrozen prerigor muscle, fixed prior to the commencement of shortening ($n = 20$).[a]

Band	Treatment	
	Unfrozen	Frozen
Sarcomere	1.15	1.09
A band	1.01	0.94
I band	[b]	[b]
Z line	0.11	0.16
H zone	0.10	0.14
M line	0.05	0.08

[a] Widths of bands, expressed in μ.
[b] No band discernible.

(pH 7.2) and finally fixed for 2 hr in Palade's osmium tetroxide. The samples were dehydrated in a series of graded alcohols and embedded in araldite. Sections 600–900 Å thick were prepared and mounted on formavar grids and stained with Karnovsky stain (1961). The sections were examined in a Siemens Elmiskop electron microscope.

Duplicate photographic prints at ×30,000 were prepared for measurement of the sizes of the contracture bands. Five individual sarcomeres from each print provided measurements of the lengths of the sarcomere, *A* band, *I* band, *Z* line, *H* zone, and *M* line.

RESULTS AND DISCUSSION

The electron micrographs showed that prerigor freezing and incubation temperature had a significant influence upon the nature and magnitude of the contracture bands. Low standard deviations (± 0.001 to ± 0.004 μ) were noted with respect to size of bands among the sarcomeres measured in one print and between the two prints and two animals.

Effect of prerigor freezing. Fig. 1 (a,b) shows the fibers of prerigor frozen and unfrozen tissue to be in a highly contracted state. Smaller mean sarcomere lengths (1.09 μ) were observed in the frozen tissue, indicating a higher degree of contraction. Variations in *A* band size were associated with

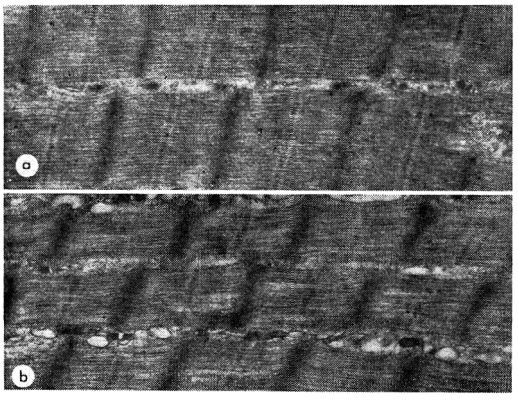

Fig. 1. Tissue fixed prior to the onset of rigor shortening (no incubation).
(a) Unfrozen. Highly contracted (sarcomere = 1.15 μ).
(b) Frozen. Highly contracted, myofibrils (sarcomere = 1.09 μ) showing broad *Z* lines.

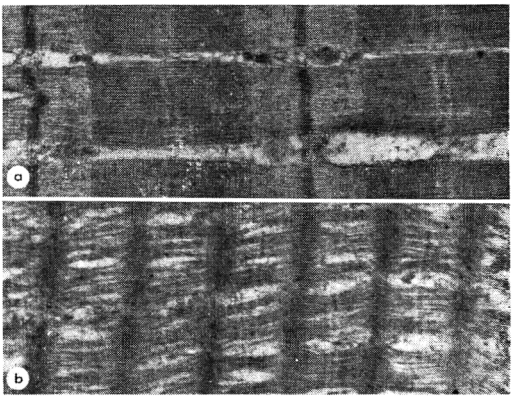

Fig. 2. Prerigor unfrozen tissue incubated at 0°C for 24 hr.
(a) Relaxed myofibrils Type *A* (sarcomere = 2.60 μ) with a clearly defined banding pattern.
(b) Highly contracted myofibrils Type *B* (sarcomere = 0.76 μ) showing broad *Z* lines and slight convexing of myofilaments within the sarcomere.

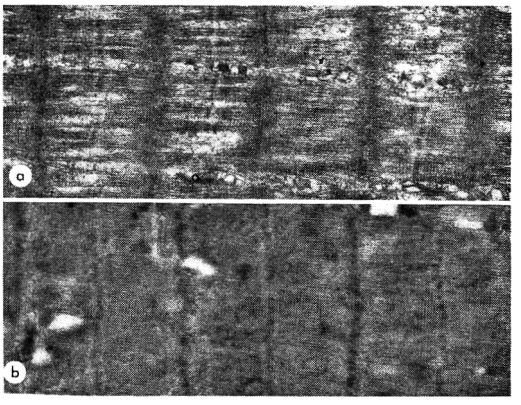

Fig. 3. Prerigor unfrozen tissue incubated at the following temperatures for 24 hr:
(a) 5°C. Highly contracted myofibrils (sarcomere = 1.10 μ) illustrating broad *Z* lines and the convex nature of the myofilaments within a sarcomere, also granulation of the myofilaments.
(b) 10°C. Slightly contracted myofibrils (sarcomere = 1.67 μ) showing poorly defined *I* band, also the appearance of irregular dense masses within the *I* band.

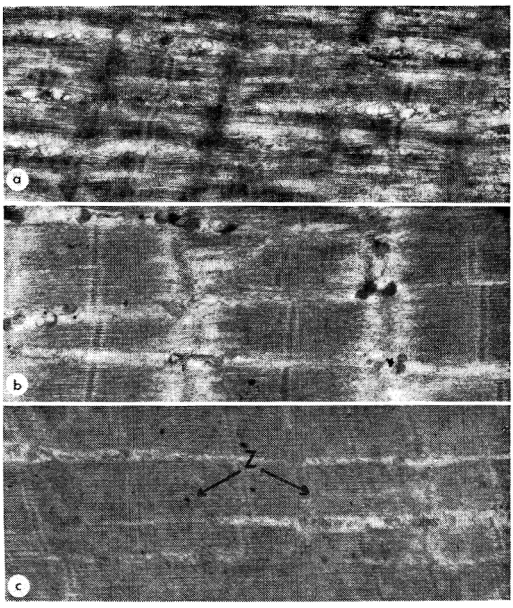

Fig. 4. Prerigor unfrozen tissue incubated at the following temperatures for 24 hr post-mortem:
(a) 20°C. Highly contracted myofibrils (sarcomere = 1.17 μ) showing the convex nature of the myofilaments within a sarcomere.
(b) 30°C. Slightly contracted myofibrils (sarcomere = 1.83 μ) showing broad Z lines and poorly defined A-I junctions.
(c) 40°C. Highly contracted myofibrils (sarcomere = 1.00 μ) showing disorientation of Z-line material (Z). H zones and M lines are clearly defined.

sarcomere length. Freezing had little effect upon the lengths of the H zones and M lines (Table 1).

Although freezing had a small effect upon sarcomere and A band length, it did not cause any violent disorientation of the actin and myosin filaments.

Effect of temperature on contracture band patterns of unfrozen muscle. The electron micrographs (Figs. 2, 3, 4) showed differences in the contracture band patterns among tissues incubated at various temperatures. The data (Table 2) show that tissues incubated at 10 and 30°C had respective mean sarcomere lengths of 1.67 and 1.83 μ and had not contracted to the same extent as tissues incubated at 5, 20, and 40°C, which manifested mean sarcomere lengths between 1.00 and 1.17 μ. Small I bands, measuring 0.53 and 0.40 μ were noted in the 10 and 30°C samples. The 10°C sample exhibited irregular, dense masses within the sarcomere.

Table 2. The mean lengths of the contracture bands in unfrozen, and prerigor frozen muscle, after incubation at various temperatures for 24 hr post-mortem ($n = 20$).[a]

Band	Incubation temperature (°C)													
	0			5		10		20		30		40		
	Type A Unfrozen[b]	Type B Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen
Sarcomere	2.60	0.76	1.07	1.10	0.87	1.67	1.63	1.17	1.33	1.83	1.23[c]	1.00	[d]		
A band	1.50	0.60	0.97	0.83	0.75	1.00	1.13	0.98	1.28	1.40	[d]	0.90	[d]		
I band	1.00	[d]	[d]	[d]	[d]	0.53	0.47	[d]	[d]	0.40	[d]	[d]	[d]		
Z line	0.10	0.13	0.12	0.26	0.10	0.10	0.10	0.17	0.07	0.13	[d]	0.10	[d]		
H zone	0.13	0.12	0.10	0.13	0.12	0.13	0.13	0.13	0.10	0.13	0.10	0.10	[d]		
M line	0.07	0.05	0.06	0.06	0.07	0.07	0.06	0.07	0.06	0.07	0.07	0.07	[d]		

[a] Lengths of bands expressed in μ.
[b] Type A relaxed fibers, Type B contracted.
[c] Measured between M lines.
[d] No band discernible.

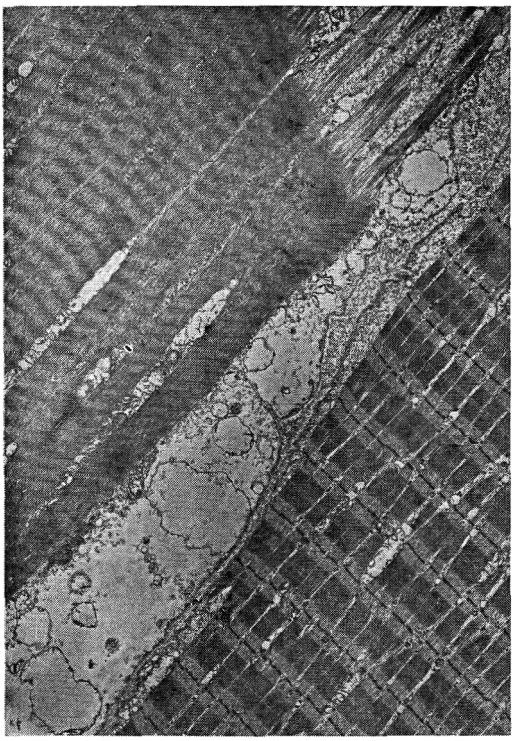

Fig. 5. Relaxed and contracted myofibrils within the same section. Sample incubated at 0°C for 24 hr post-mortem. Magnification ×6.150.

The nature of the contracture bands observed in the 0°C tissue was highly variable. Both contracted and relaxed fibers were noted among the myofibrils of the tissues. This phenomenon was demonstrated clearly in one section, showing adjacent myofibrils in relaxed and contracted states (Fig. 5).

Although there was a great variation in sarcomere length and *A* and *I* band length between treatments, no treatment effect was noted upon the lengths of the *H* zones and *M* lines.

The observation that all transverse striations were definite components of the sarcomere does not concur with findings of Cassens *et al.* (1963a,b,c), Elliot (1965), and Ramsbottom and Strandine (1949), who have reported the occurrence of irregular bands in porcine and bovine musculature. The nonappearance of irregular striations in ovine musculature subjected to elevated temperatures post-mortem may suggest the existence of differences in some of the post-mortem biochemical reactions among species.

Locker (1960), with unfrozen bovine muscle, and Cook and Langsworth (1966a), with unfrozen and prerigor frozen ovine

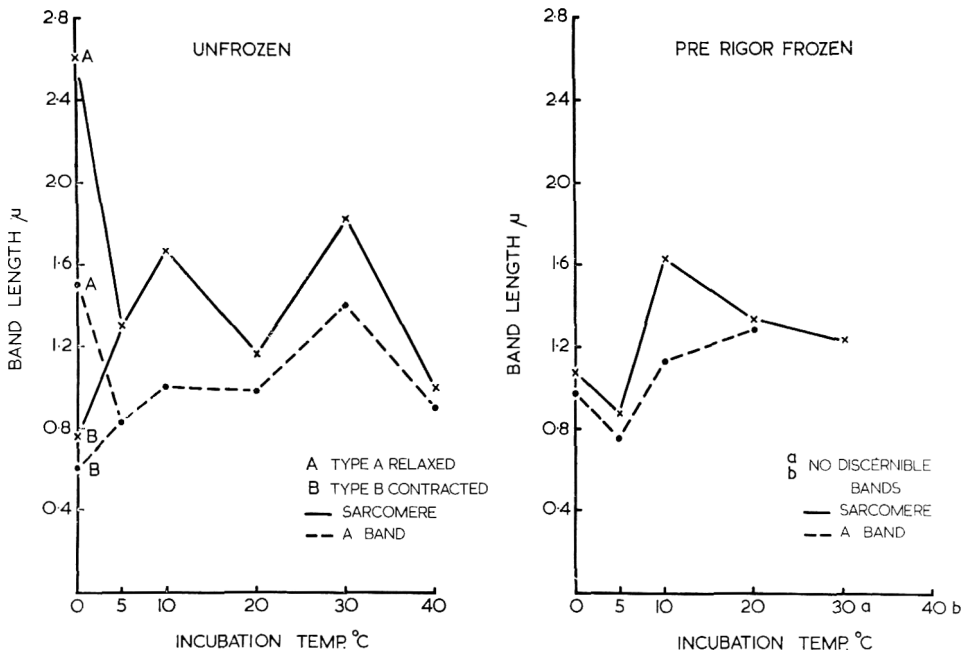

Fig. 6. Variation in the lengths of sarcomeres and *A* bands in unfrozen and frozen muscle incubated at various temperatures between 0–40°C for 24 hr post-mortem.

muscle, demonstrated differential shortening of the fibers after incubation at post-mortem temperatures between 0 and 40°C. Shortening of bovine and ovine musculature was maximum after 24 hr at 0 and 37–40°C, while shortening was minimum in the region of 15–19°C.

The sliding-filament theory of muscle contraction, as proposed by Hanson and Huxley (1953) and Huxley and Niedergerke (1954), suggests that sarcomere lengths decrease proportionately with the extent of fiber contraction. This decrease is wholly reflected by a decrease in *I* band length, rather than by an alteration in the size of the *A* band. The data presented in this and in a previous study (Cook and Langsworth, 1966) do not support these findings, since no relation was observed between sarcomere length and the percent shortening undergone by fibers subjected to various post-mortem temperatures ($r = -0.23$ and 0.54 for unfrozen and frozen respectively). The lack of a significant correlation between sarcomere length and extent of fiber contraction may possibly be associated with the phenomenon of a proportionate decrease in *A* band length with decreasing sarcomere length (Table 2, Fig. 6). Similar observations have been made in frog muscle by Galey (1964), who postulated that the proportionate decrease in *A* band length with decreasing sarcomere length manifests a transitional phase of contraction.

The significant ($P < 0.01$) positive correlation 0.74 between *A* band length and sarcomere length suggests that post-mortem temperatures other than 10 and 30°C cause the fibers to pass into a super-contracted state (Hoyle *et al.*, 1965).

Effect of temperature on contracture band patterns of prerigor-frozen muscle. Prerigor-frozen tissue exposed to different thawing temperatures between 0 and 40°C for 24 hr exhibited myofibrils which were more highly contracted than those in the unfrozen sample (Figs. 7, 8). Sarcomere

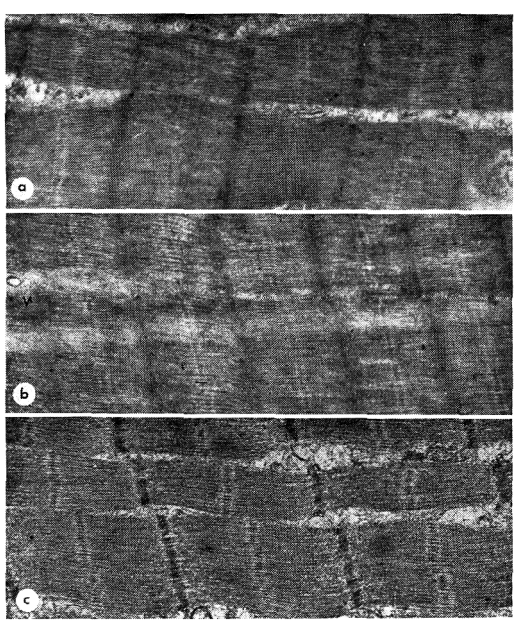

Fig. 7. Frozen prerigor and thawed for 24 hr at the following temperatures:
(a) 0°C. Contracted myofibrils (sarcomere = 1.07 μ)
(b) 5°C. Contracted myofibrils (sarcomere = 0.87 μ) showing broad and poorly outlined *Z* lines.
(c) 10°C. Contracted myofibrils (sarcomere = 1.63 μ) showing very small I band, and granulation of the myofilaments.

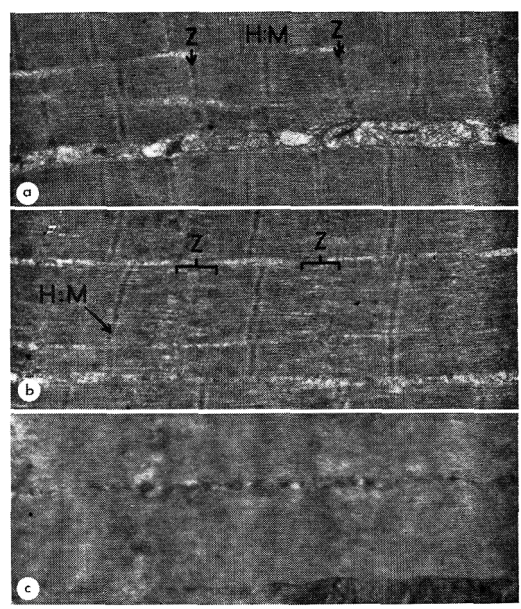

Fig. 8. Frozen prerigor and thawed for 24 hr at the following temperatures:
(a) 20°C. Highly contracted myofibrils (sarcomere = 1.33 μ) showing clearly defined *H* zones and *M* lines, with *Z* line tending to become diffuse.
(b) 30°C. Highly contracted myofibrils (sarcomere = 1.23 μ) showing clearly defined *H* zones and *M* lines. *Z* line material present in a diffused and unorientated state (*Z*).
(c) 40°C. Very highly contracted fibers. No banding patterns discernible.

lengths and *A* band lengths of the fibers varied randomly among temperature treatments. A maximum sarcomere length of 1.63 μ was noted for the samples incubated at 10°C, while the minimum value of 0.87 μ was observed in the 5°C samples. *A* band lengths varied concomitantly with sarcomere lengths. Measurable *I* bands were not noted in any tissue. Contrary to findings of Marsh and Thompson (1958) and Kaminez (1962), *Z* lines, *H* zones, and *M* lines were clearly demonstrated in the 0, 5, and 10°C samples (Fig. 7a,b,c), while *H* zones and *M* lines were observed in the 20 and 30°C samples. Although the latter two samples did not illustrate clearly defined *Z* lines, *Z*-line material was observed in a disorientated and diffused state (Fig. 8a,b). The 40°C sample had undergone severe contraction, and all longitudinal and transverse striations were absent (Fig. 8c). These observations suggest that the *Z* line may be the structural component of the myofibril that is first degraded by post-mortem heat treatment.

In agreement with the observations noted for the unfrozen tissues, no irregular transverse striations were observed for prerigor-frozen tissue subjected to similar thawing temperatures.

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Effect of Sulfite and Ascorbic Acid on Mushroom Phenol Oxidase

SUMMARY

In model systems containing mushroom phenol oxidase and o-diphenolic or monophenolic substrates in phosphate buffer, pH 6.5, sulfite initially did not protect the ascorbic acid from oxidation by the quinones formed enzymatically. The sulfite, however, gradually decreased both the diphenolase and monophenolase activity of the enzyme and thereby diminished the overall destruction of ascorbic acid. Under certain conditions, ascorbic acid counteracted the sulfite inhibition of the monophenolase activity of the enzyme.

INTRODUCTION

Sulfur dioxide and ascorbic acid are widely used as food additives to prevent browning discoloration in fruits and vegetables with active phenol oxidase systems. Phenol oxidase catalyzes the oxidation of phenolics to quinones, which then condense to form melanins. Sulfite interferes with this condensation by combining irreversibly with the quinones to form colorless addition products (Embs and Markakis, 1965). It also gradually diminishes the ability of the enzyme to oxidize o-diphenols. Ascorbic acid acts as an antioxidant, reducing the quinones to the original phenolics. It also shortens the characteristic induction period in the conversion of monophenols to diphenols by unpurified phenol oxidase (Kendal, 1949).

This study was made to investigate the behavior of sulfite and ascorbic acid when both are present in phenol oxidase systems with monophenols or o-diphenols as substrates. In addition, the effect of sulfite alone on the monophenolase activity of the enzyme is discussed; this part supplements a previous study on the effect of sulfite on the diphenolase activity of this enzyme.

MATERIALS AND METHODS

The enzyme used in this work was a tyrosinase, also called phenol oxidase or polyphenol oxidase, prepared from mushrooms (Sigma Chemical). Its activity was found to be 800 units/mg (1 unit = 0.001 absorbancy increase per min at 280 mμ in phosphate buffer pH 6.5, at 25°C, containing $10^{-4}M$ L-tyrosine). The substrates used were dihydroxyphenylalanine, abbreviated to DOPA (Eastman), L-tyrosine (Calbiochem), and p-coumaric acid (Mann). Other chemicals used were sodium bisulfite (Merck reagent), ascorbic acid (Eastman reagent), the dye 2,6-dichlorobenzenoneindolphenol (Eastman), pyrocatechol (Eastman), caffeic acid (Nutritional Biochem.), and $0.5M$ phosphate buffer pH 6.5.

The several model systems used, depending on the analytical method employed in their study, are described along with the results obtained.

The ascorbic acid was assayed by transferring 1-ml portions of the reaction mixture into Evelyn colorimeter tubes. Periodically, 1-ml portions of 4% metaphosphoric acid were added to each tube to stop the enzymatic action and reduce the pH of the system to the level suitable for the dye reaction. Subsequently, 9 ml of dye solution was added, and 45 seconds later the transmittance of the mixture was measured in the colorimeter at 515 mμ. The dye solution was prepared by dissolving 10 mg 2,6-dichlorobenzenoneindolphenol in 200 ml water and subsequently diluting 9 ml of this solution with 491 ml of 10% acetone. The acetone was necessary to bind any excess of sulfite and prevent its interference in the ascorbate-dye reaction (Mapson, 1942). A Warburg apparatus thermostated at 30°C was used for the manometric studies. Paper chromatography was performed with Whatman No. 1 paper and the organic phase of n-butanol/acetic acid/water (25:6:25) mixture as the solvent, in ascending fashion at 25°C. Spectrophotometric measurements were made with a Beckman DU spectrophotometer connected to a Ledland log-converter and a Sargent SR recorder.

RESULTS AND DISCUSSION

Diphenol systems. Ascorbic acid determinations were carried out in systems containing DOPA, ascorbic acid, and varying amounts of sulfite. After the enzyme was added to the systems their ascorbic acid content was determined at 2-min intervals, and the percent ascorbic acid remaining in the systems was plotted against time (Fig. 1). It can be seen that sulfite retarded and eventually stopped the oxidation of ascorbic acid. There are two ways by which the sulfite could induce this inhibition: a) by

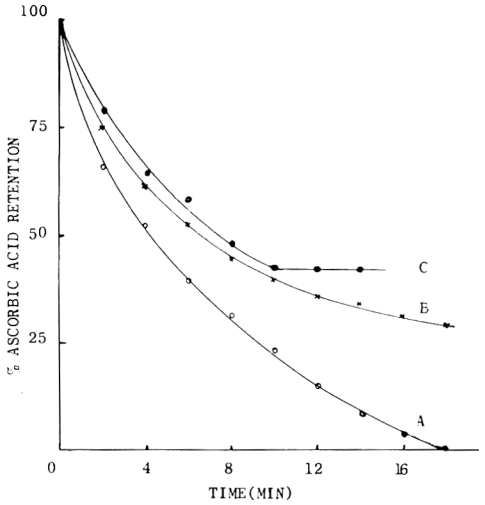

Fig. 1. Effect of sulfite on the retention of ascorbic acid in phenol oxidase-DOPA systems. Composition: $2\times10^{-3}M$ DOPA, 4 units/ml enzyme, $3.5\times10^{-4}M$ ascorbic acid, and $0.15M$ phosphate buffer, pH 6.5. Sulfite in *A*, none; *B*, $3.5\times10^{-4}M$; *C*, $7\times10^{-4}M$.

competing with ascorbic acid for the quinones, which are formed enzymatically; and b) by inactivating the enzyme. The leveling of the sulfite curves, B and C of Fig. 1, suggests that enzymatic inhibition is the dominant factor in slowing the ascorbic acid oxidation. If no enzyme inhibition occurred, the oxidation of the ascorbic acid would proceed to completion.

To test the possibility of a sulfite-ascorbic acid competition for the quinones, caffeic acid (a fluorescent o-diphenol) was allowed to react with phenol oxidase, sulfite, and various amounts of ascorbic acid. At 1-min intervals, after addition of the enzyme, portions of the systems were spotted on chromatographic paper and chromatographed (Fig. 2). The spots near the solvent front represent excess caffeic acid in the systems, and the spots near the origin represent sulfite addition products. In system *A*, with no ascorbic acid, the addition products formed in less than 1 min. In system *B*, with a small amount of ascorbic acid, product formation was delayed for about 2 min; and with twice as much ascorbic acid the delay was twice as long (system *C*). These results indicate that the caffeic quinone reacted first with the ascorbic acid, and when all the ascorbic acid was oxidized the quinone combined with

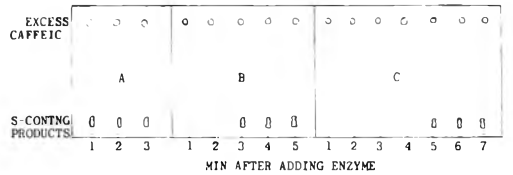

Fig. 2. Paper chromatography of phenol oxidase-caffeic acid-sulfite-ascorbic acid systems. Composition of reaction mixture: $3.3\times10^{-4}M$ caffeic acid, $3\times10^{-5}M$ $NaHSO_3$, 13 units/ml enzyme, $0.16M$ phosphate buffer, pH 6.5. Ascorbic acid in *A*, none; *B*, $1.5\times10^{-4}M$; *C*, $3\times10^{-4}M$. Paper: Whatman No. 1. Solvent: n-butanol-acetic acid-water (25:6:25).

sulfite to form the addition products.

A similar conclusion concerning the failure of sulfite to protect the ascorbic acid in apple pulp was reached by Johnson and Johnson (1952). On the other hand, Goodman and Markakis (1965) found that sulfur dioxide will protect anthocyanin pigments in mushroom phenol oxidase systems. Since it is likely that the quinones formed enzymatically from substrates better than anthocyanins oxidize the latter pigments (Peng and Markakis, 1963), it appears that the quinones react at different velocities with sulfite (k_1), ascorbic acid (k_2), anthocyanins (k_3), and themselves (k_4). The multiplicity of sulfite-quinone reaction products, made easily observable when fluorescent tyrosinase substrates are used (e.g. caffeic acid), indicates that some quinone polymerization involving quinone-type intermediates occurs (Mason, 1959; Forsyth *et al.*, 1960) while the sulfite is reacting with the original quinone. This places the reaction velocities of quinone polymerization and quinone-sulfite addition not far apart, and the following order is probable:

$$k_2 > k_1 \geqslant k_4 > k_3$$

Monophenol systems. First, the sulfite effect on the monophenolase activity of the mushroom tyrosinase was investigated manometrically, spectrophotometrically, and chromatographically. Buffered solutions of tyrosine and the enzyme were allowed to react in the presence of varying concentrations of sulfite, and the oxygen uptake was plotted against time (Fig. 3,*A*,*B*,*C*). The absorbance at 470 mμ of similar systems was also measured and recorded automatically (Fig. 4,*A*,*C*). These data indicate that sulfite caused a delay in oxygen uptake and brown-

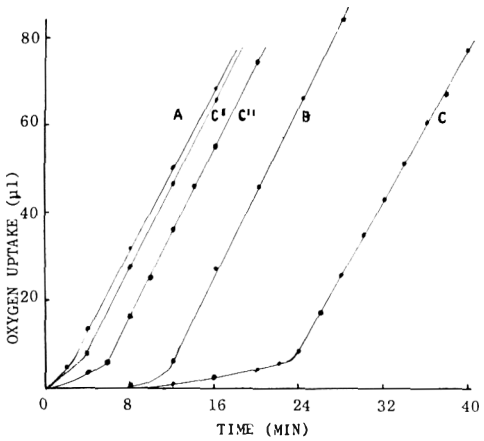

Fig. 3. Oxygen uptake by phenol oxidase-tyrosine-sulfite in the presence of ascorbic acid or pyrocatechol. Composition: $3.3\times10^{-4}M$ tyrosine, 10 units/ml enzyme, $0.32M$ phosphate buffer, pH 6.5. Sulfite in *A*, none; *B*, $8\times10^{-4}M$; *C*, $1.6\times10^{-3}M$. *C'* had the composition of *C* plus $1.6\times10^{-4}M$ ascorbic acid and *C''* was *C* plus $1.6\times10^{-4}M$ pyrocatechol. In *A*, browning started immediately; in *B*, it was delayed 10 min; in *C*, it was delayed 20 min; in *C'* and *C''*, there was hardly any delay. Total vol., 3 ml.

ing development; in fact, the onset of browning appeared to coincide with the beginning of vigorous utilization of oxygen. This is in contrast to the o-diphenol-tyrosinase systems, in which sulfite delayed the browning but did not affect the initial oxygen upake (Embs and Markakis, 1965).

When p-coumaric acid, a fluorescent monophenol, was mixed with phenol oxidase in the presence of sulfite and then subjected to paper chromatography, no fluorescent sulfite addition products were observed (Fig. 5,*A*), while fluorescent o-diphenols do give such products (Embs and Markakis, 1965).

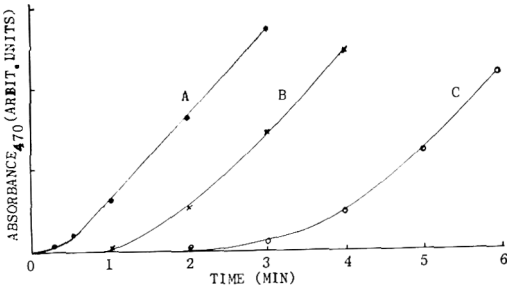

Fig. 4. Effect of ascorbic acid on the sulfite-induced delay of browning in the phenol oxidase-tyrosine system. *A*, $3\times10^{-4}M$ tyrosine; 13 units/ml enzyme; $0.16M$ phosphate buffer, pH 6.5; *B*, Same as *A*, plus $10^{-5}M$ sulfite and $3\times10^{-5}M$ ascorbic acid; *C*, Same as *B* but without ascorbic acid.

All these observations suggest that sulfite in low concentrations induces a delay in the monophenolase activity of mushroom phenol oxidase; no such lag was caused by sulfite on the o-diphenolase activity of the enzyme, although there was gradual loss of that activity (Embs and Markakis, 1965).

The tyrosine-phenol oxidase-sulfite system was then studied in the presence of small quantities of ascorbic acid and pyrocatechol. Manometric measurements showed that ascorbic acid and, to a smaller extent, pyrocatechol reduced considerably the sulfite-induced delay of oxygen uptake by the system (Fig. 3,*C',C''*). A similar decrease was observed in the delay of browning of the system when ascorbic acid was added to it (Fig. 4,*B*); and when the fluorescent monophenol p-coumaric acid was used as substrate of the enzyme, the presence of ascorbic acid or pyrocatechol resulted in the appearance of fluorescent sulfite addition products on the paper chromatograms of the systems (Fig. 5,*B,C*). These addition products were shown in an unpublished parallel study to be the same as those obtained when caffeic acid (an o-hydroxylated product of p-coumaric acid) was used as substrate of phenol oxidase in the presence of sulfite. At any rate, the chromatographic and spectrophotometric data indicate that decrease in the delay of oxygen uptake could not be due to a simple oxidation of ascorbic acid or pyrocatechol, but rather to a restoration of the sulfite-inhibited monophenolase activity.

A possible explanation of this restoration may be the fact that phenol oxidase is acti-

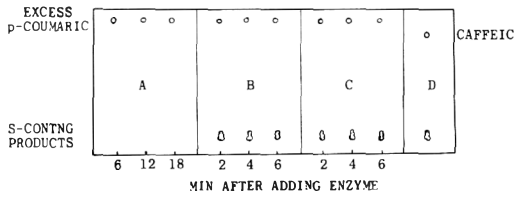

Fig. 5. Paper chromatography of phenol oxidase-p-coumaric acid-sulfite-reducing agent systems. Composition of reaction mixture: $3.3\times10^{-4}M$ p-coumaric acid; $3\times10^{-5}M$ $NaHSO_3$; 13 units/ml enzyme; $0.16M$ phosphate buffer, pH 6.5. Reductant in *A*, none; *B*, $3\times10^{-5}M$ ascorbic acid; *C*, $3\times10^{-5}M$ pyrocatechol. *D* depicts a chromatogram of the system phenol oxidase-caffeic acid-sulfite. Paper: Whatman No. 1. Solvent: n-butanol-acetic acid-water (25:6:25).

vated by reducing agents (Kendal, 1949; Mason, 1957); Bright *et al.* (1963) suggested that mushroom phenol oxidase may have a reducing site in its molecule capable of that reduction. If sulfite should block this reducing site and thereby inhibit the enzyme, the presence of a reducing agent such as ascorbic acid or pyrocatechol would itself activate the enzyme.

From these considerations one would expect that the combined effect of sulfite and ascorbic acid on monophenol systems would be similar to that on diphenol systems. Fig. 6 shows that this is the case. The systems contained tyrosine, phenol oxidase, ascorbic acid, and varying amounts of sulfite; it can be seen that sulfite slowed the oxidation of ascorbic acid. As in the o-diphenol systems, the sulfite curves tended to level off, indicating that the sulfite acted primarily by inhibiting the enzyme rather than by competing with the ascorbic acid for the quinones.

To further demonstrate the sulfite inhibition of the enzyme, a system containing tyrosine, phenol oxidase, ascorbic acid, and sulfite was allowed to react until ascorbic acid oxidation stopped (Fig. 7). Fresh enzyme was then added to the system. The oxidation resumed. When it began to slow again, more enzyme was added. By successive additions of fresh enzyme, all the ascorbic acid in the system could be oxidized.

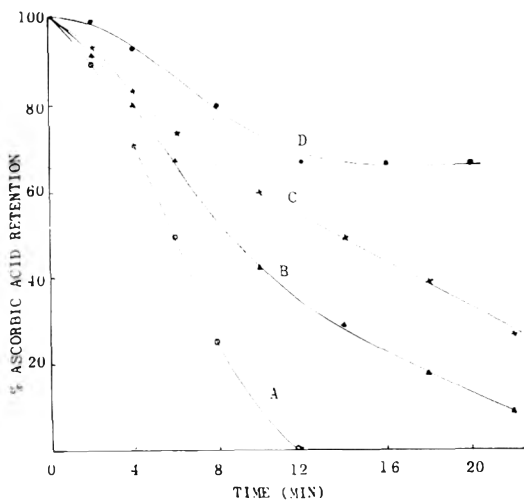

Fig. 6. Effect of sulfite on the retention of ascorbic acid in phenol oxidase-tyrosine systems. Each system contained $4\times10^{-4}M$ tyrosine, 20 units/ml enzyme, $3.5\times10^{-4}M$ ascorbic acid in $0.15M$ phosphate buffer, pH 6.5. Sulfite in *A*, none; *B*, $3.5\times10^{-4}M$; *C*, $5.3\times10^{-4}M$, and *D*, $7\times10^{-4}M$.

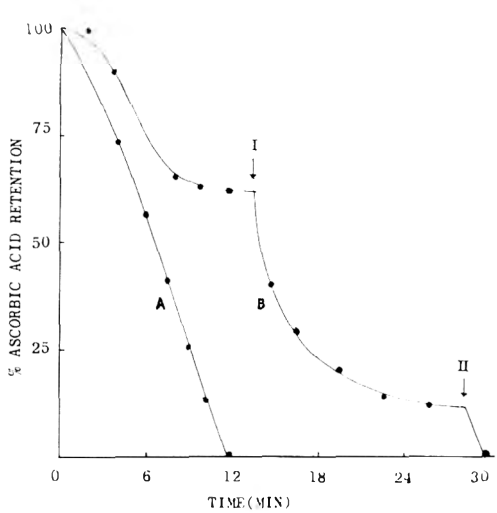

Fig. 7. Addition of more enzyme during the reaction of the system phenol oxidase-tyrosine-sulfite-ascorbic acid. Same system composition as in Fig. 6, with *A* containing no sulfite, and *B* $7\times10^{-4}M$ sulfite. At 14 min (arrow I), fresh enzyme (20 units/ml) was added to the system, and at 28 min (arrow II) more enzyme (20 units/ml) was added.

From these observations and the fact that higher sulfite concentrations are more effective in the inhibition of phenol oxidase (Embs and Markakis, 1965), it becomes understandable that ascorbic acid, by preventing the reaction of sulfite with the quinones, facilitates the sulfite inhibition of the enzyme. In turn the effective inhibition of the enzyme results in decreased total loss of ascorbic acid.

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Interaction of Low-Density Lipoprotein (LDL) from Yolk Plasma with Methyl Orange

SUMMARY

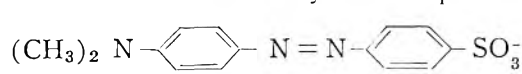
The surface characteristics of plasma low-density lipoprotein (LDL) in various buffers were investigated by determining the binding of an anionic dye, methyl orange, during equilibrium dialysis at 10°C. As the buffer pH increased from 4.5 to 8.6, the binding capacity of native LDL diminished. Additional experiments were carried out at pH 6.5, the normal value for egg yolk and plasma. A plot of dye concentration vs. dye binding by native LDL produced a curve with a shape similar to that for β-lipoprotein from blood. Native LDL, with only about 15% protein, bound as much dye as a pure protein, β-lactoglobulin. The LDL from pasteurized yolk (63 and 64.8°C) bound as much dye as LDL from native yolk. Sodium chloride and sucrose did not restrict the dye adsorption to native LDL. When yolk plasma was treated with bromelain, the dye-binding capacity of isolated LDL was somewhat reduced.

INTRODUCTION

Lipid-protein complexes with low densities make up the major portion of egg yolk solids. According to Sugano and Watanabe (1961), the yolk protein fraction consisted of 72% low-density lipoprotein (LDL) with a composition of 16% protein and 84% lipid. On a lipid basis, the LDL was made up of about 24% phospholipid, 4% cholesterol, and 72% triglyceride. For the most part, these lipoproteins are present in the yolk plasma as spherical micelles with hydrated diameters between 250 and 310Å (Sugano and Watanabe, 1961). Two LDL fractions, differing only slightly in chemical composition and density, have been isolated by Saari *et al.* (1964) and Martin *et al.* (1964). From the data of Martin *et al.* (1964), molecular weight was 10.3×10^6 for the less-dense LDL fraction, and 3.3×10^6 for the more-dense LDL. Cook and Martin (1962) postulated that low-density lipoproteins in yolk are made up of a triglyceride core with phospholipids and proteins on the surface.

The surface characteristics of low-density lipoproteins have been investigated indirectly by some investigators. Since Saari *et al.* (1964) have shown that papain can hydrolyze the protein moieties of LDL fractions, it can be assumed that the protein molecules are spread out on the surface. Martin *et al.* (1964) have estimated that if the thickness of the protein film on the micelle surfaces is assumed to be 8Å, a sufficient amount of protein is available to cover two-thirds of the lipoprotein surfaces. Burley and Kushner (1963) reported that phosphatidase D can hydrolyze 95% of the phospholipids in LDL. From this observation, it can be assumed that phosphate groups in the phospholipids are exposed at the micellar surfaces of native LDL. Paper electrophoresis indicated that yolk low-density lipoproteins are charged negatively at pH 8.6 (Evans and Bandemer, 1957; McCulley *et al.*, 1962) and positively at pH 6.5 (Powrie *et al.*, 1963).

Surface characteristics of lipoprotein micelles can be assessed by determining the binding of organic ions. Rosenberg *et al.* (1955) reported that, during equilibrium dialysis, human serum β-lipoprotein (about 23% peptide) at pH 7.4 had a marked affinity for methyl orange (I) anions. β-lipoprotein bound these anions on the alkaline side of the isoelectric point as strongly as serum albumin. Methyl orange may have been adsorbed exclusively to the protein



(I) Methyl orange

moieties of the β-lipoprotein micelles rather than to phospholipids. Ashworth and Green (1963) found that no methyl orange was bound to lipid particles in a lecithin-stabilized triolein emulsion.

Electrically-charged groups on the surfaces

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of egg yolk LDL are undoubtedly involved in the stability of the micelles in aqueous medium. Any alteration in the surface properties of the LDL micelles may lead to destabilization and aggregation. With the introduction of thermal energy into an LDL solution, lipid and protein moieties on the surface of each micelle may be reoriented in such a way as to alter the number of exposed ionic groups. Such an alteration may occur during the pasteurization of egg yolk. When yolk or plasma is frozen, LDL micelles presumably aggregate, with the consequence of increase in yolk viscosity (Powrie *et al.*, 1963; Saari *et al.*, 1964). The addition of sugars, sodium chloride, and proteolytic enzymes to yolk or plasma can inhibit viscosity change.

This study was initiated to examine quantitatively the interaction of methyl orange with LDL from native and heat-treated yolk, and thus gain more knowledge on the surface characteristics of LDL micelles. The dye-binding of LDL from bromelain-treated frozen plasma was assessed to provide an insight into the reason why proteolytic enzymes inhibit viscosity change of yolk during freezing and thawing.

MATERIALS AND METHODS

Yolk was prepared from eggs not more than 24 hr old by the method of Powrie *et al.* (1963). Yolk was pasteurized by pumping the yolk through a small heat exchanger by a Sigma pump. The pasteurizing temperatures were maintained for 3 min by passing the yolk through a temperature-controlled holding tube. The temperature of the yolk was lowered immediately to about 5°C. The yolk was held in a refrigerator at 3°C.

The plasma from native and heat-treated yolk was obtained by high-speed centrifugation (Saari *et al.*, 1964). For the preparation of bromelain-treated plasma, bromelain was added to native plasma at 25°C to form a 0.5% bromelain mixture. The plasma with enzyme was incubated for 30 min at 25°C and then frozen at −23°C for 24 hr. The thawed mass was as fluid as the unfrozen control. The untreated frozen plasma was pasty upon thawing.

Preparation of low-density lipoprotein (LDL) solutions. Native heat-treated or bromelain-treated thawed plasma was diluted with two volumes of 10% NaCl, and the resulting dispersion was centrifuged at 78,000 × G for 6 hr with refrigeration. The floating pellicle and cloudy subnatant were combined and diluted with 15% NaCl. The solution was centrifuged as described above, and the resulting floating LDL was removed for further experimentation. When a solution of the LDL was subjected to paper electrophoresis, no livetin bands could be detected.

For the preparation of 2% lipoprotein solution, enough floating LDL was dispersed in the selected buffer that the concentration of the LDL was above 2%. The solution was dialyzed against the appropriate buffer for 24 hr at about 5°C with constant stirring and frequent changing of the buffer. The LDL content in each solution was determined by subtracting the solids content of the buffer from that of the dialyzed LDL solution. The concentration of LDL was adjusted to 2% by the addition of a suitable buffer.

Equilibrium dialysis. The 2% LDL solutions were subjected to equilibrium dialysis according to a method similar to that of Klotz *et al.* (1946). In all cases, the ionic strength of the buffers was 0.1. For each experiment, a 2-ft length of Visking dialyzing tubing 8DC (0.390 in. flat width) soaked in the selected buffer was tied at one end and filled with 10 ml of 2% LDL solution. Upon tying the other end, the tubing was placed in a screw-cap culture tube containing 20 ml of methyl orange buffer solution. After the tubes were shaken gently in a bath at 10 ± 0.05°C for 24 hr, the optical density of the outside methyl orange solution was determined at 465 mμ. At this wave length, methyl orange solutions with pH values between 4.5 and 9.2 had maximum absorption. Concentration of the methyl orange dye was calculated from a standard curve. To obviate the error due to dye binding by the dialyzing tubing, control tubes were used with selected buffers in the dialyzing tubing rather than LDL-buffer solutions. By subtracting the concentration of methyl orange in the outside solution of the control tube from that of the experimental tube, the amount of bound dye was estimated. All dye-binding values are averages of at least two determinations.

Paper electrophoresis of plasma. Paper electrophoresis was conducted in a Spinco Durrum cell, Model R, using acetate buffer, pH 4.5, phosphate buffer, pH 6.5, and veronal buffer, pH 8.6. All of the buffers had an ionic strength of 0.1. The electrophoretic technique and staining procedure were similar to those of Powrie *et al.* (1963).

RESULTS AND DISCUSSION

For this study, a mild flotation procedure was selected for isolation of the LDL fraction of plasma so that the structure of LDL would not be altered markedly. The 2% lipoprotein solutions, used exclusively in this

Table 1. Influence of pH on the binding of methyl orange by native low-density lipoprotein.[a]

Buffer	pH	Moles bound dye/10^5 g low-density lipoprotein: Mean	Av. deviation
Acetate	4.5	0.180	0.001
Phosphate	6.5	0.160	0.003
Veronal	8.6	0.063	0.000

[a] Concentration of methyl orange = 2.62×10^{-5} moles/liter.

investigation, were devoid of opacity, thus indicating excellent dispersibility of the micelles in the buffer solutions, all with an ionic strength of 0.1.

At the beginning of this investigation, experiments were undertaken to determine the influence of buffer pH on the binding of methyl orange by LDL from native plasma. As shown in Table 1, the LDL micelles bound the largest amount of methyl orange at pH 4.5, while the least quantity of dye was bound at a solution pH of 8.6. The results suggest that the number of dye-binding cationic sites on the LDL micelles increased as the pH of the solution was lowered. During paper electrophoresis of yolk plasma, the LDL band migrated toward the cathode when the buffer pH values were 4.5 and 6.5, and toward the anode with a pH 8.6 buffer. It is probable that the LDL micelles in the buffers, used for dye-binding experiments, had net positive charges at pH 4.5 and 6.5. In such a situation, negatively-charged methyl orange molecules would be readily adsorbed on the micellar surfaces. In the case of pH 8.6 buffer, the net charge on each LDL micelle was probably negative, although some positively-charged dye-binding sites were undoubtedly present. Phospholipids are presumably not involved in the dye binding, since Ashworth and Green (1963) found that lecithin-stabilized triolein droplets did not bind methyl orange. The quaternary nitrogens in the proteins of the LDL could be the cationic sites. From the amino acid analysis of yolk LDL (Cook *et al.*, 1962), it is apparent that substantial amounts of histidine, lysine, and arginine are present. By increasing the pH of LDL solution, the positive charges of the quaternary nitrogens would be reduced and

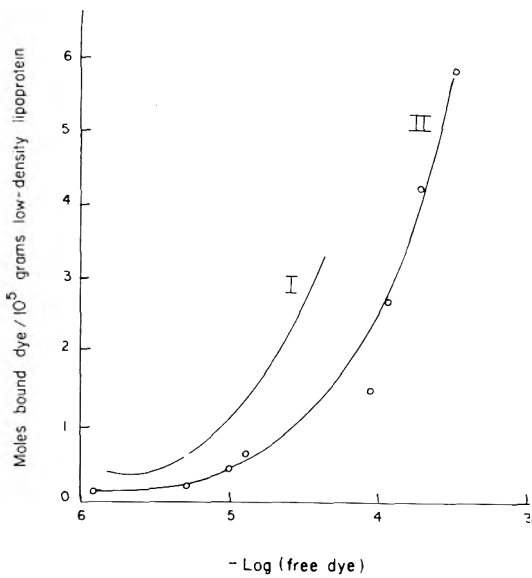

Fig. 1. The effect of methyl orange concentration on the dye-binding capacity of native low-density lipoprotein. I) Human serum β-lipoprotein (Rosenberg *et al.*, 1955). II) Yolk plasma low-density lipoprotein (LDL).

the methyl orange binding would be diminished.

Equilibrium dialysis was conducted with native LDL to determine the effect of methyl orange concentration on the dye-binding capacity of micelles at pH 6.5. This pH was selected because yolk has a similar pH value. From Table 2 and Fig. 1, it can be seen that as the concentration of the free dye was increased, more methyl orange was bound to the lipoprotein. An abrupt rise in dye binding of LDL is apparent in Fig. 1 (Curve II) when the $-\log$ (free dye) reached a value of about 4. The experimental curve II in Fig. 1 has a shape similar to curve I, obtained by Rosenberg *et al.* (1955) for human serum β-lipoprotein in solution at pH 7.4. However, the β-lipoprotein, with about 23% peptide, bound more moles of methyl orange than yolk LDL on an iso-weight basis. On the other hand, LDL, with about 15% peptide (or commonly called protein), had a dye-binding capacity comparable to that of β-lactoglobulin in phosphate buffer at pH 6.8 (Klotz and Urquhart, 1949) and greater than that of myosin in solution at pH 6.8 (Mihalyi and Ghosh, 1952). When the dye-binding of LDL is

Table 2. Effect of methyl orange concentration on the dye-binding capacity of native low-density lipoprotein.[a]

Initial methyl orange concentration (moles/liter)	Moles bound dye/10^6 g low-density lipoprotein	Free dye concentration (moles/liter) (c)	—log free dye	Moles bound dye/moles total lipoprotein [b] (r)	$r/c \times 10^{-6}$
1.0×10^{-5}	0.061	1.1×10^{-6}	5.96	2.93	2.66
2.5×10^{-5}	0.161	2.9×10^{-6}	5.28	7.74	2.67
7.5×10^{-5}	0.402	9.2×10^{-6}	5.03	19.30	2.08
1.0×10^{-4}	0.590	1.3×10^{-5}	4.90	28.36	2.23
2.5×10^{-4}	1.470	8.2×10^{-5}	4.08	70.67	0.86
5.0×10^{-4}	2.610	1.2×10^{-4}	3.94	125.48	1.09
7.5×10^{-4}	4.125	1.8×10^{-4}	3.74	198.33	1.08
1.0×10^{-3}	5.780	3.5×10^{-4}	3.46	277.88	0.80

[a] Phosphate buffer, pH 6.5

[b] Based on molecular weight of low-density lipoprotein $= 4.8 \times 10^6$ (Martin *et al.*, 1959).

expressed on a peptide basis, the binding capacity is much higher that that of β-lactoglobulin. If all of the dye-binding sites on LDL are considered to be cationic groups of the protein moieties, then the high dye-binding capacity of LDL may be accounted for by the existence of uncoiled protein molecules with a large number of exposed cationic groups on the micellar surfaces. Generally, proteins exist in the uncoiled form at oil-water interfaces (Gurd, 1960). Colvin (1952) pointed out that the binding of methyl orange by lysozyme can be increased by heat denaturation of the protein. Heat denaturation involves the rupture of intermolecular hydrogen bonds, the uncoiling of helical structures, and the exposure of reactive groups (Fox and Foster, 1957).

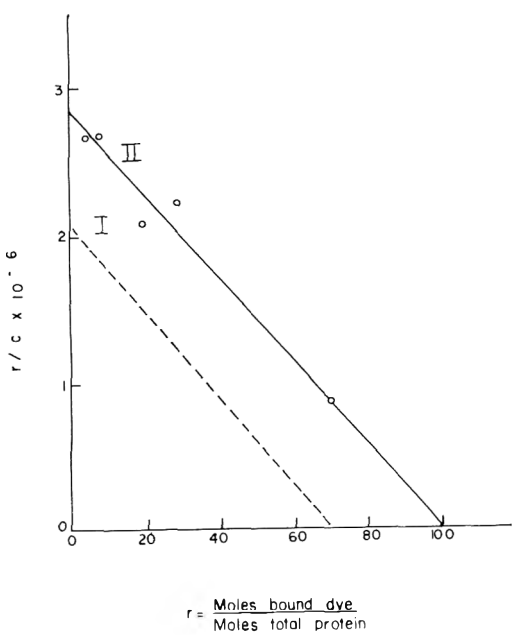

Fig. 2. Binding of methyl orange by native low-density lipoprotein. I) Human serum β-lipoprotein (Rosenberg *et al.*, 1955). II) Yolk plasma low-density lipoprotein (LDL).

An attempt was made to determine the average number of binding sites on the native LDL micelles. To accomplish this, dye-binding data from Table 2 were plotted in Fig. 2 (Curve II) as the ratio of moles of bound dye/moles of total LDL (r) against $r/c \times 10^{-6}$, where c represents the concentration of free dye. Only the first five $r/c \times 10^{-6}$ values in Table 2 were used for the plot, so that the straight-line slope could be compared with that of Rosenberg *et al.* (1955). The average molecular weight of LDL was regarded as 4.8×10^6 (Martin *et al.*, 1959). If the LDL has a specific number of dye-binding sites, n, with intrinsic association constants, K_i, and if there is no interaction between the bound anions, then the equation of Von Muralt (1930) is

$$r = \sum_i \frac{K_i c}{1 + K_i c} \quad \text{where } i = 1, 2, 3 \ldots\ldots n. \quad [1]$$

Scatchard (1949) suggested that, if all sites have the same intrinsic association constant, K, then Eq. 1 can be rearranged to

$$r/c = nK - rK \quad [2]$$

With the K_i values equal, then the plot of r/c vs. r should be linear, and the intercept on the X-axis should give the number of dye-binding sites. A straight line was drawn

in Fig. 2 as curve II with a slope similar to curve I obtained by Rosenberg *et al.* (1955) for serum β-lipoprotein. A value of 100, indicating estimated sites, was obtained at the intercept. β-Lipoprotein, with a molecular weight of 1.3×10^6, had an estimated 69 sites (Rosenberg *et al.*, 1955). If the last three $r/c \times 10^{-6}$ values from Table 2 were plotted in Fig. 2, then the LDL curve would be nonlinear. This deviation from linearity may be due to more than one site type, each type characterized by a particular association constant and/or interaction between bound anions (Scatchard, 1949). Thus, the exact number of binding sites cannot be estimated by the r/c vs. r plot method. However, it can be supposed that the average number of binding sites on the LDL micelles is at least 100.

In the next phase of this study, LDL was isolated from yolk samples which were pasteurized at 63 and 64.8°C with a holding time of 3 min. These temperatures are somewhat higher than the normal commercial pasteurization temperature of about 62°C. As shown in Table 3, the LDL from heated and native yolk had similar dye-binding values. Apparently, the dye-binding sites on LDL micelles were not altered significantly by the introduction of thermal energy. If the binding sites are considered to be only in the protein moieties, and if the protein molecules are naturally present in the denatured form, then it seems unlikely that the dye-binding capacity of LDL would be changed when the yolk was heated to these moderate temperatures.

When egg yolk is frozen below −6°C, the viscosity of the thawed yolk is much higher than that of the unfrozen sample (Powrie *et al.*, 1963). This irreversible viscosity alteration is called gelation. The yolk gelation is probably caused by the aggregation of LDL micelles. Intermicellar bonding may be electrostatic, involving positively- and negatively-charged groups of the protein moieties. Any reduction of charged groups in the LDL protein moieties may be responsible for the prevention of gelation. In the present study, it was found that the treatment of yolk plasma with bromelain inhibited plasma gelation. In all probability, the hydrolysis of surface proteins on the LDL was involved in the inhibition mechanism. Saari *et al.* (1964) reported that the protein moiety of LPL_2, a LDL fraction, was hydrolyzed by papain. With the assumption that methyl orange molecules are adsorbed to the peptide portion of LDL, experimentation was carried out to determine any change in the number of LDL cationic sites when plasma was treated with the proteolytic enzyme bromelain. As shown in Table 3, the dye-binding capacity of LDL from bromelain-treated frozen-thawed plasma was lower than that for the LDL from native plasma. These data suggest that hydrolysis of micellar proteins by bromelain was responsible for a decrease in cationic sites. Electrophoretic information indicates that enzymic hydrolysis of proteins also brings about a reduction of negatively-charged groups. As shown in Fig. 3, the migration distance during paper electrophoresis was

Table 3. Binding of methyl orange by low-density lipoprotein isolated from pasteurized yolk and bromelin-treated frozen-thawed plasma.[a]

Sample	Treatment	Moles bound dye/10^5 g low-density lipoprotein
LDL from native plasma	none	0.160
LDL from pasteurized yolk	Patsteurization:	
	145°F (63°C) for 3 min	0.162
	148°F (64.8°C) for 3 min	0.161
LDL from bromelin-treated plasma	Proteolysis: 30 min enzymic digestion at 25°C; frozen at −10°F (−23°C) for 24 hr	0.152

[a] Concentration of methyl orange = 2.6×10^{-5} moles/liter. Phosphate buffer, pH 6.5.

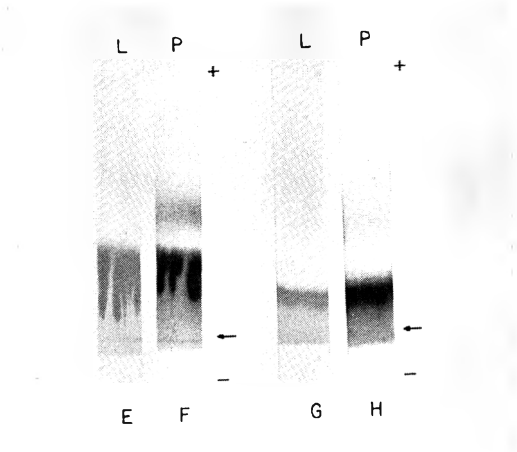

Fig. 3. Paper electrophoretograms of yolk plasma. *E* and *F*, native plasma; *G* and *H*, plasma treated with bromelain. *L* = lipid stain, *P* = protein stain. Electrophoretic conditions: veronal buffer, pH 8.6, ionic strength = 0.1, voltage gradient = 4.1 V/cm for 24 hr, 0.02 ml plasma diluted 1:1 with 10% NaCl.

much lower for LDL in bromelain-treated plasma than for LDL in native plasma. The reduction in the number of electrically-charged groups on the LDL micelles may be explained by the alteration of fairly rigid surface polypeptides to more flexible fragments during hydrolysis and, subsequently, the interaction of $\equiv NH^+$ groups with COO^- groups.

Since sucrose and NaCl are added to yolk prior to commercial freezing for the prevention of gelation, the influence of these additives on the dye-binding capacity of native LDL was investigated. A decrease in binding capacity of LDL could be interpreted as a binding of the sucrose molecules or Na^+ and Cl^- ions to the dye-binding sites. Prior to dialysis, the 2% lipoprotein solutions (pH 6.5) contained 1, 5, 10, and 15% of either sucrose or NaCl. As equilibrium dialysis progressed, solute in each case diffused out of the dialyzing sac into the outside solution. The final concentration of the solute in each sac of lipoprotein solution was about one-third of the original solute concentration. From Table 4, it can be seen that only NaCl at the 15% initial concentration level had an influence on the binding of methyl orange by native LDL. If any solute molecules were adsorbed on the dye-binding sites, then they were displaced by the methyl orange molecules during equilibrium dialysis.

Table 4. Effect of sodium chloride and sucrose on methyl-orange-binding capacity of native low-density lipoprotein.[a]

Initial solute concentration in LDL solution		Moles bound dye/10^5 g low-density lipoprotein	
NaCl (%)	Sucrose (%)	Mean	Av. deviation
0.0	.....	0.160	0.003
1.0	.....	0.160	0.003
5.0	.....	0.161	0.002
10.0	.....	0.163	0.002
15.0	.....	0.166	0.003
.....	1.0	0.160	0.003
.....	5.0	0.160	0.003
.....	10.0	0.159	0.003
.....	15.0	0.159	0.003

[a] Concentration of methyl orange = 2.62×10^{-5} moles/liter. Phosphate buffer, pH 6.5.

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Protein Alterations and Associated Changes in Porcine Muscle as Influenced by Maturity, Genetic Background, and Post-Mortem Muscle Temperature

SUMMARY

Stage of maturity, genetic background, and post-slaughter holding temperature have been studied in relation to their effects on the rate of post-mortem glycolysis, change in gross muscle morphology, and alteration of muscle protein solubility. Muscle from 5–10-kg animals tended to have more glycogen and less myoglobin as well as less total nitrogen than muscle from 250–300-kg animals. Glycolytic rate appeared to be slightly faster in the post-mortem muscle of mature animals, but differences among weight groups were minor. Muscle from mature animals exhibited a greater loss of color after slaughter. Protein solubility also decreased to a greater extent in these mature muscles. This was especially evident in the myofibrillar fraction. Post-slaughter holding temperature did not affect the rate or extent of glycolysis in the muscle, but chilling gave benefits to subjective score for color and juice-retaining capacity. It also tended to make the loss of protein solubility less severe, particularly for the myofibrillar fraction.

INTRODUCTION

Porcine muscles range in color and gross morphology from a dark, firm, dry to a pale, soft, exudative condition (Sayre *et al.*, 1964). The gross morphology of post-mortem porcine muscle has been shown to be influenced by pharmacological agents (Ludvigsen, 1957), exercise and ration (Briskey *et al.*, 1959, 1960), rate of glycolysis and temperature (Wismer-Pedersen and Briskey, 1961), and environmental and genetic background (Sayre *et al.*, 1961, 1963a,b). Rate of glycolysis has been demonstrated to be of greater importance than glycogen level or muscle pH 24 hr after slaughter in influencing gross morphological features. When pH was low (<5.9) and muscle temperature high ($>35°$) at the onset of rigor mortis, there was a marked reduction in the extractability of the sarcoplasmic and myofibrillar proteins (Sayre and Briskey, 1963). These experiments were made to: 1) study the effects of animal maturity on post-mortem changes in chemical and physical characteristics of muscle; and 2) determine the extent to which muscle protein solubility and associated juice-retaining properties were influenced by breed and carcass holding temperature.

EXPERIMENTAL

Animal source. All animals came from the University of Wisconsin Swine Farm and thus represent restricted lines of breeding. All animals were fed normal swine rations commensurate with their stage of maturity. No special preslaughter treatment was administered, and the animals were not fasted prior to slaughter. Exsanguination and evisceration were conducted in a manner and at a rate corresponding to commercial practices.

Experiment I. All animals used in the first experiment were from the Poland China breed and were grouped according to live weight as shown in Table 1. Carcasses of the 5–10-kg group were held at a constant temperature of 35° for 3 hr after slaughter, so that the rate of temperature drop would be similar in the muscle of animals from

Table 1. Live weight groups for animals used in Experiment I.

Live weight (kg)	No. of animals
5–10	4
57–107	13
125–140	6
250–300	5

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each weight group. Subsequently these carcasses were transferred to a 4° environment.

Experiment II. Six pigs from the Poland China breed and seven pigs from the Chester White breed, all weighing approximately 125 kg, were used in this experiment. Carcasses were split, and the left side was held for 4 hr at a constant temperature of 35° and the right side allowed to chill at 4°.

Sample removal and analysis. Muscle samples for certain chemical and physical measurements were removed from the lumbar region of the longissimus dorsi muscle at specified post-mortem intervals. Subjective score for color and juice-retaining capacity, surface reflectance, pH, and temperature were determined as previously described (Sayre *et al.*, 1963b). The juice-retaining capacity of the 24-hr-chilled muscle was measured by the filter paper absorption technique of Grau and Hamm (1953) as modified by Urbin *et al.* (1962), and expressed as the ratio of total area to meat film area. All samples used for protein extraction or chemical analysis were frozen in liquid nitrogen, sealed in plastic bags, and held at −25° until processed.

The frozen muscle samples used for protein extraction were ground with a mortar and pestle to a fine powder in the presence of dry ice. These powdered samples were passed through a 16-mesh sieve to remove any large particles and stored at −25°. Extraction was conducted according to the method of Helander (1957), using 0.03*M* K phosphate buffer at pH 7.4 to solubilize sarcoplasmic proteins, and 1.1*M* KI in 0.1*M* K phosphate buffer at pH 7.4 to remove total soluble proteins. Myofibrillar protein solubility was calculated by difference. Nonprotein nitrogen was measured after trichloroacetic acid precipitation. All protein extractions were conducted at 2° and were completed within 72 hr of slaughter.

Nitrogen was determined by the Kjeldahl method, and all values for the protein fractions were expressed as a percentage of total nitrogen. Glycogen was determined by the phenol-sulfuric acid method of Dubois *et al.* (1956); and myoglobin was measured by the cyanometmyoglobin procedure of Poel (1949). All values are represented graphically as means ± standard error of means.

RESULTS AND DISCUSSION

Experiment I. Although differences were not significant at the 5% level of probability, the 5–10-kg group tended to have the greatest amount of glycogen stored in the muscle at the time of slaughter (Fig. 1). Glycogen degradation during the first hour after slaughter was most extensive in the 250–300-kg group. The total amount of glycogen

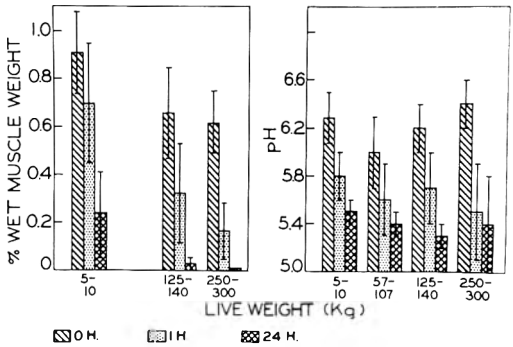

Fig. 1. Glycogen content and pH values of longissimus dorsi muscle from pigs in experiment I.

broken down during the first 24 hr was essentially the same for all groups measured. The rate and extent of pH decline (Fig. 1) did not show a consistent pattern among the four weight groups. The 250–300-kg group exhibited the most rapid rate of pH decline during the first hour post-mortem, but the 24-hr pH values were similar for all groups. These observations indicate that, although there was a tendency for more rapid glycolysis in mature animals, maturity was not a major factor influencing the rate and extent of post-mortem glycolysis.

Subjective scoring of muscle appearance or gross morphology (based on a four-point scale from 1, pale and exudative, to 4, dark and dry) did show some differences among the weight groups (Fig. 2). The 0-hr score increased with increasing level of maturity, and the muscle from 250–300-kg animals was very dark and dry. There was almost no change in the score for the 5–10-kg group during the first 24 hr post-mortem. However, there were significant decreases of subjective score in the other three groups, particularly in the 250–300-kg group. Objective evaluation of color by measurement of the intensity of 485-mμ light reflected from the muscle surface tended to correspond to the subjective scores (Fig. 2). Again, there were only minor changes with time post-mortem in the 5–10-kg group, while changes were marked during the first hour after slaughter in both the 57–107-kg and 250–300-kg groups.

Myoglobin content of the muscle was altered markedly as animal weight and maturity increased (Fig. 2). Muscle from the

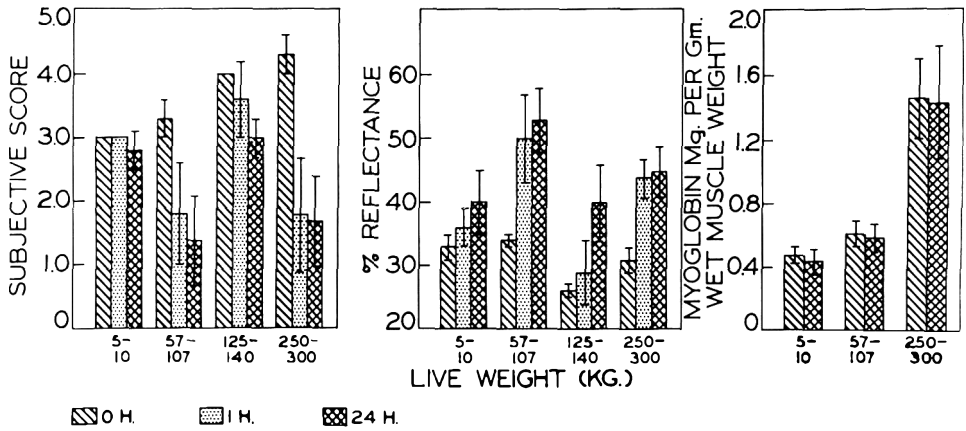

Fig. 2. Subjective score for color and juice-retaining capacity and per cent reflectance of 485-mμ light, and myoglobin concentration for longissimus dorsi muscle from pigs in experiment I.

250–300-kg group contained about three times as much myoglobin as muscle from the 5–10-kg group. The myoglobin content of muscle as measured by the cyanometmyoglobin method did not change during the first 24 hr post-mortem. The dramatic loss of color following slaughter observed particularly in the 250–300-kg group apparently was not the result of heme destruction or other loss of myoglobin. However, the myoglobin molecule must have been altered in such a manner that it no longer had the same chromogenic activity under the conditions found in these muscles. Preliminary trials with muscle extracts at various pH values and temperatures have demonstrated that the color of a low-ionic-strength extract from pale, soft, exudative muscle cannot be restored by simple pH adjustment.

The juice-retaining capacity of muscle as indicated by expressible juice ratio (Fig. 3) was not altered significantly by animal maturity. Although muscle from the 125–140-kg group appeared to be less exudative than that from the other three groups and similar to the 5–10-kg group in color at 24 hr, this change might be attributed to environmental conditions at the time of slaughter. Forrest *et al.* (1963) have noted wide seasonal variation in the incidence of exudative porcine muscle.

The total nitrogen content of the muscle was considerably lower in the 5–10-kg group than in any of the other three groups (Fig. 3). The lower nitrogen content of muscle from 5–10-kg animals may explain the rather low level of sarcoplasmic protein found in 0-hr samples from these muscles (Fig. 4). The fact that glycolysis as well as rate and extent of color change after slaughter were moderate would support the conclusion that a smaller amount of sarcoplasmic protein was present in these muscles and that the low values were not the result

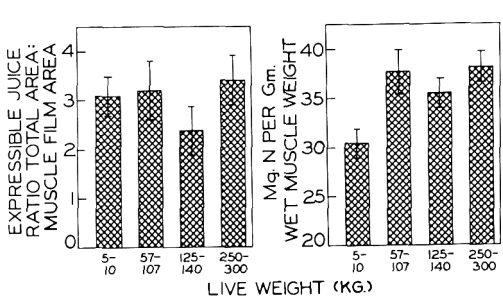

Fig. 3. Expressible-juice ratio and nitrogen content of longissimus dorsi muscle from pigs in experiment I.

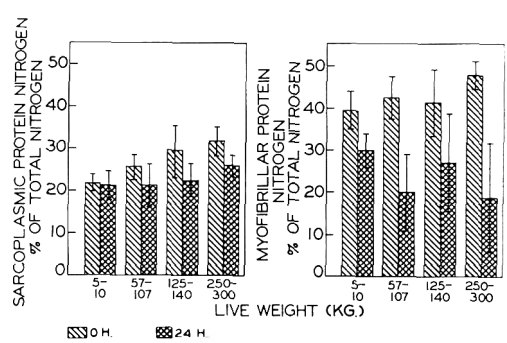

Fig. 4. Sarcoplasmic and myofibrillar protein extractability of longissimus dorsi muscle from pigs in experiment I.

of decreased extractability of the proteins. Conversely, the low 0-hr values for sarcoplasmic protein in the 250–300-kg group could be attributed to a rapid loss in extractability of these proteins. Previous work has shown that the solubility of sarcoplasmic proteins can be decreased very quickly after slaughter under conditions which eventually result in pale exudative muscle (Sayre and Briskey, 1963). It is also of interest that there was no change in the extractability of sarcoplasmic proteins from the 5–10-kg group during the 24 hr following slaughter, while sarcoplasmic protein extractability from each of the other three groups decreased throughout this period.

The amount of myofibrillar protein extracted at 0-hr appeared to be less in the 5–10-kg group than in the 250–300-kg group (Fig. 4). Previous observations that severe changes in the gross morphology of muscle were closely related to myofibrillar protein solubility (Sayre and Briskey, 1963) were further substantiated by this study. The 57–107-kg group, and particularly the 250–300-kg group, exhibited extensive losses of myofibrillar protein extractability during the first 24 hr post-mortem. As was the case for sarcoplasmic protein, the 5–10-kg group exhibited the least decrease in myofibrillar protein solubility at 24 hr.

Experiment II. Previous work has shown marked differences in certain muscle characteristics between breeds of swine (Sayre *et al.*, 1963b); in this experiment, however, no differences in the characteristics measured were apparent between the two breeds studied. Muscle glycogen levels and pH values indicated that rate and extent of glycolysis were similar for both breeds (Fig. 5). Muscle color and juice-retaining capacity were also similar, as indicated by percent reflectance, subjective score, and expressible-juice ratio (Figs. 6, 7). Solubilities of the muscle proteins apparently were not associated with the genetic background of the animals used in this experiment.

Previous results indicated that muscle temperature in an intact carcass was maintained at approximately slaughter level for 45 min post-mortem (Sayre *et al.*, 1963a). Data from this experiment show that the muscle temperature decreased approximately 1° at 1 hr and only 8° at 2 hr post-mortem in muscle from the chilled side of the carcass (Fig. 7). However, after 4 hr post-mortem, muscle temperature in the chilled sides had decreased nearly 17°, or about 11° below the temperature of the unchilled sides.

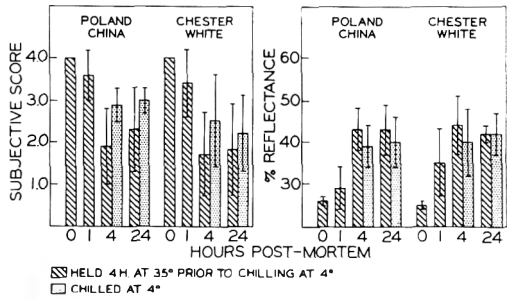

Fig. 6. Subjective score for color and juice-retaining capacity and per cent reflectance of 485-mμ light of longissimus dorsi muscle from pigs in experiment II.

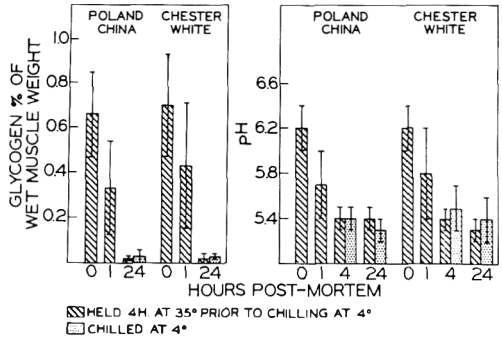

Fig. 5. Glycogen content and pH values of longissimus dorsi muscle from pigs in experiment II.

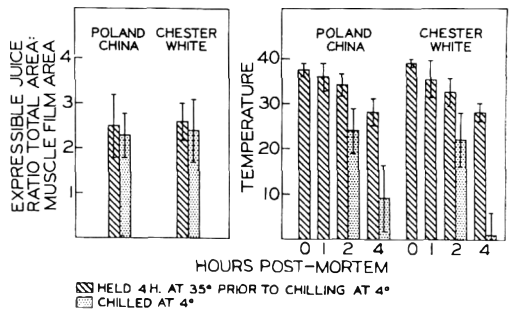

Fig. 7. Expressible-juice ratio and temperature in the carcass of longissimus dorsi muscle from pigs in experiment II.

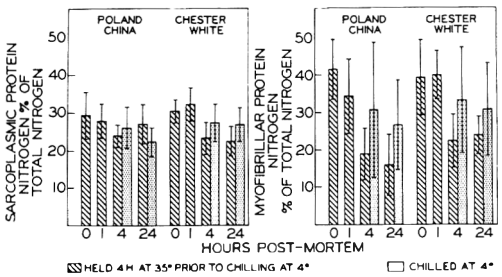

Fig. 8. Sarcoplasmic and myofibrillar protein extractability of longissimus dorsi muscle from pigs in experiment II.

Post-slaughter holding temperature had no effect on the extent of pH decline, and pH values were as low at 4 hr as at 24 hr post-mortem. Only a minute amount of glycogen remained in either chilled or unchilled muscle at 24 hr. Surface reflectance tended to be less from muscles held at 4° than from those held at 35°: the differences were small and nonsignificant, however. Subjective scores also tended to indicate that the chilled muscle appeared darker in color and less exudative than muscle from unchilled sides; but, again, variability in these data was considerable.

Muscle pH dropped to 5.8 or lower while the muscle temperature remained above 35°. These conditions of pH and temperature have been shown to decrease the solubilities of muscle proteins markedly (Sayre and Briskey, 1963). Sarcoplasmic proteins were not affected to as great an extent as myofibrillar proteins (Fig. 8); however, solubility had declined considerably at 4 hr and remained unchanged at 24 hr after slaughter. Sarcoplasmic protein extractability tended to be greater from the chilled sides, but the differences were not significant.

Myofibrillar protein extractability was markedly diminished in the chilled sides, but the loss was even more extensive in the sides which were not chilled during the initial 4-hr period. This is further evidence of the importance of the temperature-pH relationship in causing alteration of the myofibrillar proteins. Sayre and Briskey (1963) found that the myofibrillar proteins did not respond as rapidly to the physiological conditions in the muscle after death; but if a response was observed, the loss of solubility was much more severe than for sarcoplasmic proteins.

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Histological Studies of Post-Mortem Changes in Sarcomere Length as Related to Bovine Muscle Tenderness

SUMMARY

The effect of post-mortem muscle contraction on ultimate tenderness was studied in muscles of 12 beef animals of similar weight and grade. State of contraction was determined by measurement of sarcomere lengths. Longissimus dorsi and semimembranosus muscles were observed. Histological samples removed at various intervals post-mortem were treated with ethylenediaminetetraacetate (EDTA) to prevent further contraction. A phase-contrast microscope was used to observe muscle fibers, and sarcomeres were measured with a filar micrometer. Muscle contraction patterns of each animal were plotted through a 7-day aging period. State of contraction after 7 days appeared to have a greater influence on subsequent (7 days) tenderness than did state of contraction at time of maximum rigor mortis. Although contraction did not seem to be the factor most responsible for final tenderness, it did appear to have a significant influence. Considerable lengthening of sarcomeres normally occurred during the aging period. Semimembranosus muscles routinely contracted less than longissimus dorsi muscles during rigor mortis, and were more relaxed after 7 days of aging. Semimembranosus muscles were consistently less tender than longissimus dorsi muscles at slaughter, but the reverse was usually true after 7 days.

During recent years, considerable interest has been focused on relationships between post-mortem muscle contraction and tenderness. Locker (1960) observed that relaxed muscles were more tender than partially contracted muscles 2 days post-mortem. This was more evident in muscles low in connective-tissue content. Partmann (1963) impeded post-mortem actomyosin formation in strips of a diaphragm muscle by allowing them to age in a weighted, stretched condition. The weighted strips were significantly more tender than the unweighted controls. It was earlier suggested that increased tenderness during aging may be related to dissociation of actomyosin (Wierbicki *et al.*, 1956). However, there has been no conclusive proof that such a dissociation actually occurs. Wierbicki *et al.* (1956) indicated that although actomyosin appeared to play a role in meat tenderness, no evidence of actomyosin dissociation during aging was found. Similar conclusions have been reached by Neelin and Rose (1964) with chicken muscle. Yet, fiber fragments of aged muscle were shown to contract upon addition of ATP, suggesting at least some dissociation during aging (Partmann, 1963).

If sarcomere length decreases with an increase in actomyosin, or vice versa, it would appear logical that measurements of sarcomere lengths at various intervals post-mortem would reflect the degree of actomyosin dissociation during the aging period. Therefore, measurements have been made to determine the extent of maximum muscle contraction at rigor mortis, and the subsequent amount of relaxation occurring during a 7-day aging period. The relationships of these changes in bovine muscles to ultimate 7-day tenderness values were investigated.

METHODS

Selection and treatment of carcasses. Carcasses from 4 bulls, 4 heifers, and 4 steers were used. The animals were of various beef breeds but were similar in weight and grade. Carcasses weighed 500–600 lb, and graded high-good to low-choice.

In order to create different contraction patterns between the two sides of each carcass, different initial holding temperatures were employed. Immediately after dressing, the right side of each carcass was subjected to 1–2°C for rapid chilling. The left side was left at 20°C for 9 hr, and then placed with the other side. After 48 hr, both sides were transferred to a holding cooler and maintained at 3°C throughout the remainder of a 7-day aging period.

Sampling methods. Longissimus dorsi and semimembranosus muscles were sampled from each side of every carcass. Two types of samples were taken: shear samples for tenderness evaluations, and histological samples for microscopic observations.

Steaks 3.17 cm thick were excised both immediately after stunning and after 7 days' aging. In bulls, initial samples were taken approximately 1 hr after death, but heifer and steer samples were removed within 5 min of stunning. Initial longissimus dorsi samples were removed from over the 13th rib of the left side. Samples from semimembranosus muscles were taken parallel to the ischium. Corresponding muscles of the right side were also severed in order to create the same effect of cutting. All steaks were in the cooker within 10 min of removal and were cooked in deep fat at 135°C to an internal temperature of 70°C. Following a 5-min cooling period at room temperature, three 2.54-cm cores were removed from each steak and sheared twice with a Warner-Bratzler shear machine. The average of the 6 readings was used for shear value.

Samples were removed for histological examination at various intervals post-mortem. Beginning at the time of slaughter, a sample from each muscle was taken every 3 hr for the first 24 hr. Two samples were then taken at 6-hr intervals, and one was taken each day throughout the remainder of the aging period. All samples were immediately frozen in OCT compound. Frozen tissue blocks were sectioned with a microtome cryostat, and sections were collected onto slides treated with EDTA to prevent thaw contraction. Slides were observed with a phase-contrast microscope, and sarcomeres were measured with a filar micrometer. Five muscle fibers were selected at random from each section, and 5 consecutive sarcomeres were measured in each fiber. The average of 25 readings was used as sarcomere length of each muscle at any given time.

RESULTS AND DISCUSSION

Extreme contraction was noted in prerigor muscle samples when sections were collected onto clean untreated slides. Such thaw rigor has been reported to occur from calcium release into the actomyosin system (Partmann, 1961). Since it was necessary to observe muscle fibers in exactly the state of contraction as when removed from the carcass, a method was devised to prevent thaw contraction. By employing EDTA to chelate calcium ions, state of contraction of a selected muscle at time of sampling could readily be observed and measured. When frozen sections were collected onto slides treated with glycerine and saline solution saturated with EDTA, further contraction did not occur. Fig. 1A shows muscle fibers not treated with EDTA. In this case, fibers

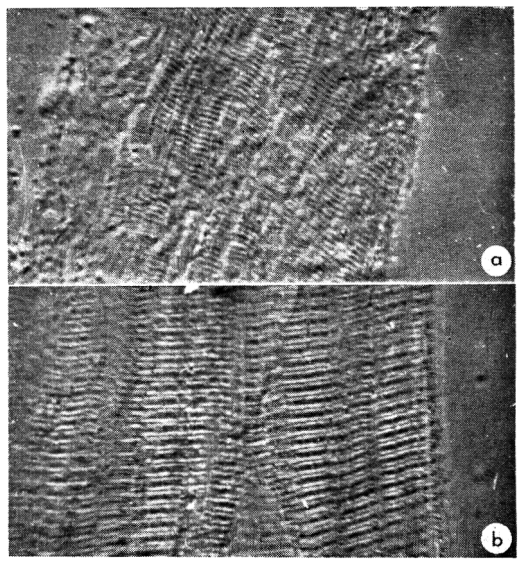

Fig. 1. A) Muscle fibers excised prior to onset of rigor mortis. The frozen section was collected onto a clean slide, and extreme thaw contraction occurred. B) An adjacent section from the same sample as *A*, but collected onto a slide treated with EDTA. Thaw contraction was prevented, as evidenced by more relaxed fibers.

contracted greatly upon thawing. Fig. 1B shows EDTA-treated fibers from an adjacent section of the same sample in which fibers were not in a contracted state.

By this procedure it was possible to observe fiber changes and to plot muscle contraction patterns of each animal. Many samples excised soon after stunning contracted greatly upon cutting, which corresponds to previous findings (Locker, 1960; Herring *et al.*, 1965). This was especially true in one heifer that showed evidence of nervousness immediately previous to slaughter. However, as rigor mortis approached, muscles lost this irritability and very little response to cutting occurred after 3 hr post-mortem.

There was no significant difference in sarcomere lengths of longissimus dorsi and semimembranosus muscles during the delay period. These values were consistently about 2 μ. Observed changes suggested that cut muscles did not recover to their original lengths prior to cutting, for considerably longer sarcomeres have been reported for psoas major (Locker, 1960) and semitendinosus muscles (Herring *et al.*, 1965).

As the rapid phase of contraction began,

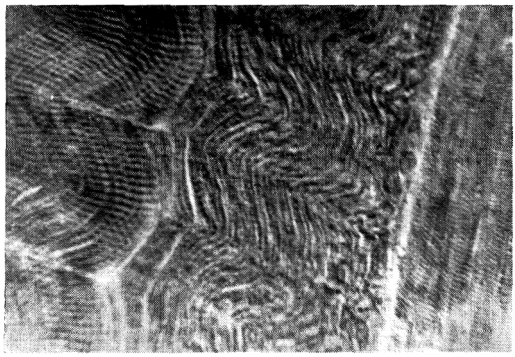

Fig. 2. Muscle fibers excised during onset of rigor mortis. The most contracted fiber, at right, is straight, whereas the less contracted ones are wavy.

muscle fibers contracted at various rates. Rapidly contracting fibers were normally straight in appearance, while adjacent slower contracting fibers were usually wavy or "kinked" in appearance. There was evidence that kinking was due to a pulling influence of rapidly contracting fibers on those more relaxed, as shown in Fig. 2.

