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176 W. Adams St., Chicago 3, Illinois 60603, U.S.A.

H. O. HULTIN and A. S. LEVINE
Department of Food Science and Technology, Massachusetts Agricultural Experiment Station
University of Massachusetts, Amherst, Massachusetts 01003

Pectin Methyl Esterase in the Ripening Banana

SUMMARY

Three pectin methyl esterase fractions were obtained from banana pulp by successive extraction with water (Fraction I), a solution of 0.15*M* NaCl (Fraction II), and a solution of 0.15*M* NaCl after adjusting the pH of the mixture to 7.5 (Fraction III). The changes in the activities of these fractions during post-harvest ripening were investigated. The activities of all three fractions increased as the banana skin began to change from green to yellow. Thereafter Fractions I and II remained relatively constant while Fraction III continued to increase in activity throughout the period of ripening studied.

The changes in pectic substances of plant tissue that occur during ripening and maturation are undoubtedly of considerable importance to texture. Many investigators have studied changes in pectic materials in products such as apples (Woodmansee *et al.*, 1959), pears (Emmett, 1929; McCready and McComb, 1954), peaches (DeHaan, 1957; Postlmayr *et al.*, 1956; Reeve, 1959), tomatoes (Stier *et al.*, 1956; Woodmansee *et al.*, 1959), and oranges (Sinclair and Jolliffe, 1958). Von Loesecke (1950) reviewed changes in pectic substances in the banana during ripening. Studies of changes in pectic substances during ripening have been much more extensive than studies of the enzymes catalyzing these changes.

One of the pectic enzymes involved is pectin methyl esterase (PME), which catalyzes the demethylation of the methyl esters of polygalacturonic acids and is widely distributed in the roots, stems, leaves, and fruits of most higher plants. Dennison *et al.* (1954) found that PME activity in the tomato was low until the fruit reached the mature-green stage, at which time the activity rose sharply until about two days after the fruit began to turn color. Jacquin (1955) reported that PME activity in the apple and pear increased with ripening, while Weurman (1954) found that the enzymatic activity in Doyenne Boussoch pears did not decrease after picking as it did in fruit left on the tree. Hobson (1963) discussed the influence of nitrogen and potassium fertilizer on pectic enzyme activity and suggested the probable involvement of the pectic enzymes in the ripening process.

We recently obtained evidence indicating the presence of 3 pectin methyl esterase-active fractions in ripe banana pulp (Hultin and Levine, 1963). One fraction is extractable with water, one with 0.15*M* NaCl, and one with 0.15*M* NaCl at an alkaline pH. The respective fractions have been labeled Fractions I, II, and III. Each fraction has a different pH-activity relationship and shows a characteristic response to the anionic surfactant sodium dodecyl sulfate (SDS). A study was undertaken to determine how the activity of each pectin methyl esterase fraction varies in the pulp of the banana during post-harvest ripening.

EXPERIMENTAL

Material. The bananas used were the Gros Michel variety of *Musa sapientum*. Green bananas were purchased locally and ripened slowly at 62–64°F. Ordinarily a large hand of green fruit containing 12–16 fingers was obtained, and 3 or 4 bananas were removed and used for testing at the various ripeness stages. In some instances riper bananas were purchased and used directly.

Extraction. The extraction procedure was as follows. The peel was removed from pre-chilled bananas, and the pulp was sliced into thin wafers. The wafers were placed in a flask and immediately frozen in a bath consisting of dry ice and 1-methoxy-2-propanol. The slices were freeze-dried overnight, pulverized in a mortar and pestle, and stored in a closed container at −20°F until used. The dried fruit was reconstituted by trituration with distilled water in an amount equivalent to what had been removed. This water ordinarily contained 2 mg of caffeine per ml; other levels of caffeine were also tried. After reconstitution, the extractions were carried out as described (Hultin and Levine, 1963) except that the same quantity of caffeine (2 mg per ml of extracting solution) was used in every extraction. Essentially the procedure consisted of extracting the reconstituted tissue five times with an equal weight of distilled water, six times with 0.15*M* NaCl, and twice with 0.15*M* NaCl after the pH of the mixture had been adjusted to 7.5. The mixture was centrifuged each

time, the supernatant decanted through cheesecloth, and the volume determined. Aliquots were then taken for assay of PME activity. All extractions were performed at 0–4°C.

Enzyme assays. The assay procedure, based on the release of free carboxylic acid groups on de-esterification of pectin by the enzyme, has been described (Hultin and Levine, 1963). The unit of activity was defined as that amount of enzyme which would hydrolyze 1 meq of ester per minute; it was measured by the amount of standard 0.1000*N* NaOH required to maintain the mixture at a given pH, which was measured by a pH meter with glass electrode. The final concentration of pectin (pure citrus pectin, N.F.) in the reaction mixture was 0.5% by weight, and the reaction mixture was adjusted to 0.15*M* in NaCl in all cases since the concentration and type of cation can greatly influence PME activity (Lineweaver and Ballou, 1945). The temperature of assay was 25°C and the pH was 8.5, which gives approximately optimal activity for all 3 fractions but is not high enough to cause alkaline de-esterification of the pectin under the conditions of assay (Hultin and Levine, 1963). Acid production in the absence of added pectin was subtracted from the total in all cases. It was negligible in all extracts except the first with distilled water. This activity is thought to be due to the presence of endogenous soluble pectins.

RESULTS

The extraction procedure was designed to prevent the inhibition of the enzymes by tannins (Young, 1962) since early work indicated that tannins might be causing excessive inhibition of the enzymes of green fruit. Freeze-drying of the tissue followed by maceration in a mortar and pestle was successful in completely disrupting the cells of fruit at all stages of ripeness as determined by microscopic examination. The caffeine was added to the water of reconstitution and the extraction media as a complexing agent for the tannins (Mejbaum-Katzenellenbogen, 1959; Young, 1962). As will be shown later, there was a significant decrease in the activities of the extracted pectin methyl esterases when fresh pulp was homogenized with a mortar and pestle or in a Potter-type homogenizer, or when the freeze-dried tissue was treated as described above but without the caffeine. A level of 2 mg of caffeine per ml of solution gave more consistent results and greater extraction than did concentrations of either 1 or 4 mg caffeine per ml.

Fig. 1 gives the total PME activity of ripening banana pulp and the activities of Fractions I, II, and III throughout the storage period. The values shown are the averages of from 3–5 determinations. Three separate runs were made with different fruit

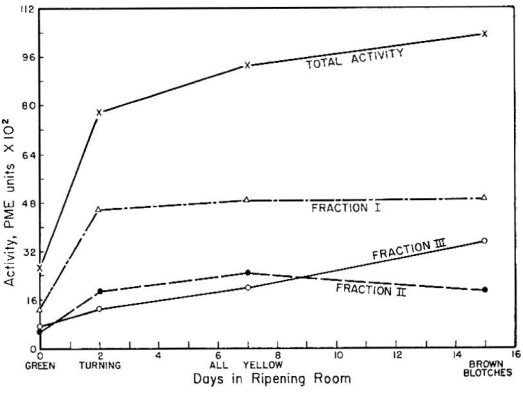

Fig. 1. Increase in total PME activity and the activity of the individual PME fractions of banana pulp during ripening. The samples were freeze-dried initially and the extracting media contained 2 mg caffeine in each ml. The extraction and assay procedures have been described (Hultin and Levine, 1963).

purchased at various stages of ripeness. Results rarely differed by more than 10–15%. Although respiratory data were not obtained on these samples, other experiments indicate that the green bananas used were just prior to, or approximately at, the beginning of the climacteric rise. These green bananas had significantly lower PME activities than did bananas of a later stage of ripeness. One of the features that often accompanies the climateric period in fruits is an increase in total protein. It may be that one of the proteins synthesized in the banana during this period is pectin methyl esterase. After the bananas reached a skin color in which considerable yellowing appeared, there was little change in either Fraction I or II. Fraction III, however, continued to increase throughout the ripening period indicated in the graph.

Two questions might be raised at this point. One is whether inhibition still occurs in green fruit even in the presence of the caffeine. The second is whether the caffeine causes a redistribution of the enzymes in the extracting media. The first question was resolved by grinding and extracting equal amounts of freeze-dried green and ripe bananas together. If the tannins of the green fruit were inactivating the enzymes, there should have been a decrease in the expected values caused by an inactivation of the enzymes of the ripe fruit by the tannins. In several samples tested, the activity of the mixed sample was equal within ±5% to the sum of the values of the green and ripe fruit treated singly. In other words, we saw no evidence for any inactivation of the enzymes of the ripe fruit by grinding the tissue of the freeze-dried ripe fruit with that of the green

fruit. We conclude that the activity of PME in the green fruit extracted by this procedure represents the actual activity.

A study as to whether there was any change in distribution of the enzymes as extracted into the water, salt, or high pH-salt solutions caused by the use of caffeine was performed by obtaining pH-activity curves for each fraction. No anomalous behavior was noted for any fraction, indicating that caffeine has little or no effect on the distribution of the enzymes in the media of extraction used in these experiments.

The results obtained when fresh tissue was extracted after grinding in a mortar and pestle without the use of caffeine are illustrated in Fig. 2.

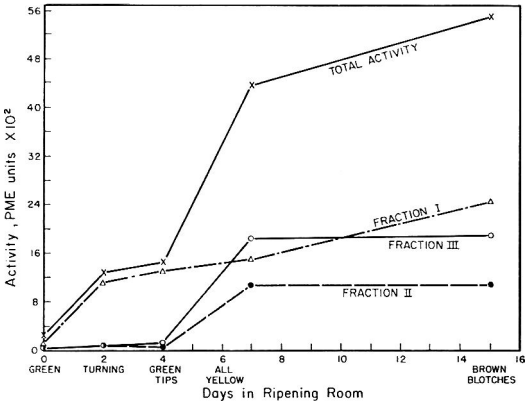

Fig. 2. Increase in PME activities in ripening banana pulp as determined on fresh tissue. The conditions of extraction and assay were as described in the legend in Fig. 1 except that the samples were not freeze-dried nor was caffeine used in the media of extraction.

These data are included here to illustrate the effect of not using a tannin-complexing agent in the extraction procedure. They also show that extraction of ground fresh tissue is much less efficient than that of the freeze-dried; this is true even in ripe banana pulp. It may be due to the greater difficulty in obtaining complete cell rupture with the fresh tissue. An even more significant difference is observed in green fruit. Whereas the total activity of the pectin methyl esterases obtained from fresh ripe fruit is slightly greater than one-half of that of the freeze-dried, caffeine-extracted samples, the total activity of the fresh fruit at the green stage is less than 1/10 that of the freeze-dried. As might be expected, the effect of the tannin-protective agent is greatest with green fruit. In addition, on a percentage basis the activities of Fractions II and III in the unripe banana are more affected by the absence of caffeine in the extracting medium than is the activity of Fraction I. This may be related to the cellular localization of Fractions II and III; conceivably they could be more susceptible to inhibition by reason of their ready accessibility to the disrupted latex cells.

DISCUSSION

Work on the pectin methyl esterases of the banana was initiated after observations were made of an acidic drift in pH when banana extracts were brought to an alkaline reaction. This phenomenon has been noticed with other plant extracts. Festenstein (1961) observed a drift in pH after adjustment to pH 8 or 9 with leaf extracts but offered no explanation. Heinicke *et al.* (1961) ascribed the acidic drift in pineapple stem extracts which had been made alkaline to the formation of micelles which, being extremely well buffered, would be slow to reach equilibrium with the ions in solution. Heintze (1961) also noticed a drift from pH 6 and above while titrating plant roots and suggested that it might be due to reaction with colloidal material in the roots, the presence of acids of varying strength, or changes in ion intake with time. Based on the heat sensitivity of the reaction and the production of methyl alcohol during the reaction, Holden (1945) reported that the acidic drift observed when minced tobacco leaves were made alkaline was caused by enzymic demethylation of pectin to pectic acid. Similar factors, together with an increase in rate of drift when additional pectin was added, indicate that a like mechanism was involved in banana extracts.

We conclude that there is an increase in the PME activities of banana pulp with ripening. The greatest part of this increase occurred during the period that the skin color began to show some yellow. This change in skin color usually occurs near the beginning of the climacteric rise under our conditions of storage. The increase is not due to inactivation of enzyme in the green fruit by tannins; this is clearly indicated by the experiments in which the pulp of unripe fruit was mixed and ground with that of ripe pulp, and total recovery was obtained.

During the ripening process there was a continuous increase in the activity of Fraction III (salt-soluble and high pH) whereas

the activities of Fractions I and II reached a maximum after about 2 days in the ripening room. When the pulp is extracted with water, both Fraction II and Fraction III sediment at low speed (approximately 500 × g); this may possibly indicate that they are associated with the cell wall fraction of the banana tissue.

We have no information at the present time that defines the relationship of the various fractions of PME to each other or to ripening. We have, of course, in these experiments measured the appearance of PME activity in banana pulp, and we do not know how this may relate to the total quantity of enzyme. It is possible that each PME enzyme fraction is synthesized by a separate pathway and that the increase in activity results from a direct synthesis of the enzyme or from the conversion of an inactive form to an active one. Conversely, the various fractions may have a common origin. One of the fractions may serve as precursor for the others. It could be that a change occurs in the protein itself, or it may relate to a change in association (i.e., either an aggregation or dissociation of the enzyme, a combination with some other component such as lipid, etc.). Isolation and purification studies are under way to clarify this problem.

One further point deserves mention. The method used for extraction and assay of the pectin methyl esterases of the banana measures only the potential activity of these enzymes in the tissue of the banana. The actual activity is controlled by pH, substrate availability, and the presence or absence of activators or inhibitors at the site of action of the enzyme. The ideal way of measuring the cellular enzymic activity would be to follow the disappearance of substrate or the appearance of product in the tissue. One would have to be sure that other coupled reactions were not involved, however. We would like to point out here that an earlier study (Hultin and Proctor, 1961) showed that there was a very significant increase in methanol content in banana pulp at approximately the same time as we now find that the increase in the pectin methyl esterases occurs. If we assume that the methanol is produced primarily by demethylation of pectin, then the appearance of "potential" PME activity correlates well with the appearance of the product of the reaction. Additional evidence for this could be obtained by following the change in methoxy content of the endogenous pectic substances.

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G. FRED SOMERS
Departments of Biological Sciences, and Agricultural Biochemistry
University of Delaware, Newark, Delaware 19711

Viscoelastic Properties of Storage Tissues from Potato, Apple, and Pear

SUMMARY

Slices of potato tubers were subjected to cyclic elongation, stress relaxation, and breaking in an Instron materials-testing instrument. Cubes of such tissues and of ripe fruits of pears and apples were subjected to cyclic compression and stress relaxation. The time required for 20% relaxation of the stress was progressively longer with each of 3 cycles. Cycling increased the apparent values of Young's modulus, *E*, for slices treated in acetone, treated in acetone and rehydrated, and frozen and then thawed, but decreased values for slices soaked overnight in H_2O at 6°C. With potato slices and cubes, treatments which wilted them resulted in shorter times for a 20% relaxation of stress. Cubes of pear and apple soaked 3 hr in H_2O tended to disintegrate and also showed much shorter times for relaxation of stress than cubes treated in 0.5*M* sucrose. The general features of all curves were qualitatively similar, except that compressing cubes of potato tubers beyond a stress of about 0.9 Kg/cm^2 resulted in a sharp, reversible increase in apparent values of *E*.

INTRODUCTION

The physical properties of the walls of cells composing the tissues of fruits and vegetables are of considerable practical importance. They determine in some measure their resistance to infection and the capacity of fruits and vegetables to resist mechanical damage in harvesting, handling, and storage. Various penetrometers, tenderometers, etc., are available to measure the breaking strength of plant tissues. However, such measurements do not yield an understanding of the ability of plant tissues to resist deformation and mechanical damage short of breaking. More likely to yield such information, as Alfrey (1948) pointed out for other viscoelastic materials, are measurements of stress-strain interrelations under a variety of conditions. A start in this direction is the present investigation with a commercially available instrument, the Instron materials-testing instrument.

LITERATURE REVIEW

A number of investigators have measured elastic and/or plastic properties of various plant tissues (for early reviews, see Brauner and Brauner, 1943; Heyn, 1940). In addition to observations based upon osmotic deformation, methods used have included various means of stretching the tissue (e.g. Brauner and Brauner, 1943; Preston and Hepton, 1960; Probine and Preston, 1962; Spark *et al.*, 1958), of bending it under static load (e.g. Heyn, 1940; Bonner, 1960; Tagawa and Bonner, 1957; Somers, 1965), or of vibrational bending (e.g. Drake, 1962; Falk *et al.*, 1958; Virgin, 1955). In most of these cases the instruments have been uniquely devised for a particular set of experiments, and hence are not readily available to other workers. Furthermore, the results obtained have usually been presented in relative terms and usually required tedious, and sometimes difficult observations over relatively long periods.

For a more thorough understanding of the viscoelastic properties of plant tissues, recourse needs to be made to an instrument, or instruments, available to several workers so that comparable measurements can be made in standard units. Moreover, since the results obtained are dependent upon time and the stress-strain history of the tissue, these factors need to be more closely measured and/or controlled, and observations need to be made over shorter periods to minimize changes in the materials under test. Such an accomplishment should lead to fuller understanding of the viscoelastic properties of plant tissues and organs, which in turn should lead to a better understanding of the viscoelastic properties of plant cell walls—a matter of interest both to those concerned with harvesting, transport, and storage of plant products and to the plant physiologist.

EXPERIMENTAL METHODS

Potato tubers, Russett Burbank variety, and pears, presumably Bartlett variety, were purchased in a local market. Apples, Golden Delicious variety, were obtained from a local garden. All were stored at about 5°C until used. About a day before being used the potato tubers were placed in H_2O at about 5°C to ensure crispness. Slices were carefully cut 1 mm thick from the central portions of the tuber on a hand microtome and were sized to 1 cm wide and about 4 cm long with sharp razor

blades fastened in special holders. Such slices were used for tension measurements. For compression measurements, cubes were cut from potato tubers, apples, and pears with razor blades spaced 0.5 cm apart. In all cases, care was taken to minimize drying by placing the slices or cubes in covered containers promptly after cutting.

For tension measurements, each slice was blotted between sheets of bibulous paper applied under a load of 1 kg, and the ends were then clamped in compressed gas-activated jaws of a table-model metric Instron materials testing instrument (Hindman and Burr, 1949; Van Wazer *et al.*, 1963). One set of jaws held one end of the slice, and another set the other end (Fig. 1). The jaws

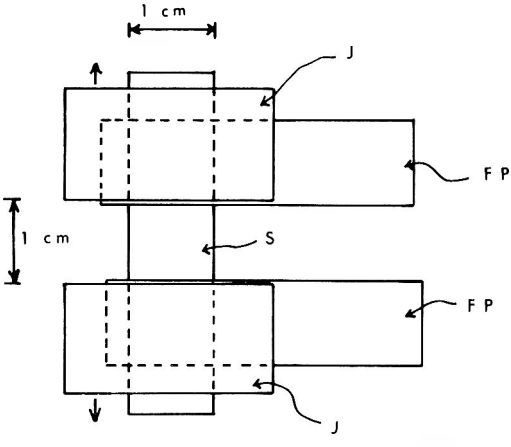

Fig. 1. Diagram of tissue slice (S) clamped in the jaws (J) of the test instrument for tension measurements. Strips of filter paper (FP) were used to hold the slice in position until the jaws were closed by gas pressure (16 lb per sq in.). The jaws were then moved apart as described in the text.

were rubber-faced, and the sets of jaws were spaced 1 cm apart. Strips of filter paper about ½ inch wide, about 3 inches long but folded in half to 1½ inches long, were used to assist in inserting the slices into the open jaws. The tissue slice could be held with a folded filter paper strip until it was appropriately oriented and clamped in the upper jaws. A second folded strip aided in placing the slice between the lower jaws and holding it in place until they were closed. The filter paper also absorbed moisture on the surface of the slices, minimizing slippage. The jaws were closed with a pressure of 16 psi.

The upper set of jaws of the testing instrument is fastened to a sensitive strain gage, and the lower set is fastened to a crosshead which is driven by a synchronous motor at closely controlled speeds. In these experiments the speed of the crosshead was uniformly 0.5 cm/min. The output from the strain gage is amplified and registered on a strip-chart recorder driven by a synchronous motor. The chart speed was 20 cm/min. The full-scale load of the recorder was usually 1000 or 500 g. The maximum load recorded never exceeded one-half the full-scale load.

The strip charts from the recorder (10 inches wide) were reduced photographically and traced to facilitate reproduction for publication. Consequently some details are obscured in the figures presented in this paper. The major features are evident, however.

For tension measurements a rather uniform cycling pattern was used (Fig. 2A). The slice was elongated (strain) by moving the crosshead downward at a uniform speed (0.5 cm/min). This produced the stress shown in the first portion (a) of each record. The motion of the crosshead was routinely stopped (b) after a desired stress had been reached at somewhat less than the breaking point of the slice. With the elongation held constant the stress was allowed to relax a desired amount, usually 20%, and the strain was again increased (c) to produce again the same stress as at b. The crosshead was stopped again, and the stress allowed to relax a second time. This cycle was repeated once more (d–e), and then the cross-

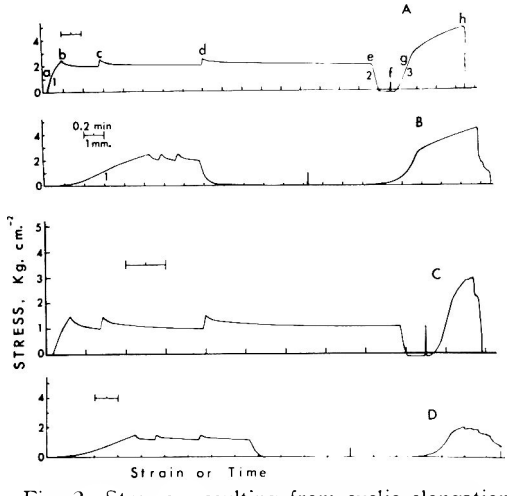

Fig. 2. Stresses resulting from cyclic elongation (strain) and stress relaxation of slices of potato tubers. See text for cycling details. In all cases the units for the abscissa are presented to the left above the tracing. In each case the line represents 0.2 min or 1 mm, depending upon whether strain or stress relaxation is being observed. The treatments were: A, in H_2O at 6°C; B, acetone −15°C and then rehydrated; C, acetone; and D, frozen in H_2O at −15°C and thawed; all overnight (about 18 hr). Attention is called to the fact that the scale for C differs markedly from that of the other portions of the figure.

head was moved back to zero at a rate of 0.5 cm/min. When the crosshead reached its initial position, i.e. jaws 1 cm apart. a short pip was recorded manually (f) and the crosshead was once again set to produce an elongation at the same uniform rate as for the initial cycles. This was continued until failure of the slice (h).

For compression measurements the general approach was similar. A cube of tissue was mounted upon a compression-load cell (strain gage), and strain (compression) was applied with an attachment mounted rigidly to the bottom of the crosshead. Used in most cases was a cyclic application of strain, with intervening periods of relaxation, etc., similar to that for slices under tension. Exceptions will be noted.

RESULTS

With crisp slices of potato tuber (Fig. 2A) the application of a strain promptly produced a stress. For about the first ¼ mm (per cm of slice) increase in strain, the stress-strain curve was essentially linear (see 1 on curve). Then the curve suggested a plastic-flow component. When the strain was stopped the stress relaxed in a curvilinear fashion. The relaxation times were progressively longer with each successive cycle (compare b–c, c–d, d–e). Each time, a very small further elongation was sufficient to restore the stress value.

When the crosshead was returned (e) to the starting position, the stress returned first to zero and then showed some pressure, as evidenced by the displacement of the stress curves below the base of the chart. The magnitude of this pressure is not recorded under the cycling pattern used, but it gives evidence of an elongation of the slice. The slice shortens to some extent under the reduced strain, as evidenced by the fact that the time required to reach the initial test length (at f) after reaching zero stress is longer than the time required to show renewed stress following f. Reapplication of a strain results in no stress until the residual excess length of the slice has been overcome. Then the curve, after a very short curvilinear portion, becomes essentially linear (see 3 in Fig. 2A). Further strain once again produces an inflection in the stress-strain curve (at g). The curve from g to h approximates an extrapolation of the curve from the origin to b.

It was observed that there was a nearly linear relationship between the stress produced during the first strain program, i.e. at point b, and that at the inflection point, g, in the stress-strain curve during the final elongation. A regression analysis of this relationship revealed that for initial peak stress values (point b) ranging from 2000 to 5650 g/cm^2:

$$b = 0.95\ g - 51 \qquad n = 42$$

where b is the initial peak stress and g is the inflection point in the second curve. The error estimate of the slope was ±0.02.

Slices treated in various ways to modify their turgidity before being subjected to a similar stress-strain program yielded curves which were qualitatively similar but differed considerably in a quantitative sense. Slices which were stored in acetone (20 slices/100 ml) at about −15°C, and then thawed and rehydrated in H_2O before being cycled were, of course, very flaccid. This is reflected in the stress-strain curve (Fig. 2B). Initially, stress appears slowly, presumably because of adjustments in the network of cell walls to accommodate the elongation—an adjustment not opposed by turgid cell contents, as with the fresh slice. Then the stress-strain curve is nearly linear for a time. When the strain is stopped, the stress relaxes rela-

Table 1. Apparent values for Young's modulus of elasticity, E, obtained by cyclic application of tension to potato tuber slices treated overnight in various ways. Five slices per treatment. The numbers in parentheses at the top of each column of apparent values for E identify portions of stress-strain curves in Fig. 2.

Treatment	Apparent value of E (10^7 dynes/cm^2) Initial strain (1)	Return to origin (2)	Final strain (3)	Breaking load (Kg/cm^2)
A) H_2O, 6°C	6.3 ±0.35[a]	5.9±0.35	4.2±0.33	5.3±0.22
B) Acetone −15°C rehydrated	0.81±0.04	5.4±0.09	3.5±0.11	4.5±0.41
C) Acetone −15°C	4.1 ±0.22	9.1±0.51	5.6±0.26	3.0±0.24
D) H_2O −15°C; thawed	0.55±0.03	3.4±0.07	2.4±0.09	2.2±0.25

[a] Mean ± standard error, $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n\ (n-1)}}$

Table 2. Time required for a 20% relaxation of stress, and the degree elongation with cyclic application of tension to potato tuber slices treated overnight in various ways.

Treatment	n	Magnitude of stress relaxation[a] (Kg/cm²)	Seconds required for 20% relaxation			Elongation (mm)	
			First	Second	Third	Origin to b	f to h
A) H_2O, 6°C	5	2.5—2.0	20±2.8	70± 8.6	114±6.1	0.79±0.07	4.4±0.26
	4	1.5—1.2	18±3.5	89±25.7	109±7.3	0.36±0.02	4.2±0.25
B) Acetone −15°C and rehydrated	5	2.5—2.0	5.4±0.38	7.9±0.97	13±2.4	4.7±0.17	8.8±0.39
C) Acetone −15°C	5	1.5—1.2	2.0±0.13	4.2±0.38	9.2±0.93	0.55±0.05	1.7±0.20
D) H_2O −15°C and thawed	5	1.5—1.2	8.2±0.48	33±10.3	71±24	4.4±0.19	6.8±0.40

[a] The first value is the stress corresponding to b on the curve, Fig. 2. The second value is the one to which the stress was allowed to relax.

tively rapidly, but the time required is longer for each successive cycle, as with the fresh slice.

Slices stored in acetone, as above, and then strained without rehydration, but after most of the acetone had evaporated, produced a stress-strain pattern similar to that for the fresh slice (Fig. 2C). However, these slices were rather stiff. This is reflected in the strain/time scale of Fig. 2C. Moreover, these slices break rather readily, and hence would not tolerate stresses applied to slices given other treatment. Slices frozen in water and cycled after thawing (Fig. 2D), gave curves similar to slices frozen in acetone and rehydrated, except that the relaxation times were somewhat longer. Data summarizing results obtained with several slices for each treatment are summarized in Tables 1 and 2. The *apparent* values for E, Young's modulus, were computed in the usual terms from the slopes of appropriate portions of the stress-strain curves, i.e.

$$E = \frac{F/A}{\Delta L/L}$$

where F is force in dynes per cm² initial cross sectional area, A (as cut), ΔL is the change in length, and L is the initial length of that portion of the slice between the upper and lower sets of jaws of the tester (*cf.* Falk *et al.*, 1958).

As judged by their weights following blotting and just prior to being inserted in the jaws of the test instrument, the slices soaked overnight in water at 6°C increased in fresh weight by some 16%. During treatment, the weights of the others decreased 28% for acetone −15°, rehydrated; 51% for acetone −15°; and 30% for thawed, frozen slices.

It is obvious that the treatments profoundly affected the properties of the slices. The breaking strength was lowered by the acetone and freezing, the latter being lowest of all. For slices stored in water at 6°C the the apparent value for E is lower during the final strain than was evidenced in the previous portions of the program. However, with other treatments the value increased markedly after the initial strain cycles, particularly in the case of the rehydrated acetone-treated slices and those which were frozen. It is interesting to note, for example, that the rehydrated acetone-treated slices gave some values for E not very different from some obtained with slices soaked only in water, even though the breaking strength had been reduced markedly.

Properties which might be associated with a plastic-flow component are summarized in Table 2. Note that slices from the water at 6°C and from acetone *without* rehydration required relatively little elongation (origin to b) to produce rather

large stresses, i.e. both were rather stiff. However, they differed markedly in the time required for a 20% relaxation of the stress. In this regard the two treatments in acetone resembled each other more closely, even though they differed markedly in the elongation required to produce the initial stress and required to cause a final breaking of the slice. The slices frozen in H_2O differed in many respects from those treated with acetone and rehydrated, particularly in times for 20% relaxation and in breaking strength. In all cases the added elongation required to restore the stress following a relaxation period was small (about 0.2 mm or less) despite a rather large elongation required to produce the stress initially with some treatments.

Some concern was felt for the possibility that some of these observations were the result of slippage of slices in the jaws, though careful observation failed to reveal any. This possibility was obviated by using compression cycling to strain cubes of tissue cut 0.5 cm on each side. Stresses were recorded simultaneously (Figs. 3, 4, 5). In general, features similar to those obtained with slices were observed. Upon interruption of strain application the stress relaxed, apparently exponentially, and the relaxation time was longer for successive cycles. With many of these tissues, using a 0.5-cm/min crosshead speed (our slowest available at the time), the stress increased so rapidly on recycling that reproducible relaxation cycles were difficult to obtain with the manual controls available on the instrument. Nevertheless, it is apparent that for pear and apple the time required for 20–25% relaxation of the initial stress is somewhat less for curves from water than for curves from 0.5*M* sucrose. With potatoes, the reverse occurred. It is probably pertinent that the cubes of apple and pear tended to disintegrate spontaneously on soaking for 3 hr in H_2O. The potatoes, of course, did not react this way.

Details regarding apparent elasticity are presented in Table 3. It is interesting to note that pear and apple tissues gave apparent values for *E* that were lower in water than in 0.5*M* sucrose. These tissues, on standing in water, became rather spongy, and sap could be expressed from them readily. This is reflected in the trace in Fig. 5A by an unsteadiness characteristic of tissue about to break. It should be remembered that these fruits were fully ripe. Once again, an increase in the apparent value of *E* was noted upon recycling.

With potato tuber cubes, an additional phenomenon was observed. This is particularly evident in Fig. 6, in which the strain was cycled without including relaxation cycles. As the strain was increased, the stress increased essentially linearly to about 900 g/cm^2 (note the arrow in Fig. 6) and

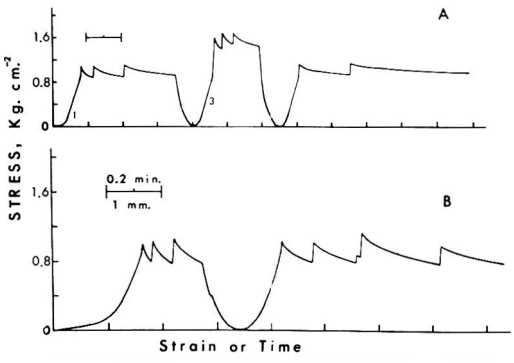

Fig. 3. Stresses resulting from cyclic compression (strain) and stress relaxation of cubes of potato tubers. See text for cycling details. Treatments *A*, H_2O; *B*, 0.5*M* sucrose.

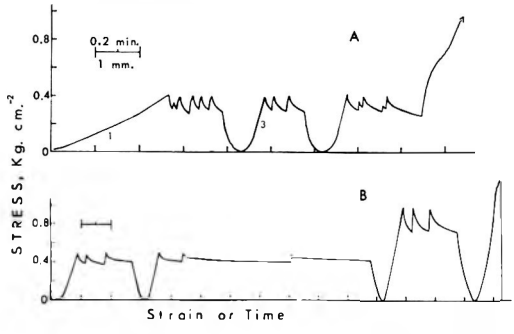

Fig. 4. Stresses resulting from cyclic compression (strain) and stress relaxation of cubes of pear fruit. Other details as for Fig. 3.

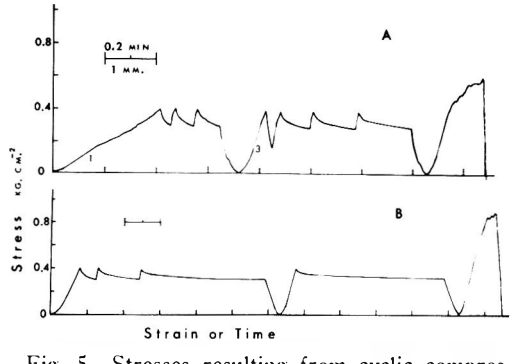

Fig. 5. Stresses resulting from cyclic compression (strain) and stress relaxation of cubes of apple fruit. Other details as for Fig. 3.

then increased sharply (compare columns 1 and 1a in Table 3). As the strain was decreased the curve was essentially linear, usually until the stress had decreased to about 700–750 g/cm^2, and then became curvilinear toward a lower apparent value for *E*. Recycling repeatedly reproduced the same inflections at nearly the same stress. The apparent *E* values computed from the steeper slopes were

Table 3. Apparent values for Young's modulus of elasticity, E, obtained by cyclic application of compression to cubes of potatoes, apples and pears. All treatments were of three hours' duration following cutting and a brief washing in tap water.

Tissue	Treatment	n	Apparent value of E (10^7 dynes/cm^2) (1)[a]	(1a)[a]	(3)[a]	(3a)[a]
Potato tuber	H_2O	5	1.00±0.06	4.3±0.27	1.12±0.05	6.0±0.28
Potato tuber	0.5M sucrose	3	0.74±0.03	2.3±0.12	0.99±0.02	3.5±0.16
Pear fruit	H_2O	2	0.13	None evident	0.63	None evident
Pear fruit	0.5M sucrose	1	0.43	None evident	0.70	None evident
Apple fruit	H_2O	2	0.11	None evident	0.52	None evident
Apple fruit	0.5M sucrose	2	0.36	None evident	0.65	None evident

[a] These numbers refer to portions of the stress-strain curve as numbered in Figs. 3, 4, and 5. Numbers 1a and 3a apply to the steeper portions of curves 1 and 3. Data are not presented for E following the second return to zero strain.

about 4 times as large as for the lower values and were more nearly comparable to those obtained for tension measurements of slices. E values computed from curves in Figs. 3 and 6 were comparable to each other.

DISCUSSION

In interpreting viscoelastic properties of plant tissues it is generally assumed that these properties reflect properties of the walls of the cells in the tissues used (see, for example, Bonner, 1960; Brauner and Brauner, 1943; Falk *et al.*, 1958; Preston and Hepton, 1960; Tagawa and Bonner, 1957; Nilsson *et al.*, 1958). It is also recognized that these walls are composed essentially of cellulose microfibrils embedded in an amorphous matrix (Setterfield and Bayley, 1961). Admittedly, the cell walls represent a structural system which is complex and difficult to analyze in terms of the properties of the cell walls themselves. Yet, in quantitative terms they are presumably the principal viscoelastic components of plant tissue. They form a network of interacting units which, by interaction with the cell contents inside the walls, give the tissues their over-all viscoelastic properties. The cytoplasm is also viscoelastic, but the cytoplasmic connections among cells are so tenuous that they probably contribute little to an interacting viscoelastic network. The cell contents, including the protoplasm, are essentially liquid, and hence are essentially incompressible at the pressures used in these studies. However, the hydrostatic pressures (turgor) of the cell contents, interacting with the viscoelastic cell walls, are of prime importance in determining the magnitude of the viscoelastic properties of plant tissues (Falk *et al.*, 1958).

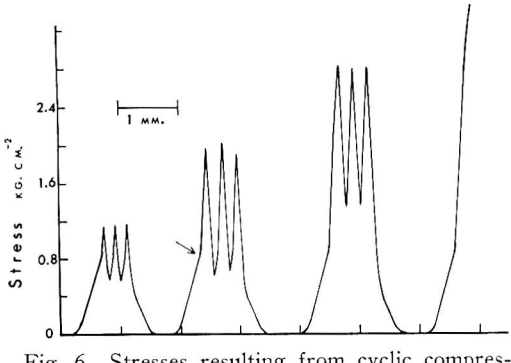

Fig. 6. Stresses resulting from cyclic compression and decompression of cubes of potato tuber. Treatment similar to the *3A*, except that no stress relaxation was included in the cycling program, and greater stresses were produced. Crosshead speed was uniformly 0.5 cm/min for both compression and decompression.

It is conceivable that some of the observations reported above could have resulted from forcing water from the cell contents. Such a concern was discussed by Brauner and Brauner (1943), who concluded that it was not a serious factor in their experiments. The slices stored in water were fully turgid, and the stresses produced in them may have forced some water from the cell contents, but this would probably not be

true for slices treated in 0.5*M* sucrose. It is difficult to see how water loss could explain the increased relaxation with repeated cycling or the increase in apparent *E* values. Increases in *E* values have been reported in the absence of such a possible complication with synthetic polymers (e.g. Hindman and Burr, 1949) in response to strain applications. Nor is it evident, if water loss is the explanation for these observations, why the relaxation times should be so much longer for H_2O-treated slices than for those frozen, killed with acetone, or wilted in 0.5*M* sucrose. Rather, it seems more likely that most, if not all, of the phenomena observed reflect the properties of cell walls and their interaction with the cell contents.

It would seem likely that the low breaking strength of the frozen slices resulted from damage to the cell wall structure by freezing. However, comparison with other treatments suggests a need for caution in drawing such an interpretation. The treatment involving only acetone also resulted in a marked reduction in breaking strength, but when such slices were rehydrated the load required to break them was not significantly lower than for the control slices in H_2O. Acetone might have been expected to remove water and lipids from the tissues. Lipids have not been reported as consistent organized components of the cell walls of tissues such as these (Setterfield and Bayley, 1961). However, in a highly hydrated system composed of cellulose microfibrils embedded in an amorphorous matrix (Setterfield and Bayley, 1961), the removal of water would be expected to affect viscoelastic properties significantly. It would seem likely that the differences between the slices hydrated and those not hydrated following acetone treatment resulted from differences in hydration. Without hydration, the slices were characterized as being stiff (high initial apparent *E*), having a very short stress relaxation time (20% relaxation), and a low breaking strength. Presumably both freezing and acetone treatment destroyed the semipermeable properties of the cell membranes. The viscoelastic properties following such treatments presumably reflect little interaction between the cell walls and the cell contents until the cell-wall network has been distorted sufficiently to reduce the volume of the cell greatly. Such a conclusion is supported by the very low apparent *E* values for the initial elongation of hydrated-acetone-treated slices and frozen ones. It would appear that without hydration the network of cell walls remained rather rigid.

It seems likely that increases in apparent *E* values resulting either from recycling of tissues or, as in Fig. 6, from a continuing increase in strain, result from structural changes in the cell wall components. The transitions seen in Fig. 6 may have resulted from shifting arrangements of the cellulose microfibril network in the amorphous matrix of the cell walls, a suggestion made by Bonner and Frey-Wyssling (see Brauner and Brauner, 1943).

A logarithmic rate of stress relaxation at constant strain is characteristic of plastics (Karas, 1961). Brauner and Brauner (1943) reported that the extension of potato slices decreased at a logarithmic rate when the load was removed. It was found with the tissues used in this study that stress relaxation could be expressed by the equation:

$$\frac{S_o - S_t}{S_o} = n \log t + C$$

Where S_o is the stress when the strain is stopped in the first elongation cycle, S_t is the stress at time t, and n and C are constants which differed with the tissue and treatment. (Only the first relaxation curve was examined for such a relationship.) Models for the relaxation of stress in viscoelastic materials predict an exponential relationship (Van Wazer *et al.*, 1963), though not necessarily this one. Stress relaxation in a heterogeneous system such as a cell wall may result from a variety of factors such as slippage of the cellulose microfibrils through the amorphous matrix of the cell wall, from a flow of the matrix, from molecular rearrangements of various polymeric cell wall constituents, especially cellulose, or various combinations of such factors. A purely elastic body does not show such a relaxation. The differences in relaxation time with various treatments may reflect properties of the amorphous matrix of the cell wall. A short re-

laxation time suggests a rapid adjustment (flow?) in the matrix. Possibly the turgid slices had such long relaxation times because the cell walls were already extended. With potatoes, short relaxation times and relatively high values for n and C were associated with dehydration (acetone, not rehydrated), wilting (acetone, rehydrated; 0.5M sucrose), and freezing. With pears and apples a tendency to disintegrate was associated with short relaxation times and high values for n and C. Note that these latter tissues were fully ripe; hence the pectic materials of the cell walls may have been rather soluble.

The apparent E values observed are of an order of magnitude similar to those found by Falk *et al.* (1958) with a vibrational method, and by Somers (1965) with a cantilever beam method. The values reported here for 1-mm-thick slices should probably be increased by a factor of about 2 to allow for cells on the surface which have been rendered ineffective by being cut (Somers, 1965).

Finally, this instrument appears to be promising as a means of studying tissue properties which presumably reflect cell wall properties. Obviously, much remains to be done, particularly since the viscoelastic parameters measured are influenced to some extent by temperature (Heyn, 1940) and considerably by the previous stress-strain history of the sample.

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F. W. KNAPP

Department of Food Technology and Nutrition
University of Florida, Gainesville, Florida 32603

Some Characteristics of Eggplant and Avocado Polyphenolases

SUMMARY

Eggplant polyphenolase oxidized chlorogenic acid much faster than it did any other substrate. In contrast, the substrate oxidized most rapidly by the avocado preparation was nordihydroguaiaretic acid (NDGA), followed by catechol and catechin. Resorcinol was a moderately effective competitive inhibitor (K_i = 0.02*M*), whereas hydroquinone (K_i = 0.005*M*) was a slightly less potent noncompetitive inhibitor. All the chlorides tested depressed eggplant polyphenolase activity to a similar but limited extent. The copper-chelating agents 1-phenyl-2-thiourea and sodium diethyldithiocarbamate were powerful inhibitors; the former (K_i = 0.01m*M*) was shown to act noncompetitively.

INTRODUCTION

Through their catalysis of enzymatic browning, attended by losses of flavor and nutritive values, the polyphenolases are among the enzymes most destructive to the quality of fresh and frozen fruits and vegetables. In their review, Joslyn and Ponting (1951) summarized the findings of investigations of the polyphenolases (also designated polyphenol oxidases or phenolases) from plant sources. To date, most of these studies have been carried out on fruits or vegetables which are plagued by enzymatic browning, most of them of major economic importance. Peach polyphenolase has been investigated by Reyes and Luh (1960), that of pears by Tate *et al.* (1964), and that of apples by Ponting and Joslyn (1948). Nelson and Dawson (1944) reported on the browning enzymes of mushrooms. Arthur and McLemore (1956) studied the polyphenolase of sweet potatoes, and Clark *et al.* (1957) studied polyphenolase in white potatoes. This list is representative rather than exhaustive.

Samisch (1937) carried out limited studies on apricot and avocado polyphenolases. Apparently no one has investigated the browning enzymes of the latter fruit further. Very little data had been published on the polyphenolase of eggplant until the first report of this investigation (Knapp, 1961). The work reported here is an extension of the study of eggplant polyphenolase and the first portion of a continuing investigation into the polyphenolase of avocado.

Because the polyphenolases are copper enzymes (Arnon, 1949), it has been found that compounds such as 1-phenyl-2-thiourea, which bind this metal, are effective inhibitors (Heymann *et al.*, 1954; Reyes and Luh, 1960). The former authors also reported that 4-chlororesorcinol inhibited potato polyphenolase, and the latter found phloroglucinol effective against peach polyphenolase.

The sensitive technique of continuous spectrophotometric assay offered a good opportunity for determining modes of inhibition and calculating Michaelis and inhibitor constants for eggplant and avocado polyphenolase. The constants for avocado polyphenolase will be presented in a later publication.

MATERIALS

Fruits were obtained from local retail or wholesale markets. Eggplants were of the "Florida Market" variety. The avocados were mainly of the "Lula" variety, although some other Guatemalan–West Indian hybrid fruit were used.

Polyphenols for enzyme assays were obtained from Nutritional Biochemicals Corporation and were used without further purification.

Enzyme preparation. Because of the well-known heat sensitivity of phenolase enzymes, all preparative operations were carried out at as low a temperature as practical, initially in a cold room at 2°C. But by the use of a refrigerated centrifuge, and by keeping the materials on ice at other times, extraction and purification could be conducted as successfully in the laboratory as in the low-temperature room, and much more comfortably.

The buffer used throughout the extraction procedure was 0.1*M* phosphate, pH 7.0. All centrifugations were at $1 \times 10^4 \times G$ and 0°C unless otherwise noted. The fruit sample (generally 100 g), freshly peeled and cubed, was blended with 2 volumes of a 3:1 mixture of cold buffer and flake ice. Most of the eggplant pulp was removed by straining through several layers of cheesecloth;

the avocado insolubles were removed by centrifugation for 15 min. The supernatant was treated with 1.5 volumes of acetone (−20°C), and centrifugated for 10 min at −15°C. The sediment was suspended in 2.0 ml of buffer per g of fruit by rubbing with a rubber "policeman." Inactive protein was removed by precipitation with $(NH_4)_2SO_4$ (0.2 saturation) followed by centrifugation for 20 min. The enzyme was recovered by making the supernatant 0.7 saturated with $(NH_4)_2SO_4$ and centrifuging for 20 min. On the occasions when the $(NH_4)_2SO_4$ precipitates failed to pack well, centrifugation was carried out for 15 min at $4 \times 10^4 \times G$. The enzyme was suspended in buffer (0.5 ml per original g of fruit), and insoluble materials were removed by a final 10-min centrifugation. The enzyme preparation was kept refrigerated for overnight storage and frozen for longer storage.

Enzyme assays. Manometric assays were conducted in the conventional way at 25°C, using a refrigerated Warburg apparatus and air atmosphere. Readings were made at 0.5-min intervals.