The two muscle types observed revealed somewhat different contraction patterns. Semimembranosus muscles normally did not reach the state of contraction of that observed in the longissimus dorsi muscles during rigor mortis (Table 1).

During rigor, semimembranosus muscles contracted to an average of 78.4% of prerigor values, while longissimus dorsi muscles were contracted to 73.7%. Muscles from sides initially exposed to the 20°C treatment usually showed greater contraction than those held at 1–2°C. Correlation coefficients (Table 2) indicated only a small relationship between state of contraction at maximum rigor mortis and the ultimate (7 days) tenderness.

Yet, within animals, more contracted muscles of the two treatments usually proved to be ultimately less tender. In 16 comparisons with heifers and steers, muscles reaching greater contraction during rigor mortis were ultimately less tender in 12 instances. This difference proved significant ($P < .05$) with semimembranosus muscles.

Table 1. Average sarcomere lengths (μ) of longissimus dorsi and semimembranosus muscles during maximum contraction.

	Longissimus dorsi		Semimembranosus	
	1–2°C	20°C	1–2°C	20°C
Bulls	1.34	1.52	1.70	1.67
Heifers	1.55	1.45	1.68	1.51
Steers	1.54	1.54	1.60	1.55
Av.	1.50	1.48	1.66	1.57
	1.49		1.62	

Table 2. Correlation coefficients of maximum contraction during rigor mortis and ultimate tenderness.

	Longissimus dorsi	Semimembranosus
Bulls	−.57	.05
Heifers	−.32	−.56
Steers	−.18	.16
Heifers and steers	−.47*	−.23
Heifers, steers, and bulls	−.38	−.11

* Significant at .05 level.

Normally, sarcomere lengths began to increase less than 3 hr after maximum contraction had been reached. This was most marked in the semimembranosus muscles, which were in most cases considerably more relaxed after 7 days than longissimus dorsi muscles. By the 7th day, values for semimembranosus muscles were 90.4% those of prerigor, and values for longissimus dorsi muscles averaged 86.5%.

Semimembranosus muscles were consistently less tender than longissimus dorsi muscles immediately after slaughter (Table 3). Average initial shear values were 18.62 kg and 12.15 kg, respectively. However, these tenderness trends were normally reversed after 7 days. Average 7-day shear value was 10.3 kg for semimembranosus muscles, and 12.18 kg for longissimus dorsi muscles. Semimembranosus muscles underwent considerable tenderization during aging, but final longissimus dorsi shear values differed very little from initial values. In three of the four heifer carcasses, longissimus dorsi muscles were considerably less tender after 7 days.

Final state of contraction appeared to be closely associated with tenderness. After 7 days' aging, more contracted longissimus dorsi muscles, between the two treatments, were significantly ($P < .05$) less tender than less contracted muscles. This was also true of semimembranosus muscles from heifers and steers.

Table 3. Initial and final shear values[a] of two muscles (in kg).

Animal	Treatment[b]	Longissimus dorsi		Semimembranosus	
		Initial shear	Final shear	Initial shear	Final shear
Bull 1	R	16.11	17.10	21.05	15.62
	C		13.88		12.02
Bull 2	R	14.40	9.01	24.29	9.61
	C		9.95		9.52
Bull 3	R	11.71	12.66	18.20	8.75
	C		13.85		7.36
Bull 4	R	13.65	12.21	14.67	11.72
	C		13.52		6.93
Heifer 1	R	11.81	12.69	18.55	11.25
	C		12.61		12.08
Heifer 2	R	13.55	12.19	17.91	9.88
	C		7.74		8.21
Heifer 3	R	9.37	12.74	19.56	10.58
	C		12.04		9.13
Heifer 4	R	10.66	21.86	20.09	17.04
	C		19.73		14.88
Steer 1	R	10.45	9.71	13.57	8.48
	C		13.73		9.39
Steer 2	R	9.77	8.20	14.86	9.61
	C		8.84		7.08
Steer 3	R	11.47	11.06	17.40	9.73
	C		9.92		10.28
Steer 4	R	12.99	9.71	23.40	8.31
	C		8.20		7.44

[a] Shear value represents mean value of six shear determinations.
[b] R = initial treatment of 20°C; C = initial treatment of 1–2°C.

It was often very difficult to section samples removed after 6 or 7 days of aging. The fibers appeared to be brittle, and were easily shattered. Shattering always appeared to occur at the levels of the I bands, which suggested occurrence of some type of degradation of the protein actin during aging. Such a degradation could possibly be associated with tenderization during the aging period. The degree of contraction after 7 days aging may be a significant factor influencing muscle tenderness, particularly in muscles where contraction occurs rather uninhibited as in the longissimus dorsi.

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Stability of Malic Enzyme in Fish Flesh at +3°C

SUMMARY

An increase in endogenous malic enzyme in tissue fluid, previously noted in frozen and thawed fish, does not occur in unfrozen fish. In tissue pasteurized by low-level gamma irradiation, malic enzyme activity remained constant for 21 days at +3°C. At higher levels of gamma radiation, activity was lost.

INTRODUCTION

A consistent observation in this laboratory has been that in the soluble portion of fish flesh, activity of the malic enzyme [L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40] in the presence of added substrate approximately doubles after a single freeze-thaw cycle of the flesh. This freezing-induced activity remains fairly constant in fish held under good (−29°C) frozen-storage conditions, but drops with time in fish held under poor (−7°C) frozen-storage conditions (Gould, 1965).

Although it seemed probable that the observed rise in activity was due to the release of a previously bound malic enzyme (ME) fraction by the physical processes of freezing and thawing, it was necessary to determine whether similar changes may also occur in the endogenous ME activity of fish held at iced-storage temperatures.

To ensure that we were working with only the endogenous enzyme in fish, we inhibited bacterial growth by subjecting the tissue to pasteurizing levels of cobalt-60 radiation (up to 1 megarad), after determining that the ME activity in such irradiated samples did not differ from the activity in the unirradiated control. Higher doses were used to measure the *in situ* lability of ME to gamma radiation.

METHODS AND PROCEDURES

Sample preparation. Fresh fillets, cut from 4-to-6-lb haddock *(Melanogrammus aeglefinus)* caught by hook 1 day prior to the experiments, were freed from skin and fatty tissue, cut transversely into roughly 1-inch strips, and separated into head and tail sections. One strip from each section made up a sample (60–100 g). The samples were vacuum-sealed in plastic pouches and placed in No. 2 cans, which were then air-sealed. During the irradiation process, each can was centered in a No. 10 can filled with ice to prevent warming. A series of samples, irradiated at 0.25-Mrad intervals up to 1.0 Mrad, was stored at +3°C and tested every few days for ME activity. A second series, irradiated stepwise from 0.25 Mrad to 4 Mrads, was tested immediately.

Enzyme preparation and assay. On the day of assay each tissue specimen was transferred whole, including in-package drip, to a polypropylene tube, capped, centrifuged at +4°C for 20 min at 20,000 × G, and its supernate withdrawn with a syringe. The supernate (CTF) served as the crude enzyme preparation. Mincing and homogenization of the tissue were avoided, both to eliminate the possibility of mechanically produced artifacts and to enable us to use undiluted tissue fluid, since ME activity in the skeletal muscle of fish is very low. The ME assay, a measure of the rate of reduction of NADP by the malic enzyme in the presence of added malate, is based on the work of Ochoa *et al.* (1948) and has been described elsewhere (Gould, 1965).

Unit of activity. The ME index, unit of activity used here, is the change in absorbance (340 mμ) × 10^3/min/0.1 ml. CTF, under the conditions of assay. The reaction rate for each sample was corrected for the NADP-dependent activity with no added substrate.

Bacterial count. Standard agar plate counts were taken at 2 weeks for the 0.25-, 0.50-, and 1-Mrad samples and for the control. The nutrient formula used was developed by Nickerson *et al.* (1961) for determining total counts of psychrophilic organisms.

RESULTS

Samples irradiated up to 1 Mrad retained a fairly uniform level of ME activity during the 3-week storage period (Table 1), despite an erratic increase in CTF volume with increasing radiation dose and with time of refrigerated storage. Because there was no change in crude ME activity in these samples even after 3 weeks, we infer that no significant damage was done by the irradiation either to the ME itself or to any endogenous system upon which its stability may depend.

The unirradiated controls, however, showed not only an increase in ME activity but also considerable bacterial contamination (see plate counts at 12 days in Table 1). Assays on these controls were

Table 1. Malic enzyme activity in irradiated and unirradiated haddock flesh stored at +3°C.

Cobalt-60 dose (megarads)	ME index ($\Delta A \times 10^3$/min/0.1 ml CTF)[a] Days of storage 0	2	8	12	15	18	21
0.	39	53	89	101 (9,000,000)[b]			
0.25	33	33	39	43 (1,130)	35	32	34
0.50	30	41	38	38 (30)	34	33	36
0.75	32	36	37	42	34	34	32
1.00	25	40	33	38 (10)	32	34	30

[a] Arithmetic mean for 3 samples.
[b] Figures in parentheses represent the number of bacteria per gram of flesh after 12 days of storage.

not continued beyond 2 weeks, as spoilage had set in by that time. The irradiated samples, by contrast, had little bacterial contamination at 12 days and still had a moderately fresh odor after 3 weeks. Because the only variable in treatment between the controls and the irradiated samples was the pasteurizing irradiation itself, and because an increase in ME activity occurred only in the bacterially contaminated controls, it seems probable that the increased ME activity is due to the organisms themselves or to some fraction of their number.

With increasing radiation doses (Table 2), there was measurable loss in ME activity at 1.5 Mrads, and at 2 Mrads the activity dropped to one-third of the original. Assay control values rose with increasing levels of radiation, peaking at 1.5 Mrads and falling off thereafter. This activity may possibly be ascribed to (NADP-linked) alcohol dehydrogenase (Shaw, 1965); alcohols and ketones have been produced in protein solutions by ionizing radiation (U. S. Army Qm. Corps, 1957). The rise to peak values could be ascribed to an increase in available substrate, and the falling off, to the gradual inactivation of the enzymes involved.

Table 2. Lability of the malic enzyme *in situ* to gamma radiation.

Cobalt-60 dose (megarads)	Malic-enzyme index[a] (activity due to added malate)	Assay control[a] (NADP-dependent activity without added malate)
0	39	6
0.25	33	11
0.50	30	14
0.75	32	21
1.00	25	22
1.50	16	33
2.00	10	25
3.00	12	11
4.00	7	19

[a] Arithmetic mean for 3 samples.

CONCLUSIONS

Malic enzyme activity in haddock flesh during iced storage. The uniform concentration of ME activity in haddock flesh held aseptically at +3°C for 3 weeks is in striking contrast to the loss in activity of this enzyme at −7°C. Although the ME index may be used to indicate quality loss in frozen-stored fish, it cannot be used for a similar purpose in ice-stored fish.

A major consideration in comparing ME stabilities at the two temperatures is that the enzyme's activity in the fluid of frozen and thawed fish is about double that in unfrozen fish. This activity, solubilized by physical processes, may be a second molecular form of the enzyme, more labile than its soluble counterpart.

Although the ME index increases in nonpasteurized fish at +3°C, it would not be a reliable criterion of "freshness," for several reasons:

1) A fillet cut from a fish held iced several days in the round would have a comparatively small bacterial population and a correspondingly low ME index. Such a fillet, "fresh" by bacterial standards, would not be so "fresh" as a fillet cut from a freshly caught fish, in which glycogen and nucleotide breakdown would be less far advanced.

2) ME activity attributable to the presence of bacteria can only reflect a degree of bacterial growth and would be no more de-

pendable than a plate count. Moreover, it is unlikely that all bacteria commonly associated with fish elaborate ME.

3) In freezing weather, if fish were partially frozen on deck before stowage, ME values would be erratically and deceptively high because of the partial release of the "bound" ME fraction. In such a case, one cannot distinguish between "bacterial" ME and the freeze-thaw released ME.

Stability of the malic enzyme to gamma radiation. The malic enzyme is relatively stable to gamma radiation *in situ,* requiring more than 1 megarad to produce significant inactivation in iced tissue.

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Influence of Processing Procedures on the Post-Mortem Permeability of Chicken Muscle Sarcolemmas to Protein

SUMMARY

The permeability of intact chicken muscle sarcolemmas was studied in relation to: a) the pH of Ringer's solution surrounding the muscle; b) bathing the muscle in distilled water; c) rigor; d) freezing and thawing; and e) processing in a factory. Change in pH of Ringer's solution between 5.5 and 7.5 had no effect, but bathing in distilled water, freezing and thawing, muscle restraint during rigor, and normal processing in a factory all made the sarcolemma permeable to protein. When isolated muscles were immersed in distilled water for 24 hr, 1.7% of total muscle protein was lost. Isolated muscles which were not restrained during rigor were not permeable to protein when immersed in Ringer's solution, but muscles which had passed through rigor on the carcass were. Intracellular protein can therefore be lost from carcasses both as a result of processing and of freezing and thawing.

INTRODUCTION

During the processing of poultry, it is usual to immerse the eviscerated carcass in slush ice to remove body heat rapidly, a procedure which results in the uptake of varying amounts of water (Brant, 1963), some of which remains within the carcass after the free water has drained away. The absorbed moisture is retained by freezing, but is later released, during and after thawing, as a viscous red fluid containing appreciable amounts of solid materials including protein (Osner and Shrimpton, 1966a). When freezing and thawing are accomplished rapidly, losses of both fluid and solids are less than when these procedures are carried out slowly (van den Berg and Lentz, 1964). Furthermore, it has been suggested (Osner and Shrimpton, 1966a) that some of the solids lost in the fluid from slowly thawed carcasses may be of intracellular origin, although part undoubtedly comes from the skin.

A recent paper (Dawson, 1966) suggested that injury to the sarcolemma (muscle-fiber membrane) may result in a rapid diffusion of intracellular proteins from muscles. The present study was undertaken to determine whether intracellular protein can be lost from the skeletal muscles of chicken carcasses as a result of processing and thawing procedures. For this purpose the permeability of muscle membranes to proteins was investigated, using isolated but intact gastrocnemius muscles bathed in Ringer's solutions, the proteins diffusing into the solution being examined qualitatively by starch gel electrophoresis for the presence of an intracellular marker-protein, myoglobin.

EXPERIMENTAL

Choice of muscle and its preparation. Sixteen 'Cobb' broiler chickens aged 6–8 weeks were used in the five experiments reported below. Unless otherwise stated, birds were killed in the laboratory by injection with nembutal.

Gastrocnemius muscles were selected as most suitable since they can be dissected from the carcass without injury to the sarcolemma membranes. For the first two experiments, prerigor muscles were dissected from carcasses and used immediately, but in the final experiments postrigor muscles were used. A muscle weighing 8 g was placed in a small perspex container, 8 × 3 × 2 cm, and bathed in 20 ml Ringer (White, 1949). After 1 hr the muscle was removed and the solution dialyzed overnight at 0°C against a buffer containing 0.5*M* sucrose, 0.001*M* citric acid, and 0.01*M* tris (Scopes, 1964). A control was prepared for electrophoresis by homogenizing the muscle in 2.5 volumes of distilled water and centrifuging the homogenate at 18,000 × G for 30 min at 0°. The supernatant was then dialyzed at 0° against the same buffer as the Ringer.

Choice and preparation of marker proteins. If the membrane becomes permeable to soluble protein, the fluid bathing the muscle will contain both intracellular and extracellular proteins. Electrophoresis will separate these different proteins, but, to be able to determine whether the membrane has become permeable, at least one intracellular protein must be identified.

As an intracellular marker, a crude preparation

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of chicken myoglobin was prepared by the method of Penny (1965), which involves the precipitation of crude myoglobin in 65% acetone, followed by fractionation with $(NH_4)_2SO_4$ according to the method used by Akeson and Theorell (1960) for the isolation of horse myoglobin.

Chicken serum albumin, for use as an extracellular marker, was prepared by the method used by Cohn *et al.* (1950) for isolating serum albumin from human plasma.

Starch-gel electrophoresis. The apparatus used was of a vertical type (Smithies, 1959), employing a discontinuous buffer system generally similar to that described by Scopes (1964). The outer gel (pH 8.6 at 20°) was made up in 0.06*M* tris–0.1*M* boric acid. The inner gel was made up in 0.012*M* tris–0.003*M* phosphoric acid and 1*M* urea (pH 8.3 at 20°). This buffer system gave similar results to the buffer used by Scopes (1964), but the solution was more easily prepared. The lower electrode (anode) buffer contained 0.1*M* tris–0.025*M* sulfuric acid, and the upper electrode (cathode) buffer of 0.2*M* boric acid–0.05*M* NaOH–0.04*M* NaCl.

Electrophoresis was carried out at 400 v. 12–15 ma for 4½ hr in a cold room at 1°. Following Scopes (1964), the gels were sliced, stained for protein with a solution of 1% Naphthalene Black 10B plus 2% Nigrosine (water soluble) in methanol–acetic acid–water (5:1:4 by volume) and photographed for a permanent record. The complementary slice was sometimes stained in a mixture of dianisidine and hydrogen peroxide (Owen *et al.*, 1958) as a test for the catalase activity of myoglobin.

EXPERIMENTS WITH RINGER'S SOLUTION

In the following experiment, muscles were bathed in Ringer at pH 7.5 or 5.5 to simulate prerigor and postrigor conditions, respectively.

To obtain a pH of 7.5, White's Ringer was modified by omission of $NaHCO_3$. This was dissolved separately, and an appropriate amount was added to the Ringer until the required pH was reached. To prepare Ringer of pH 5.5, no $NaHCO_3$ was needed, and only 0.0145 g/L Na_2HPO_4, together with 0.0053 g/L NaH_2PO_4, was used, instead of 0.145 g/L Na_2HPO_4. For both modifications, extra NaCl (0.5 g/L) was added to keep the ionic strength of the solutions within the physiological range for chicken's extracellular fluid. Prerigor muscles from 2 carcasses were placed in muscle-baths at 20°C containing 15 ml of modified White's Ringer at the appropriate pH. Thirty, 60, and 120 min later, 2 ml of solution were removed from each bath and prepared for electrophoresis, the pH being noted prior to the removal of each sample. The whole experiment was then repeated, with sampling at 0, 1, 2, and 3 hr.

Effect of distilled water and permeability of intact muscles to lactic acid. One muscle from each of 2 birds was placed in a bath containing 20 ml sterilized White's Ringer (pH 7.5), and the other in a bath containing 20 ml distilled water. Five ml samples of fluid were then taken from each bath after 0, ½, 1, 2, 3, 4, 5, 6, and 24 hr, the volume removed being replaced by an equal volume of fresh medium. After the final sample, surplus moisture was removed from the muscle with filter paper and the muscle was reweighed. The total protein content of each 5 ml sample and the soluble sarcoplasmic protein from the muscle were both measured with Folin-phenol reagent (Lowry *et al.*, 1951), using crystallized bovine serum albumin (Armour Pharmaceutical Co.) as a standard. After dialysis, the proteins were examined qualitatively by starch-gel electrophoresis. The lactic acid contents of the samples taken from the muscle in Ringer's solution were determined by the colorimetric method of Barker and Summerson (1941). The experiment was repeated with the modification that samples were taken after 0, 1, and 24 hr only.

Experiments with muscles allowed to pass through rigor-mortis on the carcass. One muscle from each of 2 carcasses was placed in a polyethylene bag and the bag sealed with tape. After 20 hr, the other muscles were dissected and all 4 muscles (including the 2 that had been held in polyethylene bags) were bathed in 20 ml of Ringer's solution (pH 7.5) for 2 hr. The proteins in the Ringer's solutions were then examined qualitatively by starch-gel electrophoresis. In a second experiment, the same procedure was followed except that the birds were killed by dislocation of the neck.

Effect of quick freezing and thawing. In this experiment, an attempt was made to discover whether the commercial practice of freezing carcasses rapidly results in muscle membranes being permeable after thawing. Four muscles were removed from 2 carcasses and allowed to go through rigor in polyethylene bags. After 20 hr, the muscles from the left legs were frozen in an air-blast of 1000 ft/min at −30°C and thawed at +30°C, and, after removal from the polyethylene bag, all four muscles were bathed for 1 hr in 20 ml Ringer's solution, which was then examined by electrophoresis.

Effect of processing in a factory. Two fresh carcasses were selected at random after mechanical chilling. A muscle dissected from each was bathed for 1 hr in 20 ml Ringer's solution, which was then examined by electrophoresis.

RESULTS AND DISCUSSION

The position of the marker protein myoglobin is shown on an electrophoretogram in relation to some other protein bands including serum albumin (Fig. 1). If a muscle

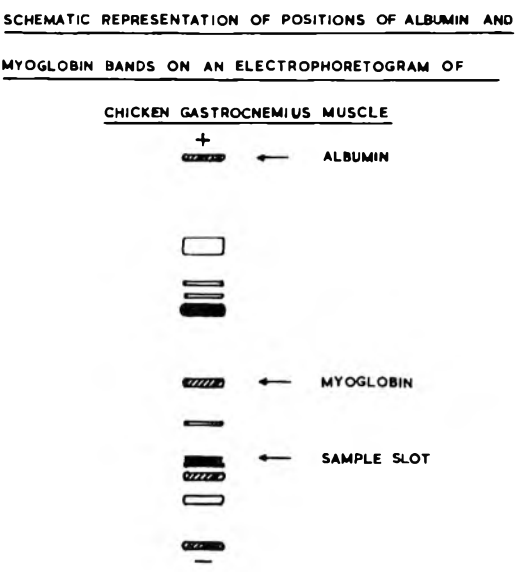

Fig. 1. Schematic representation of positions of albumin and myoglobin bands on an electrophoretogram of chicken muscle.

was cut before or during rigor, the damage was revealed by electrophoresis, the pattern of bands from Ringer's solutions used to bathe the muscles for 5 min being then similar to that of muscle extracts. Muscles on the carcass may be accidentally injured in this way during evisceration.

The pH of a rested muscle immediately post-mortem is normally within the range 7.2 to 7.4, but with birds this rapidly drops to an ultimate value of 5.7 to 6.0 within a few hours. Even after immersion of muscles in Ringer's solutions at pH 7.5 or 5.5 for 3 hr, there were no clearly defined bands of intracellular protein on an electrophoretogram although the albumin band was present. The pH of both Ringer's solutions changed during this period (Table 1). It was found that 3.4 mg lactic acid per g muscle diffused into the Ringer's solution during a 24-hr period, leaving 4.0 mg/g within the muscle, and this was probably almost entirely responsible for the lowering of the pH of the Ringer, originally at pH

Table 1. Change, with time, of the pH of Ringer's solution bathing a gastrocnemius muscle.

Time of muscle in solution (hr)	pH of solution (original pH 5.5)	pH of solution (original pH 7.5)
0[a]	6.1	7.4
½	6.3	7.3
1	6.4	7.2
2	6.4	6.7
3	6.4	6.7
Muscle homogenate[b]	6.5	6.5

[a] Value taken ½ min after immersion.
[b] Value taken 3 hr post-mortem.

7.5. The observed rise in pH of the Ringer, originally at 5.5 to 6.4, may be due to diffusion-out of the peptides carnosine and anserine, which together account for about 40% of the buffering power of muscle (Davey, 1960).

Muscles immersed in Ringer's solution for 24 hr increased their weight by 21%, compared with 36% for those immersed in distilled water for the same period. Although no *intracellular* protein could be detected in Ringer's solutions used for bathing muscles for 24 hr, a detectable amount had diffused into distilled water after ½ hr (Fig. 2). Furthermore, after 24 hr, over 5 times as much total protein had diffused from muscles immersed in distilled water as from muscles in Ringer's solution (Table 2). Some of this protein was extracellular albumin from capillaries within muscles. However, the increased amount of protein diffusing from the muscles immersed in distilled water must have been a result of permeability of muscle membranes to intracellular protein. The strain imposed by imbition of water may have caused the membranes to become permeable, and it is therefore possible for car-

Table 2. Diffusion of soluble proteins from gastrocnemius muscles into surrounding media.

Time of sampling (hr)	mg soluble protein per g muscle	
	Ringer's solution	Distilled water
0	0.038	0.057
1	0.441	0.237
24	0.635	3.33
Muscle[a]	26.7	28.5

[a] 24 hr post-mortem.

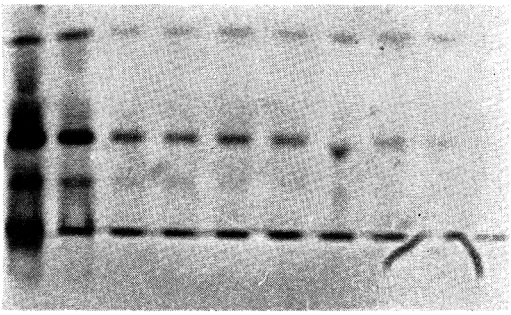

Fig. 2. Electrophoretogram of muscle and samples of distilled water used to bathe a muscle during a 24-hr period.

Order of solutions on electrophoretogram (from right to left):

1) Distilled water used to bathe muscle at 0 hr.
2) Distilled water used to bathe muscle after ½ hr.
3) Distilled water used to bathe muscle after 1 hr.
4) Distilled water used to bathe muscle after 2 hr.
5) Distilled water used to bathe muscle after 3 hr.
6) Distilled water used to bathe muscle after 4 hr.
7) Distilled water used to bathe muscle after 5 hr.
8) Distilled water used to bathe muscle after 6 hr.
9) Distilled water used to bathe muscle after 24 hr.
10) Muscle extract, 24 hr post-mortem.

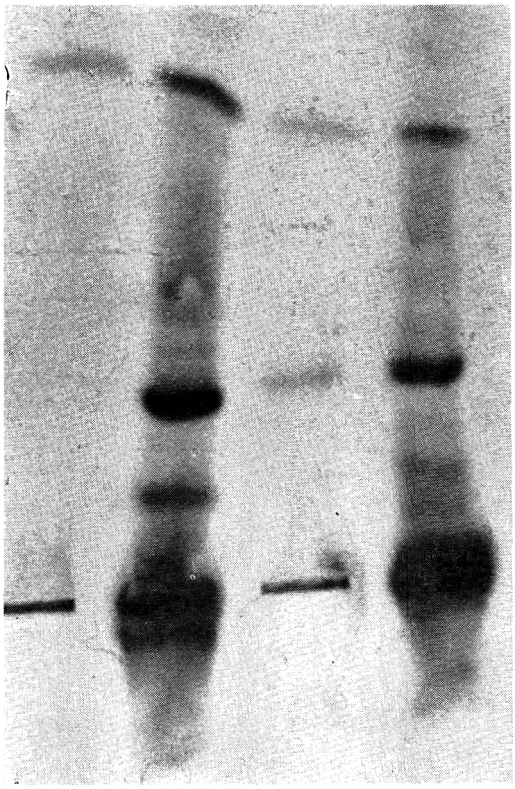

Fig. 3. Electrophoretogram of muscle extracts and Ringer's solutions used to bathe muscles dissected from a carcass either pre- or postrigor.

Order of solutions on electrophoretogram (from right to left):

1) Extract of muscle dissected postrigor.
2) Ringer's solution used to bathe above muscle.
3) Extract of muscle dissected prerigor.
4) Ringer's solution used to bathe above muscle.

casses to lose intracellular protein during wet chilling if conditions are such as to allow penetration of water as far as the skeletal muscle membranes. However, assuming that gastrocnemius muscles contain 0.2 g crude protein per g muscle on a wet-weight basis (Osner and Shrimpton, 1966b), even after 24 hr of immersion in distilled water only 1.7% of the total protein content was lost from the muscle.

After death by dislocation or by injection with nembutal, muscles left on the carcass for 20 hr were permeable to protein, as shown by a faint intracellular band on an electrophoretogram (Fig. 3). It is probable that muscles left on the carcass during rigor become permeable to protein as a result of physical injury to the membranes during the muscles' final contractions, since muscles that were dissected from carcasses immediately post-mortem and allowed to shorten freely during rigor in polyethylene bags for 20 hr were not permeable to protein. It has been shown both for poultry (Lowe, 1948) and beef (Locker, 1960) that muscles allowed to shorten freely during rigor are less tender than those which are restrained during this process. Two muscles, which were removed from a carcass immediately post-mortem and left during rigor in polyethylene bags for 20 hr, were permeable after quick freezing and thawing, as shown by the presence of myoglobin and other intracellular proteins in the Ringer's solutions used to bathe the muscles for 1 hr (Fig. 4). This confirms observations of Dawson (1966), who has shown that cytoplasmic enzymes can diffuse rapidly from chicken muscle after freezing and thawing.

Muscles from carcasses that had been processed in a factory, but not frozen, were permeable to myoglobin and other intracellu-

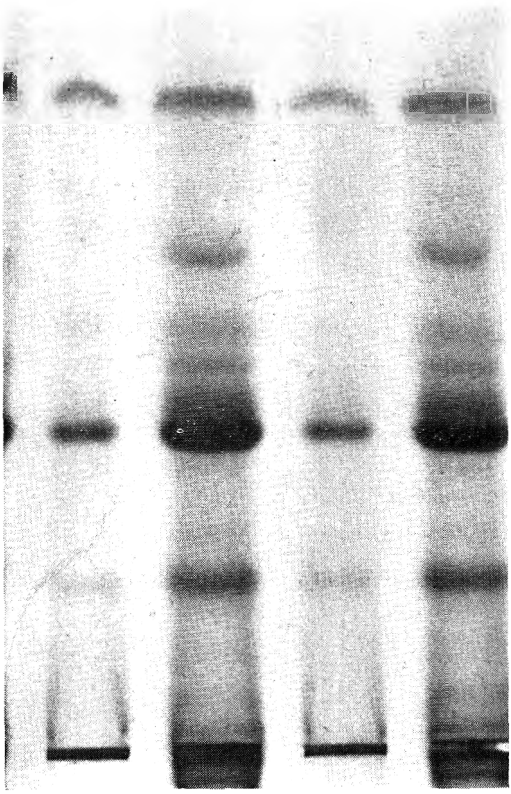

Fig. 4. Electrophoretogram of muscle extracts and Ringer's solutions used to bathe muscles after freezing and thawing.

Order of solutions on electrophoretogram (from right to left):

1) Extract of muscle that had been frozen and thawed.
2) Ringer's solution used to bathe above muscle.
3) Extract of muscle that had been frozen and thawed.
4) Ringer's solution used to bathe above muscle.

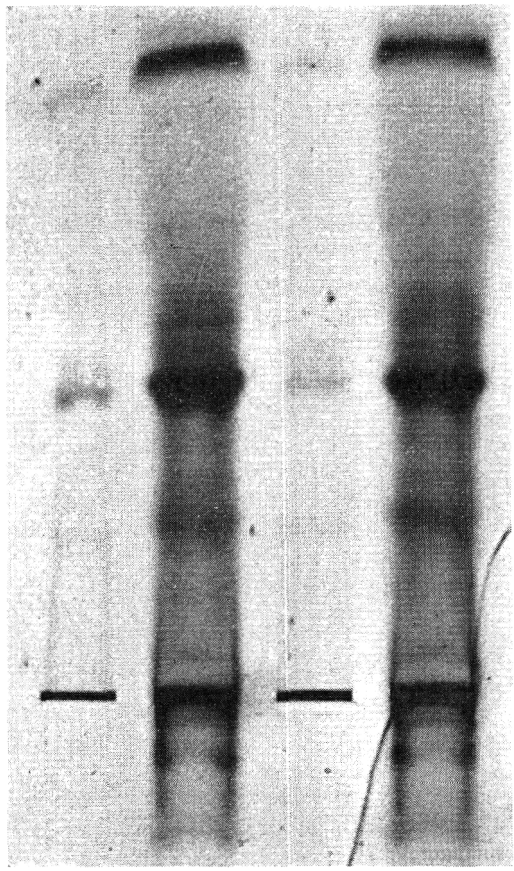

Fig. 5. Electrophoretogram of muscle extracts and Ringer's solutions used to bathe muscles dissected from processed carcasses (unfrozen).

Order of solutions on electrophoretogram (from right to left):

1) Muscle extract from processed carcass.
2) Ringer's solution used to bathe above muscle.
3) Muscle extract from processed carcass.
4) Ringer's solution used to bathe above muscle.

lar proteins (Fig. 5). It is possible that mechanical plucking and passage through rigor under tension on the carcass contributed to this effect.

To summarize, muscles from chicken carcasses are not rendered permeable to protein by passing through rigor unless under tension on the carcass. Physical injury during processing, immersion in distilled water, and freezing and thawing, all render the muscle membranes permeable to protein. However, when an isolated muscle was immersed for 24 hr in distilled water, 98% of the total protein content was retained. Thus, intracellular protein can be lost from carcasses both as a result of processing and during thawing, although the amounts involved are not large.

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A New Pectic Acid Transeliminase Produced Exocellularly by a Bacillus

SUMMARY

A new type of endo-pectic acid transeliminase was isolated from the culture medium of a strain of *Bacillus*. The enzyme attacked pectic substances randomly and produced unsaturated trigalacturonic acid as the major end product. The optimum pH on either acid-soluble pectic acid or tetragalacturonic acid was 9.3–9.7, and the enzyme required calcium ions for maximum activity. Strontium was the only other divalent cation which stimulated activity. Trigalacturonic acid was attacked very slowly. The major site of attack of tetragalacturonic acid was the glycosidic bond on the nonreducing end. Unsaturated tetragalacturonic acid was also attacked at the central bond. The cleavage of pentagalacturonic acid occurred preferentially at bond 3, followed by 4 and 2, in order. The glycosidic bond on the reducing end is called bond 1. With unsaturated pentagalacturonic acid, it was concluded that the major site of cleavage was at bond 3, with a much slower rate at bond 2.

INTRODUCTION

It is now apparent that the major mechanism involved in breakdown of pectic substances by enzymes of bacterial origin is by an elimination reaction which results in the formation of unsaturated products. On the basis of substrate specificity, these enzymes are classified into two groups, pectic acid transeliminases (PATE) and pectin transeliminases. The latter group, which attacks pectin but is inactive toward pectic acid, has been demonstrated in fungi (Albersheim *et al.*, 1960; Edstrom and Phaff, 1964a,b). Pectic acid transeliminase, which attacks pectic acid more rapidly than pectin, appears to be widely distributed in bacteria. It has been found in members of the genera *Bacillus* (Starr and Moran, 1962; Nagel and Anderson, 1965), *Pseudomonas* (Preiss and Ashwell, 1963a,b; Fuchs, 1965), *Erwinia* (Okamoto *et al.*, 1964), *Clostridium* (MacMillan and Vaughn, 1964; MacMillan *et al.*, 1964), and *Xanthomonas* (Starr and Nasuno, 1963). There are at least two different types of PATE: one attacks pectic acid randomly (Nagel and Anderson, 1965), and the other degrades pectic acid terminally (MacMillan *et al.*, 1964; Okamoto *et al.*, 1964). In all cases the enzymes have a high optimum pH and require calcium ions for maximum activity.

During a survey for production of a bacterial polygalacturonase by bacteria, we isolated a new type of PATE from an aerobic *Bacillus* isolated from soil. This enzyme produces unsaturated trigalacturonic acid from pectic acid as the major end product, while all the PATE's of other bacteria reported previously produce unsaturated digalacturonic acid. It is interesting that cell extracts of this bacterium, in addition to the transeliminase, contain a hydrolase which catalyzes the hydrolysis of only low-molecular-weight uronides (Hasegawa and Nagel, 1966).

The present publication deals with the production, isolation, and characterization of the PATE of the *Bacillus* isolate. We have shown that the enzyme degrades pectic acid and certain oligogalacturonides in a random manner, and thus is classified as an endo-pectic acid transeliminase (endo-PATE).

MATERIALS AND METHODS

Substrates. Polygalacturonic acid (No. 3491) and pectin N.F. (No. 3442) were purchased from Sunkist Growers, Corona, California, and used for most experiments without any treatment. Sodium polypectate (No. 6024), obtained from the same company, was used as the carbon and energy source in the growth medium. Acid-soluble pectic acid (ASPA) and the unsaturated oligogalacturonides were prepared by procedures reported previously (Nagel and Anderson, 1965).

Oligogalacturonides were isolated by Dowex 1-X8 ion-exchange column chromatographic separation of the products produced from pectic acid by yeast endo-polygalacturonase (endo-YPG). Pectic acid was partially hydrolyzed by the crude preparation of endo-YPG prepared by the method of Luh and Phaff (1954). The reaction was carried out in 2 L of a mixture containing 3.0% pectic acid, 0.1*M* acetate buffer, pH 6.0, and 10% (v/v) of the enzyme preparation. After 16 hr of incubation at

30°C, the reaction was halted by heating at 80°C for 10 min. The resulting mixture was filtered, and the supernatant liquid was stored in a refrigerator with a few drops of toluene as a preservative until used.

A 400-ml sample of the supernatant fluid was applied to the top of a Dowex 1-X8 column in the acetate form (450 ml of wet resin in a 3.4-cm-ID glass column). The column was first washed with distilled water and then eluted, stepwise, with increasing concentrations of sodium acetate solution, pH 6.0, by the following schedule: at first, 1200 ml of 0.20*M* followed by 1200 ml of 0.30*M*, 600 ml of 0.40*M*, 600 ml of 0.50*M*, 900 ml of 0.55*M*, 900 ml of 0.60*M*, and finally 900 ml of 0.70*M*. Fractions of 9.1 ml were collected with a flow rate of approximately 90 ml per hour. Various peaks were determined by measuring the uronic acid concentrations of every fourth fraction with the carbazole assay method (McComb and McCready, 1952) and were identified by paper chromatographic examination with authentic compounds as the standards. The results are shown in Fig. 1. The strontium salt of each compound was precipitated from pooled fractions with the addition of ethanol to a final concentration of 60–80%. The free acid was recovered from its salts by passing the aqueous solution through a Dowex 50 (H^+ form) column, concentrating in a vacuum desiccator, precipitating by the addition of an ethanol-acetone mixture, filtering, and washing with acetone. To ensure purity, the isolated substrates were refractionated a second time over Dowex 1-X8.

Characterization of substrates. All the substrates used were shown to be chromatographically homogeneous. Molecular weights of the compounds were determined by the hypoiodite method for reducing groups and also by the carbazole method. For the carbazole assay, galacturonic acid was used as the standard and was purified from a commercial product (No. 3493, Sunkist Growers, Ontario, California) by Dowex 1-X8 column chromatography and reprecipitated from water with acetone. Ratios of carboxyl to aldehyde groups were also determined, by the usual manner.

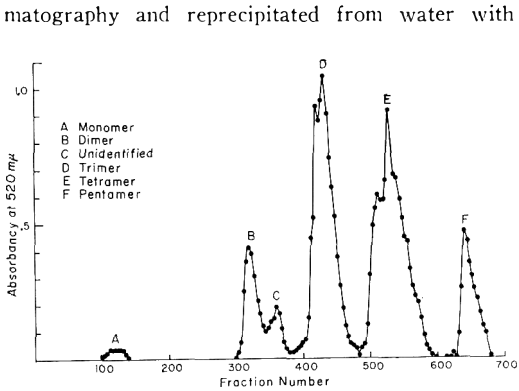

Fig. 1. Separation of oligogalacturonic acids by Dowex 1-X8 column chromatography.

Assay methods. Protein concentration was determined by the procedure of Lowry *et al.* (1951). Uronic acids were measured by the carbazole method described by McComb and McCready (1952). The reducing value of uronic acids was determined by the hypoiodite method (Patel and Phaff, 1959). Paper chromatographic analysis of uronic acids was carried out by the method described previously (Nagel and Anderson, 1965).

Unless otherwise stated, PATE activity was assayed on the basis of the enzymatic production of unsaturated products which have an absorption maximum at 232 mμ. Routinely, PATE activity was determined in 3 ml of a reaction mixture consisting of 0.1*M* glycine buffer, pH 9.5, 0.067% ASPA, 0.001*M* $CaCl_2$, and 0.5 ml of proper dilutions of enzyme solution. Absorbancy changes at 232 mμ were measured in a Beckman DU spectrophotometer equipped with thermospacers and adapted to measure absorbance changes with time at constant wave length by a Gilford automatic recording apparatus. The reaction was carried out in 1-cm cuvettes. The reaction temperature was 25°C. The absorbancy readings at 232 mμ were converted into molar concentrations using a molar extinction coefficient of 4,600 (Nagel and Anderson, 1965).

One unit of PATE activity is the amount of enzyme which will produce 0.1 millimole of unsaturated uronides per minute under the above conditions.

Enzyme production. The strain of aerobic *Bacillus* was isolated from soil on the same pectic acid medium described by Preiss and Ashwell (1963a). A crude enzyme solution was prepared by inoculating 1.0% (v/v) of a 48-hr culture into a 4-L Erlenmeyer flask containing 500 ml of a 2.0% sodium polypectate medium with the same mineral salts used for the growth of *Bacillus polymyxa* (Nagel and Vaughn, 1961a). The culture was agitated by a reciprocal shaker (76 strokes per minute, amplitude 4 cm) at room temperature. Cell production (Nagel and Vaughn, 1961a), PATE activity, and protein concentration were monitored in order to obtain maximum yield of exocellular enzyme without excessive contamination from cell protein. After 40 hr of incubation, the cells were removed by centrifugation. The supernatant solution, after dialysis against tap water for 2 hr, was used as the crude enzyme preparation.

Purification of PATE. The enzyme was partially purified by ammonium sulfate precipitation followed by diethylaminoethyl cellulose (DEAE cellulose) column chromatography. The dialyzed preparation was adjusted to 90% saturation with solid ammonium sulfate at 0°C. The precipitate

was collected by centrifugation, dissolved in a small quantity of distilled water, and dialyzed for 2 hr with running tap water followed by 2 hr more with distilled water at 2°C. Although the specific activity was not changed, preliminary experiments showed that it was necessary to concentrate the samples extensively before loading the DEAE-cellulose column in order to obtain good resolution. The concentrated enzyme preparation was carefully introduced at the top of the DEAE cellulose (20 ml of wet resin in a 1.5-cm-ID glass column) which had been equilibrated with 0.005*M* tris buffer, pH 9.0. The column was eluted, stepwise, with increasing concentrations of tris buffer, pH 7.6, and NaCl as follows: 25 ml of 0.01*M* followed successively by 25 ml of 0.04*M*, 25 ml of 0.07*M*, 50 ml of 0.10*M* *tris*, 50 ml of 0.10*M* tris + 0.1*M* NaCl, 50 ml of 0.10*M* tris + 0.30*M* NaCl, and 50 ml of 0.10*M* tris + 1.0*M* NaCl. The effluent, whose rate of flow was approximately 35 ml per hour, was collected in 2.7-ml fractions. The pectic acid cup-plate method was employed to locate the enzyme (Nagel and Vaughn 1961a), and quantitative determinations of the enzyme activity in the fractions were carried out by the standard procedure. The protein concentration was estimated by measuring absorption at 280 mμ.

Effect of pH on PATE activity. The effect of pH on PATE activity was examined with ASPA and tetragalacturonic acid as substrates. The activity was determined by measuring the initial rate under standard conditions. Reaction mixtures consisted of 0.067% ASPA or $5 \times 10^{-3}M$ tetragalacturonic acid, 0.001*M* $CaCl_2$, 0.1*M* glycine-tris buffer at various pH's, and 0.048 unit of the enzyme for the ASPA and 0.076 unit for the tetragalacturonic acid assays.

Determination of mechanism of attack. The random-splitting mechanism of PATE was demonstrated by comparing the decrease in viscosity and the percent degradation of sodium polygalacturonate as a function of time. Reaction mixtures consisted of 0.6% sodium polygalacturonate, 0.1*M* glycine buffer, pH 9.5, 0.001*M* $CaCl_2$, and 5.5 units of the enzyme in a total volume of 10 ml. Immediately after addition of the enzyme, 4 ml of the reaction mixture was placed in a capillary viscosimeter, and flow times were determined periodically. The rate of reaction was determined by removing 0.5 ml of the sample at various time intervals from the remaining 6.0 ml, treating with Dowex 50 (H^+) to inactivate the enzyme, filtering, washing, adjusting the sample to a final volume of 50 ml, and measuring absorbancy at 232 mμ. The reaction temperature was 30°C.

Effect of divalent cations on PATE activity. To determine the effect of calcium ions on PATE activity, the reaction was carried out under standard conditions in the presence or absence of 0.002*M* sodium ethylenediaminetetraacetate (EDTA). The PATE concentration used was 0.061 unit. To determine whether other divalent cations could replace calcium, the chloride salts of 6 different cations were tested. The concentration of cations was 0.001*M*, and 0.094 unit of PATE was used. The control contained no added divalent cation.

Paper chromatographic analysis of reaction products. The reaction products from saturated and unsaturated oligogalacturonides were analyzed by paper chromatography. The reaction mixtures consisted of $5 \times 10^{-3}M$ substrate or 0.067% ASPA, 0.001*M* $CaCl_2$, 0.1*M* glycine buffer, pH 9.5, and 0.221 unit of the PATE in 3 ml. After 30 hr of incubation at 30°C, the reaction mixture was treated with Dowex 50 (H^+ form), and the filtrate was evaporated to dryness and dissolved in 0.5 ml water. Twenty μl of the sample was spotted on Whatman No. 4 paper and the paper was developed by the method described previously (Nagel and Anderson, 1965). The spots were developed by the periodate-benzidine method.

Designation of bonds. In all cases the glycosidic linkage next to the terminal reducing group will be called bond 1; the next bond is 2; etc.

RESULTS

The results of purification of the enzyme are shown in Table 1. This purification produced a 3-fold over-all increase in specific activity. The degree of purification is markedly dependent upon the time of harvest of cells. Longer incubation times resulted in marked reduction of the specific activity of the crude enzyme preparation. The results from fractionation of PATE by DEAE-cellulose column chromatography are presented in Fig. 2. Two peaks of PATE activity were observed. Fifty-seven percent of the activity was recovered in fractions 60–80, 8% was found in fractions 29–33, and approximately 35% was lost. The ratio between the two peaks was approximately 1:7. This ratio, however, was not constant from column to column. In some instances, almost all the activity was collected in one peak in the vicinity of fractions 60–80. An attempt was made to determine whether the PATE consisted of a single enzyme. Paper chromatographic analysis of the products of degradation of normal and unsaturated oligogalacturonides produced by the enzymes from the two peaks showed that both fractions attacked the substrates and yielded identical end products. In addition, the reaction rates of the two peaks on ASPA and tetragalacturonic acid were compared. The reaction was carried out under standard conditions with 0.067% ASPA or $6 \times 10^{-3}M$ tetragalacturonic acid. The smaller peak, fractions 29–33, attacked ASPA 3.5 times as

Table 1. Purification of PATE.

Purification step	Total volume (ml)	PATE Units/ml	PATE Total	Protein (mg/ml)	Specific activity (units/mg protein)	Purification	Yield (%)
Dialyzed culture fluid	77	4.44	342	0.525	8.43	1	100
$(NH_4)_2SO_4$ precipitate after dialysis	10	21.1	211	2.42	8.72	1	62
DEAE cellulose fractions (62–68)	20	4.42	88	0.176	25.1	3	26

fast as tetragalacturonic acid, while the ratio of activities with the major peak was 3.4. From the above results, it was presumed that PATE consisted of only a single enzyme. Three individual fractions in the major peak were also subjected to the same method of analysis in order to determine the presence of two or more enzymes. All three fractions degraded ASPA 3.5 times as fast as tetragalacturonic acid.

The molecular weights of the substrates determined by the reducing group assay method and the carbazole method are shown in Table 2. In the case of the unsaturated compounds, the molecular weights were determined only by the reducing group assay method since preliminary experiments indicated that the presence of double bonds interfered with color formation in the carbazole reaction.

The effect of pH on PATE activity was determined at various pH values with ASPA and tetragalacturonic acid as substrate. The results (Fig. 3) show that the pH range of maximum activity was 9.3–9.7. This relatively high value is in good agreement with the pH optima of the PATE of other bacteria reported previously. The pH optimum curve on ASPA is nearly identical to the one found by Nagel and Anderson (1965) for the PATE from *Bacillus polymyxa*. It has been reported that chain length of substrate has a marked effect on pH optimum (Demain and Phaff, 1954; Nagel and Anderson, 1965). Yeast endo-PG has its optimum activity at pH 4.4 on pectic acid, but the optimum pH is shifted to pH 3.4 on tetragalacturonic acid (Demain and Phaff, 1954). Similar results have been reported on the PATE of *B. polymyxa*, where the optimum activity is at pH 9.3–9.5 on ASPA, but at pH 8.4–8.6 on tetragalacturonic acid (Nagel and Anderson, 1965). In contrast to these results, it was found that the shift of optimum toward a lower pH did not occur with our enzyme when tetragalacturonic acid was used as the substrate (Fig. 3).

The mode of attack, terminal or random, can be determined by measuring viscosity changes and percent cleavage of the substrate simultaneously. The endo-PATE of *Bacillus polymyxa* caused 50% reduction in relative viscosity of pectic acid when only about 2.0% of the bonds were broken (Nagel and Vaughn, 1961a,b), whereas the exo-PATE from *Clostridium multifermentans* had degraded 22.5% of the bonds of pectic acid when 50% reduction in viscosity had occurred (MacMillan *et al.*, 1964). As shown in Fig. 4, the PATE of this *Bacillus* caused 50% reduction in relative viscosity when only 1.6% of the bonds were degraded. These results strongly suggested that the PATE of this *Bacillus* species degraded pectic acid randomly rather than terminally.

The activity of the PATE of the bacterium, which is shown by absorbancy changes at 232 mμ in Fig. 5, was stimulated markedly by calcium ions. The addition of 0.001*M* $CaCl_2$ to the reaction mixture increased activity markedly, whereas the addition of 0.002*M* EDTA inhibited activity completely. To determine whether other divalent cations could

Table 2. Characteristics of substrates.

Substrates	Molecular weights Theoretical[a]	Carbazole assay	Reducing-group assay	COOH/CHO
Trigalacturonic acid	546	558	568	2.92
Tetragalacturonic acid	722	746	723	3.83
Pentagalacturonic acid	898	935	1012	5.02
Unsaturated trigalacturonic acid	528		618	2.99
Unsaturated tetragalacturonic acid	704		752	3.84

[a] As anhydrates.

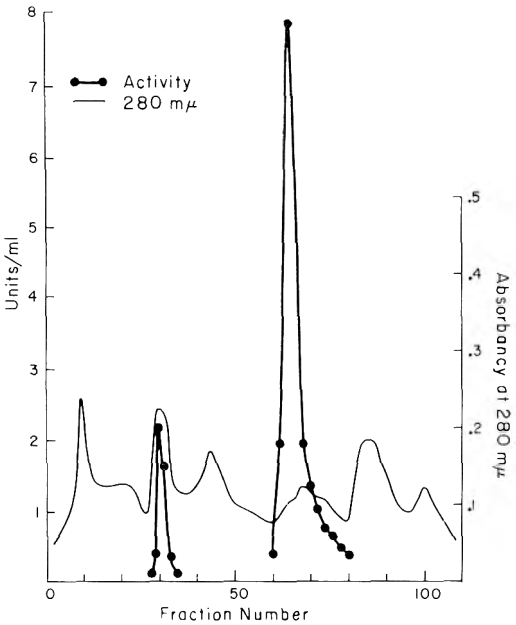

Fig. 2. Separation of PATE on a DEAE cellulose column. The assay conditions are described in the text.

replace calcium, the chloride salts of 6 different divalent cations were tested with ASPA as substrate. It was found that strontium was the only cation which had a marked stimulating effect on enzyme activity (Table 3). Strontium ions also stimulated the activity of the PATE of *Bacillus polymyxa* (unpublished data). These results appear to be in disagreement with those of MacMillan and Vaughn (1964) for the exo-PATE from *Clostridium multifermentans* and of Starr and Nasuno (1963) for the endo-PATE of *Xanthomonas campestris*. The former investigators showed that all of the divalent cations tested except zinc exhibited some degree of stimulation. Calcium showed the highest stimulation, followed in order by strontium, manganese, magnesium, and barium. In contrast, Starr and Nasuno (1963) reported that none of the ions other than calcium, including sodium, potassium, magnesium, manganese, zinc, and strontium, promoted enzyme action.

Table 3. Effects of divalent cations on PATE activity.

Cation	Reaction rate (Δ OD at 232 mμ/min $\times 10^{-3}$)
$BaCl_2$	4.3
$CaCl_2$	43.0
$CdCl_2$	4.0
$CoCl_2$	3.7
$MgCl_2$	4.5
$SrCl_2$	25.3
$MnCl_2$	4.0
Control	4.2

Reaction mixtures consisted of 0.067% ASPA, 0.1*M* glycine buffer, pH 9.5, and 0.094 unit of PATE per 3 ml. The cation concentration was 0.001*M*.

The products obtained from oligogalacturonides by PATE degradation were separated and analyzed by paper chromatography. The results are presented in Table 4. It appeared that trigalacturonic acid was not attacked. This observation was further investigated by measuring absorbancy changes of the reaction mixture at 232 mμ. With the aid of this sensitive procedure, it was found that trigalacturonic acid was attacked slowly. It was estimated that the initial rate of reaction on tetragalacturonic acid was 75 times as fast as on trigalacturonic acid. This finding differs significantly from those with PATE's of other bacteria. The latter appear to attack trigalacturonic acid at a much faster relative rate. In the case of the PATE of *Bacillus polymyxa*, the ratio of the reaction rates, trimer to tetramer, is 1 to 3.6–3.8 (Nagel and Anderson, 1965).

The major products obtained from tetragalacturonic acid were galacturonic acid and unsaturated trigalacturonic acid (Table 4). The occurrence of these two compounds indicated that the primary

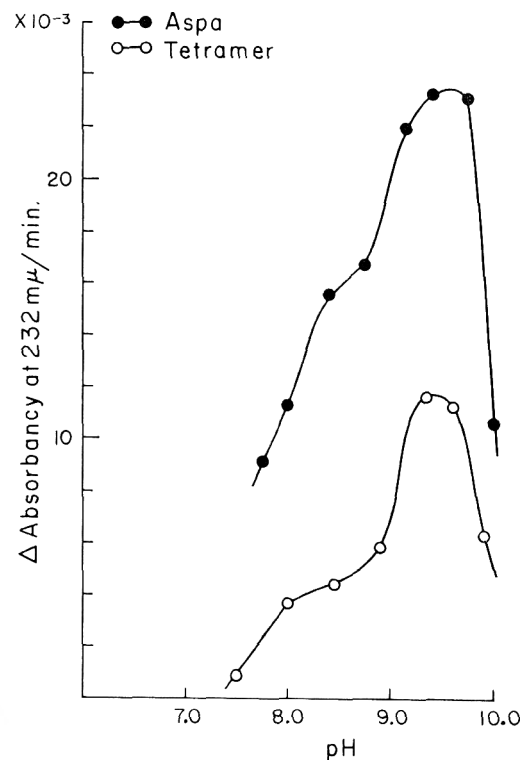

Fig. 3. Effect of pH on activity of PATE on ASPA and tetragalacturonic acid.

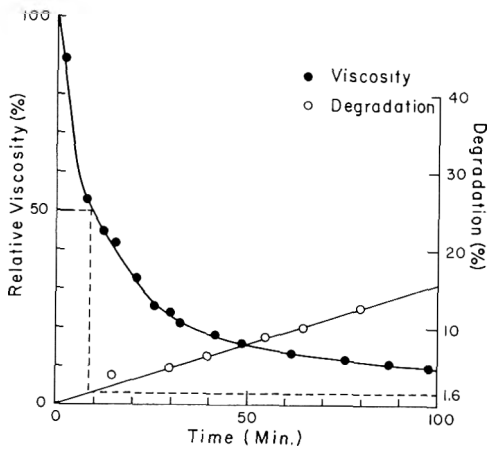

Fig. 4. Relationship of viscosity reduction to degradation of sodium polypectate by PATE.

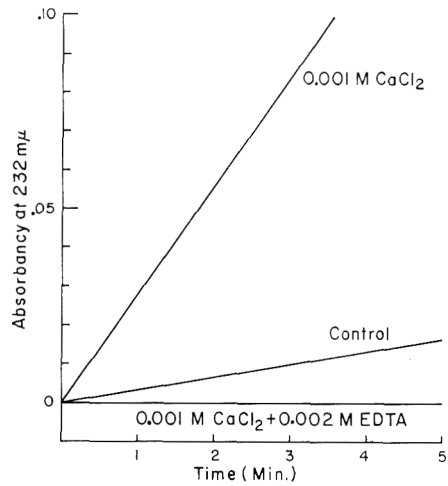

Fig. 5. Effect of calcium on activity of PATE.

attack on tetragalacturonic acid occurred at the nonreducing end of the molecule (bond 3). Although the major cleavage occurred at bond 3, bond 2 appeared to be degraded to some extent.

The fact that only a small amount of the unsaturated tetramer had been attacked although tetragalacturonic acid had virtually disappeared under the same conditions, would clearly indicate that the unsaturated bond inhibits attack of the adjacent glycosidic bond by PATE. It is apparent that the inhibition was not complete, since some unsaturated trigalacturonic acid was produced. The production of unsaturated digalacturonic acid indicates that bond 2 was also attacked.

A mixture of 6 compounds, mono-, di-, and trigalacturonic acids, and unsaturated di-, tri-, and tetragalacturonic acids were produced from pentagalacturonic acid. The cleavage of bonds 4, 3, and 2 should produce mono-, di-, and trigalacturonic acids, respectively. Thus, the quantitative estimation of these three compounds should give the relative importance of the three types of cleavage. The results indicated that the enzyme attacked at bond 3 preferentially, followed by 4 and 2, in order.

The primary products from unsaturated pentagalacturonic acid were unsautrated di- and trigalacturonic acids. Thus, the attack of unsaturated pentagalacturonic acid can occur at either one of two sites, bond 2 or bond 3. However, the major site appears to be bond 3, because the cleavage of the glycosidic bonds of tetragalacturonic acid occurs almost exclusively at bond 3 rather than bond 2.

With ASPA as substrate, six compounds were isolated. The majority of the products consisted of unsaturated di- and trigalacturonic acids.

DISCUSSION

The results show that the PATE of a strain of aerobic *Bacillus* isolated from soil attacks pectic acid in a random manner. It is therefore classified as an endo-polygalacturonic acid transeliminase (endo-PATE), in contrast to the terminal splitting PATE (exo-PATE), which cleaves unsaturated

Table 4. Paper chromatographic analysis of the reaction products from oligogalacturonides.

Substrates	Products					
	Unsat. tetramer	Unsat. trimer	Unsat. dimer	Trimer	Dimer	Monomer
Trimer	.....	.....	.....	++++	.....	.....
Unsat. trimer	.....	++++	.....	.....	.....	.....
Tetramer	.....	+++	(+)	.....	(+)	+++
Unsat. tetramer	+++	(+)	+	.....	.....	.....
Pentamer	++	+++	(+)	(+)	+++	++
Unsat. pentamer	.....	+++	+++	.....	.....	.....
ASPA	(+)	++++	++	(+)	+	+

(+), very weak spots; +, weak spots; ++++, very intense spots.
0.221 unit of PATE per 3 ml of reaction mixture containing $5 \times 10^{-3}M$ substrates or 0.067% ASPA, $0.001M$ $CaCl_2$, and $0.1M$ glycine buffer, pH 9.5.

digalacturonic acid units from the reducing end of the substrate (MacMillan and Vaughn, 1964; MacMillan *et al.*, 1964; Okamoto *et al.*, 1964).

The presence of a polygalacturonase in the crude extract is ruled out because, with unsaturated tetra- and pentagalacturonic acids as substrates, no saturated compounds were detected in the reaction mixtures. Also, the presence of an exo-PATE is unlikely, because the ratio of the reaction rates on ASPA and tetragalacturonic acid of different fractions obtained from DEAE cellulose chromatography was always constant. The enzyme of our bacterium has characteristics similar to PATE's of other bacteria in the respect that the enzme has a high pH optimum and requires calcium ions for maximum activity.

The most significant observation is that tetragalacturonic acid is the smallest oligogalacturonide which can be attacked by the PATE at a significant rate, in contrast to other PATE's which attack trigalacturonic acid. Thus, it is understandable why unsaturated trigalacturonic acid accumulates as one of the major products from pectic acid, whereas other PATE's produce unsaturated digalacturonic acid as the primary product.

It is apparent that both the reducing end group and the unsaturated moiety of the substrate hinder PATE activity. The glycosidic bond adjacent to the reducing end (bond 1) is not attacked at all, but the glycosidic bond adjacent to the unsaturated galacturonic acid group can be cleaved at a reduced rate. It is of interest to note also that the nonreducing end of the substrate interferes with PATE activity. This conclusion is based on the fact that cleavage of bond 2 occurred faster with saturated and unsaturated tetragalacturonic acids than with trigalacturonic acid. In addition, with pentagalacturonic acid as the substrate, the enzyme preferentially attacks bond 3 rather than bond 4. This is not the case for the PATE of *Bacillus polymyxa*, where the enzyme splits bond 3 of tetragalacturonic acid faster than bond 2 (Nagel and Anderson, 1965). From the above evidence, it is reasonable to assume that unsaturated trigalacturonic acid is not attacked at all.

Unsaturated trigalacturonic acid, and other low-molecular-weight oligogalacturonides are presumably metabolized by the growing organism, since they do not accumulate in the culture medium. The organism appears to preferentially utilize unsaturated rather than saturated uronides, as indicated by the presence of saturated uronides in the culture medium during the early stages of growth. The hydrolytic conversion of both unsaturated and saturated oligogalacturonides to galacturonic acid and unsaturated galacturonides most likely takes place in the organism. The enzyme responsible for this hydrolysis has been isolated from the cell extracts of our strain of *Bacillus* (Hasegawa and Nagel, 1966). This enzyme specifically catalyzes the hydrolysis of uronic acids of low molecular weight, and thus it has been called on oligogalacturonic acid hydrolase. The presence of this type of enzyme in the cells of *Bacillus polymyxa* has been reported by Nagel and Vaughn (1961b) and as an intracellular enzyme from a pseudomonad by Preiss and Ashwell (1963a).

Results of further studies on the mode of attack, and Km and Vm measurements on different oligogalacturonides of the PATE will be reported in a separate publication.

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Post-mortem Changes in Glycogen, Nucleotides, Sugar Phosphates, and Sugars in Fish Muscles—A Review

Though fish flesh is consumed largely because it supplies high-quality protein and, to a lesser extent, lipids in human diets, the compounds that occur in comparatively small amounts are almost certainly responsible for the desirable or undesirable flavors that are so important in determining consumer preferences. It is only during the past decade that research on these substances has received increased emphasis, so that quite rapid progress is now being made in separating and isolating these compounds and determining their importance. The present communication is restricted to carbohydrates and related compounds, and to nucleic acids, nucleotides, and their derivatives.

The occurrence of glycogen in fish muscles was investigated first by Dill (1921), and subsequently in more detail by MacLeod and Simpson (1927). Since those early studies, there have been further investigations concerning glycogen in fish muscles and its post-mortem degradation, and these have been reviewed by Tomlinson and Geiger (1962) and by Partmann (1965).

There has been a tendency to assume that glycogen content is considerably lower in fish muscles than in mammalian muscles. However, as Tomlinson and Geiger (1962) have pointed out, many species of fish have a muscle glycogen content which compares favorably with that of warm-blooded mammals. Since the procedures used to capture fish almost invariably involve excessive struggling, the glycogen content of the flesh of marketed fish is usually very low. However, the products of post-mortem degradation of glycogen are present and undoubtedly contribute to both the flavor and texture of fish. Glycogen may be broken down post-mortem by two routes, discussed below.

It has long been known that lactic acid accumulates in muscle of living fish as a result of exercise or struggling and may also increase after death. Several investigators have observed that there is not always a parallel between glycogen disappearance and lactic acid formation. However, there is now abundant evidence that lactic acid is formed in fish muscles by the same sequence of enzymes that is operative in mammalian muscles—the Embden-Meyerhof (glycolytic) pathway. On the other hand, glycogen is also broken down by an amylolytic route (Fig. 1).

MacLeod *et al.* (1963) identified many of the enzymes of glycolysis, using tissue homogenates of rainbow trout (*Salmo gairdnerii*) or soluble extracts of these homoge-

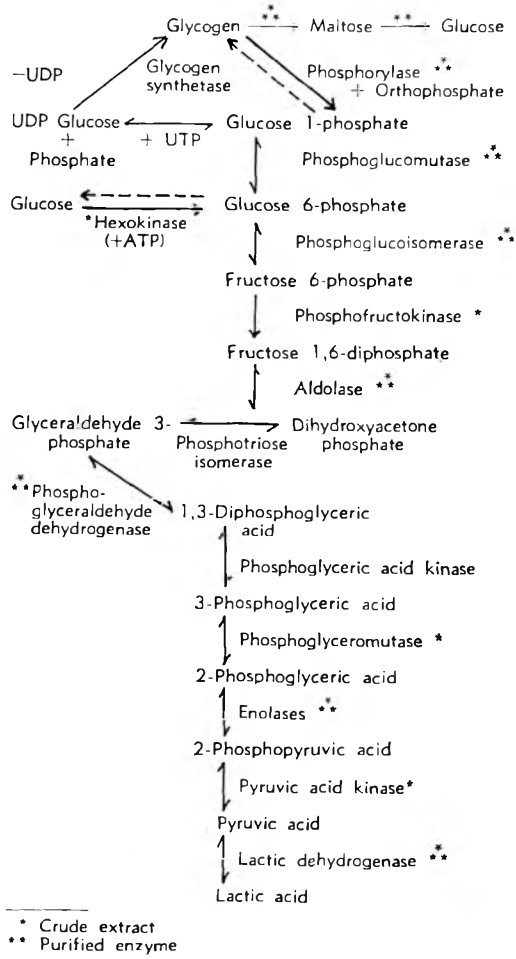

Fig. 1. Occurrence of enzymes of the Embden-Meyerhof and amylolytic pathways in fish tissues.

nates. Their findings (cited by Black *et al.*, 1961) were published in 1963. They showed that the following soluble enzymes were present: phosphoglucoisomerase, aldolase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, enolase, phosphoglyceromutase, lactic dehydrogenase, and pyruvic kinase. Several investigators have isolated either crude or partially purified glycolytic enzymes from fish. Phosphorylase was demonstrated in muscle extracts of different fish by Cordier and Cordier (1957) and by Ono *et al.* (1957), and was slightly purified by Nagayama (1961a). Martin and Tarr (1961) carried out partial purification of phosphoglucomutase and phosphoglucoisomerase from lingcod muscle employing several steps including chromatography on DEAE cellulose. Nagayama also studied phosphoglucomutase (1961b) and phosphoglucoisomerase (1961c) of sea bass muscle. The fact that several phosphoglucomutase enzymes are present in fish muscles is indicated by Roberts and Tsuyuki (1963). The multiple nature of the enolase of muscle of *Salmo* species was demonstrated by Tsuyuki and Wold (1964), and three distinct crystalline enolase "isozymes" were later prepared from trout muscle by Cory and Wold (1965). Ludovicy-Bungert (1961) prepared D-glyceraldehyde 3-phosphate dehydrogenase from carp muscle; the enzyme was homogeneous when examined by moving-boundary electrophoresis and by the analytical ultracentrifuge. Nagayama (1961c) showed that muscles of sea bass possess lactic dehydrogenase. Kaplan and his colleagues have carried out extensive studies on triosephosphate dehydrogenase (Allison and Kaplan, 1964) and on lactic dehydrogenases (Wilson *et al.*, 1964; Fondyl *et al.*, 1965). Shibata (1958) partially purified aldolase from carp muscle.

The accumulation of lactic acid in fish muscles after death is of considerable importance technologically. Thus, it is almost certainly the principal factor in determining post-mortem muscle acidity. While in livestock the post-mortem lactic acid concentration may be controlled to a significant extent by feeding and by slaughtering procedures, this is obviously difficult or impossible with fish. Certain fish such as tuna (Tomlinson and Geiger, 1963) and halibut (Tomlinson *et al.*, 1965) may exhibit high muscle lactic acid concentrations post-mortem and corresponding low pH values, while in Atlantic cod the pH of the muscle may be as high as 6.8 or 7.0 (Fraser *et al.*, 1961). It has been pointed out that low pH values tend to inhibit bacterial spoilage of fish (Tarr and Ney, 1949), and it is well-known that magnesium ammonium phosphate (struvite) formation in canned fish occurs more frequently if the muscle is above pH 6.0. On the other hand, low pH values cause muscle proteins to approach their isoelectric zones, and consequently they tend to lose their water-holding ability. This results in a tendency to a loss of free liquid (drip) on thawing frozen fish, and has been shown to be responsible for the chalky condition that quite frequently occurs in halibut (Tomlinson *et al.*, 1965).

Glucose occurs in quite variable amounts in fish muscles. Early work (Tarr, 1954) indicated that it was probably absent from muscles of living fish (liquid nitrogen frozen), but the technique used for its separation was probably not quite as sensitive as methods now available, and very small concentrations could have been missed. Burt (1961) found about 210–220 μmoles of glucose per 100 g of muscle in rested aquarium cod, and as much as 340 μmoles after 5 days at 0°. However, the fish apparently were not frozen in liquid nitrogen while still alive, and it is possible that some glucose formed by post-mortem degradation of glycogen. Quite large amounts of glucose were found in muscles of "fresh" fish of different post-mortem age, while others contained negligible amounts. Thus, several fish examined had between 100 and 400 μmoles of glucose per g of muscle (Tarr, 1954). Rather small amounts of glucose were found in muscles of lingcod and rainbow trout that were held for one or several days at 0°C after death (Tarr and Leroux, 1962).

Glucose 6-phosphate is hydrolyzed to glucose only very slowly in fish muscles after death, and available evidence indicates that glucose arises largely or entirely by hydrolysis of glycogen. Ghanekar *et al.* (1956) first

suggested that glucose was formed in fish muscles post-mortem by direct hydrolysis of glycogen. Andreev (1958) found that the amylase responsible for hydrolysis could best be prepared by autolysis of ground muscle under toluene and that unautolyzed muscle exhibited insignificant hydrolytic activity. The enzyme was active in the absence of orthophosphate, and it was obtained from muscles of several species of fish.

During studies of sugars and sugar phosphates in fish muscles, Jones (1960), Burt and Jones (1961), and Tarr and Leroux (1962) observed maltose and dextrins in addition to glucose. Thus, the latter investigators observed that radioactive glycogen when introduced into ground fish muscle post-mortem yielded radioactive glucose, maltose (identified only by its R_f value) and what was presumably dextrin(s). Andreev (1962) prepared a dialyzed extract of fish muscle similar to that used during his work with amylase. The extract hydrolyzed maltose as determined by the appearance of reducing sugars, and heated extracts were inactive. The maltase enzyme appeared to be less stable than the amylase, and was widespread among different fish species. Since grinding and autolysis stimulated amylase activity it was suggested that the enzyme might be present in the particulate fraction of muscle extracts. Burt (1966) has studied some of the glycogenolytic enzymes of cod muscle, and concluded that the amylolytic route accounted for the greater proportion of glycogen degradation post-mortem. This view is also held by other investigators (Tarr, 1965; Nagayama, 1966). The possible technological significance of glucose in fish muscles will be referred to later.

The presence of hexose and pentose phosphate esters in fish muscles was first recorded some sixteen years ago (Tarr, 1950a), and several years elapsed before active studies on the occurrence of these compounds were resumed. The more recent work has been greatly facilitated by better methods of separation and identification. Burt and Jones (1961), using ion-exchange chromatography, identified a number of sugar phosphates in cod muscle including glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, ribose 1-phosphate, and ribose 5-phosphate. They found that the total concentration of hexose phosphates was about 220 μmoles per 100 g in fresh-trawled cod muscle, and about double this value in rested cod muscle. These values were of the same order as those recorded for several species of fish previously (Tarr, 1950a). In general the amounts of hexose phosphates decreased post-mortem, though the concentrations of the pentose phosphates increased for several days and subsequently decreased. Tarr and Leroux (1962) studied acid-soluble phosphorous compounds and free sugars in muscles of several fish species using a combination of radioactive tracer techniques and a sensitive ion-exchange chromatographic method of separation. Whereas previous investigators had apparently relied entirely on ion-exchange separation alone in identification of sugar phosphates, in this investigation the sugar phosphates were further identified by preparation of their lithium (or barium) salts and paper chromatography of these with one or more solvent systems. The results with salmon species, cod, and halibut indicated that extreme quantitative variations can be expected in the hexose phosphates. Glucose 6-phosphate usually predominated, and fructose 6-phosphate and fructose 1,6-diphosphate were present in lower concentrations. Examination of fructose 6-phosphate fractions indicated that if fructose 1-phosphate were present, it accounted for less than 5% of the total fructose monophosphate fraction. It was concluded that it was almost certainly absent from the fish muscle examined. Ribose 5-phosphate was rarely found, and ribose 1-phosphate did not occur in any fish muscle studied. Since fish muscles usually possess a fairly strong phosphoribomutase enzyme which promotes a reaction which is about 90–95% in favor of ribose 5-phosphate, it is not surprising that ribose 1-phosphate is rarely found. Burt and Stroud (1966) recently investigated the occurence of 3-phosphoglyceric acid, pyruvate, dihydroxyacetone phosphate, and α-glycerophosphate in cod muscle. The technological importance of the

sugar phosphates in fish muscles will be considered later.

Work carried out some 16 years ago with a barium salt-alcohol fractionation procedure showed that muscles of several species of fish examined contained adenine nucleotides in concentrations rather similar to those occurring in rat muscle. Subsequent research by several investigators showed that the ATP content of rested fish muscles averages about 500–800 μmoles per 100 g of muscle (Saito *et al.*, 1959; Jones and Murray, 1960; Fraser *et al.*, 1961; Tomlinson *et al.*, 1961). Except in unusual circumstances ATP is rapidly degraded post-mortem by a series of enzyme reactions (Fig. 2) (Tomlinson and Geiger, 1962; Jones and Murray, 1962; Partmann, 1965; Kobayashi, 1966). The enzymes responsible have been studied to some extent. It appears that hydrolysis to the stage of inosine monophosphate (IMP) is quite rapid, and that the comparative rate of hydrolysis of IMP to inosine is slower. Thus, IMP tends to accumulate in fish muscles. The inosine that is formed is split by one or both of two different enzymes—a nucleoside hydrolase (Tarr, 1955) or a nucleoside phosphorylase (Tarr, 1958a). The comparative activities of these two enzymes post-mortem has never been accurately assessed for any fish muscle. The fact that free ribose occurs much more frequently and in much higher concentrations than does

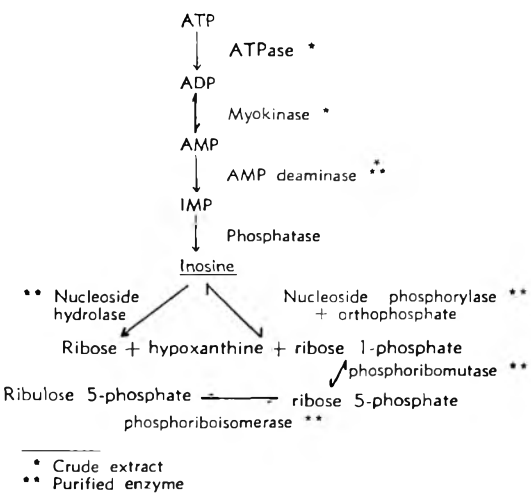

Fig. 2. Enzymes concerned in post-mortem degradation of adenosinetriphosphate in fish flesh.

pentose phosphate in fish muscles post-mortem, would indicate that the hydrolytic mechanism is much more active. In addition to ATP and related nucleotides, fish muscles contain di- and triphosphopyridine nucleotides. These occur in comparatively small concentrations, however, and are probably of little technological significance in comparison with other nucleotides. Jones and Murray (1960) found rather small concentrations of a number of other nucleotides in rested cod muscles. Thus, nicotinamide adeninedinucleotide and its reduced form, which occur in fish muscles in small concentrations (Jones and Murray, 1966), are enzymically degraded by a glycohydrolase enzyme which was first studied in some detail in carp liver (Raczynska-Bojanowska and Gasiorowska, 1963).

Fish muscles also contain ribonucleic acid (RNA) (42–142 mg/100 g) and deoxyribonucleic acid in very low concentrations (0.2–2.5 mg/100 g). Ribonucleic acid was prepared in comparatively pure form from lingcod muscle (Bluhm and Tarr, 1957).

At one time the possibility that RNA and ATP might both be precursors of free ribose in fish muscles was considered (Tarr, 1958b). Indeed, Tomlinson prepared and purified a nuclease enzyme from lingcod muscles that could hydrolyze RNA and certain simple ribo- and deoxyribomononucleotides (1958, 1959). However, experiments indicated that indigenous or added RNA was not degraded appreciably in lingcod muscle held at 0°C (Tomlinson and Creelman, 1960). The possibility cannot be entirely discounted that RNA is degraded post-mortem to yield nucleotides and free ribose in other species of fish. It is now believed that the free ribose in fish muscles arises largely from post-mortem degradation of ATP. Thus, when generally labeled (^{14}C) ATP was introduced into fish muscle, both radioactive inosinic acid and ribose were isolated after it was held 2 days at 0°C. Since uniformly labeled glucose did not cause formation of radioactive ribose 5-phosphate or ribose under such conditions, it was inferred that ribose does not arise from glucose by post-mortem operation of the hexosemonophosphate shunt pathway (Tarr and Leroux,

1962). Very small amounts of IMP and ribose may arise through degradation of nicotinamide adenine dinucleotide (Kassemsarn *et al.*, 1963). The general course of ATP breakdown in fish muscles post-mortem has therefore been established, and many of the enzymes concerned identified. It was originally stated that inosine triphosphate and inosine diphosphate might be intermediates in formation of ribose and hypoxanthine from ATP (Shewan and Jones, 1957), but this has not been borne out by subsequent investigations.

The technological significance of sugars, sugar phosphates, nucleotides, and their degradation products will now be considered. The literature concerning their importance in contributing to fish flesh flavors will first be discussed.

Jones (1961) has stated that "recent studies in our laboratory indicate that much of the sweetness of fresh fish flesh results from the high initial concentrations of glucose." He also stated that solutions of the potassium salts of the sugar phosphates commonly occurring in fish muscle were "sweetish-salty" in the maximum concentrations found in fresh or chilled fish. Unfortunately, these appear to be the only statements available concerning the effect of such compounds on fish flavor, and more work on this general aspect of fish flavors is desirable.

In contrast to the rather scanty information concerning the contribution of sugars and related compounds to fish flavor, there is much more data on the effect of nucleotides. The importance of inosinic acid (IMP) in enhancing the flavors of flesh foods is now well-known, principally as a result of Japanese investigations (Kuninaka *et al.*, 1964; Wagner *et al.*, 1962). The concentrations used are quite low (0.001–0.1%). The 6-hydroxy-substituted 5′-nucleotides, including IMP, guanylic acid, and xanthylic acid produce a "meaty" flavor under appropriate conditions. The corresponding deoxynucleotides are also effective. The various applications of these nucleotides to fish products and other foods have been reviewed (Shimazono, 1964).

The tendency for IMP to accumulate in fish muscles shortly after death has already been pointed out, and the whole subject has been discussed in two fairly recent papers (Jones, 1963; Jones and Murray, 1964); the compound is thereafter hydrolyzed to inosine and orthophosphate, the very variable rates of hydrolysis depending on the fish species and holding conditions. IMP decreases slowly at −14°C, and quite rapidly at higher temperatures. It appears that really fresh fish contains more IMP than would normally be required to cause flavor enhancement, and the slow conversion of IMP to inosine that occurs when fish flesh is held is probably an important cause of flavor loss.

Inosine, which arises from dephosphorylation of IMP, is said to be comparatively flavorless, but hypoxanthine, formed by hydrolytic or phosphorolytic splitting of inosine, is bitter and has been thought to be responsible, to a considerable extent, for the bitterness that may arise in cod held for a considerable time after death (Jones, 1963). On the other hand, Hashimoto (1965) stated that many Japanese investigators suggest that hypoxanthine is comparatively tasteless. Jones (1965) showed that the flavor score in cod and several other fish correlated quite well with the hypoxanthine concentration. Spinelli (1965), concerned about the possibility that hypoxanthine might cause bitterness due to its probable increase in stored radiation-pasteurized fish, studied the relation between this purine base and fish flavor. He found that hypoxanthine in a concentration of 8.8 μmoles per g of fish did not produce a consistently detectable change in flavor. Jones (1965) had found that much lower concentrations correlated with undesirable flavor in cod. Spinelli also found that radiation pasteurization, subsequent storage for 12 days at 32–35°F, and addition of hypoxanthine did not produce a detectable change in flavor. It was concluded that hypoxanthine does not produce an appreciable flavor change in chill-stored sole until the bacterial count exceeds one million per g. At present these results are difficult to reconcile, but they may well be due to differences in the fish that affect the "hypoxanthine flavor." More work is required in this area.

The increase in hypoxanthine concentrations in muscle of chill-stored fish has been suggested as an objective measure of quality (Jones *et al.*, 1964; Spinelli *et al.*, 1964). In fact, an automatic analytical method has been described for this purine, based on the formation of uric acid by action of xanthine oxidase (Jones *et al.*, 1965). It is still too early to predict whether this method will prove more useful than any other of the very large number of objective tests for fish quality that have been proposed from time to time (Farber, 1965; Gould, 1965).

Sugars and sugar phosphates are of considerable technological significance since they enter into Maillard-type browning reactions. Studies carried out some 16 years ago showed that the browning that usually occurred on heating muscle of white-fleshed fish 1 hour at 120°C, was largely eliminated by leaching the flesh with water. Addition of substances possessing a free aldehyde group (sugars, hexose phosphate, aldehydes, reductone, etc.) to leached flesh which was subsequently heated caused browning (Tarr, 1950b). Addition of bisulfite or hydroxylamine strongly inhibited browning. Subsequent work showed that free glucose and ribose were found in fish muscles after death, and that ribose was about five times as effective as glucose in causing browning. There was also a rough agreement between the ribose content of fish muscles and the degree of browning that occurred on heating. Adding certain ribonucleosides, ribonucleotides, RNA, and ribose 5-phosphate to fish muscles and holding them several days at 0°C caused marked increases in post-mortem formation of ribose in the majority of cases. The enzymic sequence leading to formation of ribose in fish muscles has already been discussed. Jones (1954) observed that browning reactions occurred in freeze-dried extracts of cod muscle, and later (1959) studied both loss of free amino acids and sugars in aqueous extracts of the muscle. When extracts were held three days at 40° and 60% relative humidity, free amino acids decreased 40–60%, ribose disappeared, and glucose nearly disappeared. Jones (1962) reviewed the various browning reactions that occur in dried fish products. Certain of these do not involve sugars or sugar phosphates but are due to degradation products of lipids or changes in heterocyclic ring structures such as 1-methylhistidine.

Nagayama (1960) found no linear correlation between browning of heated fish flesh and ribose or glucose content, and believed that concentrations of amino compounds were important. However, he believed that the browning was caused by sugar-amino reactions. He also found that the level of acid-soluble hexose decreased significantly in browned flesh but not in unbrowned (Nagayama, 1961; Nagayama *et al.*, 1962). Yamada and Amano (1960) found that irradiation of leached fish flesh to which various sugars were added did not cause an increase in browning.

The very variable concentrations of hexose phosphates in fish flesh have already been mentioned, and therefore it is obvious that the importance of these in causing browning reactions will be difficult to forecast. Glucose 6-phosphate supported browning of leached fish flesh that was subsequently heated (Tarr, 1954). Ribose 5-phosphate was shown to be a little more reactive than glucose in causing browning (von Tigerstrom and Tarr, 1965). Undoubtedly, certain of the hexose phosphates could be of significance in causing Maillard reactions in heated or dehydrated fish flesh, but pentose phosphates usually occur in very low concentrations and would therefore probably be of little importance in this respect. Burt (1965) has indicated that a determination of the fructose phosphates might give useful information regarding fish quality.

Removal of free sugars from fish flesh has been found to be quite effective in decreasing browning, but no practical method of removal is yet available. It was shown that addition of crude or purified fish muscle nucleoside hydrolase to fish muscle which was subsequently held several days at 0°C caused marked increase in ribose, especially in fish flesh that had very low concentrations of this enzyme. The addition of adenosine also caused a marked increase in free ribose. When free ribose was largely removed from the muscle by incorporation of washed cells of *Lactobacillus pentoaceticus,*

heating no longer caused significant browning (Tarr and Bassett, 1954). More recently, a particulate enzyme prepared from washed cells of *Pseudomonas fragi* has been found to remove much of the ribose and glucose from fish muscles, thereby decreasing browning of the heated fish considerably (von Tigerstrom and Tarr, 1965).

Though much work has been carried out on freeze-dried fish, the product still suffers from two major defects—toughness and tendency to discoloration during storage. A study has recently been made of the browning of freeze-dried sole and lingcod (Tarr and Gadd, 1965). No loss of either ribose or glucose occurred during the freeze-drying procedure. Browning occurred during storage at 25, 33, and 37° and at 0, 5, and 10% relative humidities, and was more rapid at the higher temperatures. Added ribose accelerated browning, and it was retarded when glucose and ribose were largely removed by treatment of the flesh with the particulate enzyme before freeze-drying. Storage under nitrogen retarded development of browning but did not eliminate it, and this finding indicates that oxidation, perhaps of lipids, may be responsible for some of the browning. It was observed that many of the browned samples became so tough that they could not be blended with conventional homogenizers. Only in frozen samples stored at −30°C did no appreciable discoloration occur. It thus appears that production of a satisfactory freeze-dried fish will be very difficult.

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Enzymatic Reducing Pathways in Meat

SUMMARY

Methods for measuring reducing capacity of meats are described. These include changes in oxidation-reduction potentials of ground meat and changes in oxygen tension of meat slurries, as well as reduction of metmyoglobin. Except for a small residual utilization of oxygen in meat slurries (ascribed to nonenzymatic oxidation), all reductive activity in meat can be stopped by inhibitors of DPNH oxidation via the electron transport chain. Added DPN accelerates all reductive activity. Metmyoglobin reduction does not occur until oxygen has substantially disappeared from the meat.

Meat contains little or no succinate. Added succinate greatly accelerates oxygen utilization, but affects metmyoglobin reduction only indirectly by establishing anaerobic conditions more rapidly. It is concluded that both oxygen utilization and metmyoglobin reduction in meat are normally mediated through DPN.

INTRODUCTION

The color of fresh meat is largely determined by the relative proportions and distribution of the three meat pigments, purple reduced myoglobin (M), red oxymyoglobin (MO_2), and brown metmyoglobin (M^+). The last pigment is particularly undesirable, not only from the color standpoint, but also because ferric hemes act as catalysts in the oxidation of unsaturated lipids.

The proportion of M to MO_2 is influenced by the activity of enzyme systems within the meat. That meat is capable of utilizing oxygen is evident from the simple observation that in oxygen-impermeable wrappings, surface MO_2 quickly dissociates to M. Since the oxygen tension for half saturation of M is 1 to 1.4 mm Hg (George and Stratmann, 1952), this color change obviously denotes a rapid utilization by the meat of the oxygen within the package.

M may also be oxidized to M^+ rather than oxygenated to MO_2. The rate of autoxidation is dependent on oxygen tension and is highest at half saturation (George and Stratmann, 1952). However, meat enzymes can reduce M^+ as well as oxygen (Stewart *et al.*, 1965b). The accumulation of M^+ in stored meats is the resultant of these opposing factors (autoxidation and enzymatic reduction). The purpose of this research is to explore enzymatic pathways by which meat is able to reduce both oxygen and M^+, and the relation between oxygen and M^+ reduction.

A review of the biochemistry of respiratory activity in the living muscle cells and the changes known to occur after slaughter (Bendall, 1962; Lawrie, 1962) leads to some predictions concerning probable reductive pathways in meat. In the living tissue, hydrogen, derived from the reactions of the Krebs cycle, passes to the electron-transport chain (ETC) either by way of DPN and its associated flavoprotein dehydrogenase (FD) or from succinate and the flavoprotein succinic dehydrogenase (FS) (Green and Fleischer, 1962; Hatifi, 1963).

Upon slaughter, oxygen is cut off from the tissues, and a rapid anaerobic glycolysis ensues. In postrigor meat most of the glycogen has been converted to lactic acid, leaving a large pool of lactate. ATP and other high-energy phosphates have disappeared.

On the other hand, many enzymes, including lactic dehydrogenase (LD) and all components of the glycolytic pathway, succinic dehydrogenase and all components of the ETC, remain potentially active in meats even after extended refrigerator storage (Andrews *et al.*, 1952; Bodwell *et al.*, 1965).

If oxygen becomes available again, as when meat is ground or cut surfaces are exposed, the resumption of enzymic oxidase activity would be expected provided suitable hydrogen donors are present. Any succinate present should be rapidly oxidized by way of mitochondrial succinic dehydrogenase and the ETC. This should result in a utilization of oxygen, but there is no known pathway for transfer of electrons from succinate to M^+.

On the other hand, reduction of DPN^+ to DPNH could lead to reduction of both M^+ and O_2. There is an extensive literature, not reviewed here, on the reduction of ferric hemes by DPNH in artificial systems, red

cell preparations, etc., provided suitable intermediates are present. Although the pyridine nucleotides cannot pass undamaged mitochondrial membranes, there is good evidence that DPNH, generated externally in the cytoplasm, can be oxidized by way of the mitochondrial ETC (Margreth and Azzone, 1964; Cunningham, 1964).

While there are a number of enzyme-substrate systems capable of reducing DPN in living muscle, the activity of these systems in postrigor meat is unexplored. In most cases substrates would be lacking, even if the enzymes are present. As mentioned above, lactate is present in large amounts. Lactate may be oxidized to pyruvate by the DPN-linked enzyme LD:

$$CH_3CHOHCOOH + DPN^+ \rightleftarrows CH_3COCOOH + DPNH + H^+$$

The equilibrium of this reaction lies far to the left, so that lactate oxidation would require effective removal of the pyruvate and DPNH.

With the above background in mind, the reductive pathways shown in Fig. 1 may be advanced as a hypothetical scheme for the reduction of oxygen and metmyoglobin in meat.

To test this hypothetical scheme, we have tried the effect of added DPN and succinate and of known inhibitors on reducing activities in meat. Four inhibitors have proven useful in this study. Oxalate acts as a competitive inhibitor for lactate in the LD region (Ottolenghi and Denstedt, 1958; Novoa *et al.*, 1959), thus blocking the overall reaction in position 1, provided lactate is the substrate. Ernster and Lee (1964) have reviewed the literature on the other three inhibitors. Amytal and rotenone both block reduction of DPNH in the flavoprotein region, position 2, but have no effect on succinate oxidation. Antimycin A blocks electron transport from DPNH or succinate at position 3. Unfortunately, inhibitors in the cytochrome A region are compounds such as cyanide, azide, etc., which combine with heme iron and therefore react with M or M^+ as well as with cytochrome A. Since they interfere with determination of M^+ reduction, they were not used in this work.

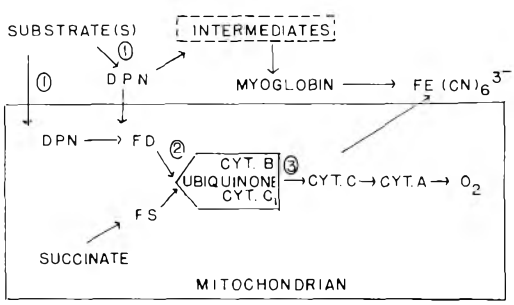

Fig. 1. Hypothetical scheme of reductive pathways in meat. Arrows indicate direction of electron flow. Electron transport is blocked at position 1 by oxalate (if the substrate is lactic acid), at position 2 by amytal and rotenone, and at position 3 by antimycin A.

METHODS

Preparation of meat. Meat was obtained from local packing houses. Both pork hams and beef (eye of round) were used. The preslaughter history of the animals was not known. All meat was trimmed of external fat and ground twice just before each experiment. The ground meat for any one experiment was mixed thoroughly, and weighed portions were treated with additives as described under each experiment. Three kinds of tests, described below, were used to measure the reductive capacity of the controls and treated samples.

M^+-reducing activity was measured as described by Stewart *et al.* (1965b). The method consists of first oxidizing all of the M to M^+ in ground meat by the addition of a slight excess of $K_3Fe(CN)_6$ and then following the reduction of the M^+ during a 15-to-90-min period by reflectance spectrophotometry (Stewart *et al.*, 1965a).

The addition of any oxidizing agent has obvious drawbacks in a study of enzymatic reducing activity. The oxidizing agent may itself be reduced enzymatically. Ferricyanide is known to be reduced by electrons, which may come off the ETC at various points, but mainly at the cytochrome *c* level (Eastbrook, 1961). Magos (1964) investigated a number of methemoglobin-forming agents and concluded that nitrite was preferable to ferricyanide because it did not oxidize sulfhydryl groups in the protein or cause denaturation. However, nitrite may be reduced, at least under anaerobic conditions, by reduced cytochrome *c* and cytochrome oxidase (Walters and Taylor, 1965).

In view of these uncertainties, and of its use in meat curing, nitrite was used as the metmyoglobin-forming agent in some of the experimental work. Preliminary work established that .006% NO_2^- was sufficient to oxidize completely the M in most samples of meat. Oxidation was less rapid by nitrite than by ferricyanide; the meat had to be stirred longer to obtain complete oxidation. The standard procedure was to add 1 ml of freshly pre-

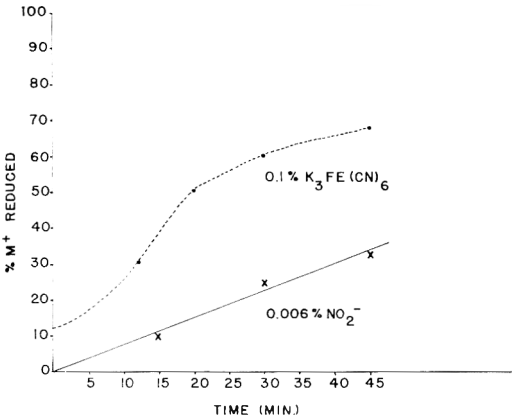

Fig. 2. $K_3Fe(CN)_6$ vs. NO_2^- as an oxidant for studying M^+-reducing activity of meat.

pared $NaNO_2$ solution (0.3% NO_2^-) to 50 g meat, stir for 12½ min, and then begin readings on the Spectronic 505 at 15 min (designated as zero time in the reduction data). The enzymatic reduction of M^+ occurred more slowly when NO_2^- was used as the oxidizing agent than when ferricyanide was used, and reduction with NO_2^- was generally linear with time, whereas sigmoid curves with variable lag periods were obtained with ferricyanide (Fig. 2).

The pigment obtained upon reduction of M^+ in the presence of NO_2^- was nitric oxide myoglobin. This pigment, like reduced myoglobin, was isosbestic with M^+ at 525 mμ, but the wavelength of greatest difference between the nitric oxide pigment and M^+ was at 550–553 mμ. The M^+ determination was therefore modified from the procedure described by Stewart *et al.* (1965a) by substituting the ratio $\frac{K/S\ 550\text{-}553}{K/525}$ for the ratio $\frac{K/S\ 572}{K/S\ 525}$, used to determine M^+ in mixtures of M and M^+. The average ratio was 0.88 for completely oxidized samples, and 1.37 for completely reduced samples. A linear relation was assumed between these limiting values and the percent of the total pigment present as M^+.

Oxidation-reduction potentials. In some of the earlier experiments, before suitable methods for measuring oxygen tension had been developed, changes in oxidation-reduction potential of ground meat were used as supplementary evidence for reducing capacity of the meat. The ground meat was brought to room temperature as in the spectrophotometric analysis and stirred for 3½ min. Test substances were then added and mixed 3½ min. The meat was packed tightly into a 50-ml beaker, electrodes were inserted, and readings were begun in another 1½ min (designated zero time).

The Beckman zeromatic pH meter, model 9600, was used for the measurements. A platinum electrode, No. 39276 was used in combination with the calomel reference electrode, No. 39170. Readings were usually taken at intervals over a period of 30 min.

The pattern of potential change was different for each lot of ground meat, but the pattern was reproducible when different portions of the same lot of meat were similarly treated. Also when the same sample of meat was removed from the beaker and restirred with air, the potential returned to its original high value and a second potential curve usually duplicated the first.

The drop in potential does not, of course, give specific information either on oxygen utilization or M^+ reduction; it is affected by the concentration of many metabolite pairs and their associated enzymes (Wurmser and Banerjee, 1964). However, under the conditions of these experiments, where the initial values are under aerobic conditions, and the packed meat utilizes the oxygen present, rate of fall in oxidation-reduction potentials of different samples of meat seemed to correlate reasonably well with enzymatic reduction by way of the ETC. The fall in potential could be completely blocked by ETC inhibitors.

Oxygen consumption. Changes in oxygen tension were measured with a polarographic oxygen analyzer, Beckman model 777. In order to obtain meaningful measurements with this instrument it is necessary to work with meat slurries which can be adequately stirred during the test period. The following technique was found to yield slurries which generally maintained a linear rate of oxygen consumption for at least a 10-min period.

A 50-g portion of ground meat plus any additives under investigation and sufficient 0.25*M* sucrose solution to give a total liquid volume of 100 ml is homogenized in a 500-ml Virtis flask for 2 min at a rheostat setting of 40. The meat and all solutions are cold, and the blending is done in an ice bath. The cold homogenate is run through a large strainer or a double layer of cheesecloth to remove strands of connective tissue, and brought quickly to 25°C. A 50-ml Erlenmeyer flask with Teflon-covered magnet is filled with the homogenate and placed on an asbestos-covered foam pad on a magnetic stirrer. The sensor of the oxygen analyzer is then placed in the flask (it fits snugly in the neck), and the recording is begun. The entire preparation time, up to the beginning of the recording, is 8 min. Deviations in preparation time, failure to keep the homogenate cold during blending, or omission of the sucrose (used to protect cell structures from osmotic damage) result in poor reproducibility and decreases in the rate of oxygen consumption during the test period.

Usually the slope of the change in oxygen tension (PO_2) was measured from 2 to 5 min. At 5 min a test substance would be added to the flask and the new slope measured from 7 to 8 min. All slopes are expressed as drop in oxygen tension in mm Hg per minute. Changes in oxygen tension could not be translated precisely into moles of oxygen consumed, since the solubility of oxygen in the slurries at any particular oxygen tension is not known. However, accepting the figure of oxygen solubility of 8.4 ppm O_2 in pure water in equilibrium with water-saturated air at 25°C and 760 mm Hg (160 mm Hg PO_2) and correcting this for the decreased solubility of oxygen in the sugar solutions used (92% of the water value) gives a value of 7.7 ppm O_2 at 160 mm Hg PO_2. A fall of 1 mm in the oxygen tension measured is thus very roughly equivalent to a loss of .048 ppm O_2 or to 1.5×10^{-4} μmoles of O_2 per g of slurry.

Addition of inhibitors. Tentative concentrations of each inhibitor necessary to give maximum inhibition were obtained from several sources in the literature and are shown in the results. Except with potassium oxalate, concentrations of each inhibitor higher than those shown were also tried in samples of meat where 100% inhibition had not been obtained. Doubling the concentration gave no additional inhibition. The oxalate was added in phosphate buffer of the same pH as the meat sample, and the same amount of buffer was used in the control. The amount of amytal needed for 50 g meat was dissolved in 1 ml propylene glycol, and rotenone and antimycin A were dissolved in 1 ml ethanol, before addition to the meat. Since both propylene glycol and ethanol increased the reductive capacity of the meat, it was necessary to use controls having the same amount of solvent.

With all of the poisons used, the inhibition disappeared after some time (usually 15 to 50 min). This is apparently due to a loss of the inhibitor itself rather than to the establishment of alternative reductive pathways, since addition of further inhibitor when reduction had begun resulted, again, in complete inhibition.

Anaerobic experiments. To test the ability of the meat to reduce M^+ in the absence of oxygen, two different experimental techniques were employed:

Procedure for ground meat. Fifty-gram portions of the ground meat were placed in Saran bags (6½ × 11¾ inches). A glass cup (1¾ × 1⅛ inches) with lid was also placed in the bag. Nitrogen inlet and outlet tubes and the sensor of the oxygen analyzer were inserted through the opening in the bag and held in place with rubber bands and a clamp.

One minute of nitrogen flushing was sufficient to bring the oxygen tension practically to zero. The meat was mixed for a 3-min period, by external manipulation of the bag, to remove entrapped oxygen from the meat. The oxidizing agents and test substances were injected into the meat mass through the Saran bag. Mixing of meat and additives, under nitrogen, was continued for another 3 min. Finally, while still in the bag and under nitrogen, the meat was packed into the glass cup and covered. After removal from the bag, the cup was sealed with masking tape around the sides and the meat pigments analyzed by spectrophotometry as previously described. The first curve was obtained 4½ min after addition of the oxidizing agent.

Procedure for meat slurries. Slurries were prepared as previously described. After blending, the oxidant, ferricyanide or nitrite, was added. After filtering through cheesecloth the slurry was brought to 25°C (1–1½ min) and transferred to a special cell (see Fig. 3) designed to allow simultaneous measurements of PO_2 and M^+ reduction while flushing with N_2. A Teflon-covered stirring magnet 2½ cm long was placed in the flask, and the calibrated oxygen sensor was inserted and secured with Parafilm. The flask was placed on a magnetic stirrer covered with asbestos screen and a petri-dish top to catch the overflow of slurry. Slurry was added through arm B to fill the cell completely, and the arm was sealed off with Parafilm. The magnetic stirrer was set at a speed of 7, and the recorder of the analyzer was started. The total preparation time from beginning of blending to the time the recorder was

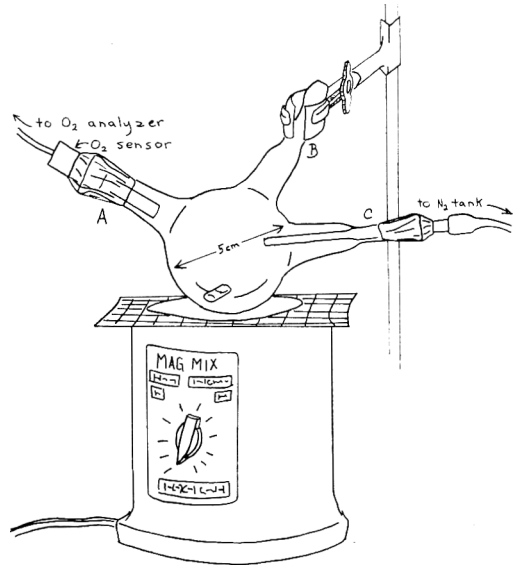

Fig. 3. Specially designed cell for simultaneous measurements of oxygen tension and M^+ reduction in meat slurries.

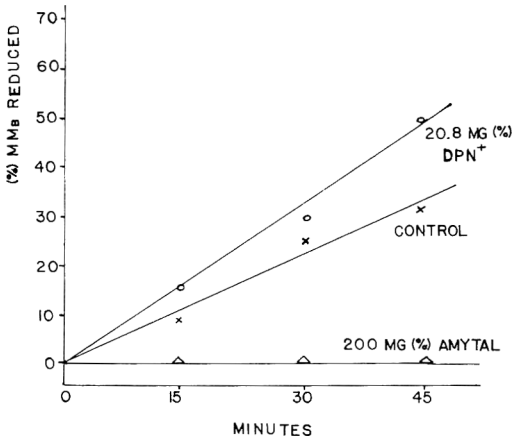

Fig. 4. Effect of DPN and amytal on reduction of M^+ in ground pork. Pigment was first oxidized with nitrite.

turned on was 10 min. The spectral analysis was carried out as with the ground meat sample.

RESULTS

Role of DPN. Evidence for the important role of DPN in the reductive activity of meat was obtained both from the use of inhibitors and also from the addition of DPN. Figs. 4, 5, and 6 show data obtained on the same sample of meat with the three tests described. In this experiment, nitrite was used as the oxidizing agent for the pigment studies, but no oxidizing agent was used in the potential or oxygen tension measurements. It will be seen that amytal blocked completely both M^+ reduction and also the drop in the oxidation-reduction potential. Oxygen consumption in the meat

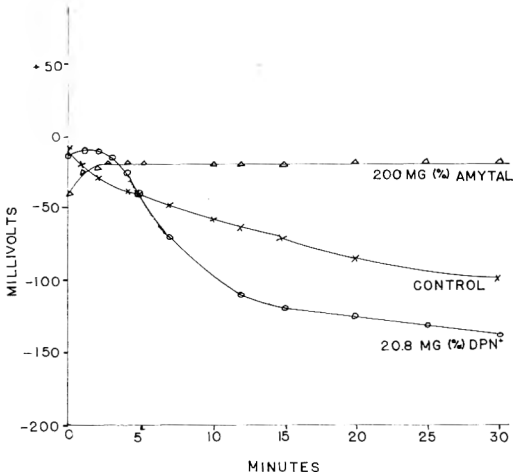

Fig. 5. Effect of DPN and amytal on oxidation-reduction potentials of ground pork.

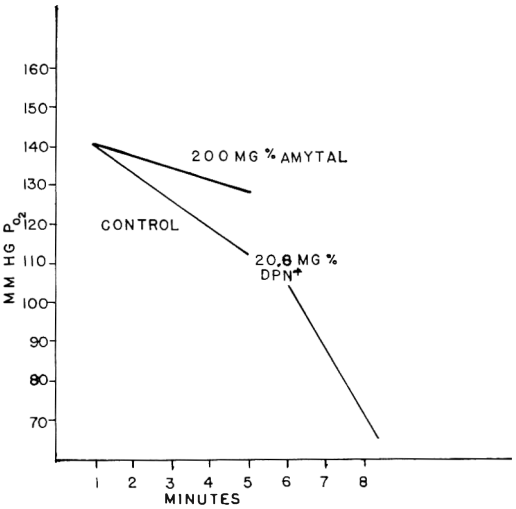

Fig. 6. Effect of DPN and amytal on oxygen utilization in a ground pork slurry.

slurries was inhibited only partially. It was found in all inhibitor experiments with the oxygen analyzer that a residual oxidation of about 2 to 5 mm Hg PO_2 per minute remained even when, as in this experiment, all pigment oxidation and potential change had been stopped in the ground meat. It is probable that this activity represents other oxidations stimulated by the homogenization. This could include nonenzymatic reactions such as heme-catalyzed lipid oxidations. In fact, oxygen consumption of this order of magnitude is observed in slurries from cooked beef.

Table 1 shows the results of five additional experiments with amytal, and one with retenone. Inhibition is generally quite high, approaching 100% with most samples of meat. In Expt. 2, where the inhibition was somewhat less, no more inhibition was obtained even with five times the amount of amytal shown. It is believed that traces of succinate account for the residual oxidation. Malonate, a specific inhibitor for succinate oxidation, was not used in this sample of meat, but was found to give slight, variable inhibition with several samples of meat.

The addition of DPN to meat resulted in increased reductive activity. This is shown in the typical experiment illustrated in Figs. 4, 5, and 6. In eight additional experiments, rate of M^+ reduction was increased 23 to 51% over that of the control. The DPN present in meat at slaughter is progressively

Table 1. Effect of ETC poisons on reducing activity of pork.

Expt. no.	Inhibitor added	M^+ reduction: Oxidizing agent	Time of reduction (min)	Control % M^+ reduced	Inhibition (%)	Potential changes: Time (min)	Control drop (mv)	Inhibition (%)
1	Amytal, 200 mg%	Ferri	30	66	100	30	91	100
2	Amytal, 200 mg%	Ferri	20	78	84	20	105	29
3	Amytal, 200 mg%	Ferri	20	43	96	20	116	88
	Rotenone, 6 mg%	Ferri	20	43	100	20	116	85
4	Amytal, 200 mg%	NO_2^-	40	29	100	.....	.....	.....
5	Amytal, 200 mg%	NO_2^-	40	31	94	30	90	100
6	Antimycin A, 6 mg%	Ferri	10	7	86	10	65	92
			20	32	90	20	110	70

destroyed by the action of several enzymes which are brought into contact with the nucleotide upon maceration of the tissue. These enzyme systems have been reviewed by Severin *et al.* (1963). The loss of reducing activity when ground meat is refrigerated, noted by Stewart *et al.* (1965b), may be ascribed to loss of DPN. Most of the original reducing activity can be restored by the addition of DPN at the end of the storage period.

The identity of the substrate or substrates (Fig. 1) which supply hydrogen to DPN^+ is not clear at this time. The addition of potassium oxalate, competitive inhibitor for lactate in the LD reaction, gave variable results with different samples of meat. At an oxalate level of 0.5 g per 100 g meat, the general pattern seemed to be an initial inhibition of reductive activity, by all three tests, for the first 10–20 min. This was followed by a marked acceleration, so that at 45 min the oxalate-treated samples showed greater M^+ reduction than the controls. Larger concentrations, up to 1%, generally increased the time of inhibition, although this varied with the sample of meat. Further work on the addition of DPN-linked substrates to meat will be reported later.

Intermediates between DPNH and M^+ are also unknown at this time. DT diaphorase (also known as menadione reductase) is a cytoplasmic flavoenzyme which catalyzes the reduction of various quinones, which in turn reduce ferric heme compounds (Ernster *et al.* 1962; Conover and Ernster, 1962). However, dicoumarol, a specific inhibitor for this enzyme, did not inhibit M^+ reduction in meat at a concentration of 6 mg%.

Relation between M^+ reduction and oxygen utilization. The fact that inhibitors of the ETC also inhibit M^+ reduction does not mean that M^+ is being reduced by way of the ETC. Rather, it indicates that oxygen must be substantially eliminated before M^+ reduction begins. This is indicated by the fact that all spectrophotometric curves obtained during the reduction of M^+ show only mixtures of M^+ and M, without any MO_2, where ferricyanide was used as the oxidant. Apparently, hydrogen or electrons from DPNH react preferentially with oxygen as long as any oxygen remains.

This is shown in Table 2, containing results of an experiment in which simultaneous

Table 2. Effect of oxygen tension and DPN on reduction of metmyoglobin in beef slurries.

Sample	Time (min)	PO_2 (mm Hg)	% M^+ reduction
Control [a]	0	54.4	0
	10	32.0	0
	20	0	10
	30	0	26
DPN [b]	0	65.6	.....
	10	9.6	0
	20	0	35
	30	0	56
Control $+N_2$ [c]	0	.....	0
	10	0	1
	20	0	14
	30	0	25
DPN $+N_2$ [c]	0	.....	.....
	10	0	26
	20	0	46
	30	0	73

[a] Control contained: 200 mg% nicotinamide, 3 mg% CTC and 30 mg% $K_3Fe(CN)_6$.
[b] DPN contained: Same as control + 40 mg% DPN.
[c] N_2 bubbled through at zero time.

O_2 tension and M^+ reduction measurements were carried out on meat slurries, with and without added DPN. For this experiment, the special flask described earlier (Fig. 2) was used.

The addition of DPN to ground meat oxidized with ferricyanide under anaerobic conditions (Table 3) usually resulted in a very rapid reduction of M^+, with no lag period. In fact, much reduction takes place during the mixing period, before the first spectrophotometric curve is obtained. On the other hand, when meat is not deaerated before the addition of DPN, there is normally a lag period before M^+ reduction begins. Oxygen and any excess ferricyanide or nitrite are presumably being reduced during the lag period. Fig. 7 shows a typical set of data on meat containing DPN under anaerobic versus aerobic conditions.

Oxygen utilization is much more rapid in the presence of succinate. Most of the samples of meat tested contained no more than a trace of succinate, as shown by the fact that malonate gave very slight inhibition of reducing activity. The addition of 100–200 mg succinate per 100 g meat increased the rate of oxygen utilization in meat slurries to 3–9 times that of the control. Thus, although there is no direct pathway from succinate to M^+, the rapid establishment of anaerobic conditions in succinate-treated samples effectively shortened or eliminated the lag period in M^+ reduction.

Table 3. The effect of DPN on metmyoglobin reduction in ground beef under anaerobic conditions.

Experiment	Time (min)	Metmyoglobin (% of total pigment) Sample: Control	Sample: (+ DPN, 40 mg%)
A	0	100	86
	10	99	51
	20	86	22
	30	75	8
	40	65	0
B	0	100	52
	10	95	10
	20	82	6
	30	68	0
C	0	99	100
	10	99	94
	20	96	68
	30	92	36
	40	86	14
	50	77	5
	60	71	0

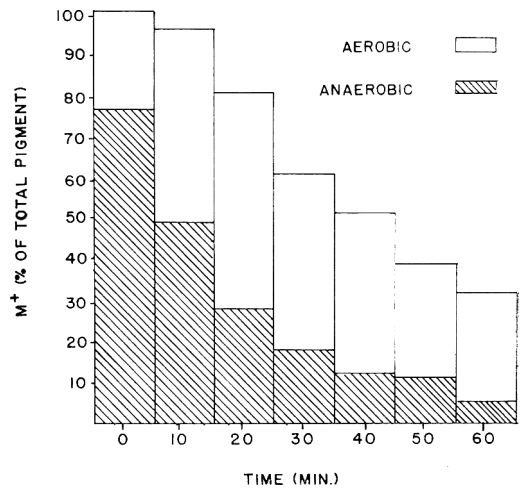

Fig. 7. Comparison of M^+ reduction in ground beef containing 40 mg% DPN under aerobic vs. anaerobic conditions.

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Abbreviations used: ATP = adenosine triphosphate, DPN = diphosphopyridine nucleotide (also known as niacin adenine dinucleotide), DPN^+ and DPNH = oxidized and reduced states, respectively, of DPN; ETC = electron-transport chain; FD = flavoprotein DPN dehydrogenase; FS = flavoprotein succinic dehydrogenase; LD = lactic dedrogenase; M = reduced myoglobin; M^+ = metmyoglobin; MO_2 = oxymyoglobin; MRA = metmyoglobin-reducing activity; PO_2 = oxygen tension; ppm = parts per million.

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Irradiation of Mangoes. I. Radiation-induced Delay in Ripening of Alphonso Mangoes

SUMMARY

Extension of storage life of unripe and mature Alphonso mangoes could be achieved at an optimum dose of 25 krads when irradiated under air, nitrogen, or carbon dioxide. Nitrogen atmosphere during irradiation is of some advantage in minimizing changes in organoleptic quality, ascorbic acid, and carotenoids, and at the same time allowing the least spoilage of fruit. Judging from skin color and fruit firmness, a six-day delay in ripening could be expected in fruits irradiated with 25 krads under nitrogen or air. Radiation effect on fruit skin is more prominent in terms of inhibition in chlorophyll disappearance and carotenoid formation than in ripening changes in the meat of the fruit.

INTRODUCTION

Transport of perishables in tropical regions contributes to high losses, except under refrigerated conditions, which are seldom used in most developing countries. Ripening therefore takes place rapidly and unevenly during transit, with attendant softening and rotting of the tissues. Irradiation could be of help in delaying ripening, as has been shown recently in a number of reports for other fruits. Thus, Maxie *et al.* (1966) observed delay in ripening of Bartlett pears at doses of gamma rays of 300 krads and higher. Retardation in red color development of tomatoes was found to increase with increasing radiation dose (Abdel-Kadar and Morris, 1963), 100 or 200 krad treatment having very little effect, and 500 krad showing marked effect. Cooper and Salunkhe (1963) found a delay in the ripening of cherries irradiated at 300 krads. Papayas subjected to 200–400 krads ripened more slowly than unirradiated control fruits (Maxie and Stallman, 1963), with no off-flavor development up to a 300-krad dose.

In contrast to the general effect of delayed ripening by irradiation, Maxie *et al.* (1965) found quicker degreening in irradiated green lemons.

This communication details work on the extension of storage life of Alphonso mangoes by delay in ripening. Data are also included on the effects of irradiation under various gaseous atmospheres.

MATERIALS AND METHODS

Alphonso mangoes (*Mangifera indica* L.), picked on the previous day and trucked from growing regions within 250 miles from Bombay, were bought from the market, selection being restricted to mature unripe fruits, uniformly olive green in color.

Irradiation was done on the same day in a gamma cell 220 (Atomic Energy of Canada Ltd., 22,500 curies) at a dose rate of 25 krads per minute. The irradiation chamber, 6 × 8 inches in size, showed axial respective dose-rate variations of 1.48, 1.60, and 1.43 Mrads/hr in the bottom, middle, and upper 2.5-inch regions. For this reason, three mangoes, stacked on each other in a beaker, were irradiated with rotation of their positions every one-third of the irradiation time. This procedure ensured uniformity of dose to all fruits, the total radiation doses administered being 12, 25, 50, 75, 100, and 200 krads. For each treatment, 24–30 fruits were used in replicates of 4–5 on each of two independent trials.

Irradiation under different atmospheres. Mangoes were kept flushed under an atmosphere of nitrogen or carbon dioxide in a desiccator for 3 hr to equilibrate with the respective gas; they were then transferred quickly to 250-gauge polyethylene bags and heat-sealed, with the appropriate gas being simultaneously blown in and exhausted from the bags, this operation taking only a few seconds. The bags were carefully examined for leaks, and only the properly sealed ones were used for irradiation studies. The bags were cut open after irradiation, and the fruits were studied for storage behavior in ordinary atmosphere.

Analyses. Used for analysis was the pulp obtained by blending the edible portion from three mangoes from each lot.

Total carotenoids. The total carotenoids were extracted by blending fruit pulp, methanol, and petroleum ether (1:1:2) in a Waring blender; washing the ethereal layer, after separation by centrifugation, with water; and reading the optical density at 440 mμ in a Beckman Spectronic 20 after making up to a known volume.

Ascorbic acid. Ascorbic acid was estimated by 2:6 dichlorophenol indophenol titration (AOAC, 1960).

Total and reducing sugars. These were estimated by the Lane and Eynon method (AOAC, 1960).

Starch. Starch was determined, after removal of the sugars from the pulp by 95% alcohol for 2 hr in a Soxhlet apparatus, by hydrolysis with 6.5*N* hydrochloric acid and subsequent analysis for total sugars by the Lane and Eynon method.

Organoleptic evaluation. A panel of six judges scored, on a nine-point hedonic scale, from 1, extreme dislike, to 9, extreme liking. Color, flavor, texture, and taste of the control and irradiated fruits were evaluated. The final rating was obtained by averaging out the markings, with nine as the highest.

Fruit pressure. Fruit pressure was ascertained by a Ballauf pressure-tester (Joslyn and Heid, 1963) by noting the pressure in lb to push the penetrometer rod 1 cm through the skin of the fruit into the fruit flesh.

Skin color. Skin color was scored for colors like green and orange by plus signs (+), between one and four, depending upon the intensity of color. For example, green (++++) stood for completely green skin, and green (+) for light green covering part of the fruit.

RESULTS AND DISCUSSION

Initial experiments were carried out to ascertain the optimum radiation dose required to delay ripening of the fruit. Mature olive-green Alphonso mangoes were therefore irradiated by different doses, *viz.* 12, 25, 50, 75, 100, and 200 krads, one dozen mangoes being taken for each treatment and for a control unirradiated lot. The fruits were stored at room temperature (25–30°C) for a period of 20 days to study the ripening process, the parameters of which are green color and fruit pressure. The percentages of fruits ripening and deteriorating are respectively depicted in Figs. 1 and 2. It may be seen that, in the control batch, all the fruits have ripened in 10 days' storage time, whereas in the batches irradiated with 12, 25, 50, and 75 krads, the respective times taken for all the fruits to ripen is 13, 16, 15 and 15 days (Fig. 1). Differences in rate of ripening are more or less similar throughout the experimental period; for example, the time required for 50% of the fruits to ripen is respectively 8, 11½, 12½, 14 and

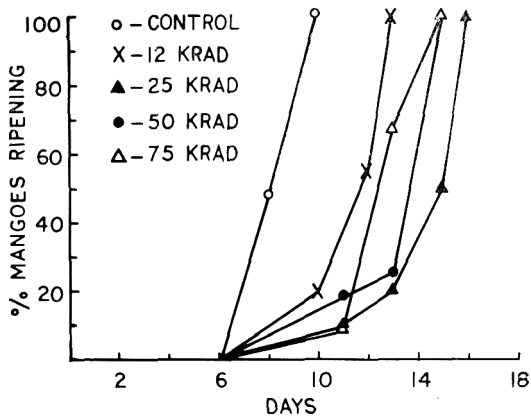

Fig. 1. Percentage of mangoes ripening during storage at ambient temperatures (25–30°C) with and without irradiation. Unirradiated mangoes and fruits irradiated at different doses (12–200 krads), were stored at room temperature, and the percentages of ripe fruits noted for each treatment. Twenty-five krads is seen to be the optimum dose with maximum delay in ripening.

15 days for radiation doses of 0, 12, 75, 50 and 25 krads. The data also show that, at 25 krads, there is maximum delay in ripening, 12 krads as well as 50 and 75 krads showing earlier ripening, which may be due to activation or distortion of the respiratory enzyme system or to differences in degree of ethylene production. Fig. 2 shows that higher irradiation doses cause deterioration in fruit quality, 25 krads being optimum since it causes least spoilage during the 20-day storage period. There is progressive

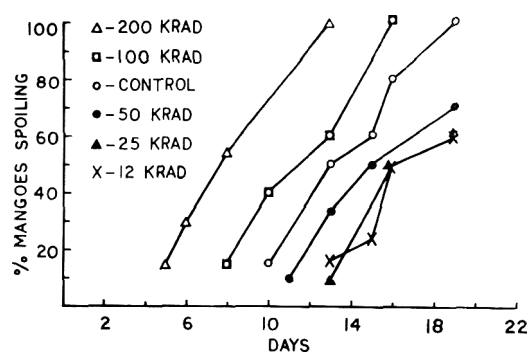

Fig. 2. Percentage of mangoes spoiling during storage at ambient temperatures (25–30°C) with and without irradiation. Percentages of spoiled fruits during storage at room temperatures after irradiation by different doses (12–200 krads) is depicted. Mangoes irradiated by 12 and 25 krads show minimum spoilage on the 19th day of storage (60%, compared with 100% for the controls). Fruits irradiated by 100 and 200 krads spoil much earlier.

deterioration with increase in radiation dose, as evidenced by more spotting and, with fruits irradiated to 200 krads, complete blackening.

There are reports pointing to a lessening of damage by irradiation under a nitrogen atmosphere (Tappel *et al.* 1955; Hollaender *et al.*, 1951; Maxie *et al.*, 1966; Martin and Tichenor, 1962). Thus, radiation-induced browning in orange juice during storage at room temperature could be considerably reduced by irradiation under a nitrogen atmosphere (Dharkar, 1964).

It was therefore of interest to carry out irradiation under gaseous atmospheres such as nitrogen and carbon dioxide and to compare with irradiation under air. Reduction, if any, in damage in the former case can best be seen by using radiation doses not tolerated by the mangoes under usual air. As observed earlier, 200 krads causes blackening and spoilage in mangoes (Fig. 2). Green mangoes, 1 dozen each, were therefore exposed to 200 krad under air or nitrogen atmosphere, as detailed under materials and methods. Fruit color and quality following irradiation were considerably better in the latter case than in the former. Thus, a photograph of typical fruits (Fig. 3) taken after 10 days of irradiation showed that the control unirradiated fruit (marked 1) was ripe and yellow, while that irradiated under air (marked 3) turned completely black;

Fig. 3. Typical photographs of Alphonso mangoes, unirradiated and irradiated under nitrogen and air, taken on the 10th day of storage. Fruits were given a dose of 200 krads under nitrogen (2) or air (3). Unirradiated mango (1) is ripe and orange yellow. Fruit 2, irradiated under nitrogen, is green in color and unripe. Fruit 3, irradiated under air, has turned black and is completely spoiled.

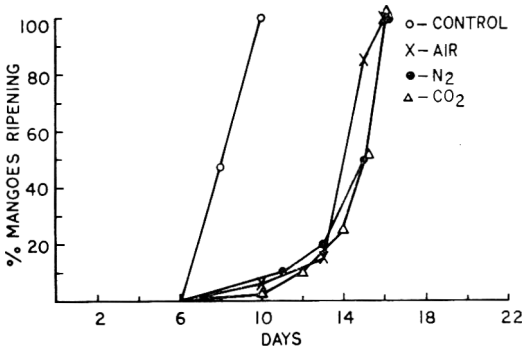

Fig. 4. Ripening of mangoes, unirradiated or irradiated under air, nitrogen, and carbon dioxide. Mangoes unirradiated and irradiated with the optimum dose of 25 krads under air, nitrogen, or carbon dioxide were stored at room temperature (25–30°C) to study delay in ripening. Ripening is seen to proceed at nearly the same rate when irradiated under the different gases.

the nitrogen-irradiated one (marked 2) was still green in color and apparently unaffected by the massive radiation dose. The inside of the fruit irradiated under air showed blackening, a white fibrous texture, and large gas fissures, whereas the unirradiated control had orange color with the taste of ripe fruit and the fruit irradiated under nitrogen had a normal appearance with the taste of unripe mango.

It seemed likely that, even with the optimum radiation dose of 25 krads, nitrogen atmosphere may exert a beneficial effect. In the next series of experiments, therefore, 15 fruits each were irradiated by 25 krads under air, nitrogen, or carbon dioxide. Data relating to ripening and physiological spoilage are respectively represented in Figs. 4 and 5.

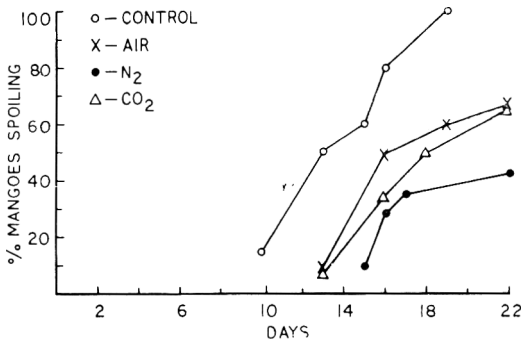

Fig. 5. Spoilage of mangoes, unirradiated and irradiated under various gaseous atmospheres. Percentages of mangoes spoiling after radiation treatment of 25 krads under air, nitrogen, and carbon dioxide are depicted. It is seen that irradiation under nitrogen shows minimum spoilage.

Ripening was delayed by irradiation, and the number of fruits ripening with time was nearly the same with the various atmospheres for irradiation (Fig. 4). However, there was a marked difference in the rate at which the fruits senesced, irradiation under nitrogen atmosphere causing minimum spoilage during storage at room temperature (Fig. 5). Carbon dioxide treatment was also slightly better than air-irradiation. Thus, as compared to spoilage of 100% on the 15th day for the control samples, spoilage was 67, 64, and 42% for air-, carbon-dioxide-, and nitrogen-irradiated samples, respectively, 22 days after storage at room temperature (25–30°C).

Unirradiated control mangoes and fruits irradiated by 25 krads under various atmospheres were evaluated organoleptically on the 12th day of storage and scored individually for color, flavor, texture, and taste. The average results of 5 independent determinations by a panel of 6 trained judges are shown in Fig. 6. Total acceptability ratings

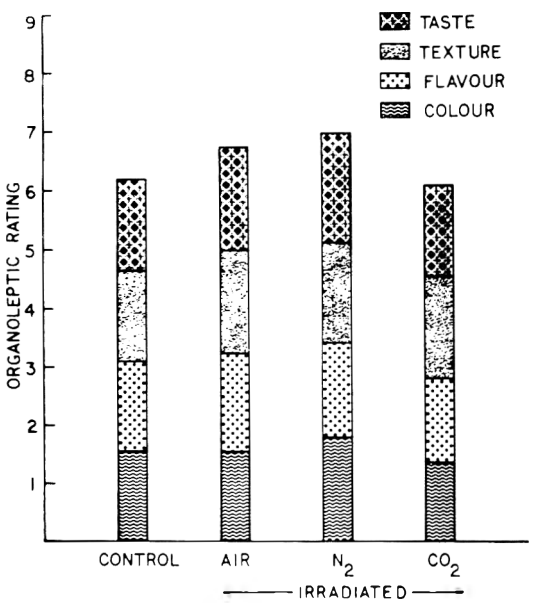

Fig. 6. Organoleptic qualities of unirradiated and irradiated mangoes (25 krads) under various gaseous atmospheres. Organoleptic tests were taken with untreated and treated fruits, on the 12th day of storage. The total organoleptic rating of 9 (like extremely) is divided into parameters of color, flavor, texture, and taste, each carrying the maximum rating of 2.25. The results show maximum acceptability for mangoes irradiated under nitrogen; carbon dioxide-irradiated fruits, although apparently acceptable, score the least.

for the different treatments varied from 6.1–7.0, and the deviations of scores by individual judges from the average were small. Although the differences in total scoring or in the scores for the various attributes, singly, were generally small between treatments, they were consistently so with the different judges and on varied lots of mangoes sampled on different days.

Total acceptability rating was higher for nitrogen-irradiated mangoes than for other treatments (nitrogen-irradiated, 7; control, 6.25; air-irradiated, 6.75; and carbon dioxide-irradiated, 6.12). An analysis of the scores for the various attributes also showed that nitrogen-irradiated mangoes rated highest for color and taste and had a high rating for flavor. Irradiation under nitrogen would therefore appear to diminish radiation damage, since there is least spoilage of fruits or change in organoleptic qualities. Irradiation under carbon dioxide, although effective in minimizing spoilage somewhat, results in low acceptability rating on storage. The lower rating for the control fruits is due to the fact that the test was done on the 12th day of storage, by which time the fruits in that group had become overripe.

Table 1 shows changes, during storage, in total carotenoids and ascorbic acid in the variously treated fruits. Ascorbic acid values are higher for nitrogen-irradiated mangoes than for other treatments and compare with those for controls. Since carotenoid development is associated with ripening, the delay in ripening due to irradiation is reflected in the lower values for total carotenoids. However, it is interesting to note their increased formation in fruits irradiated with nitrogen, especially during the latter period of storage.

Table 2 shows changes in acidity, total and reducing sugars, and starch durng ripening of control and irradiated mangoes. The delayed reduction in acidity under the stress of irradiation is as may be expected. Reducing sugars are more in the irradiated mangoes, although some delay is observed in total sugar formation and starch disappearance. The differences between the various treatments are small, however, especially after 5–8 days of storage.

Table 3 shows fruit pressure and skin

Table 1. Changes in ascorbic acid and total carotenoids during ripening of control and irradiated (25 krads) mangoes.

	Days in storage											
	0		2		5		8		12		15	
	Ascorbic acid	Carotenoids	Ascorbic acid	Carotenoids	Ascorbic acid	Carotenoids	Ascorbic acid	Carotenoids	Ascorbic acid	Carotenoids	Ascorbic acid	Carotenoids
	mg percent on fresh weight basis											
Unirradiated	91.5	0.67	89.6	0.57	86.3	—	65.6	1.35	63.1	9.1	70.2	10.5
Air-irradiated	91.5	0.67	80	0.64	83.5	—	55.1	0.55	47.3	5.0	61.3	7.9
Nitrogen-irradiated	91.5	0.67	85.6	0.79	82.6	—	60.5	1.69	68.5	10.1	64.3	9.8
CO_2-irradiated	91.5	0.67	83.5	0.54	74.6	—	68.1	0.41	54.0	2.24	52.8	7.67

Table 2. Changes in acidity, sugars, and starch during ripening of control and irradiated (25 krads) mangoes.

	Days in storage																							
	0				2				5				8				12				15			
	Acidity	Red. sugar	Total sugar	Starch	Acidity	Red. sugar	Total sugar	Starch	Acidity	Red. sugar	Total sugar	Starch	Acidity	Red. sugar	Total sugar	Starch	Acidity	Red. sugar	Total sugar	Starch	Acidity	Red. sugar	Total sugar	Starch
	Percent on fresh-weight basis																							
Unirradiated	3.3	1.3	4.5	13.8	2.9	1.9	7.0	10.5	1.2	2.4	14.4	3.8	0.3	2.4	13.8	1.5	0.2	2.2	14.6	0.6	0.2	1.9	12.8	0.6
Irradiated in air	3.3	1.3	4.5	13.8	2.2	1.7	9.4	7.3	2.1	3.3	11.4	4.8	0.5	2.9	14.7	1.0	0.3	2.8	13.8	1.0	0.3	2.2	12.8	0.7
Irradiated in nitrogen	3.3	1.3	4.5	13.8	2.1	2.0	9.4	6.8	2.1	3.4	11.3	5.1	0.5	3.4	14.0	0.8	0.2	1.9	14.0	0.6	0.2	2.2	13.8	0.5
Irradiated in CO_2	3.3	1.3	4.5	13.8	2.2	2.0	8.3	7.5	2.1	3.2	13.0	3.8	1.1	3.5	12.3	2.1	0.2	2.7	13.2	0.5	0.3	2.2	12.4	0.5

Table 3. Fruit-pressure, skin-color and other characteristics of control and irradiated (25 krads) mangoes.

	Days in storage											
	0		2		5		8		12		15	
	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage
	lbs		lbs		lbs		lbs		lbs		lbs	
Unirradiated	>30	Green (++++) Raw	>30	Green (++++) Raw	24.4	Green (++) inside yellow and juicy	12.3	Green (0) yellow skin firm yellow pulp; ripe taste and flavor	10.5	Green (0) yellow skin overripe taste and flavor	9.6	Completely overripe appearance
Irradiated In air	>30	Green (++++) Raw	>30	Green (++++) Raw	>30	Green (++++) inside whitish yellow; not juicy	19.8	Green (++) with yellow tinge. Pulp yellow, sour; no flavor developed	14.0	Yellowish green (+) Fully ripe flavor and taste	14.9	Yellowish green (+), orange pulp; firm texture, good flavor and taste
In nitrogen	>30	Green (++++) Raw	>30	Green (++++) Raw	>30	Green (++++) inside whitish yellow; not juicy	17.6	Green (++) with yellow tinge. Pulp yellow, sour; no flavor developed	12.6	Yellowish-green (+) Fully ripe flavor and taste	12.5	Yellowish green (+), orange pulp; firm texture, good flavor and taste
In CO_2	>30	Green (++++) Raw	>30	Green (++++) Raw	>30	Green (++++) inside whitish yellow; not juicy	23.3	Green (+++) with yellow tinge. Pulp more sour, no flavor developed	13.1	Green (++), inside, orange. No flavor developed, slightly firm	14	Yellowish green (++) Pulp orange with good flavor and taste

color during ripening under various conditions. The changes seen in both these attributes would tie up with the definite delay in ripening due to irradiation, with fruit pressure and green color being greater for treated fruits than for control fruits. The effect of irradiation on fruit texture would be of advantage under present handling and transport practices.

The foregoing data show that irradiation under different gaseous atmospheres delays the ripening of mangoes. From the changes in values for ascorbic acid, carotenoids, total sugars, and other fruit constituents, one may deduce a delay of about 2–3 days, although, judging from skin color and fruit firmness, a delay of even six days can be reckoned. Changes in skin color, however, are not apparently consistent and do not, at any rate, seem to correlate well with the stage of ripening of the irradiated mangoes, since fruits at eating-ripe stage still had green skin. Radiation effect on fruit skin seems to manifest itself more prominently in terms of inhibition in chlorophyll disappearance and carotenoid formation than in the ripening changes in the meat of the fruit. The higher fruit pressure of irradiated mangoes (Table 3), with green skin but ripe meat, also reflects on the hardness of the skin. It will be of interest to ascertain the effect of irradiation on the enzyme system responsible for the breakdown of the pectins in the middle lamella of the skin.

Production of ozone during irradiation is now well recognized. It may be expected that such a reaction, inside the fruit tissues, by irradiation of the oxygen present there, will have a harmful effect on the tissue cells (Kertesz and Parsons, 1963; Shah and Maxie, 1966). Replacement of intracellular oxygen by some other gas could thus be helpful in reducing radiation damage. Of the different gaseous atmospheres tested for irradiation, carbon dioxide showed minimum chlorophyll disappearance, but, from organoleptic qualities, spoilage data, and changes in ascorbic acid and carotenoids, nitrogen atmosphere would seem of definite advantage in minimizing the ill effects of radiation, with delay in ripening.

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Irradiation of Mangoes. II. Radiation Effects on Skin-coated Alphonso Mangoes

SUMMARY

The respiratory patterns of skin-coated and uncoated Alphonso mangoes, either unirradiated or irradiated under gaseous atmospheres like air, nitrogen, or carbon dioxide, were studied. The skins were coated with an emulsion made of an acetylated monoglyceride preparation. Skin-coated fruits show physiological damage presumably due to too much inhibition of respiration. This defect is rectified by a spurt in respiratory activity when fruits are irradiated in air or nitrogen. Irradiation in carbon dioxide, which also retards respiration, shows physiological damage which is reversible, the recovery of organoleptic qualities being possible to some extent. Suppression of respiration beyond an optimum level seems to be responsible for irreversible damage to the fruit. Organoleptic evaluation, analysis for fruit constituents, skin color, and pressure tests showed that storage life of the fruit can be increased by combining skin-coating with irradiation under either air or nitrogen.

INTRODUCTION

The storage life of fruits has been extended by radiation (Cooper and Salunkhe, 1963; Maxie *et al.*, 1966), cold storage (Porritt, 1964), gas storage (Workman, 1963; Edney, 1964), and coating the skin with various protective materials (Trout *et al.*, 1953; Narasingh *et al.*, 1963). A previous communication (Dharkar *et al.*, 1966) showed that radiation, preferably in nitrogen, could be employed to delay the ripening of mangoes. Radiation treatment, combined with gas or cold storage or with skin-coating, can be expected to add to the shelf life of fruits. Skin-coating does not entail big layouts, as in cold- or gas-storage installations, with their capital and recurring expenses, and therefore should be easier and more economical to use in combination with radiation. Skin-coating has been tried alone for shelf-life extension of mangoes (Subrahmanyam *et al.*, 1962).

The data presented here relate to the combined effects of radiation and skin-coating on storage life, organoleptic qualities, and control of respiration in Alphonso mangoes. It is shown that, unlike skin-coating which causes delayed ripening with loss of flavor and taste, combined use of irradiation under air or nitrogen results in additive delay in ripening, with retention of organoleptic attributes in the fruit.

MATERIALS AND METHODS

Unripe Alphonso mangoes, olive-green in color, picked on the previous day and trucked either from Ratnagiri or from Balsad, both within 250 miles of Bombay, were bought from the market, care being taken to select fruits of uniform maturity.

Skin-coating. The skin-coating emulsion for these studies was prepared from a commercial preparation of distilled acetylated monoglyceride, "Myvacet," type 7.00 (Distillation Products Industries, Rochester, N.Y., U.S.A.), 32 g; oleic acid, 2 g; triethanolamine, 2.5 g; and liquor ammonia, 4 g. The constituents were emulsified with 450 ml water in a Waring blender at 70°C. This gave an approximately 6% emulsion. The fruits were dipped in this emulsion and dried under a fan before use for irradiation studies.

Irradiation. Irradiation was done as described in the preceding paper (Dharkar *et al.*, 1966), on the second day of picking and immediately after skin-coating. Each time, a control batch was irradiated without skin-coating. All irradiation was at the optimum dose of 25 krads.

Respiration. Respiration was studied at a constant temperature of 24°C by absorption of the carbon dioxide evolved by the fruits during an hour each day in a closed system, in a known quantity of standard baryta solution kept in a Pettenkauffer tube, carbon-dioxide-free fresh air being made available to the fruits continuously by means of an aspirator. In any one experiment, the same fruit was used for this purpose each day. Carbon dioxide evolved was calculated after titration of excess baryta with standard HCl solution and expressed as mg CO_2/kg fruit/hr.

Irradiation under different gaseous atmospheres, organoleptic tests, analyses for fruit constituents, and fruit pressure determinations have all been described (Dharkar *et al.*, 1966).

All results represent the averages of at least three to four independent experiments, and the differences reported upon were consistent throughout.

RESULTS AND DISCUSSION

Typical respiration rates under different conditions of irradiation are shown in Figs. 1–4. The uncoated unirradiated control mangoes reached a climacteric peak (80 mg CO_2/kg/hr) in nine days (Fig. 1), starting from 15 mg CO_2 on the day of storage, and with the rate rising steeply with ripening from 7th day. In contrast to this, the skin-coated fruits did not show any rise in respiration above 5 mg CO_2/kg/hr for the first three days, on the 5th day reaching the initial respiration rate of 15 mg CO_2 of the uncoated fruit. Thereafter, there was a steady increase up to 35 mg on the 9th day, after which there was a slow decline. Skin-coating results in definite decrease of respiration over that of controls, throughout.

Respiration rates of mangoes irradiated under air and nitrogen are shown in Figs. 2 and 3, respectively. There was a sharp rise in respiration rate immediately after irradiation, reaching a maximum of 70 mg CO_2 24 hr after radiation treatment, after which respiration was reduced on the 2nd and 3rd days. In mangoes irradiated under nitrogen the reduction in respiration rate was much sharper and lower. However, the climacteric peak was reached on the 9th day,

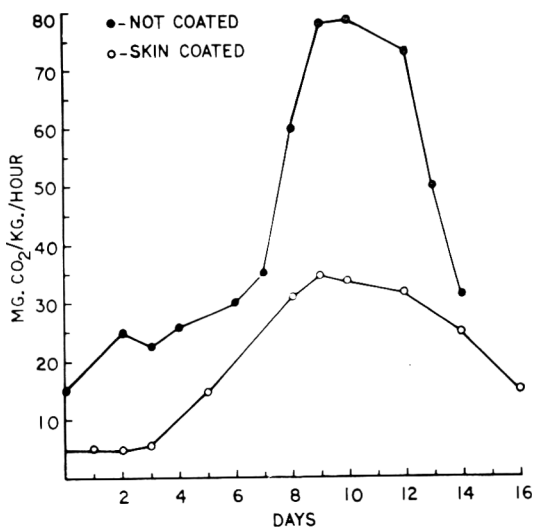

Fig. 1. Respiration of unirradiated mangoes with and without skin-coating. Respiration of a single marked mango, skin-coated or uncoated, was measured every day as described in text. Skin-coating suppressed respiration. The experiment was repeated three times, with similar trends in results.

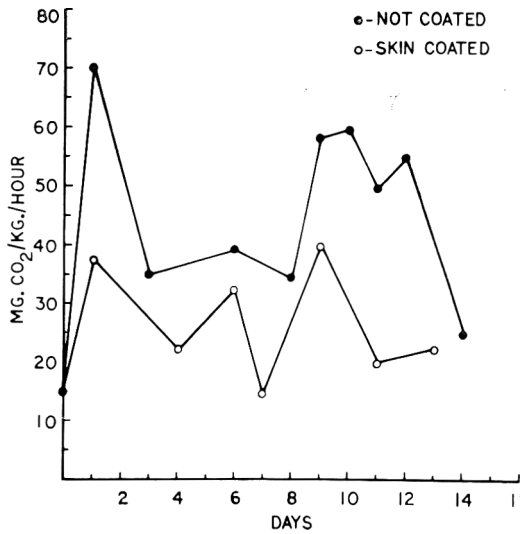

Fig. 2. Respiration of mangoes with or without skin-coating, irradiated under air. Enhanced respiration obtained under the stress of irradiation became subdued from the second day. This coincides with a decrease in total acidity and starch and increase in total sugars. These indices for enhanced ripening are more evident immediately after irradiation (*cf.* Dharkar *et al.*, 1966), some delay in ripening being observed subsequently. Skin-coated mangoes also show enhanced respiratory activity, though to a lesser extent.

as with the controls, although some fluctuations were seen in the irradiated mangoes. The skin-coated mangoes also showed rise in respiration after irradiation, but to a much smaller extent (30 and 37 mg CO_2 for nitrogen and air, respectively) than the uncoated, the climacteric peak being also on the same day as for the uncoated fruits.

The pattern of respiration with mangoes irradiated under carbon dioxide was different in that not much rise in respiration was seen immediately after irradiation (Fig. 4), the uncoated showing rise up to 30 mg CO_2, as compared to 15 mg for the skin-coated fruits. At the climacteric peak, which was about the same day as with other treatments, the respiration was no more than 50 mg CO_2 (uncoated), compared to 60 mg for irradiation under air or nitrogen.

Fig. 5 shows organoleptic ratings after 20 days' storage at room temperature of skin-coated mangoes irradiated (25 krads) under different gaseous atmospheres. The unirradiated control and those irradiated under air, nitrogen, and carbon dioxide, have respective total ratings of 2.9, 7.5, 7.5, and

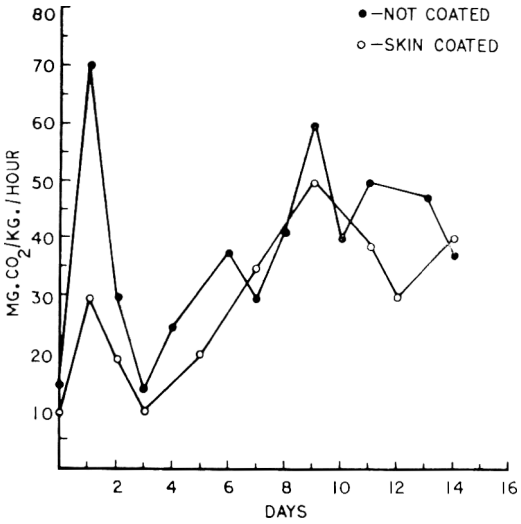

Fig. 3. Respiration of mangoes with or without skin-coating, irradiated under nitrogen. Enhanced respiration is observed immediately after irradiation in mangoes with and without skin-coating. Suppression of respiration in unirradiated skin-coated mangoes (Fig. 1), is offset by radiation treatment, conferring improved organoleptic qualities.

5.8. Ratings for color, flavor, texture, and taste are shown separately, the maximum rating for each being 2.25. The histogram shows the controls to be unacceptable, the

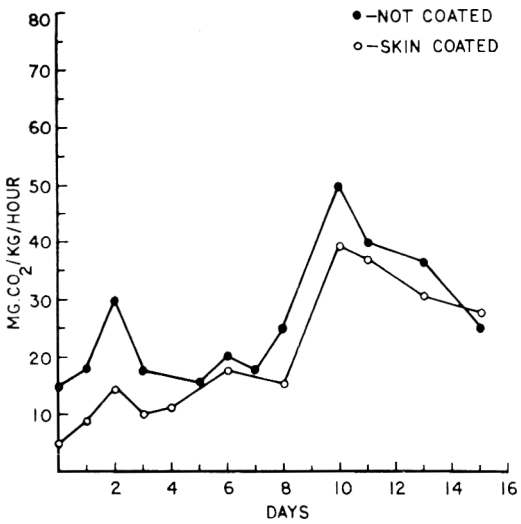

Fig. 4. Respiration of mangoes with or without skin-coating irradiated under carbon dioxide. There is a suppression of respiration by a carbon dioxide atmosphere. The initial spurt in respiratory activity due to irradiation is minimum, as compared to that in Figs. 2 and 3. Skin-coated mangoes irradiated under carbon dioxide atmosphere also show suppressed respiratory activity, resulting in impaired organoleptic qualities.

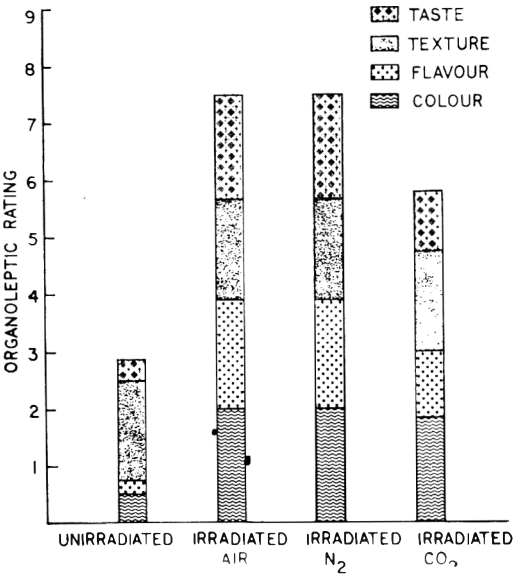

Fig. 5. Organoleptic tests for skin-coated mangoes stored for 20 days at ambient temperatures (25–30°C) after irradiating (25 krads) under different gaseous atmospheres. The maximum rating of 9 (like extremely) was further divided for the parameters of color, flavor, texture and taste, each carrying a maximum of 2.25. Results show the unirradiated skin-coated controls to be unacceptable, compared to the highly acceptable mangoes irradiated under air or nitrogen. Irradiation under carbon dioxide reduces organoleptic qualities to some extent.

irradiated ones under air or nitrogen highly acceptable, and the carbon-dioxide-irradiated fruits intermediate. The controls developed off-flavor and -taste right from the beginning of the experiment, whereas those irradiated under carbon dioxide had pronounced off-flavor and taste in the beginning of the experiment but decreasing during storage, with a corresponding increase in acceptability. Skin-coated mangoes irradiated under air or nitrogen did not develop any off-flavor throughout the experiment, and were quite acceptable. It seems therefore that physiological degradation in skin-coated control mangoes can be offset by irradiation under either air or nitrogen.

Immediately after irradiation under air or nitrogen, a respiratory peak of 35 mg CO_2 was reached, whereas the control skin-coated mangoes did not show respiration above 5 mg CO_2 for the first three days, after which the rate reached 15 mg CO_2 on the 5th day; fruits irradiated under

carbon dioxide also did not show high respiration for the first five days, after which it increased (Figs. 1–4). Organoleptic changes closely followed this trend in respiratory pattern. Mangoes which had an average respiration less than optimum for the first five days, showed degradation in taste and flavor. There is thus a possibility that organoleptic changes are profoundly influenced by inhibition of respiration in the beginning of the storage period, this latter is offset by irradiation, with corresponding improvement in taste and flavor. Trout *et al.* (1953) also found that an off-flavor in waxed apples is produced by anaerobic respiration, resulting from low internal oxygen content caused by the skin-coating. Jurin and Karel (1963) also found anaerobic respiration in apples stored in an atmosphere with less than 3.5% oxygen.

Physiological losses in control and skin-coated mangoes during 12 days are shown in Fig. 6. Untreated fruits had a loss of 12.5%, while skin-coated fruits, with and without irradiation, had respective losses of 8% and 7.8%. Losses due to transpiration and respiration are minimized by skin-coating and irradiation, with consequent increase in storage life.

Table 1 shows the effect of irradiation under different gaseous atmospheres on the ripening process of skin-coated mangoes. Control fruits did not show ripening even on the 15th day of storage, as defined by skin color, taste, and flavor. There was also off-flavor development in the pulp throughout the experiment. Mangoes irradiated under nitrogen and air, on the other hand, ripened well by the 15th day, and had good taste and flavor even on the 20th day. Fruits irradiated under carbon dioxide behaved similarly to the controls, but their flavor and taste improved considerably from 15th to 20th days of storage, though the rating obtained was not as high as for fruits irradiated in either air or nitrogen.

Table 2 shows changes in acidity, sugars, and starch in skin-coated control and irradiated mangoes. There is good conformity in the values as reported in Tables 1 and 2. Acidity, total sugars, and starch in control mangoes show that ripening does not

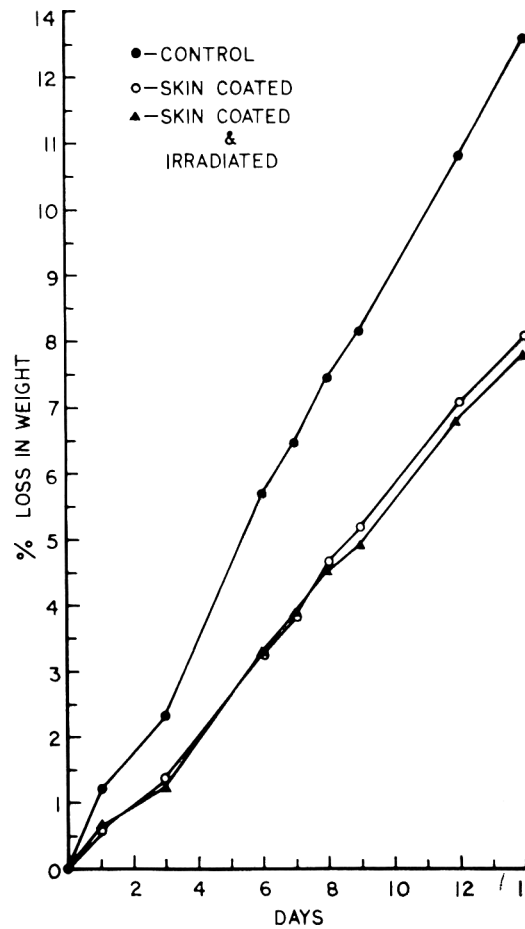

Fig. 6. Physiological losses in skin-coated mangoes, with or without irradiation, compared to controls without either treatment. Higher losses in weight of control mangoes result from higher rates of respiration and transpiration than in mangoes that were either skin-coated or skin-coated and irradiated.

progress satisfactorily even on the 20th day of storage. The changes for fruits, irradiated under air or nitrogen, on the other hand, show ripening from the 15th day onward. With uncoated mangoes, changes in acidity, sugars, and starch point to ripening during the 9th and 10th days of storage (Dharkar *et al.*, 1966). Thus, a combination of skin-coating and irradiation shows an increase in storage life. It would seem that physiological damage due to suppression of respiratory enzymes caused by skin-coating can be overcome by the spurt in respiratory activity resulting from radiation. It is of interest to note that skin-coating helps keep the re-

Table 1. Fruit pressure, skin color, and other characteristics of control and irradiated (25 krads) skin-coated mangoes, stored for various periods at room temperature (25–30°C).

	0 days		2 days		5 days		8 days	
	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage
Unirradiated	>30	Green (++++) Raw	>30	Green (++++) Raw	>30	Green (++++) Whitish pulp; raw : off-flavor developed	23.3	Green (++++) Yellowish white pulp; off-flavor
Irradiated in air	>30	Green (++++) Raw	>30	Green (++++) Raw	26.5	Green (++++) Whitish pulp; raw ; no off-flavor	20	Green (+++) Yellow pulp; no off-flavor
Irradiated in nitrogen	>30	Green (++++) Raw	>30	Green (++++) Raw	26.5	Green (++++) Whitish plup; raw ; no off-flavor	19.5	Green (+++) Yellow pulp; no off-flavor
Irradiated in carbon dioxide	>30	Green (++++) Raw	>30	Green (++++) Raw	>30	Green (++++) Whitish pulp; raw ; off-flavor developed	24.2	Green (++++) Whitish-yellow pulp ; off-flavor

Table 1—Continued

	12 days		15 days		20 days	
	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage	Fruit pressure	Ripening stage
Unirradiated	13.1	Green (+++) Whitish yellow pulp; starchy taste; off-flavor	13	Green (+++) Yellow (+) Sour; off-flavor	9	Green (+) Yellow (++) Yellow pulp; off-flavor
Irradiated in air	16	Green (++) Yellow (++) Good mango flavor; orange pulp	12.6	Green (+) Yellow (++) Good mango flavor; orange plup	9.6	Green (+) Yellow (++) Orange pulp; slightly over-ripe
Irradiated in nitrogen	12	Green (++) Yellow (++) Good mango flavor; orange plup	10.5	Green (+) Yellow (++) Good mango flavor; orange pulp	9.5	Green (+) Yellow (++) Orange pulp; slightly over-ripe
Irradiated in carbon dioxide	14.8	Green (+++) Yellow (+) Whitish yellow pulp; off-flavor	14	Green (++) Yellow (+) Yellow pulp; little off-flavor	10.9	Green (+) Yellow (++) Yellowish-orange pulp; very little off-flavor

Skin-coated unirradiated control mangoes do not ripen satisfactorily, and evince off-flavor throughout the experimental period. This off-flavor in the coated mangoes is offset by irradiation either under air or nitrogen, irradiation under carbon dioxide showing some off-flavor development in the beginning of the experiment.

Table 2. Changes in acidity, sugars, and starch of skin-coated mangoes irradiated in different gaseous atmospheres, expressed as percent on fresh weight basis.

Days in storage		Un-irradiated	Irradiated in air	Irradiated in nitrogen	Irradiated in carbon dioxide
0	Acidity	3.7	3.7	3.7	3.7
	Red. sugar	1.02	1.02	1.02	1.02
	Total sugar	2.42	2.42	2.42	2.42
	Starch	13.76	13.76	13.76	13.76
2	Acidity	3.6	2.8	3.2	3.2
	Red. sugar	1.15	1.41	1.32	1.39
	Total sugar	2.28	4.24	6.1	5.28
	Starch	12.14	11.81	9.84	11.38
5	Acidity	2.8	2.0	2.0	3.7
	Red. sugar	2.08	2.47	2.75	1.51
	Total sugar	8.0	11.34	10.95	5.81
	Starch	—	3.65	5.43	6.64
8	Acidity	1.9	1.6	1.5	1.9
	Red. sugar	2.67	2.95	2.82	3.14
	Total sugar	11.34	11.4	15.0	11.75
	Starch	4.85	—	3.3	3.44
12	Acidity	2.0	1.2	—	1.04
	Red. sugar	3.34	4.17	2.92	3.97
	Total sugar	12.75	15.35	14.23	15.35
	Starch	2.74	1.02	0.88	1.42
15	Acidity	1.2	0.6	0.5	0.8
	Red. sugar	4.1	3.63	4.16	4.46
	Total sugar	14.15	15.74	14.77	14.34
	Starch	2.13	0.92	0.66	0.59
20	Acidity	1.03	0.2	0.13	0.2
	Red. sugar	4.16	2.54	2.59	3.6
	Total sugar	13.93	14.77	13.36	13.93
	Starch	0.94	0.63	0.24	—

Skin-coated mangoes, treated as shown, were stored at room temperature (25–30°C) to study the ripening changes. Data show that ripening does not proceed satisfactorily in skin-coated unirradiated mangoes, those irradiated showing satisfactory ripening from the 15th to the 20th day of storage. The combination of skin-coating and irradiation confers more storage life to the fruit than irradiation alone.

spiratory activity of irradiated mangoes to the optimum, reducing physiological losses and increasing storage life.

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Autoxidation of Methyl Linoleate in Freeze-Dried Model Systems. I. Effect of Water on the Autocatalyzed Oxidation

SUMMARY

Oxidation of methyl linoleate was studied in a freeze-dried model system based on microcrystalline cellulose. Oxidation was followed manometrically in samples adjusted to various water activities ranging from approximately 0 to approximately 0.6.

Water was found (as determined from induction period and rate data) to have an inhibitory effect on the oxidation reaction, varying with water activity up to values of 0.5.

Evaluation of the rate data indicates that the inhibitory effect of water is most pronounced in the initial stages of oxidation, including the period during which the hydroperoxide decomposition follows monomolecular decomposition kinetics. Possible interpretations of the observed water effect and its significance to the general problem of lipid oxidation in dehydrated foods are discussed.

INTRODUCTION

One of the major problems in research on dehydrated foods is the prevention of deleterious changes during storage. It is known that many of these changes may be minimized by maintaining the moisture content at a very low level. Under these conditions, however, other changes, particularly those due to autoxidation of fat, are accelerated.

Removing water from a food by freeze-dehydration results in a product that has a porous, sponge-like matrix. The porosity of the dehydrated food permits oxygen ready access to the components of the food, thereby facilitating oxidative changes. Of primary importance is the autoxidation of lipids. The changes in the properties and acceptability of foods due to lipid oxidation are well known. Water is known to retard lipid oxidation in many dehydrated and low-moisture food products (Stevens and Thompson, 1948; Marshall *et al.*, 1945; Martin, 1958; Matz *et al.*, 1955).

Several hypotheses have been advanced to explain the protective effect of water in retarding lipid oxidation. The most important are:

1) That water has a protective effect due to retardation of oxygen diffusion (Halton and Fisher, 1937).

2) That water lowers the effectiveness of metal catalysts such as copper and iron (Uri, 1956).

3) That water is attached to sites on the surface, thereby excluding oxygen from these sites (Salwin, 1959).

4) That water promotes non-enzymatic browning, and browning can result in the formation of antioxidant compounds (Lea, 1958).

5) That water forms hydrogen bonds with hydroperoxides and retards hydroperoxide decomposition.

The effects of water observed in studies on foods do not support any single hypothesis covering its antioxidant activity. Since a food is a heterogeneous system containing both pro-oxidant and antioxidant substances, interpretation is complicated. A model system was devised, therefore, to study the effect of water under more controlled conditions. This model system was considerably less complex than a dehydrated food, even though it was not possible to eliminate all trace contaminants.

MATERIALS AND METHODS

The major components used in the model system were methyl linoleate (Chromatographic standard, Mann Research Laboratories), microcrystalline cellulose (Avicel, American Viscose Company), and water.

Methyl linoleate. Methyl linoleate was further purified by urea adduct formation in methanol. Methyl linoleate was dissolved in methanol, and

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urea was added under constant stirring. The ratio used was 1:4:6 lipid (g) : urea (g) : methanol (cc). The resultant paste was placed in a refrigerator for 2 hr to facilitate adduct formation. Excess methanol was then filtered off and the adduct washed with methanol saturated with urea (1 L saturated MeOH per 10 g linoleate). The wet adduct was then allowed to stand for an additional 2-hr period under refrigeration, and the washing procedure was repeated.

The linoleate was recovered by partitioning the product between water and benzene in a separatory funnel. The benzene layer was washed eight separate times with 200 cc fresh water per 10 g linoleate. After the third partitioning step, the benzene-linoleate was washed with a *0.1M* citric acid solution to chelate any heavy metals present.

The benzene was then removed in a rotary evaporator, and the residue was subjected to high-vacuum distillation. The center cut of the linoleate (bp 125–130°C, still-pot pressure not measured) was collected for further preparation. Care was exercised to avoid oxidation and/or contamination during purification.

Thin-layer chromatography and the measurement of diene conjugation were used to check the absence of peroxides and other contaminants. The methods used were essentially those reported by Privett and Blank (1962). Further evidence of purity was obtained by gas chromatographic analysis of the ester after purification to assure the absence of contamination by methyl esters other than linoleate. A flame ionization gas chromatograph was used, and the procedure was calibrated with standard mixtures of methyl esters of fatty acids.

Preparation of the model system. The model system was prepared as follows: Methyl linoleate, 1 g, was mixed with cellulose, 6 g, and 30 g water. The resultant slurry was mixed for 10 min in a Servall Omni-mixer. The mixing cup was equipped with Teflon bearings to prevent contamination by lubricant and was modified by attaching a Swagelock fitting to the base of the cup so that the gel-like material produced by mixing could be extruded with a Teflon plunger directly into reaction flasks. Metal contamination from the cup was minimized by a protective coating of silicone (Siliclad, Will Scientific Company). Following extrusion of the paste into reaction flasks, the flask contents were frozen in liquid nitrogen and the samples freeze-dried at 100 μ for 48 hr. Sample size varied from 7 to 10 g wet material.

Control of water content. After samples were freeze-dried, they were humidified in vacuum desiccators over salt solutions adjusted to specific water activities (Rockland, 1960). This was done by evacuating the desiccator and allowing the sample to equilibrate for 2 hr. Control samples were maintained in the dry state but were treated in the same manner and were held over magnesium perchlorate. After equilibration, the vacuum was broken with air adjusted to the proper humidity by passing through humidification flasks containing salt solutions. At this time, samples were removed for moisture analysis based on the determination of vapor pressure of water over the samples (Stitt, 1958). The apparatus used is described by Karel and Nickerson (1965).

Oxidation. The Warburg reaction flasks containing the model systems were attached to Warburg manometers, and the oxidation was conducted under air at 37°C in a water bath. Standard manometric procedures were followed for calibration of the equipment and calculation of results (Umbreit *et al.*, 1964). No carbon dioxide was detected when the atmosphere over the reaction mixture was checked for possible carbon dioxide production using gas chromatographic analysis. To eliminate the possibility of oxygen depletion, the reaction flasks were flushed at suitable intervals with air having the desired relative humidity.

The initial degree of oxidation was determined by extracting the freeze-dried material with methanol and measuring the UV absorption at 233 mμ. Following the procedure of Privett and Blank (1962) the UV absorption data were used to determine diene conjugation, which is known to give good correlation with the degree of oxidation of linoleate. The extraction procedure was previously checked and found to be both efficient and mild enough that no oxidation takes place during the extraction.

Evaluation of results. The progress of oxidation was followed by manometric measurement of oxygen absorbed. The resulting curves of oxygen absorbed vs. time showed, in all cases, a curve with the typical autocatalytic shape which results from the kinetics of a free radical reaction (Bolland, 1949; Bateman, 1954). The progress of oxidation is characterized by 3 distinct periods.

1) An initial induction period.

2) A subsequent period during which the hydroperoxides that have been formed decompose by a monomolecular reaction occurring autocatalytically, or, as proposed by Uri (1961), catalyzed by trace amounts of the heavy-metal transition elements such as cobalt, copper, and iron.

3) A period during which the hydroperoxides decompose by a bimolecular reaction. This occurs at high hydroperoxide concentrations, probably because of bimolecular association through hydrogen bonding. This association is supported by the spectrophotometric evidence of Bateman (1954) for ethyl linoleate and by Walling and Heaton (1965) for t-butyl hydroperoxide. Semenov (1959) concluded on the basis of thermodynamic consid-

Initiation

$$ROOH \xrightarrow[k_i]{[M]} RO^{\cdot} + {}^{\cdot}OH \qquad \text{Monomolecular}$$

$$\text{or } 2ROOH \xrightarrow[k_{ii}]{[M]} R^{\cdot} + RO_2^{\cdot} + H_2O \qquad \text{Bimolecular}$$

Propagation

$$R^{\cdot} + O_2 \xrightarrow[k_o]{} RO_2^{\cdot}$$

$$RO_2^{\cdot} + RH \xrightarrow[k_p]{} ROOH + R^{\cdot}$$

Termination

$$RO_2^{\cdot} \xrightarrow{k_t} \text{non-radical end products}$$

Legend:

ROOH	hydroperoxide concentration
$R^{\cdot}$, $RO^{\cdot}$, $RO_2^{\cdot}$	free radical intermediates
[M]	trace metal concentration
RH	substrate concentration
O_2	oxygen present as free oxygen
k_i	monomolecular initiation rate constant
k_{ii}	bimolecular initiation rate constant
k_o, k_p	propagation rate constants
k_t	termination rate constant
H_2O	water

Fig. 1. Equations representing bimolecular reactions when oxygen is not limiting.

erations that hydrogen bonding is a probable step in bimolecular decomposition. The bimolecular reaction can also occur in a metal-catalyzed process (Ingold, 1962).

The equations representing these reactions are presented in Fig. 1 for the case where oxygen is not limiting.

In order to discern on a molecular level the mechanism by which water inhibits the autoxidation reaction, the data were treated kinetically. Fig. 2 represents the kinetics for the initial stage of the catalyzed oxidation when monomolecular decomposition predominates. In this case, the individual rate constants k_p, k_t, and k_i, as well as the trace metal concentration term (M) and the substrate concentration term (RH), were combined to form an overall monomolecular rate constant, K_M. The inclusion of the metal and substrate terms is based on the assumption that catalyst inactivation and substrate depletion are negligible at this extent of oxidation. In the case of autocatalytic oxidation the metal term M is absent, but the rate follows the same equation. Integration of the rate equation shows that a plot of the square root of either the oxygen absorbed or of the hydroperoxide content vs. time gives a straight line. The slope of this line would be proportional to K_M.

Since the initial extent of oxidation varied and the beginning of the monomolecular period could not be measured, a composite induction time was measured from the graph of the square root of oxygen absorbed vs. time. This was taken as the time to reach 400 μL oxygen absorbed per g of methyl linoleate or a level of oxidation of approximately 0.5% (5 mM of peroxide/M substrate) in cases where the initial extent of oxidation was very low. In other cases the time to reach 1% oxidation was taken as the composite induction time.

The kinetics for the final period of oxidation, the bimolecular decomposition period, are summarized in Fig. 3. In this case, since substrate depletion becomes significant, it was corrected for by multiplying the initial substrate concentration (RH_0) by the fraction of unoxidized ester $(1 - y)$ to give the substrate concentration at any time.

$$\frac{-d[O_2]}{dt} = \frac{d[ROOH]}{dt} = \frac{k_p}{[2k_t]^{1/2}} k_i^{1/2} [M]^{1/2} [RH] [ROOH]^{1/2}$$

$$K_M = \frac{k_p}{[2k_t]^{1/2}} k_i^{1/2} [M]^{1/2} [RH]$$

$$\text{Set: } y = [ROOH] \cong [O_2]$$

$$\frac{dy}{dt} = K_m y^{1/2}$$

Integrating:

$$y^{1/2} = [O_2]^{1/2} = [ROOH]^{1/2} = \frac{K_m}{2} t$$

Legend:

$[O_2]$ = concentration of absorbed ogygen which has reacted

K_m = overall monomolecular rate constant

Fig. 2. Kinetics for the initial stage of the catalyzed oxidation when monomolecular decomposition predominates.

$$\frac{-d[O_2]}{dt} \cong \frac{d[ROOH]}{dt} = \frac{k_p}{(2k_t)^{1/2}} k_{i1}^{1/2} [RH] [ROOH]$$

Set:

$$y = [ROOH] \cong [O_2]$$

$$[RH] = [RH_o] [1 - y]$$

$$K_B = \frac{k_p}{(2k_t)^{1/2}} k_{i1}^{1/2} [RH_o]$$

$$\therefore \frac{dy}{dt} = K_B (y) (1 - y)$$

Integrating:

$$\ln \frac{y}{1 - y} = K_B t$$

Legend:

$[RH_o]$ = initial substrate concentration
K_B = overall bimolecular rate constant

Fig. 3. Kinetics of the initial stage of the catalyzed oxidation when bimolecular decomposition predominates.