Spectrophotometric assays of polyphenolase were carried out essentially as described by Sussman (1961). A Beckman DB spectrophotometer, with Sargent SRL recorder, was used to measure the increase in absorbance of reaction mixtures. In most cases, ferrocyanide was used to continuously reduce the oxidized substrate. The initial rate of ferricyanide production, as determined by increased absorbance at 420 mμ, was considered to be the reaction rate. The concentration of potassium ferrocyanide used in reaction mixtures was 0.8% initially, later increased to 1.3 or 1.6%. This amount of ferrocyanide had no adverse effect on the reaction rate and provided a ferrocyanide-substrate ratio between 10 and 100. In cases where the inclusion of ferrocyanide was undesirable, the absorbance at 390 mμ by oxidized polyphenols was followed directly.

Because change in absorbance per min, rather than absolute absorbance, was used as the measure of reaction rate, the recorder pen could be displaced by means of the variable-range control without altering the slope of the absorbance-time trace. This allowed a series of traces to be made on the same section of paper, facilitating comparisons and conserving paper (see Fig. 2).

Spectrophotometric assays were made at ambient temperature (22–25°C). The duration of the reaction was too brief for appreciable temperature change after mixing.

After all other components of the reaction mixture had been placed in the cuvette, the substrate was introduced by serological pipette; the cuvette was shaken rapidly and placed in the spectrophotometer. This procedure occupied about 5 sec but gave faster mixing than merely blowing the substrate into the cuvette.

For determination of the optimum pH of eggplant polyphenols, a series of buffers was prepared by mixing appropriate amounts of 0.1*M* citric acid and 0.2*M* Na_2HPO_4. For avocado polyphenolase the buffers were equimolar mixtures of citric acid and Na_2HPO_4, adjusted to appropriate pH values with H_2SO_4 or NaOH before dilution to a final concentration of 0.031*M* in each compound. In both series, the pH recorded was the actual pH of the reaction mixture.

Michaelis constants (K_m) were calculated by least-squares analysis of the plots of S/v vs. S (Lineweaver and Burk, 1943). Inhibitor constants (K_i) were calculated by this method and from the plots of v_o/v_i vs. inhibitor concentration as described by Laidler (1958).

RESULTS AND DISCUSSION

Although previous manometric studies had indicated that the optimum pH for the oxidation of a crude extract of eggplant polyphenolase was about 6.6 (Knapp, 1961), the spectrophotometric method using ferrocyanide showed the maximum rate of enzymatic chlorogenic acid oxidation at pH 5.0–5.2 (Fig. 1). The pH optimum for avocado polyphenolase was slightly lower, about 4.7–4.8.

All *ortho*-diphenols tested were oxidized by enzyme preparations from both avocado

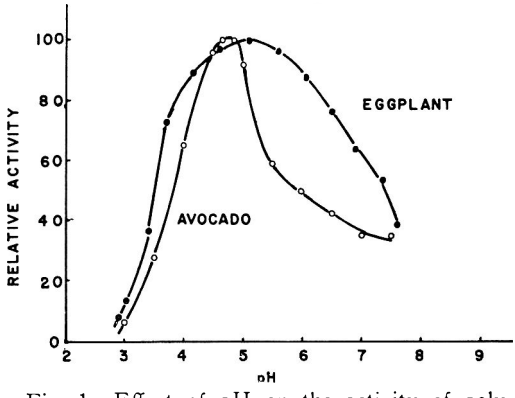

Fig. 1. Effect of pH on the activity of polyphenolase from eggplant and avocado. Activity: Increase per minute in absorbance at 420 mμ. Avocado reaction mixture contained 2 ml of equimolar citrate-phosphate (0.31*M* in each), 0.4 ml of 10% $K_4Fe(CN)_6$, 0.5 ml 0.01*M* catechol, and 0.1 ml of enzyme. Eggplant reaction mixture contained 1.3 ml of 0.1*M* citrate–0.2*M* phosphate, 0.5 ml of 5% $K_4Fe(CN)_6$, 1.0 ml of $5 \times 10^{-4}M$ chlorogenic acid, and 0.2 ml enzyme.

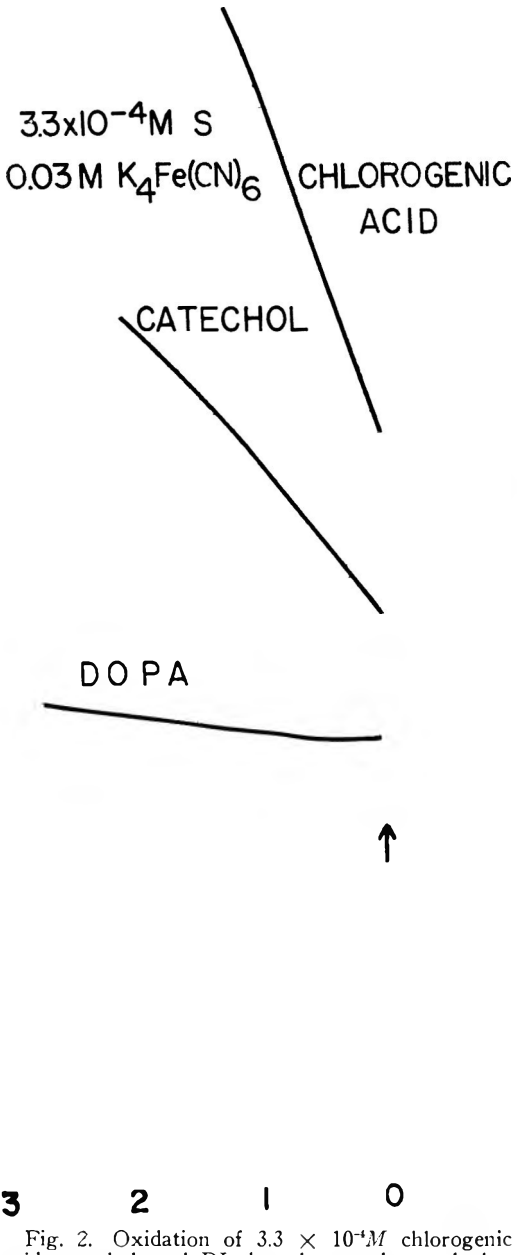

Fig. 2. Oxidation of 3.3 × $10^{-4}M$ chlorogenic acid, catechol, and DL-dopa by eggplant polyphenolase as determined in the ferrocyanide assay. The ordinate of the recorder paper represents relative absorbance at 420 mμ, and the arrow indicates the time of addition of the substrate.

and eggplant. Reaction rates varied considerably, however. Fig. 2 shows reaction traces for the oxidation of 0.0033M chlorogenic acid, catechol, and DL-dopa by equal amounts of eggplant polyphenolase. The respective rates were 0.30, 0.13, and 0.015

Table 1. Comparison of substrates for avocado and eggplant polyphenolases.

Substrate[a]	Relative reaction rates[b] Enzyme source: Avocado	Eggplant
1. Catechol	100	100
2. Chlorogenic acid	33	760
3. Caffeic acid	33	212
4. Catechin	81	31
5. Epinephrine	.....	52
6. Dopa	12	8.5
7. NDGA	250	390
per diphenol group	125	195
8. Quercetin	6	39

[a] Substrates 1 through 6 were 1.67 × $10^{-4}M$; substrates 7 and 8 were 6 × $10^{-5}M$ and incompletely dissolved.

[b] Rates calculated on equimolar substrate basis and related to rate of catechol oxidation by each enzyme, as determined by spectrophotometric assay.

absorbance units per min. A similar pattern is shown in Table 1, which presents the results of direct comparisons between all the *o*-diphenolic compounds tested. Eggplant polyphenolase consistently oxidized chlorogenic acid much faster than it did any other substrate. This is not unexpected, for chlorogenic acid has been shown to be the major polyphenol of eggplant (Sakamur and Obata, 1963). Of the other substrates, nordihydroguairetic acid (NDGA) and caffeic acid were oxidized most rapidly. The latter compound, of course, constitutes the aromatic portion of the chlorogenic acid molecule. But why NDGA, which lacks this 3,4-dihydroxycinnamic acid grouping, was oxidized at a rate (per diphenol group) comparable to that of caffeic acid is not apparent. In contrast to the eggplant enzyme, avocado polyphenolase oxidized chlorogenic and caffeic acids at only a fraction of the rates of catechin, catechol, and NDGA. The last-named compound was oxidized most rapidly, even allowing for its two diphenol groups. Dopa was a poor substrate for enzymes from both sources.

Michaelis constants (K_m) for eggplant polyphenolase and five of the substrates are shown on Table 2. Agreement was fairly good, although substrate and enzyme concentrations varied considerably between trials. The values of K_m varied with the age

Table 2. Michaelis constants of eggplant polyphenolase.

Substrate	Concentration[a]	No. of trials	K_m (mM) Range	K_m (mM) Av.
Catechol	2.7–6.7	8	1.9–6.3	4.0 ±1.3[b]
Chlorogenic acid	0.3–2.7	9	0.37–1.05	0.73±0.13[b]
Caffeic acid	.03–0.3	2	0.30–0.38	0.34
Dopa	0.4–4.0	3	4.5—6.0	5.6
Epinephrine	0.6–2.6	2	2.0—3.3	2.7

[a] Maximum concentration in any one trial; minimum was 1/10 or 1/20 of maximum in each case.
[b] Standard deviation.

of preparations and with the degree of purification. In agreement with the relatively higher rates of oxidation of chlorogenic and caffeic acids, K_m values for these substrates were the lowest of those determined. The K_m for caffeic acid was $3.4 \times 10^{-4}M$, about half the average value for chlorogenic acid. Since K_m is a measure both of enzyme-substrate affinity and of the rate of breakdown of enzyme-substrate complex, the lower value for caffeic acid may have been a reflection of the slower rate in the latter step. Although the relative rate for dopa, epinephrine, and catechol were 8.5, 52, and 100, their K_m values were all of the same order of magnitude, respectively 5.6, 2.2, and 4mM.

Neither hydroquinone nor resorcinol, respectively the *para* and *meta* isomers of catechol, was oxidized directly by polyphenolase preparations from either eggplant or avocado. No determination was made as to whether the presence of a diphenol would catalyze the enzymatic oxidation of these compounds. The color of oxidized hydroquinone or oxidized resorcinol would be difficult to distinguish from the color of other oxidized polyphenols.

Both resorcinol and hydroquinone inhibited polyphenolase from each of the two sources. Resorcinol appeared a somewhat more potent inhibitor than hydroquinone. The inhibition of chlorogenic acid oxidation by resorcinol was strictly competitive. Based on the Lineweaver-Burk plot (Fig. 3A), the dissociation constant (K_i) for the enzyme-inhibitor complex was calculated to be 0.023M. This agrees well with the value of 0.021 calculated from data obtained with two fixed chlorogenic acid concentrations and varying resorcinol concentrations. In

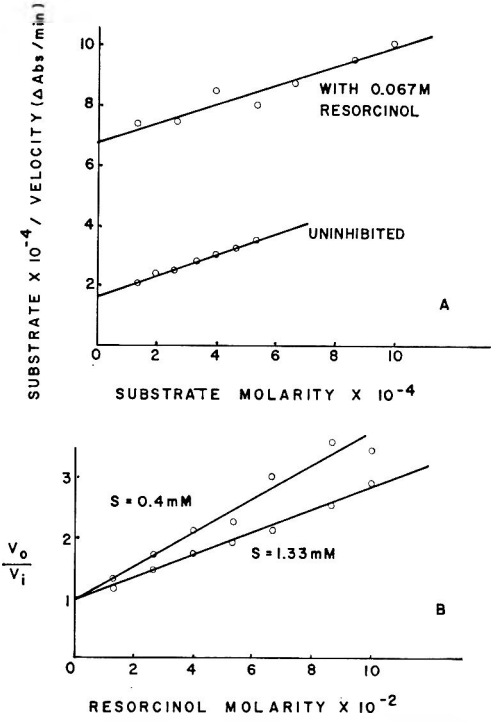

Fig. 3. Inhibition by resorcinol of oxidation of chlorogenic acid by eggplant polyphenolase. *A*) Lineweaver-Burk plot (S/v vs. S). Slope ($1/v$) unchanged by resorcinol; intercept (K_m/V) increased by a factor of 4. *B*) plot of v_o/v_i vs. inhibitor concentration. Slope increased at lower substrate concentration.

that case (Fig. 3B) the plot of v_o/v_i (uninhibited rate/inhibited rate) vs. resorcinol concentration gave a steeper slope for the lower substrate concentration. This also indicated that the inhibition was competitive.

Somewhat surprisingly, the inhibition of catechol oxidation by hydroquinone was of the noncompetitive type. This is clearly shown by the Lineweaver-Burk plot in Fig. 4A, in which both intercept and slope in-

creased by the same factor (1.72). On this basis, the value of K_i for eggplant polyphenolase and hydroquinone was calculated to be $0.0046M$. The plot of v_o/v_i vs. I (Fig. 4B) for inhibition by hydroquinone of the oxidation of two levels of chlorogenic acid indicated that the mechanism of inhibition was other than competitive. The results were somewhat anomalous in that inhibition was greatest when the substrate concentration was greatest—just opposite to the competitive case. For ordinary noncompetitive inhibition there should be no change of slope when the substrate concentration is changed.

It was conceivable that the decrease in rate of ferricyanide production might have been due to nothing more than the reducing action of hydroquinone on ferricyanide. The observation that both ferricyanide and oxidized catechol lost color after hydroquinone was added lent support to this theory. But if hydroquinone was merely acting as a reducing agent for oxidation products, it should have no effect on the rate of O_2 uptake. It was found that O_2 uptake by a mixture of enzyme, catechol, and ferrocyanide was reduced from 9.0 to 1.5 μL per min by $0.0005M$ hydroquinone, showing that this agent did in fact inhibit the oxidation of polyphenol.

The K_i values of 0.02 and 0.005 for resorcinol and hydroquinone, respectively, indicate that the latter compound is much more tightly bound to the enzyme than the former. The more effective inhibition by resorcinol must be explained by its being bound to the active site of the enzyme, whereas hydroquinone, although more tightly bound, exerts its inhibitory effect by some indirect and still obscure mechanism.

Resorcinol was somewhat more effective as an inhibitor of polyphenolase than was chloride ion. Fig. 5 also shows that KCl and NH_4Cl inhibited oxidation to about the same extent as NaCl when the same concentration of substrate was used. $CaCl_2$ exerted a somewhat greater effect in spite of the higher concentration of substrate, even when the concentration of chloride rather than molarity of $CaCl_2$ was used as the inhibitor concentration. It must be noted that relatively high chloride concentrations were needed to lower rates appreciably.

The copper-binding agents 1-phenyl-2-thiourea (PTU) and sodium diethyldithio-

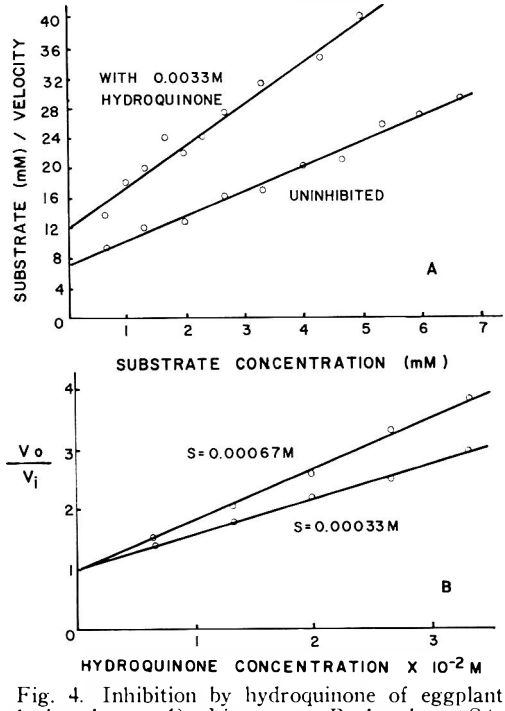

Fig. 4. Inhibition by hydroquinone of eggplant polyphenolase. *A*) Lineweaver-Burk plot (S/v vs. S) for oxidation of catechol with and without hydroquinone. Slope and intercept increased by same factor (1.7). *B*) Oxidation of two levels of chlorogenic acid. Greater inhibition at higher substrate concentration indicates other than competitive mode of inhibition.

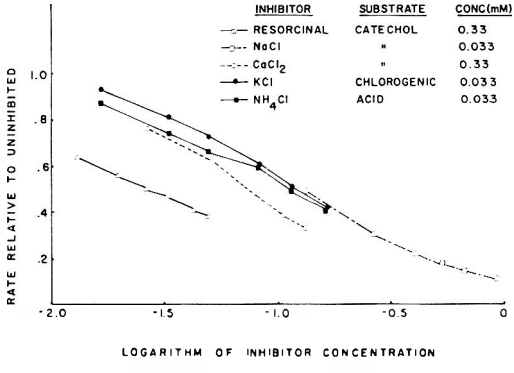

Fig. 5. Comparison of resorcinol and chloride ion as inhibitors of eggplant polyphenolase. Inhibitor concentrations are molar except for $CaCl_2$, which is calculated as chloride ion. The logarithm of inhibitor concentrations are used to facilitate graphing.

Table 3. Inhibition of eggplant polyphenolase by 1-phenyl-2-thiourea (PTU).

PTU concentration ($10^{-4}M$)	Catechol concentration $1.67 \times 10^{-3}M$		Catechol concentration $3.33 \times 10^{-3}M$	
	Rate[a]	% inhib.	Rate[a]	% inhib.
0	.173	0	.293	0
.167	.111	36	.196	33
.833	.055	68	.091	69
1.67	.039	77	.064	78
3.33	.021	88	.033	89
6.66	.014	92	.016	95
10.0	.009	95	.012	96

[a] Increase in absorbance per min.

carbamate (SDDC) strongly inhibited polyphenolases from both sources. The inhibition, by PTU at least, appeared noncompetitive on the basis of the plot of S/v vs. S shown in Fig. 6. Both intercept and slope

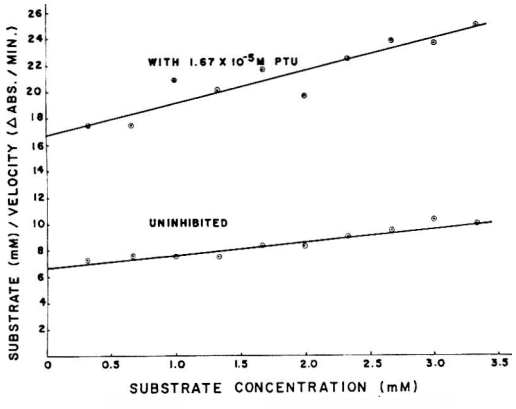

Fig. 6. Inhibition by 1-phenyl-2-thiourea of catechol oxidation by eggplant polyphenolase. Substrate and intercept both increased by a factor of 2.50.

were increased by the same factor (2.5), leading to a calculated K_i for PTU and eggplant polyphenolase of $1.1 \times 10^{-5}M$; the average value was $1.7 \times 10^{-5}M$.

These results are at variance with a previous report by Heymann *et al.* (1954) that PTU inhibition of catechol oxidation by potato phenoloxidase was largely, though not exclusively, competitive. These workers concluded that PTU might inhibit by competing for coordination sites on Cu. This seemed eminently reasonable, and caused the present finding to be viewed with reserve. The degree of inhibition by a competitive inhibitor should decrease at higher substrate concentrations. However, doubling the concentration of substrate did not lessen the degree of inhibition, as shown by the data in Table 3. It can be concluded that while PTU interferes with the ability of Cu to mediate electron transport between substrate and O_2, it does not interfere with the formation of enzyme-substrate complex in the same way that resorcinol does.

The instability of SDDC in solution made it impractical to use this inhibitor in a series of enzyme assays. However, a direct comparison was made between the inhibitory effects of SDDC and PTU on eggplant polyphenolase. The data presented in Table 4 indicate that almost ten times as much PTU as SDDC was needed in order to obtain the same inhibitory effect.

Table 4. Comparison of 1-phenyl-2-thiourea (PTU) and sodium diethyldithiocarbamate as inhibitors of eggplant polyphenolase.[a]

Inhibitor	Conc. ($10^{-5}M$)	Rate[b]	% Inhib.
None		.363	0
SDDC	0.33	.240	34
	0.66	.198	45
	1.67	.165	54
PTU	3.3	.232	36
	6.6	.188	48
	13.3	.166	54

[a] Enzyme 0.1 ml, catechol $0.0067M$, buffered at pH 5.0.
[b] Increase in absorbance per min.

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MASAO FUJIMAKI, NOBUHIKO ARAKAWA, AKIHIRO OKITANI, and OSAMU TAKAGI

Department of Agricultural Chemistry, University of Tokyo, Tokyo

The Changes of "Myosin B" ("Actomyosin") During Storage of Rabbit Muscle. II. The Dissociation of "Myosin B" into Myosin A and Actin, and its Interaction with ATP

SUMMARY

To elucidate the changes of "myosin B" during storage of rabbit muscle, investigation was made of the dissociation of "myosin B" into myosin A and actin, their content of "myosin B," and the interaction of "myosin B" with ATP. The approximate contents of myosin A and actin in "myosin B" were variable, showing a maximum content of actin in "myosin B" extracted from the muscle at 2 days after slaughter. This can also be inferred from changes of viscosity and the salting-out curve of "myosin B," as recently shown. Moreover, the interaction between actin and myosin A in "myosin B" became less strong with the progress of aging.

INTRODUCTION

Our previous report (Fujimaki *et al.*, 1965) showed that reduced viscosity, ATPase activity, ATP-sensitivity, salting-out curves, chromatographic diagrams on TEAE-cellulose column, and sedimentation diagrams among "myosin B" prepared from muscles differed considerably at different stages of aging.

Inferring that these differences might be mostly dependent on the content of myosin A and actin in "myosin B," their approximate contents in "myosin B" from the muscle during aging were determined.

It has been generally accepted that myosin B extracted from muscle with Weber-Edsall solution is essentially a complex of actin and myosin A.

Earlier results (Dainty *et al.*, 1944) showing a decrease in the viscosity and flow birefringence of myosin B solution on addition of ATP were interpreted in terms of the dissociation of actomyosin into actin and myosin A. However, the light-scattering measurements of Blum and Morales (1953), suggesting elongation of myosin B particles on the addition of ATP without any apparent change in the molecular weight, cast a serious doubt on the validity of the above view and prompted a series of investigations of this subject.

Weber (1956) demonstrated the separation of free myosin A from myosin B on the addition of ATP, but her results concerning the simultaneous liberation of F-actin were not quite conclusive. Gergely (1956) and Gergely and Kohler (1957), in light-scattering experiments, found that the molecular weight of both reconstituted and natural actomyosins decreased on the addition of ATP or pyrophosphate, suggesting the dissociation of actomyosin into actin and myosin. Moreover, Martonisi *et al.* (1960) pointed to the liberation of F-actin from natural or reconstituted actomyosin in the presence of pyrophosphate when fluorescent or radioactive materials were used to label the actin component.

Laki *et al.* (1952) postulated that such an agent as ATP under reduced temperature dissociated actomyosin by shifting to the left the position of the following equilibrium, F-actin + myosin A $\rightleftharpoons$ actomyosin, the hypothesis having not been universally accepted.

Further, Johnson and Rowe (1962), in ultracentrifugal studies, concluded that the F-actin combined into actomyosin was not normally liberated into solution as F-actin upon dissociation of the actomyosin, and the actin moiety might appear in three forms, depolymerized actin, G-actomyosin, and gel actin. The complexity and polydispersity of actomyosin solutions render hazardous the interpretation of results obtained by many physicochemical methods.

The analytical ultracentrifuge is a particularly useful tool for such a system, in which, even if a gel material occurs, the sedimentation diagram often enables the various species to be characterized by the sedimentation rates. However, since it is quite erroneous to calculate the relative content of myosin A and actin in "myosin

B" from the area measurement of sedimentation diagrams in an analytical ultracentrifuge and, moreover, no peaks corresponding to F-actin seem to appear, the approximate content of myosin A in "myosin B" was determined by the method of Weber (1956), and that of actin by the method of Maruyama and Gergely (1962b).

In addition, since Kominz *et al.* (1954) pointed out that one of the obvious differences between actin and myosin A was the amino acid composition, that of "myosin B" from the muscle during aging was also analyzed.

Moreover, the changes of the strength of the actin-myosin A interaction in "myosin B" were inferred by the modified method of Weber (1956) with varying ATP concentration

These results are presented in this paper.

EXPERIMENTAL

Preparation of "myosin B." "Myosin B" was prepared by extracting ground rabbit muscle (longissimus dorsi, immediately after slaughter [myosin B], and stored at about 4°C for 2 or 7 days after slaughter ["myosin B"]) with Weber-Edsall solution, purified tarice by the dilution-precipitation method, and successively clarified by ultracentrifugation for 1 hr at 35,000 × G.

Preparation of myosin A. Myosin A was prepared by extracting ground rabbit muscle with Hasselbach and Schneider solution (1951), purifying by the method of Perry (1955), and successively clarifying by ultracentrifugation for 1 hr at 35,000 × G.

Preparation of F-actin. G-actin was extracted at low temperature from the acetone-dried muscle (prepared from the residue after myosin A was extracted with Hasselbach and Schneider solution) by the method of Drabikowski and Gergely (1962). Crude extracts were polymerized with KCl (final concentration of 0.1*M*) for 2 hr at room temperature, and crude F-actin was sedimented in the preparative ultracentrifuge for 2 hr at 70,000 × G. Then, G-actin was obtained from F-actin pellets by dialysis with stirring for 40 hr against 2×10^{-4} *M* ATP solution at pH 8.0, and clarification by centrifugation for 30 min at 18,000 × G. It was transformed to F-actin by polymerization in 0.1*M* KCl + 1m*M* $MgCl_2$.

Content of myosin A. The approximate content of myosin A in "myosin B" from the muscle during aging was determined by the method of Weber (1956). "Myosin B" preparations (0.6*M* KCl, 1m*M* $MgCl_2$, 20m*M* tris-maleate buffer, pH 7.0) containing 5m*M* ATP were centrifuged for 3 hr in an average gravitational field of 100,000 × G at 5°C. The initial protein concentration ranged from 0.3 to 0.4%. After centrifugation, the tubes contained a water-clear solution and a small dense pellet. Centrifugation of "myosin B" solution in the absence of ATP as control resulted in a quite different appearance. Here, a water-clear supernatant was separated by a sharp boundary from a turbid solution in the lower third of the tube and a large opaque precipitate was formed at the bottom. This water-clear supernatant has been recognized as myosin A in "myosin B" as an inseparable contaminant. On centrifugation after the addition of ATP, the upper half of the supernatant contained 20–30% of the total amount of the protein present. It has been confirmed by Weber that this protein exhibited properties characteristic of L-myosin, not those of "myosin B." Therefore, the twofold values of the protein concentration in the upper half of the supernatant were reckoned as the relative amount of the dissociated myosin A. Since Weber has reported that, on centrifugation of L-myosin itself under the same condition, the protein concentration in the supernatant was 70% of the original, the approximate content of myosin A dissociated from "myosin B" on the addition of ATP was calculated from the protein content in the supernatant, using the corrected value by Weber.

Content of actin. The approximate content of actin in "myosin B" was measured by the method of Maruyama and Gergely (1962b). "Myosin B" preparation (0.05*M* KCl, 1m*M* $MgCl_2$, 10m*M* tris-maleate buffer, pH 7.0) containing 1m*M* ATP and 1×10^{-4} *M* PES (polyethylene sulfonate as interaction inhibitor) was incubated for 10 min at 25°C after addition of ATP, and was centrifuged for 5 min at 10,000 × G. After centrifugation the protein concentration in the supernatant was determined by the method of Lowry *et al.* (1951). Bárány and Jaisle (1960) found PES as one of a group of charged compounds that caused the inhibition of actomyosin formation in the presence of small amount of ATP. Maruyama and Gergely, (1962b), in the experiment of the ATP-PES system at low ionic strength, showed that, although myosin A was precipitated and actin became soluble, about 90% of constituted actin in actomyosin dissolved in the supernatant. Accordingly, the approximate content of actin in "myosin B" was calculated from the protein content in the supernatant, using the corrected value by Maruyama and Gergely (1962b).

Adenosine triphosphatase assay. ATPase assay was carried out at 25°C by the modified method of Maruyama and Ishikawa (1963). The final

incubation mixture usually contained: 1m*M* ATP, 1m*M* $MgCl_2$, 10m*M* tris-maleate buffer (pH 8.0), and 0.14*M* KCl. ATPase activity was expressed as μg of phosphorus liberated in 1 min by 1 mg of protein.

Protein concentration. Protein concentration was determined by biuret or Lowry's method, standardized by micro-Kjeldahl procedure.

Amino acid content. Hydrolysis was carried out for 18 hr in 6*N* HCl at 120°C in sealed glass ampoules. The hydrolysate was filtered to remove the humin, evaporated under vacuum to dryness, and redissolved in water. The analysis was carried out with an Amino Acid Analyzer (Shibata Chem. Co., Ltd, Tokyo).

Strength of the actin-myosin A interaction. The strength of the actin-myosin A interaction in "myosin B" from the muscle during aging was measured with varying ATP concentration for the dissociation of "myosin B." "Myosin B" preparation (0.6*M* KCl, 1m*M* $MgCl_2$, 20m*M* tris-maleate buffer, pH 7.0) containing various concentrations of ATP (0.01–5m*M*) was centrifuged for 3 hr at 5°C in an average gravitational field of 100,000 × G, similarly as in Tables 1 and 2. The protein concentration in the supernatant was determined and reckoned as the relative amount of the dissociated myosin A.

RESULTS AND DISCUSSION

Approximate content of myosin A in "myosin B." As shown in Tables 1 and 2, myosin A content in "myosin B" seemed to be maximum from the muscle immediately after slaughter, to decrease at 2 days after slaughter, and then successively to increase at 7 days after slaughter. The myosin A content dissociated from "myosin B" on the addition of pyrophosphate is shown in parentheses, and the results were similar to those obtained with the addition of ATP.

However, from the result of the determination of the amount of the dissociated myosin A, it remained doubtful whether myosin A content in "myosin B" from the muscle during aging varied actually or it appeared to vary because of the differences in the degree of dissociation. Accordingly, the content of actin in "myosin B" from the muscle during aging was determined.

Approximate content of actin in "myosin B." As shown in Table 3, the actin content in "myosin B" seemed to be minimum from the muscle immediately after slaughter, to increase at 2 days after slaughter, and then successively to decrease at 7 days after slaughter. This result showed precisely the reverse one of Table 2. Moreover, each sum of the actin (shown in Table 3) and the myosin A contents (in Table 2) in "myosin B" from the muscle during aging showed a value of approximately 100%.

Therefore, it was possibly conceivable that the variations of the amounts of actin and myosin A dissociated from "myosin B" were not due to the change of the degree of dissociation, but to the differences of the contents of actin and myosin A in "myosin B" from the muscle during aging. To obtain further evidence for this fact, the binding ratio of F-actin to myosin A was investigated.

Table 1. Ultracentrifugal separation of myosin A dissociated from "myosin B"[a] in the presence of ATP or pyrophosphate.

	Storage period (days)		
	0	2	7
"Myosin B" (mg)	35.2±0.7	44.2±0.8	38.3±0.6
Protein in the supernatant[b] (mg)	19.2±0.6 (20.0±0.6)	19.3±0.5 (18.2±0.5)	19.9±0.5 (19.9±0.5)
$\frac{\text{Protein in the supernatant}}{\text{"Myosin B"}} \times 100\%$	54.8±2.7 (56.8±2.8)	43.6±2.2 (41.2±2.1)	51.9±2.5 (51.9±2.5)
$\frac{\text{Myosin A}^{c}}{\text{"Myosin B"}} \times 100\%$[d]	78.3±3.8 (81.3±4.0)	62.3±3.1 (58.8±3.0)	74.2±3.6 (74.2±3.6)

The figures in parentheses show the values in the presence of pyrophosphate.
[a] Prepared from the muscle (sample A) immediately after slaughter and 2, 7 days post-mortem.
[b] Calculated from the twofold value of the protein content in the upper half of the supernatant.
[c] Calculated from the protein content in the supernatant by the method of A. Weber (1956).
[d] The values considered from the biological variation were 75.9±2.5 immediately after slaughter (0 day), 61.5±3.9 at 2 days, and 70.6±4.2 at 7 days after slaughter.

Table 2. Ultracentrifugal separation of myosin A dissociated from "myosin B"[a] in the presence of ATP.

	Storage period (days)		
	0	2	7
"Myosin B" (mg)	41.3±0.7	35.2±0.8	37.0±0.9
Protein in the supernatant (mg)	21.2±0.6	15.8±0.4	17.2±0.3
$\frac{\text{Protein in the supernatant}}{\text{"Myosin B"}} \times 100\%$	51.4±2.5	45.0±2.2	46.5±2.1
$\frac{\text{Myosin A}}{\text{"Myosin B"}} \times 100\%$	73.4±3.6	64.3±3.1	66.4±3.0

[a] Prepared from the muscle (sample B) immediately after slaughter and 2, 7 days post-mortem.

Binding ratio of F-actin to myosin A. The binding ratio of F-actin to myosin A, prepared from the muscle immediately after slaughter and at 8 days after slaughter, was estimated from the effect of F-actin on the ATPase activity of the reconstituted actomyosin and the relative protein concentration of the supernatant (pH 8.0, $\mu = 0.2$) in the reconstitution of myosin A and F-actin. The results are shown in Fig. 1.

When F-actin was added to a constant amount of myosin A, ATPase activity of the reconstituted actomyosin increased up to a definite ratio of F-actin to myosin A, essentially proportional to the amount of actin added, and on further addition of actin its activity remained at a definite level. The binding ratio of F-actin to myosin A prepared from the muscle immediately after slaughter, which showed the highest ATPase activity, was 0.26:1, corresponding nearly to the optimal ratio at 0.1 μ for superprecipitation found earlier by Spicer and Gergely (1951), and to the ratio found by Szent-Györgyi (1951), who produced a maximal viscosity response on addition of ATP at 0.6 μ, and moreover to the ratio confirmed by Maruyama and Gergely (1962a), who investigated the effect of F-actin on the ATPase activity of myosin. However, the binding ratio of F-actin to myosin A prepared from the muscle at 8 days after slaughter was 0.31:1.

From these results, the binding ratio was found to vary with aging and corresponded to that in "myosin B" obtained from the results of the dissociation of "myosin B" shown in Tables 1–3. Therefore, it was most likely that the contents of actin and myosin A in "myosin B" from the muscle during aging were variable.

Amino acid analysis. Kominz *et al.* (1954) pointed out that one of the obvious differences between actin and myosin A was amino acid composition, the content of proline being the most remarkable one, and, on the other hand, the content of arginine being almost same for both proteins.

Table 3. The amount of actin dissociated from "Myosin B"[a] in the ATP-PES[b] system.

	Storage period (days)		
	0	2	7
"Myosin B" (mg)	6.13±0.12	5.22±0.08	5.50±0.09
Protein in the supernatant (mg)	1.27±0.10	1.33±0.05	1.37±0.06
$\frac{\text{Protein in the supernatant}}{\text{"Myosin B"}} \times 100\%$	20.8 ±2.1	25.3 ±1.3	24.0 ±1.5
$\frac{\text{Actin}^{c}}{\text{"Myosin B"}} \times 100\%$	23.1 ±2.7	28.1 ±1.4	27.2 ±1.7

[a] Prepared from the muscle (sample B) immediately after slaughter and 2 and 7 days post-mortem.
[b] Polyethylene sulfonate (interaction inhibitor).
[c] Calculated from the protein content in the supernatant by the method of Maruyama and Gergely (1962).

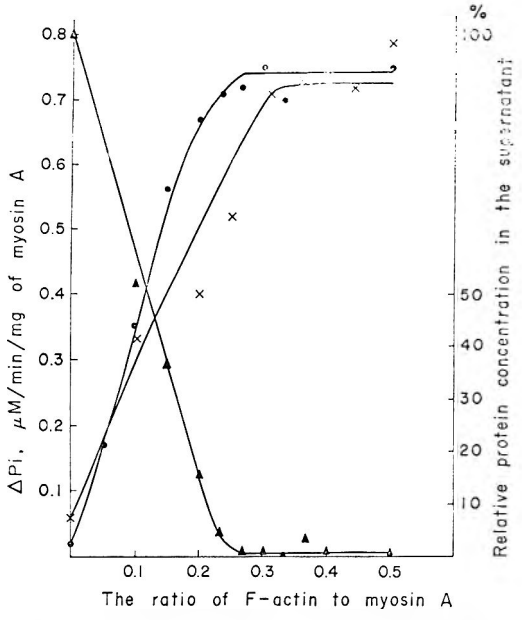

Fig. 1. Effect of F-actin on the reconstitution of actomyosin from myosin A and the ATPase activity of the reconstituted actomyosin
▲ Relative protein concentration of the supernatant (pH 8.0, 0.2 μ) in the reconstitution of myosin A and F-actin prepared from the muscle immediately after slaughter
● ATPase activity of the actomyosin reconstituted from myosin A and F-actin prepared from the muscle immediately after slaughter
× ATPase activity of the actomyosin reconstituted from myosin A and F-actin prepared from the muscle at 8 days after slaughter

The molar ratio of proline to arginine was 0.53 for myosin A, 1.15 for actin (Kominz *et al.*, 1954), and 0.74 for myosin B (Noda and Maruyama, 1960), but this last value may be slightly too high. Calculating from the ratio of actin to myosin A in myosin B as 1:3.7, the molar ratio of proline to arginine for myosin B amounted to 0.66. The ratios for "myosin B" from the muscle during aging were 0.60 immediately after slaughter, 0.70 at 2 days, and 0.64 at 7 days after slaughter (Table 4). These values may be slightly low, but the results obtained corresponded satisfactorily with those in Tables 1–3 and Fig. 1. From all the above-mentioned results, therefore, it could be concluded that there existed considerable differences in the contents of actin and myosin A in "myosin B" from the muscle during aging.

Interaction of "myosin B" with ATP. As shown in Fig. 2, myosin A was fully dissociated with 0.6mM, 0.2mM, or 0.1mM (considerably even with 0.05mM) concentration of ATP for myosin B from the muscle immediately after slaughter, at 2 days, or at 7 days after slaughter, respectively.

This result may show that the interaction between actin and myosin A in "myosin B" from the muscle during aging changes generally with the progress of aging.

Post-rigor changes of muscle associated with the increasing tenderness have been widely studied for many years, but the mechanism of the post-rigor tenderization is not well elucidated. Many researchers have often attempted to demonstrate whether the increase in tenderness is due to proteolysis by the enzymes in the tissue. But, under the aging conditions usually applied (low temperature, limited time, good hygienic conditions), the proteolytic changes may be extremely small. Accordingly, proteolysis seemed not to play an important part in post-mortem aging. Though it may be assumed that the changes in tenderness brought about by aging are due to conversions of the connective tissue, Steiner (1939) and Whitaker (1959) came to the conclusion that changes in connective tissue did not appear to be closely associated with aging.

It has been generally accepted that it is impossible to expect meat tenderization due to dissociation of the actomyosin complex into actin and myosin A during the resolu-

Table 4. Molar ratio of proline to arginine in "myosin B."[a]

Myosin B from the muscle immediately after slaughter	0.60	
"Myosin B" from the muscle at 2 days after slaughter	0.70	
"Myosin B" from the muscle at 7 days after slaughter	0.64	
Myosin A	0.53[b]	
Actin	1.15[b]	
Myosin B	0.74[c]	0.66[d]

[a] Prepared from the muscle (sample C) immediately after slaughter and 2, 7 days post-mortem.
[b] Kominz *et al.* (1954).
[c] Noda *et al.* (1960).
[d] Calculated from the ratio of actin to myosin A as 1:3.7.

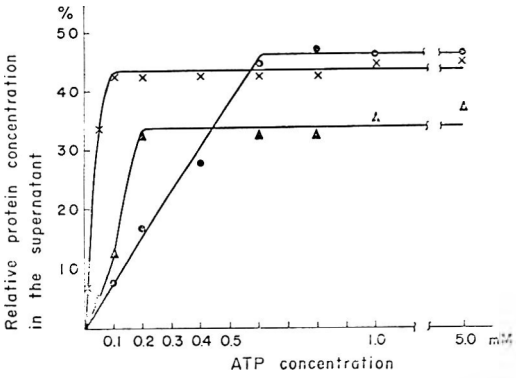

Fig. 2. The dissociation of "myosin B" in the presence of ATP.
- ● myosin B prepared from the muscle immediately after slaughter
- ▲ "myosin B" prepared from the muscle at 2 days after slaughter
- × "myosin B" prepared from the muscle at 7 days after slaughter

tion of rigor, from considering the rapid phase of ATP breakdown immediately after slaughter, and that, probably, the increased hydration of protein (caused principally by the increase in pH and movements of cations) may influence meat tenderization. But, coming back to the sliding-filament model, another possibility may explain the tenderness change, at least in part. It is well known that relaxed muscles are more tender than partly contracted muscles (Locker, 1960). Partmann (1963) stated that it might be possible to get a tender meat from dissociation of the actomyosin complex or if the association of actin and myosin during rigor development is impeded fully or partly. Weinberg and Rose (1960) expected that actomyosin formed during rigor development was dissociated into actin and myosin, though Bendell (1963) emphasized that rigor mortis was never resolved unless by bacterial decomposition.

Those researchers discussed only the possibility of the dissociation of actomyosin into actin and myosin, but the authors found that there was the possibility of the dissociation of the actomyosin formed during rigor development even with the residual amount of ATP from the decrease of the interaction between actin and myosin A in "myosin B," as shown in Fig. 2, although further investigation in detail would be needed. This inference is supported by the electron microscopic study of Takahashi *et al.* (1965) indicating that the myofibril prepared from the muscle in isometric state at the stage of the resolution of rigor had the phase of the relaxing band.

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C. E. BODWELL,[a] A. M. PEARSON, and R. A. FENNELL
Departments of Food Science and Zoology, Michigan State University, East Lansing, Michigan 48823

Post-Mortem Changes in Muscle III. Histochemical Observations in Beef and Pork

SUMMARY

The activity of 17 or 18 specific enzymes in the longissimus dorsi muscle of five beef and 18 pork carcasses was followed by histochemical procedures. Beef samples were removed from carcasses within 10 min up to 20 days port-mortem, and pork samples within 16 min up to 24 and/or 48 hr post-mortem. The beef carcasses were submitted to so-called normal cooling procedures. However, one side of each of 13 pork carcasses was placed at −29°C, while the other side was subjected to 37°C for the first 4½–5 hr post-mortem.

In beef muscle, the histochemical activity of lactate dehydrogenase, alpha-glycerophosphate dehydrogenase, succinate dehydrogenase, isocitrate dehydrogenase, TPN diaphorase, and DPN diaphorase showed a steady decrease with increasing time post-mortem. Reactions for both alcohol dehydrogenase and glutamate dehydrogenase were very weak or entirely absent at 48 hr post-mortem and all subsequent sampling periods. Positive reactions for glucose-6-phosphate dehydrogenase and beta-hydroxybutyrate dehydrogenase were observed in the initial samples only. No acid phosphatase, leucine amino peptidase, or 6-phosphogluconate dehydrogenase activity was detected in any of the samples.

In pork muscle, the activity of lactate dehydrogenase, alpha-glycerophosphate dehydrogenase, succinate dehydrogenase, isocitrate dehydrogenase, and DPN diaphorase was slightly weaker at both 0 and 24 hr post-mortem than similar activity in beef muscle. Alcohol dehydrogenase activity was weaker than that observed for the above enzymes, while the activity of glutamate dehydrogenase was of intermediate intensity. Traces of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity were observed in only a portion of the initial samples. Similarly, weak reactions for beta-hydroxybutyrate dehydrogenase were observed only at 0 hr post-mortem. None or only weak activity was observed for malic dehydrogenase, and TPN diaphorase activity was unexplainably absent from all samples. Moderate levels of cytochrome oxidase activity were observed at both 0 and 24 hr post-mortem. UDPG-glycogen transferase was completely inactivated by the 37°C treatment post-mortem, but the treatment had a less marked effect upon phosphorylase and branching enzyme. The presence of acid and alkaline phosphatase activity in muscles of 7 carcasses suggested that a degenerative condition existed in these muscles.

INTRODUCTION

Although data are available on various post-mortem chemical and physical changes in both pork and beef muscle (Marsh, 1954; Howard and Lawrie, 1956, 1957; Wismer-Pedersen, 1959; Whitaker, 1959; Swift *et al.*, 1960; Lawrie, 1962; Bendall, 1962; Sharp, 1962; Bendall *et al.*, 1963; Partmann, 1963; Briskey, 1963, 1964; Goll *et al.*, 1964; Bodwell *et al.*, 1965, 1966), causal factors have been relatively unexplored. Post-mortem activity of the oxidative and respiratory enzymes in muscle would appear to be potentially relevant. Andrews *et al.* (1952) studied ATP-ase, succinic dehydrogenase, aldolase, and total glycolytic activity in beef muscle at 48 hr, 2 weeks, and 4 weeks post-mortem. They found that ATP-ase, succinic dehydrogenase, and total glycolytic activity did not decrease on storage, but aldolase activity decreased to about two-thirds of its 48-hr value at 2 wk and to one-half at 4 wk. Thus, they concluded that the limiting factor in post-mortem muscle metabolism was the lack of adequate substrates.

British studies (Scopes and Lawrie, 1963; Lawrie *et al.*, 1963; Scopes, 1964) have demonstrated that creatine kinase and several unidentified enzymes of sarcoplasmic origin are labile under conditions of low pH and high muscle temperature in beef, pork, and rabbit. Likewise, Charpentier and Goutefonga (1964) noted differences in the electrophoretic patterns of lactic dehydrogenase from normal and from soft, watery pork muscle.

[a] Present address: Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Disease, National Institutes of Health, Bethesda, Maryland.

Histochemical observations in beef and pork muscle are virtually limited to the investigations of Ogata and Mori (1963, 1964), who utilized histochemical techniques to observe the activity of some 6 enzymes in unspecified muscles. The present study was undertaken to follow histochemically the activity of a number of different enzymes in the longissimus dorsi muscle of beef and pork carcasses from immediately after death through various post-mortem storage periods.

EXPERIMENTAL

Animals. Five beef carcasses and 18 pork carcasses were utilized. The treatment of these carcasses, as well as complementary chemical and physical data thereon, have been reported (Bodwell *et al.*, 1965a,b).

Histochemical procedures. All beef samples were removed within 10 min of death. Immediately after removal from the carcass, samples for histochemical analysis were frozen in isopentane cooled to approximately −158°C with liquid nitrogen, wrapped in Handi-Wrap, and stored at −29°C. The frozen blocks of tissue were trimmed and mounted on chucks with distilled water in the cold (−29°C), and sections 10–12 μ thick were cut on a Slee-Pearse cryostat at −16°C within one week of sampling. The sections were mounted directly onto coverslips.

TPN and DPN diaphorase activity were observed by methods of Nachlas *et al.* (1958a,b). Acid and alkaline phosphatases and thiamine pyrophosphatase were detected by using lead nitrate methods as described by Pearse (1960). Mg^{++}-activated and Ca^{++}-activated ATP-ase were respectively observed by the procedures of Wachstein and Meisel (1957) and Padykula and Herman (1954). Leucine amino peptidase and succinic dehydrogenase activity were respectively observed by the methods of Nachlas *et al.* (1957, 1960).