This method assumes that each mole of oxygen absorbed forms a mole of hydroperoxide, which is reliable for this level of oxidation. Combining this term (RH_0) with the individual rate constants gives the overall bimolecular rate constant, K_B. Integration of the rate equation shows that a semilog plot of $y/1 - y$, the corrected oxygen absorbed per mole of substrate left vs. time, should give a straight line. The slope of this line is then equal to K_B. This analysis can apply to either catalyzed or autocatalytic autoxidation.

RESULTS AND DISCUSSION

The freeze-dried system described above was used to study the retardation effect of water on the autoxidation of methyl linoleate. Oxygen absorption was measured at water activities of less than 0.015 (hereinafter referred to as "dry"), 0.145, 0.185, 0.277, 0.425, 0.488 and 0.601. All activities were determined experimentally on duplicate samples prepared from the same starting materials. Duplicate determinations agreed to within ±0.005. The corresponding moisture contents may be obtained from Fig. 5.

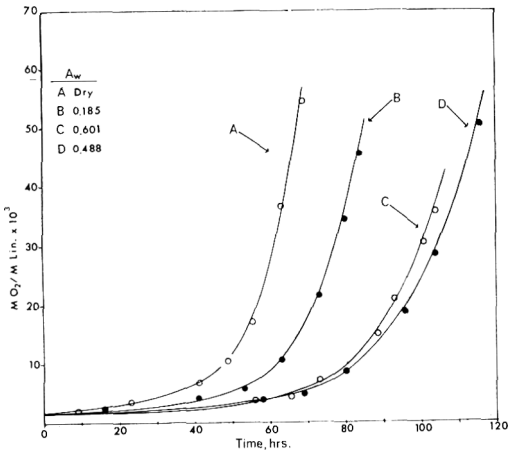

Fig. 4. Oxygen absorption by model system at various relative humidities.

The effect of increasing the water activity on the oxidation of methyl linoleate in the freeze-dried system is shown in Fig. 4, with curves representing mean values of the absorbed oxygen for duplicate reaction flasks, plotted against time. This plot shows the early stage of oxidation up to a value of 45mM of oxygen per mole of linoleate, which corresponds to a level of oxidation of 4–5%. It can be seen that water effectively retards oxidation, and that the extent of this retardation increases with increasing activity. However, the effectiveness of water appears to level off at higher activities.

Previously, the isotherm for the freeze-dried system was determined; and the monolayer value for water was calculated using the B.E.T. equation. The isotherm as shown in Fig. 5 is of a typical sigmoid type. The monolayer coverage was found to occur at an activity of 0.195 and at a moisture content of 2.66 g of water per 100 g dry solids. The activity of 0.185 in Fig. 4 is close to that calculated for the monolayer, and shows a considerable effect over the dry samples.

The effects of water activities above and below the monolayer are shown in Fig. 6. The curves in Fig. 6 represent average values for duplicate samples. The dry control and water activity of 0.185, the same curves shown in Fig. 4, are presented for

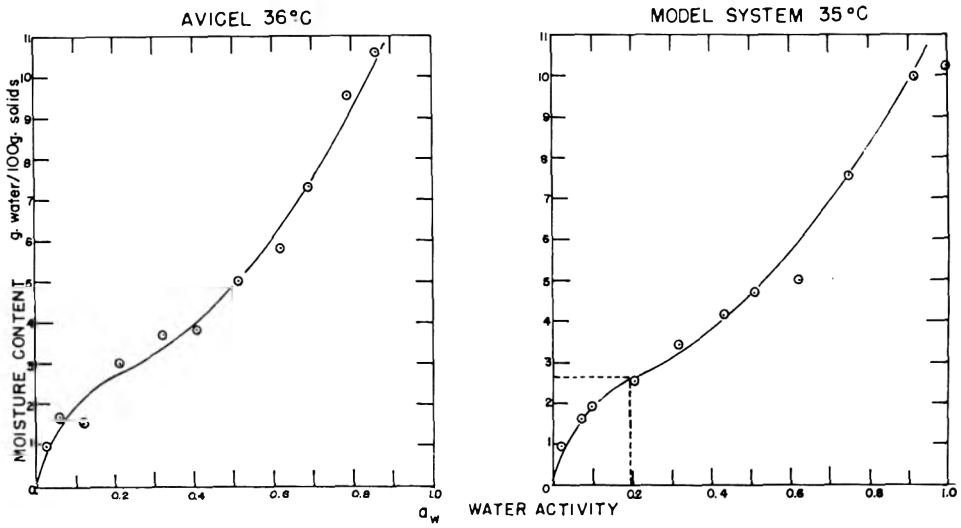

Fig. 5. Water adsorption isotherms for Avicel and the model system

comparison. A decrease in activity from the approximate monolayer value of 0.185 to an activity of 0.145 results in a slight decrease in the effect. At a value of 0.277 a significant increase occurs over the approximate monolayer value.

In the more advanced stage of linoleate oxidation (above approximately 1% oxidation), the initiation of the chain reaction is due to bimolecular decomposition of hydroperoxides. Kinetic plots for the bimolecular period of oxidation based on the equations presented in Fig. 6 are shown in Fig. 7.

The log of oxygen absorbed in moles of oxygen per mole of linoleate ($y/1-y$) is plotted against time for the dry controls, a water activity of 0.185, and an activity of 0.601 with duplicates for each activity plotted to show the variation occurring between identical samples. It is noted that the effect of water is to displace the lines along the time axis. The magnitude of this displacement increases with increasing water activity, and the slopes of these lines are proportional to the bimolecular rate constant, K_B. Table 1 shows the value of K_B for all activities studied.

The lack of significant differences in K_B indicates that water has no significant effect in the bimolecular period in the uncatalyzed model system tested. The lack of a water effect in the bimolecular period indicates that the effect of water is mainly in the initial stage of autoxidation, i.e. up to approximately 10m*M* of oxygen, or the 1% level of oxidation. For this case we have taken

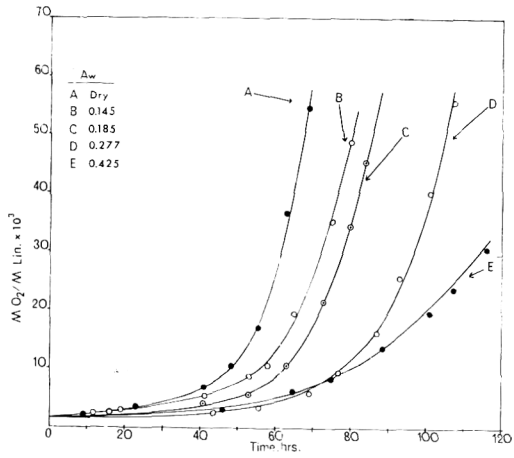

Fig. 6. Oxygen absorption by model system at selected relative humidities.

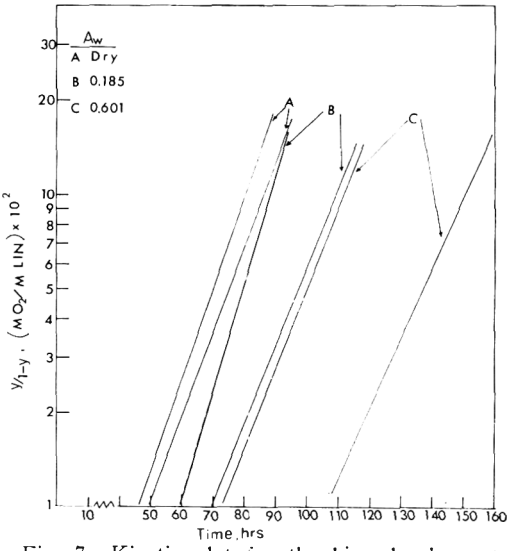

Fig. 7. Kinetic plot for the biomolecular rate period.

Table 1. Effect of humidification on bimolecular rate constants.

Water activity	$K_B \times 10^2$ (hr^{-1})	
	Sample 1	Sample 2
Dry	5.8	6.9
0.145	6.3	6.8
0.185	6.0	7.9
0.277	5.1	6.8
0.425	3.3	...
0.488	5.0	5.2
0.601	4.9	6.3

the time required to reach the 1% level of oxidation as an index of the magnitude of the water effect as shown in Table 2. All activities were tested on duplicate samples, and the values for the water activities of 0.145 and 0.277 are the same for both duplicates.

The time required to reach the 1% level of oxidation increased with increasing activity. It is apparent that there is considerable variation between duplicates and that the variation increases with increasing activity.

To compare the differences between the dry controls and the humidified samples, the ratio of time needed by the dry samples to reach 1% oxidation, to the time required by the humidified to reach 1% oxidation, has been calculated. Thus, values smaller than unity indicate prolongation of the true induction period, a decreased rate of oxidation in the early stage of oxidation, or both. The results show lowering of the ratios with increasing water activity, and, therefore, an inhibitory effect increasing with water activity.

Table 2. Effect of humidification on time required to reach 1% oxidation.

Water activity (A_w)	t (hr) to 1% oxidation	$\frac{t_d}{t_h}$
Dry	45.0	1.0
Dry	48.1	...
0.145	55.5	0.84
0.185	58.3	0.80
0.185	70.3	0.66
0.277	77.8	0.60
0.425	75.9	0.61
0.425	84.7	0.55
0.488	76.0	0.61
0.488	93.6	0.50
0.601	73.2	0.64
0.601	103.2	0.45

t_d = time required by "dry" samples to reach 1% oxidation.

t_h = time required by humidified samples to reach 1% oxidation.

CONCLUSIONS

Water has an antioxidant effect on methyl linoleate in our model system. The protective effect of water is operative at an activity below the calculated monolayer and increases with increasing water activity up to a value of approximately 0.5, where a leveling-off occurs. The increasing variability between duplicates with increasing activity causes this value to be somewhat uncertain.

Only tentative interpretations of the observed effects can be advanced at this time. However, hydrogen bonding of hydroperoxides with water, with a resultant prevention of hydroperoxide decomposition by bimolecular reaction, may be operative in this system. As a result, the amount of hydroperoxide available for initiation would be less than that predicted from the total oxygen absorbed, since a fraction of the hydroperoxides is concentrated at a lipid-water interface. The removal of a fraction of the hydroperoxides would be most effective in early stages of oxidation, which is in agreement with our experimental results. Work on this aspect is currently in progress.

The lack of an effect in the later stages of oxidation (bimolecular phase), at least in the system used in the present study (in which only trace metal contaminants were present), makes it probable that metal inactivation is not the only, and probably not the major, effect. Also, since there were no browning precursors present, it is evident that promotion of browning by water is not necessary to retard autoxidation.

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Autoxidation of Methyl Linoleate in Freeze-Dried Model Systems. II. Effect of Water on Cobalt-Catalyzed Oxidation

SUMMARY

The oxidation of methyl linoleate catalyzed by various salts of cobalt was studied in a model system based on microcrystalline cellulose. The freeze-dried model system was adjusted to various water activities, and the effect of water on the oxidation kinetics was determined using manometric measurements and measurements of diene conjugation.

It was found that water had an inhibitory effect on the metal-catalyzed oxidation of the fatty ester, as well as on oxidation in the absence of added metals. The kinetics of the reactions were evaluated in terms of the previously established hydroperoxide decomposition mechanisms. The effect of water on the metal-catalyzed oxidation was found to exist in the monomolecular decomposition period as well as in the more rapid phase of the reaction, during which the hydroperoxide decomposition is known to follow bimolecular decomposition kinetics.

The inhibition of the reaction by water is interpreted as due to deactivation of added, as well as of originally present, metal catalysts by hydration of the coordination shells; and also possibly as due to hydrogen bonding between hydroperoxides and water, and therefore to interference with the normal bimolecular decomposition reaction.

INTRODUCTION

Part I of the present series (Maloney *et al.*, 1966) reported studies of the oxidation of methyl linoleate in a freeze-dried model system based on microcrystalline cellulose. Oxidation was followed manometrically in samples adjusted to various water activities ranging from approximately 0 to approximately 0.6.

Water was found (as determined from induction period and rate data) to have an inhibitory effect on the oxidation reaction. The magnitude of this inhibitory effect was found to depend on water activity up to values of 0.5.

Evaluation of the rate data indicated that the inhibitory effect of water was most pronounced in the initial stages of oxidation, including the period during which the hydroperoxide decomposition follows monomolecular decomposition kinetics. The effect could be explained by hydrogen bonding of hydroperoxides with water. The specific objective of the present study was to determine the effect of water on the catalytic activity of salts of cobalt added to the model system, as well as to trace metals present in the system as contaminants.

MATERIALS AND METHODS

Model system. The model system was described in detail by Maloney *et al.* (1966).

Humidification. One half of each of the freeze-dried samples was placed in a desiccator containing magnesium perchlorate; the other half was placed in a desiccator above a saturated salt solution of known water activity. The desiccators were evacuated, and the samples equilibrated for 2–3 hr at the given relative humidity and then removed after breaking the vacuum with air at the proper relative humidity.

Oxidation. Oxygen absorption was measured at 37°C with Warburg manometers. The initial extent of oxidation was determined by measurement of diene conjugation at 233 mμ according to the method of Privett and Blank (1962).

Metal catalysts. Cobalt stearate, acetate, and nitrate (reagent-grade) were normally added in aqueous solution during the mixing step in the preparation of the system. In one test, the metal salt was added in a separate step by mixing it initially with the cellulose to form a catalyst-impregnated support. This was done by adding the cobalt salt in aqueous solution to the cellulose, mixing thoroughly at high speed, and then freeze-drying the cellulose to remove the water. This impregnated cellulose was then used in making the model system, the metal having been added in the proper concentration. This method was compared with the other method described above. Studies of water adsorption by the catalysts showed that, at the humidities used, the salts were completely hydrated. In the dry state, cobalt acetate has no water of hydration, and cobalt nitrate 3 moles of water per mole of salt.

Metal analysis of model systems. For each

model-system preparation, duplicate samples were ashed at 450°C for 18–24 hr. The ash was dissolved in dilute acid, and iron, copper, and cobalt analyses were performed on the solution.

1) Iron analysis. The standard colorimetric procedure of Sandell (1950) with 1-10-o-phenanthroline was used. The optical density for the ferrous ion complex was measured at 510 mμ on a Coleman Jr. Spectrophotometer, which was also used for the other metal tests.

2) Copper analysis. Copper was determined by the modified procedure of Sandell (1950) using the ammonia buffer method with sodium diethyldithiocarbamate as the colorimetric reagent. The optical density was measured at 436 mμ.

3) Cobalt analysis. The Nitroso-R-Salt method was used for cobalt analysis as described by Sandell (1950) after thorough evaporation of the sample to remove all acid. Optical density was measured at 420 mμ.

Metal analysis of lipid. A direct solvent colorimetric procedure was developed for iron and copper analysis of lipids, which eliminated the need for ashing and minimized losses. The details of the method will be published elsewhere. Copper and iron analyses were made on each batch of lipid before and after purification.

RESULTS

A number of pertinent experimental conditions, referred to as run parameters, are shown in Table 1. These parameters allow a comparison of the effects of purification for different runs. It is evident that purification was effective in decreasing the initial extent of oxidation and the metal content of the lipid.

The variation in the total metal content of the model system is also evident from the results of metal analyses (shown in Table 2). The analyses were performed on the Avicel® and on the model system prepared from it; and the results are expressed as the metal concentration based on the weight of lipid in the model system (1:6 methyl linoleate and cellulose). It can be seen that the cellulose itself is not completely free of trace metals, and that they are present in variable amounts. Extraction of the cellulose with solutions of either concentrated hydrochloric acid, water, acetone, or EDTA showed that the iron is firmly bound and the copper is partially free. The availability of the cobalt was not determined. It can be seen also that, in every case, the preparation of the model system increased the concentration of the trace metals. This increase occurred in spite of the rigorous techniques used in the preparation procedure and the glassware washing process used to prevent contamination. Lastly, because of the variability of the trace metal concentration of each of the runs, the absolute values of kinetic data between runs could not be compared. Insofar as possible, each run contained all the possible treatments for correct interpretation of the results.

Kinetic analysis of the results is explained in detail in Part I of this series (Maloney *et al.*, 1966).

The results of two oxidation experiments are presented in Figs. 1 and 2. Oxygen absorption as a function of time for Run 5 is shown in Fig. 1. Cobalt acetate was used as the catalyst at 20 μg cobalt per gram of methyl linoleate. The "wet" samples were humidified above saturated sodium nitrate and gave a relative humidity of 59% with a moisture content of 5.5 g water/100 g solids, which is in the multilayer absorption regions as determined from the sorption isotherm for the model system (Maloney *et al.*, 1966).

Table 1. Run parameters

Run no.	Relative humidity of sample [a] (% RH) Dry [d]	Humid	Cobalt catalyst PPM [b]	Salt	Oxidation extent [c] (moles O_2/mole) × 10^3 Unpurified	Purified	Trace-metal analysis of lipid: Iron (μg/g) Unpurified	Purified	Copper (μg/g) Unpurified	Purified
2	0.28		0.13	Stearate	1.43	0.62	.....	.....	0	0
			9.40	Stearate						
3	0.45		9.48	Acetate	1.35	0.15	0.10	0	0	0
4	0.33		10.2	Acetate	1.39	0.65	0.20	0.1	.....	.....
5	0.92	58.7	20.1	Acetate	5.15	2.47	0.14	0.03	.....	.....
6	0.94	45.0	19.95	Nitrate	5.02	2.23	0.20	0.15	0.88	0.27
7	0.85	29.4	19.90	Nitrate	1.48	0.30	0.12	0.08	0	0
9	0.50	44.6	10.03	Nitrate	1.58	0.76	0	0.05	.....	.....
10	0.87	30.2	10.15	Nitrate	1.81	0.24	0	0	0.17	0.17

[a] Determined from isotherm.
[b] μg cobalt/g lipid.
[c] From diene conjugation.
[d] Water activity less than 0.01.

Table 2. Trace-metal content based on weight of added methyl linoleate.

Run	Iron (μg/g lipid)		Copper (μg/g lipid)		Cobalt (μg/g lipid)	
	Avicel	Model system [a]	Avicel	Model system	Avicel	Model system
2	10.14	13.51	15.84	8.19	<0.01	3.57
	10.98		29.40		<0.01	
3	1.26	17.99	3.60	12.88	0.66	11.97
	4.08		4.08		1.50	
4	0.60	8.40	0.96	4.13	1.38	5.60
	2.10		1.62		1.08	
5	0.72	3.01	n.d.	n.d.	n.d.	n.d.
6	19.92	23.52	1.20	5.95	0.60	1.82
	23.70		1.38			
7	15.96	19.67	1.38	1.54	0	2.73
	8.64		2.40		1.14	
9	3.66	3.99	<0.01	0.42	0	1.75
	1.86		<0.01		0.78	
10	22.80	21.91	0.78	1.89	1.02	n.d.
	20.70		0.90		1.26	

[a] Control model system.
n.d., not determined.

It can be seen that the dry-catalyzed treatment oxidizes much more rapidly than the dry control, as would be expected from the kinetics. The effect of humidification can also be seen distinctly in Fig. 1. With the humidified control treatment (curves labeled 2), the initial portion of the curve is extended, showing a definite increase in the induction period over that of the dry control. The period of rapid oxidation can be seen to begin about 20–30 hr later than in the dry treatments. Humidified samples containing catalyst also show an increase in induction period over that of the dry catalyzed samples and oxidize somewhat more slowly than the dry control (uncatalyzed samples).

Fig. 2 shows the oxygen absorption data for Run 7, in which cobalt nitrate was used at 20 μg cobalt/gram lipid. The samples were humidified above saturated magnesium chloride to 29% relative humidity. This gave a moisture content of 3 g water/100 g solids, which is just above that of the monolayer value of 2.68 g/100 g solids. As in Fig. 1, the curves follow the typical autocatalytic shape, a large difference existing between the dry control and catalyzed treatments. In this run, the two methods of catalyst addition were tested, as represented by curves 3 and 5 for the dry treatments. The difference between these treatments was negligible, indicating that the method of addition does not affect the results.

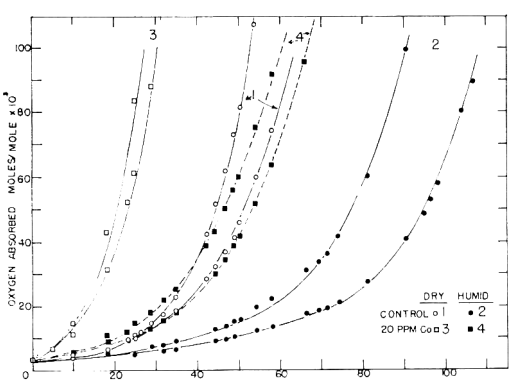

Fig. 1. Effect of humidity and metals on oxygen absorption by model system: Run 5.

The effect of humidification was similar to that observed in Run 5 (Fig. 1). Uncatalyzed samples humidified to 29% relative humidity showed an increased induction period and delays in reaching the rapid phase of oxidation. The effect on catalyzed samples containing cobalt nitrate was even more pronounced than the effect on cobalt acetate observed in Run 5. The humidification resulted in an oxidation curve showing slower oxidation than that of the uncatalyzed dry control.

DISCUSSION

Kinetic plots of the data as described in Part I of this series (Maloney *et al.*, 1966) were made for each duplicate sample in all of the runs in order to determine t_i, the composite induction time; K_M, the monomolecu-

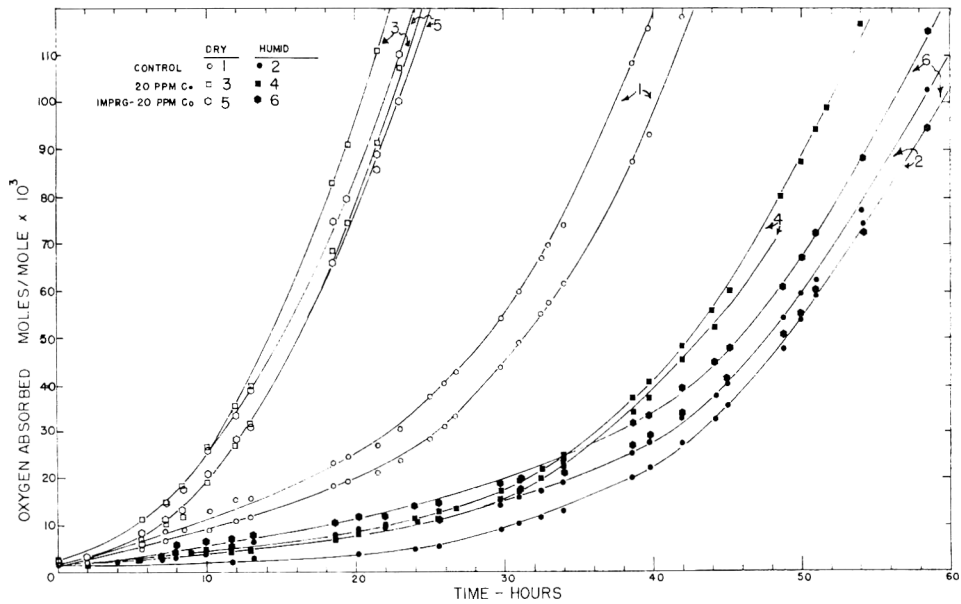

Fig. 2. Effect of humidity and metals on oxygen absorption by model system: Run 7.

lar rate constant; and K_B, the bimolecular rate constant. Fig. 3 represents some of the data of Run 7 plotted in this manner for the monomolecular rate period. The reduction in slopes due to humidification is evident from the figure. It can also be seen that the dry catalyzed samples had the larger slope and, therefore, the larger K_M, as would be expected from the kinetics. The increases in induction times due to humidification are also evident. Fig. 4 shows the data of Run 7 plotted for the bimolecular rate period. Again, the slopes of the humidified samples are smaller; but the differences in slopes between the treatments are not as large as for the monomolecular period.

In order to compare the data between samples as well as between runs, the individual kinetic constants for each run were compared with the average values for their respective dry control samples. Table 3 lists the average values of the rate constants for the dry

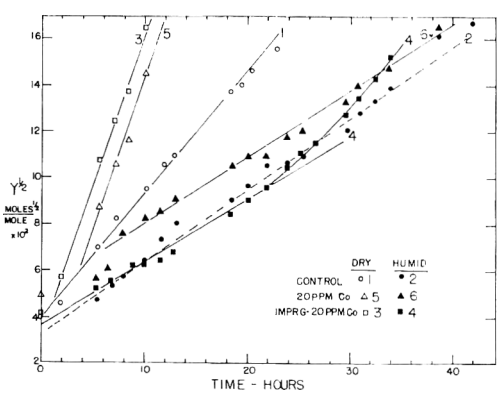

Fig. 3. Kinetic plot for the monomolecular rate period: Run 7.

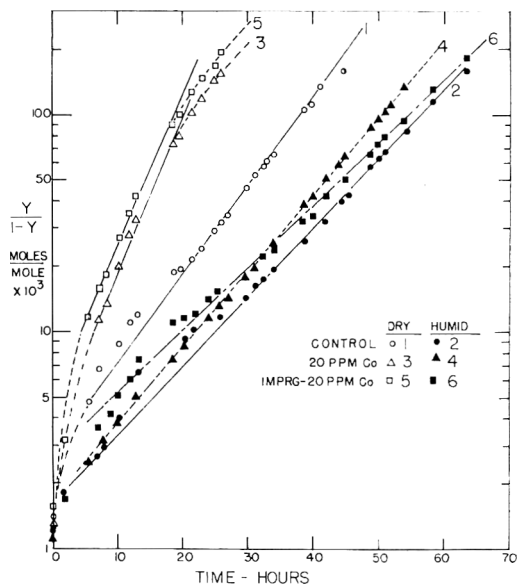

Fig. 4. Kinetic plot for the bimolecular rate period: Run 7.

Table 3. Average kinetic constants for dry control model system 37°C.

Run	T_i (hr)	$K_M \times 10^3$ (moles/mole)$^{1/2}$ hr^{-1}	$K_B \times 10^2$ hr^{-1}
2	27.9	3.28	5.25
3	33.7	7.91	8.06
4	26.8	7.10	9.49
5	17.25	7.73	7.13
6	9.75	7.92	9.36
7	5.15	10.81	8.48
9	25.35	5.41	9.20

control treatments of each run. Table 4 shows the ratios of the kinetic values of each treatment as compared to the dry control. The ratios are presented in such a way that a value below 1.0 demonstrates an inhibitory effect and one above 1.0 a catalytic effect, as compared with the dry control.

In Table 4, the results for the "dry-catalyzed" samples indicate that in almost every case the addition of cobalt shortens the induction time (time to reach 0.5% oxidation). In addition there was an increase in both the monomolecular and bimolecular rate constants due to an increase in the respective rates of initiation. This would be expected from the kinetics. With the humidified control treatments, which have no added cobalt, the induction time is increased significantly. For this case the monomolecular rate constant was reduced by almost one-half with respect to the control. The bimolecular rate constant was reduced by 40%. With the catalyzed humidified treatments, in most cases the induction period was increased, while there were significant reductions in K_M and K_B. It is interesting to note that addition of EDTA, which has a strong metal-chelating capacity, was much more effective in the wet state although it also significantly reduced the catalysis in the dry samples. The discrepancies found for some of the catalyzed treatments can probably be explained by catalyst inactivation, very likely occurring early in the oxidation (for example, in the rate constant ratios in Run 4).

To explain the observed effects of addition

Table 4. Comparison of kinetic constants of the model system with respect to the dry control.

		Catalyzed dry			Catalyzed humidified			Control humidified		
Run	Cobalt concentration	$1/T_i$	K_M	K_B	$1/T_i$	K_M	K_B	$1/T_i$	K_M	K_B
		$1/T_{i_c}$	K_{M_c}	K_{B_c}	$1/T_{i_c}$	K_{M_c}	K_{B_c}	$1/T_{i_c}$	K_{M_c}	K_{B_c}
2	0.13 ppm	0.75	0.91	1.45						
		0.85	1.47	1.27						
	9.4 ppm	0.96	2.11	1.69						
		2.39	2.56	1.73						
3	9.48 ppm	2.81	2.17	1.62						
		2.05	2.32	1.43						
		2.65	1.83	1.23						
4	10.2 ppm	2.34	0.76	0.91						
		2.68	0.94	0.91						
5	20 ppm	9.08	1.46	1.33	2.13	0.82	0.83	0.55	0.43	0.65
		7.50	1.07	1.45	1.69	0.61	0.79	0.73	0.50	0.62
6	20 ppm	1.32	2.07	1.26	0.86	0.51	1.12	0.94	0.44	0.60
		1.36	2.17	1.25	0.92	0.63	0.84	0.81	0.63	0.60
7	20 ppm	1.12	1.94	1.92	0.36	0.50	0.96	0.21	0.38	0.85
		1.59	2.38	2.28	0.38	0.46	0.99	0.39	0.57	1.02
	20 ppm	1.06	2.45	2.17	0.48	0.63	0.79			
	Impreg	1.64	2.34	2.45	0.55	0.57	0.77			
9	10 ppm	1.71	1.78	1.36	0.47	0.72	0.55	0.48	0.77	0.59
		1.45	2.11	1.22	0.55	0.75	0.58	0.43	0.77	0.60
	10 ppm	0.95	1.26	1.03	0.25	0.12	0.54			
	+ EDTA[a]	0.87	1.31	1.12	0.25	0.46	0.57			

Subscript c refers to dry control model system.
[a] 10 moles EDTA/mole cobalt.

of water on the three kinetic values, a dual mechanism was proposed and is shown in Fig. 5. The results indicate that there was a substantial increase in the induction period due to humidification. Also, it was suspected that the change from monomolecular to bimolecular kinetics took place at a slightly higher hydroperoxide concentration in the humidified samples than in the dry samples. The model proposes that some of the hydroperoxides produced in the propagation reaction, $(ROOH)_B$, are not available for chain initiation but rather are drawn to the water/lipid interface because of their amphipolar nature. There, they are held by the water through hydrogen bonding, which causes a lowering of the effective hydroperoxide concentration. This process increases the time required to reach the point of change-over from monomolecular to bimolecular kinetics, which in turn increases the measured composite induction period. That this was the case can be seen from the last table, and has been shown by Spetsig (1959) to occur with mixtures of methyl linoleate and water. Only when the hydroperoxides saturate the water/lipid interface on the solid surface can their concentration in the bulk be built up sufficiently for bimolecular kinetics to take over.

This postulate explains the increased induction period found in humidified treatments. It could also account, at least partially, for the decrease in the observed K_M, if one also postulates that the hydroperoxide concentration available for the monomolecular decomposition is lower than that calculated from the measured oxygen absorption.

However, the reduction of the effective ROOH does not explain the observed decrease in K_B. It is proposed, therefore, that a second mechanism of inhibition by water is also operative in reducing K_B as well as K_M. This mechanism is based on hydration of both added and unintentionally present metal catalysts.

Hydration would decrease the value of K_M by reducing the effective metal concentration term contained in it. Similarly, hydration of the metals should decrease the value of K_B by changing the bimolecular initiation rate constant, k_{ii}, from that typical of a metal-catalyzed two-step decomposition (Ingold, 1962) to a value closer to that for bimolecular decomposition of hydroperoxides in the absence of catalysts. Support for this proposal has been found in the work of Uri (1956), who showed that metal salts were much less active catalysts in polar solvents. Kamiya *et al.* (1963) showed that in the cobalt-catalyzed oxidation of tetralin, addition of 10% of water by volume reduced the rate by a factor of five. Further support for the hypothesis is seen in the work of Dean and Skirrow (1958). Addition of water to a cobalt acetate: *t*-butyl hydroperoxide: acetic acid system gave a retarding effect of 25-fold, which leveled off at 3% added water. Spectroscopic data verified that the water pushed the acetate out of the coordination sphere of the metal.

Other mechanisms were tested in which water was assumed to participate directly by reacting with reaction intermediates. However, the observed data did not fit the integrated equations developed on the basis of these assumptions.

These two mechanisms can undoubtedly occur in a food system and should explain in part the observations found in practice. It is certainly possible, however, that in a system as complex as that of an actual food, other reactions may be involved, including non-enzymatic browning.

Initiation

$$[ROOH]_A \xrightarrow[k_i]{H_2O,\ [M]} RO^{\cdot} + {}^{\cdot}OH \longrightarrow \text{free radicals}$$

Propagation

$$R^{\cdot} + O_2 \xrightarrow{k_o} RO_2{\cdot}$$

$$RO_2{\cdot} + RH \xrightarrow{k_p} [ROOH]_A + R^{\cdot} + [ROOH]_B$$

Inhibition

$$[ROOH]_B \xleftarrow[\text{interface}]{\text{water-lipid}} [ROOH]_{aB}$$

Termination

$$2RO_2{\cdot} \longrightarrow \text{non-radical products}$$

Fig. 5. Proposed mechanism for antioxidant effect of water.

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Autoxidation of Methyl Linoleate in Freeze-Dried Model Systems. III. Effects of Added Amino Acids

SUMMARY

Oxidation of methyl linoleate in a model system based on microcrystalline cellulose was studied in the presence and absence of added amino acids in concentrations ranging from 10^{-1} to 10^{-3} moles of additive per mole of linoleate. All experiments were conducted in freeze-dried model systems, in absence of water; the oxidation was followed manometrically and by determination of diene conjugation. Parallel experiments were conducted on the same systems in the presence of conventional antioxidants including propyl gallate.

Certain amino acids, including histidine, β-amino-butyric acid, lysine, and cysteine, had substantial antioxidant activity. The nature of this activity was found to be different from that observed with propyl gallate, since the main, if not sole, effect of the amino compounds was to prolong the induction period and to affect the initial rate of oxidation. No effect was present in the more rapid, bimolecular phase of oxidation; whereas propyl gallate had an inhibitory effect in this later stage also.

Kinetic interpretation of the data and the significance of the findings to stability problems in dehydrated foods are discussed.

INTRODUCTION

Amino acids and proteins have been known to affect the course of autoxidation of lipids in foods, but systematic studies of their pro-oxidative and antioxidative effects have been few (Bishov *et al.*, 1961; Janicki and Gogolewski, 1958; Tappel *et al.*, 1961; Pokorny *et al.*, 1964). In 1961, Marcuse reported on a study of the antioxidative effects of several amino acids added to aqueous solutions of linoleate at pH 7.5. He experienced the usual difficulties in reproducing, in repeated experiments, the magnitudes of the antioxidative effects, but concluded the following:

1) Histidine, alanine, methionine, and lysine reduced oxygen absorption by linoleate by as much as 50–80%.

2) Each amino acid had an optimum concentration for the antioxidant activity, and at high concentrations showed an activity inversion, becoming pro-oxidative rather than antioxidative in its action.

3) The antioxidative activity depended on pH, the presence of other antioxidants or synergists, and the state of oxidation of the linoleate.

More recently, several studies have been reported on the pro-oxidative effects of amino acids, especially those of histidine, on linoleate in aqueous emulsions. The same factors which affected the antioxidant activity of the amino acid were shown to be involved in the pro-oxidant activity (Saunders *et al.*, 1962; Coleman *et al.*, 1964).

Among food products particularly susceptible to autoxidation are dehydrated foods, in which the oxidation of lipids may have serious consequences on quality. Oxidation leads to deterioration of lipids, and secondary reactions between lipid oxidation products and proteins may cause browning, loss of protein quality, and impaired organoleptic quality. The present study was undertaken in order to explore, in a model system designed to approximate conditions in freeze-dehydrated foods, the interaction of amino acids and lipid oxidation.

MATERIALS AND METHODS

Model system. The model system used is described in part I of the present series (Maloney *et al.*, 1966). Samples were freeze-dried in reaction flasks, which could be attached directly to manometers for determination of the progress of oxidation. The original wet mix was at pH 4.5, and was buffered primarily by the presence of acidic groups on the cellulose. The addition of amino acids did not change this pH significantly. The significance of this pH measurement for the actual conditions in the completely dry system is not known.

Amino acids. All of the amino acids used in the present study were of the highest grade available commercially (Cal. Biochem., Los Angeles, California). All compounds were grade A, except for DL-β-amino-n-butyric acid, and ε-amino-n-caproic acid, which were grade C. The amino acids having no guarantee of purity (grade C) were

tested for homogeneity by chromatographic procedures of Fahmy *et al.* (1961).

The following compounds were used as the free base: DL-alanine, DL-β-amino-n-butyric acid, α-amino-n-butyric acid, ϵ-amino-n-caproic acid, and L-histidine. The following were used as the monohydrochlorides: DL-lysine and L-cysteine.

Oxidation. Oxygen absorption was measured at 37°C, manometrically and by measurement of diene conjugation at 233 mμ according to the method of Privett and Blank (1962). Procedural details have been discussed previously (Maloney *et al.*, 1966).

RESULTS AND DISCUSSION

The results of individual experiments using model systems containing the amino acids, in concentrations of 10^{-3} moles of the amino acid per mole of linoleate, showed considerable variation in the rates of oxidation of the control samples (containing no amino acid) and also in the magnitude of the effect of the additive. In particular, the extent to which the model system was capable of exhibiting a significant induction period was different in different experiments.

In comparing the effect of individual amino acids, therefore, it is necessary to relate this effect to the behavior of the control samples used in the same experiment, under identical conditions with the treated sample.

Typical results for runs in which a very pronounced delay in oxidation of the linoleate was achieved by the addition of trace amount of amino acid are shown in Figs. 1, 2, and 3. Fig. 1 shows the effect of addition of histidine; Fig. 2, the effect of lysine and of β-amino-n-butyric acid; and Fig. 3, the effect of addition of cysteine and of ϵ-amino-n-caproic acid.

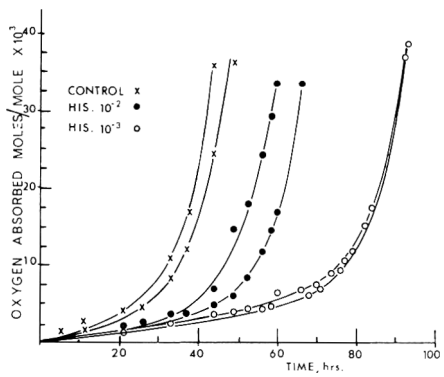

Fig. 1. Oxygen absorbed by model system in initial stages of oxidation. Run I (additive: histidine, 10^{-2} and 10^{-3} moles/mole of linoleate).

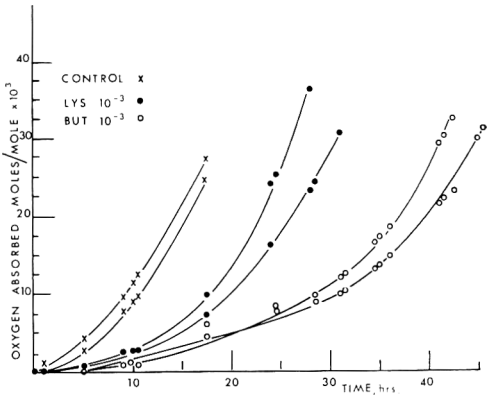

Fig. 2. Oxygen absorbed by model system in initial stages of oxidation. Run VII (additives: lysine, β-amino-n-butyric acid, 10^{-3} moles/mole linoleate).

The results presented in these figures were based on oxygen absorption measured by a manometric technique. Similar data for oxygen absorption calculated from the increase in diene conjugation as measured by UV absorption at 233 mμ are presented in Table 1.

The amino acids mentioned—histidine, lysine, β-amino-n-butyric acid, cysteine, and ϵ-amino-n-caproic acid—showed the greatest retardation of oxidation in the model system studied. As mentioned previously, however, the magnitude of this effect varied from experiment to experiment. For instance, in another run in which the effectiveness of histidine addition was compared with that of propyl gallate addition in the same concentration, histidine showed considerably less antioxidant activity than that obtained in the

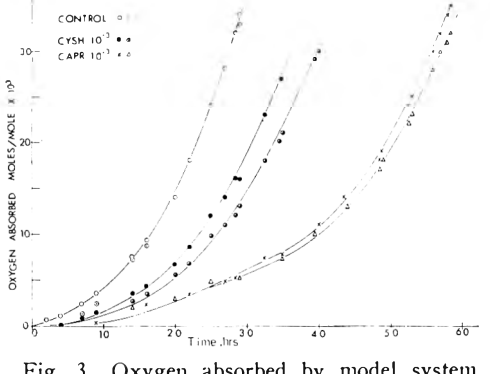

Fig. 3. Oxygen absorbed by model system in initial stages of oxidation. Run VIII (additives: cysteine, ϵ-amino-n-caproic acid, 10^{-3} moles/mole linoleate).

Table 1. Oxygen absorption of methyl linoleate based on UV analysis of diene conjugation (run VII).

Time (hr)	(M oxygen/M linoleate) × 10^3		
	A	B	C
0	3.1	2.6	1.3
5	7.6	4.9	2.1
9	13.1	9.0	3.5
21	42.2	21.2	9.0
26.5			8.8
34.5	116.5	87.7	17.9

A = control; B = 10^{-3} M lysine/M linoleate; C = 10^{-3} M β-amino-n-butyric acid/M linoleate.

Table 2. Oxygen absorption of methyl linoleate based on UV analysis of diene conjugation (run VI).

Time (hr)	(M oxygen/M linoleate) × 10^3		
	A	B	C
0	3.5	2.3	1.7
4	5.2	2.9	1.9
8	6.7	4.6	2.4
20	21.9	11.0	4.7
24	37.6	19.6	8.0

A = control; B = 10^{-3} M histidine/M linoleate; C = 10^{-3} M propyl gallate/M linoleate.

experiment shown in Fig. 1. The results of the experiment comparing the phenolic antioxidant with histidine are shown in Fig. 4 and in Table 2. It is evident that the effect of histidine is less pronounced in its antioxidant action than either that of propyl gallate or that of histidine in a previous run in which the histidine addition resulted in a very substantial increase of induction time.

Part of the difficulties in reproducing the magnitude of antioxidant activity results from the observation which we have confirmed in a number of experiments, namely that all of the antioxidant activity of the amino acids manifests itself only in the induction period, or in the very early stages of autoxidation. Once the rapid stage of oxidation is reached, the amino acid gives practically no protection. Thus, if there are differences in inherent capabilities of the samples to exhibit induction periods at the time of initiation of the oxidation experiment, these differences will be magnified in the presence of amino acids.

The autocatalyzed oxidation of unsaturated fatty acids, and of their esters, is known to

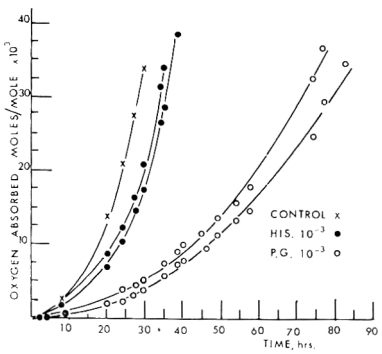

Fig. 4. Oxygen absorbed by model system in initial stages of oxidation. Run VI (additives: histidine, propyl gallate, 10^{-3} moles/mole linoleate).

have at least two stages separable on the basis of kinetic considerations (Lundberg, 1961). In the initial stage, following an induction period, the oxidation is catalyzed by hydroperoxides decomposing into radicals by a scheme involving monomolecular decomposition.

In the subsequent rapid phase of oxidation, the reaction is catalyzed by bimolecular decomposition of hydroperoxides. Under these conditions, as has been discussed previously (Maloney *et al.*, 1966), the rate of oxidation is given by Eq. 1.

$$\frac{dy}{dt} = K\,y\,(1-y) \qquad [1]$$

where k = a rate constant, which includes many factors held constant in an experiment; y = amount of oxygen absorbed (moles of oxygen per mole of linoleate); t = time.

On integration it becomes obvious that a plot of log $(y/1-y)$ vs. t should give a straight line. This method of plotting allows graphical representation of the beginning of the bimolecular decomposition period, and changes in slopes of the straight lines indicate the existence of inhibitory or catalytic effects in this period. Plots of oxygen absorption in the rapid oxidation phase in the presence and absence of amino acids have been made and show invariably that amino acids do not affect the rate of oxidation in this period. Fig. 5, for instance, presents oxidation in the rapid oxidation period for the run in which histidine was found to extend the induction period of linoleate by some 40 hr. The slopes of the oxidation curves in this period, however, remain unaffected by the presence of the amino acid. This behavior may be contrasted with that of a phenolic antioxidant, which, as seen in Fig. 6,

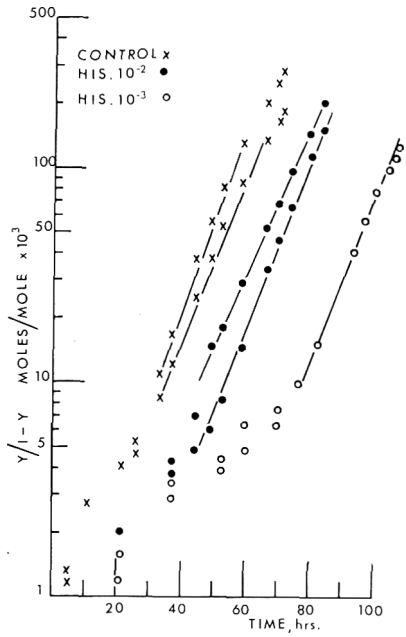

Fig. 5. Oxygen absorption plot for the bimolecular decomposition. Run I (additives: histidine, 10^{-2} and 10^{-3} moles/mole linoleate).

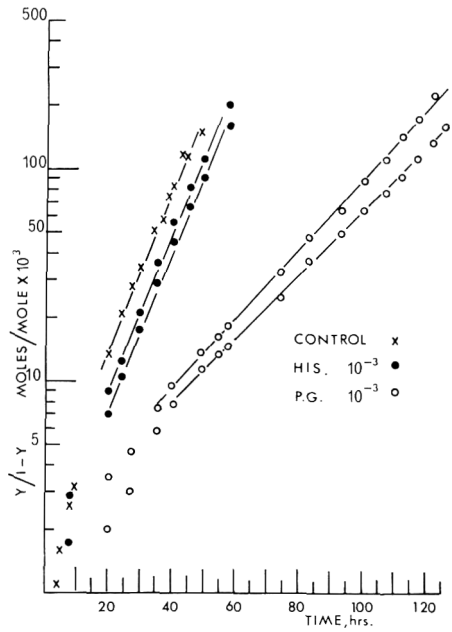

Fig. 6. Oxygen absorption plot for the bimolecular decomposition. Run VI (additives: histidine, propyl gallate, 10^{-3} moles/mole linoleate).

not only extends the induction period but also decreases the rate of oxidation in the later stages of oxidation.

To compare the effects of various amino acids and to compare different experiments, calculations were made of relative rates based on absorption by the controls in the individual experiments. These relative rates are expressed as ratios of the amount of oxygen absorbed by the treated samples to that absorbed by the controls. It was felt that the best possible comparison between runs would be achieved by calculating these relative values at a fixed level of oxidation of controls, rather than at a fixed time after starting the experiment. Table 3 shows the results for the various amino acids studied. The comparisons were made at a time when the level of oxidation of controls was 10 moles of oxygen per mole of linoleate. In addition to the experimental values, the values obtained by Marcuse (1961) in his study on amino acids are included for comparison.

The table also presents a comparison of the time required by the control to reach a 1% level of oxidation, with that required by the treated samples. The comparison is made by calculation of the ratio t_c/t_t, that is, ratio of time for control sample to time for treated sample.

The results indicate that all of the amino compounds listed in the table showed antioxidant activity as great as or greater than the effects reported by Marcuse, and that some of the amino compounds had activity comparable with that of propyl gallate, a phenolic antioxidant.

On the other hand, we failed to observe in our system such activity with methionine, phenylalanine, isoleucine, and arginine.

Table 3. Antioxidant activities of different amino acids.

Additive (10^{-3} M/M lin.)	Relative induction time (t_c/t_t)	Relative oxygen absorption (Y_t/Y_c)	
		Present study	Marcuse 1961
Histidine	0.70	0.54	0.68
Cysteine	0.73	0.50	>1
Lysine	0.52	0.18	0.81
β-Amino-n-butyric acid	0.33	0.13	n.a.[a]
ε-Amino-n-caproic acid	0.41	0.18	n.a.
α-Amino-n-butyric acid	0.89	0.74	n.a.
Alanine	0.77	0.48	0.89
Propyl gallate	0.39	0.18	n.a.

[a] n.a. = not available.

Further studies on the mechanism and kinetics of the amino compounds will be reported at a later date.

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Distribution of Lipids in Air-Fractionated Flours

SUMMARY

Lipids were extracted with petroleum-ether, water-saturated butanol following petroleum-ether, or directly with water-saturated butanol from a composite hard red winter wheat flour and from five flours separated from the original flour by air classification. The lipids were fractionated by silicic acid column and thin-layer chromatography. Lipid contents increased as the protein contents of the flour fractions increased. The original flour contained a higher concentration of total and bound lipids than the residual coarse flour; the two flours contained comparable amounts of protein. It is postulated that shifts in particle size and in lipids during air fractionation might be responsible for differences in storability of various flour fractions as such, or in combination with other perishable food ingredients.

INTRODUCTION

Wheat flour produced by conventional roller milling contains particles of different sizes: large endosperm chunks, small particles of free protein, free starch granules, and small chunks of protein with starch granules. Fine grinding followed by air classification can separate such a mixture into three main fractions: 1) a high-protein fraction with particle size under 15 μ (by a sedimentation method); 2) a low-protein fraction with particle size of 15–40 μ; and 3) a coarse residue size of 40–120 μ with protein content approximating that of the original flour. Repeated grinding to free more protein and starch particles, followed by air-classification, increases the protein content of some fractions and the starch content of other fractions, and is the basis for numerous patents for the production of "tailor-made" flours from a single parent flour.

Air classification is relatively inexpensive, and its advantages are numerous (Griffin, 1962): 1) manufacture of more uniform flours from varying wheats; 2) increase of protein content of bread flours and decrease of protein in cake and cookie flours; 3) controlled particle size and chemical composition; and 4) production of specialty flours for specific uses.

Most studies on air classification of wheat flours have been concerned with the distribution of protein and mineral matter and with the effect on maltose, gassing power, amylograph viscosity, and over-all breadmaking characteristics (Wichser, 1958; Jones *et al.*, 1959; Grosh *et al.*, 1959; Sullivan *et al.*, 1960; Ponte *et al.*, 1963; Stringfellow and Pfeifer, 1964). Jones *et al.* (1960) determined vitamin contents of air-classified flours; and Wilson *et al.* (1964) studied distribution of chlorine in chlorine-treated soft wheat flours dry-fractionated by a four- or five-stage air classification.

Data on lipids in air-classified flours are limited and generally fragmentary. Houston (1961) reported preliminary results of studies on simple and compound lipids in air-classified flours. Ponte *et al.* (1963) determined fat (by acid hydrolysis) of starch and gluten; and Gracza (1965) studied the petroleum-ether extract of air classified flours. This study was made to determine total, free, and bound lipid content of air-classified flours.

MATERIALS AND METHODS

Wheat flours. Flour used for air-fractionation was experimentally milled on an Allis Mill from a composite grist of several hard winter wheat varieties grown in 1962 at several locations throughout the Great Plains. The flour was stored at 4°C in closed containers until used. The flour was fractionated into high- and low-protein streams, according to the scheme given in Fig. 1. Fractionations were made in a Pillsbury Laboratory Model No. 1 classifier, employing indicated feed rates, speeds, and internal set-up. The fractionation procedure involved removing fine fractions *B*, *C*, *D*, and *E* from the original flour, *A*; residual coarse flour was designated *EE*. Particle size (average diameter in μ) was determined in a Fisher Sub-Sieve Sizer (No. 14-311), as described by Croteau (1960).

Bread-making. Baking properties were determined as described previously (Pomeranz *et al.* 1966b). Loaf volumes were determined immediately after the bread was taken from the oven.

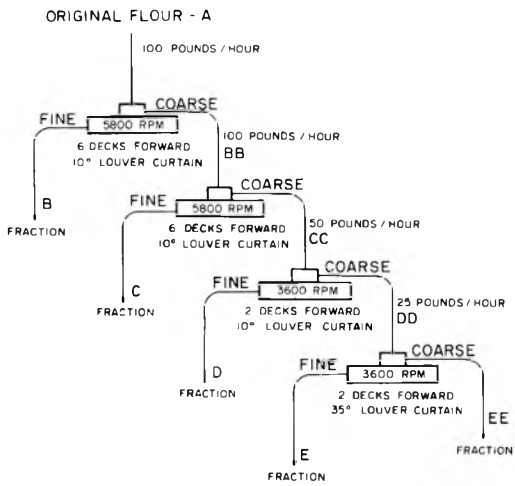

Fig. 1. Flow diagram of the hard red winter wheat flour fractionation procedure.

After the loaves had cooled, they were cut and their crumb grains evaluated by the code S = satisfactory, Q = questionable, and U = unsatisfactory.

Lipid extraction. Lipids were extracted exhaustively (8–10 hr) with petroleum-ether (bp 35–60°) in a Goldfish extractor. Petroleum-ether in the flour was evaporated at room temperature, and the flour was re-extracted with water-saturated 1-butanol, as described previously (Daftary and Pomeranz, 1965a). In addition, lipids were extracted directly with water-saturated 1-butanol. All butanol extracts were purified by redissolving the butanol extract with petroleum ether. Total lipids extracted directly from the flour with water-saturated 1-butanol were also washed with a dilute aqueous calcium chloride solution (Pomeranz *et al.*, 1966a). All determinations were made in triplicate.

Silicic acid column chromatography. Lipids in the water-saturated 1-butanol total extract were fractionated into nonpolar and polar fractions on silicic acid columns (Daftary and Pomeranz, 1965a).

Thin-layer chromatography. Extracted lipids were fractionated by thin-layer chromatography (TLC), as described elsewhere (Chiu and Pomeranz, 1966). The solvents used for one-dimensional ascending development of 125-γ lipids were: chloroform for nonpolar lipids, and a mixture of chloroform-methanol-water (65:25:4) for polar lipids. The spots were visualized by exposure to iodine vapor, or by spraying with a saturated solution of $K_2Cr_2O_7$ in 70% (by volume) aqueous sulfuric acid. More specific spraying methods included ninhydrin, modified Dragendorff reagent, and molybdenum spray. In addition, TLC plates were sprayed with α-naphthol to identify glyco-

Table 1. Certain physical, chemical, and bread-making characteristics of air-fractionated wheat flours.

Sample	Part of whole (%)	Av. diam. (μ)	Moisture (%)	Ash (%)	Protein ($N \times 5.7$) (%)	Mixing time (min)	Water absorption (%)	Bromate requirement (mg/%)	Loaf volume (cc)	Crumb grain
A	100	21.0	12.6	0.44	12.9	3	61.9	4	933	*S*
B	3.9	3.1	9.5	1.05	28.5	2½	95.0	....	.....	.....
C	9.2	5.9	10.3	0.74	18.0	2⅞	76.4	6	1085	*Q-S*
D	9.6	11.5	11.6	0.47	8.9	3⅞	59.3	1.5	675	*Q-U*
E	12.7	14.6	10.7	0.41	8.2	3⅝	54.8	1.5	670	*Q-U*
EE	64.7	28.4	11.3	0.35	12.8	2⅞	59.3	3.5	952	*S*

Table 2. Relative amounts of petroleum-ether and water-saturated-butanol-extractable lipids in air-fractionated flours.

Sample	Lipid distribution		Phosphorus in lipid	
	Petroleum-ether (%)	Butanol following petroleum-ether (%)	Petroleum-ether extract (%)	Butanol following petroleum-ether (%)
A	0.74	0.69	0.32	1.00
B	2.22	1.60	0.31	0.94
C	1.32	0.76	0.32	1.05
D	0.63	0.34	0.31	1.01
E	0.61	0.30	....	....
EE	0.64	0.42	0.33	1.39

lipids (Feldman *et al.*, 1965). For identification of polar lipids separated by TLC, standards used were phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine (from Applied Science Labs., Inc., State College, Pa.) and mono- and digalactosyl glyceride (gift from Dr. D. H. Hughes, Procter and Gamble Co., Cincinnati, Ohio).

Phosphorus in lipid extracts was determined by a modified Fiske-Subbarow method (Oser, 1965).

RESULTS AND DISCUSSION

Table 1 summarizes certain chemical and bread-making characteristics of the flours. The data do not include baking results of flour *B*, since its protein content was too high for baking tests by conventional methods. Samples *A* and *EE* were comparable for all practical purposes from the standpoint of protein content and bread-making.

Tables 2 and 3 compare the lipid content of the flours. The TLC of the lipids is shown in Figs. 2 and 3. In comparing results in Tables 2 and 3, note that the water-saturated butanol extracts (in Table 3) were washed with a dilute calcium chloride solution. Such washing removes nonlipid impurities and, at the same time, causes losses of polar components.

Table 3. Total, polar, and nonpolar lipids in water-saturated butanol extracts of air-classified flours.

	Water-saturated butanol extract			
	Total (%)	Nonpolar (%)	Polar (%)	Recovery (%)
A	1.15	49.7	48.8	98.5
B	3.32	50.8	46.5	97.3
C	1.92	49.9	45.5	95.4
D	1.01	49.9	48.3	98.2
E	0.90	49.2	48.1	97.3
EE	1.08	46.9	50.1	97.0

Comparing actual lipid content of the original flour with total lipid content, calculated from the contribution of the various fractions, showed good agreement for petroleum-ether and direct water-saturated butanol extracts. The calculated butanol extract following the petroleum extract was, however, lower than the actual extract of the unfractionated flour. That probably resulted from losses during extraction and purification of small amounts of lipids extracted with butanol after a major part was extracted with petroleum-ether.

Differences in recovery of nonpolar and

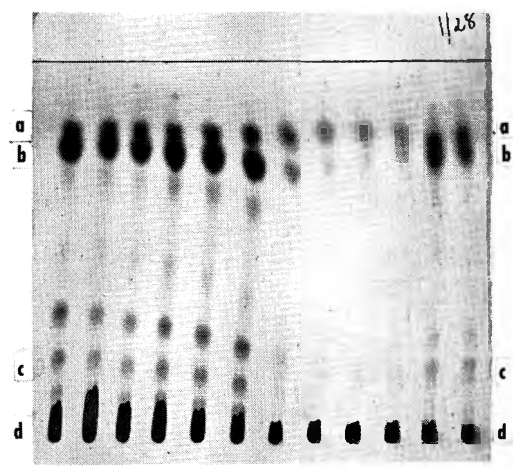

Fig. 2. TLC of nonpolar lipids in air-fractionated flours. Samples 1–6 extracted with petroleum, and 7–12 extracted with water-saturated butanol following petroleum ether. Order of samples in each extract corresponds to the order in Tables 1 and 2. Developed with chloroform; spots visualized by charring with sulfuric acid, picture taken under UV light. a) hydrocarbons and sterol esters; b) triglycerides; c) mono- and diglycerides; and d) non-migrating polar lipids.

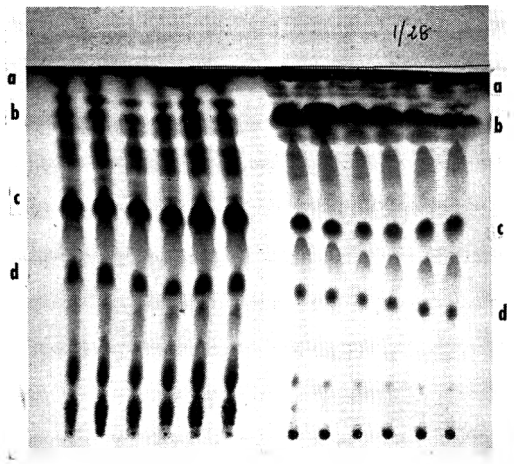

Fig. 3. TLC of polar lipids in air-fractionated flours. Samples 1–6 extracted with water-saturated butanol after extraction with petroleum ether (7–12). Developed with chloroform mixture; spots visualized by charring with sulfuric acid, picture taken under UV light. a) nonpolar lipids; b) monogalactosyl glyceride; c) digalactosyl glyceride, and d) phosphatidyl choline.

polar lipids make it difficult to determine precisely the ratio of nonpolar and polar lipids from the empirical method of fractionation on silicic acid columns. No consistent or significant differences in the polar-nonpolar lipid ratio were found for the tested flours.

Total lipid content increased as protein content of flour increased. The lipid-protein quotient was, however, much higher in the high-protein *B* flour than in the other flours. Consequently, residual flour *EE* contained less lipids than the original flour (*A*), despite a comparable protein content of both flours. Flour *EE* was lower than flour *A* in total, free (petroleum-ether soluble), and bound (butanol after petroleum-ether extract) lipids.

TLC of nonpolar lipids (Fig. 2) shows that triglycerides, free fatty acids, and diglycerides were present almost exclusively as free lipids, extractable with petroleum ether. Small amounts of triglycerides were present in a bound form in the low-protein fraction, *E*, and in the residual flour, *EE*. Both the petroleum ether extract and butanol following petroleum ether contained substantial amounts of polar lipids (Fig. 3). The concentration of polar lipids in the butanol extract was substantially higher than in the petroleum ether extract. Visual observations indicate possible differences in concentrations of specific polar components in various lipid extracts and in various flour fractions. Additional work will be necessary to evaluate the significance of those differences.

The results seem to indicate that, during air-fractionation, lipids are shifted along with proteins. The lipid shift, accompanying a particle size shift, may explain the fact recognized by industry that the residual flour, *EE*, low in lipids, stores better than the original flour (*A*). Residual flour (*EE*) is especially low in bound polar lipids, previously found to be more susceptible than nonpolar lipids to enzymatic degradation during storage under adverse conditions (Daftary and Pomeranz, 1965b). Currently being investigated are changes in lipids in air-fractionated flours from various classes and varieties, and effects of lipid shifts on storage.

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Mention of a trade product, equipment, or commercial company does not imply endorsement.

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Identification of Some Trace Lipids in Honey

SUMMARY

The trace lipids of cotton honey were extracted with Skellysolve B and diethyl ether. The crude lipid extract was subjected to transesterification, purified on a silicic acid column, and analyzed by gas-liquid chromatography. For further confirmation the crude lipid extract was saponified and the fatty acids identified by reverse-phase chromatography. The infrared spectrum of the crude lipid extract and methyl esters showed absorption bands typical of alkane and ester groups. Palmitic and oleic acids were respectively found in relative concentrations of 26.7 and 60.3%, along with small amounts of lauric, stearic, and linoleic acids.

INTRODUCTION

Research on the composition of honey is quite extensive, as evidenced by the reviews of White (1957) and Pryce-Jones (1950). A considerable number of sugars, vitamins, minerals, and proteins have been identified. Many workers have examined the free or volatile acid content of honey, but none have studied the release of acids after saponification, except Heiduschka and Kaufmann (1911), who reported that more volatile acids and more formic acid were obtained in every case by steam distillation after saponification. Dörrscheidt and Friedrich (1961) recently separated the odorous substances of several honeys by gas-liquid chromatography and identified, by retention time, methyl formate, methyl acetate, methyl propionate, methyl butyrate, methyl valerate, and methyl caproate. The present investigation was undertaken to study the extractable long-chain fatty acids in honey.

METHODS AND MATERIALS

Preparation of crude extract. Cotton honey was diluted to 50% (w/w) with distilled water, filtered through cheesecloth, and extracted three times with redistilled Skellysolve B (bp 60–70°C) and diethyl ether. Emulsions were broken with absolute ethanol. The extract was washed at least three times with distilled water, dried over sodium sulfate, and concentrated by rotary evacuation at 30–40°C. The residue was weighed, taken up in dry Skellysolve B, and stored at 5°C. This procedure yielded 0.015% of a yellow lipid residue. This fraction is referred to as the crude extract.

The Molisch test for carbohydrate, a spot test for glycerol or glycerides, a spot test for phosphorus, the Liebermann-Burchard test for sterols, and the ninhydrin test for protein were performed on the crude extract (Weissberger, 1954; Feigl, 1946).

Determination of infrared spectrum. The infrared absorption spectrum was determined on the crude extract and subsequent fractions. For analysis, a 2% solution of the sample in spectral-quality chloroform was placed in a sodium chloride cell, and the spectrum was recorded on a Perkin-Elmer Infracord, model 137.

Preparation of samples for gas-liquid chromatography. The crude extract of cotton honey was subjected to transesterification by treatment with 0.4*N* sodium methoxide in excess methanol and eluted from a silicic acid column to obtain methyl esters free of unsaponifiables or other impurities (Luddy *et al.*, 1960).

Gas-liquid chromatography. The "purified" methyl esters were analyzed with an Aerograph gas chromatograph, model A90C. The columns, 20% diethyleneglycol succinate (DEGS) on acid-washed fire brick (60/80-mesh), were 5–10 ft long and ¼–⅛ in. in diameter. Identification of components on the gas chromatograms was based on the equivalent chain length (ECL) which canceled variations in column dimensions, temperature, and carrier gas flow rate (Miwa *et al.*, 1960). A gas chromatograph (Research Specialties Co., model 604) with an ionizing detector and a 10% DEGS column on chromosorb W, acid-washed fire brick (60/80), was used to determine the relative concentration of the purified methyl esters.

Paper chromatography. To obtain the free fatty acids for paper chromatography the crude extract was saponified with 0.5*N* alcoholic potassium hydroxide by refluxing 2 hr and standing overnight in the dark (Hilditch, 1956). Diethyl ether was added in an amount equal to the total sample volume, and distilled water was then added until two layers formed. The diethyl ether layer was decanted in a separatory funnel and extracted three times with distilled water to remove any fatty acid salts. These washings were combined with the original aqueous layer, the mixture was acidified with hydrochloric acid, and the free fatty acids were extracted three times with Skellysolve B. This extract was washed once with 1% sodium

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carbonate and three times with distilled water, and dried over sodium sulfate. Before chromatography the sample was concentrated on a rotary evaporator at 35–40°C.

For chromatography of the fatty acids, Whatman No. 1 paper was impregnated with 5% silicone (Dow Corning 555) in diethyl ether (Mangold *et al.*, 1955). After application of the unknown sample and appropriate standards, the chromatogram was developed 15–18 hr in 85% acetic acid. The paper was then air-dried, washed 3 times for 3 min in distilled water, soaked 5 min in 1% lead acetate, washed 3 times in distilled water, air-dried, and finally exposed to hydrogen sulfide. The presence of fatty acids was indicated by brown spots on a white background. To detect unsaturated fatty acids a chromatogram was washed in distilled water after development, air-dried, and exposed to iodine vapors. The unsaturated fatty acids produced yellow spots on a white background.

RESULTS AND DISCUSSION

The spot tests for glycerides, phosphorus, and sterols on the crude extract of cotton honey were all positive. However, the blank of the phosphorus spot test also gave a slightly positive test. The ninhydrin reaction for protein or amino acids and the Molisch test for carbohydrate were negative. The tests indicated the presence of triglycerides, sterols, and possibly phospholipids.

The infrared absorption spectrum of the crude extract and the purified methyl esters shown in Fig. 1 revealed sharp absorption bands at 3.5, 5.9, and 6.9 μ which were typical of alkane and ester groups (Markley, 1960). On the other hand, bands indicating carboxyl groups were absent. The presence of a sharp band at 2.8 μ in the crude extract indicated a free hydroxyl group. The purified methyl esters eluted from silicic acid lacked the band at 2.8 μ, but gave the characteristic infrared spectra of fatty acid methyl esters. The slight shifting of some bands was presumably caused by mechanical error.

Table 1 shows the results of gas-liquid

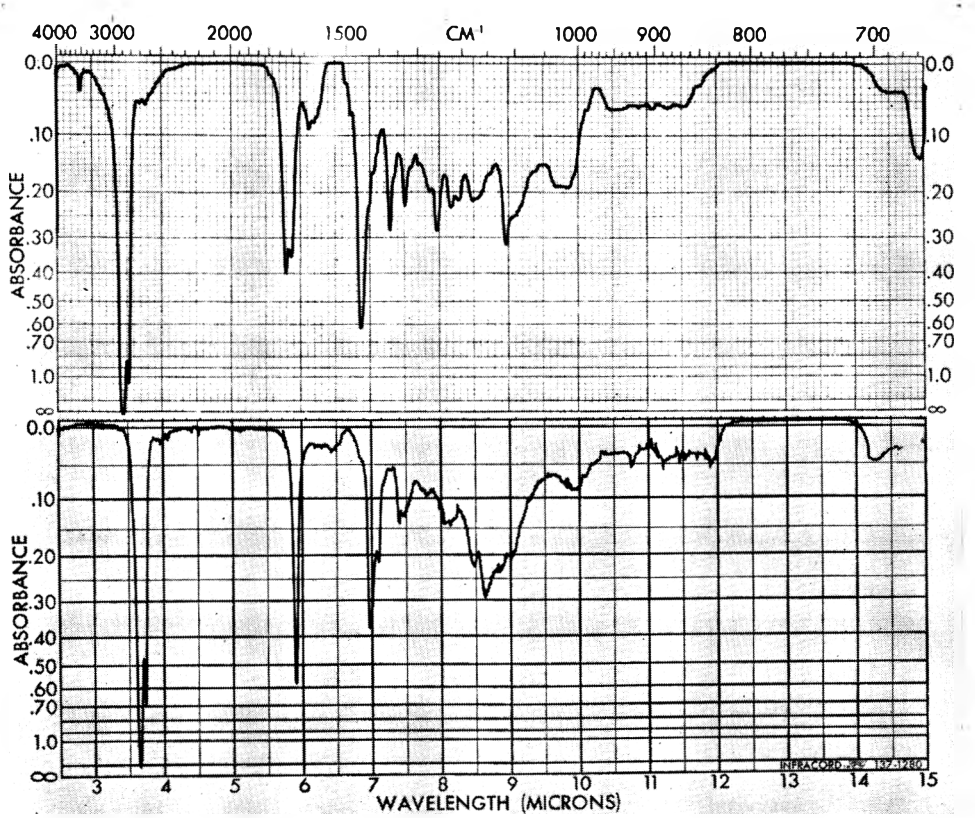

Fig. 1. The infrared spectra of cotton honey crude extract (top) and purified methyl esters (bottom).

Table 1. Gas chromatography of fatty acids isolated from cotton honey.

	Methyl ester standards		Honey methyl esters	
	ECL		ECL	
Caprylate	8.1±.30[a]	(3)[b]	8.6[c]±.21[a]	(6)[b]
Caprate	10.0±.10	(3)		
Laurate	11.9±.10	(4)	11.5 ±.45	(5)
Myristate	14.0±.09	(7)	13.7[c]	(1)
Myristoleate	14.7±.07	(7)	14.8 ±.10	(2)
Palmitate	16.0±.08	(7)	16.0 ±.20	(7)
Palmitoleate	16.6±.11	(7)	16.7	(1)
Stearate	18.0±.11	(7)	18.0 ±.25	(4)
Oleate	18.6±.11	(7)	18.6 ±.25	(7)
Linoleate	19.2±.15	(7)	19.1 ±.18	(5)
Arachidate	20.0±.15	(5)	20.0 ±.29	(5)
Linolenate	20.0±.17	(3)		
Vaccinate	18.3	(1)		
Behenate	21.9	(1)		

[a] Standard deviation.
[b] Number of observations.
[c] Significantly different from the corresponding methyl ester at the 5% level.

chromatography on the purified methyl esters from honey. Definitely shown to be present by comparison with known methyl esters were methyl laurate, myristoleate, palmitate, stearate, oleate, and linoleate. The ECL of 8.6 in the purified methyl esters may be the unsaturated analog of caprylic acid, octenoic acid. Methyl myristate and palmitoleate may be present, but not enough observations were made to be certain. The purified methyl esters contain either methyl arachidate or linolenate. Since the ECL's are similar, it was not possible to determine whether one or both were present. Fig. 2 is a typical gas chromatogram of the honey methyl esters.

The percent concentration of the purified methyl esters was determined with a gas chromatograph equipped with an integrator. By multiplying the integrator value times the molecular weight of each methyl ester, a factor directly proportional to the relative percent composition was obtained. The results of these calculations are shown in Table 2. The methyl ester found in highest concentration was oleate (60.3%), and the second was palmitate (26.7%).

After saponification, the fatty acids of the crude extract were subjected to reverse-phase chromatography. The fatty acids had the same R_f as that of palmitic and oleic acid, as seen in Table 3. Use of iodine vapor confirmed the presence of oleic acid at R_f

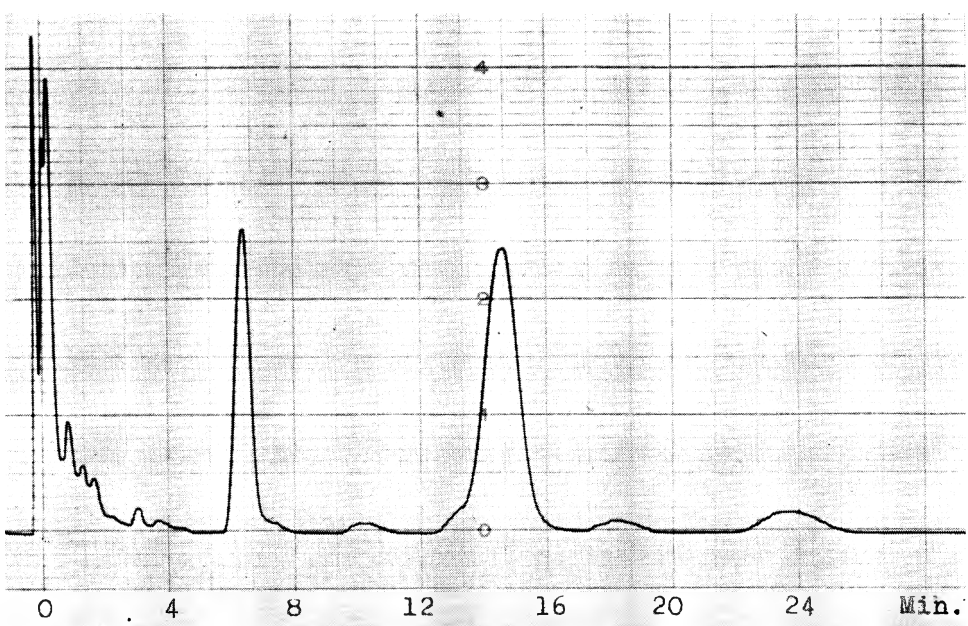

Fig. 2. Chromatogram of purified methyl esters from cotton honey. Research Specialties Co. gas chromatograph model 604; column temperatures, 170°C; pressure, 3.7 lb.

Table 2. Relative concentration of honey fatty acids by integrator technique.

Methyl ester standards		Honey methyl esters		
	ECL	Retention time (min)	ECL	Relative % concentration
Methyl myristate	14.0	3.3	13.9	1.0
Myristoleate		4.0	14.5	1.1
Palmitate	16.0	6.7	16.0	26.7
Palmitoleate	16.5	7.7	16.5	1.0
		10.5	17.4	
Stearate		13.3	18.1	1.7
Oleate	18.5	14.9	18.5	60.3
Linoleate	19.2	18.5	19.2	1.8
Arachidate	20.0	24.0	20.0	6.5

Table 3. Paper chromatography of honey fatty acids.

Sample	R_f
	Indicator: H_2S
Honey fatty acids	0.40 (.35–.45)
Stearic acid	0.29 (.26–.32)
Palmitic acid	0.43 (.41–.46)
Oleic acid	0.40 (.38–.42)
Palmitic and oleic acid	0.40 (.36–.44)
	Indicator: I_2
Honey fatty acids	0.37 (.43–.39) and 0.55 (.52–.58)
Palmitic and oleic acid	0.37 (.33–.42)

0.37. Palmitic and oleic acid cannot be separated by this method; however, since palmitic acid gives a slightly darker spot than oleic, the color of the spots and comparison of R_f values indicate the presence of both palmitic and oleic acid. The fatty acid revealed by iodine vapors at R_f 0.55 may be linoleic acid. Increased concentration did not yield additional fatty acids.

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Kinetic Behavior and Mechanism of Inhibition in the Maillard Reaction. I. Kinetic Behavior of the Reaction Between D-Glucose and Glycine

SUMMARY

The kinetic behavior of the Maillard reaction between D-glucose and glycine is discussed, and apparent rate constants—k_1, from the induction period of the reaction, and k_{st}, from the steady-phase formation of melanoidins—are estimated. The free energies of activation for two steps with corresponding rate constants k_1 and k_{st} are respectively found to be 26.5 and 26.1 kcal mole^{-1}. Both steps have unfavorable negative entropy of activation. The overall rate expression is

$$d(B)/dt = [k_2(k_1(G)_o/k_2'(I))^{1/2} + k_3(g_o - B)]\ (I - B),$$

where I represents intermediates, g and G respectively glycine and glucose, B the brown products, and k's the rate constants. On the basis of kinetic considerations, hydroxymethyl furfural is ruled out as a major intermediate in the Maillard reaction.

INTRODUCTION

Maillard (1912, 1913, 1916, 1917) systematically studied the reactions of aldehydic monosaccharides with amino acids in aqueous solution under various conditions. General reactions leading to brown-colored products from sugar and amino acids are named after Maillard. These thermal reactions (Maillard reaction) produce uncharacterized brown products, sometimes called melanoidins. Despite vast amounts of work representing various laboratories and approaches since Maillard, the mechanism of the reaction remains obscure, mainly because of the complexity of the reaction and the number of variables in the experimental conditions.

We became interested in elucidating this complex reaction in terms of kinetics and inhibition. The present series of papers uses the term "Maillard reaction" since our main concern is the reaction of aldehydic monosaccharides with glycine under conditions similar to those in Maillard's work, thus excluding thermal reactions of monosaccharides alone, which under extreme conditions (such as very acidic or alkaline pH) yield visually similar brown products (for example, see Deschreider, 1954). The complexity of the reaction, as seen from work of Hodge (1953), makes it almost impossible to study the reaction by means of simple analytical or organic chemical methods. A kinetic approach, however, can be used in attacking the problem.

One interesting kinetic facet of the reaction has been noted (Haugaard *et al.*, 1951; Haas *et al.*, 1948). In starting with colorless reactants—glucose and glycine, for example—brown melanoidins are formed only after an induction period. Haugaard *et al.* (1951) and Haugaard and Tumerman (1956) were able to obtain a linear kinetic relationship in the plot of absorbance versus t^2 and to give a graph with a line through the origin. However, as will be pointed out, such a treatment of the induction period is not always applicable. The present series of papers presents work on the kinetic behavior of the reaction.

EXPERIMENTAL

Preparation of reaction mixture. A reaction mixture was made up of 5 ml of aqueous solution of 1*M* D-glucose (obtained from Merck and Co.) and 0.25*M* glycine (obtained from Nutritional Biochemicals Co.), and the pH of the reaction mixture was adjusted to 5.5–5.6 with 0.1*N* HCl or 0.1*N* NaOH.

Kinetic runs. Colorimetric tubes (Pyrex, 1.1 × 10 cm) containing 5 ml of the reaction mixture were flame-sealed to prevent evaporation during long reactions at elevated temperature. The sealed tubes were placed in either a temperature-controlled oven or a water bath at desired temperatures (±1°C). The kinetics of the reaction were followed by reading the color intensity of the solution at wavelength 490 mμ with a Bausch and Lomb colorimeter as a function of reaction time. The reaction time varied from a week to several weeks, depending on experimental conditions and the concentration of the reactants. Evaluation of rate constants for the reaction is described in the next section.

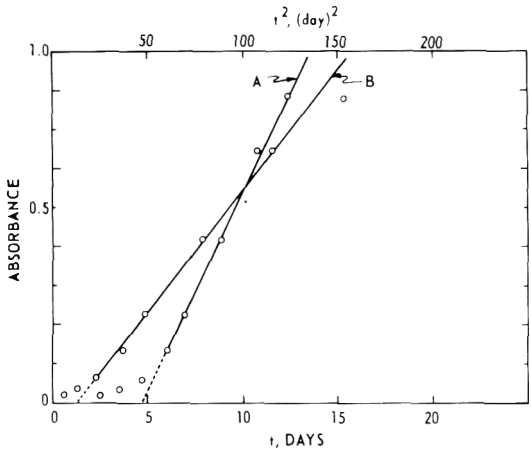

Fig. 1. Kinetics of the reaction for 1.0M D-glucose plus 0.25M glycine at pH 5.6 and 55°C. Plot A, plot of absorbance versus t; and plot B, plot of absorbance versus t^2.

RESULTS AND DISCUSSION

Fig. 1 is a typical kinetic plot. It is possible to draw a straight line for the steady-state phase of production of colored melanoidins up to absorbance 0.8, above which a positive deviation from linearity begins to appear. Extrapolation of the straight-line portion to the time axis gives a value for the induction period, τ. Rate constants obtainable from a plot such a Fig. 1 are approximately evaluated later in this section.

Fig. 1 also gives a plot of absorbance versus t^2. According to Haugaard *et al.* (1951), a graph with a line through the origin can be obtained by plotting absorbance against t^2 instead of t. However, such a graph is possible only under experimental conditions that match those of Haugaard *et al.* with respect to concentrations of reactants, temperature, and pH. For example, one would obtain no significant reaction under very unfavorable conditions, such as low concentrations of reactants and low pH. Curve B in Fig. 1 shows an apparent induction period and initial curvature even under moderate conditions.

To resolve this difficulty, an attempt was made to evaluate an apparent initial rate constant representing the induction period of the reaction. It turned out that the rate constant calculated from the induction period was independent of glycine concentration under pseudo-first-order condition and independent of the wavelength employed for the color measurement. This latter point is particularly noteworthy because melanoidins absorb visible light over wide ranges of wavelength.

In establishing an approximate kinetic scheme fitting the data in Fig. 1, certain approximations were unavoidable because of the formidable complexity of the reaction and the unavailibility of information on the structure of intermediates and products. The following mechanism appears to be reasonable as an over-all picture of the reaction, excluding later steps of extensive polymerization which result in the formation of dark-brown insoluble products.

Using G for D-glucose, g for glycine, I for intermediates, B_f for colored by-products, and B_m for major products (melanoidins):

$$G + g \xrightarrow{k_1} I \qquad [1]$$

$$I \xrightarrow{k_2} B_f \qquad [2]$$

$$I + b \xrightarrow{k_3} B_m \qquad [3]$$

where b represents glycine or its degradation product, such as ammonia. Reaction 1 can be defined as the initial stage of the reaction, during which no coloration takes place. Since the recovery of D-glucose from an intermediate, for example, 1-glycine-D-glucose or its rearrangement products, is practically zero (Chichester, 1954; Hannan and Lea, 1952; Mackinney and Chichester, 1952; Gottschalk and Partridge, 1950), the reverse reactions are not written in the above scheme, although the apparent rate constants k_1, k_2, and k_3 include terms such as K_{eq} intrinsically wherever the reverse reactions actually occur. In the assumed mechanism, I should be the sum of all possible intermediates which can arise from the reactants.

Keeping one of the reactants in excess, glycine for example, the following rate expression can be written for reaction 1:

$$-d(G)/dt = d(I)/dt = k_1(G_o - I) \qquad [1]$$

From the kinetic behavior of the reaction shown in Fig. 1, it can be assumed that major intermediates accumulate up to a steady-state concentration, followed by the formation of colored products as represented

by a straight line. Experimental findings indicate that the following two assumptions may be made to define the kinetic behavior of the reaction.

First, some of the intermediates or by-products of side reactions contribute to "browning" as measured by the method used for following the rate of the reaction. These include aldose fragmentation products such as glycolaldehyde (Taeufel and Iwansky, 1952) and hydroxymethyl furfural (HMF) (Chicester *et al.*, 1952; Nomura and Kawano, 1954; Petit, 1957) and Strecker degradation products of amino acid (Stadtman *et al.*, 1952).

Second, it may be assumed that α,β-unsaturated enolic aldimines are being built up until a steady-state concentration is reached. These aldimines are considered to be intermediate in the Maillard reaction (McWeeny and Burton, 1963; Burton *et al.*, 1963). The appearance of UV absorption in the reaction mixture has been suggested to result from the formation of enolic unsaturated compounds (Hannan and Lea, 1952; Chichester *et al.*, 1952; Friedman and Kline, 1950; Singh *et al.*, 1948; Wolfrom *et al.*, 1949, 1962; Kato, 1960; Mednick, 1962). Anet (1963) maintained the view that unsaturated sugars such as enolic 3-deoxy D-glucose are true intermediates in the formation of HMF and Maillard-reaction end products. Evidence for the importance of α,β-unsaturated intermediates in the reaction will be presented in Part II of this series.

Although sugar fragmentation products such as glycolaldehyde and glyceraldehyde may yield brown products in the presence or absence of amino acids, it seems unlikely that the fragmentation occurs to an appreciable extent during the induction period at the moderate temperature and pH used in our experiments. Autoradiographic evidence to be presented later in the series also indicates that fragmentations do not occur significantly during the induction period. Although these secondary aldehydes may be present in our system and contribute to "browning," they are not considered to be major intermediates in the Maillard reaction, as pointed out above. The formation of HMF in the D-glucose-glycine reaction mixture has been reported and accepted as the main pathway in the Maillard reaction (see references given in Ellis, 1959). We have also identified its presence in the brown mixture spectroscopically (see Part II) and chromatographically (Table 1).