DPN- and TPN-linked dehydrogenase activity were detected according to procedures of Hess *et al.* (1958) and Nachlas *et al.* (1958a,b) as modified by Pearse (1960). MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) was used as the electron acceptor for beta-hydroxybutyrate, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases. Similarly, Nitro BT (nitro blue tetrazolium chloride) was used in the detection of isocitrate, glutamate, alpha-glycerophosphate, alcohol, and lactate dehydrogenases.

The activity of glucose-6-phosphate dehydrogenase was also observed in the initial samples with a Nitro BT procedure in which sections were incubated for 30 min at 37°C in a reaction mixture containing 0.1 ml di-Na-glucose-6-phosphate (1.0*M*, pH 7.0), 0.1 ml 0.1*M* amytal (pH 7.0), 0.25 ml Tris-buffer (0.2*M*, pH 6.8–7.0), 0.05 ml of a 1 mg/ml Nitro BT solution (pH 7.0), 0.05 ml 0.01*M* NaF (pH 7.0), 0.1 ml 0.1*M* TPN, 0.1 ml 0.05*M* $MgCl_2$, and 75 mg polyvinyl pyrrolidone. The sections were fixed in 10% formol-saline or formol-calcium for 10 min, and mounted in glycerin jelly.

The methods described for beef were utilized to observe TPN and DPN diaphorase, as well as isocitrate, glutamate, beta-hydroxybutyrate, succinate, alpha-glycerophosphate, alcohol, and lactate dehydrogenase activity in the initial and 24-hr-post-mortem samples of pork carcasses 1–10 and 11Y. Glucose-6-phosphate dehydrogenase activity was observed by the Nitro BT method described for beef muscle. An analogous Nitro BT method was also used to observe the activity of 6-phosphogluconate dehydrogenase. Malate dehydrogenase and cytochrome oxidase activity were respectively detected by methods of Nachlas *et al.* (1958a) and Burstone (1960). The modified coupling azo dye and the naphthol AS-BI phosphate methods as described by Pearse (1960) were respectively used to detect alkaline and acid phosphatase activity in carcasses 1–5. The activity of phosphorylase, BE, and UDPG-glycogen transferase was observed on some of the initial samples of carcasses 6–10 according to the methods of Takeuchi (1958) and Takeuchi and Glenner (1960).

With pork carcasses A-G, only four, and in some cases five, enzymes were studied. These included phosphorylase, BE, and UDPG-glycogen transferase, as well as succinic dehydrogenase and/or glutamate dehydrogenase. The activity of these enzymes was observed on all the initial samples at approximately 3 hr post-mortem and on a limited number of samples removed 24 hr after death.

Sections from all samples removed 3 hr post-mortem and on a limited number of 24-hr samples of carcasses A-G were subjected to an HCl-Orange G-Anilin Blue staining procedure as modified by Guyer (1953). Sections were fixed 10 min in 10% formalin, stained 6 min in acid ironhematoxylin, washed in distilled water, rinsed in 1% acetic acid, stained in anilin blue-orange G-HCl solution, washed in 3 changes of acetic acid, dehydrated, and cleared by an acetone-xylene sequence and mounted in Permount.

In all cases, two or more sections were treated as outlined above and as specified for both beef or pork. Additional sections were utilized for substrate blanks, i.e., reaction mixture containing no substrate. The diazotate of 5-chloro-*O*-toluidine

was obtained from Bordon Co., Chemical Division; rabbit muscle glycogen and thiamine pyrophosphate (Cl) from Mann Research Laboratories; and 1-hydroxy-2-naphthoic acid and *N*-phenyl-*p*-phenylene diamine from Eastman Kodak Co. All other chemicals were obtained from Sigma Chemical Co.

Over 1200 and 1350 mounted sections of beef and pork muscle resulted from application of the above procedures. The intensity of the reactions was evaluated by microscopic observation and rated as: −(none), ±(traces), +(weak), ++(moderate), +++(moderately strong), and ++++(strong).

Photomicrographs were made of representative sections with a Leitz Dialux microscope equipped with a Kodak colorsnap 35-mm camera and a Vickers Instruments photo-multiplier and automatic integrating timer unit. Ansco Veraspan (135 mm, A.S.A. No. 125) film was used.

RESULTS AND DISCUSSION

Observations in beef muscle. The relative activity of the 17 enzymes studied at 0 and 480 hr is tabulated in Table 1. The differences in intensity of enzyme activity observed between small red, large white, and intermediate fibers were in agreement with established patterns of distribution (Dubowitz and Pearse, 1960, 1961; Stein and Padykula, 1962).

Glycolytic enzymes. Photomicrographs of typical reactions at 0, 48, and 480 hr post-mortem for lactate dehydrogenase, alpha-glycerophosphate dehydrogenase, and alcohol dehydrogenase are shown in Fig. 1. Of these three glycolytic enzymes, lactate dehydrogenase and alpha-glycerophosphate dehydrogenase were more uniformly reactive at all times post-mortem than alcohol dehydrogenase. Alcohol dehydrogenase activity was highly variable from carcass to carcass, especially with increasing periods post-mortem. Furthermore, beyond 24 hr post-mortem, reactions for alcohol dehydrogenase were usually either very weak or completely absent (Fig. 1, III B and C).

Enzymes of the TCA cycle. Typical isocitrate dehydrogenase, succinate dehydrogenase, and glutamate dehydrogenase reactions observed in samples 0, 48, and 480 hr post-mortem are shown in Fig. 2.

Table 1. Relative enzyme activity in longissimus dorsi muscle from beef.

Enzyme	Activity[a] 0 hr	480 hr
Glycolysis		
Lactate dehydrogenase	++ to ++++	++
α-glycerophosphate dehydrogenase	++ to +++	+ to +++
Alcohol dehydrogenase	+ to +++	+ to ++
TCA cycle		
Isocitrate dehydrogenase	+ to ++++	+ to ++++
Succinate dehydrogenase	++ to +++	+ to ++
Glutamate dehydrogenase	+ to ++	− to ±
Electron transport		
DPN diaphorase	>+++	+++
TPN diaphorase	>+++	+ to +++
Hexose monophosphate cycle		
Glucose-6-phosphate dehydrogenase	− to +	−
6-phosphogluconate dehydrogenase	−	−
Fatty acid oxidation		
Beta-hydroxybutyrate dehydrogenase	± to ++	−
Others		
Acid phosphatase	−	−
Alkaline phosphatase	+++	+++
ATP ase (Ca^{++})	++++	++++
ATP ase (Mg^{++})	++++	++++
TPP ase	++	++
Leucine amino peptidase	−	−

[a] The following code was used for expressing the amount of activity: − none, ± traces, + weak, ++ moderate, +++ moderately strong, ++++ strong.

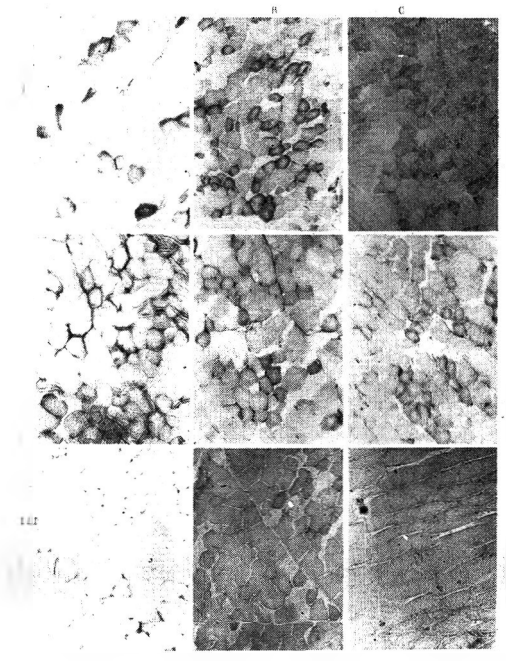

Fig. 1. Photomicrographs showing activity of three glycolytic enzymes from beef muscle. Lactate (I), alpha-glycerophosphate (II), and alcohol dehydrogenase (III) at 0 (A), 48 (B), and 480 (C) hr post-mortem (×225).

Under post-mortem conditions, isocitrate dehydrogenase appeared to be the most active enzyme of this group as well as the most stable (Table 1). Succinate dehydrogenase activity was detected at all times post-mortem but was highly variable in intensity. The lower level of glutamate dehydrogenase activity detected at 0 hr as compared to that observed for succinate dehydrogenase is in agreement with the observations on beef muscle of Ogata and Mori (1964). Reactions for glutamate dehydrogenase were very weak or absent at 48 hr post-mortem and all subsequent sampling times (Fig. 2, III B and C).

Other enzymes. Fig. 3 shows reactions for TPN and DPN diaphorase at 0, 48, and 480 hr post-mortem, beta-hydroxybutyrate dehydrogenase at 0 hr, and glucose-6-phosphate dehydrogenase at 0 hr. The two diaphorases were both generally quite active throughout 480 hr post-mortem. However, the DPN diaphorase reactions were uniformly stronger than the corresponding TPN reactions (Table 1).

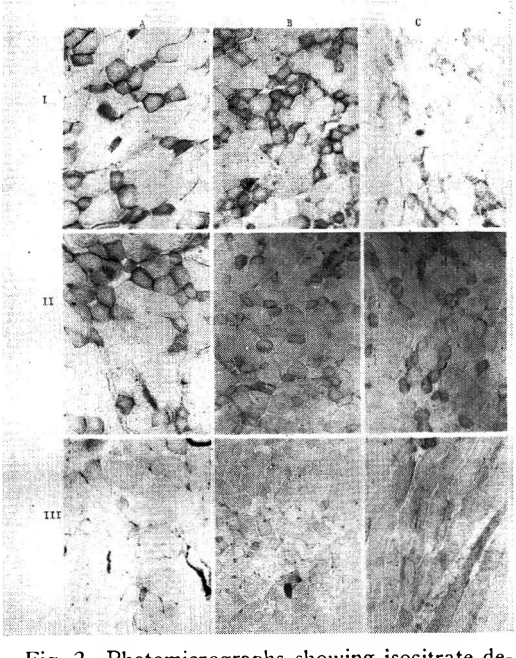

Fig. 2. Photomicrographs showing isocitrate dehydrogenase (I), succinate dehydrogenase (II), and glutamate dehydrogenase (III) activity of beef muscle at 0 (A), 48 (B), and 480 (C) hr post-mortem. (×225).

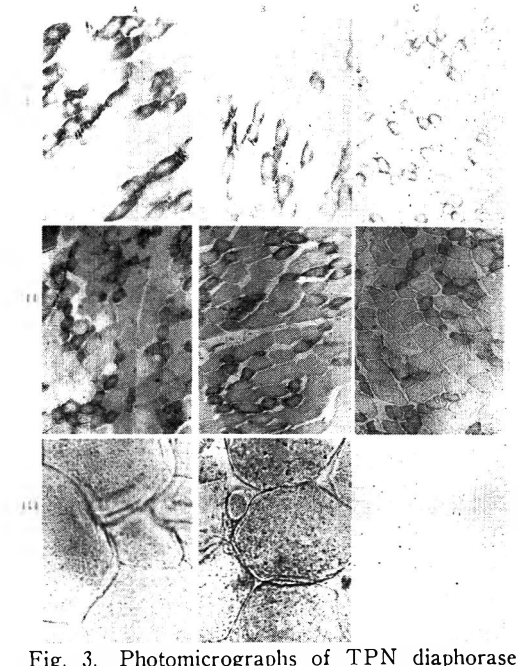

Fig. 3. Photomicrographs of TPN diaphorase (I) and DPN diaphorase (II) activity at 0 (A), 48 (B), and 480 (C) hr post-mortem and glucose-6-phosphate dehydrogenase (III-A) and beta-hydroxybutyrate dehydrogenase (III-B) at 0 hr post-mortem in beef (×225).

Muscle from two of the five beef carcasses studied showed a weak glucose-6-phosphate dehydrogenase activity at 0 hr (Fig. 3, III A). Very weak to moderate reactions were observed for beta-hydroxybutyrate dehydrogenase at 0 hr (Fig. 3, III B). However, no activity was detected for these enzymes in either the 24-hr-post-mortem or subsequent samples. This would suggest that these enzymes are more labile under post-mortem conditions than the majority of the enzymes in the current study (Table 1).

The presence of detectable glucose-6-phosphate dehydrogenase activity was unexpected in view of work of Ogata and Mori (1964). Those investigators did not detect any activity in normal mouse, cat, or human muscle. However, the activity detected for this hexose monophosphate shunt enzyme in the current study was confirmed by the Nitro BT procedure previously described. It is of interest that no activity was observed for another hexose monophosphate cycle enzyme, 6-phosphogluconate dehydrogenase (Table 1). The latter may be less stable immediately after death than glucose-6-phosphophosphate dehydrogenase. The presence of beta-hydroxy-butyrate dehydrogenase activity supports the suggestion of Howard and Lawrie (1956) that fatty acid oxidation may play an important role in the metabolism of beef muscle.

Acid phosphatase and leucine amino peptidase activity was not detected in any of the muscles from the five carcasses (Table 1). These results are in agreement with results of Ogata and Mori (1964), who found no acid phosphatase or leucine amino peptidase activity in normal human, cow, pig, cat, or mouse muscle.

The methods used to detect alkaline phosphatase, the two ATP-ases, and TPP-ase resulted in moderate to strong reactions for all samples studied. Activities were similar throughout the post-mortem period (Table 1). However, the Gomori (1952) method, used for alkaline phosphatase, resulted in a very diffuse general reaction and consequently had no significance.

General observations. In general, the enzymes of the glycolytic and TCA cycles and of the electron transport system, which were observed in the current study, exhibited a steady decrease in activity with increasing time post-mortem. Results support the observation of Andrews *et al.* (1952) that the lack of substrates is the limiting factor in post-mortem metabolism in beef muscle. However, the possible lack of activity of enzyme(s) other than those studied here by the above workers cannot be precluded. The post-mortem breakdown of residual glycogen can be performed by an alternate system to the glycolytic cycle (Sharp and Rolfe, 1958; Sharp, 1962). The latter researchers concluded that the alternate system resulted in the accumulation of glucose and glucose-6-phosphate post-mortem. That observation has been supported by previously reported findings in the current study (Bodwell *et al.*, 1965, 1966). Whether use of such an alternate system of glycogen degradation as well as the accumulation of intermediates post-mortem is due to the inactivity of some enzyme(s) in the glycolytic cycle or whether the functioning of the latter is merely prohibited by the lack of a readily available energy source (i.e., adenosine triphosphate) has not been established.

Observations in pork muscle. The relative activities at 0 and 24 hr post-mortem of 15 of the enzymes studies in carcasses 6 through 10 and 11Y are shown in Table 2. In general, the variation observed in activity at 24 hr post-mortem did not appear to be related to the temperature treatment and/or to the pH level of a given muscle at the time of removal of a given sample.

TCA cycle enzymes. Succinate dehydrogenase, isocitrate dehydrogenase, and glutamate dehydrogenase exhibited similar decreases in activity between 0 and 24 hr post-mortem (Table 2). Photomicrographs of typical reactions obtained for isocitrate dehydrogenase and succinate dehydrogenase at 0 hr and 24 hr post-mortem from carcasses subjected to the −29°C and 37°C treatments are shown in Fig. 4. However, malate dehydrogenase was observed to be either weakly active or completely inactive at 0 hr post-mortem. In all cases no activity for this enzyme was detected at 24 hr post-mortem.

Table 2. Relative enzyme activity in longissimus dorsi muscle of pork carcasses 1 through 10 and 11Y.

Enzyme	Activity[a] 0 hr	24 hr
Glycolysis		
Lactate dehydrogenase	+ to +++	± to ++
α-glycerophosphate dehydrogenase	± to +++	± to +++
Alcohol dehydrogenase	− to ++	− to ±
TCA cycle		
Succinate dehydrogenase	+ to +++	± to ++
Isocitrate dehydrogenase	± to +++	− to ++
Glutamate dehydrogenase	± to +++	− to ++
Malate dehydrogenase	− to ±	−
Electron transport		
DPN diaphorase	+ to +++	+ to ++
TPN diaphorase	−	−
Hexose monophosphate cycle		
Glycose-6-phosphate dehydrogenase	− to ±	−
6-phosphogluconate dehydrogenase	± to +	−
Fatty acid oxidation		
Beta-hydroxybutyrate dehydrogenase	− to +	−
Others		
Cytochrome oxidase	± to ++	± to ++
Acid phosphatase[b]	positive	positive
Alkaline phosphatase[b]	positive	positive

[a] The following code was used for expressing the amount of activity: − no activity, ± very weak, + weak, ++ moderate, +++ moderately strong, ++++ strong.
[b] Observed on carcasses 1 through 7 only.

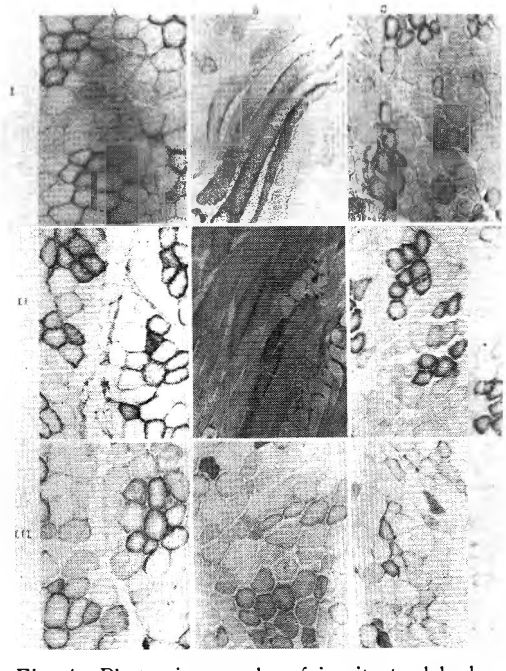

Fig. 4. Photomicrographs of isocitrate dehydrogenase (I), succinate dehydrogenase (II), and DPN diaphorase (III) at 0 (A) hr and at 24 hr post-mortem from pork carcasses subjected to the −29°C (B) and 37°C (C) treatments (×225).

Glycolytic enzymes. Lactate dehydrogenase and alpha-glycerophosphate dehydrogenase activities were generally similar at both 0 and 24 hr post-mortem (Table 2). However, individual muscles showed higher 24-hr levels of alpha-glycerophosphate dehydrogenase activity than that found for lactate dehydrogenase in any of the muscles studied. Photomicrographs typical of the reactions observed for these two enzymes are shown in Fig. 5. Alcohol dehydrogenase reactions were weaker than the reactions observed for either of the other two glycolytic enzymes studied (Table 2).

Electron transport enzymes. Weak to moderately strong reactions were obtained when DPN diaphorase activity was observed (Table 2). Typical DNP reactions are shown in Fig. 4. However, TPN diaphorase activity was not detected in any of the muscles studied.

Other metabolic enzymes. Glucose-6-phosphate dehydrogenase activity was detected in traces in some of the muscles studied. Reactions for 6-phosphogluconate dehydrogenase and beta-hydroxybutyrate de-

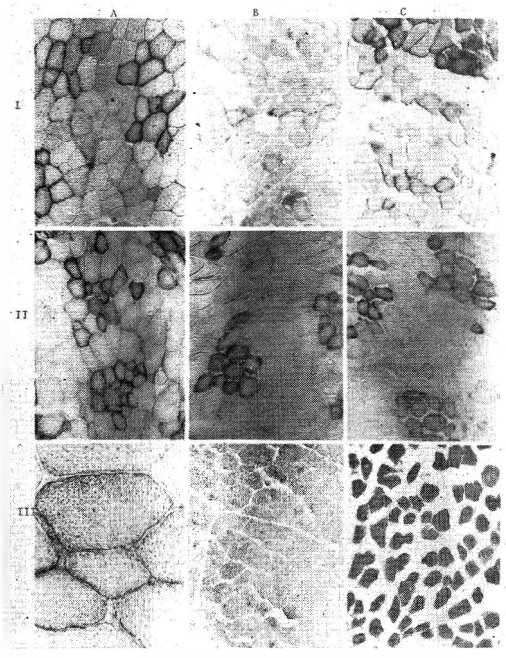

Fig. 5. Photomicrographs showing reactions of longissimus dorsi muscle from pork for alpha-glycerophosphate (I) and lactate dehydrogenase (II) activity at 0 hr and at 24 hr post-mortem at −29°C (B) and 37°C (C) (×225); cytochrome oxidase (III) at 0 hr (A) post-mortem (×860); and Aniline blue-orange C stained sections from 24 hr post-mortem muscle in −29°C (III-B) and 37°C (III-C) treatments (×225).

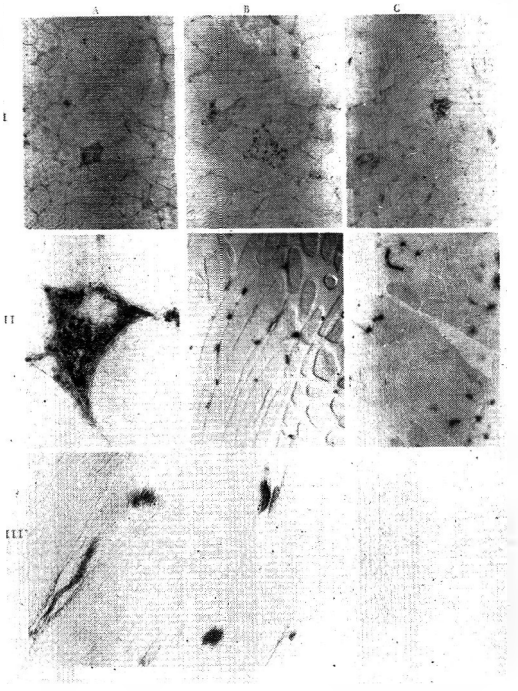

Fig. 6. Photomicrographs of longissimus dorsi muscle of the pig showing varied levels of acid phosphatase activity in isolated fibers (I A, B and C—×225); strong acid phosphatase activity in degenerating fiber (II A—×860); alkaline phosphatase activity (II B and C—×225); alkaline phosphatase activity in fragmented fiber (III A—×860); and alkaline phosphatase activity showing development of cross-linkages between fibers at points of high activity (III B—×860).

hydrogenase were slightly more positive (Table 2). Cytochrome oxidase activity was detected at levels which varied from very weak to moderate a both 0 and 24 hr post-mortem. A 0-hr reaction is shown in Fig. 5.

Acid and alkaline phosphatases. Positive areas were observed for acid and alkaline phosphatase activity in all muscles in which these enzymes were studied (carcasses 1–7). In all cases, the activity was not affected by any of the temperature treatments. The incidence of positive areas did not appear to be related to the appearance of the muscles at 48 hr post-mortem.

Various levels of acid phosphatase activity are shown in Fig. 6 (I A, B, and C; II A). It was concluded that the fiber showing an intense level of acid phosphatase activity (II A) was in an active state of degeneration. This conclusion was supported by the results of other workers. Fennell and West (1963) concluded that a striking feature of dystrophic muscle in mice was the strong acid phosphatase reaction of atrophic fibers and the interfibrillar connective tissues. They further suggested that acid phosphatase may function in catabolic processes. This suggestion was supported by results of Klamer and Fennell (1963), who observed increased acid phosphatase activity in specimens of Tetrahymena during the declining phase of growth. Furthermore, Beckett and Bourne (1958, 1960) observed that groups of inflammatory cells invading degenerating fibers often showed a moderate to strong histochemical reaction for acid phosphatase.