Rice *et al.* (1947) proposed that furans play an important role in the Maillard reaction. Similar proposals have been made by many workers (Ellis, 1959, and references therein). However, the formation of HMF may not be considered as a main pathway in the Maillard reaction, for the following reasons:

1) If the steady-state approximation, $d(\text{HMF})/dt = 0$ at $t = \infty$, is applied to the present system, the concentration of HMF should be approximately maximum at the end of the induction period extrapolated from the steady-phase line to the abscissa (see Fig. 1). However, it was observed in spectrophotometric experiments (to be reported in Part II) that the concentration of HMF increases steadily throughout the entire period of the reaction, as measured by absorbance at 283 mμ of the compound of R_f 0.78 (Table 1). This suggests that HMF is being accumulated rather than being incorporated into the steady-state phase of the reaction.

2) The induction period was not eliminated by adding to the reaction mixture at the start of the reaction, a steady-state concentration of HMF (9.3×10^{-5} M^{-1}, calculated from $\epsilon_{284\ m\mu} = 16{,}830$ and assuming that absorbance at 284 mμ of the reaction

Table 1. Identification of HMF by paper chromatography (solvent *n*-butanol–ethanol–water, 4:1.1:1.9, see Experimental section in Part II of this work).

Reaction time	R_f Commercial HMF	R_f Sample	Benzidine test	λ_{max} (mμ)
10 days	0.80	0.78	+	282.5[a]
				283[b]

[a] Commercial HMF.
[b] Sample from reaction mixture.

mixture at the end of induction period was due entirely to HMF; of course, the latter assumption would yield a much higher value for the concentration of HMF than the actual concentration, since other substances in the reaction mixture undoubtedly absorb at the same wavelength region) or its tenfold concentration (9.3×10^{-4} Ml^{-1}).

3) No furan derivatives are possible from sugars such as tetroses and trioses, all of which are common substrates of the Maillard-type reaction. In fact, any aldehydes undergo apparently similar browning reaction in the presence of amino acids.

4) Although the formation of HMF is strongly acid-catalyzed (Pigman and Goepp, 1948), the Maillard reaction is extremely slow under these conditions, as will be shown later.

However, since HMF yields a yellowish color in the presence of amino acid in aqueous solution and copolymerizes to form brown products under thermal condition, the coloration due to HMF may be called a secondary "browning reaction." The reaction scheme must therefore include contributions from the formation of HMF as well as of other possible color precursors via side reactions.

An assumption that the α,β-unsaturated enolic intermediates play a role in the reaction has been partly noted in the literature, and will be justified in a succeeding paper.

We now account for the Maillard reaction and "browning reaction" as two competitive reaction pathways—3 and 2—in which B_f and B_p are now identified as B as far as measurement of the absorbance of the solution is concerned. Since the final stage of the Maillard reaction can be considered to be condensation polymerization, the molecular weight of B products would increase. B products can be formed via reactions 2 and 3 by consecutive mechanisms. Within the range of absorbance measured (1.0), all the colored products were dialyzable. After a considerable period (20–30 days), some products precipitated as nondialyzable polymers and aggregates. From two assumptions made above and experimental observations, the following rate expression can be written for reaction 2:

$$d(B_f)/dt = k_2(I-B_f) \qquad [2]$$

For reaction 3 we have:

$$d(B_p)/dt = k_3(b-B_p)(I-B_p) \qquad [3]$$

Combining Eqs. 2 and 3 yields an approximate rate law for the steady phase in Fig. 1:

$$d(B)/dt = [k_2 + k_3(b-B)]\,(I-B) = K_{st}(I-B) \qquad [4]$$

where $k_{st} = k_2 + k_3(b-B)$ and designates an apparent rate constant of the steady-phase reaction.

From Eq. 1 in its pseudo-first-order form with respect to glycine instead of glucose, Eq. 5 is obtained after integrating Eq. 1 and evaluating the integration constant at $t = 0$ and $I = 0$;

$$\ln(g)_o/(g-I) = k_1 t = k_1 \tau \quad \text{at } t = \tau \qquad [5]$$

where t equals the induction period thus,

$$(I) = g(1-e^{-k_1 t}) \qquad [6]$$

Substitution of Eq. 6 in Eq. 4 yields an overall rate expression for the steady-phase formation of brown products:

$$d(B)/dt = [k_2 + k_3(b-B)]\,[g(1-e^{-k_1 t})-B] \qquad [7]$$

On the other hand, a plot of induction period against $\ln(g)_o$ according to Eq. 5 yields a straight line with the slope equal to k_1 (Fig. 2). A similar relationship can be obtained by plotting τ against $\ln(G)_o$ at constant g (Table 2).

As shown in Fig. 1, a straight line during the steady-state phase can be drawn after the induction period. In the presence of relatively constant G, the rate of the steady-state coloration can be expressed as follows:

$$-d(I)/dt = d(B)/dt = k_{st}(I-B) \qquad [8]$$

where $k_{st} = k_2 + k_3(b-B)$. To account for the induction period in the overall rate of the reaction, a first-order Eq. 8 can still be useful by integrating the equation and introducing τ into the equation:

$$\ln(B)/(I-B) = k_{st}(t-\tau) \qquad [9]$$

However, a plot of log A against t for the data shown in Fig. 1 showed negative deviation from linearity, nor did an apparent second-order plot of $1/A$ against t yield a straight line. Under these circumstances, a relative rate constant is evaluated from the slope of Fig. 1 as an approximation, and these constants (or rate) designated K_{st} are

Table 2. Dependence of induction period (τ) on concentrations of glycine and D-glucose at a constant concentration (1M) of either of the reactants, respectively, and at different temperature.

Concentration (M^{-1})	τ (min × 10^{-3})					
	99°C	89.5°C	80.5°C	69°C	57°C	53°C
Glycine						
0.025	0.66	1.24	2.80			
0.050	0.54	1.02	2.04	4.02		
0.125	0.36	0.66	1.44	3.01	6.02	19.90
0.250	0.21	0.38	0.78	1.89	4.26	8.74
0.500	0.061	0.18	0.36	0.95	2.40	5.26
Glucose						
0.02	0.262	0.462	0.864	2.14	5.05	
0.05	0.204	0.368	0.680	1.56	4.02	
0.10	0.148	0.278	0.550	1.24	3.30	6.20
0.20	0.112	0.200	0.361	0.92	2.40	3.30

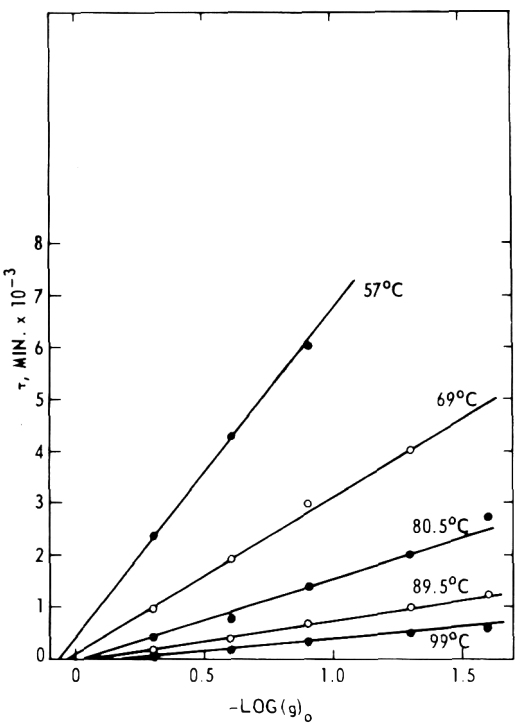

Fig. 2. Plot of induction period against log $(g)_o$ at 1M D-glucose and pH 5.6 at different temperatures.

then evaluated from the type of plot shown in Fig. 1. In turn, these K_{st} values are used to find the correct kinetic order for the steady-phase coloration.

If log K_{st} is plotted against log $(g)_o$ or log $(G)_o$, a linear relationship is obtained (Figs. 3, 4). While the values of the slopes of the plots of log K_{st} against log $(g)_o$ can be taken as approximately an integer of unity over the temperature range studied, the slopes of the plots of log K_{st} against log $(G)_o$ appear to be a non-integer value of about ½, which suggests the complexity of the reaction in both kinetics and mechanism (Table 3). A probable kinetic explanation is given later for ½-order term with respect to D-glucose.

It is now possible to establish a kinetic rate law consistent with the above observation. As will be discussed in Part II, it is

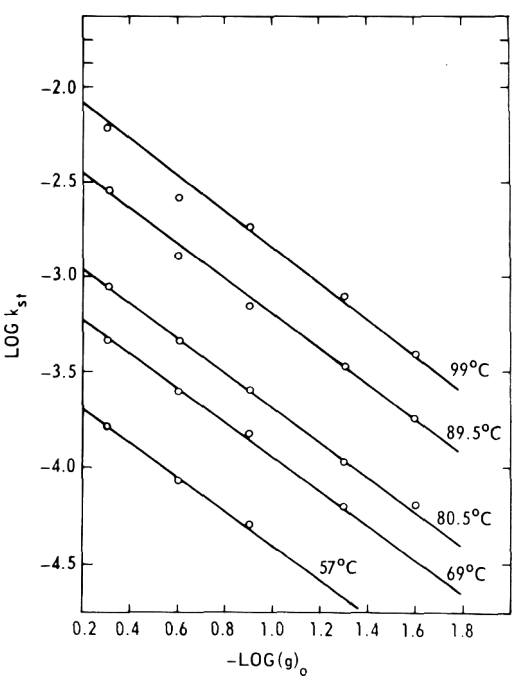

Fig. 3. First-order plot of log K_{st} versus log $(g)_o$ at different temperatures.

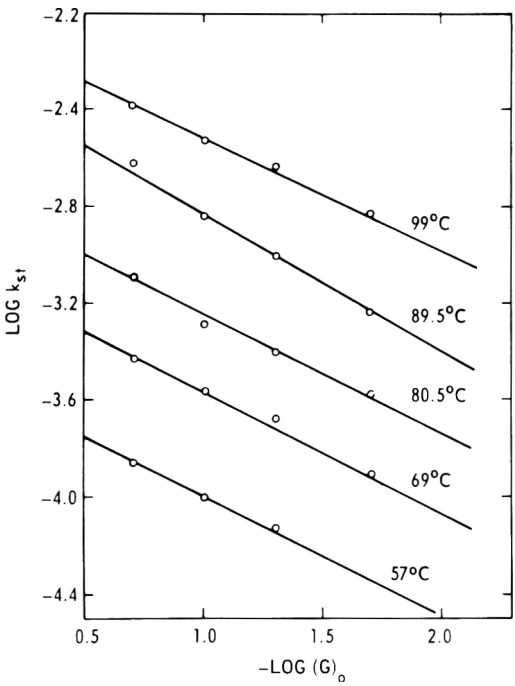

Fig. 4. First order plot of log K_{st} versus log $(G)_o$ at different temperatures.

very likely that only a conjugated base of the glycine amino group will react with D-glucose in the initial step. Eliminating the pseudo-first-order condition, Eq. 1 may be rewritten as:

$$-d(G)/dt = -d(g)/dt = d(I)/dt = k_1(G_o - I)[(g^+/K_b(H^+)) - I] \quad [10]$$

where

$$K_b = \frac{(CH_2-COO^-)}{NH_3^+}\Big/\frac{(CH_2-COO^-)}{NH_3^+}\,(H^+) = g^+/(g)\ (H^+).$$

Rearranging Eq. 10,

$$d(I)/(G_o - I)(g^+/K_b(H^+)) = k_1\,dt$$

and integrating this expression via partial fraction, and evaluating the integration constant at $I = 0$ and $t = 0$,

$$\frac{1}{(G)_o[(g^+/K_b\ (H^+)) - I]} \ln \frac{(g^+/K_b(H^+))(G_o - I)/[G_o - (g^+/K_b(H^+))]}{} = k_1 t \quad [11]$$

From Eq. 11, I can be expressed as follows:

$$I = \frac{(G)_o(g^+/K_b(H^+)(e^{-k_1[G_o-(g^+/K_b(H^+))]t} - 1)}{[(g^+/K_b(H^+))e^{-k_1[G_o-(g^+/K_b(H^+))]t} - (G)_o]} \quad [12]$$

In the presence of excess glycine, Eq. 3 takes the following form, b being now identified as glycine:

$$d(B_p)/dt = k_3(I - B_p)(G_o - B_p)^{1/2} \quad [13]$$

where ½-order term appears in accordance with the result in Table 3. Under the condition of comparable concentrations of G and g, we have the following rate expression for the rate of formation of total B products:

$$d(B)/dt = k_2 + k_3((g^+/K_b(H^+)) - B)(G_o - B)^{1/2}$$

$$\left[\frac{(G)_o(g^+/K_b(H^+))(e^{-k_1\{G_o-(g^+/K_b(H^+))\}t} - 1)}{(g^+/K_b(H^+))e^{-k_1\{G_o-(g^+/K_b(H^+))\}t} - (G)_o}\right] \quad [14]$$

Under pseudo-first-order condition and keeping G relatively constant, Eq. 14 can be simplified to give:

$$d(B)/dt = [k_2 + k_3\{(g^+/K_b(H^+)) - B\}][(g^+/K_b(H^+))\{(1 - e^{-k_1 t}) - B\}] \quad [15]$$

Eq. 15 can then be used to account for the induction phenomena. K_{st} can be used to evaluate k_{st} from the following relationship, where g is approximately equal to the original concentration, as will be evidenced from a tracer experiment to be reported in Part IV of the series, K_{st} being equal to the right-hand side of Eq. 15:

$$k_{st} = \frac{K_{st}}{(g)\ (1 - e^{-k_1 t})} \cong \frac{[k_2 + k_3\ (g)][(g)(1 - e^{-k_1 t})]}{(g)(1 - e^{-k_1 t})} = [k_2 + k_3(g)] \quad [16]$$

where t is equal to the induction period, τ, and B is equal to approximately zero at the end of the induction period. In principle, values of k_2 and k_3 can be determined from the plot according to Eq. 16 if accurate data are available.

Table 3. Slopes of the plots in Figs. 3 and 4.

Temperature (°C)	Slope of plot of log K_{st} vs. log $(g)_o$	Slope of plot of log K_{st} vs. log $(G)_o$
99.0	0.97	0.47
89.5	0.92	0.57
80.5	0.92	0.49
69.0	0.91	0.50
57.0	0.91	0.50
Av.	0.93	0.51

The fact that a ½-order term with respect to D-glucose in the rate equation may indicate complexity is a manifestation of the complex nature of the Maillard reaction. It is difficult to see how D-glucose participates

Table 4. Thermodynamic quantities of the activation in the Maillard reaction at 330°K.[a]

k ($sec^{-1} \times 10^6$)	A (sec^{-1})	E_a (kcal $mole^{-1}$)	$\Delta H^{\ddagger}$	$\Delta F^{\ddagger}$	$\Delta S^{\ddagger}$
$k_1 = 5.92$	2.64×10^5	16.1	15.3	26.5	−33.98
$k_{st} = 7.8$	2.46×10^6	22.1	21.5	26.1	−13.87

[a] A is Arrhenius frequency factor; E_a is Arrhenius activation energy; $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$, and $\Delta S^{\ddagger}$ are respectively enthalpy, free energy (in kcal/mole), and entropy (in cal/deg/mole) of activation.

in the steady-phase reaction. However, it can be speculated upon by assuming the following scheme:

$$G \xrightarrow{k_1} S_1 + S_2 \qquad [4]$$

assuming that both S_1 and S_2 contribute to the formation of B by termolecular reaction:

$$I + 2S \xrightarrow{k_2'} B_I \qquad [5]$$

Steady-state treatment then yields the following over-all rate expression:

$$d(B)/dt = k_2(k_1(G)/k_2'(I))^{1/2} - k_3(g_\infty - B)(I - B) \qquad [17]$$

where I is given by Eq. 12. No attempt can be made to suggest the nature of the intermediates and S's, and the mechanism of reactions 4 and 5 must be highly speculative.

The validity of the observed rate constants, k_1 and k_{st}, was established by the linearity of the plot of log k against $1/T$ (Fig. 5). Thermodynamic quantities calculated from the data on temperature dependence of the reaction are given in Table 4.

It appears from Table 4 that both initial and later steady-phase steps of the reaction have negative entropy of activation, especially for k_1, although E_a's are relatively low. It was shown that both k_1 and k_{st} of the reaction were independent of ionic strength, suggesting that the rate-determining transition-state complex consisted of oppositely charged species with equal magnitude.

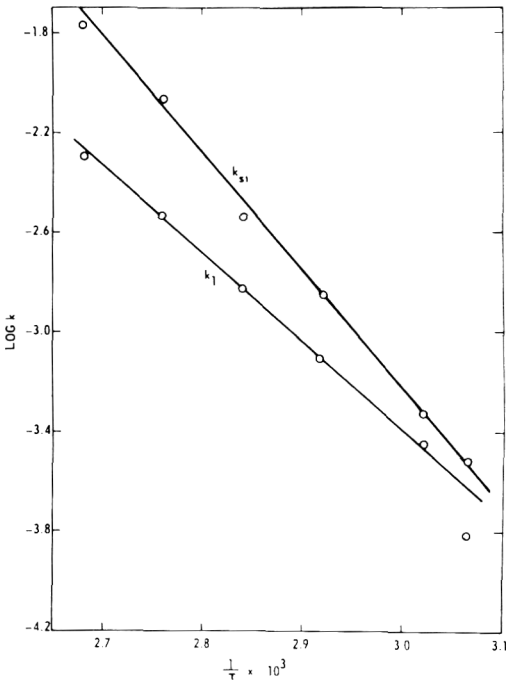

Fig. 5. Temperature dependence of rate constants k_1 and k_{st}. Apparent rate constants (k_{st}) were evaluated for the reaction mixture consisting of 1.0M D-glucose and 0.25M glycine.

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Kinetic Behavior and Mechanism of Inhibition in the Maillard Reaction. II. Mechanistic Considerations in the Reaction Between D-Glucose and Glycine

SUMMARY

The mechanism of the initial Maillard reaction was investigated in kinetic, autoradiographic, spectroscopic, and model studies. Results indicate that the initial reaction involves the formation of glucosylglycine from aldehydic glucose and the free amino group of glycine, followed by base-catalyzed conversion of glucosylglycine into α,β-unsaturated aldimine, with the steady-state concentration of the latter accumulated during the induction period and undergoing subsequent reactions to yield melanoidins. Evidence to support this mechanism is presented and discussed. Also proposed is a possible mechanism for the melanoidin formation in the reaction. Side reactions giving rise to products of "browning reaction," such as from hydroxymethyl furfural, were not considered.

INTRODUCTION

The early stage of the Maillard reaction has been proposed to involve the formation of an N-substituted D-glucosyl amine, namely 1-glycine-D-glucose (Hannan and Lea, 1949; Micheel and Klemer, 1952; Weygand, 1939). A compound similar to 1-glycine-D-glucose has been chromatographically isolated in crystalline form by passing a reaction mixture through a cellulose column (Mackinney and Chichester, 1952; Lea and Hannan, 1950).

Katchalsky and Sharon (1953) proposed the subsequent formation of Schiff's base from N-substituted D-glucosyl amine. Hodge (1953) and others (Bayne and Holms, 1952; Pigman *et al.*, 1951) postulated that the Amadori rearrangement of N-substituted D-glucosylamine occurs as a subsequent step in the reaction, finally yielding the brown melanoidins. One of the rearrangement products, N-substituted 1-deoxy-1-amino-D-fructose, has been isolated by Richards (1956). The Amadori rearrangement is acid-catalyzed (Weygand, 1940); and the rearrangement products undergo browning decomposition at alkaline pH, and the rate of the color development is considerably enhanced by additional amino acids (Hodge and Rist, 1953). The participation of amino acids in the browning stage of the reaction was kinetically confirmed in the present studies (Part I).

Evidence for the formation of enolic intermediates was discussed by Ellis (1959). Enolic compounds were recently reported to react readily with amines to form colored polymers (Dubourg and Devillers, 1962). We support the importance of enolic intermediates in the Maillard reaction, since the over-all Maillard reaction is accompanied by a pH decrease. It is likely that the enol form of an N-substituted glycosyl amino acid lowers the pKa of the amino acid carboxyl group, in addition to pH changes that can be attributed to the change in pKa of the carboxyl group as the zwitterionic amino acid becomes N-substituted D-glucosyl amino acid derivatives other than the enol. A general mechanism will be proposed in which the α,β-unsaturated enolic intermediate plays an important role in the Maillard reaction.

The present paper (a successor to Part I) presents the results of studies on the mechanistic aspect of the Maillard reaction, and reviews some mechanisms proposed by others in the light of our findings and the general mechanism applicable to systems of reactants other than D-glucose and glycine.

EXPERIMENTAL

Materials. The reagent-grade compounds used were as follows: D-glucose from Merck and Co.; methyl-α-D-glucoside, D-arabinose, glycine, DL-serine, α-alanine, and β-alanine from Nutritional Biochemicals Co.; D-fructose, 2-amino-D-glucose, propionaldehyde, n-heptaldehyde, capronaldehyde, p-hydroxybenzaldehyde, and 2-propenal from Eastman Organic Chemicals; paraformaldehyde from Aloe Scientific Co.; ascorbic acid, USP, from Hoffmann–La Roche; and aniline from J. T. Baker Chemical Co.

Synthesis of glucosyl glycine. Twenty g of D-glucose and 2.8 g of glycine in 140 ml of dried methanol were refluxed for 5 hr. Fifty ml of methanol and 50 ml of acetone were added to the reaction mixture, which was then reheated to dissolve the mixture. After filtration the filtrate was mixed with 300 ml of acetone and kept in the refrigerator overnight. Small amounts of precipitates were then collected to be washed with methanol-acetone (1:4) several times. The washed precipitates were dried in a desiccator under reduced pressure and were recrystallized from methanol-acetone as described above. Needle-like crystals were obtained with a yield of 0.11 g. An attempt to prepare this compound by the method of Micheel and Klemer (1952) was not successful. Analysis: N, 5.95% (calculated), for $C_8H_{14}O_7N.N$, 5.79% (found), mp, 258–259° (found).

Preparation of reaction mixtures. Reaction mixtures were prepared as described previously (Part I). Reactants other than D-glucose and glycine were prepared in the same way.

Kinetic run. The reaction was followed as described previously (Part I).

Spectroscopic examination of reaction mixtures and products. *Spectra of reaction mixtures.* To follow the reaction more closely, UV spectra of reaction mixtures were measured occasionally with a Beckman DK2 spectrophotometer. Spectrum *A* in Fig. 1 shows a typical spectrum of the reaction mixture during reaction.

The spectra of the samples that follow were measured in the same manner unless otherwise specified.

Spectra of ether or ethylacetate extracts of reaction mixture. Extraction with ether or ethylacetate was done in an attempt to isolate some of the unsaturated intermediates which may form during the reaction. UV absorption spectra of the extracts were taken. Usually, 5–10 ml of aqueous brown reaction mixture were extracted with 50–100 ml of ether or ethylacetate, and the extracts were then concentrated *in vacuo* for spectral measurement in any desired solvent.

Separation of intermediate(s) on aluminum oxide deposits. An attempt was made to isolate intermediate(s), which may be so reactive that isolation from the reaction mixture is practically impossible. Isolation would be possible if intermediate(s) are adsorbed (trapped) on an adsorbent in the reaction vessel as soon as they are formed in the solution, thereby making the reaction stop. About 5 g of aluminum oxide granules were deposited at the bottom of the reaction vessel, containing 100 ml of reaction mixture at 55°C. The reaction was retarded to some extent by the presence of the adsorbent, although some malanoidin products

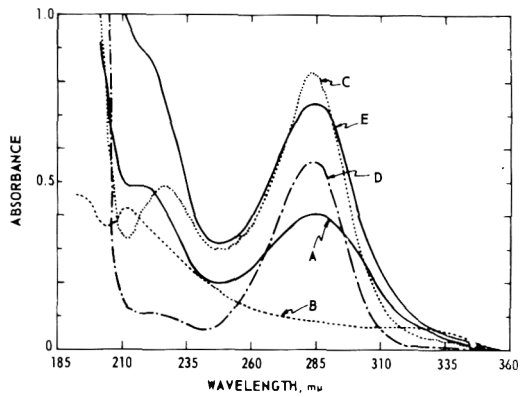


Fig. 1. Ultraviolet spectra in water.
A: Reaction mixture of 1*M* D-glucose and 0.25*M* glycine after 144 hr, diluted 100-fold.
B: Melanoidins after a thorough dialysis.
C: Commercial hydroxymethyl furfural (HMF).
D: The eluate from the spot of chromatographic R_f 0.78 of reaction mixture.
E: The ether extract of the reaction mixture (the extract is identified mainly as of HMF by the test shown in Table 1, Part I).

were formed at a later stage of the reaction. The absorption spectrum of ether extract from the adsorbent was taken in water (Fig. 5). Some melanoidins were also adsorbed on the deposits, but they were not eluted by either ether or ethylacetate extraction.

Preparation of melanoidins. After sufficient color was developed (more than absorbance of 2.0), 200 ml of 1*M* D-glucose plus 0.5*M* glycine mixture were dialyzed against running water for a week, during which most of the reactants as well as low-molecular-weight brownish-yellow compounds were dialyzed out. The dark-brown polymers (melanoidins) were then collected, and their UV (Fig. 1) and visible spectra (Fig. 2) were recorded.

Bromination test. To obtain qualitative information on the structure of the intermediate(s) of the reaction, the bromine addition test was carried out occasionally for the reaction mixture. Since enolic compounds have been suggested as intermediates in the Maillard reaction, and bromine adds to enolic double bonds instantly, the test would indicate their presence in the mixture. The procedure of the test is given by Feigl (1960).

Paper chromatography and autoradiography. An aliquot of reaction mixture was chromatographed to follow the reaction patterns. Filter papers used were Whatman No. 1 for most aliquots of the reaction mixture, and Whatman No. 4 for gluconic acid. The solvent systems employed for chromatography were as follows:

Solvent system	*Composition (V/V)*
I	n-butanol–acetic acid–water (4:1:1)
II	isopropanol–water (4:1)
IIIa	n-butanol–ethanol–water (4:1.1:1.9)
IIIb	n-butanol–ethanol–water (4:1:5)
IV	ethanol–pyridine–water–aqueous ammonia (30:10:8:2)
V	n-butanol–formic acid–water (4:1:5)
VI	isopropanol–isoamylalcohol–water (3:1:1).

Autoradiography was done for the radioactive reaction mixture chromatographed on Whatman No. 1 paper. The paper chromatograms were placed on 35.3 × 42-cm Kodak Medical X-ray films and covered with a film exposure folder for the required time.

Preparation of radioactive reaction mixtures. D-Glucose-C_1^{14} and glycine-C_1^{14} were obtained from New England Nuclear Co. The following systems containing tracer reactants were studied:

I. D-Glucose ($1M$) plus D-glucose-C_1^{14} ($5.33 \times 10^{-3}M^{-1}$, specific activity 10.4 mc/mg) and glycine ($0.5M$);

II. D-Glucose ($1M$) plus glycine ($0.5M$) and glycine-C_1^{14} ($7.97 \times 10^{-3}M^{-1}$, specific activity 2.49 mc/mmole).

Radioactivity counting. Radioactive strips from paper chromatograms corresponding to radioactive areas determined by autoradiography were counted on an automatic scanner using a gas-flow counter (Tracer Lab Chromatogram Autoscanner). Spots located by autoradiography were cut out and counted in a liquid scintillation spectrometer (Packard Tricarb Model 314 FX-2) immersed in a scintillation liquid (0.6% 2,5-diphenyloxazole plus 0.05% 1,4-bis-2-(5-phenyloxazolyl)-benzene in toluene) (Davidson, 1962).

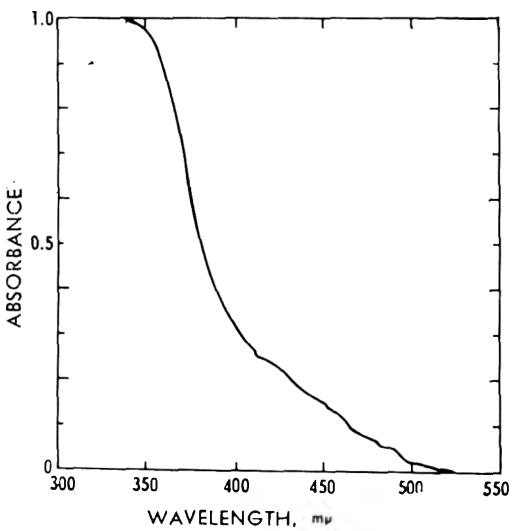


Fig. 2. A typical visible spectrum of melanoidins in water.

RESULTS

pH profiles of the reaction. Fig. 3 shows pH profiles of the reaction and of the reaction between D-fructose and glycine. pH profiles indicate that the reaction is strongly catalyzed by base (perhaps general acid-base catalysis). The similarity of pH profiles of glucose-glycine and fructose-glycine systems suggests that the behaviors of the two systems toward base catalysis are identical.

Comparative rate data on the reactions with various analogs. Table 1 shows for various reactants the induction periods from which was calculated (Part I) the rate constant k_1, and rate K_{a1} and k_{a1}. It is assumed that the reaction with different analogs follows a general reaction scheme analogous to that of the D-glucose-glycine system, since brown products produced with these analogs always showed broad UV and visible spectra and kinetic behaviors that were apparently the same. All reactions were enhanced by higher pH and inhibited by sodium bisulfite, as in the reaction of the D-glycose–glycine system.

From Table 1 and additional experiments the following results can be summarized:

a) D-Fructose is considerably more reactive than

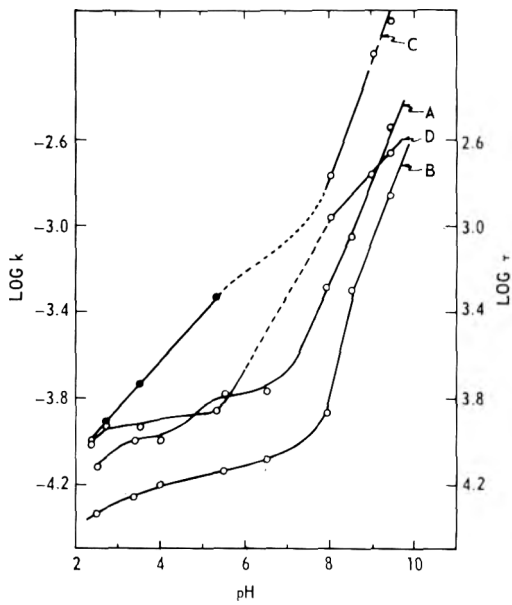


Fig. 3. pH profiles of the reactions of glucose-glycine and fructose-glycine systems.
A: Plot of k_1;
B: Plot of K_{a1} evaluated at $1M$ D-glucose and $0.25M$ glycine;
C: τ in $1M$ D-fructose plus $0.25M$ glycine reaction;
D: K_{a1} evaluated at $1M$ D-fructose plus $0.25M$ glycine.

Table 1. Comparative rate data for different reactants at 55°C. The pH's of aqueous solutions are 5.3–5.5 unless stated otherwise.

Reactants (M^{-1})	τ (min $\times 10^{-3}$)	τ_G/τ_A	τ_g/τ_a	$K_{st} \times 10^5$ (M^{-1})	$\frac{K_{stG}}{K_{stA}}$	$\frac{K_{stg}}{K_{sta}}$
D-Glucose (1)						
Glycine (0.25)	6.91	1	1	7.1	1	1
D-Glucose (1)						
α-Alanine (0.25)	5.33		1.3	12.8		0.56
D-Glucose (1)						
β-Alanine (0.25)	1.76		3.9	38.8		0.18
D-Glucose (1)						
Aniline (0.25)	ca. 0.8		ca. 8.5	[a]		[a]
D-Fructose (1)						
Glycine (0.25)	2.30	3.0		14.0	0.51	
D-Arabinose (1)						
Glycine (0.25)	1.08	6.4		41.9	0.17	
α-Methyl-D-glucoside (0.5)						
Glycine (0.5)	13.0			1.6		
2-Amino-D-glucose (0.5)						
pH 2.5	10.6	0.7		3.2	2.2	
2-Amino-D-glucose (0.5)						
Glycine (0.25), pH 2.8	0	∞		36.8	0.19	
2-Amino-D-glucose (0.5)						
pH 6	0.04	168		942	0.007	
Ascorbic acid (0.5)						
Glycine (0.5), pH 3.4	1	8.4[b]		21.7	0.29[b]	
Capronaldehyde (2)						
Glycine (0.25)[c]	3.61			0.9		
Heptaldehyde (1)						
Glycine (0.25)[c]	1.44			4.5		
Propionaldehyde (1)						
Glycine (0.25)[c]	20.4			0.8		
Paraformaldehyde (10%)						
Glycine (0.25)[d]	0.52			6.9		

τ_G and τ_g are the respective induction periods for D-glucose and the glycine system, and K_{stG} and K_{stg} are steady-phase rate of coloration; subscripts A and a respectively refer to D-glucose analogs and amino acid analogs.

[a] Color measurement was not possible, because of turbidity developed during the reaction.

[b] Ratios obtained from data using 1.0M glucose and 0.5M glycine at pH 3.4.

[c] In 70% ethanol.

[d] In 50% ethanol.

D-glucose. pH profiles of the reaction closely resemble that of glucose-glycine system (Fig. 3). The reaction mixture of D-fructose and glycine at the end of the induction period was strongly positive to the bromination test for enolic compounds.

b) The induction period is practically nonexistent in the 2-amino-D-glucose reaction. Since the induction period is kinetically a reciprocal function of the formation of intermediates in the reaction, minimization of induction period in 2-amino-D-glucose reaction is of great significance for elucidating the reaction mechanism. During the reaction, ammonia was released as detected by Nessler's reagent, and bromination for the reaction mixture at the start of color development was positive.

c) α-Methyl-D-glucoside has a considerably longer induction period and slower K_{st}, indicating that the aldehydic form of glucose is the reactant to be attacked by the amino nucleophile.

d) The rate of formation of brown products from ascorbic acid and glycine is greater than that of the D-glucose and glycine system. It is to be noted that ascorbic acid is a reactive enol.

e) D-Arabinose appears to be more reactive than D-glucose, while aliphatic aldehydes do not seem to be particularly reactive (although a comparison of the rate of reaction of the latter with that of D-glucose-glycine is not meaningful, because of the different solvent and concentration used).

Chromatography and autoradiography of reaction mixture during induction period. An aliquot of colorless reaction mixture containing C^{14} reac-

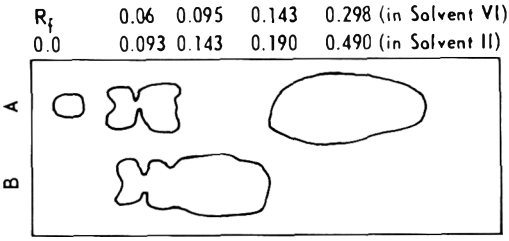


Fig. 4. Autoradiogram of the reaction mixtures after 3 days:
A: Aliquot of D-glucose-C^{14} plus glycine (0.25M);
B: Aliquot of D-glucose plus glycine-C^{14}.
The solvent used was VI, and R_f's of the respective spots in the solvent system II are also indicated.

tants during the induction period was chromatographed and autoradiographed (Fig. 4). There appear to be only two initial products of relatively high concentration from D-glucose (R_f 0.298) and glycine (R_f 0.143). Separate chromatograms with the two spots (R_f 0.06 and 0.095) and reactants were sprayed with ninhydrin, benzidine, and m-phenylenediamine reagents. Qualitative tests showed that the compound with R_f 0.095 was slightly ninhydrin-positive and benzidine- and m-phenylenediamine-positive, and that with R_f 0.06 (synthetic N-D-glucosylglycine has R_f 0.07) was benzidine- and m-phenylenediamine-negative but slightly ninhydrin-positive.

From the results of qualitative tests and cochromatography with synthetic N-D-glucosylglycine, it is concluded that the compound of R_f 0.06 is probably N-D-glucosylglycine, which may undergo consecutive reaction to yield the compound of R_f 0.095. Qualitative tests for the compound with R_f 0.095, as well as the ratio of radioactivity counting of the spot from glucose-C^{14} and glycine-C^{14} reaction mixtures, are shown in Table 2. Bromination test for the eluate from R_f 0.095 in solvent VI or R_f 0.11 in solvent IIIa in Table 2 was only slightly positive, owing to low concentration.

Although further proofs are needed, it appears from Table 2 that the compound of R_f 0.095 is probably α,β-unsaturated enolic aldimine which may be in equilibrium with the keto form in water, similar to the compound isolated by Richards (1956), since α,β-unsaturated aldehydes or aldimines give a rapid positive test with m-phenylenediamine whereas saturated compounds are less reactive (McWeeny and Burton, 1963). As discussed in the section on inhibition, the reaction mixture at the end of the induction period consumes a significant amount of bromine. These results suggest that reactive intermediate(s) and malanoidin precursors are N-substituted enolic derivatives.

Identification of hydroxymethyl furfural. Paper chromatography of a reaction mixture revealed the presence of HMF, as identified by cochromatography with authentic HMF. The chromatogram showed a number of spots with lower R_f values than HMF, which also has a characteristic yellow color with benzidine spray on the paper. The result is shown in Table 1, Part I. The same spot can be obtained from ether or ethylacetate extract of the reaction mixture. UV spectra of ether and ethylacetate extracts resemble that of authentic HMF (see spectra C and D, Fig. 1).

Adding a considerable amount of HMF to an initial reaction mixture did not shorten the induction period significantly. The kinetic result concerning the role of HMF is discussed in the previous paper (Part I).

Separation of reactive intermediate(s) and absorption spectra. Since α,β-unsaturated aldehydes (for example, 2-furfuraldehyde) are readily adsorbed on aluminum oxide from organic solvents (Khol'kin and Reznikov, 1959), we attempted to isolate some α,β-unsaturated aldehyde(s) or aldimine(s) from the reacting mixture of glucose and glycine by means of adsorption on an aluminum oxide granule deposit as described earlier. Although hydration was expected to be extensive, it was possible to elute adsorbed substance on the deposit with ether, and an absorption spectrum was taken (Fig. 5).

The ethereal eluate from the adsorbent gave self-browning on standing at room temperature

Table 2. Qualitative tests of paper chromatograms for the compound of R_f 0.095 and equivalent spot in other solvent systems.

Solvent system	R_f	Incorporation ratio[a] of glucose-C^{14} to glycine-C^{14}	Ninhydrin	Benzidine[b]	m-Phenylenediamine[c]
VI	0.095	1.198	±	+	+
II	0.143	1.320	+	+	+
IIIa	0.110	1.076	+	+	+
	0.11[d]		+[d]	+[d]	

[a] Calculated from percent incorporation of radioactive glucose and glycine.
[b] Prolonged heating at 55–60°C was required.
[c] At room temperature.
[d] Reported (Richards, 1956).

for two days, indicating that the compound(s) isolated are of reactive nature. Aqueous solution of the eluate was also positive in the bromination test. The eluate had maximum absorption at 276–278 mμ, whereas authentic 2-furfuraldehyde has absorption maxima at 276 and 229 mμ, and HMF at 283 mμ. Thus, the compound in the eluate cannot be HMF. Although the absorption maxima of the eluate and of 2-furfuraldehyde are close together, the shapes are somewhat different, especially in wavelength regions higher and lower than the maximum absorption region, suggesting that the eluate has either substance(s) different from 2-furfuraldehyde or some other compound along with 2-furfuraldehyde. However, it seems unlikely that 2-furfuraldehyde (C_5) is formed from D-glucose (C_6) under the mild conditions employed. Paper chromatography further showed no 2-furfuraldehyde in the reaction mixture of the D-glucose-glycine system, although HMF was detectable. The absorption spectrum obtained from the reaction mixture of the 2-amino-D-glucose-glycine system is also compared to show a similarity of the mechanism with respect to the structure of the intermediate (Fig. 5), as is discussed later. The brown products from 2-furfuraldehyde and glycine after ether treatment and dialysis showed a difference in absorption spectrum from that of melanoidins from the D-glucose-glycine reaction, again suggesting that 2-furfuraldehyde, if any, is not a significant contributing intermediate in the D-glucose-glycine Maillard reaction (Fig. 6).

Fig. 6 shows that the absorption spectrum of brown products from 2-furfuraldehyde and glycine has a small spectral shift in 80% ethanol from that in water. The spectrum of melanoidins from the D-glucose-glycine reaction, however, was almost

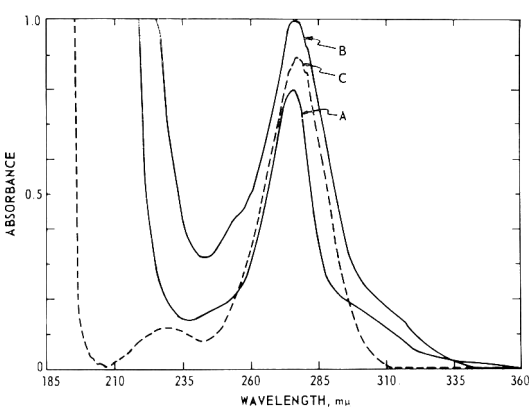


Fig. 5. Ultraviolet spectra of the ether eluate in water:
A: From 2-amino-D-glucose-glycine reaction mixture;
B: From aluminum oxide deposits in the D-glucose-glycine reaction mixture;
C: 2-Furfuraldehyde.

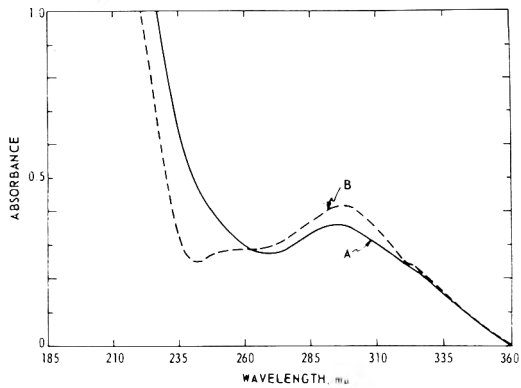


Fig. 6. Ultraviolet spectra of the brown products from 2-furfuraldehyde and glycine:
A: In water;
B: In 80% ethanol.

unchanged in 80% ethanol. The shift from 295.5 mμ in water to 297.5 mμ in 80% ethanol in Fig. 6 may be partially due to an $n \rightarrow \pi^*$ transition, and the shift toward shorter wavelength at lower wavelength region in 80% ethanol can be due to $\pi \rightarrow \pi^*$ transition.

From these results, it is thus difficult to identify the spectrum of ether eluate from the aluminum oxide adsorbent as that of 2-furfuraldehyde. Further examination of the spectrum will be presented later.

Reactions with model compounds. It is not unreasonable to generalize that the "browning" of a solution of aldehyde and amino compound shown in Table I follows a general mechanism analogous to that of D-glucose-glycine system, as can be seen from the comparable kinetic behaviors and spectra of the respective products in each model reaction.

Absorption spectra of 2-amino-D-glucose reaction mixture. 2-Amino-D-glucose was chosen as a model compound because of the similarity of its structure to that of D-glucose. Drozdova (1957) reported the formation of brown products from 2-amino-D-glucose solution.

UV spectra of the reaction mixture and dialyzed products were shown to closely resemble those of the D-glucose-glycine reaction, as shown in Fig. 7. The absorption spectrum of an ether eluate from the reaction mixture is also shown in Fig. 5.

Reaction of 2-propenal with glycine. As a model compound analogous to a possible intermediate with a chromophore $-C{=}C{-}C{=}N-$, the reaction of 2-propenal with glycine was determined, and the kinetics of the formation of brown products was followed. A plot of absorbance increase against time gave biphasic curves from which the initial rate (K_1) and the rate of subsequent steady-phase reaction (K_{st}) can be evaluated. Biphasic curves appear more distinctive in the plot of first-order

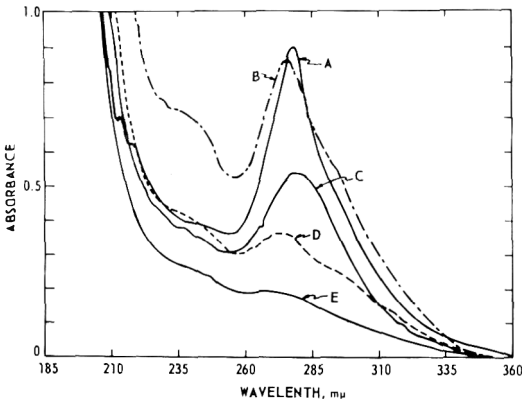


Fig. 7. Ultraviolet spectra of 2-amino-D-glucose reaction mixtures in water:
A: Before ether extraction;
B: After crude ether extraction;
C: The mixture in the presence of glycine before ether extraction;
D: After re-extraction of mixture *B* with ether;
E: The products dialyzed for a week.

kinetics, from which values of two apparent pseudo-first-order rate constants (k'_1 and k'_{st}) can be approximately evaluated (Fig. 8).

Rate data for this reaction are given in Table 3.

UV spectra of the 2-propenal reaction mixture and products are shown in Fig. 9. While unreacted 2-propenal absorbs at 313.5 mμ by $n \rightarrow \pi^*$ transition (*cf.* 327.8 mμ in alcohol, Evans and Gillam, 1943) and at 206.5 mμ by $\pi \rightarrow \pi^*$ in water, the ether-treated reaction mixture with brown color shows no absorption peak at 313.5 mμ but has a characteristic absorption maximum of semicarbazone chromophore at 269 mμ (Gillam and Stern, 1957.

DISCUSSION

The initial step of the reaction can be deduced from the pH profile of the reaction shown in Fig. 3, in which the rate increases considerably as the pH approaches the pK value of glycine ($pK_2 = 9.7$). A reagent with a high pK value is thus expected to react faster, as is confirmed by a higher rate of reaction with 0.25*M* β-alanine ($pK_2 = 10.19$) over that with α-alanine ($pK_2 = 9.87$), as shown in Table 1.

It is also found that α-methyl-D-glucoside is far less reactive than glucose, whereas D-arabinose, which forms less stable hemiacetal, is more reactive, suggesting that the free aldehydic form of D-glucose is being attacked by a nucleophile (free NH_2-group).

Since C^{14}-incorporation into the intermediates shown in Fig. 4 can be taken as approximately a ratio of unity, the following reaction appears reasonable:

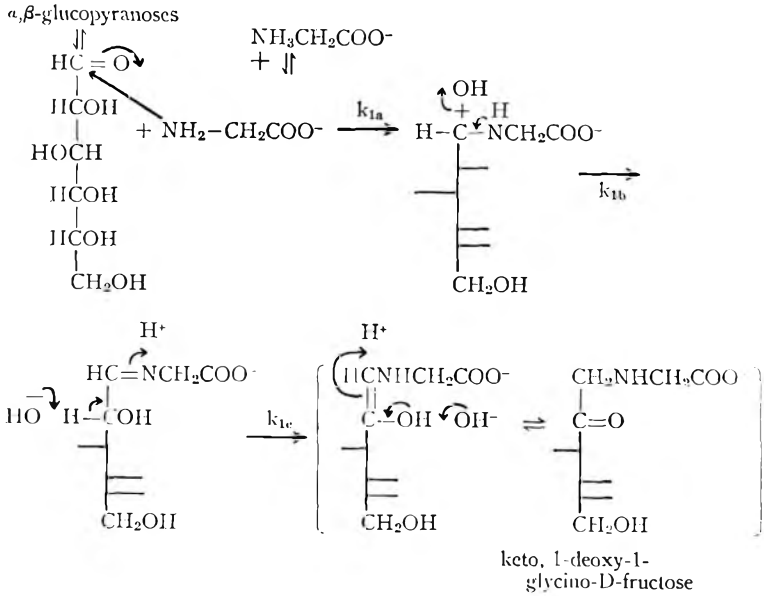
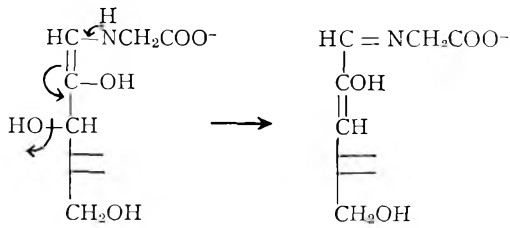


Table 3. Kinetic data on reaction of 2-propenal and glycine to yield brown products at 55°C.

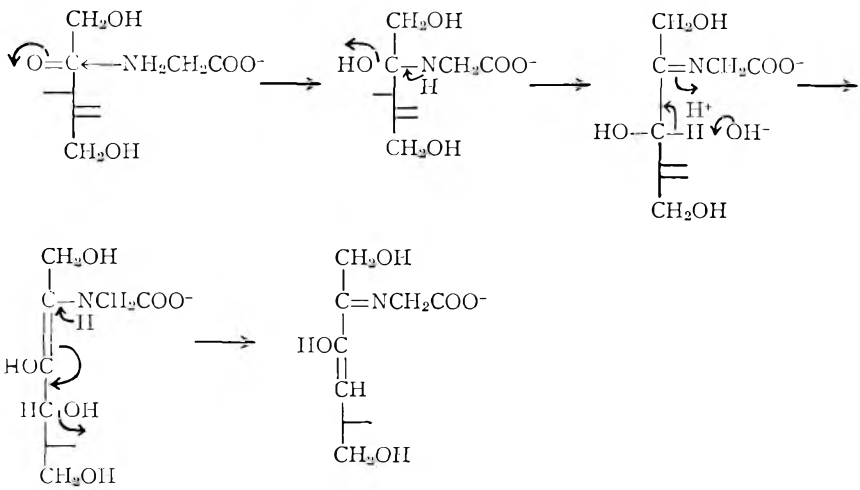
2-Propenal ($M1^{-1}$)	Glycine ($M1^{-1}$)	pH	K_1 ($M1^{-1}$ min^{-1})	K_{st} ($M1^{-1}$ min^{-1})	k'_1 (min^{-1})	k'_{st} (min^{-1})
1.0	0.25	5.6	4.97×10^{-2}	4.14×10^{-3}	0.195	3.76×10^{-3}
0.1	0.5	5.4			0.121	6.40×10^{-3}

Katchalsky and Sharon (1953) also assumed a mechanism analogous to the above with the initial rate-determining step k_{1a}. Subsequent reaction may involve formation of an α,β-unsaturated aldimine from the enol above, as is indicated by results from C^{14}-incorporation ratio, bromination, absorption spectra of the reaction mixture, and the eluate from aluminum oxide adsorption.



It is to be noted that enolization of D-glucose is base catalyzed (Wolfrom *et al.*, 1947). Indirect evidence to support the above mechanism is now available from the present studies.

First, the fact that D-fructose reacts faster with glycine can be explained by the following mechanism:



The compound obtained above is analogous to the structure of α,β-unsaturated aldimine from D-glucose and glycine. γ,δ-Unsaturated aldimine so formed would account for bromine consumption by the D-fructose-glycine reaction mixture at the end of the induction period. Both reactions of glucose-glycine and fructose-glycine systems were also inhibited by added bromine, as will be discussed in Part IV.

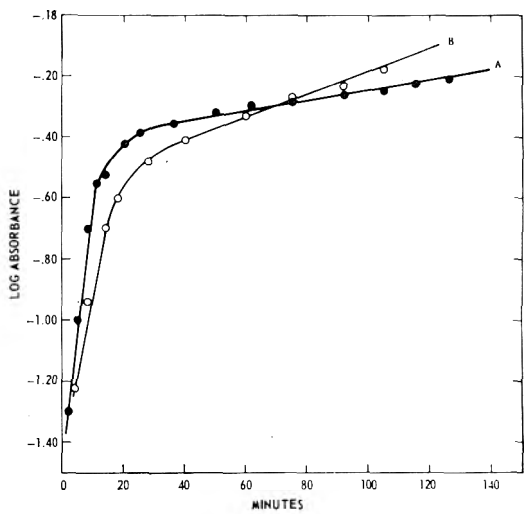
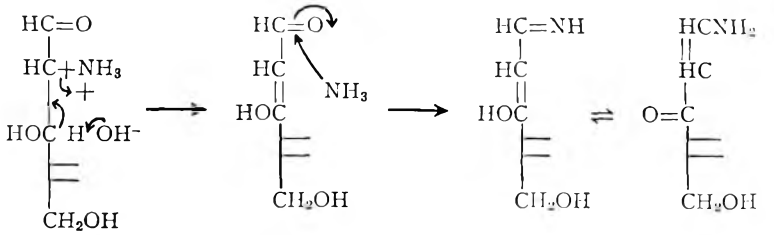


Fig. 8. Kinetics of the formation of brown products from 2-propenal and glycine at 55°C.

A: 1*M* 2-propenal plus 0.25*M* glycine at pH 5.6;

B: 0.1*M* 2-propenal plus 0.5*M* glycine at pH 5.4.

Second, 2-amino-D-glucose was chosen as a model compound because of its structural similarity to D-glucose. Results showed that 2-amino-D-glucose is much more reactive than D-glucose, the induction period being practically eliminated even in the absence of glycine at about pH 6.0. The mechanism of this reaction, again, is explainable in terms of the formation of an α,β-unsaturated aldimine intermediate:



The observation that the Nessler's test and bromination were positive is consistent with the above mechanism. As can be seen from Fig. 7, UV spectra of the reaction mixture and dialyzed products closely resemble those of the D-glucose-glycine reaction system. Fig. 5 also suggests that the intermediate in the 2-amino-D-glucose reaction is analogous to that isolated from the D-glucose-glycine reaction mixture.

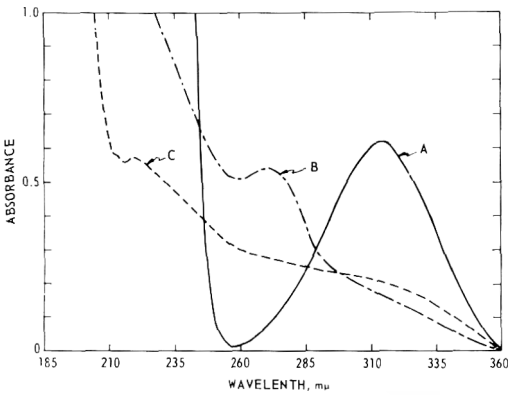
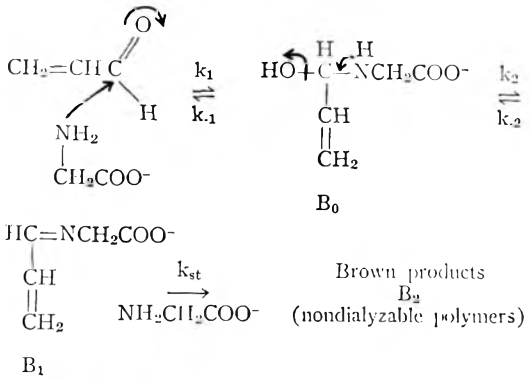


Fig. 9. Ultraviolet spectra of 2-propenal (A) and the reaction mixture of 1*M* 2-propenal plus 0.25*M* glycine in water:
B: After removal of 2-propenal with ether;
C: Brown products after dialysis for 3 days.

Since α,β-unsaturated aldehydes and their semicarbazone chromophores (−C=C−C=N−) show their respective absorption maxima at 210–245 mμ and 264–274 mμ (Gillam and Stern, 1957), the spectrum of the eluate from aluminum oxide adsorption may well be due to a semicarbazone chromophore such as α,β-unsaturated aldimine in the present mechanism (Fig. 5). It is noteworthy that the colorless reaction mixture before the induction period is over absorbs at 273–277 mμ, which, again, suggests that an intermediate such as α,β-unsaturated aldimine with a semicarbazone-type chromophore is being accumulated during the induction period (see Part IV).

From the results, it is suggested that α,β-unsaturated aldimine plays a definite role as an intermediate in the Maillard reaction, followed by further reactions to yield melanoidins. Although Amadori rearrangement is significant in the glucose-glycine reaction, it is not a general reaction common to systems of analogs such as 2-amino-D-glucose, which, because of the presence of the 2-amino group, does not undergo Amadori rearrangement.

Further evidence in favor of the α,β-unsaturated intermediate is obtained from studies with model compounds in which an α,β-unsaturated bond is present. From the biphasic behavior of the kinetics of the "browning" of 2-propenal in the presence of glycine shown in Fig. 8, and from the fact that no induction period appears in the reaction, the following mechanism can be deduced under pseudo-first-order condition:



If B_1, B_2, and intermediates between B_1 and B_2 contribute to coloration of the reaction mixture being followed by absorbance increase, and if accumulation of a steady-state concentration of aldimine (B_1, for example) must be required prior to any further condensation of aldimine (log of steady-state concentration can be estimated

by extrapolating the upper straight line to the absorbance axis in Fig. 8), the above hypothetical mechanism would give two straight lines, analogous to data in Fig. 8:

$$d(\Sigma_n B_n)/dt \cong k_2(B_0) + [k_{st}(g) - k_{-2}]\ (B_1)$$

From steady-state approximations with respect to $d(B_0)/dt$ and $d(B_1)dt$, the following expressions can be obtained:

$$(B_0) = \frac{k_1[k_{st}(g) + k_{-2}]\ (A)}{[1 + k_2k_{-2}]k_{-1}k_{st}}$$

$$(B_1) = \frac{k_1\ k_2(A)}{[1 + k_2k_{-2}]k_{-1}k_{st}}$$

where (A) is concentration of 2-propenal. Thus, the following over-all rate law can be written:

$$d(\Sigma B_n)/dt \cong \frac{k_1k_2\ [k_{st}(g) + k_{-2}]}{[1 + k_2k_{-2}]k_{-1}k_{st}}\ (A) + \frac{k_1k_2\ [k_{st}(g) + k_{-2}]}{k_{st}\ k_{-1}[1 + k_2k_{-2}]}\ (A)$$

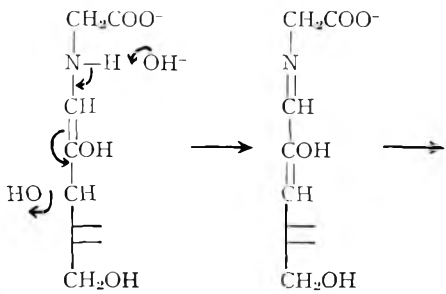
$$= k'_1\ (A) + k'_{st}\ (A) = (k'_1 + k'_{st})\ (A).$$

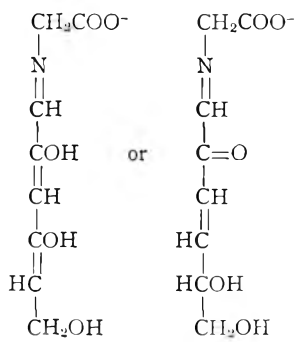
Approximate values of k'_1 and k'_{st} are given in Table 3.

The mechanism above is consistent with UV spectra of the reaction mixture (Fig. 9). The solution of the aliquot for spectra B in Fig. 9 (which may be due to an intermediate such as B_1 in the above mechanism) is brown-colored. It is to be noted that propionaldehyde (saturated analog of 2-propanal) is unreactive with respect to the induction period and formation of brown products. It is interesting that ascorbic acid with α,β-unsaturated bond also produces browning, but much faster than D-glucose. These model studies, again, suggest that the formation of α,β-unsaturated aldimine is responsible for the intermediate step in the Maillard reaction.

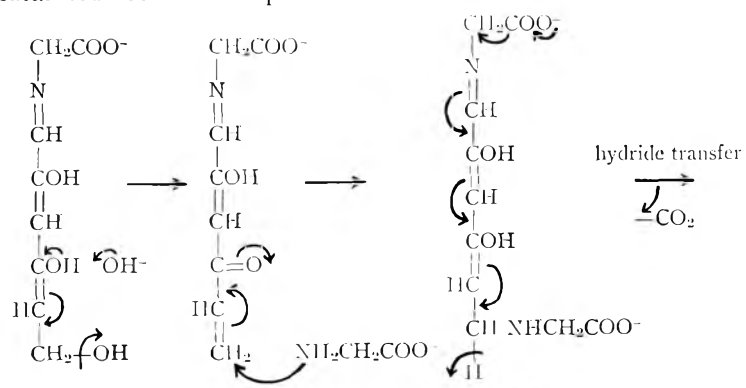
Information is lacking on the subsequent stage of the reaction. It is possible, however, to suggest a hypothetical, although speculative, mechanism based on the present findings and the findings of others.

Base-catalyzed elimination of water from α,β-unsaturated aldimine appears to be reasonable:

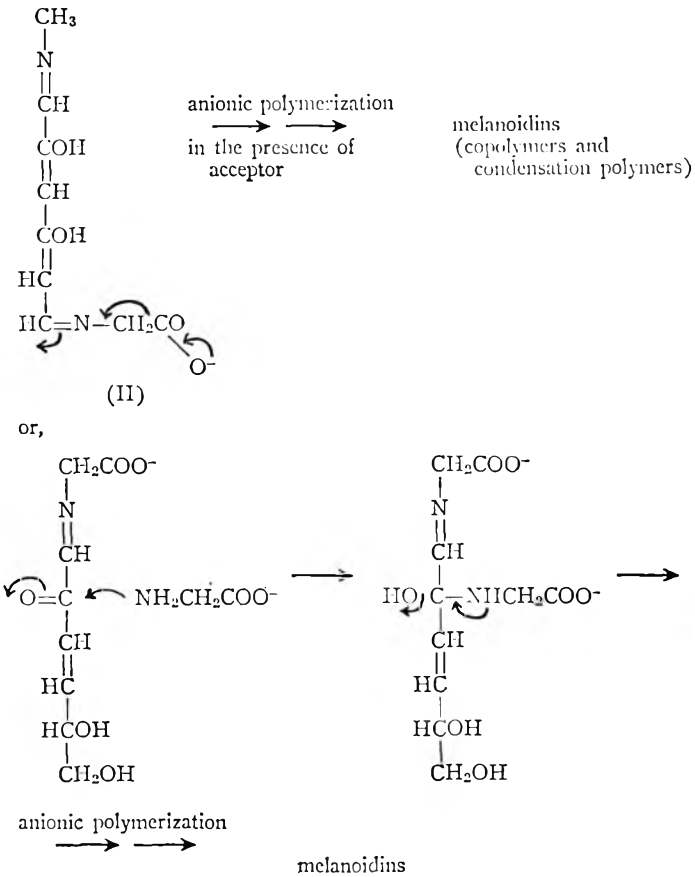




A subsequent attack on these unsaturated compounds by another amino group would occur:



(I)



It must be emphasized that the structures of the intermediates in the above mechanism do not necessarily represent actual structures, but the attempt here is to show a probable type of reaction mechanism occurring in the Maillard reaction. Some indirect evidence confirming the proposed mechanism for the later stage of the reaction is presented below:

a) Elimination of several molecules of water from D-glucose during the reaction has been reported by Maillard (1912a,b, 1916), which is consistent with dehydration in the present proposal.

b) The formation of reductones in the reaction was suggested and was confirmed in the studies of the reactions of galactosyl piperidine and model Amadori rearrangement product 1-deoxy-1-piperidine-D-fructose with glycine, and the formation of reductone was inhibited by the reducing agent (Hodge and Rist, 1953). The melanoidins were also positive toward the reductone test by Tillman's reagent and were reduced by methylene blue. Ascorbic acid, which readily gives rise to reductone, reacted faster than D-glucose to yield brown products in the present studies. All of these results indirectly support the mechanism proposed.

c) Products whose structures are similar to (I) and (II) have been isolated from a 4-(D-xylosyl-amino)-benzoic acid model system (Kato, 1958) and an aniline-furfuraldehyde system (Foley *et al.*, 1952).

d) Both base-catalyzed imine formation (Layer, 1963; Campbell *et al.*, 1944) and polymerization of α,β-unsaturated imine or aldimine (Layer, 1963; Royals, 1954) are well known, which is central to the pH profiles of the reaction and the proposed mechanism.

e) Hodge (1953) suggested that the repeating unit of melanoidins is the following

type: $-(-C=C-C=C-)_{\overline{n}}$. In fact, infrared spectra of the products indicated the presence of $-C=C-$, $-OH$, and $C=O$ (Reynolds, 1963). These results favor the proposed mechanism.

(f) In alkaline pH where HMF is little formed, the absorption at 283–284 mμ of the reaction mixture appears to be due to $\pi \rightarrow \pi^*$ rather than $n \rightarrow \pi^*$ transition, indicating a considerable conjugation along the backbone of the D-glucose molecule, as in the proposed mechanism. The assignment of $\pi \rightarrow \pi^*$ was tentatively established by the effect of several solvents on the absorption maximum (for example, 275 mμ in 50% ethanol, and 283 mμ in water).

g) In reactions of any aldehydes, especially α,β-unsaturated aldehydes, with amines, the mechanism of reaction and structures of the products are comparable to the present proposed mechanism (Sprung, 1940).

h) Addition of bromine and reducing agents (for example, sodium bisulfite) to the melanoidin preparations bleached the brown color to some extent, indicating that the structure of the product(s) and mechanism are of enolic and oxidative nature, respectively, resulting in unsaturation. This is also in accordance with the proposed mechanism, in which unsaturated intermediates and products with enolic structure are important compounds involved in the reaction.

i) Oxidation (hydride ion transfer) and decarboxylation in the proposed mechanism may be significant in initiating anionic polymerization. Decarboxylation of amino acid during the Maillard reaction is well known (see, for example, Hodge, 1953).

In conclusion, a general mechanism of the Maillard reaction leading to the formation of brown products (melanoidins) has been proposed on the basis of results of intermediate studies. Unsaturated aldimines have been found to be key compounds during and after the induction period of the Maillard reaction, as was established from kinetic, spectroscopic, and qualitative studies. Further aspects of the mechanism will be presented in forthcoming papers on inhibition of the reaction.

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The Anthocyanin Pigments of Black Raspberries

SUMMARY

The chemical structure of the major red pigments of the black raspberries, Monger variety, was elucidated. These components were cyanidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-diglucoside, and cyanidin-3-rhamnoglucoside-5-glucoside. The pigments were isolated, purified, concentrated, and then separated into individual anthocyanins by column and paper chromatography. The separated anthocyanins were identified by paper chromatographic and spectrophotometric analysis of the individual pigments and their products after specific chemical degradations.

INTRODUCTION

Black raspberries are frozen and used primarily for remanufacture into punch, jellies, and pies. The color of the whole berries is dark red, almost black. There is limited information in the literature on the composition of the pigments. It has been reported that a cyanidin-3-bioside is present in the cultivated red raspberry (Willstätter and Bolton, 1916). Harib and Brown (1956) showed that the pigment of red raspberries was composed of four components. Lamort (1959) reported that the anthocyanin pigments in raspberries (Newburgh variety) were anthocyanins, and separated them into four fractions by paper chromatography. Fouassin (1956) also found four major pigments in red raspberries. Nybom (1960) identified cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-diglucoside, and cyanidin-3-diglucosyl-rhamnoside in red raspberries (*R. idaeus*). He also reported a xylose-containing glycoside in black raspberries (*R. occidentalis*). Harborne and Hall (1964), in a survey of *Rubus* species and hybrids, reported that black raspberry species contained cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-sambubioside, and cyanidin-3-xylosyl-rutinoside. However, Luh *et al.* (1965) found that the major pigments of boysenberries are cyanidin-3-glucoside, cyanidin-3-diglucoside, cyanidin-3-rhamnoglucoside, and cyanidin-3-rhamnoglucosido-5-glucoside. A relationship would be expected to exist between the pigments of boysenberries and raspberries since boysenberries originated from crossing blackberries, raspberries, and loganberries (a cross of red raspberry and blackberry).

This paper presents identification of the major anthocyanin pigments of black raspberries, Monger variety, and new evidence of glycosidation in the 5-position in the pigments of this American commercial variety. Differences in the pigmentation of American and European varieties of black raspberries remain to be confirmed.

EXPERIMENTAL

Solvents and reagents. Solvent systems for chromatography of the anthocyanidins, anthocyanins, phenolic compounds, and sugars are given in Table 1.

Chromogenic spraying reagents for location of sugars and phenolic compounds in paper chromatography were Partridge's (1949) and Lindstedt's reagent (1950).

Isolation of the pigments from berries. Mature black raspberries, variety Monger, were washed, individually quick-frozen, freeze-dried, and stored under nitrogen at −18°C. Seventy grams of the crushed freeze-dried berries were stirred with 200 ml 1% HCl-MeOH for 24 hr at 1°C. After a 3-min blending, the mixture was filtered under suction through Whatman No. 5 paper. The residue was re-extracted with 150 ml of 1% HCl-MeOH, and the combined filtrates were concentrated under nitrogen to one-half volume in a rotary evaporator at 33±2°C. An isomolar amount of lead acetate was added, and the white lead chloride precipitated at 1–2°C was removed by filtration. The anthocyanin was then precipitated by gradual addition of lead acetate, separated by centrifugation, and dissolved in 5% HCl-MeOH. The precipitation was repeated, and the anthocyanin solutions were concentrated to one-sixth volume as above.

The concentrate was extracted with petroleum ether, and the aqueous phase was dried to a shiny, microcrystalline violet-colored crude pigment.

Column chromatographic separation of crude pigment. The columns (5 × 62 cm) were packed

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Table 1. Solvent systems used in chromatography.

Solvent notation	Composition	Phase used	Used for
BAW	n-Butanol:acetic acid: water, 4:1:5 by vol.	upper	Anthocyanidins Anthocyanins Sugars Phenolics
BuHCl	n-Butanol:2*N* HCl, 1:1 by vol.	upper	Anthocyanidins Anthocyanins
HAc:HCl	Acetic acid:HCl:water, 15:3:82 by vol.	miscible	Anthocyanidins Anthocyanins
1% HCl	Water:12*N* HCl, 97:3 by vol.	miscible	Anthocyanidins Anthocyanins
15% HAc	Water:acetic acid 85:15 by vol.	miscible	Anthocyanidins Anthocyanins
HAcHCl:H_2O	Acetic acid:HCl:water, 5:1:5 by vol.	miscible	Anthocyanidins Anthocyanins
Forestal	Water:acetic acid:HCl 10:30:3 by vol.	miscible	Anthocyanidins
Formic	Formic acid:HCl:water 5:2:3 by vol.	miscible	Anthocyanidins
m-Cresol	m-Cresol:acetic acid:water 50:2:48 by vol.	upper	Anthocyanidins
BuPy	n-Butanol:pyridine:water 10:3:3 by vol.	miscible	Sugars
BuEt	n-Butanol:ethanol:water 40:11:19 by vol.	miscible	Sugars
EtAc	Ethyl acetate:acetic acid: water, 3:3:1 by vol.	miscible	Sugars
Collidin	Collidin saturated with water	miscible	Sugars
BeAW	Benzene:acetic acid:water 2:2:1 by vol.	upper	Phenolics
EtAcW	Ethyl acetate:acetic acid: water, 9:2:2 by vol.	miscible	Phenolics

with Whatman standard-grade cellulose powder under suction, then washed successively with HAc-HCl (Table 1), water, and methanol, and air-dried. The crude pigment was adsorbed from 0.1% HCl-MeOH on 5 g cellulose powder and dried under vacuum. This was placed on the chromatographic column, covered with 8 g cellulose, and topped with glass wool. Development was carried out for 7–8 hr with HAc-HCl, and four distinct bands were obtained. The cellulose was then extruded, and each band was vacuum dried under nitrogen. The pigment was extracted from the cellulose with 0.1%HCl-MeOH and rechromatographed individually by the same procedure to produce purified pigment fractions.

Paper chromatographic separation of crude pigment. Ten Whatman no. 3 MM filter papers were streaked along the short axis with 0.30-ml portions of the crude pigment. Development into four individual bands required 10 hr at 21±0.5°C in the ascending system using BAW. These bands were cut from the paper, and the pigment was removed by extraction with 1%HCl-MeOH. After concentration to less than 1 ml, the individual bands were rechromatographed in the same manner, and the isolated pigments were dried under nitrogen.

Hydrolysis of pigments. Five mg of each of the pigments obtained by column chromatography were dissolved in 10 ml HCl-MeOH (1:2) and refluxed under nitrogen at 105°C for 1 hr. After cooling to −10°C, the mixture was filtered and extracted with n-pentanol to remove the aglycone moiety resulting from hydrolysis. The aqueous phase, containing the sugars, was stored at −18°C.

The n-pentanol solution was then mixed with petroleum ether to precipitate the aglycone. The amorphous mass was dissolved at 1% HCl-MeOH and stored in the dark under nitrogen at −18°C until chromatographic and spectrophotometric analyses could be carried out.

Identification of sugar moiety. The aqueous phase was neutralized with Dowex 1-X8 anion-exchange resin which had been converted to the hydroxyl form with 0.8% sodium hydroxide. The resin was removed by filtration and washed with methanol. The combined aqueous and methanol solutions were concentrated and spotted on Whatman No. 1 chromatographic paper along with xylose, rhamnose, glucose, and galactose. Separate papers were developed in BAW, BuPy, EtAc, and

Collidin (Table 1) in ascending systems. The sugar spots were located with Partridge reagent sprays.

Identification of sugars in 3-position. The technique of Karrer and de Meuron (1932), modified by Chandler and Harper (1961) and adapted to microscale by Zapsalis and Francis (1965), was applied. The sugar moieties were chromatographed in BAW, BuEt, and Collidin (Table 1) in ascending systems.

Identification of dissacharidic glycoside. The method of Bendz *et al.* (1962) was used to split the glycosidic bond. The sugar and aglycone moieties were separated as previously described. The sugar solution was chromatographed on Whatman no. 3 MM filter paper using BAW, and the disaccharides were located by spraying just the ends of the paper with Partridge's reagent. The disaccharide strips were then cut from the chromatogram, and the sugars were eluted with water, concentrated, and hydrolyzed with 4*N* HCl for 5 min. After neutralization, the resulting sugars were chromatographed on Whatman no. 1 chromatographic paper in EtAc, BuEt, BAW, and Collidin in ascending systems (Table 1).

Identification of aglycones. The aglycone portion of the hydrolyzed fraction and the aglycone from the total crude pigment were chromatographed on Whatman no. 1 chromatographic paper in ascending systems with solvents listed in Table 1. Known anthocyanidins were used as standards.

Alkaline degradation. The technique of Karrer and Widmer (1927) as adapted to microscale by Albach *et al.* (1963) was followed. The isolated phenolics and phenolic standards were chromatographed on Whatman no. 1 chromatographic paper using the solvents listed in Table 1 in ascending systems. Spots were made visible by coupling with diazotized benzidine (Lindstedt, 1950). To alter the color of the spots, 20% sodium carbonate solution was applied.

Chromatography of anthocyanins. The original purified mixture, the individual anthocyanins, and their hydrolysates (obtained by the procedure of Abe and Hayashi, 1956) were chromatographed on Whatman no. 1 chromatographic paper using the solvents of Table 1 in ascending systems at 21±0.5°C (Harborne, 1959).

Spectra of black raspberry pigments. The Beckman DK1 spectrophotometer was used to record the spectra of the individual anthocyanin pigments, anthocyanidins, and their complexes with aluminum chloride. The pigments were carefully purified and dissolved in absolute methanol containing 0.01% HCl and in absolute ethanol containing 0.1% HCl. The spectral shift on the addition of aluminum chloride was measured by adding three drops of a 5% solution of the salt in absolute ethanol to the cuvette, mixing, and recording the spectrum at 5-min intervals up to 15 min. The ultraviolet spectrum was also obtained.

RESULTS AND DISCUSSION

The individual pigments of black raspberries, Monger variety, were separated on a cellulose column using HAc-HCl solvent (Table 1). A second separation was carried out using BAW on Whatman no. 3 MM paper in order to reverse the sequence of separated anthocyanins (Harborne, 1959). Four distinct bands were obtained from the column and paper chromatography.

Aglycones. The aglycones obtained by

Table 2. R_f values of black raspberry pigment aglycones.

Pigments	Solvents							
	HAc:HCl	m-Cresol	BAW[a]	Forestal	Formic	HAc:HCl:H₂O	1% HCl	15% HAc
Standards								
Cyanidin [b]	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.14
Pelargonidin [c]	.....	.....	0.80	0.78	0.32	0.57	0.03	0.20
Petunidin [d]	.....	.....	0.52	0.46	0.20	.....	.....	.....
Delphinidin [e]	0.02	.....	0.43	0.33	0.13	0.23	0.00	0.05
Hydrolyzed crude pigment	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.14
Band *A* aglycone	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.13
Band *B* aglycone	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.13
Band *C* aglycone	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.13
Band *D* aglycone	0.09	0.18	0.69	0.49	0.23	0.37	0.01	0.13

[a] Acid-washed paper.
[b] From hydrolyzed pigment of Chrysler Imperial Rose.
[c] From hydrolyzed pigment of strawberry.
[d] From hydrolyzed pigment of petunias.
[e] From hydrolyzed pigment of egg plant fruit peel.

hydrolysis of the total crude pigment were chromatographed on paper along with known anthocyanidins of the aglycones obtained from the hydrolysis of the four separate bands. The results are shown in Table 2. The isolation of natural anthocyanidins was based on the plant sources reported by Hayashi (1962).

The aglycone from Chrysler Imperial rose pigment, which is cyanidin (Albach *et al.*, 1963), gave consistently the same R_f values as the aglycones of the individual and total pigment in all solvent systems used. Consequently, cyanidin is suggested as the aglycone moiety of the four black raspberry anthocyanins isolated. The hydrolyzed crude pigment did not show separation of an aglycone other than cyanidin. The R_f values from BAW, Forestal, and formic solvents were in agreement with those reported by Harborne (1959) and Hayashi (1962). The R_f values from HAc-HCl-H_2O were also in agreement with those reported by Hayashi (1962).

Paper chromatography of the alkaline degradation products (Karrer and Widmer, 1927) confirmed the above chromatographic data that cyanidin is the aglycone moiety of the anthocyanin pigments of these berries (Table 3). The chromatograms were replicated three times, and the reported R_f values are the mean of the R_f values of duplicate spots in each replicated chromatogram. The color response is also in agreement as to the degradation products of cyanidin, and both R_f value and color are in good agreement with those reported by Akiyoshi *et al.* (1963) and Swain (1952). It was not possible to detect the phloroglucinol or any second phenolic compound as spots on the papers developed in Et-AcW.

Spectral data cited in Table 8 likewise confirm that cyanidin is the aglycone part of all anthocyanins isolated from black raspberries.

Products of mild alkaline hydrolysis were not detectable, which suggests that the pigments were free of acylation.

Sugars. Determination of the sugar was carried out in triplicate in pigment fractions from both column and paper chromatography (Table 4).

On the basis of R_f values and the color response with Partridge's reagent, Band *A* contained glucose and rhamnose, and Bands *B*, *C*, and *D* contained only glucose.

A difficulty arose in identification of the sugars by column chromatography because

Table 3. R_f values of degradation products of black raspberry pigments.

Compound	Solvents			Color[a]	
	BeAW	BAW	EtAcW	Diazotized benzidine	20% sodium carbonate
Standards					
p-Benzoic acid	0.38	0.89	0.94	Y	V-Bl
Protocatechuic acid	0.06	0.80	0.93	Y-Or	Y-Wh
Gallic acid	0.00	0.03	0.71	Y-Or	Br
p-Coumaric acid	0.46	0.89	0.97	Y-Br	V-Bl
Phloroglucinol	0.00	0.69	0.82	Y-Br	V-Br
Mixed pigment	0.05	0.80	0.93	Y-Or	Y-Wh
	0.00	0.70	.....	Y-Br	V-Br
Fraction *A*	0.05	0.80	0.94	Y-Or	Y-Wh
	0.00	0.70	.....	Y-Br	V-Br
Fraction *B*	0.06	0.80	0.94	Y-Or	Y-Wh
	0.00	0.70	.....	Y-Br	V-Br
Fraction *C*	0.06	0.80	0.94	Y-Or	Y-Wh
	0.00	0.70	.....	Y-Br	V-Br
Fraction *D*	0.05	0.80	0.92	Y-Or	Y-Wh
	0.00	0.70	.....	Y-Br	V-Br
Product from acylation test	.....	.....	.....	.....	.....

[a] Color notations: Y = yellow; Wh = white; V = violet; Br = brown; Bl = blue; Or = orange.

Table 4. R_f values of sugars from hydrolyzed black raspberry bands isolated by column chromatography.

Sugars	Solvents					Color[a]
	EtAc	Collidin	BuEt	BuPy	BAW	
Standards						
Arabinose	0.43	0.47	0.30	0.13	0.25	R
Xylose	0.44	0.58	0.34	0.15	0.27	R
Rhamnose	0.59	0.64	0.40	0.30	0.37	Br
Glucose	0.35	0.42	0.28	0.07	0.20	Br
Galactose	0.33	0.39	0.25	0.05	0.18	Br
Sugars from	0.36	0.42	.....	0.07	0.20	Br
crude pigment	0.59	0.62	.....	0.27	0.38	Br
Band *A*	0.35	0.42	0.28	0.07	0.20	Br
	0.56	0.64	0.40	0.26	0.38	Br
Band *B*	0.35	0.42	0.28	0.06	0.20	Br
Band *C*	0.35	0.43	0.27	0.07	0.19	Br
Band *D*	0.35	.....	0.21	0.07	0.19	Br

[a] Color developed with Partridge's reagent: Br = brown; R = red.

xylose was detected along with Bands *A* and *B*. It was demonstrated that xylose was an artifact produced by the action of the mineral acid on cellulose or was simply a contaminant originating from the berry pigments or other glycosides (Harborne, 1959; Forsyth, 1952). The same phenomenon appeared also in the pigments isolated by the paper chromatographic technique. In the second case, xylose was present in all fractions and in the blank run throughout the entire procedure. This is the first reported evidence of xylose as an artifact in paper chromatography. This finding may explain why xylose was not detected in the glycosides of the Monger raspberry pigments, which is in agreement with work of Luh *et al.* (1965) but in opposition to work of Nybom (1960) and Harborne and Hall (1964). The sugars detected by paper chromatography of the

Table 5. R_f values of sugars from black raspberry pigments hydrolyzed for disaccharide analysis.

Sugars	*Solvents*				Color[a]
	EtAc	BuEt	BAW	Collidin	
Standards					
Glucose	0.34	0.23	0.18	0.45	Br
Galactose	0.32	0.26	0.17	0.42	Br
Xylose	0.44	0.34	0.24	0.55	R
Rhamnose	0.57	0.43	0.35	0.66	Br
Sucrose	0.16	0.23	.....	0.40	Br
Lactose	0.14	0.16	0.06	0.26	Br
Maltose	0.20	0.20	0.09	0.36	Br
Sugars from hydrolyzed					
crude pigment	0.57	0.44	0.35	0.68	Br
	0.34	0.29	0.18	0.45	Br
	0.18	0.16	0.08	0.28	Br
	0.09	0.08	.....	.....	Br
Hydrolyzed Band *A*	0.16	0.23	0.09	0.30	Br
	0.57	0.43	0.35	0.66	Br
	0.34	0.29	0.18	0.45	Br
Hydrolyzed Band *B*	0.14	0.21	0.08	.....	Br
	0.34	0.23	0.18	.....	Br
Hydrolyzed Band *C*	0.34	0.28	0.18	0.45	Br
Hydrolyzed Band *D*	0.34	0.28	0.18	0.45	Br

[a] Color developed with Partridge's reagent: Br = brown; R = red.

hydrolyzed bands correspond in an opposite manner to those detected by column chromatography, because of the solvent differences (Harborne, 1959).

From Table 5 one can deduce the manner in which the sugar is attached to the aglycone (Bendz *et al.*, 1962). After mild acetic acid hydrolysis, Band *A* gave a disaccharide which after isolation, hydrolysis, and rechromatography yielded rhamnose and glucose in the ratio of 1:3, which suggested the presence of a quadriglycoside. However, the R_f values of the pigment on paper chromatography (Table 7) suggested that Band *A* pigment is a triglycoside. Band *B* disaccharide yielded only glucose when hydrolyzed; therefore, two molecules of glucose must be connected and both attached to the aglycone. The known disaccharides sucrose and lactose gave R_f values near those of the disaccharides of Bands *A* and *B*. Bands *C* and *D* did not give any spots corresponding to the known disaccharides chromatographed.

Another sugar detachment, specific for sugars attached in the 3-position, was carried out (Karrer and de Meuron, 1932; Chandler and Harper, 1961). Protocatechuic acid was detected in the hydrolysate and was removed with repeated ethyl ether extraction. The expectation is that the extraction would completely remove the 2,4,6-trihydroxyphenylacetic acid and partially remove its glycoside in the 5-position. The aqueous solution was then chromatographed (Table 6).

These data suggest that in Bands *A* and *B* there is an oligosaccharide attached to the 3-position of the aglycone. The R_f values cited in Table 6 for the oligosaccharide suggest in both cases a disaccharide: rhamnosyl-glucose in Band *A*, and glucosyl-glucose in Band *B*. When the isolated disaccharides were hydrolyzed, Band *A* yielded predominantly glucose and a smaller amount of rhamnose, while Band *B* produced only glucose.

Small amounts of free rhamnose and glucose were found in Band *A* (predominantly glucose), and free glucose in Band *B*. These were probably due to a low degree of hydrolysis of the oligosaccharide.