The distribution of alkaline phosphatase activity is shown in Fig. 6 (photomicrographs II B, C; III A, B). The general distribution of activity is shown in the two photomicrographs at the lower magnification (II B, C). The photomicrographs III A

and B are higher magnifications of the section shown in II B (Fig. 6). In photomicrograph III A, alkaline phosphatase activity is shown in a fragmented or possibly degenerating fiber. The positive area in the upper part of photomicrograph III B shows alkaline phosphatase in adjacent areas to two separate fibers. In the lower part of the same photomicrograph, a similar area is observed, but in this case the fibers appear to be joined together at the point of phosphatase activity.

It is suggested that the alkaline phosphatase activity observed is indicative of an abnormal condition in these muscles. Such a conclusion is supported by observations of Beckett and Bourne (1958, 1960), who have noted that alkaline phosphatase activity is limited to the walls of the capillaries and the endothelial lining of the larger blood vessels in normal muscle. Those workers have observed positive alkaline phosphatase activity in fibers from human muscle taken from patients with various muscular or neuromuscular disorders. However, occurrence of the alkaline-phosphatase-positive fibers observed by Beckett and Bourne (1960) was not correlated with the general histological state of the muscle or with the diagnosis.

UDPG-glycogen transferase, phosphorylase, and branching enzyme activity. Histochemical procedures for observing phosphorylase and BE activity were carried out on some of the samples of carcasses 6–10. Some differences appeared to be present, but these differences were highly variable. Furthermore, the tissue blocks had been stored at −29° for 2½–3 weeks. The generally weak reactions as well as excessive ice crystal growth precluded any conclusions concerning the differences observed.

Consequently, phosphorylase, BE, and UDPG-glycogen transferase activity was observed in the initial sample and in the samples removed at approximately 3 and 24 hr post-mortem for pork carcasses A–G. Results of the observations on the samples removed at 3 hr are in Table 3.

In the muscles from the −29°C treatment, considerable variation existed in the amount of activity observed between different carcasses (Table 3). However, no relationship was apparent between activity, pH, and ultimate muscle condition.

There was no detectable UDPG-glycogen transferase activity present at 3 hr post-mortem in any of the muscles of the sides subjected to the 37°C treatment. Thus, it would appear that a combination of a low pH value and high muscle temperature completely inactivates this specific enzyme.

The variable effect of the 37°C treatment on phosphorylase and phosphorylase + BE activity (Table 3) could not be explained on the basis of muscle pH or ultimate 48-hr muscle condition.

The reactions observed in the initial samples were generally similar to those found for the −29°C treatment at 3 hr post-mortem. The reactions observed for all muscles at 24 hr post-mortem generally reflected the activity at 3 hr post-mortem. However, all reactions at 24 hr were considerably weaker than those observed in the −29°C treatment at 3 hr post-mortem. Photomicrographs showing an extreme effect of the heat treatment on the histological condition of muscle from carcass A have been included in Fig. 5 (III B, C).

General observations. Generally, enzyme activities in initial and the 24-hr-post-mortem samples were weaker in pork muscle than in beef muscle. This observation may merely reflect a much faster pace of post-mortem chemical changes in pork muscle. The apparent absence of detectable malic dehydrogenase or TPN diaphorase activity in pork muscle may suggest that these two enzymes are quite labile under the conditions extant immediately after death in pork muscle. However, it is suggested that this observation requires further substantiation.

Results suggest that UDPG-glycogen transferase may be one of the "unidentified enzymes" of Scopes and Lawrie (1963). A study would seem to be warranted of the relationship between the activity of this enzyme and the rate of post-mortem pH fall in muscle eventually appearing soft and watery. Although the degree of acid and alkaline phosphatase activity did not appear to be related to ultimate muscle condition, the detection of such activity, together with

Table 3. Levels of pH, and UDPG-glycogen transferase, phosphorylase and branching enzyme activity in pork carcasses A-G at 3 hr post-mortem.[a]

Treatment	Carcass	pH	UDPG-glycogen transferase	Phosphorylase +BE[b]	Phosphorylase[c]	Phosphorylase[d]
−29°C	A	6.4	+ to ++	++ to +++	+++ to ++++	++++
	B	6.1	± to +	++	++	+ to ++
	C	6.5	++++	+++	++++	+++++
	D	6.6	++	++ to +++	+++ to ++++	+++
	E	6.6	+ to ++	++	++ to +++	++++
	F	6.7	++++ to +++++	++	+++	+++
	G	6.7	+++ to ++++	+++	+++ to ++++	++ to ++++
37°C	A	5.4	−	+	+	±
	B	5.7	−	±	−	−
	C	5.4	−	+++	++++	++ to ++++
	D	5.5	−	++ to +++	+++	+++
	E	5.4	−	±	±	±
	F	5.3	−	++	++	++
	G	5.4	−	++	+++	++ to ++++

[a] The following code was used for expressing the amount of activity: − none, ± very weak, + weak, ++ moderate, +++ moderately strong, ++++ strong, and +++++ very strong.

[b] Branching enzyme.

[c] Reaction mixture contained 20% ethanol which strongly inhibits branching enzyme activity.

[d] Reaction mixture contained $10^{-4}M$ level of $HgCl_2$, which completely inhibits BE.

the implications previously discussed, would seem to warrant further elucidation.

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Abbreviations used are as follows: ATP and ADP: adenosine tri- and diphosphate. ATP-ase: adenosine triphosphatase. DPN and TPN: di- and triphosphopyridine nucleotide. UDPG: uridine diphosphate glucose. BE: branching enzyme or amylo-1,4↔1,6-transglucosidase. Nitro BT: 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride. MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide.

R. C. EISENHUT, R. G. CASSENS, R. W. BRAY, and E. J. BRISKEY
University of Wisconsin, Madison, Wisconsin 53706

Fiber Arrangement and Micro-Structure of Bovine Longissimus Dorsi Muscle

SUMMARY

An experiment was conducted to establish the fiber arrangement and micro-structure at three sites in the longissimus dorsi of horizontally placed and vertically suspended bovine carcass sides. Fiber angles, from transverse processes, increased markedly from anterior to posterior positions. Conversely, the fiber angles, from the spinous processes, decreased from anterior to posterior positions. In comparison with horizontal placement, vertical suspension released some of the tension on the longissimus dorsi and allowed rearrangement of its internal construction. This rearrangement was noted by larger angles, from both the transverse and spinous processes, and shorter sarcomeres in the vertically suspended than in the horizontally placed sides. Differences were also observed in muscle shape and marbling coarseness.

INTRODUCTION

The attachment of muscle to the skeleton as well as intramuscular fiber arrangement, both of which may influence tension on muscles post-mortem, may be important factors in controlling the amount of post-mortem change in the length of individual sarcomeres as well as influencing associated characteristics of the muscle.

The present study was designed to determine: 1) the variation in fiber arrangement within the longissimus dorsi; 2) the importance of carcass position on fiber arrangement; 3) the relation of fiber arrangement to post-mortem shortening of the muscle; and 4) the importance of carcass position on muscle shape and marbling characteristics.

EXPERIMENTAL

Sample source and preparation. Twelve bovine animals (400–450 kg) were used in this portion of the study. All were stunned with a captive-bolt pistol, exsanguinated, eviscerated, and transferred to a 4°C holding cooler. All right sides were suspended vertically according to normal procedure. The left sides were placed horizontally in either of two positions: 1) eight sides placed horizontally, bone down, on a flat surface with the flexible flank area supported by metal supports to facilitate cooling and the limbs oriented and fixed perpendicular to the long axis of the sides; 2) four sides placed horizontally, but with their limbs securely fastened together, producing arching of the vertebral column. All 24 sides were retained in the described positions for 24 hr at 4°C without any further physical disturbance.

Then the longissimus dorsi (including all surrounding tissue), from the fifth thoracic vertebra to the fifth lumbar vertebra, was removed from each side and placed immediately into a −30° blast freezer. A full-length 2.5-cm-thick sagittal section was sawed from this frozen section with the extreme lateral edge 7.5 cm from and parallel to the spinous processes. These 2.5-cm-thick pieces were placed on a flat surface and allowed to thaw at approximately 20°C.

Angle determination. The base line (Fig. 1) for the angle determination at the 13th rib was established by connecting the 7th rib (center) and the transverse process (center) of the 3rd lumbar vertebra with a straight line and subsequently drawing a parallel line through the center of the 13th rib. The base line for the angle determination at the 7th rib was established by connecting the 7th rib and 13th rib with a straight line passing through the center of each rib. Likewise, the base line for the angle determination at the 3rd lumbar vertebra was established by connecting the center of the 13th rib and the center of the transverse process of the 3rd lumbar vertebra with a straight line. Tracings were made of the surfaces 7.5 cm lateral of the spinous processes. These tracings were detailed with sketches of the fibers at the 7th rib, 13th rib, and 3rd lumbar vertebra areas. The line sketches of the muscle fibers were extended to the base lines, and the angles (hereinafter referred to as angle from transverse process) were subsequently measured at the three reference points described above. The fiber angles, in relation to the spinous processes (hereinafter referred to as angle from spinous process) were also measured in these 2.5-cm sagittal sections using the inner surface, which was parallel to the natural plane of the spinous processes, as the base line at each of the three locations.

Sarcomere length determination. After all angle determinations were made, samples (1 g) of muscle were removed from the center of each of the three previously specified anatomical sites in

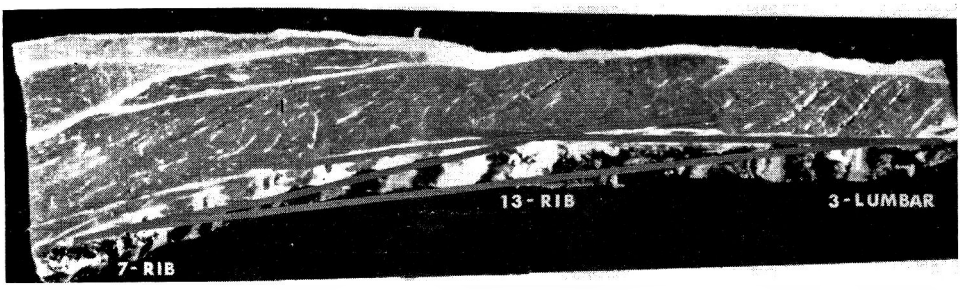

Fig. 1. Illustration of base lines for determining fiber angles from the transverse processes.

each sagittal section. These samples were homogenized in 10 ml of 0.08*M* KCl in a chilled blender (Locker, 1960). The suspension of myofibrils was examined directly in a phase-contrast microscope, and the sarcomere length was determined on 25 myofibrils from each sample.

Additional observations of associated physical characteristics. An additional eight bovine animals of similar size were used to evaluate the influence of carcass position on gross muscle shape and marbling characteristics. All right sides were suspended vertically in the normal manner, four left sides were placed horizontally with limbs perpendicular to the long axis of the sides, and four left sides were placed horizontally with the limbs, fastened together as previously described. After a 24-hr chilling period at 4°C the longissimus dorsi and surrounding tissues from the 5th thoracic to the 5th lumbar vertebra were removed. These sections were then cut into 2.5-cm-thick portions (steaks), coded, and displayed for independent, subjective evaluations of the degree of marbling (USDA, 1956) and variation in muscle size, by three judges within 30–45 min of separation of the portions. Photographs were also obtained from the anterior surfaces of the portions at the 7th thoracic, 13th thoracic, and 3rd lumbar areas from both sides of all eight animals.

Statistical analyses. All data were analyzed by paired-difference analysis as outlined by Steel and Torrie (1960).

RESULTS AND DISCUSSION

Fiber angles and sarcomere lengths in vertically suspended and horizontally placed carcasses. The fiber angles, from transverse processes, in sides which were suspended vertically according to normal procedure increased markedly from anterior to posterior positions (Fig. 2). Conversely, the fiber angles, from the spinous processes, decreased from anterior to posterior positions (Fig. 3). However, no consistent difference was noted in sarcomere length

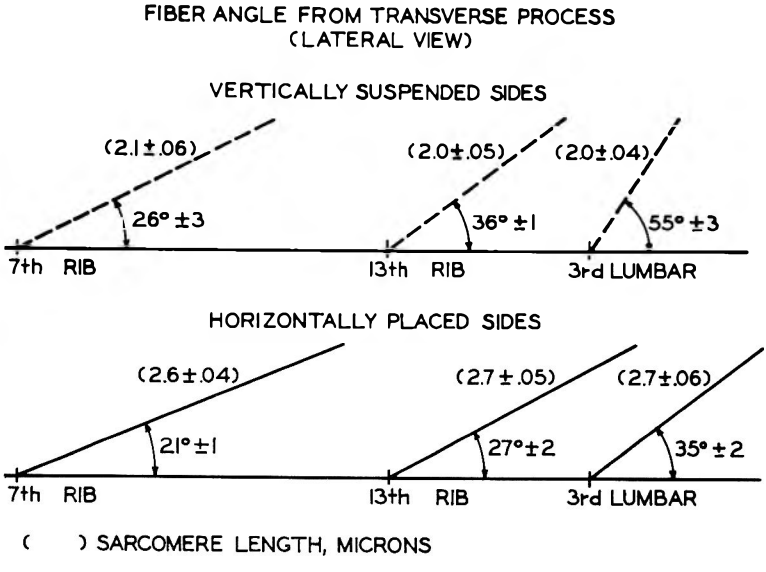

Fig. 2. Average fiber angles from the transverse processes for the vertically suspended and horizontally placed sides. The sarcomere lengths are indicated by the numbers in parentheses. (± standard errors; 8 carcasses)

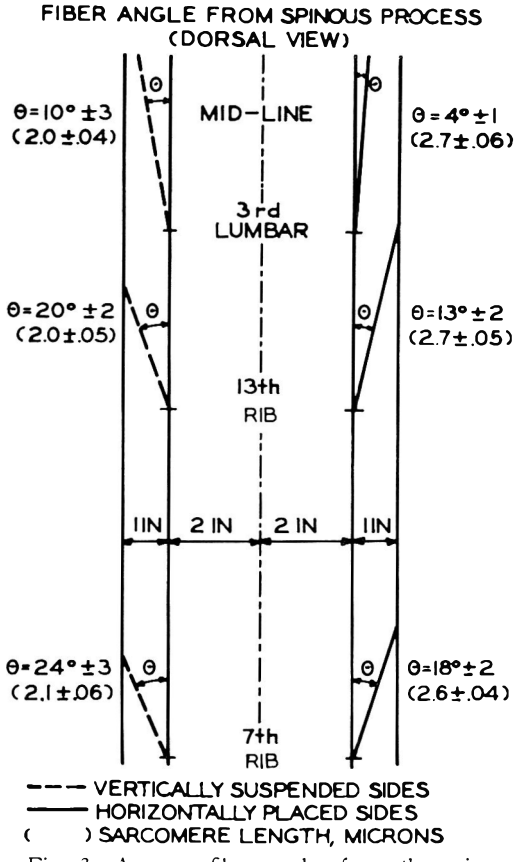

Fig. 3. Average fiber angles from the spinous processes for the vertically suspended and horizontally placed sides. The sarcomere lengths are indicated by the number in parentheses. (± standard errors; 8 carcasses)

at the three positions. When only the differences among positions in the vertically suspended sides are considered, it is not possible to postulate whether these fiber angle differences are due to: 1) inherent differences at each position; or 2) variations in tension, as suggested by Locker (1960) and Marsh (1963).

In comparison with horizontally placed sides (legs oriented perpendicular to long axis) the vertically suspended sides had larger fiber angles from both the transverse (Fig. 2) and spinous processes (Fig. 3). This indicates that vertical suspension of the carcass probably released some of the tension on the longissimus dorsi and allowed rearrangement of its internal construction. Additionally, the sarcomere length (Fig. 2) at all three positions decreased approximately 23% when the sides were suspended vertically. The fiber angle (transverse process) decrease due to horizontal placement was four times as great at the 3rd lumbar vertebra as at the 7th rib; however, no corresponding change in sarcomere length was evident. The fiber angles from the spinous processes at the 13th rib and 3rd lumbar vertebra were significantly smaller in the horizontally placed sides than in the vertically suspended sides. This indicates that the release of tension through vertical suspension permitted some lateral (from spinous process) movement of the fibers.

Two of the most important factors contributing to ultimate sarcomere length may be: 1) tension on the muscle, imposed by skeletal and connective tissue attachments; and 2) the combination of glycolytic rate, time course of rigor mortis, and immediate post-mortem temperature conditions. The data from this experiment suggest that a 2-μ average sarcomere length may represent a practical lower limit, but this suggestion is in disagreement with the earlier work of Locker (1960) and no satisfactory explanation can be given at present. If this is, in fact, a lower limit, the sarcomere length in the lumbar area would not necessarily be shortened further because of the apparent additional tension release, which may be the contributing factor to the greater fiber angle at this position. The connective-tissue sheet surrounding the longissimus dorsi is strongly attached to proximate bones and other tissues and may be extremely important in this phenomenon and especially important in the posterior portion in regard to fiber-angle and sarcomere-length interrelationships.

The origin of the longissimus dorsi is at the tubera coxae crest and adjacent part of the vertebral surface of the ilium, the first 3 sacral spines and lumbar and thoracic spines (Sisson and Grossman, 1962). The fibers commence at their posterior extremity and extend forward with successive fibers inserting and terminating on the transverse processes and lateral surfaces of the costal bones (Chauveau, 1910).

The heavy, fibrous sheet of connective tissue (aponeurosis) which covers the

longissimus dorsi is strongly entwined with the supraspinous and sacroiliac ligaments. These ligaments are connected directly to the bones serving as the ultimate origin for the longissimus dorsi (Sisson and Grossman, 1962). Any gross movement of lumbar vertebra and/or the sacroiliac joint, prior to the onset of rigor mortis, would therefore produce correspondingly large movements within the longissimus dorsi.

Effect of horizontal placement in arched position. It is readily apparent (Fig. 4) that arching decreased fiber angle markedly from the transverse process at all 3 anatomical sites studied. The arching, however, had little effect on fiber angle from the spinous process (Fig. 5) except at the 3rd lumbar vertebra, where it was increased because of distortion. The sarcomere length was increased at all positions because of increased tension resulting from arching of the back, which in effect stretched the sarcomeres of the longissimus dorsi.

Locker (1960) suggested that bovine longissimus dorsi may be stretched by variations in carcass handling. Our results indeed show that this can be accomplished by horizontal placement. Arching of the spine in horizontal position stretches the individual sarcomere further. Conversely, the common vertical suspension of the carcass contributes to a grossly different fiber ar-

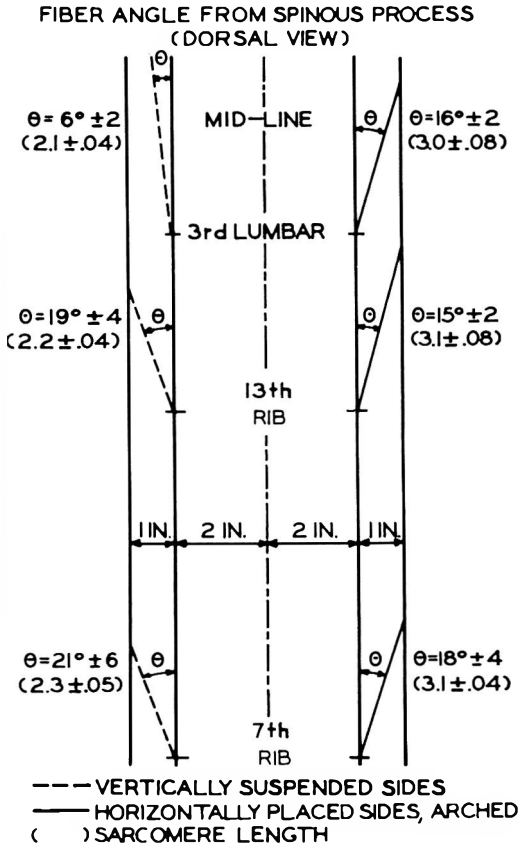

Fig. 5. Average fiber angles from the spinous processes for the vertically suspended and horizontally placed and arched sides. The sarcomere lengths are indicated by the numbers in parentheses. (± standard errors; 4 carcasses)

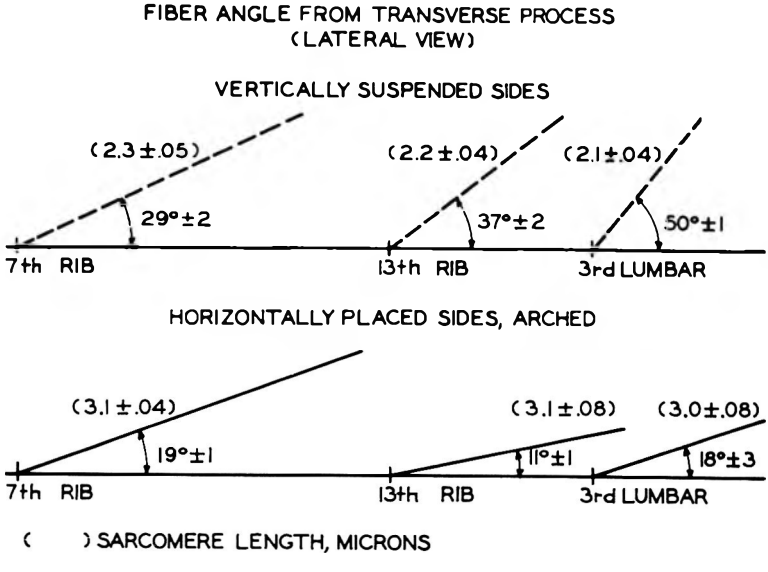

Fig. 4. Average fiber angles from the transverse processes for the vertically suspended and horizontally placed and arched sides. The sarcomere lengths are indicated by the numbers in parentheses. (± standard errors; 4 carcasses)

rangement and allows the fibers to shorten.

Associated physical characteristics. Average marbling scores 24 hr post-mortem were approximately one degree higher in vertically suspended sides than in horizontally placed sides. Generally, the higher degree of marbling in the vertically suspended sides was considered to be of a coarser nature. Subsequent observations indicated that much of the difference in magnitude of marbling (between sides) may disappear if the sides are chilled for 72 hr; nevertheless, the coarseness difference still prevailed. Monitoring of the rate of temperature fall (thermocouples) in vertical and horizontal sides revealed no measurable difference. Therefore, the apparent difference in marbling at 24 hr may be due to a shift in fiber angle and a shortening of the sarcomere in the vertically suspended sides, causing the formation of larger globules of intercellular fat. This supposition is further supported by the fact that a major change in muscle shape was noticed at the area of the 3rd lumbar vertebra, where the change in fiber angle was the greatest.

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PAULINE C. PAUL
Department of Home Economics, University of California, Davis 95616

Storage- and Heat-induced Changes in the Microscopic Appearance of Rabbit Muscle

SUMMARY

A study was made of the effects of length of cold storage and of heating on histological appearance of muscles from 4–5-month-old rabbits. The rabbit muscles appeared to contain little if any elastin except in and around blood vessels. The changes in histological appearance with increasing cold storage and with heating resembled in general those found in beef, although there were several exceptions. The rabbit muscle fibers showed less tendency to granulate, but the contraction nodes tended to disappear with longer storage, especially in the heated tissue. Also, at the longer storage times, heating tended to destroy even the granulated structure of collagen, reducing it to an amorphous state. Within the muscle bundles, rabbit muscles appeared to have more fine strands of collagen in the endomysium than did beef. Also, within the bundles, heating appeared to produce some granulation from the muscle fibers themselves, from the fine collagen strands, and from the endomysial reticulum, rather than primarily from the muscle fibers as in beef.

INTRODUCTION

Investigations have been continued as to the suitability of rabbit muscle as experimental material for meat studies. An earlier paper (Paul, 1964) gave information on degree of variation among carcasses treated alike, and on the time course of the development of rigor and storage changes, with respect to yield, moisture, fat and nitrogen content, cooking time and losses, and tenderness as measured by Warner-Bratzler shear. This paper gives data on the microscopic structure of various rabbit muscles, and changes due to length of cold storage and to heating.