No spot corresponding to a triglycoside (linear or branched) was observed in any of the oligosaccharides hydrolyzed from the

Table 6. R_f values of sugar hydrolyzed by ammonolysis of the sugars attached to the 3-position of black raspberry pigment.

Sugars	Solvents			Color[a]
	BAW	BuEt	Collidin	
Standards				
Glucose	0.20	0.26	0.41	Br
Galactose	0.17	0.24	0.39	Br
Xylose	0.24	0.34	0.54	R
Rhamnose	0.35	0.43	0.64	Br
Lactose	0.06	0.16	0.24	Br
Maltose	0.09	0.20	0.34	Br
Sucrose	0.13	0.23	0.38	Br
Sugars from hydrolyzed crude pigment	0.10	0.14	0.24	Br
	0.13	0.21	.....	Br
	0.19	0.25	0.43	Br
	0.35	0.44	0.62	Br
Band *A* disaccharide	0.13	0.21	.....	Br
Band *A* hydrolyzed disaccharide	0.20	0.26	.....	Br
	0.35	0.43	.....	Br
Band *B* disaccharide	0.10	0.14	0.24	Br
Band *B* hydrolyzed disaccharide	0.20	0.26	0.41	Br
Band *C* sugar	0.20	0.26	0.41	Br
Band *D* sugar	0.20	0.26	0.41	Br

[a] Color developed with Partridge's reagent: Br = brown; R = red.

3-position. However, the hydrolyzed crude pigment gave a spot with R_f values corresponding to a higher glycoside (Table 5, 6), which may be the 3-position triglycoside found by Nybom (1960). This pigment could not be isolated, and probably existed as a contaminant of Band *A* pigment.

In the chromatographic analysis of sugar, some R_f values in different runs were not precisely reproducible, but nevertheless close to each other. If the R_f values with respect to xylose or glucose are calculated, then they are reproducible and in agreement with those reported in the literature (Block *et al.*, 1958; Hough, 1962).

Chromatography of the pigments. Paper chromatography of the purified and isolated pigment bands as well as of the purified original pigment was carried out with both aqueous and alcoholic solvents in order to classify the pigments according to their R_f values with respect to known anthocyanins isolated and purified, as suggested by Robinson and Robinson (1931, 1932) and Hayashi (1962). The results of the chromatographic analysis of the total pigments are presented in Table 7. The individual pigments obtained from the column chromatographic technique as well as the known pigments were partially hydrolyzed in order to give evidence of their glycosidic structure.

The pigment of Band *A*, corresponding to that of I (Table 7), a very small amount, gave R_f values suggesting a glycosidation pattern higher than C-3-di-G or C-3,5-di-G. Sugar analysis and partial hydrolysis suggest that the pigment is C-3-RG-5-G.

Although a 3-position triglycoside was not isolated from Monger black raspberries, the sugar hydrolysis of the crude pigment (Table 5, 6) suggested the possibility of a cyanidin-3-xylosyl-rutinoside as reported by Nybom (1960) and Harborne and Hall (1964). The xylose, which was detected but attributed to artifacts, might have been present in a contaminating 3-triglycoside in Band *A* since a 3-triglycoside and the identified 3-RG-5-G would have similar R_f values. The xylose present in the blank could have masked the xylose derived from such a contaminant, thus preventing identification of cyanidin-3-xylosyl-rutinoside while the C-3-RG-5-G was readily identified. The existence of C-3-RG-5-G in black raspberries is in agreement with Luh *et al.* (1965), who reported this pigment in boysenberries (Rubus hybrid). It is probable, then, that an additional glycosidation pattern exists in the pigmentation of the American Rubus varieties which has not been identified in the European types.

Unfortunately, a known sample of pigment was not available, and the tentative identification is based on the comparison of R_f values observed in alcoholic and aqueous solvents of known anthocyanins and the R_f values reported by Harborne (1959), Hayashi (1962), and Luh (1963). The possibility that the pigment is a 3-triglycoside is not solidly supported, in view of the data obtained by partial hydrolysis and sugar analysis. For this to be true, partial hydrolysis should have yielded six spots, corresponding to C-3-RG, C-3-RG-5-G, C-3,5-di-G, C-3-G, C-5-G, and cyanidin, but only five spots were obtained from the alcoholic solvents and four from the aqueous solvents. This may be explained by the similarity of R_f values, and thus lack of resolution, of some of the hydrolytic products (i.e. in aqueous solvents, C-3-G and C-5-G have almost identical R_f values, whereas in alcoholic solvents they can be resolved). The C-3-RG, C-3,5-di-G, and C-3-G in alcoholic solvents and the first two pigments in aqueous solvents likewise did not appear as separate spots as reported by Harborne (1959).

The R_f values of the pigment of Band *B* and of the cyanidin-3-gentiobioside (C-3-di-G) are in agreement and suggest that C-3-di-G is the compound responsible for this band. Partial hydrolysis, aglycone analysis, and sugar analysis are in complete agreement with findings of Willstätter and Bolton (1916).

Band *C*, on the basis of R_f values of the pigment as well as of hydrolysis products, corresponds to a C-3,5-di-G. This suggestion is in accordance with the aglycone analysis as well as with the sugars detected for this band.

Band *D* pigment is composed of C-3-G on the basis of the same positive evidences as above, namely, R_f values obtained in six sol-

vent systems, and results of aglycone and sugar analysis.

A small, just noticeable, diffused band was obtained, namely Band *E*, which, from the data of Table 7, apparently corresponds to cyanidin. The existence of the anthocyanidin even in fresh unpurified pigment extracted with 1%HCl-MeOH was confirmed repeatedly. The presence of the anthocyanidin, even in traces, in the berries was unexpected.

Table 7. R_f values of black raspberry pigments isolated by paper chromatography.

Pigments	Solvents						Known or proposed compound
	BAW	BuHCl	1% HCl	HAc:HCl	15% HAc	HAc:HCl:H_2O	
Original crude pigment							
I	0.17	0.13	0.40	0.61	0.84	0.88	C-3-RG-5-G
II	0.29	0.22	0.34	0.58	0.67	0.82	C-3-di-G
III	0.21	0.10	0.18	0.43	0.51	0.70	C-3,5-di-G
IV	0.35	0.25	0.08	0.28	0.31	0.58	C-3-G
V (trace)	0.58	0.68	0.01	0.09	0.11	0.36	Cyanidin (just noticeable)
Band *A*	0.17	0.11	0.40	0.61	0.83	0.88	C-3-RG-5-G
Hydrolyzed *A*	0.17	0.11	0.40	0.61	.....	.....	C-3-RG-5-G
	0.23	.....	0.16	0.42	.....	.....	C-3,5-G
	0.35	0.25	0.08	0.26	.....	.....	C-3-G
	0.41	0.45	.....	.....	.....	.....	C-5-G
	0.59	0.68	0.01	0.09	.....	.....	Cyanidin
Band *B*	0.28	0.22	0.40	0.59	0.67	.....	C-3-di-G
Hydrolyzed *B*	0.28	0.22	0.40	0.59	.....	.....	C-3-di-G
	0.35	0.28	0.08	0.28	.....	.....	C-3-G
	0.59	0.68	0.01	0.09	.....	.....	Cyanidin
Band *C*	0.21	0.12	0.16	0.41	0.51	0.70	C-3,5-di-G
Hydrolyzed *C*	0.35	0.31	0.08	0.27	.....	.....	C-3-G
	0.41	0.40	.....	.....	.....	.....	C-5-G
	0.59	0.68	0.01	0.27	.....	.....	Cyanidin
Band *D*	0.59	0.31	0.08	0.27	0.31	0.35	C-3-G
Band *E* (trace)	0.59	0.68	0.01	0.09	0.11	0.36	(just noticeable)
Chrysler Imperial Rose pigment partially hydrolyzed	0.22	0.18	0.17	0.43	0.51	0.68	C-3,5-di-G
	0.35	0.25	0.08	0.27	0.31	0.59	C-3-G
	0.40	0.44	0.18	0.27	0.31	0.59	C-5-G
	0.58	0.69	0.01	0.09	0.11	0.36	Cyanidin
Dianthus caryophylus Red petal pigment partially hydrolyzed	0.35	0.25	0.08	0.27	0.31	0.58	C-3-G
	0.59	0.69	0.01	0.09	0.11	0.36	Cyanidin
Antirrhinum majus Flower pigment	0.32	0.26	0.19	0.43	0.51	0.69	C-3-RG
	0.35	0.25	0.08	0.28	0.31	0.58	C-3-G
	0.59	0.69	0.01	0.09	0.11	0.36	Cyanidin
Hibiscus rosea sinensis Flower pigment	0.30	0.24	0.36	0.40	0.60	0.66	C-3-di-G
	0.35	0.25	0.08	0.27	0.31	0.57	C-3-G
	0.59	0.69	0.01	0.09	0.11	0.35	Cyanidin

Notations: R = rhamnose, G = glucose, C = cyanidin.
Example: C-3,5-di-G stands for cyanidin-3,5-diglucoside.

Possibly it results from a low-degree hydrolysis of the C-3-G during extraction and chromatography by means of HAc-HCl.

A previous communication indicated the relative concentrations of pigments in raspberries (Daravingas and Cain, 1965). The pigment of Band *A* was found in very small amounts, while Bands *B*, *C*, and *D* were present in greater amounts.

The R_f values herein obtained were in general agreement with those reported for the corresponding compounds by other workers using the same solvent systems. The minor differences in R_f values were probably due to slight changes in conditions. This may account for the differences in the R_f values reported by several workers (Harborne, 1959; Hayashi, 1962; Albach *et al.*, 1963; Akiyoshi *et al.*, 1963).

Spectrophotometry. The spectrophotometric data for both anthocyanins and anthocyanidins are in Table 8. The absorption maximum of the aglycone obtained from the hydrolyzed crude pigment, as well as that from the hydrolyzed bands, corresponds to the wavelength of absorption of cyanidin isolated from Chrysler Imperial rose. This is in complete agreement with Harborne (1958) in 0.01% HCl-MeOH and 0.1% HCl-EtOH solvents. Shifts of the proper magnitude for cyanidin were also observed upon addition of $AlCl_3$. This spectrophotometric approach confirmed conclusions previously drawn from other methods of analysis.

The pigments of all bands isolated also gave shifts with $AlCl_3$ which were of higher magnitude than those of cyanidin.

The wavelength of maximum absorption of the band pigments was generally in agreement with Harborne's (1958) finding that glycosidation of the anthocyanidin gives rise to a hypsochromic shift. The magnitude of this shift was not observed to be as great as Harborne's. As suggested, Band *E*, found in trace amounts on the column, appeared to be cyanidin. As glycosidation was increased toward C-3-G, a hypsochromic shift of only 5 mμ was observed. No difference was revealed between pigments of Band *B* and *C*, which both gave hypsochromic shifts of 6 mμ. The pigment of Band *A*, which is proposed as a triglycoside, gave a shift of 10 mμ.

The E_{440}/E_{max} values (as percent) obtained from spectrophotometric analysis of the four pigments agree with the literature values for these anthocyanins. The ratio of 22 for cyanidin-3-glucoside and cyanidin-3-diglucoside agrees with Harborne's value of 22 (1958). However, the value of 17 calculated for the anthocyanins glycosidated in the 5-position is between the 13 reported by Harborne (1958) and the 23 reported by Luh *et al.* (1965); and it is smaller, as expected, than the value for the pigment with a free hydroxyl at the 5-position.

Table 8. Spectrophotometric data of the black raspberry pigments and their aglycones.

	0.01% HCL:MeOH			0.1% HCl:EtOH		
Pigments	Visible max (mμ)[a]	UV max (mμ)	Shift with $AlCl_3$ (Δλ)	Visible max (mμ)	UV max (mμ)	Absorption for acylation[b]
Band *A* aglycone	535	279	+20	546	270	no
Band *B* aglycone	536	280	+20	546	269	no
Band *C* aglycone	536	280	+19	546	269	no
Band *D* aglycone	535	279	+20	545	269	no
Band *E* aglycone	535	279	+19	545	270	no
Aglycone from total hydrolyzed pigment	535	279	+20	546	269	no
Cyanidin from Chrysler Imperial Rose	535	279	+20	545	269	no
Band *A* pigment	525	280	+20	.....	.....	no
Band *B* pigment	529	279	+22	.....	.....	no
Band *C* pigment	529	279	+22	.....	.....	no
Band *D* pigment	530	279	.....	.....	.....	no

[a] Measured maximum wavelength of absorption correct to ± 1 mμ.
[b] Absorption at 312, 326, 328 mμ.

No absorption peaks were observed at 312, 326, or 328 mμ. This confirmed that the pigments are free of acylation, at least with regard to the most usual acyl residues, namely, p-coumaric, caffeic, and ferrulic acids.

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An Apparatus for the Isolation of Volatile Compounds from Foods

SUMMARY

Volatile compounds that may contribute flavor to a wide variety of foods can be isolated in an apparatus which is described. It utilizes the principle of flash evaporation and vaporization from a continuous thin heated film. Liquid foods, such as fruit juices or beer, need no preparation, but foods such as meat and potato chips are first made into a fine water slurry. Vaporized food constituents and water are recovered by condensation in a series of specially designed cold traps. Features and performance of the apparatus are discussed.

INTRODUCTION

Of the steps that lead to knowledge of the chemical compounds that may contribute to the flavor of foods, identification procedures have seen enormous advances, primarily because of improvements in the design, operation, and performance of instruments for micro-separation of mixtures, such as the gas chromatograph, and for micro-identification of pure compounds, such as infrared, mass, and NMR spectrometers. By contrast, methods for the isolation of volatile trace flavor compounds have often had to be tailored to the requirements of the particular food under investigation; as one result, advances in design, operation, and performance of such apparatus have not been nearly so spectacular and have not approached the more universal usefulness of the procedures and instruments employed for identification.

Apparatus for the isolation and recovery of volatile constituents from oils without the creation of artifacts during isolation has been reported (Chang, 1961). This apparatus has served well in studies of soybean oil (Smouse and Chang, 1965), hydrogenated soybean oil (Kawada *et al.*, 1966), and cottonseed oil (Mookherjee and Chang, 1965). The present apparatus was designed to isolate volatile flavor compounds from beef. Its performance in this application points to a general usefulness with any food that is an aqueous solution or that can be dissolved or dispersed homogeneously in water. Experiments that have been conducted, though not reported here, have demonstrated this performance capability for such widely different foods as potato chips and beer. In the present apparatus, volatile substances are separated under reduced pressure by a combination of flash evaporation and vaporization from a thin heated film without significant creation of artifacts or decomposition of the food sample.

EXPERIMENTAL

Apparatus. The apparatus (Fig. 1) may be considered as consisting of three systems. The first system is designed to bring the liquid food or water slurry of solid food to conditions optimum for metering it into the second system, designed to separate trace volatile substances and water by vaporization under reduced pressure. In the third system, volatilized material is condensed.

The first system includes a 3-neck 5-L flask (*B*) which holds either the food or a fine water slurry of the food. The contents of the flask can be heated with a Glascol heating mantle and stirred with a precision-ground stirrer (K-78100, Kontes Glass Company, Vineland N. J.). Significant loss of volatile substances to the atmosphere is prevented by a cold-finger trap (*A*) filled with dry ice. The slurry in flask *B* is pumped through capillary borosilicate tubing into the suction port of the positive-displacement rotating and reciprocating pump (*C*) (FMI Lab Pump, supplied by Heat Systems Company, Melville, New York).

The second system begins at the exit port of pump *C*, which is connected to the modified Alihn-type vaporizers (*D*) through a stainless-steel ball joint. The two vaporizers, connected in a series, are initially evacuated to about 0.01 mm Hg. Depending upon the rate of feed of slurry or liquid, the actual pressure during a run is considerably higher. The bulbs of the two vaporizers are bathed by a suitable circulating liquid heated to a temperature that will afford maximum assistance in vaporizing without risking decomposition of the food.

After the slurry leaves the exit port of pump *C*, there occurs a combination of flash evaporation and vaporization from the thin heated film passing down the walls of the bulbs. The portion of the

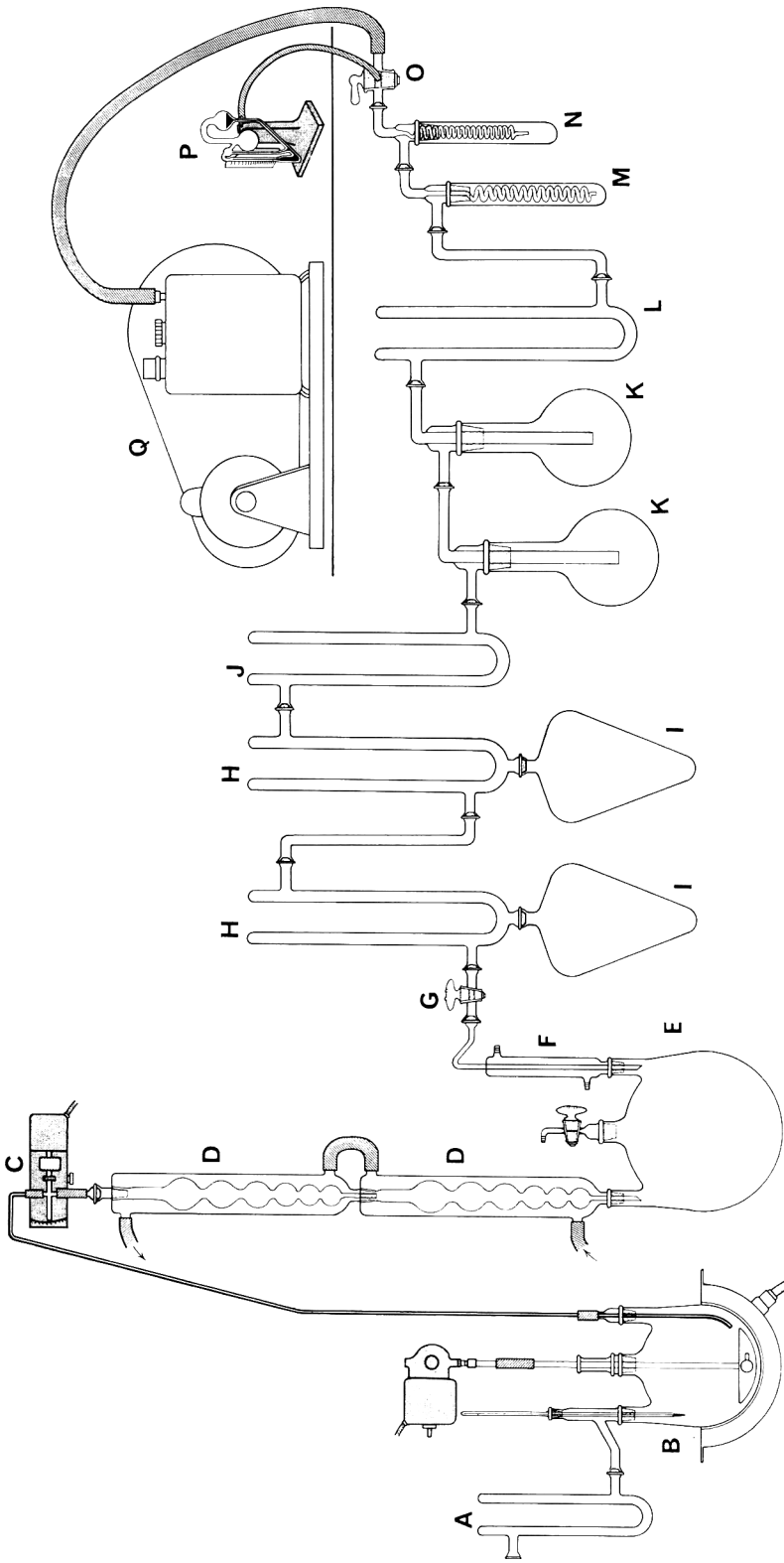

Fig. 1. Apparatus for the isolation of trace volatile constituents from foods.

food or beverage that is not volatile under these conditions drops into the 12-L round-bottom flask (*E*). The flask is normally not heated at the bottom, but mild heating of its top surface may assist in transferring vaporized material out of the flask. The exit tube (*F*) passes through a condenser jacket that may be utilized either for hot bath fluid (which assists the removal of the vaporized material toward the cold traps), or for cold liquid (which effects some slight preliminary cooling of very hot and rushing vapor).

The vaporizing system is separated from the condensing system by the stopcock (*G*), 15 mm bore, permitting the former to be maintained under reduced pressure while the latter is dismantled, cleaned, and reassembled for a second run. After reassembly, pressure in the vaporizing system may be reduced by evacuating through the adapter mounted in the center neck of flask *E*.

The kind of arrangement of cold traps employed in the condensing system may differ with the nature of the food and the conditions of vaporization. The arrangement shown in Fig. 1 has been found suitable for use with a water slurry of boiled meat. The first two dry-ice-cooled traps (*H*) and the 4-L conical receiving vessels (*I*) serve to condense the bulk of the vapors. Conditions can be found without difficulty which will result in condensation of vapor to liquid on the walls of the cold fingers of traps *H*; the condensed liquid drips off the tip of the cold finger into the conical flask *I*, which is set in a pail containing dry ice. Cold-finger trap *J* may be replaced by another set of the *H-I* combination if additional capacity is desired. The volume of liquid collecting in the different receiving vessels can be controlled by varying the level of fill and the frequency and intensity of stoking of the dry ice in the cold fingers above them. The remaining cold traps (*J* and *K*) are cooled by dry ice, and traps *L*, *M*, and *N* by liquid nitrogen.

The specially designed glass parts of the apparatus can be obtained from either Kontes Glass Co., Vineland, N. J., or from Scientific Glass Apparatus Co., Bloomfield, N. J.

Procedure. The isolation of volatile flavor compounds from boiled beef is used to illustrate this apparatus. With certain modifications of procedure, the apparatus has been similarly used for the isolation of flavor compounds from potato chips and from beer.

Cold boiled meat, 1 kg, was ground in a meat grinder (⅛-inch plate), and subsequently made into a fine slurry by comminuting in a Waring blender with just enough cold water (3400 ml), to permit effective cutting action of the blades. The flavor characteristics of the meat were found not to be altered by the grinding and blending. The fine slurry was held in flask *B* for 1 hr under moderate agitation at 70–75°C in order to develop the desirable flavor. During this time, the vaporization and condensation systems of the apparatus were evacuated to less than .03 mm Hg.

Pump *C* was then started to feed the slurry at a relatively rapid rate (80 ml/min) into the vaporizer units (*D*), the bulbs of which were bathed in circulating glycerine kept at 105–110°C. The temperatures of slurry and vapor inside flask *E* were respectively 28°C and 50°C. A typical pressure reading above the slurry was 15 mm Hg, and that at the end of the cold traps was 3 mm. A total of 710 ml of condensate was collected in the cold traps. The condensate had a strong true boiled-beef aroma. The condensate in the two coiled traps cooled with liquid nitrogen had a strong pungent odor suggestive of sulfur compounds.

To ensure as complete as possible a removal of unaltered volatile flavor compounds from the beef slurry, the isolation process was repeated. Stopcock *G* was closed, and vacuum in flask *E* was released. The slurry residue was returned from Flask *E* to *B*, reheated to 70–75°C, and held at this temperature for 1 hr. Meanwhile, the vaporization system was evacuated through the adapter fitted in the center neck of flask *E*. Stopcock *G* was then opened, and the slurry was fed into the vaporizers at a rate of 35 ml/min. A total of 590 ml of condensate was collected. Since it still had a strong true boiled-beef aroma, the slurry residue was again submitted to the isolation process at a rate of 50 ml/min. The condensate collected the third time, 520 ml, had only a weak aroma.

The total condensate may be extracted with ethyl ether, and the ether extract concentrated according to the method of Chang (1961). The concentrated ether solution can then be fractionated by gas chromatography and the pure gas chromatographic fractions identified by infrared, and mass spectrometry. Exceptionally large compounds may yield fractions sufficient for micro NMR analysis. The volatile flavor compounds isolated from 56 lb of boiled lean beef were found sufficient for a systematic study of their gas chromatographic fractions by infrared and mass spectra.

DISCUSSION

The apparatus has been used successfully for the isolation of volatile flavor compounds from foods as different as meat, potato chips, and beer. The volatile compounds isolated from each food had a strong flavor characteristic of that food as evaluated organoleptically. Furthermore the intensity of the flavor of the food after it had been processed was greatly reduced. The apparatus is therefore

flexible and may have a wide range of potential applications.

Conditions that must be determined experimentally for each food include the temperature and time of holding for flavor development, the rate of metering into the vaporizers, and the temperature of the liquid circulated through the vaporizer jacket. As the slurry enters the evacuated system, flash evaporation occurs, and the portion of the slurry that is not vaporized undergoes an instantaneous considerable drop in temperature. The effect of this drop is substantially mitigated as the slurry travels along the heated vaporizer bulb walls in a thin, continuous film. Bubbling due to vaporization from the film occurs throughout the length of the vaporizers.

Conditions must be chosen so that the largest possible quantity of flavor compounds is stripped from the food without causing alteration of the food with respect to flavor and without the creation of artifacts. In some cases, for more complete isolation of the volatile flavor compounds, the food may pass through the apparatus more than once.

Two difficult problems encountered in developing the apparatus were selection of a metering pump and design of the cold traps. The pump must be able to feed the food from a flask under atmospheric pressure into a system under high vacuum. The positive-displacement reversible pump operating on the rotating and reciprocating piston principle serves the purpose well. The train of cold traps must accomplish condensation of both the relatively large volume of water vapor and the low-boiling flavor compounds volatilized in the vaporizers. Most of the water vapor is effectively removed by condensation to the liquid state on the cold-finger traps. The liquid condensate drips off the cold finger into a conical receiving flask. The conical shape is necessary to prevent breakage when the liquid freezes. The remaining cold traps are more conventional design for condensation of the vapor to solid state. Liquid nitrogen is used as coolant for the last three traps to ensure virtually complete trapping of condensable flavor compounds.

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The Fluoride Content of Some Foods and Beverages—a Brief Survey Using a Modified Zr-SPADNS Method

SUMMARY

An analytical method is described that estimates up to 2.0 ppm of fluoride in a 10-g sample of food, with a standard deviation of 0.093 ppm F per test. A brief survey of beverages and canned vegetables indicates that the use of fluoridated water (i.e., 1.0 ppm F^-) by food or beverage processors will increase the fluoride content of the products by 0.34–0.75 ppm (av. increase: 0.50 ppm). On the basis of food analyses and a concomitant study of the fluid intake of laboratory personnel, it is estimated that the total ingestion of fluoride per day by healthy "indoor" workers will range from 2 to 5 mg per day. Implications of these findings are discussed.

INTRODUCTION

As more cities accept the practice of adjusting the fluoride content of municipal water supplies to a concentration of 1.0 part-per-million (i.e., 1 ppm), more food and beverage processing plants will utilize water containing this level of fluoride, and more fluoride will be present in commercially prepared foods and beverages. Although studies of the dental benefits derived from fluoride have been based on the level of fluoride in the drinking water, an important factor to be considered relative to general health and potential fluoride-induced injuries is the total fluoride intake from all sources (Cholak, 1960; Krepkogorsky, 1963; Walbott, 1963).

McClure (1949) reviewed the fluoride content of foods prior to extensive fluoridation of municipal water supplies, but, in the opinion of the present authors (Rose and Marier, 1964), too little attention has been paid to the probable increases that have occurred as an indirect result of water fluoridation. Several surveys (Danielsen and Gaarder, 1955; Elliott and Smith, 1960; Ham and Smith, 1950, 1954; Krepkogorsky, 1963; Pisareva, 1955), have reported the amounts of fluoride ingested with foods, but none has included foods processed with water containing 1 ppm of fluoride. Martin (1951) has shown that simulated home-cooking of vegetables increased their fluoride content, but he did not study commercial processing procedures. Some authors (Bratton, 1953; Weir, 1953) have discussed the effect of fluoridated water on specific processes (wet-milling of corn; yeast cultures), while others (Cholak, 1960; Waldbott, 1963) have reviewed the subject of foodborne fluoride in general terms without presenting experimental data on the specific increases caused by the use of fluoridated water.

This paper presents a method for the estimation of fluoride in a 10-g sample of food, and also presents the results of analyses of some Canadian foods and beverages processed in areas using fluoride-adjusted (1.0 ppm) and low-fluoride waters. Also, the fluid intake of several individuals has been recorded and estimates of total fluoride ingestion by these individuals are presented.

MATERIALS AND METHOD

An assortment of canned vegetables was obtained from two plants in close geographic proximity in Southwestern Ontario; one plant used a municipal water containing 1.0 ppm of fluoride; the other used a low-fluoride well water. Additional samples were obtained from other firms operating canneries in Southwestern Ontario. Samples of soft drinks and beer were collected from local producers before and after the fluoridation of Ottawa's water supply.

In food items comprising both a liquid and a solid fraction (e.g., canned peas), each fraction was analyzed for fluoride content; separation was achieved by simply allowing the liquid to drain from the vegetable. Foods intended for "in toto" consumption (e.g., pork and beans) and all "solid" food items were mashed in a blendor prior to sampling. All results are reported as ppm of fluoride in the blended puree, or in the liquid.

For the analysis of fluoride in foods, a modification of the micro-distillation method of Singer and Armstrong (1959) was combined with a colorimetric method based on the procedure of Bellack and Schouboe (1958). Details of the entire procedure are as follows:

Drying. A 200-mg quantity of reagent-grade MgO is transferred into a 20 ml capacity Vitrosil

crucible, followed by either: a) 10.0 ml of standard sodium fluoride solution containing 5–20 μg F^-; b) 10.0 ml of liquid food material; or c) 10.0 g of blended food puree. By means of a polyethylene stirring rod, the MgO is thoroughly suspended in the liquid sample, or thoroughly intermixed with the blended food puree. Each crucible is then immediately placed in a hot-air oven (85–100°C), where the samples are held overnight.

Incineration. The crucibles are covered with aluminum foil, with a small opening at one side to allow for escape of volatile gases. The samples are then placed in a cold muffle-furnace, and the temperature raised to 220, 320, 420, and finally 500°C, during successive 1-hr intervals. After 3 hr at 500°C, the furnace is turned off and allowed to cool overnight.

Distillation. The distillation apparatus is essentially the same as described by Singer and Armstrong (1959), but has a distilling-chamber of 2.4-cm diameter instead of 1.5 cm. The ashed material is transferred quantitatively to the disconnected distilling-chamber, and the crucible is rinsed with 5.0 ml of fluoride-free water.

The distilling-chamber is then connected to the condenser and burette assembly (Singer and Armstrong, 1959, Fig. 1), and lowered into the preheated (90°C) mineral-oil bath, immersing the distilling-chamber to the level of the side-arm. A graduated cylinder (25-ml capacity), containing 2.0 ml of 3.0*N* NaOH, is then raised into position as a distillate receiver with the tip of the delivery tube beneath the level of NaOH solution. Nitrogen flow is adjusted to give a sustained series of individual bubbles in the receiver cylinder. A 3.0-ml volume of concentrated sulfuric acid (*cf.* Bellack, 1958) is introduced into the distilling-chamber, dropwise, until effervescence subsides, then more rapidly. The bath temperature is raised to 150°C and, to maintain a fairly constant volume during distillation, water is introduced into the distilling-chamber at a rate of approx 0.2 ml per minute, by means of a motorized syringe inserted into the nitrogen line. The distillation is complete when the total volume in the receiving cylinder reaches 12 to 13 ml (i.e., at least 10 ml of distillate has been collected). The cylinder is then removed, and the distillation unit is raised from the bath.

One drop of phenolphthalein indicator is added to the distillate, which is then neutralized with 50% v/v hydrochloric acid (0.6–1.0 ml) and back-titrated to a permanent pink color by dropwise addition of 1.0*N* NaOH. The volume is adjusted to 15.0 ml with fluoride-free water, and the sample is transferred to a polyethylene container and retained for analysis.

Analysis. a) *Zirconium-HCl reagent.* First, 0.266 g of zirconium oxychloride octahydrate is dissolved in 350 ml of concentrated hydrochloric acid; then the volume is adjusted to 1 L with fluoride-free water. This reagent is kept at room temperature.

b) *SPADNS reagent.* A 0.2% w/v solution of SPADNS [4,5-dihydroxy-3-(p-Sulfo Phenyl Azo)-2,7-naphthalene disulfonic acid, trisodium salt; compound No. 7309 in the Eastman Organic Chemicals catalogue] is prepared with fluoride-free water. This reagent is stored at 5°C.

c) *Standard reference solutions.* Solutions of sodium fluoride, containing 0, 0.5, 1.0, 1.5, and 2.0 μg of F^- per ml, are prepared in 0.4*N* NaCl, so that 10-ml portions contain from 0 to 20 μg F^-.

A 10-ml portion of distillate (or standard F^- solution) is pipetted into a 19 × 175-mm test tube, and thoroughly mixed with a 2.0-ml addition of zirconium-HCl reagent, then 2.0 ml of SPADNS reagent. Spectrophotometric readings can be made immediately, or after several hours (Bellack and Schouboe, 1958). Approx. 3 ml of the test solution is decanted into 12 × 75-mm cuvettes for reading at 580 mμ on a Coleman Jr. spectrophotometer. The instrument is adjusted to 100% Transmission with the 20 μg F^- standard, and all other samples are read with this setting. The optical density varies as an inverse linear function of the fluoride content.

Samples containing added MgO require a correction for the fluoride content of the MgO. Because results tended to vary somewhat between incineration treatments, this correction was determined with each group of food samples by analyzing "standards" (containing 200 mg of MgO plus known amounts of fluoride) that had been dried and ashed simultaneously with the food samples.

RESULTS

The reproducibility of the analytical procedure was assessed with samples of known fluoride content and, also, by an examination of the data obtained with foods. A statistical analysis of 39 standard curves, comprising levels of 20, 15, 10, 5, and 0 μg of fluoride, revealed a standard deviation of 0.010 optical density units, with no appreciable difference in error among the various fluoride levels. Because the optical density change, per μg fluoride, averaged 0.0194 in the series of standard curves, the standard deviation was thus 0.5 μg F^- per test. In the food samples surveyed, the fluoride concentration ranged from 0.02 to 1.05 ppm (Table 1), and all results represent averages of single determinations performed on duplicate samples of the various foods or beverages. Although the standard deviation tended to increase slightly with increasing fluoride concentration, an over-all value of 0.093 ppm was calculated. Despite the doubtful

Table 1. Fluoride content of various foods and beverages (all values reported in parts per million).

	Unfluoridated		Fluoridated		Δfluoride
Pork and beans	0.27		0.77		0.50
Tomato soup	0.04		0.38		0.34
Ginger ale [a]	0.02		0.77		0.75
Beer	0.30		0.68		0.38
	Liquid	Solid	Liquid	Solid	Solid
Mixed vegetables	0.30	0.37	1.03	1.05	0.68
Green beans	0.14	0.20	0.71	0.89	0.69
Whole potatoes	0.13	0.38	0.87	0.76	0.38
Diced carrots	0.30	0.19	0.55	0.61	0.42
Kernel corn	0.10	0.20	0.48	0.56	0.36
Green peas	0.15	0.10	.....	.....	.....
Wax beans	.....	.....	(0.49	0.60	.....
			(0.77	0.73 [b]	

[a] Analyses were attempted on two cola-type soft drinks, but severe losses were encountered during incineration.

[b] This sample of wax beans was sent as an unfluoridated "control" sample. However, subsequent investigation revealed that the product had been processed in a cannery using well-water containing 1.2 ppm F^-.

significance of results below 0.1 ppm F^-, all results have been reported to the second decimal place, to enable calculation of fluoride increments between "unfluoridated" and "fluoridated" samples. (In our survey, green peas were not available from a cannery using water containing 1.0 ppm of fluoride, and similarly, wax beans were unavailable from a control area.)

The data of Table 1 indicate that the use of artificially fluoridated water in the processing of foods and beverages increased the fluoride content of these products by 0.34–0.75 ppm. The close agreement between the fluoride content of the "liquid" and "solid" components of most of these foods suggests that equilibration has occurred. Data for samples from plants using low-fluoride waters are in good agreement with those reported by other workers (Danielsen and Gaarder, 1955; Martin, 1951; McClure, 1949; Pisareva, 1955). Also, the increase in the fluoride content of foods processed with water containing 1.0 ppm of fluoride is in accord with the increments reported by Martin (1951) for home-cooking of vegetables (Table 2).

The relative importance of the foodborne fluoride increment must be considered in terms of fluoride intake from all sources, including all forms of beverages (Walker *et al.*, 1963). Since such data are not readily available from the literature, a survey was conducted among seven male members (age 30–50) of the research staff during a one-month winter period. Total fluid intake (Table 3) ranged from 1020 to 3150 ml per day, and this 3-fold variation between individuals was still evident when expressed on a basis of "kg body weight." The estimated *total* fluoride intake includes a foodborne estimate of 1.0–2.0 mg of fluoride per day (see Discussion), and varied from about 2 to 5 mg per day among the individuals studied.

DISCUSSION

The analytical method described in this paper permits the determination of fluoride in a 10-g sample of food, and thus has considerable advantage over earlier procedures in which 200–2000 g of material was required (e.g., Bellack, 1958; Danielsen and Gaarder, 1955; Ham and Smith, 1954).

The use of MgO as a fluoride fixative has been recommended by others (Singer and Armstrong, 1959; Venkateswarlu and Rao, 1954) and assures an easily transferable non-vitreous ash (the only exception was pork

Table 2. Increase in fluoride content of vegetables after treatment with water containing 1.0 ppm of fluoride (all values reported in parts per million).

	Martin (1951)		
	Open saucepan	Pressure-cooker	Commercial processing [a]
Beans	0.75	0.33	0.69
Carrots	0.64	0.33	0.42
Corn	0.26	0.25	0.36
Tomatoes	0.38	0.13	0.34 [b]
Av.	0.51	0.26	0.45

[a] Data from Table 1.

[b] Tomato soup.

Table 3. Daily fluid intake of male laboratory personnel; fluid intake per day, milliliters.[a]

Subject no.	Body weight (kg)	Tea at 2.0 ppm	Coffee at 1.0 ppm	Milk at 0.2 ppm	Reconst. juices at 1.0 ppm	Beer, or soft drink at 0.7 ppm	Drinking Water at 1.0 ppm	Total fluid intake	ml fluid per kg body weight	Estimated Total fluoride intake[b] mg. per day
1	47	200	Nil	1000	300	Nil	Nil	1500	31.9	1.9–2.9
2	63	400	800	200	Nil	280	Nil	1680	26.7	2.8–3.8
3	76	170	850	Nil	Nil	Nil	Nil	1020	13.4	2.2–3.2
4	78	150	600	325	75	250	1750	3150	40.4	4.0–5.0
5	80	Nil	700	500	50	300	Nil	1550	19.4	2.1–3.1
6	82	240	780	600	150	300	480	2550	31.1	3.2–4.2
7	84	Nil	300	860	Nil	350	580	2090	24.9	2.3–3.3
									Av.	2.6–3.6

[a] The fluoride content of these beverages is based on the use of fluoridated water (1.0 ppm) in processing or preparation.
[b] Assuming 1.0–2.0 mg of fluoride ingested with food in fluoridated (1.0 ppm) areas (see text).

and beans, in which the high sulfur content apparently caused a deleterious interaction with the MgO; it was subsequently found that pork and beans could be analyzed *without* any additive). The present procedure reduces the error involved in correcting for the fluoride content of the MgO by including "standards" with each group of samples incinerated.

The distillation apparatus is as described by Singer and Armstrong (1959), except that we recommend a distilling-chamber of 2.4 cm diameter (instead of 1.5 cm) to reduce "bumping," and thus permit the use of larger amounts of ashed material. Sulfuric acid is used instead of perchloric acid, because our large amounts of ashed material caused severe decomposition of perchloric acid.

For the actual determination of fluoride, the advantages of the Bellack and Schouboe method (1958) have been emphasized by several authors (Chang and Thompson, 1964; Pool *et al.*, 1965; Wharton, 1962). Sulfate did not interfere with our analytical procedure, even though Bellack and Schouboe (1958) had stated that sulfate interference was more serious at reduced hydrochloric acid concentrations (i.e., in the zirconium reagent). Although the final hydrochloric acid concentration in our modified procedure is 14% lower than that recommended by Bellack and Schouboe (1958), our levels of SPADNS and zirconium are 81 and 73% higher, respectively. Presumably, the intense color obtained with the higher levels of these reagents counteracted any deleterious effect of sulfate in the determination; it also necessitated the use of an unorthodox "reverse reading" procedure which, however, proved to be very reproducible. The increased concentration of reagents may also explain the linearity of our standard curves throughout the recommended range, whereas Bellack and Schouboe (1958) obtained agreement with Beer's Law only in the lower two-thirds of this range. Although the ranges covered by both methods are similar, Bellack and Schouboe's results (1958) were obtained with a 2-cm light-path, whereas our comparable sensitiv-

ity was achieved with a light-path of only 1 cm.

Previous surveys have shown that the fluoride content of most foods is in the range of 0.2–0.3 ppm (Danielsen and Gaarder, 1955; Martin, 1951; McClure, 1949; Pisareva, 1955) and, on this basis, it has been estimated that the daily intake of foodborne fluoride would be 0.2–0.3 mg (Danielsen and Gaarder, 1955; McClure, 1949). Some analyses of entire meals have agreed with this estimate, reporting a value of 0.3 mg as the daily intake of fluoride with food (Armstrong and Knowlton, 1942; Ham and Smith, 1950). However, other investigations (Cholak, 1960; Ham and Smith, 1954) have reported a foodborne fluoride intake of 0.34–0.80 mg per day in areas where the water was essentially fluoride-free. More recently, Hodge and Smith (1965) have estimated that "for individuals whose drinking-water contains low levels or essentially no fluoride and for whom there are no special fluoride exposures, a 'normal' daily dietary intake in the United States probably lies in the range of 0.5–1.5 mg."

The present survey indicates that food processed with fluoridated water (1.0 ppm) will contain 0.6–1.0 ppm of F^-, instead of the "normal" 0.2–0.3 ppm. Therefore, the widespread use of fluoridated water (1.0 ppm) in food processing and preparation will probably mean a foodborne fluoride intake of *ca* 1.0–2.0 mg per day, i.e., an increase of about 0.5 mg per day over the 0.5–1.5-mg range estimated by Hodge and Smith (1965) for "fluoride-free" areas.

The observed fluid intakes of seven individuals ranged from 1 to 3 L per day, even though all of them were healthy males engaged in similar occupations in a similar environment. The variation reported is probably less than would have been observed between individuals with differing occupations and environments, and total fluid intake would undoubtedly be higher during warm-weather periods of the year.

The estimated *total* fluoride intake per day for fluoridated (1.0 ppm) communities varied from 2 to 5 mg per day, averaging 3.1 mg per day. Krepkogorsky (1963) has compiled data from districts where 1.0 ppm of fluoride is present in the drinking water; he reports an average daily total fluoride intake of 2.5 mg in England, 3.3 mg in the Ukraine, and up to 2.1 mg in other regions of the Soviet Union. However, traditional dietary habits can markedly affect the level of fluoride ingested with food; thus, Elliott and Smith (1960) have shown that the staple diet of Newfoundlanders could contribute 2.74 mg of fluoride per day in an area where the drinking-water was fluoride-free. Krepkogorsky (1963) has recommended that the total fluoride intake by adults should not exceed 3.2 mg per day.

Although our survey was of limited scope, it nevertheless indicates that the use of fluoridated water in food processing will cause a significant increase in the fluoride content of foods and beverages. Our data suggest that some healthy individuals will ingest up to 5 mg of fluoride per day, under normal indoor vocational conditions for North America. Laborers exposed to outdoor summer conditions would undoubtedly ingest still more, as would individuals subject to chronic polydipsia (Adams and Jowsey, 1965; Sauerbrunn *et al.*, 1965). A need is clearly indicated for more extensive data. The total fluoride intake by individuals in a fluoridated community should therefore be monitored, and its medical significance carefully considered (Adams and Jowsey, 1965; Burgstahler, 1965; Krepkogorsky, 1963; Marier *et al.*, 1963; Rose and Marier, 1964; Sauerbrunn *et al.*, 1965; Waldbott, 1963).

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A Thin-Layer Chromatographic-Colorimetric Method for Determining Naringin in Grapefruit

SUMMARY

An analytical method for determining naringin in grapefruit is presented. The procedure involves preparing the sample by filtration, evaporation, and coagulation followed by centrifugation. The naringin is then separated from its tasteless isomer by thin-layer chromatography, and the naringin is recovered from the chromatoplate. A modified Davis test is employed for colorimetric analysis.

INTRODUCTION

The principal flavanone glycoside in grapefruit is naringin, the bitter 7β-neohesperidoside of naringenin (Horowitz and Gentili, 1963; Horowitz, 1964). This is the compound primarily responsible for the bitterness of grapefruit. Its isomer, the 7β-rutinoside of naringenin, also present in grapefruit, is tasteless (Dunlap and Wender, 1962; Mizelle *et al.*, 1965; Horowitz, 1964). Other compounds present in grapefruit, such as limonin (Maier and Dreyer, 1965) and poncirin (Horowitz, 1964) are secondary contributors to bitterness.

Because bitterness is frequently cited as the principal deterrent to the profitable marketing of grapefruit products, several procedures have been reported for the determination of naringin. The Davis test (Davis, 1947), when applied to whole grapefruit juice, suffers from the disadvantage of not being able to differentiate between naringin and its tasteless isomer. Therefore, it is not a reliable measure of naringin bitterness. The chromatographic-fluorometric method reported by Hagen *et al.* (1965), appears to be reliable. However, it is somewhat involved for routine determination of grapefruit bitterness.

This paper reports a relatively simplified quantitative determination of naringin employing a modification of both the thin-layer chromatographic system reported by Hagen *et al.* (1965) and Mizelle *et al.* (1965) and the Davis test.

EXPERIMENTAL

Unless otherwise stated, the grapefruit used throughout this work was the Florida Duncan variety, harvested November 1965 through January 1966.

Apparatus and equipment. Colorimetric measurements were obtained with an Evelyn photoelectric colorimeter using a blue filter (420 mμ). An adapter, to accommodate matched test tubes, 10 × 75 mm, was placed in the tube holder. A minimum volume of 0.8 ml of sample was required.

Disposable micro-pipettes, "Microcaps," accurate to within 1% or less, available from the Drummond Scientific Company, 502 Parkway, Broomall, Pennsylvania, were used to apply all samples onto the polyamide adsorbent. An International Clinical centrifuge model CL No. 457A, was used.

Reagents. The test reagent, used for eluting the naringin from the polyamide and in which the yellow color was developed, consisted of 125 ml methyl alcohol, 112 ml diethylene glycol, 13 ml water, and 5 ml of 4*N* aqueous sodium hydroxide. The aluminum chloride spray was a 1% solution of $AlCl_3$ in ethyl alcohol.

Preparation of thin-layer chromatoplates. A firm, non-flaky polyamide adsorbent was prepared by the method of Nordby *et al.* (1966). A mixture of 0.8 g of rice starch, 0.4 g of silica gel (Fisher No. 1 impalpable powder), and 9 ml of water in a covered 20-ml beaker was heated for 40 min on a steam bath with occasional stirring. Water (1–2 ml) was added during heating as needed to prevent caking. This mixture was rinsed with 3 ml of water into a 100-ml beaker containing 5.5 g of Woelm polyamide powder and 35–40 ml of methanol. This mixture was stirred and then blended in a Waring blender microcup for 3 min. The resulting mixture was spread as a 250-μ-thick layer on 20 × 20-cm glass plates and allowed to dry 2 hr at room temperature before use.

Procedure. 1) A 100-ml sample consisting of both grapefruit juice and sacs was blended for one minute in a Waring blender and then filtered through glass wool.

2) A 50-ml sample of the filtrate was placed in a 250-ml round-bottomed flask and concentrated with a rotary vacuum evaporator and filter pump (aspirator) at 45° to a viscous residue.

3) This residue was readily transferred to a

15-ml centrifuge tube with a disposable pipette, 15 cm long. The volume of residue in most cases was found to be about 8 ml. The flask was rinsed with just enough water to adjust the volume in the centrifuge tube to 10 ml. The flask was then rerinsed with several portions of methyl alcohol to give a 15-ml sample of juice-water-methyl alcohol in the centrifuge tube.

4) The contents of the centrifuge tube were well mixed and centrifuged at top speed for 5 min.

5) A 15-μl sample of the supernatant from step 4 was streaked with a disposable micro-pipette along a 16-cm-long pencil line drawn on the firm, non-flaky polyamide adsorbent. The pencil line does not destroy the layers. The firmness of this adsorbent layer helped ensure against mechanical loss.

6) The chromatoplate was developed twice in the 5:2 nitromethane-methyl alcohol system reported by Hagen *et al.* (1965). By developing the chromatoplate twice, a better separation was achieved between naringin and naringenin-7β-rutinoside. Each development required about 45 min at 25° in a rectangular thin-layer chromatographic tank with a filter-paper liner.

7) After development, the plate was allowed to dry and lightly sprayed with $AlCl_3$ and exposed to UV light (3660 Å). Both naringin and its tasteless isomer appear as bright-yellow fluorescent bands.

8) The naringin band, located by its yellow fluorescence in step 7 and also by the use of an authentic naringin marker, R_f 0.31, was outlined with a pencil, and the area was lightly sprayed with water. The naringenin-7β-rutinoside has an R_f value of 0.38. The damp polyamide-naringin area was scraped from the plate and placed in a 10 × 75-mm test tube (a small soft brush was used for brushing the scraped area clean). The water spray prevents the formation of an electrostatic charge, which could cause a loss while scraping.

9) Into the test tube containing the scrapings was added from a burette 1.50 ml of the test reagent. The scrapings and reagent were well mixed by syringing with a disposable pipette. The yellow color starts developing during this period and should be allowed to develop for 10 min before reading in the colorimeter.

10) The test tube was stoppered and centrifuged at top speed for 3 min. The clear supernatant was removed with a disposable pipette and transferred to a clean 10 × 75-mm test tube.

11) The intensity of color was determined with an Evelyn colorimeter (any colorimeter that can handle micro samples could be used) against a blank carried through the procedure from step 6.

12) The galvanometer reading, T (% transmittance), was converted to A (absorbancy) by ($A = 2 - \log T$), from which the micrograms of naringin were determined from a standard curve.

Preparation of standard curve. A standard solution of chromatographically pure naringin in methyl alcohol-water (1:2, v/v) containing 1 μg naringin per microliter was prepared. Nine light pencil lines, each 16 cm long, were drawn on a TLC plate composed of the same thin-layer adsorbent used in the procedure (step 5). Aliquots from 5 to 40 μl in 5-μl increments of the standard naringin sample were applied with disposable micro-pipettes along the entire length of 8 of the pencil lines. The first line contained 5 μg of naringin, the second 10 μg, and so on through 40 μg along the eighth line. The ninth line was used in preparing the blank. The plate was lightly sprayed with $AlCl_3$, and the procedure, starting with step 8, was carried through step 11.

The galvanometer reading was converted to absorbancy as in step 12 of the procedure and plotted along the ordinate versus the corresponding μg of naringin along the abscissa (Fig. 1). Standard curves were also prepared by developing the standard naringin samples in the nitromethane-methyl alcohol solvent system and scraping from the plates in the same manner as the grapefruit samples, as well as, by direct addition of the various standard naringin samples to the test reagents both with and without the addition of $AlCl_3$ and polyamide. In all cases the standard curves were essentially superimposable.

Precision and accuracy. The precision of the method can be seen in Table 1, which shows the results of five 50-ml portions from the same 500-ml sample of grapefruit juice carried through the procedure by different investigators. Also, a series

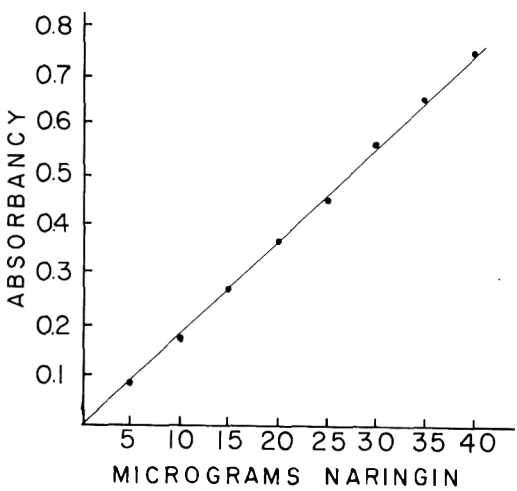

Fig. 1. Standard curve. For experimental details, see text.

Table 1. Determination of two flavanone glycosides in five identical aliquots of grapefruit juice.

Aliquot no.	μg of flavanone glycoside/15 μl of grapefruit juice chromatographed	
	naringin	naringenin-7β-rutinoside
1	15.3	5.4
2	15.4	5.3
3	15.4	5.3
4	15.6	5.4
5	15.7	5.5
Mean	15.5	5.4

Table 3. Concentration of naringin in the juice of three different varieties of fresh grapefruit and a commercial canned grapefruit juice.

Grapefruit	μg naringin per 15 μl of sample chromatographed
Duncan	15.4
Mott [a]	trace
Marsh seedless	10.8
Canned juice	16.6

[a] The Mott grapefruit matures earlier than the Duncan or Marsh seedless and is, in general, considerably less bitter.

of seven aliquots from a different sample of grapefruit juice gave a mean value of 10.4 μg of naringin per 15 μl of sample chromatographed. The percent standard deviation was 1.6.

The accuracy of the procedure was tested by investigating the percent recovery of various amounts of naringin added to a "base" sample of grapefruit juice found in the precision studies to contain 10.4 μg of naringin per 15 μl of sample chromatographed. The enriched samples were carried through the procedure. Table 2 shows the recovery of added naringin.

Application of the method. Experiments were conducted to determine the concentration of naringin in three different varieties of fresh grapefruit and in a commercial canned grapefruit juice. The samples were carried through the procedure, and the results are shown in Table 3.

DISCUSSION

This analytical method for naringin can be performed on a routine basis in both research and plant laboratories. The use of both juice and sacs in step 1 was to obtain a realistic commercial juice sample. The sample sizes in steps 1 and 2 are arbitrary. The addition of methyl alcohol (acetone also works well) in step 3 coagulates the suspended solids, thereby aiding their separation by centrifugation. The precipitated solids and any residue remaining in the flask after rinsing in step 3 were examined by sodium borohydride-hydrochloric acid; magnesium-hydrochloric acid, and thin-layer chromatography. All the solid residues were virtually free of flavonoid material. The addition of methyl alcohol also affords a supernatant which has better streaking qualities, such as lower viscosity and higher volatility, than juice alone.

The sample in step 5 should be applied as a smooth, even streak. This will aid in obtaining a clean separation of naringin from naringenin-7β-rutinoside. With care and experience, one can effect a clean separation with only one development. The R_f values are 0.31 for naringin and 0.38 for naringenin-7β-rutinoside. However, two developments are recommended.

Various experiments were conducted in which both the naringin area and the naringenin-7β-rutinoside area were removed and separately rechromatographed to see if either

Table 2. Recovery of naringin added to a "base" sample of grapefruit juice.

Sample no.[a]	μg of naringin added per 15 μl of juice	μg of naringin found in 15 μl of juice[c]	μg of recovered naringin minus "base" (10.4 μg)	% recovery of added naringin
1[b]	0	10.4	0.0	0.0
2	5	15.7	5.3	106.0
3	10	20.3	9.9	99.0
4	15	25.4	15.0	100.0
5	20	30.5	20.1	100.5
6	25	35.1	24.7	98.8
7	30	40.0	29.6	98.6
8	35	45.5	35.1	100.2

[a] Samples 1–8 contained an average of 10.4 μg of naringin in addition to the known amounts of added naringin.
[b] "Base" sample.
[c] Average of triplicate determinations.

area contained any of the corresponding isomer. The separation was adequate in all cases where the application and removal of sample had been properly performed and overloading had not occurred.

The diethylene glycol in the test reagent enhances the intensity of the yellow color over that seen in methyl alcohol. Diethylene glycol was the most effective of several glycols tested for this color enhancement. The ratio of diethylene glycol to methyl alcohol in the test reagent was chosen to afford the best compromise between color enhancement and viscosity. Clarification of the sample in step 10 is partially dependent upon a system of low viscosity.

The yellow color, which is due to the naringin chalcone formed by the action of sodium hydroxide on naringin (Horowitz, 1964), is fully developed after 10 min and is stable for at least 20 hr. The adsorbent system was shown not to develop color with the test reagent.

This accurate and relatively rapid method for naringin would be applicable in investigations where the amounts of naringin and naringenin-7β-rutinoside are to be compared and in correlation studies for bitterness.

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The Analysis of Volatile Components of Jamaica Rum

SUMMARY

The application of combined capillary-column gas chromatography and mass spectrometry in analysis of the volatile components of a heavy-body Jamaica rum is described. Prefractionation of the complex essence—containing components in widely differing concentrations—was performed on a packed column previous to further analysis on the capillary column. A large number of esters, aldehydes, acetals, and alcohols were identified.

INTRODUCTION

The aroma of distilled alcoholic beverages has not been studied extensively. Only a few data are available in the literature on the composition of cognac, brandy, and whisky. Most studies on rum were concerned with fusel oil components and some of the lower esters and carbonyl compounds (Baraud and Maurice, 1963; Bober and Haddaway, 1963; Fouassin, 1959; Kepner *et al.*, 1964; Maurel and Lafarge, 1963; Maurel, 1964; Suomalainen, 1965). Recently, certain higher boiling esters have been tentatively identified, on the basis of their retention times (Stevens and Martin, 1965).

For studying the aroma of certain dairy products, essential oils, and fruit essences, use has been made of combined capillary-column gas-liquid chromatography and mass spectrometry (CGLC-MS). The application of this technique has accelerated the development of aroma research to a great extent (Buttery *et al.*, 1963; Day and Libbey, 1964; McFadden and Teranishi, 1963; McFadden *et al.*, 1963, 1965).

This paper presents the first results of analysis of the aroma of a heavy-body Jamaica rum with the aid of gas chromatography and infrared and mass spectrometry. Fractionation of an aroma extract on a packed GLC column was applied preceding the CGLC-MS analysis.

EXPERIMENTAL

Preparation of the extract. A Jamaica heavy-body rum containing 70 volume % ethanol was diluted with water to 25 volume % ethanol before extraction, to reduce the amount of ethanol in the extract. Four hundred ml of the diluted rum was extracted with pentane-ether (2:1) in a modified Kutscher-Steudel extraction apparatus for 5 hr. After drying over Na_2SO_4 overnight at 4°C the bulk of the extractant was removed by distillation through a Vigreux column until a residue of 0.5 ml was left.

Apparatus. *Gas chromatographs.* A Becker (Delft, Holland) instrument, equipped with two katharometers, was used for trapping the fractions for infrared (IR) and mass spectral (MS) analysis. The instrument was fitted with two 4-meter × 4-mm-ID aluminum columns (in the form of loops), filled with 25% by weight of silicon oil (Embaphase) on Chromosorb W, 60–70-mesh and with 25% by weight of Lac-1-R-296 on Chromosorb W, 60–70-mesh, respectively. A second instrument (Perkin-Elmer F 6) was set up for capillary columns joined to the mass spectrometer. Two 50-meter × 0.25-mm-ID stainless-steel capillary columns, coated with polypropylene-glycol and Castor wax, respectively, were used.

Mass and infrared spectrometers. The infrared spectrometric measurements were made on a Perkin-Elmer spectrometer, model 13, and the mass spectral measurements on a conventional single focusing 60° magnetic sector field mass spectrometer (Atlas, CH 4, Bremen, Germany). For the entry of the effluent emerging from the capillary columns the mass spectrometer was equipped with a double ion source (Brunnée *et al.*, 1963). The components emerging from the column are detected in one ionization chamber working at 20 eV, so that the carrier gas (He) is not ionized. The total ionization current obtained from this chamber is recorded as a concurrent strip-chart gas chromatogram. The mass spectra are obtained from the material entering the other ionization chamber working at 70 eV. The magnetic scanning speed, used for the measurements described in this paper, was set at 2 sec for a mass range of 20–250 m.u. An ultraviolet galvanometer recorder (A.B. Electrisch Malmleting, Stockholm) registered the spectra at a chart speed of 25 cm/sec. The capillary column was connected with the mass spectrometer by means of a 70-cm × 0.15-mm-ID stainless-steel capillary, to reduce the bleeding at the end of the column. Since no split is applied at this position, the whole effluent enters the ion source, resulting in a very high sensitivity of the CGLC-MS arrangement (10^{-10} g dodecane gave an interpretable mass spectrum). The flow of the carrier gas, He,

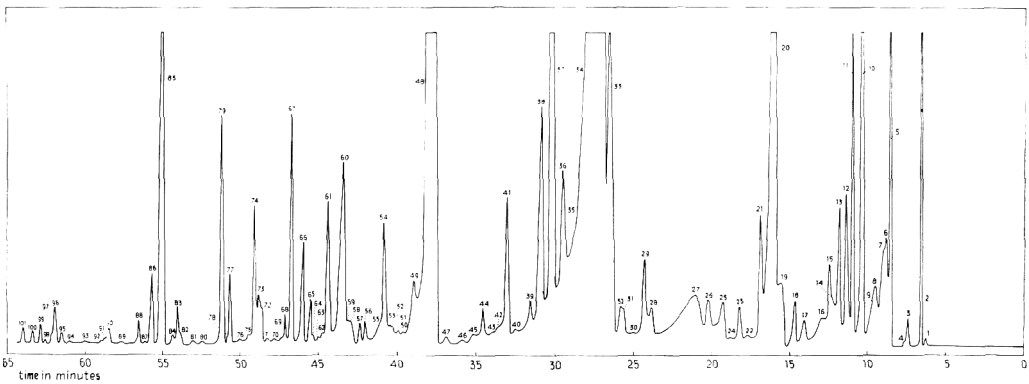

Fig. 1. Chromatogram of a rum extract. Capillary column, 50-m × 0.25-mm-ID coated with polypropyleneglycol; isothermal at 55°C for 16 min, then temperature-programmed at 2.5°C/min to 125°C.

was restricted by the capacity of the pumping system to a rate of 0.5–0.7 ml/min. The vacuum measured with a Penning gauge at the top of the analyzer tube was 3.10^{-6} Torr during the entry of the carrier gas.

Syringes. The injections were made with 1-, 10-, or 100-μl Hamilton syringes.

Condensing traps. For the condensation of components leaving the outlet of the katharometer, traps as described by Badings and Wassink (Badings and Wassink, 1965) were used.

Procedure. *Separation and trapping of components for IR analysis.* In order to obtain the components of the extract sufficiently pure for IR analysis, two columns with different stationary phases were used many times. Reinjection of the trapped components from one column on another was accomplished with the help of a syringe after adding a minute amount of solvent to the Badings trap.

Prefractionation for capillary column MS analysis. Prefractionation of the whole extract for further analysis on the capillary column was achieved by condensing component fractions emerging from the packed Lac column in Badings traps. Fractions consisting of components present in minor amounts were condensed in the same trap from replicate runs; amounts of not more than 100 μl were injected at one time. The condensate was injected for further analysis and identification on a capillary column coupled to the mass spectrometer with a 1-μl syringe.

Analysis of 2,4-dinitrophenylhydrazones. The carbonyl compounds were isolated from the rum as 2,4-dinitrophenylhydrazones and analyzed by thin-layer chromatography (TLC) according to Dhont and Dijkman (1966).

RESULTS AND DISCUSSION

The chromatogram obtained by injecting 1 μl of a rum extract on the polypropyleneglycol capillary column is given in Fig. 1. Each peak on the chromatogram was assigned a number. If a peak contained more than one component as revealed by mass spectrometry, one number was still given. Of many components (numbers 22, 30, 42, 43, 50, 51, 53, 55, 62, 69, 70, 71, 75, 76, 81, 87, 89, 91, 92, 93, 94), although they appeared as a peak on the chromatogram, the mass spectra were too weak or too much obscured by contributions of preceding major peaks for interpretation.

By fractionation on a packed column preceding CGLC-MS analysis it is very possible to obtain spectra of components present in the extract in very low concentrations. An example is given in Fig. 2. Three 90-μl portions of a rum extract were separated successively on the Lac column (Fig. 2a). From these runs a fraction consisting of three small peaks (shaded in Fig. 2a) was collected in a trap according to Badings.

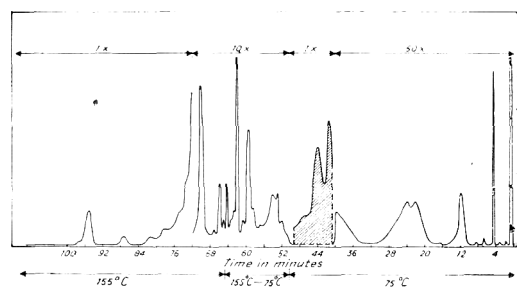

Fig. 2a. Chromatogram of a rum extract. Packed column, 4-m × 4-mm-ID filled with Lac-1-R-296 (25% by weight) on Chromosorb W, 60–80-mesh; isothermal at 75°C for 50 min, then temperature-programmed at 6°C/min to 155°C. Trapped fraction is shaded.

Table 1. Gas chromatographic, mass spectral and infrared spectral identification of components in a rum extract.

Peak no.	Identity (mass spectral)	Confirmed by IR [a]	Confirmed by KI [b]	Ref. [c]
1, 2	isopentane + pentane (solvent)	—	—	—
3	ethyl formate	+	+	+
4	2-methyl propanal	—	—	—
5	ethyl acetate	+	+	+
6	ethanol	+	+	+
7	diethoxymethane	—	+	—
8	2-methylbutanol	—	+	—
10	1,1-diethoxyethane	—	+	+
11	ethyl propionate	+	+	+
12	n-propyl acetate	—	+	+
13	n-propanol	—	+	+
	butanol-2	—	+	+
15	ethyl isobutyrate	+	+	—
17	1,1-diethoxypropane	—	—	—
19	isobutanol	+	+	+
20	ethyl butanoate	+	+	+
21	1,1-diethoxybutane	—	—	—
23	1-ethoxy-1-(2-methylpropoxy)-ethane	—	—	—
25	ethyl 2-methylbutanoate	—	—	—
26	ethyl 3-methylbutanoate	+	+	—
27	n-butanol	—	+	+
28	1-ethoxy-1-butoxyethane	—	—	—
29	3-methylbutyl acetate	+	+	+
31	n-propyl butanoate	—	—	—
32	2-methyl-furanidone-3	+	+	—
33	ethyl pentanoate	+	+	—
34	3-methylbutanol-1	+	+	+
35	1,1-diethoxy-2-methylbutane	—	—	—
36	1-ethoxy-1-(2-methylbutoxy)-ethane	—	—	—
37	1-ethoxy-1-(3-methylbutoxy)-ethane	—	—	—
48	ethyl hexanoate	+	+	+
	furfural	+	+	+
49	1-propoxy-1-(3-methylbutoxy)-ethane	—	—	—
54	1-ethoxy-1-(3-methylbutoxy)-butane	—	—	—
57	1-butoxy-1-pentoxyethane	—	—	—
60	2-acetylfuran	+	—	—
66	n-propyl hexanoate	—	—	—
67	ethyl heptanoate	+	+	—
	benzaldehyde	+	+	—
68	1,1-butoxy-pentoxyethane	—	—	—
72	1,1-ethoxy-pentoxy-butane	—	—	—
73	5(?)-methylfurfural	+	—	—
74	1-ethoxy-1-pentoxypentane	—	—	—
77	1-(2-methylbutoxy)-1-(3-methylbutoxy) ethane	—	—	—
79	1,1-di-(3-methylbutoxy)-ethane	—	—	—
83	2-methylpropyl hexanoate	—	—	—
85	ethyl octanoate	+	+	+
90	2-methylbutyl hexanoate	—	—	—
95	ethyl benzoate	+	+	—
96	diethyl succinate	—	+	—
101	ethyl nonanoate	—	+	—

[a] Also identified by means of IR measurements after trapping from a packed column (+ = yes, — = no).
[b] Kovats Index (KI) checked on packed column (+ = yes, — = no).
[c] Already found in rum by other investigator(s) (+ = yes, — = no).

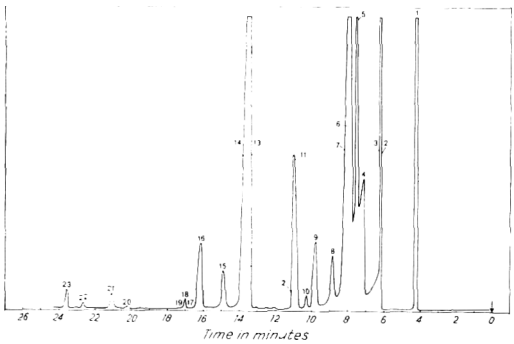

Fig. 2b. Chromatogram of a fraction of a rum extract (see Fig. 2a). Capillary column, 50-m × 0.25 -mm-ID coated with castor wax; isothermal at 85°C.

The combined fractions were reinjected on the same column and trapped again. The material was then injected on the Castor wax capillary column, and the chromatogram of Fig. 2b was obtained. Twenty-three spectra were recorded, 12 of which were not observed during the total extract run. Four of these additional spectra were interpreted (Fig. 2b, peak numbers 11, 13, 14, and 16, Table 2).

Often, mass spectra cannot be interpreted without the information obtained from an IR spectrum of the same material. Correlation of an IR spectrum obtained from a component trapped from a packed column with the corresponding mass spectrum of the large number of mass spectra of the components separated on a capillary column is sometimes difficult. When the extract is first fractionated on a packed column and the resulting fractions are then separated on a capillary column connected to a mass spectrometer this correlation of mass and IR spectra can be established easily.

Identification of components. Table 1 gives identification of the components in the total-extract run (Fig. 1). When IR spectra of components were recorded after trapping from a packed column this is also indicated in the table. Table 2 lists a number of components which were identified in rum by one

Table 2. Components identified in a rum extract with IR, MS, GLC, TLC, and UV.

Identity	Confirmed by MS	Confirmed by IR	Confirmed by KI	Confirmed by Rf and UV[a]	Ref.
2-ethoxypropanal	—	+	—	—	—
n-butyl acetate	—	+	+	—	—
pentanedione-2,3	—	+	+	—	—
n-pentanol	—	+	+	—	+
ethyl lactate	—	+	+	—	—
ethyl-3-hydroxybutanoate	—	+	+	—	—
hexyl acetate	+	—	+	—	—
isobutyl valerate	+	—	—	—	—
3-methylbutyl butanoate	+	—	—	—	—
1,1-ethoxy-hexoxyethane	+	—	—	—	—
formaldehyde	—	—	—	+	—
acetaldehyde	—	—	+	+	+
propanal	—	—	+	+	—
pentanal	—	—	—	+	—
n-hexanal	—	—	—	+	—
crotonaldehyde	—	—	—	+	—
acrolein	—	—	—	+	—
acetic acid	—	+	+	—	+
propionic acid	—	+	+	—	+
n-butanoic acid	—	+	+	—	+
n-pentanoic acid	—	+	+	—	—
n-hexanoic acid	—	+	+	—	—
n-octanoic acid	—	+	+	—	—
4-methylguaiacol	—	+	+	—	—
eugenol	—	+	+	—	—

[a] Dinitrophenylhydrazones separated by thin-layer chromatography; UV maxima were measured (9).

of the methods mentioned under "Procedure." The method of identification used is indicated. Retention time checks were made on the packed column when pure compounds were available. It is not particularly surprising that a large number of C_2—C_5 esters of the lower fatty acids were found. Apart from ethanol, the alcohols propanol, isobutanol, and isoamylalcohol occur in rum in relatively large amounts. It is interesting that such a large number of acetals could be identified. Whether these acetals are present in such large amounts in the rum itself or whether part are formed during concentration of the extract (Galetto *et al.*, 1966) will be investigated. In strawberries (McFadden *et al.*, 1965) and in a submerged-culture flor sherry (Galetto *et al.*, 1966), a number of acetals have been found recently. The presence of furan compounds in this beverage obtained by fermentation of sugar-cane molasses could be expected. The most interesting of these compounds, 2-methyl-furanidone-3, was found in coffee (Gianturco *et al.*, 1964).

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Relation Between Scald of Montmorency Cherries and Oxygen Content in Soak Tanks

SUMMARY

In studies of oxygen concentration and scald in commercial soak tanks, scald developed in bruised cherries in tanks with low oxygen concentration in the water. Laboratory experiments with bruised cherries in water or in a nitrogen atmosphere showed that 2 ppm of oxygen is the critical level below which scald develops. No scald developed on bruised cherries kept in water with an almost constant oxygen level of 8.6 ppm, obtained by saturating the water with air at room temperature (23° C) for 9½ hr.

In 1965 the Air-Aqua aeration system was tested under controlled tank storage conditions in Pennsylvania. It proved to be an effective means of increasing the oxygen level of water, thus controlling cherry scald. Aeration also provided more uniform temperatures in the tanks.

INTRODUCTION

Common practice in the handling of Montmorency cherries for processing is to store them in soak tanks prior to pitting and processing. These soak tanks provide a convenient means of cleaning, cooling, and storing large quantities of fresh cherries. Processors aim to soak the fruit long enough to obtain a firm product, but not long enough to encourage the development of cherry scald (surface discoloration). Early studies attributed the firming of cherries to soaking or to cooling. Firming is now known to occur faster at elevated temperatures (Hills *et al.*, 1953; LaBelle and Moyer, 1960; LaBelle *et al.*, 1964). However, the use of high temperatures is generally impractical because it results in discoloration from cherry scald and oxidation. Cherry scald (Fig. 1) is often a problem to processors and develops during handling and conditioning of the fruit. Scald is a localized translocation of the red pigment from the skin to the flesh of the cherry.

LaBelle (1956), Whittenberger and Hills (1956), and LaBelle *et al.* (1958) observed that only bruised cherries scald; fruit is initially bruised during harvest, whether by hand or machine. In laboratory experiments, Whittenberger and Hills (1956) prevented bruised cherries from scalding by maintaining a low temperature for 24 hr.

Fig. 1. Comparative appearance of unscalded (*A*) and scalded (*B*) Montmorency cherries. The photograph was taken while the cherries were under water.

In spite of general efforts to handle and cool the cherries as swiftly as possible, scald develops. LaBelle *et al.* (1958) found that, despite low temperature and lack of further bruising, scald continues to develop in the soak tank. Another factor observed by Dekazos (1966) was that lack of oxygen (100% nitrogen atmosphere) during holding caused scald of bruised cherries. To ascertain whether low (or lack of) oxygen could be related to scald in commercial processing, oxygen concentrations in soak tanks were studied in 1964 at a processing plant in Pennsylvania and in the laboratory at Belts-

ville, Maryland. The findings of 1964 encouraged the work of 1965 (in Pennsylvania): tests under controlled simulated tank storage conditions of the efficiency of mechanical aeration of water in preventing scald development in bruised cherries.

MATERIALS AND METHODS

Montmorency cherries grown and harvested commercially in the Biglerville area of Pennsylvania were brought to the C. H. Musselman Co. plant in wood lug boxes by truck. The 1964 and 1965 crops were used for this study.

A YSI (Yellow Springs Instrument Company) oxygen meter, model 51, with a YSI 5023 probe was used to measure dissolved oxygen and temperatures (°C) in the soak tanks. The oxygen meter was calibrated to read directly in ppm dissolved oxygen.

Commercial tests in soak tanks, 1964. The soak tanks were approximately 16 ft long, 6½ ft wide, and 4 ft high. Fruit was dumped into the tanks which were partially filled with cold water. While the tanks were being filled with cherries, water was allowed to circulate through the fruit and overflow. The temperature of the water after filling with cherries was above 16°C. With the incoming 5.5°C refrigerated water, the temperature slowly declined to 5.5–7.0°C; soak time was 8–22 hr.

Oxygen content was measured initially when each tank was half filled with cold water, before the cherries were placed in the tank. More than an hour was required to fill the tank with cherries. When the washing was completed, oxygen content was measured at the front, middle, and back of the tank at depths of 2 in., 1½ ft, and 3 ft for each position.

During fluming to the processing plant, cherries from 15 soak tanks, which were surveyed for temperature and oxygen, were scored for scald development. For a representative sample, a handful of cherries was picked at 1-min intervals from the time the molasses valve was opened until the soak tank was empty. The cherries were drained, and a 100-oz sample was weighed. Scalded cherries were separated by visual examination and weighed.

Laboratory tests, 1964. A series of laboratory experiments were conducted with bruised red tart cherries immersed in water at 23°C (73.4°F) for 9½ hr. The cherries were stemmed and bruised (softened), but their skins were not broken. Bruised cherries were placed in 1000-ml flasks containing 500–600 ml water and the oxygen and thermistor temperature probes. The water was either fully saturated with air by vigorous agitation or half-saturated by removing almost half of the oxygen from saturated water by bubbling nitrogen gas through it. Each flask contained 70 cherries of approximately 310 cc volume and was sealed with a rubber stopper to be airtight but allowing the cords of the oxygen and temperature probes to be connected to the oxygen meter. No air space was left between stopper and water. Oxygen contents were recorded at various times for each experiment.

In similar experiments but with an open flask, compressed air, saturated and clean, was used. This air, 1250 cc per minute, entered the flask through a Tygon tube which was spiral shaped at the bottom of the flask, had a closed end, and was perforated every inch with two opposite 1/32-in. holes. Prior to reading the oxygen level, the compressed air was turned off, the probe unit was shaken and stable oxygen indicated. In this manner the oxygen level in water was maintained around 8.6 ppm during the 9½ hr of the experiment.

Another series of experiments were conducted to find the critical levels of oxygen concentrations for scald development of bruised cherries in air. Cherries were placed on their unbruised stem ends so that bruised areas were in contact neither with each other nor with the flask. Airtight flasks (1000-ml each) containing bruised cherries (12 per flask) at levels of 1, 2, 3, 4, and 5 ppm of oxygen in a nitrogen atmosphere were incubated at 90°F (32.2°C) for 18 hr and examined for scald development.

Controlled simulated soak-tank tests, 1965. Two soak tanks (3×4×4 ft) were constructed of heavy-gauge sheet metal equipped with a Kennedy gate valve no. 4. Both tanks were insulated with Armstrong Armaflex 22 sheet insulation to protect the fruit from excessive warm-up, since no recirculation of water was planned. An Air-Aqua aeration system, no. 2399, manufactured by Hinde Engineering Company, was used to aerate the water.

The weighted Air-Aqua tubing was laid in the bottom of the tank in a grid pattern. There were 12 lines, 34 ft of weighted polyethylene tubing on 3.5-in. centers, connected to the 4-ft headered feeder tube. The aeration tubing has small nonclogging check valves die-formed into it during molding. These check valves release air bubbles of optimum size for maximum oxygen transfer. A flat expanded metal diamond-shaped mesh (7/10 × 4/10 in.) screen was placed over the Air-Aqua tubing, alleviating pressure and allowing the compressor to operate. Air was supplied to the tubing by a special oil-less air compressor of ¼ hp.

Fifty boxes of cherries were dumped, one box at a time, into each soak tank which was partially filled with refrigerated water at 5.5°C. Besides the normal bruising expected in commercial picking and in transport, cherries for two tests (July 21, 22) were also dropped 1½ ft prior to dumping into

the soak tanks. Cherries for another test (July 27) were flushed with refrigerated water of 5°C for 30 min to obtain a lower water temperature.

When the tanks were filled with cherries, measurement of oxygen content and temperature were taken for the front and back of the tank at depths of 2 in., 1½ ft, and 2½ ft. Soaking time varied from 7 to 17 hr. The cherries in the aerated and unaerated tanks were scored on the basis of scald development and browning. Sampling was achieved in the manner described under commercial tests. Scald and browning were separated by visual examination and weighed. Besides the composite scald, samples were taken from the front and back of the top, center, and bottom levels of each tank.

RESULTS AND DISCUSSION

Commercial tests in soak tanks. The weight of the cherries, range of oxygen concentration, range of temperature, total soaking time, and percentage of scalded cherries in the tanks are presented in Table 1. The data indicate that there is a trend towards increasing scald with decreasing oxygen concentration in the water.

The observed deficiency of oxygen during soaking of the cherries can be explained by the following: a) Heat is released from fruit when dumped into the soak tanks, which in turn decreases the solubility of oxygen in water, causing oxygen to escape. It has been found that Bing cherries in lug boxes in the field exposed to the sun quickly reached 20°F above the temperature of the surrounding air (Micke *et al.*, 1965). b) The respiration process of cherries removes oxygen. The rate of respiration depends upon temperature and bruising (Pollack *et al.*, 1958), granted that cherries are subjected to different levels of bruising in normal commercial picking and in transport. Thus, the readily available oxygen in the recirculated refrigerated water used during the cooling process of cherries can be diminished since cherries absorb oxygen.

Laboratory tests. Bruised cherries were placed in flasks containing either water fully saturated with air or water half-saturated with air. Since cherries absorb oxygen, the oxygen content of the water in these sealed flasks decreased progressively and finally became exhausted; the cherries then showed distinct scald.

The holding time, oxygen utilization, and scald development of red tart cherries at 2 levels of dissolved oxygen in water is presented in Table 2.

When the oxygen level reached 1 ppm, cherries showed apparent scald. Complete scald was attained in 5 hr in half-saturated water and in 8 hr in fully saturated water. Thus, bruising followed by oxygen deficiency during soaking caused the typical scalded condition. Scald formation of bruised cherries at low oxygen concentrations was also observed by Pollack *et al.* (1958) while con-

Table 1. The weight of cherries, range of oxygen concentration and temperature of water, total soaking time and percent of cherry scald in commercial soak tanks.

No. of tank	Date 1964	Weight of cherries (lb)	Range of oxygen concentration (ppm)	Range of temperature (°C)	Total soaking time (hr)	Scald (%)
10	July 14	15966	2.5 –5.5	17.0–6.0	8	1
13	July 14	15899	2.3 –7.0	17.0–6.0	11	1
9	July 14	15821	2.0 –5.5	14.5–6.0	8	1
8	July 7	15610	2.0 –6.2	16.0–6.0	12	1
18	July 14	17542	2.0 –6.0	19.2–6.0	9	2
8	July 14	15710	2.0 –4.0	14.0–7.2	8	2
1	July 13	17910	1.5 –4.0	19.3–7.0	8	3
2	July 13	16876	1.5 –4.0	18.0–6.0	7	3
15	July 28	14173	1.5 –5.0	18.0–6.5	22	4
6	July 27	17292	1.5 –2.4	19.0–6.5	21	4
2	July 27	21411	1.5 –2.25	20.0–6.2	18	7
2	July 28	17216	1.0 –2.0	19.0–6.0	20	7.5
5	July 27	17483	1.25–2.25	19.5–6.0	18	9
15	July 7	16914	1.0 –2.0	18.0–6.0	18	9
1	July 28	17041	1.0 –2.0	18.2–6.2	21	9.5

Table 2. Oxygen utilization and scald appearance of bruised cherries placed in sealed flasks with either water fully saturated with air or half-saturated with air at 23°C.

Water fully saturated with air			Water half-saturated with air		
Recording time	Oxygen concentration (ppm)	Scald appearance	Recording time	Oxygen concentration (ppm)	Scald appearance
10:15 A.M.	8.6	———	9:45 A.M.	4.0	———
11:00	7.4	———	10:15	3.5	———
11:55	5.9	———	10:55	3.0	———
12:55 P.M.	4.6	———	11:30	2.5	A few cherries are mottled
2:00	3.5	———			
3:00	2.5	A few cherries are mottled	12:45 P.M.	2.0	About half of the cherries are mottled
4:00	2.0	Many cherries are mottled	1:45	1.2	Most of the cherries are mottled and a number of scald areas appear
5:00	1.6	Small scald spots are beginning to appear on many cherries	2:55	1.0	Scald appears on practically all the cherries
6:30	1.0	Scald appears on practically all the cherries	3:45	0.8	Scald is more prominent on the cherries
7:45	0.6	Scald appears on practically all the cherries	4:45	0.6	Area of scald on cherries increases
			5:55	0.6	Area of scald on cherries increases
			7:15	0.5	All the cherries are scalded

ducting respiration studies of cherries in water at two different temperatures.

Bruised cherries held in water at a ratio of 1:2 (cherries to water) and supplied with compressed air in a continuous flow so to maintain the oxygen concentration at approximately 8.6 ppm for the 9½ hr of the experiment, appeared like the fresh, unbruised cherries. No scald was observed. However, 2% of the cherries showed brown spots on the skin. This oxidative browning normally follows disruption of cellular structure under aerobic conditions.

Bruised cherries were also placed at various levels of oxygen in a nitrogen atmosphere for 18 hr. Severe scald developed at low oxygen concentration: at 1 ppm, all were scalded; at 2 ppm, half were mottled with small spots of scald. At 3, 4, and 5 ppm cherries did not scald. It was also observed that unbruised cherries placed in a nitrogen atmosphere or in water with low oxygen levels did not scald.

Controlled simulated soak tanks. The aerated water tanks (Figs. 2–5) maintained a high oxygen level, 6.0–9.0 ppm, because of the effective aeration system, whereas the unaerated water tanks (Figs. 2–5) were progressively being depleted of oxygen, reaching 1.5 ppm or less after the fourth hour of soaking. Water in the latter tanks lost oxygen rapidly at the beginning of the experiment and leveled off somewhat after 4 hr. The cherries consumed oxygen rapidly at the beginning, but the rate of consumption decreased as oxygen was depleted. Pollack *et al.* (1958) found that the rate of respiration of cherries in water is related to the amount of oxygen present.

Unaerated water temperature (Figs. 2–5) was slightly higher than that of the aerated water, which maintained its original, uniform, low temperature. The bottom of the unaerated water tanks had a lower water temperature than the top. Temperature of aerated water tanks was uniform and showed an increase of only 1°C or less for each test. During the soaking period, the difference in the average temperature between aerated and unaerated water for the various water

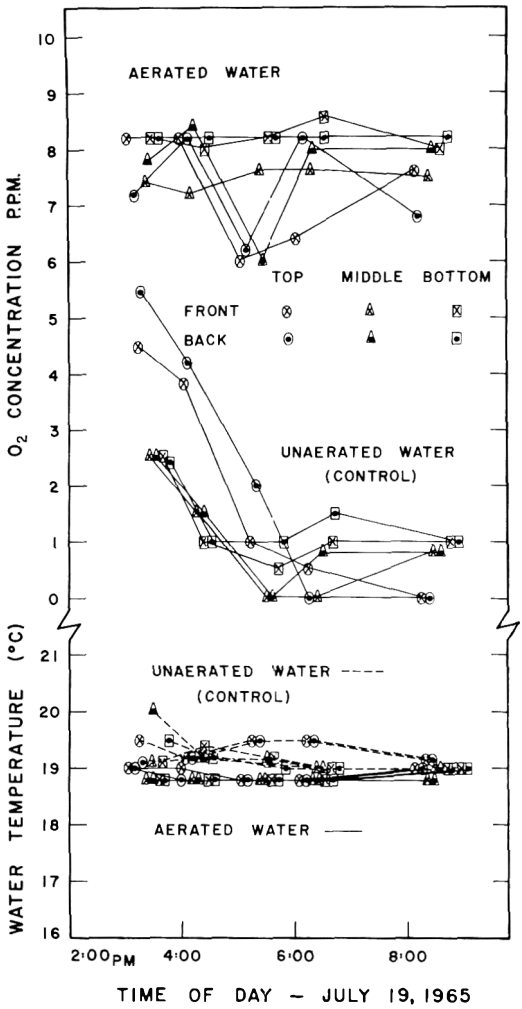

Fig. 2. Oxygen levels and temperatures of aerated and unaerated water in soak tanks. Readings were taken at the front and back sections of the top (2 in.), middle (1½ ft) and bottom (2½ ft) depths of the tanks.

depths ranged from 0.4 to 1.9°C. This difference of less than 2°C cannot account for the extreme difference in the percent of scald between these tanks. Figs. 2–5 and Table 3 show that the oxygen level is the primary factor, after bruising, involved in scald development.

Uniform and low temperatures of the aerated water are possible because the water was gently and completely mixed. Air, released through the check valves as pinpoint bubbles, rises, causing counter-rotating currents which constantly rotate the water (Hurwitz, 1963). Water is efficiently oxygenated by the aeration system because the small air bubbles released singly are in the water long enough for the oxygen to be absorbed. The uniform temperaures of the aerated water (Figs. 2–5) should eliminate possible hot spots in holding tanks, which are also a problem to processors (Baker, 1965).

The comparison of cherry scald (Table 3) between the aerated and unaerated water tanks is practically incontrovertible. In the former, scald was neglible, whereas in the latter, scald runs high and a visual difference between cherries in the two tanks was readily apparent. Cherries of the aerated tank had a deep-red color with occasional browning, while those of the unaerated tank had a light red color with considerable scald. Cherry scald was controlled effectively, even when cherries were held in water at ratios of 1:1.5,

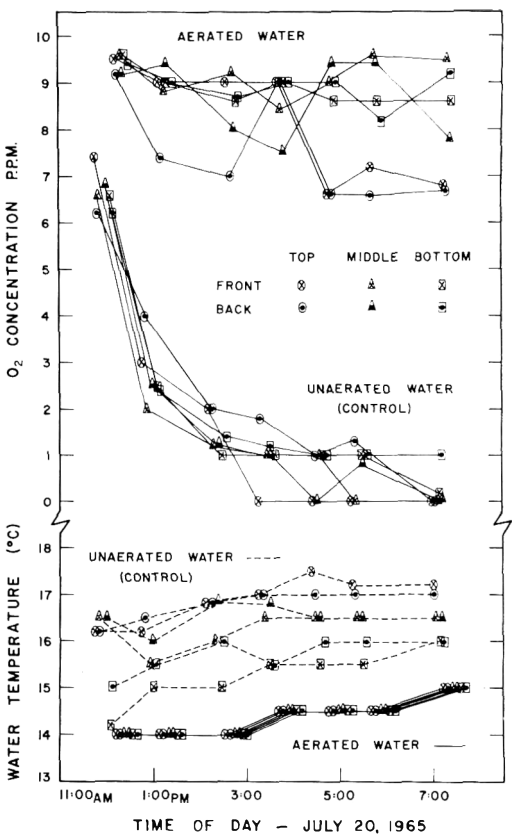

Fig. 3. Oxygen levels and temperatures of aerated and unaerated water in soak tanks. Readings were taken at the front and back sections of the top (2 in.), middle (1½ ft) and bottom (2½ ft) depths of the tanks.

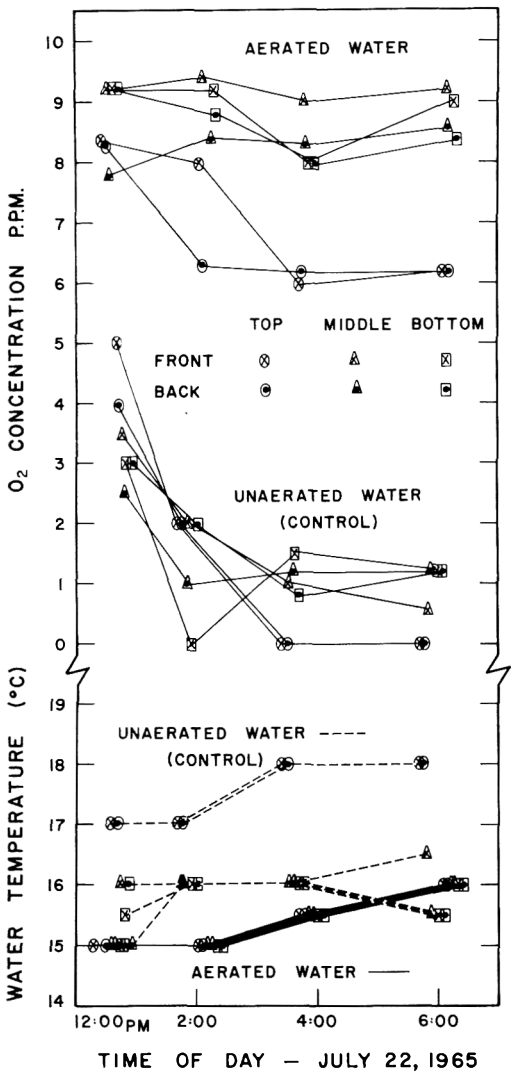

Fig. 4. Oxygen levels and temperatures of aerated and unaerated water in soak tanks. Readings were taken at the front and back sections of the top (2 in.), middle (1½ ft) and bottom (2½ ft) depths of the tanks.

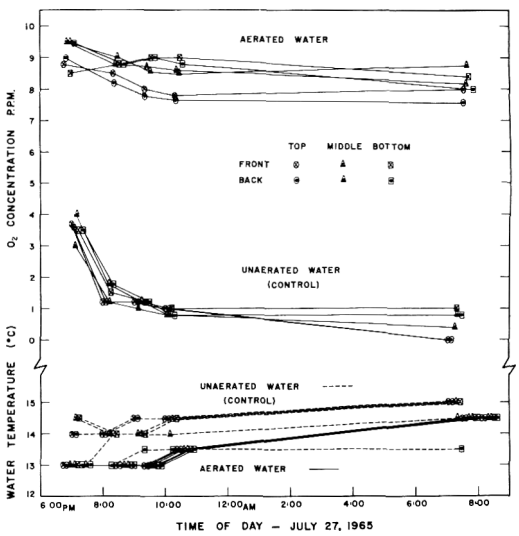

Fig. 5. Oxygen levels and temperatures of aerated and unaerated water in soak tanks. Readings were taken at the front and back sections of the top (2 in.), middle (1½ ft) and bottom (2½ ft) depths of the tanks.

2:1, 2.1:1, and 2.5:1 (cherries to water), as long as air was supplied to maintain a high oxygen level.

The scald development of cherries and the leveling of the oxygen concentration at a low value in the unaerated water tanks indicate that scald conditions are essentially anaerobic, as in the nitrogen atmosphere (Dekazos, 1966). This anaerobic process can be initiated by subjecting the bruised cherries to an atmosphere which is devoid of oxygen, or to water or an atmosphere in which the oxygen concentration is below a certain relatively low critical value. Two ppm of oxygen is the critical level below which scald develops in the experiments.

The aerated water tank of July 22 maintained a high oxygen level, but 9.6% scald was recorded for the tank. Pockets may have been formed by the severely bruised and crushed fruit, causing oxygen to be inaccessible to the cherries. The cherries were large, relatively immature, and excessively bruised. However, the percentage of scald for the aerated water tank was much lower than the 25% scald for the unaerated water tank.

Cherries of the aerated tanks showing browning must have severe cell injury. The browning of fruit is oxygen-dependent. Severe cell injury of plant tissues (Geismann, 1958) leads to polyphenoloxidase activity, which is generally regarded as being responsible for the browning of plant products. In all but one test, July 26, browning was negligible. Excessive bruising and especially severe cell injury should be avoided during the harvest operation, whether done by hand or machine, and in post-harvest handling.

The relationship between scald and oxygen content in laboratory tests has been verified by controlled field tests. This study has been successful in maintaining a high oxygen level

Table 3. The percentage of cherry scald, weight of cherries and water, oxygen concentration and temperatures of aerated and unaerated water in soak tanks.

Date 1965	Tank	Water data (initial) Weight (lb)	Oxygen content (ppm)	Temperature (°C)	Fruit (lb)	Av. water temperature during soaking time (°C)	Total soaking time (hr)	Final temp. of water (°C)	Scald in tank (%) Front (depth) 9 in.	18 in.	Back (depth) 9 in.	18 in.	Composite (%)
July 17	Unaerated	1810	8.5	6.0	1200	14.6	10	15.7	8.0	5.5	6.0	8.5	8.0
	Aerated	1810	8.5	6.0	1200	13.6	10	15.0	1.6	1.0	2.5	1.4	1.0
									(depth) 2 in.	1½ ft	2½ ft	2 in.	1½ ft	2½ ft	
July 19	Unaerated	690	8.6	5.0	1705	19.2	8	19.0	12.0	11.8	11.0	10.0	13.5	10.5	9.5
	Aerated	690	8.6	5.0	1705	18.8	8	19.0	3.0	3.0	3.5	3.2	4.9	4.0	3.0
									Browning (%) 3.5	1.2	1.2	4.2	3.0	0.5	2.0
July 20	Unaerated	850	8.6	5.0	1715	16.2	9	16.5	Scald (%) 5.5	6.2	5.6	6.8	6.6	6.0	7.0
	Aerated	850	8.6	5.0	1715	14.4	9	15.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0
									Browning (%) 0.0	0.0	0.1	0.0	0.1	0.0	0.1
July 21	Unaerated	850	8.5	6.0	1582	16.0	9	16.1	Scald (%) 8.0	6.5	6.2	7.7	6.4	4.2	7.7
	Aerated	850	8.5	6.0	1582	14.1	9	14.7	1.0	1.5	0.0	0.5	0.8	0.6	1.0
July 22	Unaerated	850	8.5	6.0	1730	16.4	7	16.5							29.0
	Aerated	850	8.5	6.0	1730	15.4	7	16.2							9.6
July 26	Unaerated	850	6.0	7.0	1805	18.4	17	18.5	22.5	25.0	28.5	24.0	15.5	23.4	25.0
	Aerated	850	6.0	7.0	1805	17.3	17	18.0	0.6	3.0	3.2	2.0	3.0	2.9	2.2
									Browning (%) 17.2	9.8	12.8	15.5	3.0	10.7	12.3
July 27	Unaerated	850	8.6	5.0	1697	14.1	15	14.7	Scald (%) 10.2	8.9	4.8	10.0	11.0	5.4	10.8
	Aerated	850	8.6	5.0	1697	13.4	15	14.5	0.0	0.6	0.0	0.0	0.0	0.3	0.9
									Browning (%) 4.4	2.0	2.0	3.0	2.6	2.2	2.0

by mechanical aeration of soak tank water, thus controlling tank scald of red tart cherries.

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Mention of specific instruments, trade names, or manufacturers does not imply endorsement.

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Proanthocyanidins in the Apple

SUMMARY

A study was conducted on the acetone- and ethyl-acetate-extractable flavan polyphenols of Waldhöfler apples, an astringent-tasting juice variety. The extracts were examined by cellulose thin-layer chromatography with 5% n-butanol and n-butanol–acetic acid–water (BAW) as the developing solvents. (−) Epicatechin was the major monomeric flavan found; (+) catechin was present in smaller amounts. A large number of unknown flavans were also detected; most of these are thought to be dimeric or oligomeric in composition, since their R_f values did not correspond to those of any known flavans. The predominating unknown substance, R_f 0.58 in 5% n-butanol and 0.46 in BAW, was purified from ethyl acetate extracts by polyamide chromatography followed by solvent fractionation. The pure compound had a marked astringent taste. The IR and UV spectra were typical of flavans. On hydrolysis, cyanidin and epicatechin were produced. Methylation with diazomethane yielded a compound with the empirical formula $C_{36}H_{42}O_{12}\cdot H_2O$ with a methoxyl content of 33.7%. NMR spectra indicated that condensations involved the A ring. Hydrolysis of the methylated derivative yielded tetramethyl epicatechin and a substance corresponding to tetramethyl flavan-3,4-diol and other unidentified intermediate products. It is thought that the purified proanthocyanidin is a dimer, or possibly an oligomer, containing (−)epicatechin and 5,7,3′,4′-flavan-3,4-diol as constituents.

The other unknown flavans, not isolated in pure form, yielded cyanidin, (−)epicatechin, and a small amount of pelargonidin on hydrolysis.

INTRODUCTION

The proanthocyanidins, or leucoanthocyanidins, have been the object of extensive research, yet our knowledge of their structure and composition is far from complete, particularly for those of an oligomeric nature. A recent review on the proanthocyanidins has been presented by Clark-Lewis (1962). The similarities of the conditions for conversion of oligomeric proanthocyanidins and monomeric flavan-3,4-diols into cyanidins (Joslyn and Goldstein, 1964b) have led to the general assumption that flavan-3,4-diol units are present in oligomeric proanthocyanidins. This view is reinforced by the demonstration by Creasy and Swain (1965) of the proanthocyanidin nature of a condensation product of 5,7,3′,4′-flavan-3,4-diol and (−)epicatechin. Further, the elemental composition of isolated proanthocyanidins (Weinges, 1961; Geisman and Dittmar, 1965; Weinges and Freudenberg, 1965) is consistent with the condensation of flavan-3,4-diols with other flavans to form oligomeric proanthocyanidins.

Oligomeric proanthocyanidins belong to the large class of natural products known as condensed tannins. The predominant characteristic of the condensed tannins is their ability to combine with proteins, presumably by means of hydrogen bond formation between the phenolic hydroxyl groups of the tannins and the peptide linkage of the proteins (Gustavson, 1954). This property of protein binding may also be related to the astringency of the condensed tannins (Joslyn and Goldstein, 1964a). Purified naturally occurring proanthocyanidins have been reported to have an astringent taste (Geisman and Dittmar, 1965).

Proanthocyanidins are widely distributed in nature, being associated to some degree with plants having a woody habit of growth (Bate-Smith and Lerner, 1954). Apples contain these compounds, and in this fruit they contribute to the astringent taste and oxidative browning. Williams (1960) reported four distinct proanthocyanidins of apples. Ito and Joslyn (1965) described a number of chromatographically mobile apple proanthocyanidins, and also nonmobile proanthocyanidins. These latter materials are probably highly polymerized compounds. Hydrolysis of their preparations yielded cyanidin, pelargonidin, (−)epicatechin, and (+)catechin. There are no reports of mono-

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meric flavan-3,4-diols in apples, although other monomeric flavans, (+)catechin and (−)epicatechin, are found (Williams, 1960; Herrmann, 1958).

Our investigation of the apple proanthocyanidins was made to prepare and purify these constituents for study of their chemical structure and behavior. We found that the number of proanthocyanidins was quite large, but that one was present in relatively large amounts. This paper describes the purification of this compound and its chemical characteristics.

EXPERIMENTAL METHODS

The apples used in this study were of the Waldhöfler variety, an astringent apple used for juice and cider. They were harvested as indicated and held frozen until used. For the extraction of polyphenols the skins and flesh were utilized, and the cores were discarded. Extractions were carried out by homogenizing with cold solvents and with the addition of 50 mg of ascorbic acid per 100 g of apple flesh.

Chromatography on cellulose thin layers was carried out with 5% n-butanol and n-butanol–acetic acid–water (6:1:2.2) used as the solvents. Detection of flavans was done routinely with Echtschwarzsalz K (diazotized 4-amino-3,6-dimethoxy-4'-nitro-azobenzol; Chroma Gesellschaft, Stuttgart, Germany). This reagent gives a blue color with flavans. R_f values showed slight variations from time to time, and average values are given in this paper. Used as standards were chromatographically pure (+)catechin and (−)epicatechin (Senn Chemical Laboratory, Dielsdorf, Switzerland).

IR spectra were made with KBr disks. NMR spectra were obtained in carbon tetrachloride with tetramethyl silane as an internal standard.

Hydrolysis of proanthocyanidins was carried out in 95% ethanol, 0.3*N* with respect to HCl, at 70°C. Cyanidin formation was evaluated from the extinction at 550 mμ (Hayashi, 1962). The pigments produced were identified by cellulose chromatography using Forestal's solvent (Bate-Smith, 1954). Polyphenol determinations were by means of the Folin-Denis procedure (AOAC, 1960).

For column chromatography, polyamide powder (Woelm) was pretreated in 95% ethanol and stirred occasionally over a 2-or-3-day period. The alcohol was filtered off, and the polyamide was washed 2 times with 95% ethanol. It was then suspended in 95% ethanol and poured into the chromatography column. The powder settled rapidly, and the excess alcohol was allowed to drain from the bottom until about 1 mm of alcohol remained above the polyamide surface. Samples were applied as ethanolic solution to the top of the column. Samples placed on a 4 × 40-cm column should not contain more than 1 g of polyphenolics in order to get good separations.

RESULTS AND DISCUSSION

A large number of polyphenolic compounds can be extracted from Waldhöfler apples by acetone. Some of these are known substances that can be readily identified by cellulose chromatography, such as (−)epicatechin, (+)catechin, quercetin glycosides, and chlorogenic acid, but a large proportion do not correspond to any well-characterized polyphenol. These unknown polyphenols can be roughly divided into two classes: those that move on chromatograms developed with 5% n-butanol, and those that do not move. Both groups contain proanthocyanidins and produce a blue color characteristic of flavans when sprayed with Echtschwarzsalz K. The immobile substances are probably polymeric flavanoids, as indicated by their high affinity for cellulose and their low solubility in ethyl acetate.

A good part of the mobile materials are probably oligomers, since many of them were proanthocyanidins but did not have the same R_f value as monomeric proanthocyanidins. Fig. 1 presents a composite 2-dimensional chromatogram of these substances. Some of these spots could not be detected in

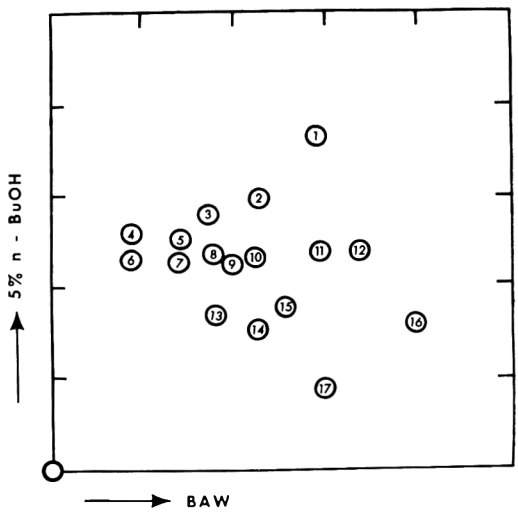


Fig. 1. Cellulose thin-layer chromatogram of Waldhöfler apple polyphenols.

crude extracts but were found after fractionation of extracts by column chromatography or solvent partition followed by concentration of the fractions. The difficulty in detecting these compounds in original extracts was due to their low concentration in comparison with (−)epicatechin (spot no. 15), (+)catechin (spot no. 12), and the unknown substance of spot no. 2, since the application of too large an amount of polyphenol at the origin tends to make all the spots more diffuse, causing minor components to appear as faint smudges. It is also possible that a few spots may be due to artifacts arising during fractionation procedures.

All the spots in Fig. 1 except chlorogenic acid, spot no. 1, are presumed to be flavans because of their lack of fluorescence under ultraviolet light and similar color reactions with diazonium salts, vanillin-HCl, and Ehrlich's reagent. To avoid confusion, the flavone glycosides are deliberately excluded from this figure.

The concentration of polyphenols and proanthocyanidins in Waldhöfler apples decreases as the apples mature (Table 1). The more mature fruit had more polyphenol per fruit than the July apples, and the polyphenols had a greater potentiality for conversion into cyanidin by hydrolysis. The high polyphenolic content of the July fruit may be due in part to the high levels of chlorogenic acids found in immature apples (Walker, 1963). Chromatographic examination showed that the proportion of (−) epicatechin to (+) catechin and substance no. 2 was greater for July apples than for the September or October apples.

The concentration of some of the polyphenols in September fruit was estimated by comparing the color development on cellulose chromatograms with standards of known amounts of (−)epicatechin, (+)catechin, and substance no. 2 (Table 1). This suggests that, even though substance no. 2 is the predominant discrete proanthocyanidin in Waldhöfler apples, it composes only about 2% of the total phenolics present.

Isolation of substance no. 2. Since substance no. 2 was a major component of the mobile polyphenols, we were interested in isolating and characterizing this material. For this purpose we found it advantageous to extract the apple flesh by homogenizing with cold ethyl acetate, thus eliminating the more highly condensed polyphenols at the beginning. After the evaporation of most of the ethyl acetate, the viscous residue was extracted with chloroform in order to remove the lipid material. The residue was dissolved in ethanol or ethyl acetate and further separated. Our first procedure of purification involved countercurrent distribution between 1% NaCl and ethyl acetate followed by cellulose column chromatography. The partition coefficient between 1% NaCl and ethyl acetate was 1.2, indicating a slight preference of substance no. 2 for the organic phase. We were able to obtain enough pure material for elemental analysis, spectral characteristics, and confirmation that it was a proanthocyanidin. The procedure was quite long, however, and the yield was low. Subsequently, we found that we could secure substance no. 2 almost pure by chromatography of relatively crude extracts on polyamide columns with ethyl alcohol used as the eluting solvent.

Table 1. Amount of acetone-soluble polyphenols in Waldhöfler apples of different maturity.[a]

Picking date	Cyanidin formed (mg/g fruit)	Polyphenol content (mg/g fruit)	Apple size (g/fruit)
July	.82	15.1	11.2
September	.56	6.7	53
October	.43	4.5	55

[a] Estimated concentration of particular polyphenols in September fruit (mg/kg): (−) epicatechin, 250; (+) catechin, 120; substance no. 2, 120; substance no. 3, 20; substances no. 8 plus no. 9, 50.

Good results in isolating substance no. 2 on the polyamide column are due to the happy circumstance that substance no. 2 was eluted long after the monomeric polyphenols but considerably before other polymeric polyphenols present in the apple extract. The elution pattern with ethyl alcohol first showed unabsorbed sugars and acids, chlorogenic acid, flavonone glycosides, and (+)catechin, and then (−)epicatechin. After a considerable volume of eluate contaning only a trace of epicatechin, there appeared substance no. 2. After substance no. 2 had been eluted, there was again an interval where only traces of epicatechin were present in the eluate. Then, together, substances nos. 3, 8, and 9 appeared. Other polyphenols,

originally present in the extract applied to the column, could not be eluted with ethanol. These tenacious polyphenols could be removed with dimethyl formamide.

The eluates that contained substance no. 2 were evaporated under vacuum, and the residue was dissolved in ethyl acetate. Chromatography of the solution indicated the presence of (−)epicatechin. The addition of 2 volumes of chloroform to the ethyl acetate solution caused substance no. 2 to precipitate, while the (−)epicatechin remained in solution. The precipitate was collected by filtration, the residual solvent was evaporated, and the dry residue was collected, as a slightly tan amorphous powder. Two-dimensional cellulose chromatography of the product showed the presence of only one component.

The infrared spectrum of purified substance no. 2 was quite similar to that of (+)catechin and (−)epicatechin. In the sensitive region of 1700–1300 cm^{-1} the spectra were identical. Absorption bands indicated the presence of aromatic ether bands, aromatic rings, and phenolic and nonphenolic hydroxyl groups. The absence of an absorption peak at 3250 cm^{-1} indicated a strong interaction of hydroxyl groups, probably with other hydroxyl groups. Carbonyl functions were absent, as well as nonaromatic C = C bands.

The ultraviolet spectrum was also similar to that of the catechins, with an absorption peak at 280 mμ. There were no absorption peaks above 300 mμ or in the visible range.

Color reactions of the purified substance gave further indications of its structure. Treatment with dichloroquinone chlorimide (Roux, 1963) yields the gray-blue color indicative of ortho dihydroxy groups. When treated with Ehrlich's reagent (Roux, 1963), the rapid appearance of pink suggested the presence of meta dihydroxy groups. Spraying with dilute p-toluenesulfonic acid resulted in the slow development of a reddish-brown coloration more typical of proanthocyanidins than of catechins. The development of this reddish-brown was considerably slower than that found for synthetic 5,7,3′,4′-flavan-3,4-diol. Vanillin (Roux and Mains, 1960) produced a pink color, and bis-diazotized benzidine a brown color. Substance no. 2 was not detectable by ultraviolet light when 1-cm-diameter spots containing 2 μg were tested.

Hydrolysis of the purified substance in ethanolic HCl resulted in the formation of cyanidin, as confirmed by chromatography with Forestal's solvent. Maximum cyanidin formation required about 1 hr of heating at 70°C. During the early stages of hydrolysis, one can also detect the formation of other phenolic hydrolysis products. Chromatograms obtained after 10 min of hydrolysis indicated (−)epicatechin as the principal product. A trace of the original substance no. 2 could be seen. The identity of four other spots has not been determined. The relative amounts of the hydrolysis products, as estimated by the intensity of color developed with the spray reagent, indicated that the amount of epicatechin found was equal to or slightly greater than the combined amounts of the other products. The later disappearance of these unknown products on further hydrolysis, together with the continued formation of cyanidin, suggests that the unknown hydrolysis products are intermediates in the formation of cyanidin or epicatechin from substance no. 2.

Comparison of the purified substance no. 2 with synthetic 5,7,3′,4′-flavan-3,4-diol (Freudenberg and Weinges, 1958), a monomeric proanthocyanidin, showed that the 2 compounds were dissimilar. They are chromatographically different, the monomer formed cyanidin much more rapidly at 70°C in 0.3*N* ethanolic HCl, and the monomer did not form catechins during the hydrolysis.

Elemental analysis of the purified apple proanthocyanidin gave C 56.5% and H 4.7%. Calculated for a dimer of 5,7,3′,4′-flavan-3,4-diol and epicatechin, $C_{30}H_{26}O_{12} \cdot 3H_2O$ was C 56.9% and H 5.06%.

Substance no. 2 forms acetates with acetic anhydride and methyl derivatives with diazomethane. The acetate proved of little value for characterization of the compound, since their chromatographic behavior was very similar to that of acetylated flavan monomers, and, when hydrolyzed, the acetyl groups were split off. The methyl derivative was, however, quite useful. The methylated

derivative was prepared by treating substance no. 2 in methanol with excess diazomethane at −10°C until excess diazomethane persisted 24 hr in the reaction flask, and chromatographic inspection of the reaction mixture showed that most of the material had been converted to a single product. The solvent and excess diazomethane were removed, and the derivative was purified by chromatography on a silicic acid column with a 1:1 mixture (v/v) of chloroform and ethyl acetate. After solvent removal, methylated substance no. 2 was dissolved in methanol, and from this solution was precipitated by the addition of water.

The elemental analysis and methoxy group content was C 64.4%, H 6.27%, and (OCH_3) 33.69%. Calculated for an octomethyl dimeric proanthocyanidin, $C_{38}H_{42}O_{12}\cdot H_2O$ was C 64.3%, H 6.22%, and (OCH_3) 35%.

The infrared spectra of the methylated product showed the presence of nonphenolic hydroxyl groups. This corresponds to the experience of Weinges (1961), who also found that nonphenolic hydroxyl groups of proanthocyanidins could not be readily methylated with diazomethane.

The NMR spectra of our methylated derivative were run with tetramethyl silane used as an interval standard. The resulting spectra were virtually identical to that found by Geisman and Dittmar (1965) for a similar derivative of avocado proanthocyanidin. We obtained a ratio of 2 hydrogens on the B ring to 1 hydrogen on the A ring. The broadening of the signals compared with those obtained from methylated monomeric flavans was also seen, and this we interpret as being due to the oligomeric nature of the proanthocyanidin.

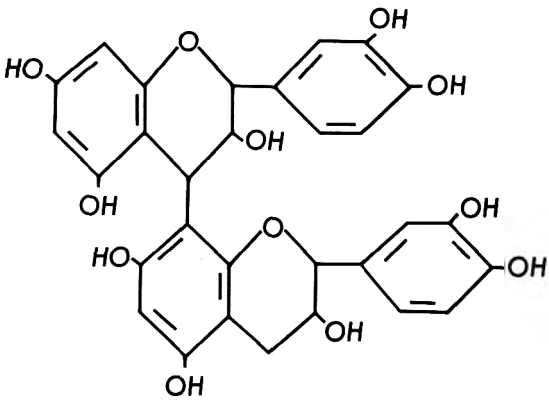
The methylated proanthocyanidin was hydrolyzed in ethanolic 0.3*N* HCl until a slight amount of red color developed. After exhaustive methylation, the mixture was chromatographed on silica gel with a 1:1 (v/v) mixture of chloroform and ethyl acetate as the solvent. Among the products found were those corresponding to tetramethyl epicatechin and 5,7,3′,4′-tetramethyl flavan-3,4-diol. Hydrolysis of the chromatographically separated products showed that only those components with R_f lower than 0.4 produced red color.

The optical rotation, $(\alpha)^{D}_{20}$, of the methyl derivative in acetone was +45°.

The dried compound no. 2 was fairly stable over several months at room temperature. There was only a slight formation of materials running slower than the parent compound on chromatograms, and a slight increase in the tan color. The compound was markedly astringent.

The purification of substance no. 2 provided an opportunity to gain some more information as to the nature of some of the other unknown flavans. We obtained a number of fractions containing various proportions of flavans corresponding to spots nos. 3, 8, and 9. Hydrolysis of these fractions yielded cyanidin and pelargonidin. A comparison of the relative amounts of cyanidin and pelargonidin formed with the relative amounts of the three substances in the fractions indicated that substance no. 3 was the precursor of pelargonidin, and substances no. 8 and no. 9 were precursors of cyanidin. These substances were held more firmly on polyamide columns than substance no. 2, and they were eluted together. The large number of substances corresponding to spots nos. 4, 5, 6, 7, and 13 were held even more strongly on polyamide columns. Hydrolysis yielded a mixture of 1 part pelargonidin and 5 parts cyanidin. It appears that at least 2 substances capable of forming pelargonidin are present in the group of mobile flavans. Most of the mobile flavans, in number as well as in quantity, are, apparently, precursors of cyanidin. Mild hydrolysis of the 3, 8, and 9 group and the 4, 5, 6, 7, and 13 group produced epicatechin.

The simplest conclusion as to the structure of purified substance no. 2 is that it is a dimer of 5,7,3′,4′-flavan-3,4-diol and (−) epicatechin. A low molecular weight of the oligomer is indicated by the mobility of the substance on cellulose chromatograms, its elution from polyamide columns following the monomers (+)catechin and (−)epicatechin, and its relatively high partition coefficient between ethyl acetate and water. The NMR spectra indicate participation of the A ring in the formation of the dimer. Elemental analysis indicates that H_2O is



SUGGESTED STRUCTURE OF AN APPLE DIMERIC PROANTHOCYANIDIN

Fig. 2. Suggested dimeric structure of substance no. 2, a proanthocyanidin in apples.

eliminated as a result of the dimerization. The ratio of 8 methyl groups to 12 *O* indicates 2 aliphatic hydroxyls for each C_{30} unit. Because of the greater chemical reactivity of C_4 than of C_3, it may be assumed that the missing hydroxyl group was the one at the 4 position. A proposed structure for the dimer is given in Fig. 3. It is probable that such a structure arises from the condensation of (−)epicatechin and 5,7,3′,4′-flavan-3,4-diol. In fact, such a condensation has been observed by Creasy and Swain (1965). The possibility, however, also exists that such a structure might arise through an as yet unknown mechanism involving the condensation of (−)epicatechin and catechin. A structure of the type shown in Fig. 2 has been presented recently (Geisman and Dittmar, 1965; Weinges and Freudenberg, 1965) for other purified proanthocyanidins. These substances, however, do not appear to be identical to the substance no. 2 that we have isolated, since they do not have the same R_f values as found for the apple proanthocyanidin. They may, however, be isomeric forms, differing in the configuration on the 2, 3, or 4 carbon atoms.

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Gas Chromatographic Determination of Anthocyanins and Other Flavonoids as Silyl Derivatives

SUMMARY

Gas chromatographic methods were developed for the separation of apigeninidin compounds, pelargonidin, cyanidin, delphinidin, petunidin, and malvidin. Protocatechuic, p-hydroxybenzoic, gallic, vanillic, and syringic acids—which are useful for the identification of particular anthocyanidins—were also amenable to gas chromatographic analysis. Phloroglucinol could likewise be measured. Separation of rhamnose, ribose, xylose, fructose, galactose, and glucose was good. Except for fructose, the others are common glycosidic sugars and aid in differentiating anthocyanins. Retention times were determined for the monoglucosides of delphinidin, petunidin, pelargonidin, and malvidin; for a delphinidin glycoside extracted from wild grape hyacinths; and for arbutin, hesperidin, rutin, and quercitrin. Betanin likewise was volatile as the silyl ether. Rhamnetin, catechin, and quercetin yielded good gas chromatographic waves. Phenolphthalein and naphthorescorcinol were sometimes used as internal standards. The silyl ethers or esters above were prepared in pyridine or dimethyl sulfoxide. The data suggest that gas chromatography might be used for preparative as well as for identification purposes.

INTRODUCTION

Paper chromatography is probably the most useful single method for the identification of anthocyanin compounds. In its effectiveness and simplicity it is already a classic among methods though it is but 22 years old (Consden *et al.*, 1944). It is not without its faults, however. For full resolution of R_f values, one must usually wait 12–18 hr. Furthermore, a second solvent may be needed to separate compounds yielding similar R_f values in a single solvent. Because of the time element and the fact that an alternate method of analysis based on a different principle or technique often has merit to avoid errors inherent within a given method, this study was undertaken. The purpose was to learn whether anthocyanin compounds could be determined gas chromatographically.

REVIEW OF LITERATURE

Prior to the finding of Sweeley *et al.* (1963) that silylation of the hydroxyl groups on sugars and certain other nonvolatile compounds made them sufficiently volatile for gas chromatographic analysis, methylation and acetylation of polyhydroxyl compounds had been tried (VandenHeuvel and Horning, 1961; Jones *et al.*, 1962; Gee and Walker, 1962; Narasimhachari and von Rudloff, 1962). Though separation could often be effected, the results were not nearly as spectacular as separation through silylation has proved to be. Since the publication of Sweeley *et al.* (1963) there has been a remarkable flowering of methods for gas chromatographic determination of nonvolatile compounds. Among these substances are: steroidal compounds (Kirschner *et al.*, 1964), bile, Krebs, and amino acids (Makita and Dells, 1963; Horii *et al.*, 1965; Smith and Sheppard, 1965), alcohols (Friedman and Kaufman, 1966) and a few flavonoids (Furuya, 1965a,b).

Friedman and Kaufman (1966) used dimethyl sulfoxide (DMSO) as a vehicle for silylation. Most prior studies had used pyridine. Furuya (1965a) observed that retention times were the same whether silylation took place in pyridine or tetrahydrofuran. Waiss *et al.* (1964) studied the trimethylsilyl ethers of catechin, apigenin, and quercetin by nuclear magnetic resonance. They showed that all the hydroxyl groups were silylated. Harkiss (1965) measured gas chromatographically the combustion products generated upon pyrolyzing rutin, quercetin, cyanidin, and pelargonidin.

EXPERIMENTAL

Materials. Small samples of pelargonidin and cyanidin of high purity were kindly donated by Dr. Leonard Jurd, Western Utilization and Research Lab., U. S. Dept. Agr., Albany, Calif. These samples were used as reference compounds. Dr. A. M. Neubert, U. S. Dept. Agr. Fruit and Vegetable Lab., Prosser, Washington, had supplied high purity delphinidin, petunidin, and malvidin for an earlier study (Somaatmadja and Powers, 1963). These compounds were also used for reference. Delphinidin was purchased from City Chemical Co., N. Y.; cyanidin, delphinidin, poenidin, and pelargonidin from K & K Laboratories, Plainview, N. Y.; and cyanidin and malvidin from Aldrich Chemical Co., Milwaukee, Wisc. The two commercial cyanidins yielded only one spot when paper

chromatographed to check for purity. The delphinidin purchased from City Chemical Co. was also pure. All the other compounds gave two or more spots, with the second and third spot sometimes being of considerable intensity. The pelargonidin was particularly refractory to handle because it was gummy rather than crystalline in nature and quite impure. Anthocyanidins were also prepared in our laboratory and purified by paper chromatography (Somaatmadja and Powers, 1963).

The Hoffman-LaRoche Co. donated generous samples of five apigeninidin compounds. These compounds had been previously used by us (Powers *et al.*, 1960; Somaatmadja and Powers, 1964). The apigeninidin-chloride furnished was very pure. The samples of apigeninidin-chloride-4′-methyl-ether and 7-hydroxy-4′-methoxyflavylium chloride showed a trace of a second substance each. The 5,7-dihydroxy-3′,4′-dimethoxyflavylium chloride and 7-hydroxy-3′,4′-dimethoxyflavylium chloride each showed the presence of two substances aside from the main compound. The impurities were present in small amounts.

Arbutin, rutin, catechin, quercetin, quercitrin, and rhamnetin were kindly donated by S. B. Penick & Co., New York. The hesperidin used was from a sample donated by the Sunkist Co., Ontario, Calif., approximately 7 years ago. It had been kept sealed and under refrigeration since 1959.

Vanillic, protocatechuic, gallic, syringic, and p-hydroxybenzoic acid, and glucose, fructose, galactose, ribose, rhamnose, and xylose were purchased from regular chemical supply houses.

Malvidin-3-monoglucoside (M-3-G), petunidin-3-monoglucoside (Pt-3-G), and delphinidin-3-monoglucoside (D-3-G) were extracted from Cabernet Sauvignon grapes. Delphinidin glycoside was extracted from wild grape hyacinths, *Muscaria racemosum*. Pelargonidin-3-monoglucoside (P-3-G) was extracted from strawberries. The procedure of Somaatmadja and Powers (1963) was used to purify the compounds.

Aglycones were prepared as described by Somaatmadja and Powers (1963) or by a modification of their procedure. The modification consisted of acidifying 90 ml of a methanolic solution of the purified anthocyanin with 10 ml of glacial acetic acid. The solution was then refluxed 1 hr, next it was heated 15 min in an autoclave at 121°, and promptly cooled to room temp. The anthocyanidins were precipitated with lead acetate, washed free of sugars, and the procedure of Somaatmadja and Powers (1963) was followed thereafter. Both the glycosides and the aglycones were powdery, crystalline masses.

Gas chromatographic conditions. The most useful column was a 6-ft ¼-inch stainless-steel column packed with 0.05% SE-52 on No. 203 glass beads (Microbeads, Jackson, Miss.). Other columns used were: 3% SE-52 coated onto 60/80-mesh Chromosorb-AW-DMCS, 6-ft ¼-inch stainless steel; 3% SE-30 coated onto the same support except the ¼-inch column was 4 ft long; and 3% QF-1 dissolved in chloroform, then coated onto the wall of stainless-steel tubing, 0.025 inch ID, 50 or 100 ft in length. For the latter, no support material was used.

Model 609 or 810 F & M gas chromatographs equipped with flame ionization detectors were used. Temperatures employed ranged from 50 to 260°. Nitrogen was the carrier gas.

Silylation. Initially, silylation was always carried out in pyridine (1.0 ml pyridine, 0.2 ml hexamethyldisilazane, and 0.1 ml trimethylchlorosilane). Sample size ranged from 6 to 10 mg. The reaction mixture usually heated up spontaneously when the trimethylchlorosilane (TMCS) was added, but, to be sure that a reaction took place, the test tubes were heated for 10 min in water at approximately 50°. Later on in the study, DMSO was also used as a reaction medium. Five to 30 mg of sample were dissolved in 0.5 ml DMSO; 0.2 ml HMDS and 0.1 ml TMCS were then added. An exothermic reaction usually took place, but when it did not, heating was employed to be sure silylation occurred.

RESULTS

Anthocyanidins. Retention times for pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin are given in Table 1. The 6-ft ¼-inch SE-52 (0.05%) stationary phase on glass beads was the best column for separation. The resolution obtained is shown in Fig. 1. Petunidin and delphinidin could not be readily separated on the 0.05% SE-52 glass-bead column, but they could be separated on the 4-ft ¼-inch SE-30, 60/80-mesh Chromosorb-AW-DMCS column. Peonidin was always most difficult to silylate. Delphinidin was easiest to silylate, and the silyl ether was stable for a week or more in pyridine and for 2–5 days in DMSO. In the DMSO, the ethers appeared to hydrolyze, for the original red color ultimately returned. Malvidin was also easy to silylate and chromatograph. Pelargonidin was somewhat more difficult to silylate. Some of the difficulties with pelargonidin were a result of the poor quality of the first batch purchased. Little difficulty was encountered with a second batch which was purer and more crystalline. Cyanidin was

Table 1. Retention times for anthocyanidin compounds as silyl ethers.

Compounds	Column	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
Cyanidin	1[a]	80	—	30.0
	2	140	250	19.2
	3	80	250	18.4
Delphinidin	1	80	210	17.8
	2	140	250	16.4
	3	80	250	10.0
Pelargonidin	1	80	210	6.0, 10.0
	2	140	250	3.8
	3	80	250	1.8
Malvidin	1	80	210	23.0
	2	140	250	12.0
	3	80	250	12.8
Petunidin	1	80	210	19.2
	2	140	250	7.0
	3	80	250	13.8
Peonidin	1[b]	70	—	22.0

[a] Programmed at 15°/min from 210–255° with a PI of 24 min.
[b] Programmed at 15°/min from 158–255° with a PI of 16 min.
Column 1 = 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads.
Column 2 = 6-ft stainless-steel, ¼-inch 3% SE-52 on Chromosorb AW-DMCS.
Column 3 = 4-ft stainless-steel, ¼-inch 3% SE-30 on Chromosorb AW-DMCS.

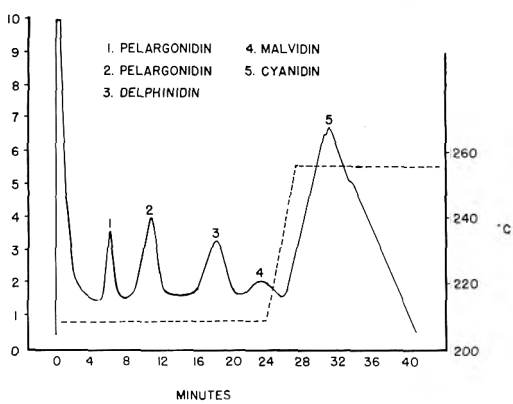

Fig. 1. Gas chromatogram of anthocyanidins. The column was a 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads with 80-ml-nitrogen/min flow rate. The initial temperature of 210° was held for 24 min, then programmed at 15°/min up to 255°.

almost as difficult as peonidin to silylate and to secure good gas chromatographic waves. Cyanidin was difficult to chromatograph on the SE-52 glass-bead column primarily because of its long retention time and the fact that breakdown seemed to take place if too long a time of column residence occurred. This was the limiting factor in separating delphinidin and petunidin in mixtures with cyanidin on the 0.05% SE-52 glass-bead column. If the temperature was low enough to separate delphinidin and petunidin, then cyanidin yielded a low, flat wave. While the 0.05% SE-52 column was the best column for most purposes, other columns were more suitable for cyanidin.

Retention times for the 5 apigeninidin compounds are in Table 2.

Glycosides. The monoglucosides of delphinidin, petunidin, pelargonidin, and malvidin each yielded chromatographic peaks. Their retention times are listed in Table 3. Fig. 2 shows the peak which resulted when the coloring matter of wild grape hyacinths was extracted with methanol, acidified with HCl, precipitated with lead acetate, washed free of sugar on a sintered glass filter, redissolved in acidified methanol, then evaporated to dryness. The pigment was a delphinidin glycoside.

Nonanthocyanin glycosides and aglycones were also determined. Retention times for these compounds are listed in Table 4. In general, glycosides could be determined at a lower temperature than their aglycones.

Sometimes certain glycosides or aglycones yielded more than one peak even though the compound appeared to be pure. This may have been the result of resonance (Jurd, 1963; Jurd and Geissman, 1963). In studies

Table 2. Retention times for apigeninidin compounds as silyl ethers.

Compound	Column[a]	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
Apigeninidin-chloride	1	140	250	9.0
	2	70	250	6.0
Apigeninidin-chloride-4'-methyl-ether				
	1	140	250	21.5
	2	70	250	4.8
5,7-Dihydroxy-3',4'-dimethoxyflavylium chloride				
	1	140	250	12.0
7-Hydroxy-4'-methoxyflavylium chloride				
	1	140	250	14.2
	2	140	240	7.0
7-Hydroxy-3',4'-dimethoxyflavylium chloride				
	1	140	250	9.8

[a] Column 1 = 6-ft stainless-steel, ¼-inch 3% SE-52 on 60–80-mesh Chromosorb AW-DMCS.
Column 2 = 4-ft stainless-steel, ¼-inch 3% SE-30 on 60–80-mesh Chromosorb AW-DMCS.

Table 3. Retention times for anthocyanin.

Compound	Column[a]	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
D-3-G	1	80	210	8.0
	2	80	210	23.6
M-3-G	1	80	210	9.6
	2	80	210	34.0
Pt-3-G	1	80	210	8.8
	2	80	210	26.8
P-3-G	2	80	215	7.0
Cyanidin glycoside	2	80	198	39.6
Delphinidin glycoside	2	70	245	18.8

[a] Column 1 = 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads.
Column 2 = 6-ft stainless-steel, ¼-inch 3% SE-30 on 60–80-mesh Chromosorb AW-DMCS.

on the polarography of anthocyanins, Somaatmadja *et al.* (1964) and Keith and Powers (1965a) observed double or triple polarographic waves for some anthocyanins at certain pH levels, which is another indication of variation in structure or oxidation state. Gas chromatography is superior to paper chromatography in being able to distinguish anomeric forms. See Fig. 3 for the mixture of sugars analyzed. Very likely, some of the di- and multiple-peaks encountered with compounds of known purity reflect differences in configuration. Another possibility is that the gas chromatographic analysis was picking up impurities not detected by paper chromatography. Furuya (1965b) encountered di- or multi-peak responses for some of the flavonoids he studied. When the benzoic acids (discussed below) yielded more than one peak, this was assumed to be due to the formation of silyl esters as well as ethers.

The separation of a mixture of aglycones and glycosides is shown in Fig. 4. The fact that various types of aglycones and glycosides may be determined by gas chromatography is important, for such compounds as rutin (DeEds and Couch, 1948), quercetin (Williams and Wender, 1952a,b), and arbutin (Bate-Smith, 1959) occur in foods and possess physiologic action.

The retention times for beet pigment at 230° on the 4-ft 3% SE-30 column were 11.6 and 12.8 min. Another strong and sharp peak occurred at a retention time of 4.7 min, but this compound appeared not to be derived from the pigment. On a column composed of 30% diethylene-glycol succinate on Chromosorb W, 4 ft long, retention times for beet pigments at 160° were 14.8

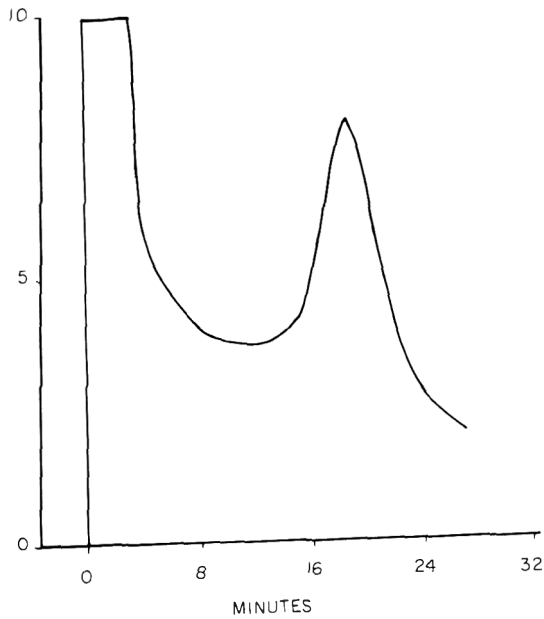

Fig. 2. Gas chromatogram of delphinidin diglucoside from grape hyacinths (*Muscaria racemosum*). The column was a 6-ft stainless-steel ¼-inch 3% SE-30 on 60–80-mesh Chromosorb AW-DMCS at 245° and a flow rate of 70 ml nitrogen/min.

and 21.2 min. Two strong peaks occurred earlier in the run (at 5.4 and 6.4 min), but these peaks seemed not to be related to the red colored compounds (Peterson and Joslyn, 1960).

Sugars. Of aid in the identification of anthocyanins is the determination of the kind and amount of glycosidic sugars which are liberated when anthocyanins are hydrolyzed. Retention times for sugars are shown in Table 5. Fig. 3 shows that these sugars can be readily separated. These sugars have been previously gas chromatographed by others (Sweeley *et al.*, 1963; Sawardeker and Sloneker, 1965), but not always in the same combination.

Benzoic acids. The kind of phenolic acid which is liberated when the side ring is split from the main ring of an anthocyanin by alkaline degradation or through the use of H_2O_2 (Geissman, 1962) is also of diagnostic aid. Clean separation of phenolic acids typical of those resulting from H_2O_2 degradation of anthocyanidins was achieved (Fig. 5). Retention times are given in Table 6. Not only should gas chromatographic analysis simplify identification of phenolic acids resulting from degradation of anthocyanins, but it might have value in the analysis of metabolic end products. Keith and Powers (1965b) have shown that these phenolic acids inhibit organisms invading the urinary tract. Horii *et al.* (1965) reported that acids of the Krebs cycle could be measured as trimethyl silyl derivatives and that the silane reacted both with the hydroxyl and carboxyl groups. Since silylation of amine and amide groups occurs (Fishbein and Zielinski, 1965) as well as hydroxyl and carboxyl groups, silylation of urinary excretion products derived from flavonoids might be a possible way to follow metabolic changes in a more sensitive manner than at present.

Table 4. Retention times for nonanthocyanin glycosides and aglycones.

Compound	Column[a]	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
Quercetin	2	60	240	28.0
Rutin	2	60	255	4.2
	3	220	250	2.2
Rhamnetin	2	70	250[b]	30.0
	3	220	250	13.6
Catechin	1	80	255[c]	22.0
Arbutin	1	80	255[c]	14.7
Hesperidin	1	80	255[c]	16.4
Quercitrin	1	80	255[c]	27.7
	3	220	250	13.8

[a] Column 1 = 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads.
Column 2 = 4-ft stainless-steel, ¼-inch 3% SE-30 on 60–80-mesh Chromosorb AW-DMCS.
Column 3 = 6-ft stainless-steel, ¼-inch 3% SE-52 on 60–80-mesh Chromosorb AW-DMCS.
[b] The temperature was programmed from 170 to 250 at 2°/min.
[c] The temperature was held 10 min at 170°, then programmed at 4°/min up to 255°.

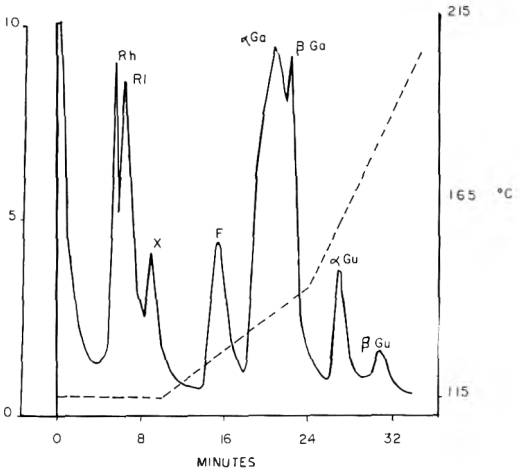

Fig. 3. Gas chromatogram of sugars. Rhamnose, ribose, xylose, fructose, α-galactose, β-galactose, α-glucose, and β-glucose were held at 115° for 10 min, programmed at 2°/min for 16 min, then at 6°/min for 10 min on a 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass-bead column.

Sensitivity and applications. Generally, 5 μL of sample were injected for each determination. When DMSO was the reaction vehicle, the ether dissolved in the upper silane layer, the total volume of which was 0.3 ml. At the maximum level of 20 mg of sample, 5 μL would contain approximately 0.3 mg of ether provided the reaction was 100% efficient (which it probably seldom was). Fig. 6 shows the kind of chromatograms obtained when 2 μL of mixed anthocyanin pigments were injected onto a 4-ft ¼-inch stainless-steel column composed of 30% diethylene glycol succinate on 60-mesh Chromosorb W. The three samples were prepared by precipitating the anthocyanins from a pint of commercial Concord grape juice, from 50 ml of an eightfold wine burgundy concentrate (Vie-Del Co., Fresno, Calif.), and from 50 ml of a true fruit elderberry extract (Seeley & Co., Nyack, N. Y.). The temperature was programmed at 6°/min from 170 to 250°. During the first 4 min of the runs, 6 to 10 peaks were observed. These are not depicted, because the drawing would then be too confusing. The glycosides and aglycones began to emerge at 6 min and on-

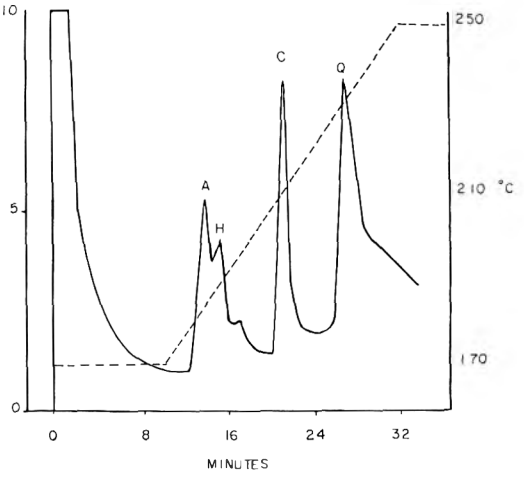

Fig. 4. Gas chromatogram of aglycones and glycosides. Arbutin, hesperidin, catechin, and quercitrin were held at 170° for 10 min, programmed at 4°/min up to 250° and held at that temperature. A 6-ft stainless-steel ¼-inch 0.05% SE-52 on No. 203 glass-bead column was used. The nitrogen flow rate was 80 ml/min.

Table 5. Retention times of sugars.

Sugar	Column[a]	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
Rhamnose	1	75	115	5.6
Ribose	1	75	115	6.2
Fructose	1	75	119	8.8
	2	60	175	9.6
Xylose	1	75	115	9.0
α-Galactose	1	80	119	11.0
β-Galactose	1	80	119	12.8
α-Glucose	1	80	119	14.0
	2	140	175	13.0
	3	35	135	4.4
β-Glucose	1	80	119	15.0
	3	35	135	6.4

[a] Column 1 = 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads.
Column 2 = 6-ft stainless-steel, ¼-inch 3% SE-52 on 60–80-mesh Chromosorb AW-DMCS.
Column 3 = 4-ft stainless-steel, ¼-inch 3% SE-30 on 60–80-mesh Chromosorb AW-DMCS.

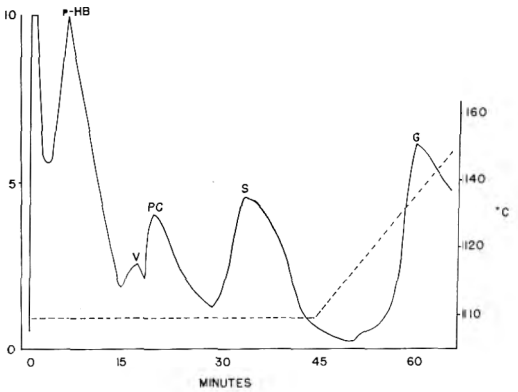

Fig. 5. Gas chromatogram of phenolic acids. p-Hydroxybenzoic, vanillic, protocatechuic, syringic, and gallic acid were held at 110° for 45 min, then programmed at 2°/min to 150°, and held. The column was a 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass-bead column with a nitrogen flow rate of 80 ml/min.

ward. One may see not only that gas chromatography is sensitive for anthocyanin and related compounds but that the pattern is different for three foods known to differ in type and amount of anthocyanin content. All of the peaks shown are not anthocyanins. No attempt was made to identify the compounds in detail other than to be sure the major ones were anthocyanins or anthocyanidins. The anthocyanidins emerged toward the end of the determinations.

Difficulties. One problem with the particular reagents used is their corrosivity. The authors have not noticed this mentioned in previous publications. The authors' experience was that syringes deteriorated far more rapidly than with most other chemicals. Sometimes the authors have used the same syringe for 2–3 years with food flavors or carbonyl compounds (Dornseifer *et al.*, 1965; El'Ode *et al.*, 1966) without difficulty. The metal components of syringes become worn rapidly and then leaked, apparently from the corrosive action of the silane materials. With DMSO, the corrosive action was even greater. The silicon materials

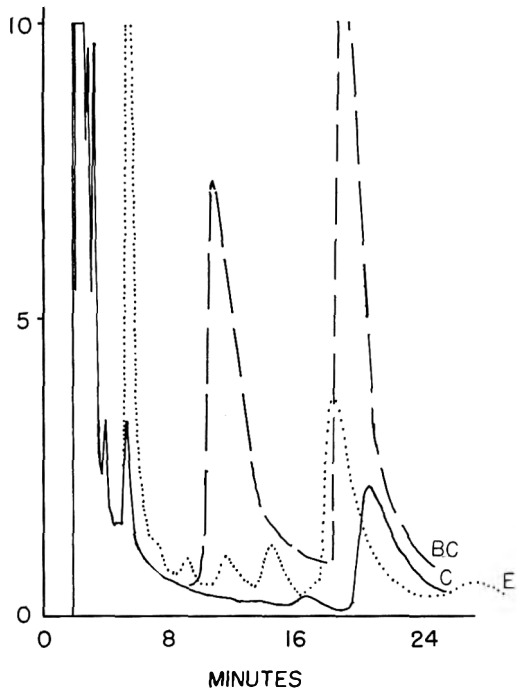

Fig. 6. Gas chromatogram of mixed anthocyanins. C = Concord grape juice, BC = California wine concentrate, and E = true fruit elderberry extract. The pigments of the three samples were measured on a 4-ft ¼-inch stainless-steel column loaded at a 30% level with DEGS on 60-mesh Chromosorb W.

Table 6. Retention times of phenolic acids as silyl derivatives.

Phenolic acid	Column [a]	Flow rate (ml/min)	Temp. (°C)	Retention time (min)
p-Hydroxybenzoic	1	80	105	4.2
Vanillic	1	80	105	12.5
	2	40	105	8.4
Protocatechuic	1	80	105	14.0
	2	40	105	9.9
Syringic	1	40	105	32.5
	2	40	105	17.5
Gallic	1	110	125	7.0
	2	40	90–210	14.2
			6°C/min	

[a] Column 1 = 6-ft stainless-steel, ¼-inch 0.05% SE-52 on No. 203 glass beads.
Column 2 = 100-ft stainless-steel, 0.025-inch ID, 3%QF-1.

formed upon burning of the effluent also coated the detector jets and collecting rings. This is one reason quantitative results are not given here. It was very difficult to measure compounds quantitatively unless an internal standard (phenolpthalein, naphthoresorcinol, sucrose, or some similar compound) was used, because sensitivity of the instrument changed drastically in the course of 2–3 determinations, as a result of fouling of the detector and collecting rings. In fact, for the most difficult compounds to measure, the detector was swabbed with acetone after the solvent peak had emerged. This procedure could be used, of course, only for compounds having a retention time considerably removed from the injection time. To destroy excess silane reagents, ethanol was added to some samples on the theory that the ethanol would react with excess silane materials, cutting down the solvent peak and minimizing their action of fouling the detector. It was assumed that if ethanol ended up in excess, it—being volatile—would pass through the column quickly and thus would not interfere with the compounds of interest. The ethanol seemed to perform a second function. Not only was the solvent peak reduced in size, but the silyl anthocyanin peak was often increased in size. The only explanation the authors have for this action is that the ethanol silyl ether possibly acted as a solvent for the anthocyanin ether. Especially in the DMSO tubes there sometimes seemed to be droplets of an oil-like material floating at the interface between the silanes and the DMSO. When ethanol was added, the bead-like drops disappeared. Control injections of ethanol silyl ether were injected, i.e., without any other hydroxy-containing substances, to be sure gas chromatographic peaks from an ethanol silyl ether were not interfering with other peaks observed. No interference was observed, but this possibility must be kept in mind. Ethanol was not always used. In fact, it was used only when the solvent peak from the DMSO tubes seemed to be excessive.

A difficulty encountered, which is mentioned as a note rather than an analytical problem, was the action of the 7-hydroxy-4′-methoxyflavylium chloride on the individuals who frequently weighed out samples. It induced sneezing for several minutes thereafter.

Preparative gas chromatography. The data suggest that gas chromatography might serve as a preparative means for separating anthocyanin or other flavonoid materials of high purity. In the first place, gas chromatographic analysis often revealed the presence of impurities (or resonance forms) which paper chromatography did not reveal. Secondly, the anthocyanin silyl ethers spontaneously decomposed upon standing for some time. This suggests that the compounds could be separated as silyl ethers, then regenerated to the original anthocyanin compounds. Friedman and Kaufman (1966) were able to recover the tertiary alcohol with which they started. Waiss *et al.* (1964) described conditions of hydrolysis for the compounds they studied.

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Use of Carbazole to Determine 5′-Ribonucleotides in Meats

The 5′-ribonucleotides have attracted considerable interest in recent years because of their flavor effects in a variety of foods (Kuninaka, 1960; Caul and Raymond, 1964; Kurtzman and Sjöström, 1964; Shimazono, 1964). One of them, inosine-monophosphate (IMP), has been listed among the compounds associated with the pleasant meaty aroma in red meats (Batzer *et al.*, 1960, 1962) and fish muscle (Jones, 1961).

Quantitative determination of the 5′-ribonucleotides is usually based on measurement of ultraviolet absorption after separation by ion-exchange chromatography, paper partition chromatography, or paper electrophoresis (Chargaff and Davidson, 1960; Markham, 1955; Lento, *et al.*, 1964). Solms (1964) suggested use of the reaction between carbazole and sugars in concentrated sulfuric acid as a colorimetric method for quantitative determination of 5′-ribonucleotides. The 5′-nucleotides give rise to a color with a characteristic absorption maximum at 685 mμ. When the method is applied to meat products, the nucleotides must previously be separated by chromatography or electrophoresis. This note presents an observation suggesting that useful results can be obtained without the rather laborious separation of the nucleotides.

EXPERIMENTAL

The experimental material was samples of longissimus dorsi from pork and beef carcasses. Of the sample to be analyzed for 5′-ribonucleotides, 5–15-g portions were homogenized in an Ultra Turrax mixer with 20 ml distilled water. The homogenate was heated to 90°C to precipitate heat-coagulable protein. The sample was transferred quantitatively to centrifuge tubes, and the precipitate was removed by centrifugation. The precipitate was resuspended in a minimum amount of water and centrifuged. The supernatants from the two centrifugations were combined. To precipitate the remaining proteins, 10 ml of a 20% solution of trichloroacetic acid and 30 ml of 96% ethanol was added to the aqueous extract. The mixture was centrifuged, and the trichloroacetic acid and ethanol were removed by three successive extractions with equal volumes of diethyl ether. The aqueous phase was diluted with water to exactly 250 ml.

Carefully added to 5 ml concentrated sulfuric acid (150 ml 98% H_2SO_4 and 50 ml H_2O mixed and chilled) in a test tube was 1 ml of the extract and 1 ml of a freshly prepared 0.1% solution of carbazol in 96% ethanol. The mixture was shaken and heated for exactly 5 min in a vigorously boiling water bath. After heating, the mixture was chilled for 3 min in an ice-water bath, and the absorption of the resulting color was measured with a Hitachi Perkin-Elmer UV-VIS spectrophotometer between 440 and 700 mμ against a blank without extract. Standard solutions were prepared with IMP (Takeda Chemical Industries) and glucose (P.D.). The contents of reducing sugars in the extracts were determined by the method of Hagedorn *et al.* (1946).

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectrum of the color developed with an extract of 7 g pork muscle (curve A). It has a maximum at 565 mμ. The same absorption maximum is obtained when the color is developed with a solution of pure glucose. Curve *B* in Fig. 1 depicts the spectrum for a solution

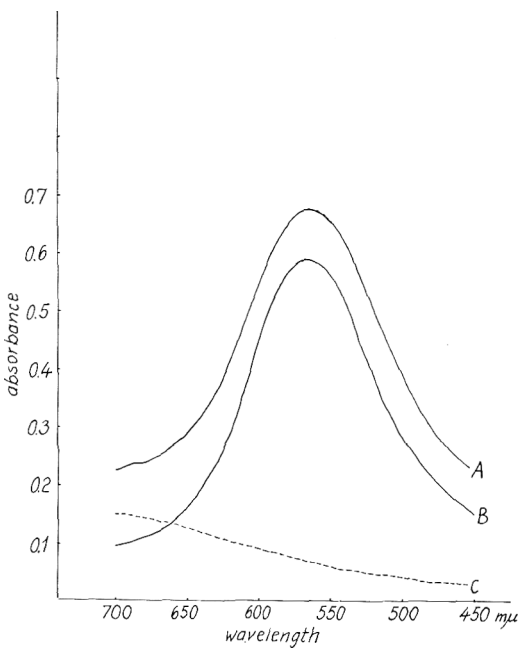

Fig. 1. Absorption spectrum of color from carbazole reaction with 1 ml A) extract of 7 g meat, B) 75 mg glucose in 250 ml H_2O, and C) 12.5 mg IMP in 250 ml H_2O.

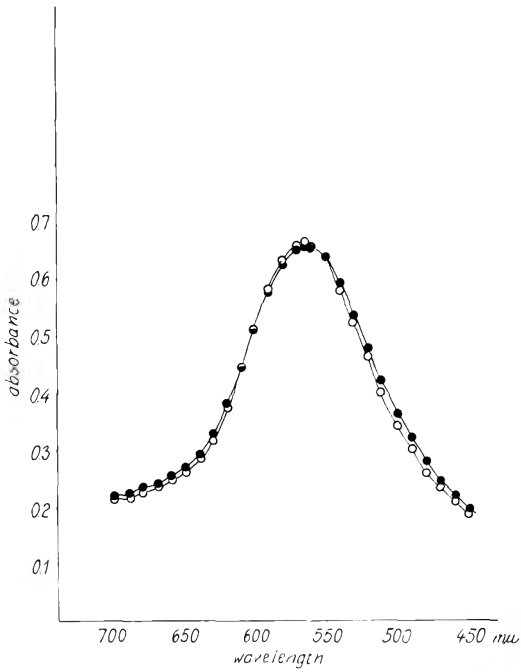

Fig. 2. Absorption spectrum of color from carbazole reaction with 1) extract of 7 g meat –●——●– and 2) 75 mg glucose and 12.5 mg IMP –○——○–.

of 75 mg glucose in 250 ml. We see that it follows the same pattern as curve *A* except at wavelengths above 600 mμ, where it has a steeper fall. The color developed with IMP has, however, an increasing absorption at wavelengths above 600 mμ, as curve *C* shows. When a mixture of 75 mg glucose and 12.5 mg IMP in 250 ml is used, the absorption spectrum matches that of the meat extract, as shown in Fig. 2. When the content of reducing sugars in the sample to be analyzed is known, the absorption spectrum can thus be compared with that of corresponding glucose solutions containing various concentrations of IMP. When the absorption spectrum matches that of the meat extract, the IMP concentration corresponds to the concentration of 5′-ribonucleotides in the meat extract. The method allows approx. 100% recovery of IMP added to meat samples in amounts corresponding to naturally occurring concentrations. Ribose-5-phosphate may interfere with the determination, for it gives the same absorption maximum as the 5′-nucleotides. It occurs in only relatively minor concentrations in meats, however.

Table 1. Content of 5′-ribonucleotides in pork and beef loin muscles.

	mg nucleotide per 100 g meat
Raw pork	180
Roasted pork	165
Raw beef	210
Roasted beef	160

The table shows determinations of 5′-ribonucleotide content in samples of pork and beef muscles. The reproducibility of the method is around 10 mg per 100 g of sample.

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Resistant Bacteria on Antibiotic-Treated Fish

SUMMARY

Fillets of English sole (Parophrys vetulus) dipped in solutions containing 5 μg/ml of chlortetracycline (CTC) or oxytetracycline (OTC) showed a gradual increase in number of antibiotic-resistant organisms under storage at 5°C. There was a strong selection for bacteria able to tolerate up to 5 μg of antibiotic, although organisms showing greater resistance also appeared. There was marked cross-resistance to both antibiotics. Fillets which had been heavily contaminated with resistant bacteria at the time of antibiotic treatment still had a shelf life considerably longer than that of untreated controls. When fillets carried a mixed population of sensitive and resistant bacteria, the latter exhibited no selective growth advantage in the absence of antibiotic.

INTRODUCTION

Tetracycline antibiotics are not widely used at present, so far as we know, to retard the spoilage of fish and poultry. Since they are definitely useful under certain conditions, however, the results reported here may be of interest to food processors. Most of the many publications about the relative merits of different antibiotics for the preservation of a variety of foods have dealt with quantitative changes affecting the bacterial flora of treated food. More seldom (e.g. Ng *et al.*, 1957) has attention been paid to the emergence of antibiotic-resistant bacteria and to the practical significance thereof.

While investigating the effect of tetracycline antibiotics on the bacterial flora of fish fillets we observed and followed the appearance of resistant organisms. Because they were able to grow in the presence of the antibiotic, they would, theoretically, tend to negate the advantages of antibiotic treatment. In view of the poor sanitary conditions often found in fish packing plants, these antibiotic-resistant bacteria could become a potential source of trouble should antibiotics become more widely used in processing. This report deals with the appearance of resistant bacteria on antibiotic-treated fish and with the effects of heavy recontamination by these bacteria on the efficacy of antibiotic treatment.

MATERIALS AND METHODS

Fillets of English sole (*Parophrys vetulus*) cut from fish within 20 hours of catching were obtained from local processors. They were used only when no subjective or objective signs of spoilage could be detected..

Treatment. Fillets were immersed 5 min in a 5% NaCl solution in tap water containing 5 μg of antibiotic per ml. Control fillets were dipped in a 5% salt solution. The temperature of the dipping solutions was approximately 18°C. When desired, various bacteria were added to the dipping baths.

Storage. The treated fillets were wrapped in groups of four in wax paper and stored at 5°C. Samples consisting of at least 2 fillets were taken daily.

Bacterial counts. Bacterial counts were made on juice obtained from the fillets with a mechanical press. With a sterile bent glass rod, 0.01 ml of appropriate dilutions was spread on the surface of Difco Penassay Base agar plates. Colonies were counted after 24 hr of incubation at 25°C. Resistant bacteria were counted in the same manner on agar to which the desired amount of a sterile solution of antibiotic had been added aseptically at 50°C. The concentrations of antibiotic thus obtained in the agar ranged from 1 to 50 μg/ml. These values were checked by bioassay.

Assessment of spoilage. Organoleptic examination was complemented by estimations of volatile reducing substances (VRS) and of trimethylamine (TMA) nitrogen, the latter by the Conway microdiffusion procedure (1947). Both determinations were carried out on press juice from the fillets. The VRS content was determined by a modification of the method originally described by Farber and Ferro (1956); the degree of reduction of the permanganate was estimated colorimetrically by reading the difference in OD at 610 mμ between the unreacted and reacted reagent and obtaining the value in microequivalents of reduction from a standard curve. Details of this method are being described in a separate communication.

Antibiotics and assays. Most of the experiments were done with chlortetracycline (CTC) kindly supplied by the Lederle Laboratories Division of the American Cyanamid Company. Also included in some was oxytetracycline (OTC) obtained

through the courtesy of Chas. Pfizer and Co. From time to time the medium used to estimate the number of resistant bacteria was assayed for antibiotic by the method of Grady and Williams (1953) as modified by Broquist and Kohler (1953).

RESULTS AND DISCUSSION

Rate of appearance of resistant bacteria. Two groups of sole fillets were respectively treated with CTC and OTC and stored at 5°C. The percentage of antibiotic-resistant organisms was estimated daily by making duplicate bacterial counts on plain agar and on antibiotic agar. The number of colonies on the antibiotic agar was expressed as a percentage of the number of those on the antibiotic-free agar. The bacterial resistance levels given are, of necessity, approximate as under our conditions the initial concentration of both CTC and OTC decreased by about 50% after 24 hr at 25°C. The initial amount of antibiotic added was adjusted accordingly and the levels given are average values over the 24–30 hr period of incubation. Bearing the above in mind, the basic level of resistance, unless noted otherwise, was to 1.5 μg/ml of antibiotic.

The initial microflora of untreated fillets was found to include between 0 and 10% of bacteria that could tolerate varying amounts of CTC. On storage, this proportion remained approximately constant or decreased. When the fillets were treated with CTC, however, the proportion of resistant organisms increased rapidly until it reached 100%, after 4 or 5 days. A similar increase was observed when fillets were treated with OTC. Table 1 shows a typical pattern of the appearance of resistant organisms. The resistance of the population on the treated fillets remained at the 100% level until the fish spoiled, on the ninth day.

Table 1. Increase in organisms resistant to CTC in fillets of sole at 5°C.

Treatment	% of total population resistant to 1.5 μg/ml CTC after: 0 days	1 day	3 days	4 days
Dipped in 5% NaCl in tap water	7.2	9.0	4.5	[a]
Dipped in 5% NaCl in tap water + 5 μg CTC/ml	7.2	21.0	75.7	100.0

[a] Spoiled.

Table 2. Development of cross-resistance to CTC and OTC by bacteria on antibiotic-treated fillets at 5°C.

Treatment	% of total organisms resistant after: 2 days	6 days	7 days	9 days
Resistant to CTC				
No antibiotic	2.0	0.93	[a]	[a]
CTC	6.2	100	100	100
OTC	8.1	100	100	100
Resistant to OTC				
No antibiotic	8.7	7.1	[a]	[a]
CTC	16.2	100	100	100
OTC	13.9	100	100	100

[a] Spoiled.

Cross resistance. Groups of fillets were treated with OTC and CTC, and duplicate counts were made on agar containing CTC, OTC, and no antibiotic. The experiment summarized in Table 2 is typical of the series. The results are expressed as a percentage of the total population and show that although the percentage of resistant bacteria in the untreated groups remained approximately constant, the proportion of resistant cells in the treated groups rose sharply. There was cross-resistance between CTC and OTC: the bacteria which grew on fillets containing CTC also became resistant to OTC, and vice versa.

Levels of resistance of the CTC-tolerant bacteria. To determine the levels of resistance of the bacteria growing on CTC-treated fillets, two types of experiments were carried out. In the first, two lots of fresh fillets were used. One was treated with CTC in the usual manner (5 ppm for 5 min); the other was left untreated. The lots were placed at 5°C and stored for 5 days. Samples were taken at the beginning and end of the storage period; platings were

Table 3. Bacterial resistance to CTC in treated and untreated fillets of sole at 5°C.[a]

Group[b]	% of total bacterial population resistant to μg CTC per ml: 1	5	10	25	50
1	5.3	0.75	0.40	0.20	0.095
2	4.8	0.46	0.36	0.07	0.056
3	100	32.5	34.3	10.4	5.9

[a] Each figure represents the average from 6 experiments.

[b] 1, fresh, untreated; 2, untreated, stored 5 days (spoiled); 3, treated with 5 μg/ml CTC for 5 min and stored 5 days (not spoiled).

made on agar containing various concentrations of CTC and on CTC-free medium. Table 3 gives the averages of six determinations. As expected, fewer bacteria resisted the higher than the lower levels of CTC. The proportion of resistant bacteria in untreated fillets did not increase with spoilage; moreover, some organisms that were highly resistant to CTC were present in populations that supposedly had never been exposed to the antibiotic.

In the second type of experiment, the changes in degree of resistance during storage were followed in both treated and untreated fillets. The results (Table 4) are, again, expressed as the percentage of the total population resistant to a given level of CTC. In the untreated fillets the proportion of resistant bacteria either decreased or remained approximately the same. As expected, the absence of antibiotics did not appear to favor the growth of resistant organisms. In the treated groups, the percentage of bacteria resistant to 1 and to 5 μg of CTC per ml rapidly reached 100, although the latter group tended to lag slightly. The proportion of bacteria resistant to the other levels of antibiotic showed no definite trend through the period of storage. The sharp difference between the percentages of bacteria resistant to 5 and to 10 μg of CTC is noteworthy. This, together with the apparent link between resistance to 1 and to 5 μg, can probably be attributed to the actual concentration of CTC at the surface of the fillet, where most of the bacteria were found; although assay of whole fillets treated with 5 μg of CTC showed a concentration of antibiotic between 1 and 1.5 μg/g, the actual concentration at the surface was probably much closer to that of the dipping bath. It is therefore likely that there was a selection for organisms that could tolerate up to 5 μg of CTC, whereas there was no selective environment for organisms possessing higher resistance.

Effect of large numbers of resistant organisms. Could the build-up of a large resistant population, through poor sanitation, reduce the effectiveness of CTC treatment under practical conditions? An experiment was designed to simulate the worst possible conditions in a commercial plant: heavy recontamination of fresh fillets with CTC-resistant bacteria over a long period.

A dipping bath containing 10 μg of CTC per ml (the commercially recommended concentration) was prepared, and resistance was tested on agar containing 10 ppm of antibiotic. The experimental procedure was as follows: 2 lots of fresh fillets of sole were dipped in a 5% NaCl solution, with and without 10 ppm CTC, and stored at 5°C. The progression of spoilage was followed organoleptically, bacteriologically (by total aerobic plate counts), and chemically (by volatile reducing substances and trimethylamine nitrogen) at daily intervals. On the

Table 4. Development at 5°C of CTC-resistant organisms in fillets of sole treated with 5% NaCl in tap water with and without CTC.

Days stored	Total population	% of total population resistant to μg CTC per ml: 1	5	10	25	50
Untreated fillets						
0	1.2×10^5	5.0	2.0	0.1	0.1	0.1
1	1.3×10^5	2.3	2.2	0.13	0.7	0.7
2	8.1×10^5	2.0	0.1	0.12	0.02	0.03
3	1.2×10^7	0.25	0.1	0.02	0.02	0.03
4	1.8×10^7	0.1	0.09	0.01	0.08	0.01
5	5.5×10^7	0.1	0.07	0.08	0.03	0.01
5-μg/ml-CTC-treated fillets						
0	1×10^4	9	3	2	1	1
4	1×10^5	30	17	12	12	13
5	3.1×10^5	80	45	27	9.4	7.1
6	2.6×10^6	100	100	19	18.5	11.2
7	3.5×10^6	100	100	17	23	6.7
9	2×10^8	100	100	17	12.5	4.3

day that each group spoiled, a last plate count was made, and after 24 hr of incubation the whole growth was washed off and resuspended in sterile physiological saline. The turbidity of the suspension was read in a Weston colorimeter and an appropriate amount was added to fresh dipping baths to give a final concentration of approximately 2.0×10^6 cells per ml. A fresh lot of fillets was then subdivided for dipping in one of the following solutions:

Group code	Solution
C	5% NaCl in tap water
CTC	C + 10 μg CTC/ml
BS	Sensitive bacteria in 5% NaCl solution
BS+CTC	BS + 10 μg CTC/ml
BR	Resistant bacteria in 5% NaCl solution
BR+CTC	BR + 10 μg CTC/ml

The six groups were then stored at 5°C. Upon spoilage, bacteria from two of the inoculated groups (BS and BR + CTC) were again collected and reinoculated, as shown in Fig. 1. Thus, a continuous line of reinoculation with resistant bacteria (BR + CTC) was maintained, its direct counterpart being group BS (reinoculation with sensitive bacteria). All other groups served as additional controls. Six reinoculation cycles were carried out in this manner. With the resistance feature as a marker, it was shown that an exchange occurred whereby about half of the fillet flora was replaced by bacteria from the dipping bath. Table 5 shows the effect of these reinoculations on the preservation time of the fillets and on the effectiveness of CTC treatment. The inoculation of sensitive bacteria onto untreated fillets did not speed up spoilage (BS vs. C); neither did the inoculation of CTC-resistant bacteria (BR vs. C). Within the pivotal BR + CTC group there was no definite trend toward a diminishing effectiveness of CTC treatment from the first

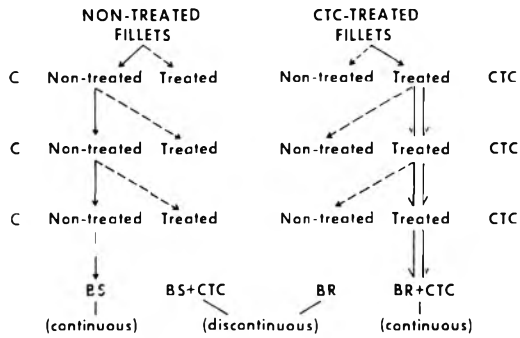

Fig. 1. Schematic representation of the reinoculation of fillets of sole with CTC-sensitive (BS) and CTC-resistant (BR) bacteria.

C: fillets dipped in 5% NaCl; no bacteria added

BS: serial inoculations of lots of fillets with sensitive bacteria from previously spoiled batch

BS+CTC: same as BS, but with CTC dip in addition

BR+CTC: CTC-treated fillets serially inoculated with resistant bacteria from previously spoiled CTC-treated fillets

BR: same as above, but with CTC dips omitted

CTC: CTC-treated fillets used as controls; no bacteria added

Groups BR + CTC and BS represent continuous lines of serial inoculations with resistant and sensitive bacteria (solid arrows). All other groups have no continuity within each series (dotted arrows), and serve as controls for the first two groups.

Table 5. Effect of various treatments on the shelf-life of fillets of sole at 5°C (based on the average of six experimental runs). On the days shown the material was organoleptically judged to be spoiled.

Group code + treatment	Av. days to reach: VRS[a] value of 15	Av. days to reach: TMA[b] value of 1.0
C : 5% NaCl dip	2.6	2.2
CTC : 5% NaCl + 10 μg/ml CTC	6.6	6.0
BS : 5% NaCl + BS[c]	2.7	2.3
BS + CTC : 5% NaCl + 10 μg/ml CTC + BS	6.4	5.9
BR : 5% NaCl + BR[d]	2.9	2.1
BR + CTC : 5% NaCl + 10 μg/ml CTC + BR	4.2	3.3

[a] VRS, volatile reducing substances as microequivalents of reduction per 5 ml of press juice.
[b] TMA, trimethylamine as mg N per 100 ml of press juice.
[c] BS, bacteria sensitive to CTC.
[d] BR, bacteria resistant to CTC.