EXPERIMENTAL PROCEDURES

As described in more detail in the first paper (Paul, 1964), the 4-5-month-old animals were slaughtered in the laboratory, and the carcasses held in cold storage at 5°C for 48 hr for Series I, and for 0, 2, 4, 6, 12, or 24 hr for Series II. Half-carcasses were heated in an oven at 163±3°C to an internal temperature of 80°C measured in the center of the anterior thigh muscles.

Histological samples were fixed in neutral formalin, dehydrated in alcohol and xylene, embedded in paraffin, sectioned 5 μ thick, mounted and stained with Weighert's triple connective-tissue stain. This stains the muscle fibers yellow, the collagenous connective tissue red, elastic connective tissue dark blue-green, and the nuclei blue. The endomysial reticulum surrounding each muscle fiber stains yellow-brown, but cannot be readily distinguished unless there has been some muscle fiber shrinkage.

Histological appearance. The size and shape of the muscle fiber bundles, as seen in cross section, and the amount and distribution of connective tissue are described in Table 1. Figs. 1 and 2 show the appearance

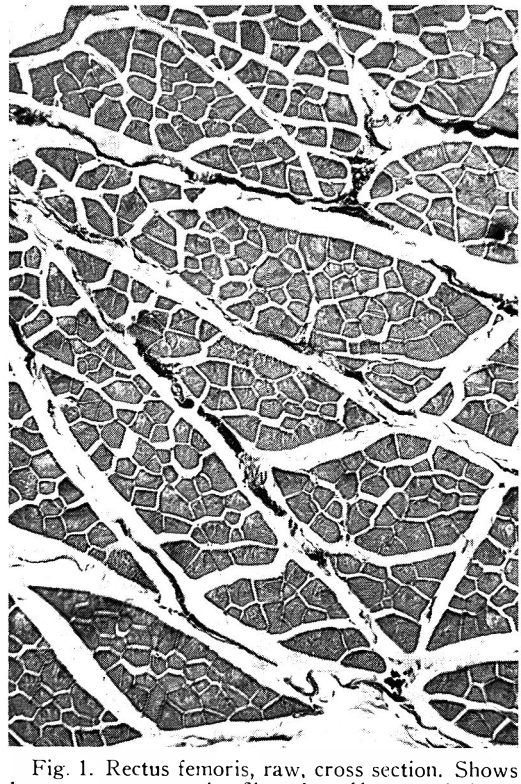

Fig. 1. Rectus femoris, raw, cross section. Shows long narrow muscle fiber bundles, and large amount of collagen (black strands between bundles). 100×.

of the rectus femoris and the psoas major, as examples. In evaluating microscopic sections, it must be remembered that there is considerable variation from place to place in the same slide, between different sections from the same muscle, and among samples of the same muscle from different animals. So the comments in Table 1 are intended to indicate a general pattern rather than a precise description. All the muscles showed elastin present only in and immediately around the blood vessels except for a very few elastin strands in the perimysium of the biceps femoris.

Table 2 summarizes the changes in appearance of the muscle fibers with increasing cold storage, and with heating. The separation between bundles and between fibers is a reflection of different degrees of shrinkage (Figs. 3, 5, 6). This may be related to the previously published findings (Paul, 1964) that cooking losses were less at 0 and at 24 hr of storage than at the intermediate times, suggesting changes in the degree of water binding of the muscle proteins after different lengths of storage.

The rabbit muscles give somewhat the same picture as beef muscle for effect of cold storage, although the time span is shorter (Figs. 3, 4, 5). However, the nodes of contraction tended to persist in beef during storage and through cooking (Paul *et al.*, 1944). Also, the rabbit muscle fibers showed much less tendency than did beef fibers (Paul, 1963) to granulate with increasing storage or with heating (Figs. 5, 7).

The cross-fiber cracking and breaking appears to start in the *I* band, as shown in Fig. 8. This suggests that the actin filaments are more easily broken than the myosin ones, or that the sarcomeres are separating at the *Z* line. The detail visible on these slides is not sufficient to distinguish between these possibilities.

Measurements were made on raw and cooked muscle at 0, 6, and 24 hr of storage, to ascertain changes in the spacing of the cross striations. Average lengths of the

Table 1. Microscopic appearance of rabbit muscles after 24 hr of cold storage.

Muscle	Muscle bundles, cross sections	Connective tissue	
		Perimysium	Endomysium
Rectus femoris	Long and narrow, triangular or lenticular	Large amount of collagen, widely distributed	Moderate number of fine strands of collagen
Longissimus dorsi	Large bundles, roughly rectangular	Small amount of collagen	Occasional fine strands of collagen apparent
Psoas major	Bundles roughly square or triangular, tend to be widely separated	Very little collagen except around blood vessels, collagen strands thin	No collagen observed
Biceps femoris	Bundles variable in shape and size, some very large	Moderate amount of collagen, some large strands. Also shows few strands of elastin	Moderate number of fine strands of collagen
Triceps brachii	Bundles tend to be small, variable in shape	Moderate to large amount of collagen not as widely distributed as in rectus femoris	
Semimembranosus	Bundles tend to be fairly large, but considerable variation in size and shape	Moderate amount of collagen	

Table 2. Changes in histological appearance of muscle fibers of psoas major (PM), longissimus dorsi (LD), and biceps femoris (BF) muscles.

Description	Changes in raw during cold storage after slaughter	Changes due to heating	Differences among muscles
Separation between fibers	At 0 storage, fiber bundles are distinct, but fibers within bundles are poorly differentiated. By 6 hr, fibers are usually separate and distinct.	Heated samples show good differentiation with individual fibers and endomysial reticulum distinct at 0 storage. After 6–12 hr storage, fiber bundles appear to be closer together.	Differentiation develops more rapidly in PM (4-hr storage) than in LD or BF (6-hr storage)
Separation of fibrils within fibers	Poorly differentiated fibers (0 storage) show considerable separations between fibrils. These disappear as fibers separate with storage (at 4–6 hr), then reappear but are narrower at 12 and 24 hr.	Heated samples show much less separation of fibrils at shorter storage times, but an increased number at 12–24 hr storage.	No difference
Shape of muscle fibers	At 0 storage, fibers generally straight to slightly wavy. By 6 hr, some are straight, some contain nodes and stretched areas, most show moderate to tight *z-z* waves. At 24 hr, *z-z* have relaxed to gentle waves, most of nodes have disappeared.	Heating produces nodes in early storage, especially at 0 and in BF. Nodes tend to disappear in heated samples after 6-hr storage.	BF shows more nodes than PM or LD
Cracks and breaks	Few appear after 12–24 hr of storage.	Heating produces few breaks even at 0 storage. Number increases with length of storage. Start in *I* band.	Appear at shorter storage times in BF and LD than in PM—either raw or cooked.
Granulation	None	Small amount appears after 2 hr storage, amount increases with longer storage, but is not large even at 24 hr. Some separation of nuclei after 12 hr.	BF shows more granulation than PM or LD

muscle sarcomeres are summarized in Table 3. These averages should not be interpreted as specific lengths of sarcomeres in unfixed tissue, since the processes of embedding probably caused some shrinkage of the fibers. However, the data illustrate the changes due to storage and heating.

The sarcomere lengths were generally quite uniform in the raw fibers at 0 and at 24 hr of storage, but much more variable at 6 hr, because of the presence of *z-z* waves. In these cases, the variations were due to changes in the length of the *I* bands. In the *z-z* waves, the *I* bands were alternately stretched and contracted along the length of the fiber, depending on whether they were on the outside or the inside of the turn. This suggests the possibility that these waves might be produced by localized contraction of fibrils, rather than being passive waves produced by contraction of adjacent noded fibers.

Heating produced shortening of the sarcomeres in a number of cases, and both the *A* and the *I* bands appeared to be shortened. At the shorter storage periods, the hind legs of the rabbit carcasses tended to straighten during cooking, probably account-

Table 3. Average lengths of sarcomeres, in microns, for longissimus dorsi, psoas major, and biceps femoris muscles.

		Storage time (hr)					
		0		6		24	
Appearance of fibers	Muscle	Raw	Cooked	Raw	Cooked	Raw	Cooked
Poorly differentiated	LD	1.72					
	PM	1.77					
	BF	1.73					
Straight, well differentiated	LD	1.77	1.09	1.48	1.44	1.76	1.61
	PM		0.676	1.45	1.43	1.73	1.38
	BF		2.14	1.44	1.89	1.82	1.60
In nodes	LD			1.07			
	PM			0.77			
	BF		1.20	1.00	1.00		
In stretched area adjacent to nodes	LD			2.00			
	PM			1.60			
	BF		3.00	3.02	2.29		
In *z-z*, stretched part,	PM			1.37			
contracted part	PM			1.00			

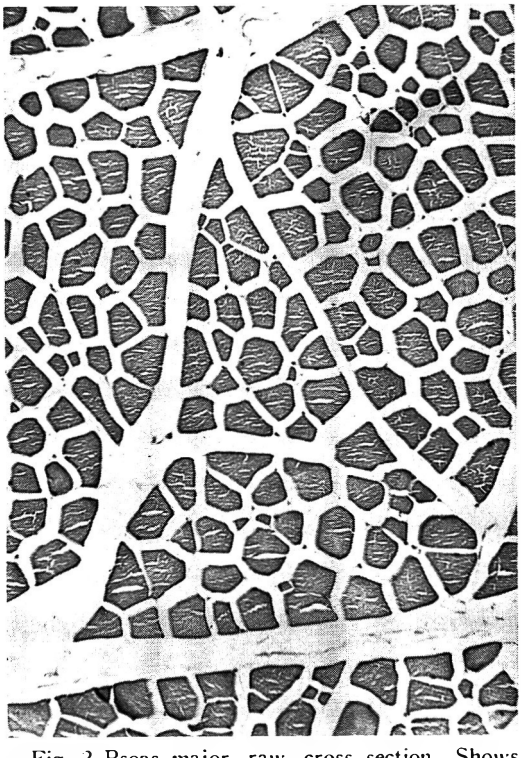

Fig. 2. Psoas major, raw, cross section. Shows wide separation between bundles, few thin collagen strands between bundles. 100×.

ing for the increase in length of the sarcomeres in the biceps femoris in the cooked samples at 0 and 6 hr of storage.

The changes in connective tissue with storage and heating are summarized in Table 4. Changes were noted in both the perimysium, the connective tissue surrounding the muscle fiber bundles, and the endomysium, the fine network or reticulum of connective tissue enclosing each muscle fiber within a bundle. The color and structural changes in the perimysium were generally similar to those found in beef, with the color changing from the bright red of raw collagen fibers, through pink-purple and blue-purple fibers, and finally to a granulated form which stained yellow. However, after storage of 12 or 24 hr, much more of the rabbit muscle collagen lost even the granular structure when heated, and became just a yellow amorphous mass.

The endomysium is generally considered to consist largely of reticulin, a form of connective tissue of composition somewhat different from that of collagen. With this staining procedure, reticulin stains yellow-brown. The rabbit muscles showed more fine red collagen fibers in the endomysium of the raw muscles than had been observed in beef. Some of these retained the fibrous structure during heating; some of them granulated. There was also some evidence of granulation of the reticulin itself. This

Fig. 3. Psoas major, 0 storage, raw, longitudinal section. Shows poor differentiation of the muscle fibers, and longitudinal splitting within the fibers. 100×.

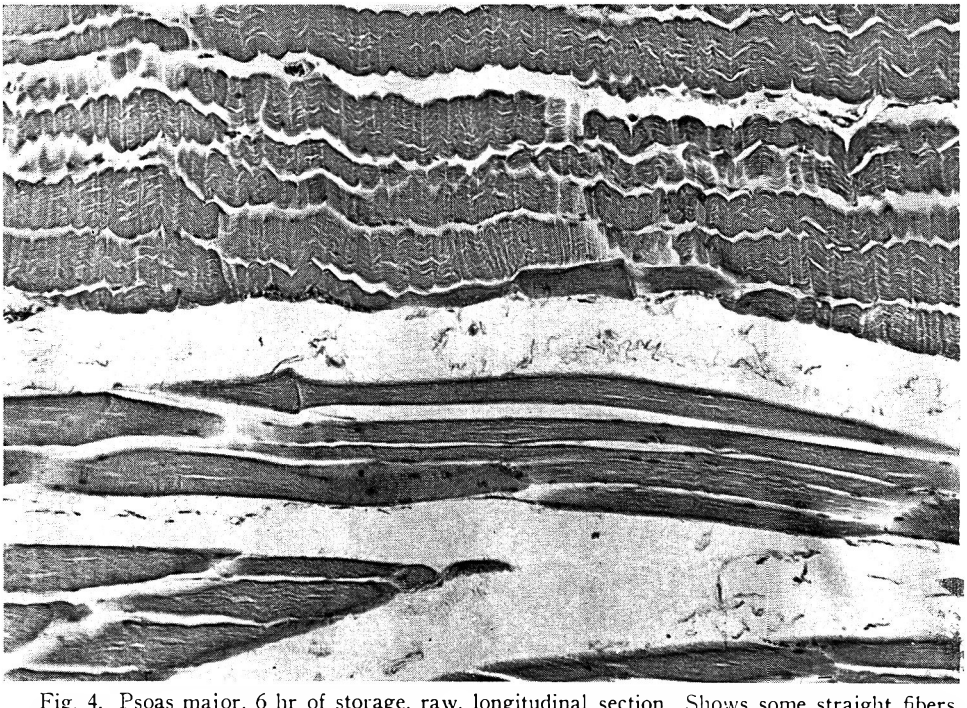

Fig. 4. Psoas major, 6 hr of storage, raw, longitudinal section. Shows some straight fibers, some with contraction nodes and adjacent stretched areas, some in tight *z-z* contraction. 100×.

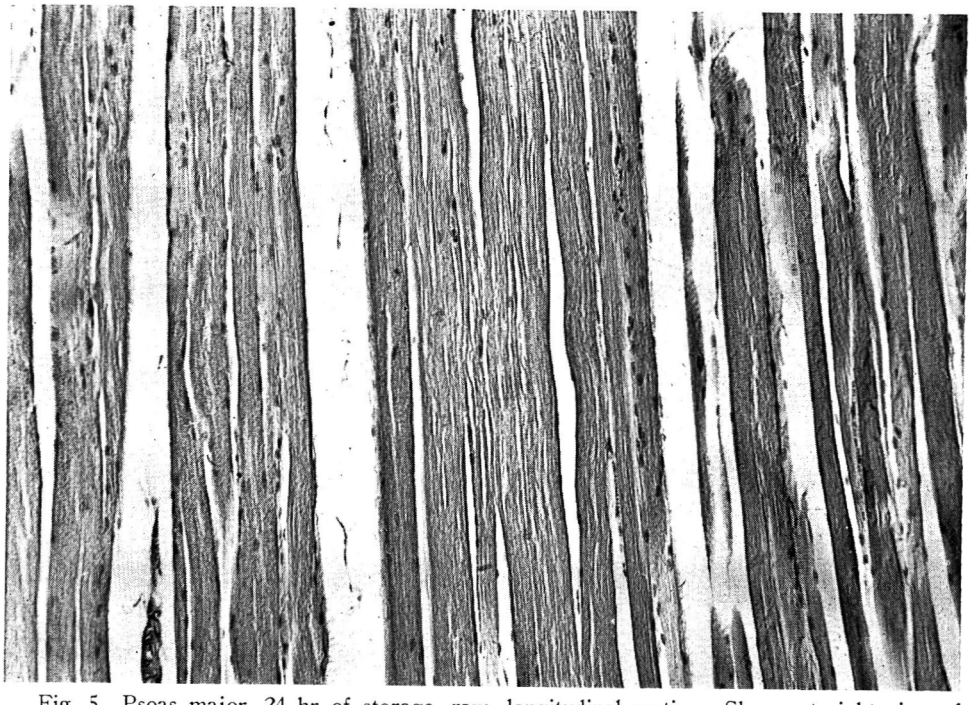

Fig. 5. Psoas major, 24 hr of storage, raw, longitudinal section. Shows straightening of fibers, improved differentiation, some longitudinal splitting within fibers. 100×.

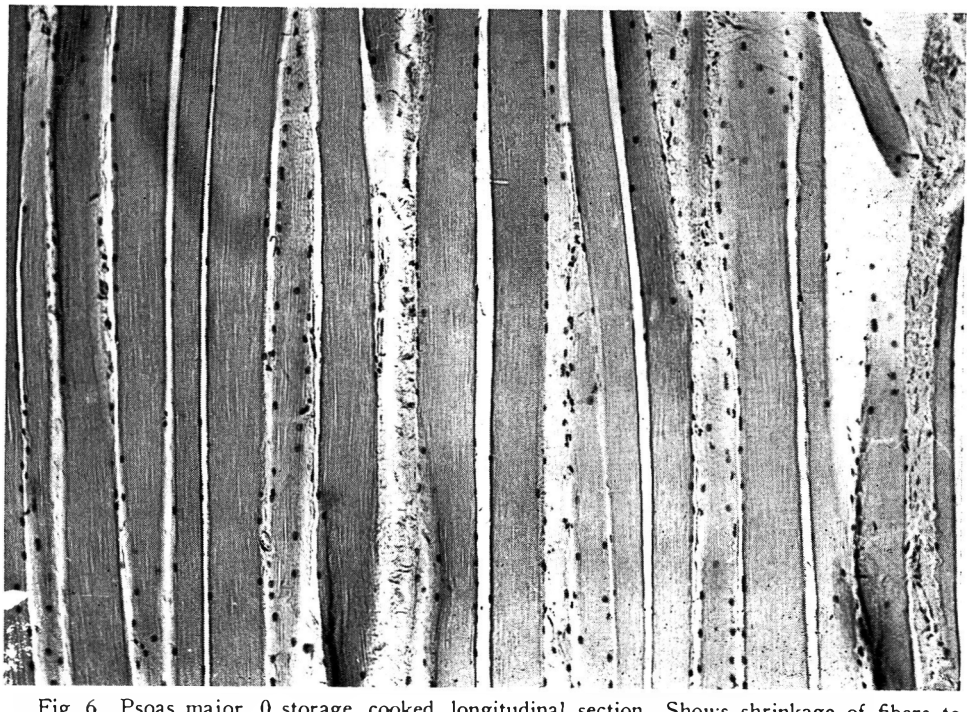

Fig. 6. Psoas major, 0 storage, cooked, longitudinal section. Shows shrinkage of fibers to give good differentiation and disappearance of longitudinal splitting within fibers. 100×.

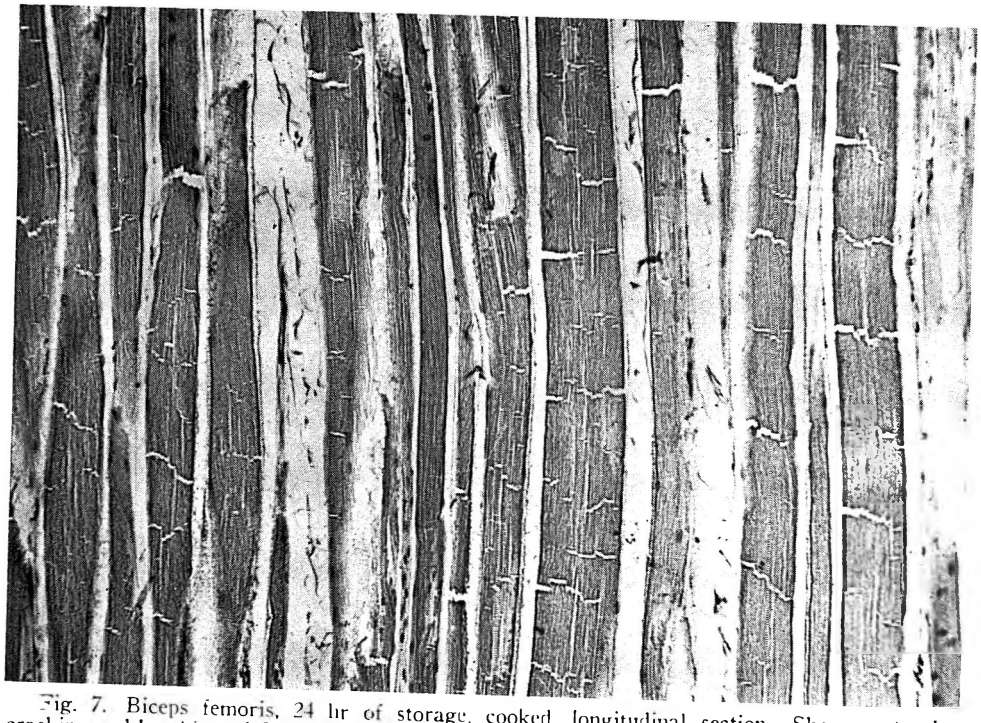

Fig. 7. Biceps femoris, 24 hr of storage, cooked, longitudinal section. Shows extensive cracking and breaking of fibers. 100×.

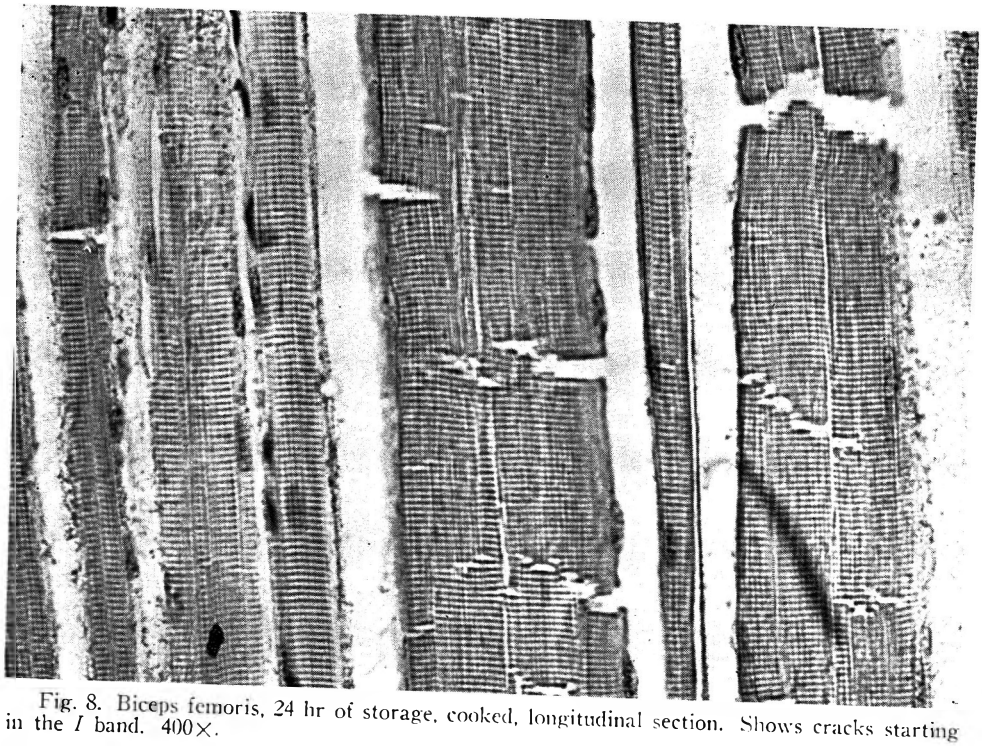

Fig. 8. Biceps femoris, 24 hr of storage, cooked, longitudinal section. Shows cracks starting in the *I* band. 400×.

Table 4. Changes in histological appearance of connective tissues of longissimus dorsi (LD), psoas major (PM), and biceps femoris (BF) muscles.