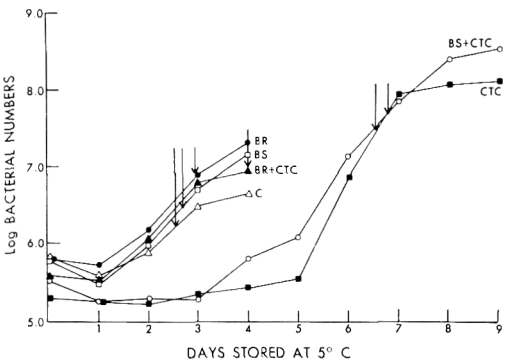

Fig. 2. Patterns of bacterial growth on variously treated fillets of sole. The values on the ordinate represent the logs of bacterial numbers per ml of press juice. The arrows indicate onset of definite spoilage (VRS = 15, TMN = 1.0).

Group Code	Treatment
C	5 min in 5% NaCl
CTC	5 min in 5% NaCl + 10 μg/ml CTC
BS	5 min in 5% NaCl + CTC-sensitive bacteria
BS+CTC	5 min in 5% NaCl + 10μg/ml CTC + CTC-sensitive bacteria
BR	5 min in 5% NaCl + CTC-resistant bacteria
BR+CTC	5 min in 5% NaCl + 10 μg/ml CTC + CTC-resistant bacteria

to the last run. This group had an average storage life midway between those of the BS and BS + CTC groups.

Fig. 2 shows the average bacterial growth curves for the various groups during the six runs. As indicated by the arrows, the times of onset of spoilage for groups C, BS, and BR were similar. Groups BR + CTC took an average of 24 hr more to spoil, whereas groups CTC and BS + CTC lasted considerably longer.

Table 6 shows the proportion of resistant bacteria in the various groups at the time of spoilage. Each value represents the average of six runs. Resistant bacteria (group BR) persisted through the onset of spoilage in about their original proportion. In the case of bacteria newly exposed to 10 μg CTC per ml, 100% resistance was not attained within the given period (about 6 days). This is in contrast to the regular appearance of a fully resistant population when 5 μg of the antibiotic per ml were used in previous experiments.

Although the bacterial population of the BR + CTC fillets was overwhelmingly resistant to 10 μg CTC (Table 6), this concentration of antibiotic still produced a significant retardation of spoilage in this group (BR + CTC vs BR, Table 6). Therefore, although the CTC-resistant bacteria can grow and multiply in the presence of the antibiotic, their metabolic activities, as measured by spoilage production, appear to be depressed. Similar observations had previously been made. Vaughn *et al.* (1957) noted that despite the predominance of a strongly resistant bacterial flora in poultry plants using CTC, the antibiotic treatment still was of some benefit. Tomiyama (1962) found that highly CTC-resistant bacteria were inhibited by 10 μg/ml of the antibiotic. These observations are interesting especially since Jay *et al.* (1958) noted that this phenomenon could not be duplicated on cooked meat. Our experimental results confirm the above findings and show that while unsanitary conditions, as expected, greatly reduce the effectiveness of antibiotic treat-

Table 6. Number of bacteria resistant to CTC in various experimental groups at the onset of definite spoilage (VRS = 15, TMN = 1). Results expressed as viable cells per ml of press juice.

Group code + treatment	Number of bacteria		
	Total	Resistant	% resistant
C : 5% NaCl dip	2.2×10^6	3.3×10^4	1.5
CTC : 5% NaCl + 10 μg/ml CTC	1.9×10^7	7.2×10^6	38.0
BS : 5% NaCl + BS[a]	3.8×10^6	7.6×10^4	2.0
BS + CTC : 5% NaCl + 10 μg/ml CTC + BS	7.3×10^6	3.0×10^5	41.0
BR : 5% NaCl + BR[b]	1.8×10^7	8.6×10^6	48.0
BR + CTC : 5% NaCl + 10 μg/ml CTC + BR	1.1×10^7	1.1×10^7	100

[a] BS, bacteria sensitive to 10 ppm CTC.
[b] BR, bacteria resistant to 10 ppm CTC.

ment, there is still some benefit even under the worst conditions.

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Bacterial Flora Associated with Rapid-Processed Ham

SUMMARY

Recent work at Oklahoma State University has shown that ham can be satisfactorily processed in less than 15 hr from time of slaughter by accelerated processing techniques. Since these techniques by-pass the conventional 24-hr chilling treatment, the number and types of bacteria present are of concern.

A total of 40 (20 right and 20 left) hams were alternately assigned to either rapid or conventional processing methods following separation from their respective sides. Total bacterial counts were determined from representative samples of both fresh and cured muscle tissue aseptically removed from each ham at specified sampling points. The selective effects of processing temperature, pH, and processing techniques were evaluated in terms of bacterial flora common to the rapid and conventionally processed hams. Aerobic bacteria and pH measurements were statistically analyzed. Anaerobic spore populations were estimated by the most probable number method. The low incidence of anaerobic spores quantitated (4 of 38) limited the analysis of variance to evaluating differences in the total bacterial population. A comparison of the final mean pH values indicates that rapid processing does not appreciably affect the ultimate pH value. Because of the curing ingredients, pH, smoking, cooking, and the rapidity of processing, results indicated a major reduction in associative vegetative bacteria during the various stages of processing. Results reveal no significant bacteriological problems in the "new" concept on complete processing prior to initial chilling.

INTRODUCTION

The widespread availability of refrigeration for the merchandising and domestic handling of cured meat products has effected a considerable change in ham processing. With the evolutionary advances made in curing and preservative techniques, the modern cured boneless, smoked, precooked hams are enjoying wide acclaim and acceptance by the convenience-minded consumer. In contrast with earlier methods, today's commercially processed hams are subjected to a relatively light cure of short duration. Weiner *et al.* (1964) studied the effect on porcine muscle and fat characteristics when processed prior to completion of rigor mortis. Researchers at Oklahoma (Henrickson *et al.*, 1965) have shown that hams can be satisfactorily processed in less than 15 hr from time of slaughter by an accelerated, hot-processing technique. Since this technique by-passes the conventional 24-hr chilling treatment, several bacteriological considerations of the product become apparent. Of primary concern are the number and type of bacteria present and the environmental influences inherent to rapid processing. The quantitative results and methodology of a bacteriological evaluation of hot-processed and conventionally cured, boneless, fully cooked hams are reported. In addition, those factors believed to influence the relative loads and ultimate disposition of certain bacteria in the final product are discussed.

METHODS

Twenty market-weight pigs were obtained from the Experiment Station herds and slaughtered by procedures established at the Oklahoma Station Meat Science Laboratory. Following conventional evisceration, the carcasses were split into right and left halves. This operation facilitated the paired-ham experimental design as each half was alternately assigned to either hot or cold processing methods. The cold processing method implies a 24-hr initial chilling period at 1.7°C with the side still intact and hanging from the rail. Hot processing implies fabrication prior to initial chilling of the hams. Hams assigned to the cold method were designated as the control simulating conventional handling. Cutting procedures followed those currently employed by the industry for physically separating the hams from their respective treatment sides (Fig. 1). All hams were stitch-pumped with a 60° salinometer brine composed of: salt, sucrose, dextrose, nitrate, nitrite, erythorbate, bicarbonate, and polyphosphates. The corresponding brine concentration was 15.8% NaCl. Required amounts of brine were prepared fresh daily from the above commercially available curing ingredients with cold tap water in a manner consistent with approved industry standards and with current meat inspection regulations of the USDA. Spot analyses of the brine revealed less than 10 organisms/g. After the hams were skinned and the outside fat removed, they were weighed, sampled, muscle pH deter-

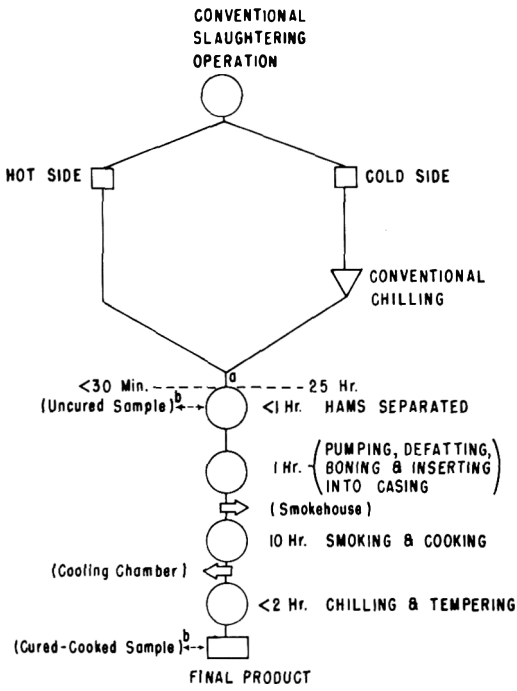

Fig. 1. Product flow diagram.

mined and by means of a percentage scale injected to 110% of green weight. They were immediately boned, defatted, stuffed into fibrous casings and placed into individual ham molds and placed in the smokehouse. After a one hour initial drying period, smoking was commenced and continued for eight hours at 55°C. The smoke was generated from hardwood sawdust that had been dampened prior to smoldering. After smoking, the hams were cooked in-place to an internal temperature of 60°C for a 2-hr period. The smoked and fully cooked hams were removed from the smokehouse and rapidly cooled. The air movement in the cooling chamber was turbulent and was determined by Smith *et al.* (1965), in concurrent heat transfer studies of the hot-processing concept, to have an effective approach velocity of 1200 fpm. Temperatures were obtained by means of thermocouple probes that indicated the temperature at the center and at ½ and 1 inch from the center of the hams. The temperature of the cooling chamber air was varied from −23 to −62°C. A 10-point potentiometer was utilized to record the changing internal temperature of each ham. Cooling time was adjusted so that when the internal temperature reached 10°C, the hams were removed from the cooling chamber to a chill storage compartment maintained at 1.7°C. When the hams had adjusted to an average internal temperature of 7°C, the cured-cooked samples were aseptically removed. Residual moisture of the adjacent tissue was determined (AOAC, 1960), and the means and standard deviations calculated. Means and standard deviations of pH measurements which were taken immediately after each sampling were also calculated.

All bacteriological samples were aseptically withdrawn by a mechanical boring device powered by a portable ¼-hp electric drill which was operated at reduced speed. Each stainless steel bore was physically cleaned with detergent and sterilized.

Bacteriological samples of the deep muscle tissue were removed from the hams immediately after separation and from the final product (Fig. 1). Prior to coring a sample, the exposed muscle tissue was heat-seared at the sampling locations (illustrated in Fig. 2) with an electric searing iron. A pH determination was made immediately following each sampling by placing a standardized (pH 7.0) probe electrode from a Beckman meter, model 72, into the deep muscle tissue. Fresh, uncured samples were given priority for immediate bacteriological analysis on days on which cured-cooked samples were taken. The samples were placed under immediate refrigeration (15°C) until all fresh (uncured) samples had been prepared for bacteriological plating. The average elapsed time for uncured samples from searing until all aerobic

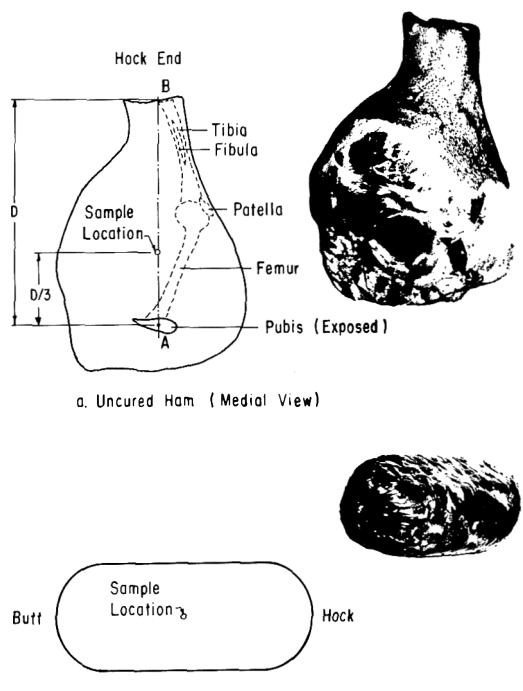

Fig. 2. Diagram showing sampling locations.

platings were completed was approximately 30 min. All uncured samples were cored with the posterior portion of the shank (hock) pointing away from the sampler and with exposed pubic bone oriented in full view. From the mid-point of the exposed pubic bone (diagram 2a, point *A*) the edge of an 18-inch rule was aligned in such a manner to form a straight-line distance (D) to a mid-point (point B) on the hock. One-third the distance from point *A* to point *B* (D/3) became the common vertical coring location for all uncured samples. Coring at this location allowed the bore to be inserted to the desired depth without difficulty. All cured-cooked samples were cored horizontal to the flat face at the midpoint of the longest side of the boneless ham (Fig. 2,b). Each core was aseptically removed from the stainless steel boring device by plunging the core into a sterile screw-top glass jar.

Ham tissue homogenates were prepared by placing an 11-g (inner) portion of the cored sample into a sterile glass Waring blender jar containing 99 ml of phosphate-buffered saline diluent (Sulzbacher, 1953; Lewis and Angelotti, 1964). All samples were blended for 2½ min at the low speed setting (approx. 12,000 rpm) on a standard laboratory model, two-speed Waring blender. Prior to pipetting the required sample aliquots for platings, each dilution blank containing the homogenate with glass beads (6) was shaken vigorously for 2 min.

Each sample was analyzed for total aerobic bacteria by standard quadruplicate platings at dilutions of 10^{-2} to 10^{-4} using Bacto tryptone glucose beef extract agar (TGEA). Coliform bacteria (*Escherichia* and *Aerobacter* species) were plated with Bacto violet red bile agar (VRBA) at dilutions of 10^{-1} to 10^{-4} and incubated at 37°C for 24 hr.

Anaerobic bacteria were quantitated using the most-probable-number (MPN) technique as outlined by Cochran (1950) for estimating total bacterial densities. The procedures generally followed those employed by Steinkraus and Ayres (1964) with the exception of choice of media and dilution ratios. Enumeration of the clostridia and heat resistant facultative anaerobes was based upon survival of a pasteurization treatment given the remaining portion of each homogenized sample from which total aerobe and coliform counts had been plated. A pasteurization treatment, as recommended by Greenberg (1965), of 60°C for 55 min was utilized for both the fresh and cured sample portions. After pasteurization, MPN tubes containing Bacto fluid thioglycollate medium modified with a sulfide detecting agent (0.02% ferrous ammonium sulfate) were seeded in 5-tube replicates using dilutions corresponding to volumes of 10, 1.0, and 0.1 ml of the original 1:10 meat-saline homogenate. All tubed media were steamed for 15 min and rapidly cooled prior to seeding in order to expel residual oxygen. The tubes were incubated 5 days at 37°C. All tubes showing visual indication of growth were subcultured for the qualitative phase of the survey. Only those tubes showing a black precipitate (FeS) and producing a putrefactive odor were recorded. The combination of positive tubes was ascertained for each sample, and the most probable number of anaerobic spores estimated by referring to MPN tables from Am. Public Health Assoc. (1960). In addition to the MPN counts, duplicate pour plates were made with Bacto-SPS (sulfite-polymixin-sulfadiazine) agar as an additional check for *Clostridium perfringens*. The procedure was reported by Angelotti *et al.* (1962). Anaerobiosis for the Bacto-SPS plates was obtained using an anaerobic incubator under an atmosphere of 90% nitrogen and 10% carbon dioxide. An oxidation-reduction indicator agar was utilized to assure anaerobiosis throughout the culturing period. All anaerobic plates were checked after 24 hr of incubation at 37°C. Negative plates were reincubated for 24 hr prior to their disposal.

Total counts were made on TGEA, VRBA, and SPSA plates without regard to the statistical limitation commonly imposed by standard plate counts. Analysis of variance was used to evaluate differences in means between treatments.

RESULTS AND DISCUSSION

Data presented are based on 19 pairs of hams of a total of 20 pairs processed. Since one pair had extremely high counts probably attributable to a bacteremia, counts obtained from these hams were removed from the statistical analyses. Total aerobic counts are presented in Table 1. Survey data in Table 2 show that there were no hot-processed cured ham samples exceeding 300 colonies per plate, whereas 2 control hams yielded samples with high counts (1,350 and 1,650 bacteria per g) in the ready-to-eat tissue. Categorically, 68% (13) of the hot-processed hams gave samples in which either no colonies or insignificant low numbers appeared (0 to 9 per plate). In the conventionally processed hams only 53% (10) of the samples were in this category. It is interesting to note that otherwise the hot and conventionally processed hams gave essentially the same data at the mesophilic range. Incubation of TGEA plates at psychrophilic temperatures yielded erratic low counts in all but 2 samples, one of which resulted in less than one psychrophile per gram of tissue. The other sample showed an unusually high initial load which was too numerous to count (TNTC) in the uncured samples from the same hot and conventional treatment pair. Analysis of variance for the two treatments with 19 pairs of hams is

Table 1. Total aerobic bacterial colonies and AOV summary.

	Sample treatment			
	Hot		Cold	
	Uncured	Cured	Uncured	Cured
Colonies/gram [a] (N = 19)	203.05	15.37	217.47	107.79
Std. dev.	502.04	27.41	337.04	303.54

	df	SS	MS	F ratio
Animal	18	3,335,467.03	185,303.72	2.03[b]
Treatment	3	503,154.26		
Hot vs. cold	(1)	54,222.37	54,222.37	<1
Uncured vs. cured-cooked	(1)	420,032.89	420,032.89	4.60[b]
Interaction	(1)	28,849.00	28,899.00	<1
Error	54	4,926,974.24	91,240.26	
Total	75	8,765,595.53		

[a] Plated on TGEA and incubated at 37°C.
[b] $P < .05$.

shown in Table 1. The summary of variance indicates significant ($P < .05$) processing reduction in total aerobic counts by uncured versus cured-cooked treatment. Thus, treatment hams injected with curing brine, smoked, cooked, and then rapidly cooled had fewer aerobic bacteria per gram of ready-to-eat tissue than the conventionally processed hams due to significantly greater reduction of bacteria during the various stages of processing. Significant animal source of variation indicates that not all tissue responded in the same manner to the two treatments. This would be reasonable since the experimental hams, while obtained from animals of uniform breeding, feeding and weight range, were slaughtered over a four-week schedule. All animals were exsanguinated in the same abattoir by the same equipment and personnel, but responded individually at death to various stresses as well as to uncontrollable fluctuations in temperature and humidity. The high standard deviations (Table 1) for the comparative flora (uncured hot-uncured cold) are attributed to variation in the initial bacterial load of individual fresh hams as well as the differing growth environments common to the two processing treatments. Also apparent are the enumeration problems inherent when sampling low bacterial populations.

A comparison of final mean pH and residual moisture values (Table 3) indicates that the ultimate pH was not affected by the hot processing treatment. The initial pH values were understandably different due to time-temperature effects on anaerobic muscle glycolysis in the two treatments. In pilot studies of hot processing techniques, unpublished data (Henrickson *et al.*, 1965) from 20 pork sides weighing approximately 34 Kg each showed that the internal temperature of the whole hams ranged from 39 to 43°C (30 min post-slaughter) without conventional chilling. The internal temperature of hams undergoing conventional processing ranged from 7 to 9°C at the time of physical separation from the side. Since treatment hams following the hot processing flow (Fig. 1) were separated and injected within 1 hr of slaughter, muscle temperature and mean pH (6.46 ± 0.24) were relatively high. Consequently, the greater reduction in bacterial flora associated with the hot treatment could be partially explained in terms of greater availability of nitrous acid and other curing ingredients due to more uniform per-

Table 2. Colony incidence of aerobic bacteria in hot- and cold-processed hams.

	Types of ham					
		Hot			Cold	
Colonies/sample		Uncured	Cured		Uncured	Cured
Greater than 300	(3)	[a]2	0	(6)	[a]5	2
Greater than 10 <300		11	6		8	7
< 10		6	9		5	7
Undetected		0	4		1	3
Total		19	19		19	19

[a] One paired set of hams removed from survey.

Table 3. Residual moisture and pH determinations.[a]

	Sample treatment			
	Hot		Cold	
	Uncured	Cured	Uncured	Cured
Moisture %	.....	65.75	.....	66.49
Std. dev.	.....	3.01	.....	3.77
pH	6.46	5.83	5.70	5.84
Std. dev.	0.24	0.17	0.16	0.20

[a] Sample size, N = 19.

meation of the curing ingredients in the fresh (hot) tissue (Mullins *et al.*, 1958; Shank *et al.*, 1962). In addition, the initiation of processing (lethal factors) relative to its effect on the normal logarithmic development of bacteria is apparently more critical. According to Partmann (1963), the breakdown of adenosinetriphosphate (ATP) and its resynthesis by the glycolytic cycle will normally take place in the muscle tissue until the depletion of the glycogen reserve or a pH value of 5.4 is reached. Briskey *et al.* (1962) showed that at increased temperatures (43°, compared to 37°) both the onset phase and total time for completion of rigor were markedly reduced. In addition, the early work of Callow (1947) relative to the effects of increased lactic acid concentrations on porcine tissue microstructure may also have bacteriological significance. Shank *et al.* (1962) demonstrated the antibacterial characteristics of nitrous acid on both vegetative bacterial cells and spores. The significant treatment effects on bacteria shown in Table 1 for cured versus uncured indicated that the combined factors associated with hot processing (i.e., pre-rigor injection, higher muscle temperature, and rapidity of processing) were effective in reducing the bacterial load of the final product. No significant differences in the percent residual moisture were found (Table 3).

Quantitation of coliform bacteria in both treatments revealed an extremely low incidence. Uncured samples from hot processed hams had an average of 4.4 confirmed coliforms per gram of the original sample. However, the presence of these aerobic and facultative anaerobic bacilli in uncured (fresh) hot processed hams compared with the conventionally processed controls is reasonable since they had longer exposure to elevated temperatures and muscle pH had not reached an inhibitory value when sampled. This level was subsequently reduced to less than one coliform per gram by processing methods and thus no particular significance is attached to this group of bacteria.

Clostridia were isolated from the tissue of four different ham samples of the total of 38 surveyed (4 of 38, or 10.5%). Quantitations pertaining to the low incidence of anaerobic sporeforming bacteria detected in the two treatments agreed with the findings of Steinkraus and Ayres (1964) and Hall and Angelotti (1965). The comparative anaerobic spore counts are shown in Table 4. All anaerobic SPS plates were negative for *Clostridium perfringens* in both treatments; however, since four samples did yield positive MPN counts, a general level of contamination by anaerobic organisms of less than 10 per gram is indicated. Burke *et al.* (1950), Strong *et al.* (1963), and Hall and Angelotti (1965) have stated that frequently the level of contamination by anaerobic spores is too low to be detected by means other than enrichment. The presence of *Clostridium* spores in low numbers in the cured-cooked tissue when they were not detected in the same fresh (uncured) tissue is consistent with the view presented by Frank (1963) for sporeforming bacilli. Frank (1963) pointed out that in addition to the destructive effects normally associated with thermal processing, heat above the growth range of some spore-bearing bacilli can stimulate or "activate" germination. Since the normal exponential development of vegetative cells would be exposed to the additive lethal effects of pH, curing ingredients, smoking, and cooking in the hot treatment, it appears that this treatment would be equally effective in reducing in-processing contamination. The low numbers of *Clostridium* spores in the cured-cooked samples probably represent those spores that did not germinate during processing (Riemann, 1963).

Preliminary findings with respect to terminal cooling and tempering (Fig. 1), indicate that of the two temperatures utilized to reduce the internal heat (68°C) as rapidly as possible to the conventional storage or holding temperature (4°C), the −62°C was most efficient with regard to time (1.9 hr, compared to 3.6 hr). No apparent quantitative bacteriologic advantage was revealed at the two temperature ranges studied. However, the employment of the shorter time would appear more desirable from the standpoint of precluding potential food poisoning and/or spoilage problems. Also, the shorter time would facilitate the production line flow currently being employed in other modern food processing operations.

Table 4. Incidence of clostridia in rapid- and conventionally processed ham.

Type	MPN series 10 1.0 0.1	Tabular MPN value[a]	Anaer-obes/g
Uncured (hot)	1 0 0	2	<1
Uncured (cold)	1 0 0	2	<1
Cured (hot)	2 0 0	4.5	4
Cured (cold)	1 0 0	2	<1

[a] Adapted from Am. Publ. Health Assoc. (1960).

CONCLUSIONS

The bacterial populations studied in the two treatments involving 38 boneless, cured and fully cooked hams revealed no significant bacteriological problems in the "new" concept of eliminating the initial chill treatment. A comparison of the final mean pH values and residual moisture indicates that rapid processing does not appreciably affect the ultimate pH of the product. Both fresh and cooked tissues were sampled on a total count basis in the survey. The incidence of aerobic bacteria in rapid-processed ham is comparable to that in the conventional processed product. However, greater reductions in total bacterial numbers were obtained with rapid processing techniques. From a bacterial standpoint, the combined effects of pH, curing ingredients, smoking, cooking, and the rapidity of handling facilitate the exposure of associative bacteria to maximum lethal factors, thus effecting a greater reduction in the bacterial load of the final product. The public health aspects as well as the economic advantages of rapid processing are self-evident.

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Composition of Raw and Roasted Lamb and Mutton
I. Physical and Proximate Composition

SUMMARY

Sixty cuts each of raw and roasted rib-loin and of raw and roasted leg of lamb and mutton, obtained from 118 carcasses, were separated into their physical components. Proximate composition was determined on the resulting separable lean, separable fat, and drippings. Correlation coefficients between weight of cut and each physical and proximate component were determined, and the effects of weight of cut and of roasting on the partitioning of nutrients among the separated portions were examined.

With increasing weight of cut, percentage of separable fat and of drippings increased, and percentage of separable lean and of evaporation loss decreased. For rib-loins, as weight of cut increased, the proportion of bone and waste, and the percentages of moisture, protein, and ash in all separable portions decreased, but the percentage of lipids increased. Relations were similar in leg cuts, except that percentage of bone and waste, percentage of protein in raw or roasted separable lean, and moisture content of drippings did not change significantly.

Separable lean, raw or roasted, contained about 90% of the protein and moisture of the entire cut, but only about a quarter of the total lipids. Lean from raw cuts contained about 90% of the ash of the total cut, but lean from cooked cuts contained only about two-thirds of the ash of the entire cut. As weight of cut increased, the amounts of proximate components in the lean decreased in proportion to those amounts in the total cut.

INTRODUCTION

Lamb is a muscle meat generally available to consumers, yet nutritive composition data for this meat are very sparse. In particular, separate composition data on the lean and fat portions of lamb cuts are needed in order to evaluate more accurately the potential contributions of this important meat to the nutrient intake of the consumer. Such data have been obtained for raw and cooked beef (Toepfer *et al.*, 1955) and for a few cooked cuts of beef, pork, lamb, and veal, purchased as carcasses on the wholesale market and cut and trimmed in the laboratory according to common retail practice (Leverton and Odell, 1958). Existing information on nutrient content of the principal kinds of meat, including lamb, has been summarized for raw carcasses and raw or cooked separable portions or retail cuts of different grades (Watt and Merill, 1963).

Data on the proximate composition of lamb carcasses and cuts have been reported as parts of studies aimed at finding a portion of the carcass which could be considered representative of the whole, or a carcass measurement which could predict composition with reasonable accuracy (Barton and Kirton, 1958a; Hankins, 1947; Jordan *et al.*, 1964; Kirton *et al.*, 1962; Kirton and Barton, 1962; Shorland *et al.*, 1947). Composition values for cooked lamb have also been reported (Barton and Kirton, 1958b; Leverton and Odell, 1958; Schuck *et al.*, 1963; Iyengar *et al.*, 1965). Kirton and Barton (1962) reported that carcass weight of wether lambs was significantly related to the chemical composition of lamb carcasses, and that protein and water were more highly correlated with carcass weight than was fat. However, little information is available concerning possible relationships between weight of cut and its physical or proximate composition (Hammond, 1932). Neither have any appreciable data been reported in which composition was determined for comparable raw and cooked meat from paired cuts from the same animal. This paper reports data on physical and proximate composition of raw and cooked lamb and mutton cuts (hereinafter referred to as lamb), as affected by weight of cut, the measure usually used by the consumer in selecting meat for purchase, and by oven-roasting. A companion paper reports data on amino acids (Blum *et al.*, 1966). In addition, palatability evaluations and selected quality measures on other cuts from these animals

have been published (Batcher *et al.*, 1962). A preliminary report of part of the findings included in these papers was made by Weir (1962) to the National Wool Growers Association.

EXPERIMENTAL

Description of carcasses and cuts. For this research, 118 lamb carcasses were obtained from three sources—54 from the Agricultural Research Center flock at Beltsville, Maryland; 16 from the Mississippi Agricultural Experiment Station flock; and 48 from the wholesale markets of New York and Chicago. The Beltsville animals were from a long-term experiment of crossbreeding of Hampshire, Shropshire, Southdown, and Merino breeds. Half of the animals from Mississippi were Mississippi-bred Dorsets and the remaining eight animals were crossbreeds from Western range ewes and mutton-type rams. Age of the animals at slaughter ranged from 3 to 80 months. Of the 98 carcasses given maturity ratings, 9 (2 Beltsville and 7 Mississippi) were classified yearling, and the remainder, including all market carcasses, were rated lamb. The remaining 20 Beltsville animals not rated for maturity fell into the following age groups: 6–8 months, 4 animals; 11–14 months, 8 animals; 19–20 months, 4 animals; and 80 months, 4 animals. Animals were divided nearly evenly between ewes and wethers. The dressed carcasses weighed 22.5–84.5 lb and varied widely in visible fatness. Carcasses were chilled and cut at Beltsville by procedures typical of the wholesale market, rather than anatomical dissections made from the carcass. Further detail on carcasses from the Beltsville and market animals has been reported previously (Batcher *et al.*, 1962).

Selected for intensive study from each carcass were the rib-loin, consisting of the loin plus the 10 adjoining ribs, and the leg. These cuts compose about 45% of the carcass weight (USDA, 1957), are considered fairly representative of the edible portion of the carcass, and afforded sufficient material for the many determinations scheduled. A total of 240 cuts, half raw and half cooked by roasting, were analyzed.

Paired cuts of legs or rib-loins were available for half the carcasses and could be used to study changes and retentions in nutrient composition resulting from roasting. For the other carcasses, cuts were available from either the left or the right side, but not both. Cuts from these carcasses which were not analyzed for nutrient content were used in palatability and quality evaluations (Batcher *et al.*, 1962).

Preparation and physical separation of cuts. Each cut was weighed, packed in a plastic freezer bag, overwrapped in freezer paper after evacuation of air from the bag, and stored at −40°C until time of separation. Median time of freezer storage was 54 days. Cuts to be separated raw were thawed in the refrigerator (38–40°F) for approximately 24 hr. Cuts to be cooked were thawed at room temperature for 6–8 hr, then placed in the refrigerator overnight. Cuts were unwrapped at the end of the thawing period. Thaw juices were negligible in nearly all cases, and were disregarded.

Thawed cuts were placed fat side up on stainless-steel racks in weighed aluminum roasting pans and roasted in household-type ovens at 325°F. The meat was cooked to an internal temperature of 180°F (well done). The hot cooked roasts were covered with plastic film to minimize moisture loss, cooled, and refrigerated overnight prior to physical separation.

All cuts, raw and cooked, were weighed and physically separated while cold into lean, fat, and waste including bone. Stainless-steel and enamel equipment was used in making the separation, to minimize mineral contamination. Each separable portion was weighed, and percentage yields were calculated.

Raw lean or fat was ground to pass through a plate having openings of 3/16 inch, and then blended in an electric blender to the consistency of a fine slurry. Cooked lean, which was drier than raw lean, was ground three times before blending. Separable fat of cooked cuts was soft, and needed no preliminary grinding before blending. Subsamples of lean or fat were wrapped in aluminum foil or plastic film and stored in sealed cans at −40°C until analyzed.

Drippings were transferred quantitatively to weighed enameled cans, using hot ion-exchange-treated distilled water to aid in the transfer. The sealed cans were stored at −40°C until analyzed. Cooking pans were weighed before and after quantitative transfer of the drippings, and the difference in weight was recorded as drippings weight. Evaporation loss was defined as the difference between weight of cut before roasting and weight of the cold cooked cut plus drippings.

Proximate composition. Proximate composition of separable lean was determined by methods of the AOAC (1960), modified as previously described (Hopkins *et al.*, 1961). Briefly, these methods were as follows: moisture by vacuum oven at 50°C; lipids (ether extract) by overnight Soxhlet extraction of residues from the moisture determination, using petroleum ether as solvent; total nitrogen by micro-kjeldahl; and ash by heating to constant weight in a muffle furnace at 600°C. Moisture in separable fat was determined by drying in a forced-air oven at 95°C for 5 hr. Weighed samples of fat for nitrogen determinations were tightly

wrapped in cigarette paper and defatted by washing with 3:1 ethanol-ether before digestion. Test checks revealed no measurable loss of nitrogen resulting from the wash. Otherwise, analytical procedures were the same as for separable lean.

The complete drippings sample from each roast was dried to constant weight. This weight was subtracted from the original weight of drippings, before addition of transfer water, and the difference was recorded as moisture. The entire dried residue was extracted in a large Soxhlet apparatus with petroleum ether. Total nitrogen and ash were determined on the dry fat-free residues.

Calculations and analysis of data. Nitrogen data were multiplied by the factor 6.25 to obtain protein values. Much of the nitrogen in the drippings of these lamb cuts is undoubtedly present as nonprotein nitrogen although protein-related, as has been established for beef (Ginger *et al.*, 1954; Grindley and Mojonnier, 1904). Therefore, values reported here for protein in the drippings should be considered a convenient way of expressing the data rather than a close estimate of actual protein content. The composition of whole cuts was calculated from analytical data on the composition and weights of separated portions. Simple correlation coefficients were calculated relating weight of cut to each proximate component of the lean, fat, and drippings, and of the whole cuts.

RESULTS AND DISCUSSION

Physical composition. Correlation coefficients between weight of untrimmed cut and percentage of each separable portion and, for roasted cuts, of drippings and of evaporation loss show that, as the weight of cut increased, the percentage of separable fat also increased, and the percentage of separable lean decreased (Table 1). With increasing weight of cut, the percentage of bone and waste decreased in rib-loin cuts, raw or roasted, but did not change significantly in leg cuts. In nearly all instances, of course, actual weights of separable lean and of bone and waste were greater in heavy than in light-weight cuts. However, the increase in weight of separable fat was so much greater than that of lean or bone as to make the change for the latter portions appear negative or unchanged when expressed as a

Table 1. Physical composition of raw and roasted lamb cuts: relation of weight of cut to percentage of separable portions and cooking losses.

Cut and separable portion	Weight of cut[a] (g)	Separable portion[a] (%)	r[b]
Rib-loins			
Raw	2596 ± 901		
Separable lean		44.0 ± 7.4	−0.79**
Separable fat		28.8 ± 13.2	0.78**
Bone and waste		25.3 ± 6.3	−0.65**
Roasted	1962 ± 706		
Separable lean		44.6 ± 7.1	−0.84**
Separable fat		27.7 ± 11.5	0.77**
Bone and waste		26.0 ± 5.6	−0.47**
Drippings	2542[c] ± 949	6.8 ± 4.1	0.79**
Evaporation	2542 ± 949	15.0 ± 3.0	−0.64**
Legs			
Raw	2252 ± 654		
Separable lean		63.8 ± 4.1	−0.55**
Separable fat		12.8 ± 6.0	0.50**
Bone and waste		21.6 ± 3.6	−0.19
Roasted	1782 ± 450		
Separable lean		62.3 ± 2.9	−0.35**
Separable fat		11.6 ± 4.3	0.32*
Bone and waste	1786[d] ± 453	25.0 ± 3.6	−0.01
Drippings	2584[c] ± 676	8.8 ± 2.9	0.67**
Evaporation	2584 ± 676	21.9 ± 2.8	−0.98**

[a] Mean and standard deviation for 60 cuts.
[b] Correlation coefficient; *, ** respectively significant at P = 0.05, and 0.01.
[c] Weight of cut before roasting.
[d] Values based on 59 cuts.

percentage of the cut weight. These increases in proportion of fat were more pronounced in the rib-loin cuts than in the leg cuts, as indicated by the higher mean values and correlation coefficients for rib-loin separable fat (Table 1).

For roasted cuts, percentage of drippings increased and percentage of evaporation losses decreased as cut weight increased. The correlation coefficient between weight of cut and percentage evaporation loss was nearly perfect (−0.98) for leg cuts. Evaporation losses are usually considered to be dependent on cooking time, which in turn partly depends on weight of cut if the cut is being cooked to a definite internal temperature or degree of doneness. The average cooking time for the leg roasts was 195 min, or 35.1 min per lb. Mean evaporation loss for leg roasts, which had an average weight of 2584 g before cooking, was 561 g or 21.9%. Rib-loin roasts, although on the average weighing nearly the same as leg roasts before cooking, had a greater surface area than did the leg cuts. They averaged only 125 min to cook, or 23.8 min per lb, and evaporation losses, which averaged 375 g or 15.4%, were considerably smaller than losses for leg cuts.

Proximate composition. As weight of untrimmed cut increased, the percentages of moisture, protein, and ash decreased for all separable portions and the whole cuts, and the percentage of lipids increased (Table 2). These relationships were significant in all cases for rib-loin cuts, and in all but three instances for leg cuts. Only for protein in the separable lean of raw and cooked leg cuts and moisture in drippings from cooked leg cuts were no significant relationships found between nutrient content and weight of cut. These findings are similar to findings of Clarke and McMeekan (1952) on caloric content and percentage of protein in lamb carcasses and cuts as related to weight within a quality grade, and also to findings of Barnicoat and Shorland (1952) on fat content of carcasses and of dissected muscles as related to maturity of the animal.

Partitioning of nutrients among separated portions. The content, in g, of each proximate component in each separable portion was calculated and expressed as a percentage of the total component in the whole cut. These percentages were then correlated with the weight of cut. Mean values and correlation coefficients are given in Table 3. These data show that separable lean, raw or roasted, contained about 90% of the protein and moisture contents of the entire cut, but only about a quarter of the total lipid contents. Separable lean from raw cuts contained about 90% of the ash of the total cut, but lean from cooked cuts contained only about two-thirds of the ash content of the entire cut.

For raw cuts, the proportion of the total nutrient that was found in the separable fat increased with increasing weight of cut, and conversely, that in the separable lean, decreased. This finding was expected, because the percentage of separable fat increased with weight of cut. The only instance in which this relationship was not significant for raw cuts was for lipids in leg cuts. Roasted cuts were partitioned among drippings as well as lean and fat. For these cuts, the proportion of nutrient found in the lean decreased significantly as cut weight increased, except for protein and ash of roasted leg cuts. For separable fat and drippings of roasted cuts, in most instances, changes in cut weight resulted in significant increases in proportion of total nutrient found in one portion but not in the other. The correlation coefficient for the affected portion was nearly equal to that for the separable lean, although opposite in sign. Partitioning of protein and ash among the separable portions of cooked leg cuts did not change significantly as cut weight increased.

For both rib-loin and legs, 31 of the 60 raw cuts were paired with roasted cuts from the same carcasses, making it possible to examine the effects of cooking. Mean values for the partitioning of nutrients for these paired cuts were so close to the means for 60 cuts given in Table 3 as to make it unnecessary to report the data separately. The most striking change with roasting was the change in partitioning of ash. The proportion of total cut ash that was found in the cooked lean decreased markedly for both rib-loin and leg cuts. The proportion of ash found in

Table 2. Proximate composition of raw and roasted lamb cuts as related to weight cut.

Cut and separable portion	Weight of cut[a] (g)	Moisture		Lipids		Protein (nitrogen × 6.25)		Ash	
		Mean (%)	r^b	Mean (%)	r^b	Mean (%)	r^b	Mean (%)	r^b
Rib-loins, raw	2596 ± 901								
Separable lean		69.8 ± 2.0	−0.72**	9.5 ± 2.5	0.73**	19.3 ± 0.7	−0.42**	0.97 ± 0.05	−0.70**
Separable fat		12.7 ± 4.0	−0.64**	83.7 ± 5.6	0.66**	3.2 ± 1.3	−0.71**	0.23 ± 0.08	−0.74**
Whole cut		34.0 ± 5.3	−0.80**	28.7 ± 12.6	0.79**	9.3 ± 1.5	−0.80**	0.49 ± 0.09	−0.79**
Rib-loins, roasted	1962 ± 706								
Separable lean		58.1 ± 3.1	−0.66**	16.0 ± 3.6	0.72**	24.6 ± 1.1	−0.38**	0.96 ± 0.06	−0.75**
Separable fat		14.6 ± 4.8	−0.70**	81.2 ± 6.3	0.71**	3.9 ± 1.2	−0.69**	0.31 ± 0.10	−0.69**
Whole cut[c]		28.7 ± 5.8	−0.82**	33.6 ± 13.0	0.79**	13.3 ± 2.2	−0.83**	0.56 ± 0.11	−0.84**
Drippings		19.7 ± 13.3	−0.58**	72.7 ± 17.9	0.64**	5.1 ± 3.9	−0.68**	1.62 ± 1.14	−0.72**
Legs, raw	2552 ± 654								
Separable lean		73.2 ± 1.1	−0.62**	4.9 ± 1.3	0.64**	20.1 ± 0.6	−0.19	1.03 ± 0.04	−0.64**
Separable fat		17.1 ± 4.4	−0.32*	78.4 ± 6.0	0.37**	4.0 ± 1.4	−0.50**	0.28 ± 0.07	−0.44**
Whole cut		48.8 ± 2.9	−0.60**	13.4 ± 5.7	0.54**	11.3 ± 0.9	−0.54**	0.69 ± 0.50	−0.69**
Legs, roasted	1782 ± 450								
Separable lean		60.7 ± 1.9	−0.41**	9.6 ± 2.0	0.44**	28.3 ± 1.1	−0.10	1.00 ± 0.05	−0.50**
Separable fat		18.4 ± 4.8	−0.37**	75.4 ± 6.6	0.40**	5.6 ± 1.5	−0.38**	0.43 ± 0.96	−0.45**
Whole cut[c]		39.8 ± 2.3	−0.47**	14.8 ± 4.6	0.39**	18.2 ± 1.1	−0.32*	0.67 ± 0.52	−0.52**
Drippings		29.5 ± 7.6	−0.21	56.9 ± 11.4	0.39**	9.2 ± 3.7	−0.56**	2.78 ± 1.06	−0.61**

[a] Mean and standard deviation for 60 cuts.
[b] Correlation coefficient; *, ** respectively significant at P = 0.05 and 0.01.
[c] Without drippings.

Table 3. Partitioning of total proximate components among the separable edible portions of raw and roasted lamb cuts: relationship to weight of raw cut.

Cut and separable portions	Weight of cut[a] (g)	Moisture		Lipids		Protein (nitrogen × 6.25)		Ash	
		Mean (%)	r[b]	Mean (%)	r[b]	Mean (%)	r[b]	Mean (%)	r[b]
Rib-loins, raw	2596 ± 901								
Separable lean		90.0	−0.73**	17.1	−0.65**	91.0	−0.65**	87.2	−0.63**
Separable fat		10.0	0.73**	82.9	0.65**	9.0	0.65**	12.8	0.63**
Rib-loins, roasted	2542[c] ± 949								
Separable lean		83.8	−0.74**	22.1	−0.67**	89.4	−0.75**	71.0	−0.74**
Separable fat		12.0	0.68**	60.8	0.18	8.2	0.70**	13.1	0.72**
Drippings		4.2	0.37**	17.1	0.67**	2.4	0.07	15.9	0.13
Legs, raw	2552 ± 654								
Separable lean		95.7	−0.46**	26.1	−0.19	96.4	−0.27*	95.1	−0.42**
Separable fat		4.3	0.46**	73.9	0.19	3.6	0.27*	4.9	0.42**
Legs, roasted	2584[c] ± 676								
Separable lean		87.1	−0.50**	28.6	−0.37**	91.5	−0.17	63.3	−0.20
Separable fat		4.6	0.11	38.8	−0.20	3.2	0.13	4.8	0.20
Drippings		8.3	0.54**	32.6	0.65**	5.4	0.09	31.9	0.13

[a] Mean and standard deviation of 60 cuts.
[b] Correlation coefficient: *, ** respectively significant at P = 0.05 and 0.01.
[c] Weight of cut before roasting.

the drippings of either rib-loins or legs could be fully accounted for by the loss from the lean. The proportion of total cut lipids found in separable fat also showed a marked decrease with roasting, and there was some indication that lipids may have melted from the separable fat into the lean as well as to the drippings during roasting. However, analytical data for this nutrient were variable, as indicated by standard deviations for lipids in separable fat and drippings (Table 2). Therefore, further investigation on more paired cuts would be needed to validate this last finding.

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Composition of Raw and Roasted Lamb and Mutton II. Amino Acids

SUMMARY

Microbiological assay values are reported for content of 18 amino acids in separable lean of raw and roasted rib-loin and leg cuts of lamb and mutton. These values agreed well with values obtained in other laboratories. The amino acid content of lamb protein tended to be constant and was not affected by cut or by roasting. Retentions of amino acids in lamb lean after roasting were essentially complete.

INTRODUCTION

Lamb and mutton (hereinafter referred to as lamb), among the major red meats in the American diet, are valuable sources of both essential and nonessential amino acids. While considerable data have been reported on the essential amino acid content of lamb muscle, fewer have been reported on the nonessential. Furthermore, a search of the literature revealed no instance in which as many as 18 amino acids were determined on the same cut, nor any instance of a study on the retention of as many as 18 amino acids in paired cuts.

This study reports microbiological assay data on 18 amino acids in lean meat from raw and roasted rib-loin and leg cuts of lamb. The data comprised one part of the results of a many-faceted study, other parts of which are reported elsewhere (Murphy *et al.*, 1966).

EXPERIMENTAL METHODS

Details of selection of lamb carcasses, oven-roasting, sample preparation and storage, and proximate composition analyses have been previously described (Murphy *et al.*, 1966). For the work reported here, 32 cuts were selected from 16 carcasses from the previously described Beltsville group of animals. At time of slaughter the animals were 6–8, 11–14, or 80 months old. Cold carcass weights ranged from 25.5 to 84.5 lb.

The 32 cuts analyzed included 8 each of raw and roasted legs and of raw and roasted rib-loins. Raw rib-loin cuts ranged in weight from 1119 to 4385 g, cooked rib-loins from 1125 to 2855 g, raw leg cuts from 1734 to 3791 g, and cooked leg cuts from 1017 to 2812 g. Paired cuts of legs or rib-loins were available from half of the carcasses and were used to calculate the amino acid retentions in the roasted lean. Separable lean from all 32 cuts was analyzed for content of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Lean from 16 of these cuts—4 each of raw and cooked rib-loin, and raw and cooked leg—was analyzed for content of alanine, aspartic acid, cystine, glutamic acid, glycine, proline, serine, and tyrosine.

Samples of homogenized ground separable lean were stored in sealed containers at −40°C prior to hydrolysis. For determination of most of the amino acids, samples of lean containing 16–25 mg of nitrogen were hydrolyzed by refluxing with 35–40 ml of 20% hydrochloric acid for 18–24 hr. After hydrolysis, excess hydrochloric acid was removed from each hydrolysate by evaporation, following which the hydrolysate was adjusted to pH 4.0 and filtered through sintered glass (Pyrex M). The humin precipitate was washed with water of pH 4.0 (Horn *et al.*, 1953; 1955), and the combined filtrate and washings were adjusted to pH 6.8, then diluted to 1 L. Samples intended for cystine and tyrosine determinations were treated as outlined above after hydrolysis by refluxing with 20% hydrochloric acid for only 2 hr. For tryptophan determinations, samples containing approximately 32 mg of nitrogen were hydrolyzed in flat-bottomed alkali-resistant glass flasks with 12-ml portions of 5*N* sodium hydroxide by autoclaving for 5 hr at 15 psi (250°F). Before alkali was added to a sample, the sample was completely wetted with 1.5 ml of 95% ethanol. The hydrolysate was then adjusted to pH 6.8 and diluted to volume. All hydrolysates were stored under toluene at room temperature until used for assay, usually within 3 weeks.

Microbiological assays for arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine were made by published procedures (Horn *et al.*, 1950; Horn and Blum, 1956), as was that for alanine (Warren *et al.*, 1963). The remaining amino acids, aspartic acid, glutamic acid, glycine, proline, tryptophan, tyrosine, and serine, were determined by unpublished procedures of M. J. Horn, H. W. Warren, and A. E. Blum.

With certain modifications the same basal medium was used in all of the assays (Horn *et al.*, 1950). Arginine was increased to 416 mg, and

[a] Deceased.

cystine to 600 mg, but methionine was decreased to 100 mg per L of basal medium. In addition, for the determination of phenylalanine the amount of tyrosine was tripled; and for the determination of tryptophan the amounts of tyrosine and phenylalanine were tripled. In the assay for cystine the amino acids of the basal medium were replaced by an acid hydrolysate of "decystinized" casein supplemented with arginine and tryptophan (Horn and Blum, 1956). Pyridoxine was the form of vitamin B_6 used in the basal media of all assays except those for threonine, valine, and arginine, in which pyridoxamine was used.

The assay test microorganisms were: *Leuconostoc mesenteroides* ATCC 8042 for aspartic acid, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tryptophan, and tyrosine; *Streptococcus faecalis* ATCC 9790 for arginine, threonine, and valine; *Leuconostoc citrovorum* ATCC 8081 for alanine and serine; and *Lactobacillus plantarum* ATCC 8014 for glutamic acid.

DL-tryptophan was used for the preparation of standard curves in the tryptophan assay. In all of the other assays the appropriate L-isomers were used. Precautions were taken to ensure the high quality and purity of amino acids used for standards.

RESULTS AND DISCUSSION

Table 1 presents means and standard deviations for the amino acid content of lamb separable lean, expressed as mg of amino acid per g of nitrogen; Table 2 presents the same data calculated to g per 100 g separable lean. Examination of the values for any amino acid in Table 1, comparing rib-loin with leg cuts or raw with roasted cuts, reveals such agreement of values as to indicate that the amino acid composition of lamb muscle protein was essentially constant.

Data reported here were compared with corresponding literature values calculated to the same basis. Our rib-loin values were compared with literature values for cuts variously labeled chop, rib chop, loin chop, loin, and sirloin, and our leg values were compared with literature values for cuts labeled leg and shank (Alexander *et al.*, 1953; Beach *et al.*, 1943; Greene and Black, 1944; Greenhut *et al.*, 1947, 1948; Kuiken *et al.*, 1947; Lyman *et al.*, 1946, 1947; Lyman and Kuiken, 1949; Schweigert *et al.*, 1945, 1949, 1951; Sirny *et al.*, 1950; Violante *et al.*, 1952). The ranges of most of the values here reported either overlap the ranges of the corresponding literature values or differ from them by less than 10%. A few differences as high as 13% were found,

Table 1. Amino acid content of separable lean of lamb expressed as mg per g of N.

Amino acid	Rib-loin		Leg	
	Raw	Cooked	Raw	Cooked
Essential amino acids [a]				
Arginine	413 ± 18	413 ± 17	406 ± 15	409 ± 13
Histidine	198 ± 9	180 ± 8	189 ± 8	180 ± 7
Isoleucine	329 ± 18	321 ± 14	319 ± 11	319 ± 11
Leucine	498 ± 22	504 ± 20	494 ± 19	500 ± 21
Lysine	583 ± 33	589 ± 39	578 ± 17	584 ± 28
Methionine	172 ± 12	166 ± 8	171 ± 7	172 ± 7
Phenylalanine	258 ± 13	265 ± 15	255 ± 9	262 ± 13
Threonine	285 ± 13	294 ± 8	283 ± 13	292 ± 9
Tryptophan	75 ± 4	73 ± 3	74 ±5	73 ± 5
Valine	333 ± 8	333 ± 10	320 ± 16	328 ± 15
Nonessential amino acids [b]				
Alanine	435 ± 33	443 ± 33	432 ± 30	434 ± 47
Aspartic acid	599 ± 16	625 ± 17	604 ± 32	626 ± 25
Cystine	73 ± 3	75 ± 6	73 ± 3	77 ± 2
Glutamic acid	1020 ± 15	1000 ± 22	1001 ± 18	1019 ± 33
Glycine	299 ± 15	321 ± 12	284 ± 18	322 ± 32
Proline	299 ± 16	288 ± 8	294 ± 23	282 ± 15
Serine	277 ± 17	302 ± 17	277 ± 15	304 ± 13
Tyrosine	210 ± 17	234 ± 14	212 ± 12	238 ± 17

[a] Mean and standard deviation of 8 replicates.
[b] Mean and standard deviation of 4 replicates.

Table 2. Amino acid content of separable lean of lamb expressed as g per 100 g of separable lean.

Amino acid (and nitrogen)	Rib-loin		Leg	
	Raw	Cooked	Raw	Cooked
Nitrogen	3.07	4.16	3.26	4.44
Essential amino acids				
Arginine	1.27	1.72	1.32	1.82
Histidine	0.61	0.75	0.62	0.80
Isoleucine	1.01	1.34	1.04	1.42
Leucine	1.53	2.10	1.61	2.22
Lysine	1.79	2.45	1.88	2.59
Methionine	0.53	0.69	0.56	0.76
Phenylalanine	0.79	1.10	0.83	1.16
Threonine	0.87	1.22	0.92	1.30
Tryptophan	0.23	0.30	0.24	0.32
Valine	1.02	1.39	1.04	1.46
Nitrogen	3.15	4.24	3.33	4.58
Nonessential amino acids				
Alanine	1.37	1.88	1.44	1.99
Aspartic acid	1.89	2.65	2.01	2.87
Cystine	0.23	0.32	0.24	0.35
Glutamic acid	3.21	4.24	3.33	4.67
Glycine	0.94	1.36	0.95	1.47
Proline	0.94	1.22	0.98	1.29
Serine	0.87	1.28	0.92	1.39
Tyrosine	0.66	0.99	0.71	1.09

as well as one of 22%. In general, the amino acid values obtained in this study agree well with those reported by other laboratories.

Values were calculated for the percentages of amino acids retained in roasted lamb of amounts originally present in the corresponding raw cuts from the opposite sides of the same animal. These values were calculated on the basis of the amino acid content per total weight of separable lean of each cut, adjusting for differences in raw weight between the paired cuts. These data show that the essential amino acids were retained after cooking to the extent of 90–104%, and the nonessential amino acids to the extent of 95–121%. The retention percentages as a whole indicate that the 18 amino acids tested in lamb lean are retained virtually in their entirety after roasting. Other laboratories have reported (Greenhut *et al.*, 1947, 1948; Schweigert *et al.*, 1945, 1949; Sirny *et al.*, 1950) retention percentages for the essential amino acids in lamb ranging from 84 to 104%, and have concluded that the cooking of lamb results in practically no loss of amino acids.

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Organoleptic Evaluation of Three Phenols Present in Wood Smoke

SUMMARY

Guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol, components of wood smoke condensates, were evaluated organoleptically. Mean panel thresholds of the taste and odor in water and the odor in mineral oil were determined for each compound. The majority of the panel characterized the compounds as being smoky in odor and taste, although phenolic characteristics were also present. The relative effectiveness of each compound in the over-all flavor picture was shown by the ratio (concentration in smoke/threshold concentration). Guaiacol had the largest index, whereas 2,6-dimethoxyphenol, which was present in smoke condensate in greatest concentration, had a considerably lower value. Mixing the three compounds in the approximate concentrations found in the smoke condensate did not give the desirable characteristic aroma. Trace components must also be considered for their effect in the over-all flavor pattern.

INTRODUCTION

Food flavors are complex blends of the taste and aroma of the chemical constituents of the products. Modern methods of instrumental analysis have made it possible to separate and identify many flavor components. Although the concentrations of these components may be determined, with a few exceptions these have not been correlated with the effect of the compounds on the sensing apparatus of observers. The relative effectiveness of a compound in the over-all flavor profile is strongly influenced by its limits of sensory detection. Burr (1964) suggested that the concentration of a component in the headspace over a food product, divided by its olfactory threshold, will provide an index of the probable contribution of a compound to total flavor. Thus, many trace components that seem unimportant on a quantitative basis may be key flavor notes, because of their strong odorant qualities.

Meat products exposed to wood smoke acquire a characteristic aroma which is of economic importance. The desirable organoleptic qualities are considered to be due to the phenolic components in the smoke. The taste and odor thresholds of a phenolic fraction chemically trapped from smoke have been determined (Tilgner *et al.* 1962). These values, however, expressed the total effect of all the components, identity and concentration unknown, in the fraction. Since there are many phenolic compounds in wood smoke, their relative importance in the over-all flavor will be governed by their concentration in the smoke, the concentration required for detection, and their possible interaction with other compounds. Thus, the information needed on the role of the phenolic components in smoke aroma requires analyses dealing with individual phenols. The phenols of a smoke condensate were recently separated in our laboratory, and many were identified (Fiddler *et al.*, 1966). Three phenols—guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol — were present in largest concentration, according to peak areas on the chromatograms. These compounds had been reported previously as products of the dry distillation of wood (Goos, 1952). Guaiacol has been used as the type or class compound for the basic "burnt" aroma in several odor classifications (Crocker and Henderson, 1927; Schutz, 1964), and 4-methyl guaiacol is known as "creosol," a component of wood tar. The present investigation was undertaken to determine the threshold concentrations of these compounds and to evaluate their contribution to the smoky flavor of meat products.

EXPERIMENTAL

Materials. The test compounds—guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol—were obtained from commercial sources. Paraffin oil, N.F., from Fisher Scientific Company was used in experiments requiring mineral oil. Deionized water was prepared initially by passing distilled water through Barnstead Universal Resin. Complaints of off-flavors, however, led to further passage of the water through a charcoal filter. All glassware was washed first with acetone (this step was necessary for removal of the mineral oil but was also

included for aqueous solutions), and then with hot Alconox solution. The glassware was rinsed well with clear, hot water, and finally with deionized water. Distilled water was not used, because it was found to leave an odorous residue in the flasks. The glassware was inverted between rows of wooden rods and allowed to drain dry. Suspending the glassware on the usual draining racks, inverting on towels, and several other methods of drying were tried and discarded because residual odors were noted. All glassware was checked by smelling before use, and odorous pieces discarded.

Gas chromatographic studies were made with a Perkin-Elmer Dual Flame Ionization Chromatograph, model 800, using a single 6-ft × ¼-inch-OD column packed with 15% Carbowax 20M on 60–80-mesh Gas Chrom P. The temperature was programmed from 70 to 170° at 5°/min. Carrier gas was helium, flowing at 80 ml/min. Injector and detector temperatures were respectively 210 and 200°. Peak areas were determined with a planimeter.

Test panel. The panel consisted of 27 professional members of the staff; approximately 20 members of the panel were available for each test period. Several of the panel had had previous experience with test-panel studies.

Test procedure. Samples of the substance to be tested were freshly weighed each day of testing and immediately diluted with deionized water or mineral oil. Concentrations for testing were obtained by further dilution in glass-stoppered bottles. One-hundred-ml quantities of aqueous solutions and 50 ml of mineral oil solutions were distributed into 250-ml glass-stoppered Erlenmeyer flasks. Several sets of solutions were prepared, each set containing a known water or mineral oil reference blank, a coded hidden blank, and four or five coded concentration levels of the test substance. The solutions were allowed to equilibrate at room temperature for 2 hr before use.

Tests were carried out in the afternoon of the same day each week, and each panelist reported at approximately the same time of day for the tests. For odor tests, panelists were requested to select coded flasks randomly, swirl the contents well, sniff the headspace vapor, and record the absence or presence of odor as compared with the reference blank. With aqueous solutions, the panelist then tasted the solution and indicated the absence or presence of oral stimulus. Retesting was permitted, and no time limit was imposed. Resin- and charcoal-treated water was used for rinsing, and salt-free crackers were available for use between samples to remove after-taste, if necessary.

Calculations. The data were decoded and arranged in order of increasing concentration with appropriate indication of absence or presence of stimulus. If a taste or odor was indicated in the hidden blank by a panelist, all of his results were eliminated from the computations of that series. Although each determination required a separate, unrelated decision, it was felt that the panelist should be able to identify the hidden blank correctly.

Thresholds of individual panelists for the test substances were determined as the lowest concentration of solution at which taste or aroma was detected. Occasionally a skip occurred, with the panelist recording absence of a stimulus at a concentration higher than his previous positive response. Threshold concentration was then taken as the next-highest concentration above which two or more consecutive positive responses were obtained.

Mean threshold concentrations for the panel were determined by plotting the percent of panelists having thresholds at each concentration level vs. the concentrations of the test solutions and locating the concentration at the 50% response.

Five or six series of analyses were made of each test substance in water or in oil. The first two or three series were considered to be training analyses, to allow panelists to become familiar with the odor and taste of the substances and to determine proper range of concentrations. Training did occur, as shown by the decrease in threshold concentrations reported by the panel. The values of the last three series of determinations, made at constant concentration levels, were used for computation of results. All tests were run at room temperatures (25 ± 1°C).

RESULTS AND DISCUSSION

Table 1 shows the mean threshold concentrations of guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol for taste and odor in aqueous solution and odor in mineral oil. The lowest concentration of material in aqueous solution detectable by smelling was 0.021 ppm ($1.7 \times 10^{-7} M$) guaiacol. Approximately 5 times as much 4-methyl guaiacol (0.09 ppm), and 90 times as much 2,6-dimethoxyphenol (1.85 ppm), were required for detection by 50% of the panel. Taste thresholds of the aqueous solutions had the same relationship toward each other that was observed for odor thresholds—e.g., the threshold for 2,6-dimethoxyphenol was approximately 100 times that for guaiacol. However, sense of taste appears to be more acute than sense of smell for these compounds, and taste threshold values were somewhat lower. Although stimulation was

Table 1. Mean threshold concentrations of guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol in water and mineral oil.

Phenol	Mean threshold concentration		
	In water		In oil
	Taste	Odor	Odor
Guaiacol	0.013 ppm (1×10^{-7} *M*)	0.021 ppm (1.7×10^{-7} *M*)	0.07 ppm (5×10^{-7} *M*)
4-Methyl guaiacol	0.065 ppm (4.7×10^{-7} *M*)	0.09 ppm (6.5×10^{-7} *M*)	0.4 ppm (2.9×10^{-6} *M*)
2,6-Dimethoxyphenol	1.65 ppm (1×10^{-5} *M*)	1.85 ppm (1.2×10^{-5} *M*)	0.34 ppm (2×10^{-6} *M*)

applied orally in these experiments, about 90% of the panel reported a loss of taste when they pinched their noses closed during sampling. Thus, the results reported as taste appear to be attributable to stimulation of the olfactory area by passage of vapor through the pharynx and posterior nares. The lower threshold concentrations observed on oral testing may be due to an increase of the vapor pressure of the test substances when raised to body temperature. The number of molecules required to evoke a response should be furnished by a less concentrated solution at body temperature (38°C) than at room temperature (25°C). Increased sensitivity of response to stimulus at higher temperatures has been reported by Baker (1962).

Since meat products contain fat, the effect of interaction of smoke components with this phase must also be considered in the overall flavor picture. Meat fats do not lend themselves as diluents for substances tested in threshold determinations. Vegetable fats such as peanut and soybean oil have characteristic odors that could interfere with flavor determinations. Mineral oil was selected as a bland vehicle for the test compounds, with the reservation that mineral oil, composed of hydrocarbons, may react differently from the fat of meats, with its content of saturated and unsaturated fatty acids. These may undergo chemical reactions with smoke components that modify threshold values. Mean odor thresholds for the test substances in mineral oil are shown in the last column of Table 1. Thresholds for guaiacol and 4-methyl guaiacol were only 3 and 4.5 times as great in mineral oil as in aqueous solution. The threshold concentration of 2,6-dimethoxyphenol, however, was less in oil than in water.

Although many foods contain fat and aqueous phases, little work has been done on the effect of these conditions on flavor. Skramlik (1926) found that taste intensity was greater in aqueous medium because of viscosity effects and the solubility of his test compounds in oil. The thresholds of aliphatic aldehydes decreased in water with increasing chain length to C_{12} (Lea and Swoboda, 1958), while thresholds in oil were not affected through the C_{12} compounds. There was little difference in threshold concentrations with paraffin or peanut oil as solvents. The partition coefficient of flavor components between oil and water may be important in determining flavor thresholds. Patton (1964) found that thresholds of fatty acids decreased with increasing chain length in water, and increased in oil. Volatilization of stimulus is essential for olfactory perception, and bonding to solvent molecules tends to reduce volatilization. Thus, the number of molecules of a compound in the headspace may be greater over a solution in which it is less soluble than over one in which it is more soluble. Guaiacol and 2,6-dimethoxyphenol, which differ in structure by one methoxy group, are slightly soluble in water to the same extent [approximately 1.7 g/100 ml at 16° (Anon.,)]. However, the taste and odor thresholds of 2,6-dimethoxyphenol were about 90 times as great. It is suggested that the volatility of 2,6-dimethoxyphenol is reduced as a result of interaction of the additional methoxy group with the polar solvent. In the nonpolar oil solvent the threshold for 2,6-dimethoxyphenol was of the same order of magnitude

as those for guaiacol and 4-methyl guaiacol. These latter compounds appear to be somewhat more soluble in oil than in water, and, as a result, the concentration required for detection increased.

Threshold concentration is the level at which the presence of a stimulus can be detected. The nature of the stimulus in the present work, however, could not be identified at the threshold level. At somewhat higher concentrations it became possible to characterize the test substance. Initially, the panelists were asked to describe the compounds in their own terms. A dictionary of approximately ten most commonly used adjectives was compiled, and panelists were required to select the most appropriate term(s) from this list. The results of the characterizations are shown in Table 2. The terms "smoky" and "phenolic" were used most commonly, but several additional adjectives of similar nature were grouped with these—e.g., "woodsy" and "bacon" were counted as "smoky," while "medicinal" and "creosote" were listed as "phenolic." Although approximately 33% of the panelists described the aqueous solutions as having a phenolic taste and aroma, the majority felt the compounds were smoky. The smoky odor was most noticeable for 2,6-dimethoxyphenol, but guaiacol had a more smoky taste. A number of panelists detected a sweet note in the odor and a bitter note in the taste of the three compounds. Characterization of the test compounds was affected by concentration. Many panelists responded differently to high concentrations than they did to lower ones, but a more detailed study of the variations was not made.

The test substances in these experiments were commercial compounds, used without further purification. It has been reported, however, that impurities may influence the response to a compound (Kendall and Neilson, 1963; Guadagni *et al.*, 1963). The three phenol standards were shown by gas chromatography to contain only traces of unidentifiable contaminants. Triangle tests were used to determine whether there was any difference between commercial and purified materials in odor and taste. The phenols were passed through the gas chromatograph, and sufficient pure material was collected to prepare aqueous solutions containing the mean threshold concentration of each compound. The panel received two test sets for each compound: Set 1 contained two flasks of commercial and one flask of purified preparation; Set 2 consisted of two flasks of purified and a flask of commercial solutions. The panelists were requested to select the odd flask in each set for both odor and taste. There was no significant difference between responses to the commercial and purified preparations of any of the three test compounds. Thus, trace quantities of impurities did not appear to have affected the overall flavor values of test phenols.

Although the effect of any compound in the overall flavor of a complex mixture may depend on a number of chemical and physical parameters, an important consideration is that the concentration be great enough to be detected. The concentrations of the three test compounds were investigated in a representative smoke condensate prepared and fractionated by gas chromatography (Fiddler *et al.*, 1966). The ratio of the areas of the peaks identified as 4-methyl guaiacol, guaiacol and 2,6-dimethoxyphenol was 1:3:4.6. The relationship of peak area to concentration was determined with a standard solution of guaiacol. By calculation,

Table 2. Characterization of the taste and odor of guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol in water.

	% response[a]					
	Odor			Taste		
Compound	Smoky	Phenolic	Sweet	Smoky	Phenolic	Bitter
Guaiacol	53.5	37.5	5.2	58.7	33.5	9.5
4-Methyl guaiacol	51.8	35.5	13.8	43.2	36.3	26.0
2,6-Dimethoxyphenol	71.5	31.5	.....	46.5	34.0	17.0

[a] The responses may add up to more than 100% because some judges indicated more than one characteristic for the odor or taste of a phenol.

Table 3. Calculation of flavor index for guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol.

Compound	Conc. in smoke condensate	Flavor index[a] In water Taste	In water Odor	In oil Odor
4-Methyl guaiacol	3×10^{-3} *M*	6400	4600	1000
Guaiacol	9×10^{-3} *M*	90000	58800	18000
2,6-Dimethoxyphenol	14×10^{-3} *M*	1400	1200	7000

[a] $\frac{\text{Conc. in smoke condensate}}{\text{Mean threshold conc.}}$.

the concentration of guaiacol in the smoke condensate was found to be 9×10^{-3} *M*. Assuming that the response of the flame-ionization detector was the same for all three compounds, the concentrations of 4-methyl guaiacol, guaiacol, and 2,6-dimethoxyphenol in the smoke condensate were respectively 3×10^{-3} *M*, 9×10^{-3} *M*, and 14×10^{-3} *M*. A flavor index was obtained from the ratio of smoke condensate concentration to mean threshold concentration (Table 3). The most effective compound was guaiacol, in both water and oil. The odor value for 4-methyl guaiacol was somewhat greater than that for 2,6-dimethoxyphenol in water, whereas the latter was more effective in an oil base. An index value of this type is more meaningful than the concentration of a component in a flavor blend, as suggested by Burr (1964).

The flame-ionization gas chromatograph is extremely sensitive, but it has been shown that the nose will often detect odors at lower concentrations than the instrument (Weurman, 1963).

The sensitivity of the nose was greater than the response of the gas chromatograph to the odor of the three smoke components. Aqueous solutions were prepared at odor threshold levels of the three compounds, and 10-μl quantities were injected into the gas chromatograph. At maximum sensitivity of the detector-recorder combination, no response was observed. To determine the lowest concentration that could be detected by the instrument, several levels of pure guaiacol were injected and the areas of the peaks determined. By calculation, it was established that a minimum detectable response would be obtained from 1.8×10^{-10} moles of guaiacol. The 10 μl of threshold concentration guaiacol solution (1.7×10^{-7} *M*) contained 1.7×10^{-12} moles, or 100-fold less material than that required for minimal response. If headspace vapor over the threshold solution had been used, in closer analogy to the odor-nose relationship, the concentration of guaiacol would be considerably less than 10^{-12} moles. (These computations are limited to the conditions set forth for the gas chromatographic analysis. The flame ionization detector is responsive to the number of carbon atoms present in the sample, so a detectable response could be reached by using a large enough sample).

Although guaiacol, 4-methyl guaiacol, and 2,6-dimethoxyphenol are present in smoke as the most plentiful of the phenols, have threshold levels indicating that they are involved in the total flavor, and have odors described as smoky, mixing the three in the proportions found in smoke condensate yielded a solution only slightly reminiscent of smoke condensate. To evaluate total smoke aroma properly it may be necessary to apply the procedure described herein to all the components in smoke.

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Mention of commercial names does not imply endorsement.

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A Classification of Objective Methods for Measuring Texture and Consistency of Foods

SUMMARY

The numerous methods for objectively measuring the textural properties of foods are classified on the basis of the variable or variables that are the basis of measurement. Physical methods of measurement are classified under the headings force-measuring, distance-measuring, time-measuring, energy-measuring, ratio-measuring, multiple-measuring, and multiple-variable instruments. There are also some chemical methods for measuring texture objectively. Methods that do not fit into any of the above categories make a special, miscellaneous group. Examples are given of the kinds of instruments found in each category.

One of the important quality factors of foods is their texture or mouthfeel. The property of texture has been the subject of considerable research over the years, and a substantial part of this effort has consisted of a search for objective methods for measuring texture. A great number of objective methods for measuring texture have been developed, and these measure many different properties of foods. With this multiplication of methods for measuring texture it becomes desirable to classify them to achieve a degree of order.

Scott Blair (1958) classified objective methods of texture measurement under the headings: fundamental, empirical, and imitative. According to Szczesniak (1963), who discussed Scott Blair's classification system at length, the fundamental tests measure properties such as elastic modulus and viscosity; the empirical tests measure properties that are usually poorly defined but which have been shown by practical experience to be related to textural quality; and the imitative tests measure various properties under conditions similar to those to which the food is subjected in practice.

Matz (1962) classified foods into the categories: liquids, gels, fibrous foods, agglomerates of turgid cells, unctuous foods, friable structures, glassy foods, agglomerates of gas-filled vesicles, and combinations of the above. He described instruments that are used to measure textural quality of foods in each of these categories. Amerine *et al.* (1965), in discussing texture measurement, classified foods into four groups—a) liquids; b) fruits and vegetables; c) meats; and d) other foods—and described instruments that are used on each group. A weakness in this kind of classification is that many instruments are used on more than one of these groups of foods.

According to Stevens (1957), measurements may be classified as nominal (numbering things), ordinal (classifying in order of greatness but with no certainty of uniformity in the scale), interval (classifying according to greatness on a known uniform scale that has an arbitrary zero), and ratio (classifying according to greatness on a known uniform scale with a true zero). Scott Blair (1960) has discussed Stevens' classification in relation to measurement of textural properties of foods.

Drake (1961) developed a classification system based on the geometry of the rheological apparatus. This classification contains the following headings and subheadings: 1) rectilinear motion (parallel, divergent, convergent); 2) circular motion (rotation, torsion); 3) axially symmetric motion (unlimited, limited); 4) defined other motions (bending, transversal); and 5) undefined motions (mechanical treatment, muscular treatment). Each subheading in Drake's classification is subdivided further on the basis of the geometry of the apparatus.

Another method of classification that can be used is described in this paper and is based upon the variable (or variables) that constitute the basis of the measurement. The classification system in Table 1 is based on this fundamental principle as far as possible. This system stresses the nature and dimensional units of the property being measured and is therefore a natural and logical means of bringing order to the great number of diverse methods used for the

Table 2. Dimensional analysis of the G.F. texture profile.

Mechanical parameter	Measured variable	Dimensions of measured variable
Hardness	force	$m\ l\ t^{-2}$
Brittleness	ratio	dimensionless
Chewiness	distance	l
Gumminess	work	$m\ l^2\ t^{-2}$
Viscosity [a]	force	$m\ l\ t^{-2}$
Cohesiveness	work	$m\ l^2\ t^{-2}$
Elasticity	force	$m\ l\ t^{-2}$
Adhesiveness (fluids only)	flow	$l^3\ t^{-1}$

[a] Viscosity is frequently measured as volume flow per unit time and the measured variable has dimensions l^3t^{-1}. The property of viscosity has the dimensions $ml^{-1}\ t^{-1}$.

objective measurement of food texture. The most commonly encountered variables are listed in Table 1. This list is not exhaustive. It is possible to use other combinations of force, distance, and time as the measured variable.

The word *objective* is stressed in this classification. The considerable amount of work that has been done on *subjective* measurement of food texture belongs in the field of sensory evaluation and is not considered here. A limited number of instruments are cited in each category in order to illustrate the principle of the classification system with concrete examples. The listing of all known instruments in their respective categories not only would make this paper excessively long but would be unnecessary, because the reader can quickly classify any instrument once the principle of the system has been grasped.

Group 1. Force-measuring instruments. This is certainly the most common method used for measuring food texture. The measured variable is force, usually maximum force, and distance and time are held constant or at least replicatable. The force measurement has the dimensions $m\ l\ t^{-2}$ (mass, length, time^{-2}). Examples of this instrument are the Tenderometer (Martin, 1937), Magness-Tayor Pressure Tester (Magness and Taylor, 1925), Warner-Bratzler Shear (Bratzler, 1949), Maturometer (Lynch and Mitchell, 1950), Shear Press (Kramer *et al.*, 1951), Christel Texture Meter (Christel, 1938), and the Bloom Gelometer (Bloom, 1925). A force measurement is not restricted to compression force. Several tensile tests have been described for foods, e.g. Platt and Kratz (1933) describe a tensile test on standard pieces of cake. The force required to pull the cake apart was found to be a measure of toughness of the cake.

Group 2. Distance-measuring instruments. This group can be subdivided into distance, area, and volume measurements. Force and time are held constant or replicatable while some function of distance is measured.

a. Distance-measuring. Distance, with the dimension l, is measured directly. Examples are: the consistometers that measure the distance an unrestrained semi-fluid product flows in a horizontal plane in a given time; various Penetrometers that are allowed to sink into soft products such as fats and mayonnaise; and the Ridgelimiter (Cox and Higby, 1944), which measures the sag of standard jellies. The applied force in these instruments is frequently gravity. The ball compressor that measures the depth of penetration of a hemispherical ball into cheese (Caffyn and Baron, 1947) is an example of a distance-measuring instrument that uses an applied force greater than gravity.

b. Area-measuring. Area, with the dimension l^2, is measured. An example is the Adams Consistometer, where the area covered by a semifluid product that has been allowed to flow over a horizontal plane for a given time is measured. The Adams Consistometer, however, is usually used as a distance-measuring instrument (dimension l) by averaging the distance of flow at four quadrant points. Incidentally, the Adams Consistometer should be known as the Grawemeyer and Pfund Consistometer if priority is recognized (Adams and Birdsall, 1946; Grawemeyer and Pfund, 1943).

c. Volume-measuring. Volume, with the dimension l^3, is measured. The best known application of this method is the measurement of bread volume by displacement of seeds. Another example is the Succulometer (Kramer and Smith, 1946), which measures the volume of juice expressed by pressing 100 g of sweet corn in a special cell.

Group 3. Time-measuring instruments. In this group, time (dimension t) is measured, while force and distance are held constant (or replicatable). The Ostwald-type viscometers are an example of this type of instrument. Some consistometers have been used as time-measuring instruments by measuring the time taken for a semi-fluid product to flow a standard distance. Consistometers are more commonly used as distance-measuring instruments.

Group 4. Energy-measuring instruments. This group of instruments measure work or energy, which is the product of force and distance and has the dimensions $m\ l^2t^{-2}$. An example is the Farinograph (C. W. Brabender Instruments Inc., South Hackensack, N. J.), which is used to measure the physical properties of dough. Another example is

the meat grinder used by Miyada and Tappel (1956). A number of instruments have been described in recent years that utilize a chart-recorder on which a force-distance curve is drawn during a test. The area under the force-distance curve, which is a measure of work, can be obtained from these charts, so such instruments may be classed in this group.

Group 5. Ratio-measuring methods. This method requires at least two measurements of the same variable, from which a ratio is calculated. This technique yields a figure which is dimensionless. The measured variable may be any one of the variables listed above. An example of this method is the factor of cohesiveness in the G. F. Texture Profile (Friedman *et al.*, 1963). In this test, the ratio of the work done during the first and second bites on the food is taken as an index of cohesiveness. The work is measured from the area under the force-distance curve, and cohesiveness is obtained from the ratio A_2/A_1. The dimensional unit of cohesiveness is $m\ l^2t^{-2}/m\ l^2t^{-2}$, and cohesiveness as defined in the GF Texture Profile, is therefore dimensionless. Another dimensionless ratio that sometimes correlates with texture is relative density.

Group 6. Multiple-measuring instruments. In this group more than one variable is measured. Instruments in this group usually use a chart on which is drawn a force-distance curve or an approximation to a force-distance curve. It is possible to measure various forces, distances, and areas with these instruments. Examples of this instrument are the G. F. Texturometer (Friedman *et al.*, 1963), the MIT Denture Tenderometer (Proctor *et al.*, 1955), and the Instron universal testing machine (Bourne *et al.*, 1966).

Group 7. Multiple-variable instruments. These instruments have more than one uncontrolled variable, but only one variable is measured. The variables may or may not be interrelated, and this relationship may or may not be linear. Any variable may be measured, and it is often difficult to establish the nature of the dimensional units. Sometimes these instruments correlate well with subjective measurements. However, it is preferable to use other instruments where possible, because of the difficulty of relating the measurement that is obtained to measurements by other instruments. An example of the multiple-variable type of instrument is the Durometer (Shore Instrument Co., Jamaica, N. Y.), which was designed for measuring softness of rubber but is sometimes used on foods. The Durometer consists of a small spring-loaded hemispherical ball that protrudes above the surface of an anvil. The Durometer is pressed against the product until the anvil makes contact with the product, and the depth of penetration of the ball is read off on an arbitrary scale that is calibrated 0–100. With this instrument a variable force is exerted over a variable area, resulting in a variable depth of penetration of the ball. The operation and limitations of the Durometer have been discussed by Bourne and Mondy (1966).

Group 8. Chemical analysis. Chemical analysis is not a physical measurement but is an objective method that is sometimes used for determining the texture of foods. The methods of measurement listed in this group and in group 9, below, are indirect methods for measuring texture. They are used because there is a correlation between their measurement and a subjective measurement, although they do not measure texture directly. A chemical analysis is usually expressed on a weight/weight basis, as percent or parts per million, and under these conditions the result is a ratio measurement and is dimensionless. If the analysis should be expressed on a weight/volume basis or volume/weight basis, the result would have the dimensions $m\ l^{-3}$ and $m^{-1}l^3$, respectively. Probably the best known chemical measurement of food texture is the alcohol-insoluble solids test (Kertesz, 1935; A.O.A.C., 1965) which is accepted as a standard of quality on raw green peas. Another example is the pericarp content of sweet corn (Kramer *et al.*, 1949) which is a partial measurement of the texture of sweet corn.

Group 9. Miscellaneous. It usually happens in any classification system that a few examples can be found that do not fit into the carefully planned scheme of order. A classification of objective methods for measuring texture and consistency is no exception to this rule. Therefore a ninth group is included in the classification. The three requirements for this group are: a) the method must be an objective one; b) it must correlate well with texture or consistency of a food; and c) it does not fit into any of the eight groups listed above. The measured variable might be any property at all, and consequently the dimensional units can vary just as widely.

The first example in this group is an optical method (Love, 1958; Love and Mackay, 1962). A standard weight of fish is homogenized in dilute formaldehyde solution, and the optical density of the homogenate is measured. A tough piece of fish breaks up less readily than a tender piece and gives a lower optical-density reading. The second example in this group is an electrical method (Nybom, 1962). The firmness of berry fruits is found by measuring their ability to transmit vibrations. A 50-cycle alternating current is supplied to an earphone in contact with a single raspberry. The vibrations from the earphone are transmitted through the fruit, and their intensity is measured in a second earphone on the opposite side. A third

example involves sound (Drake, 1963). The sounds of chewing various foods are analyzed for amplitude, frequency, and duration. Although more work is needed with this method, significant correlations between texture of the food and the characteristics of chewing sounds have already been obtained.

FINAL COMMENTS

The great diversity of methods mentioned above serve to emphasize the varied problems that the food technologist faces in attempting to measure food texture by objective methods. The breaking down of a food in the mouth into a state suitable for swallowing involves complex combinations of the variables force, distance, and time. This is shown in the dimensional analysis of the G.F. Texture Profile in Table 2 (Friedman *et al.*, 1963). The Texture Profile for solids requires three different force measurements (hardness, brittleness, and gumminess), two different work measurements (adhesiveness and chewiness), one distance measurement (elasticity), and one dimensionless ratio (cohesiveness). (In this discussion the author has adhered to Szczesniak's conception of the G. F. Texture Profile as being derived from a force-distance curve although, in fact, it is actually derived from a force-time curve. Strictly speaking, only force, time, and force-time integrals can be obtained from the G. F. Texturometer. However, the construction of the G. F. Texturometer is such that the force-time curve approximates a force-distance curve. Therefore it can be used to measure force exactly, and distance and force-distance integrals (work) approximately.

Scott Blair (1949) has proposed that intermediate entities are needed to describe the rheological nature of foods and that dimensions of the type $ml^{-1}\ t^{(k-2)}$ are needed, where the exponent $(k-2)$ is not an integer.

Although the practicing food technologist may not want to go to these lengths he should at least be aware that different variables are used in different texture measuring devices. If he is designing a new instrument he should understand which dimension is the measured variable and which dimensions are the constants in his system. Taking this into account should assist in designing optimum performance for an instrument and should assist in understanding the usefulness and limitations of the measurements that are obtained from that instrument.

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Table 1. Classification of objective methods for measuring texture and consistency.

Method	Measured variable	Dimensional units	Examples
1. Force-measuring	force F	$m\ l\ t^{-2}$	Tenderometer
2. Distance-measuring	(a) distance	l	Penetrometers
	(b) area	l^2	Grawemeyer Consistometer
	(c) volume	l^3	Seed Displacement (bread volume)
3. Time-measuring	Time T	t	Ostwald viscometer
4. Energy-measuring	work F × D	$m\ l^2t^{-2}$	Farinograph
5. Ratio-measurnig	F, or D, or T, or F × D, measured twice	Dimensionless	Cohesiveness (G.F. Texture Profile)
6. Multiple-measuring	F, and D, and T, and F × D	$m\ l\ t^{-2}$, l, t, $m\ l^2t^{-2}$	G.F. Texturometer
7. Multiple-variable	F, or D, or T (all vary)	unclear	Durometer
8. Chemical analysis	concentration	dimensionless (% or ppm)	Alcohol insoluble solids
9. Miscellaneous	anything	anything	Optical density of fish homogenate

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