Description	Changes in raw during cold storage after slaughter	Changes due to heating	Differences among muscles
Color and form of collagen in perimysium	Red	Pink-purple, blue-purple, or yellow granular. Mostly yellow granular at 0–6 hr of storage. At 12–24 hr, still a few fibers of dull pink to blue-purple, but most of yellow granular had disintegrated so no structure was visible.	Granulated most readily in PM, least in BF.
Endomysium-reticulin	Not visible until 4 hr storage had induced sufficient shrinkage of muscle fibers. Persists intact through 24 hr of storage.	Some granulation after 2 hr storage. Amount increases with longer storage, but most of reticulin is still intact at 24 hr of storage.	LD and PM show more granulation than BF.
-collagen	Some fine collagen fibers in endomysium	Occasionally see fine blue-purple fibers in reticulum.	BF shows more red collagen in raw than do LD and PM.

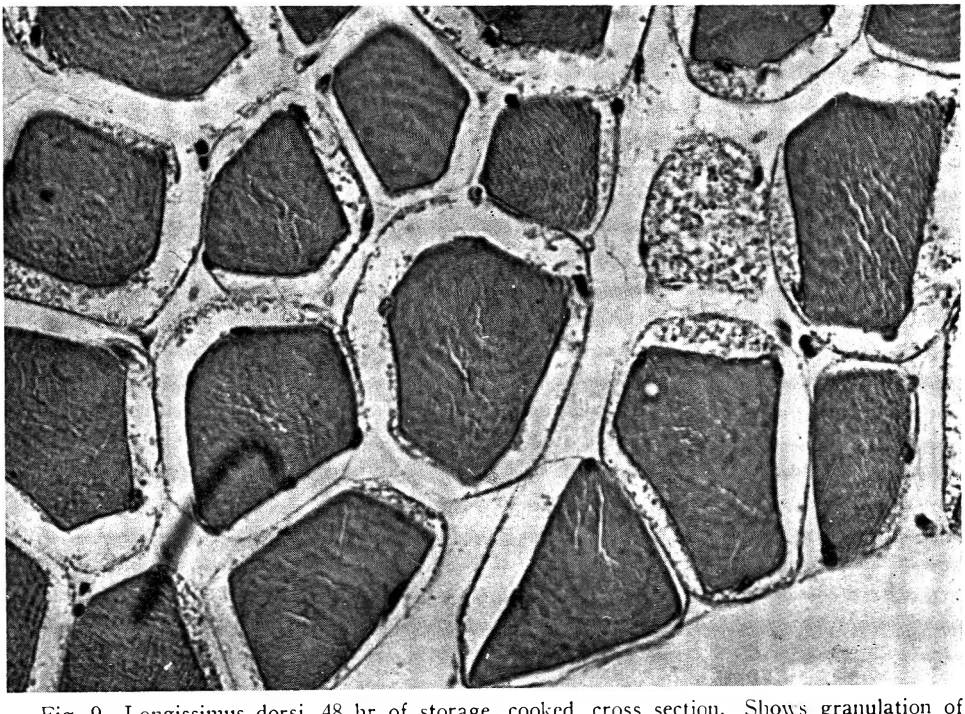

Fig. 9. Longissimus dorsi, 48 hr of storage, cooked, cross section. Shows granulation of muscle fibers and of endomysial reticulum. 400×.

differs from beef, where the reticulin remained essentially intact after heating (Paul, 1963).

In different places, evidence could be seen for granulation of the edges of the muscle fibers, of the collagenous fibers, and of the endomysial reticulum, as shown in Fig. 9. All the granular material within the muscle bundles tended to migrate to the reticulum. Since all of them stain the same color and form granules of the same size, the techniques used furnish no method of distinguishing clearly between granules from the several sources. This will require additional investigation with other staining methods.

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G. R. BEECHER, R. G. CASSENS, W. G. HOEKSTRA, and E. J. BRISKEY
University of Wisconsin, Madison, Wisconsin 53706

Red and White Fiber Content and Associated Post-Mortem Properties of Seven Porcine Muscles

SUMMARY

The red fiber content of several porcine muscles was estimated by a histochemical method (affinity for Sudan Black B due to high intracellular lipid content) and a biochemical method (high succinic dehydrogenase activity). Even though the muscles varied widely in total lipid content, the histochemical method was comparable to the biochemical method in categorizing porcine muscles as red or white. Red muscles (> 40% red fibers) contained greater myoglobin concentrations and generally had longer post-rigor sarcomeres than white (< 30% red fibers) muscles. Interrelationships of percent red fibers, succinic dehydrogenase activity, fat content, glycolytic rate, and post-rigor contraction state were discussed.

INTRODUCTION

Early work (Ranvier, 1874) revealed that striated muscles could be categorized as either red or white. Numerous reports on porcine muscle have since shown a wide range in visible color, myoglobin concentration, and fat content (Scaife, 1955; Lawrie, 1960; Briskey *et al.*, 1960; Cassens *et al.*, 1963[b]; Beecher *et al.*, 1965). It has been established (Denny-Brown, 1929; Ogata, 1958) that muscle color is dependent upon the proportion of red fibers which the muscle contains. Red muscles have high oxidative enzyme activity, whereas white muscles have high glycolytic activity. Variations in these red and white muscle interrelationships have been noted by Lawrie (1953) and Tappel and Martin (1958). Glycolytic activity is of particular interest in view of a recent report (Sink *et al.*, 1965) that post-mortem glycolytic rate was related to the extent of post-mortem contraction.

The percentage of red and white fibers in porcine muscles has not been previously considered. Since the histochemical method (Ogata, 1958) of estimating red fibers is based upon the lipid content of the fiber, it seemed particularly important to employ this technique in porcine muscle, which has a considerably higher lipid content than the mammalian species previously studied, and to compare the results with a biochemical estimate of red fibers.

On the basis of this information, an experiment was designed: 1) to estimate the percentage of red fibers in several porcine muscles with both a histochemical and biochemical method; 2) to determine the association of red fiber content with myoglobin concentration; and 3) to ascertain the association of red fiber content, post-mortem glycolytic rate, and related post-mortem properties.

EXPERIMENTAL

Sample isolation. Eight porcine animals (Poland China) were used. Immediately after exsanguination (0 hr), samples of the semitendinosus, gluteus medius, biceps femoris, rectus femoris, trapezius thoracalis, serratus ventralis, and longissimus dorsi were excised from the left side of the carcass, and the semitendinosus and biceps femoris were divided into their respective light (outside: in proximity to the subcutaneous fat layer) and dark (inside: in proximity to the femur bone) portions. Initial (0 hr) pH and succinic dehydrogenase activity were determined on all muscle samples immediately after excision. Muscle samples were also frozen in liquid nitrogen, pulverized (Borchert and Briskey, 1965), and stored (−25°C) for subsequent glycogen and lactic acid analyses. Two-hr pH was also determined on samples of all muscles.

After a 24-hr post-mortem chilling (4°C) period, muscle samples were excised from the right sides of the carcasses. Sarcomere lengths and ultimate (24 hr) pH values were determined on all muscles. Small samples (3×3×10 mm) were also placed in fixative (calcium-formol) for subsequent histochemical analysis. Samples of all muscles were frozen and stored (similar to 0 hr) for subsequent myoglobin, glycogen, lactic acid, fat, and moisture analyses.

Methods. The red and white fiber content was determined in muscle samples which were fixed (calcium-formol), freeze-sectioned (18μ), and stained with Sudan Black B (Ogata, 1958). The results are reported as the percent red fibers of the total fiber content in 10 muscle bundles.

Succinic dehydrogenase activity was determined

by a modification of the method suggested by Bocek (1964). The modified method consisted of preparing a 1% (w/v) muscle homogenate (all glass homogenizer) in 0.2*M* phosphate buffer (pH 7.5). One ml of the homogenate was added to an incubation medium (3 ml total volume) containing 0.2m*M* phosphate buffer (pH 7.5), 0.1m*M* succinate (sodium), and 2.0 μM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). The reaction mixture was incubated for 15 min at 37°C and was stopped by adding 1.8m*M* of trichloracetic acid (TCA). The formazan produced was extracted into ethyl acetate (7 ml) by vigorous shaking followed by centrifugation. The optical density of the ethyl acetate layer was measured at 490 mμ. A standard curve was determined daily with a commercial preparation of INT formazan (Nutritional Biochemicals Corporation) and found to be linear from 5 to 50 μg of INT formagan per 7 ml of ethyl acetate. The results are reported as mg of INT formazan produced per 15 min per g fresh tissue.

Myoglobin concentrations were determined by the method of Poel (1949). Muscle samples were analyzed for total moisture by measuring the weight loss after drying for 24 hr at 105°C. Fat (ether extract) was determined as the weight loss resulting from 24 hr of continuous Soxhlet extraction with diethyl ether. Fat-free dry tissue was determined by difference.

Muscle pH was determined by placing a combination glass-calomel electrode directly on the freshly cut surface (cross-section) of the muscle (Briskey, 1964). Glycogen was determined by the method of Dubois *et al.* (1956), and lactic acid by the method of Hohorst (1963). The sarcomere length of each muscle was determined microscopically on muscle homogenates (Locker, 1959) by calculating the mean length of 25 sarcomeres from each muscle.

Results were analyzed by Duncan's new multiple-range test (Steel and Torrie, 1960) to detect significant ($P < .05$) differences between muscles. Simple and multiple correlations over all muscles were also calculated.

RESULTS AND DISCUSSION

Red and white fiber content of various muscles and association with pigment and lipid concentration. *Histochemical method.* The porcine muscles studied were divided into two groups (red and white) on the basis of the percent red fibers contained in each muscle. White muscles (semitendinosus light portion, outside biceps femoris, longissimus dorsi, and gluteus medius) contained less than 30% red fibers, whereas red muscles (serratus ventralis, rectus femoris, inside biceps femoris, semitendinosus dark portion, and trapezius) contained more than 40% red fibers (Fig. 1). These data confirm work with other species (Denny-Brown, 1929; Ogata, 1958; Sréter and Woo, 1963). The red fiber content of all muscles in this study ranged from 19.5% (semitendinosus light portion) to 47.7% (trapezius). Sréter and Woo (1963) reported wide ranges (0.0–65.5%) in the red fiber content in several muscles of the rat. The apparent difference in range between the two species may be explained on the basis of the muscle used or possibly a species difference.

Biochemical method. Results from succinic dehydrogenase activity determinations, a measure of the oxidative enzyme activity of muscle, followed trends similar to the red fiber content. Histochemical staining established definite white and red groups of muscles, respectively containing less than and more than 1.7 units (Fig. 2) of succinic dehydrogenase activity. All muscles of the red muscle group, except the rectus femoris, had succinic dehydrogenase activities which were significantly ($P < .05$) higher than in the muscles of the white group. The rectus

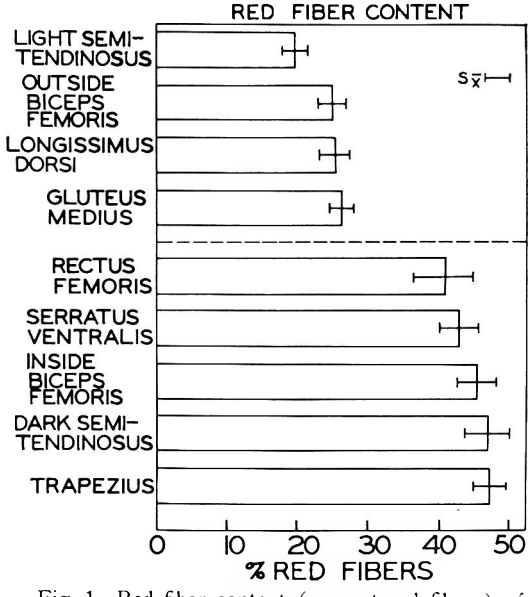

Fig. 1. Red fiber content (percent red fibers) of several porcine muscles. Percent red fibers based on Sudan Black B staining procedures of Ogata (1958). Dotted line divides muscles into red and white muscle groups.

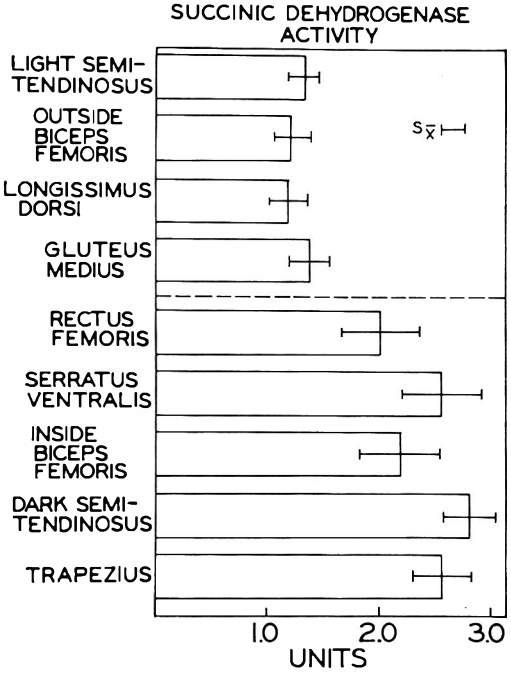

Fig. 2. Succinic dehydrogenase activity of several porcine muscles. One unit of activity equals 1 mg of INT formazan produced per 15 min of incubation per g of fresh tissue. Dotted line divides muscles into red and white muscle groups.

femoris (red muscle group) contained a significantly ($P < .05$) higher succinic dehydrogenase activity than the outside biceps femoris and longissimus dorsi (white muscle group). However, differences in succinic dehydrogenase activity between the rectus femoris (red muscle group), and semitendinosus light portion and the glutens medius (white muscle group), also approached significance ($P < .05$).

Recent observations on small laboratory animals by several workers (Ogata, 1958; Romanul, 1964; Dawson and Romanul, 1964) have also indicated a close relationship between the red fiber content of a muscle, the visual muscle color, and succinic dehydrogenase activity. However, Davey (1960) has discussed inconsistencies in the relation of myoglobin content to succinic dehydrogenase. Krebs (1950) indicated that the problem of relating myoglobin concentration to muscular activity is complicated by the fact that tissue respiration tends to increase with a decrease in body size of animal. In the present study, however, a simple correlation coefficient over all muscles of 0.65 ($P < .01$) between percentage of red fibers and succinic dehydrogenase activity indicates a close relationship between these two traits in porcine muscle.

Such information is interesting not only from a purely basic aspect, but may also be of importance to certain practical areas of meats research. Post-mortem changes convert muscle to meat, and it has been established (Briskey, 1964) that glycolytic rate often affects ultimate and economically important meat characteristics. Red porcine muscles, which usually do not undergo rapid post-mortem glycolysis and resultant changes in properties, have been considered to lead a more aerobic existence in the animal. The disclosure that the so-called red muscles do indeed possess a higher succinic dehydrogenase activity and a greater intrafibrillar affinity for Sudan Black B dye, adds further support to the previous suppositions. It appears probable that the red and white muscles of the porcine animal have different *in vivo* characteristics. Further research will elucidate *in vivo* differences existing between the two groups of muscle and determine how such factors influence post-mortem behavior.

Pigment content. Myoglobin content of the muscles studied generally increased with an increase in the percentage of red fibers (Fig. 3). Red muscles contained significantly ($P < .05$) higher concentrations of myoglobin than white muscles (Fig. 3). Similar results have been reported by Briskey *et al.* (1960) and Cassens *et al.* (1963a). Myoglobin content varied slightly between individual muscles within the white muscle group, whereas large variations were observed between muscles in the red muscle group. Within the red muscle group, the rectus femoris and inside biceps femoris had significantly ($P < .05$) lower myoglobin concentrations compared with the serratus ventralis, semitendinosus dark portion, and trapezius (Fig. 3). The significant ($P < .01$) multiple correlation ($r = .73$) observed between myoglobin content and succinic dehydrogenase activity and percent red fibers indicates a close relationship between these parameters.

The semitendinosus dark portion and in-

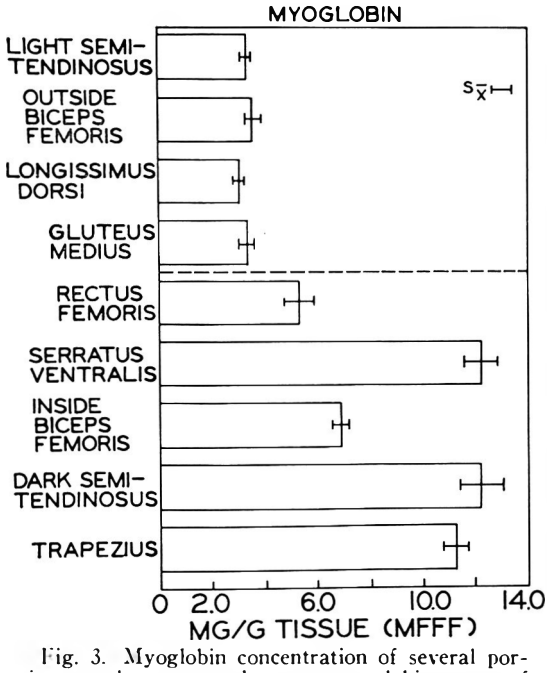

Fig. 3. Myoglobin concentration of several porcine muscles expressed as mg myoglobin per g of moisture-free, fat-free tissue. Dotted line divides muscles into red and white muscle groups.

side biceps femoris, which are internal muscle portions, contained significantly ($P < .05$) higer values for percent dark fibers (Fig. 1), succinic dehydrogenase activity (Fig. 2), and myoglobin (Fig. 3) than their respective light outside portions. Recent work with other species has also indicated that internal portions of muscles, compared with superficial layers of the same muscles, contained greater proportions of red fibers (Sréter and Woo, 1963; Ogata, 1958) and higher succinic dehydrogenase activities (Ogata, 1960; Romanul, 1964; Dawson and Romanul, 1964).

Generally, high myoglobin content is associated with high succinic dehydrogenase activity, suggesting that myoglobin ensures high oxygen tension for the muscle oxidase system (Lawrie, 1952; Chinoy, 1963). However, muscles containing low myoglobin concentrations, despite high succinic dehydrogenase activities (rectus femoris and inside biceps femoris), may possess superior muscle circulatory systems, thereby having an excellent supply of oxygen and partly dispensing with the need for an oxygen store (Lawrie, 1952).

Fat content. The fat content of the muscles studied was variable and not significantly ($P > .05$) associated ($r = .17$) with the percent red fibers of the same muscles (Fig. 4). However, significant ($P < .05$) but low correlations between lipid content and myoglobin values ($r = .50$) and also between lipid content and succinic dehydrogenase activity ($r = .30$) were observed. It is pertinent to note that Sudan Black B, the stain used in this study to determine red fibers, stains selectively for lipid material. It would appear, from the present observations, that the total lipid content (ether-extractable) of the muscle (extra- and intracellular) had little effect on the efficiency of detecting red fibers by the method used in this study (Sudan Black B). Pearse (1960) suggested that Sudan Black B stained phospholipids preferentially to neutral lipids. Thus it would appear that the phospholipid content of red fibers is higher than the phospholipid content of white fibers. These observations are in agreement with preliminary findings on porcine muscle (Allen, 1965).

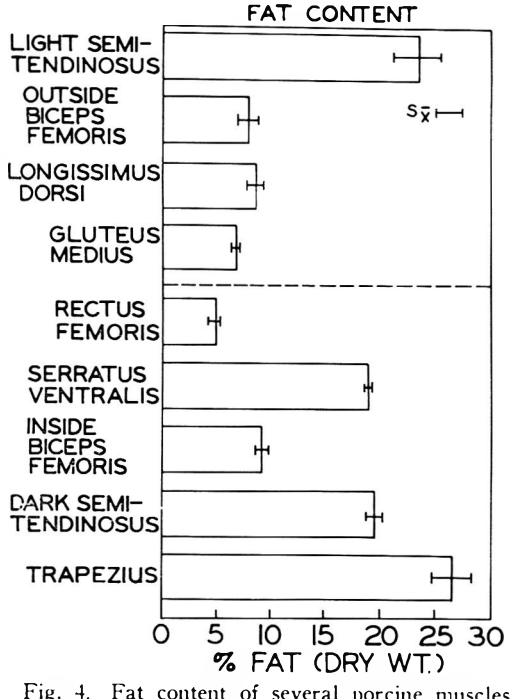

Fig. 4. Fat content of several porcine muscles expressed as percent fat of dry tissue weight. Dotted line divides muscles into red and white muscle groups.

George and Bhakthan (1961) reported that red (slow) muscles, compared with white (fast) muscles, contained higher lipid concentrations. These data suggest that although the rectus femoris and inside biceps contained high percentages of red fibers, these two muscles are not typical red porcine muscles in regard to total lipid content. It is also pertinent that both the rectus femoris and inside biceps femoris, compared with other red muscles, had lower myoglobin values ($P < .05$) but similar succinic dehydrogenase activities, suggesting increased circulation in these two muscles (Lawrie, 1952), thereby decreasing the requirement for reserve energy in the form of fat.

Association of red and white fiber content, glycolytic rate and post-mortem contraction state in various muscles. Muscle pH values, at the time of death, were similar for all muscles (Fig. 5) and not significantly ($P > .05$) associated with percent red fibers, myoglobin content, or succinic dehydrogenase activity ($r = .03$, .09, and .19, respectively). These pH values appear to be slightly lower than those reported by some other investigators; however, the differences have been discussed by Bendall *et al.* (1963). Cassens *et al.* (1963b) also reported low correlations between 0-hr pH and myoglobin values in distinctively white (longissimus dorsi) and red (trapezius) porcine muscles. Two-hr pH values were not associated with the percent red fibers, whereas ultimate (24-hr) pH values tended to be higher in red muscles than in white muscles, except for the inside biceps femoris which attained a 24-hr pH value similar to white muscles (Fig. 5). Ultimate (24-hr) pH values were not highly associated with percent red fibers ($r = .26$), myoglobin content ($r = .40$), or succinic dehydrogenase activity ($r = .14$). Briskey *et al.* (1960) also noted low correlations between ultimate (24-hr) pH values and myoglobin concentrations within 8 porcine muscles.

Initial (0-hr) glycogen concentration was similar in all muscles studied except for the outside biceps femoris, which tended to have greater quantities of glycogen than the other white muscles (Fig. 6). It should be noted that large variations in glycogen concentrations within each muscle were observed at the time of exsanguination. Ultimate (24-

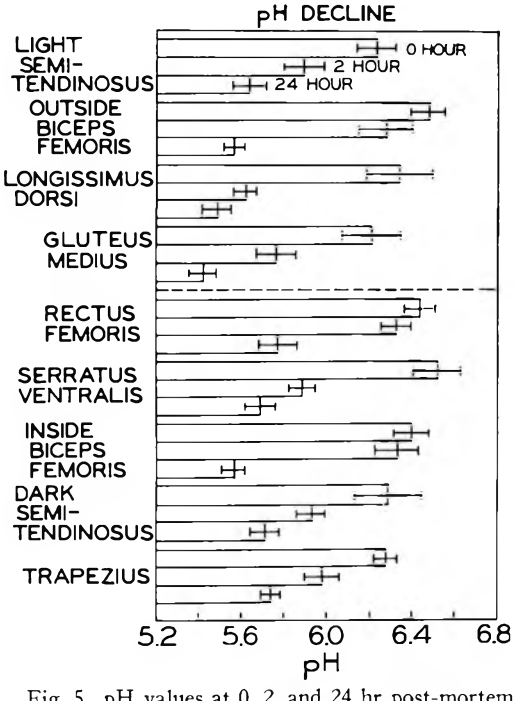

Fig. 5. pH values at 0, 2, and 24 hr post-mortem of several porcine muscles. Dotted line divides muscles into red and white muscle groups.

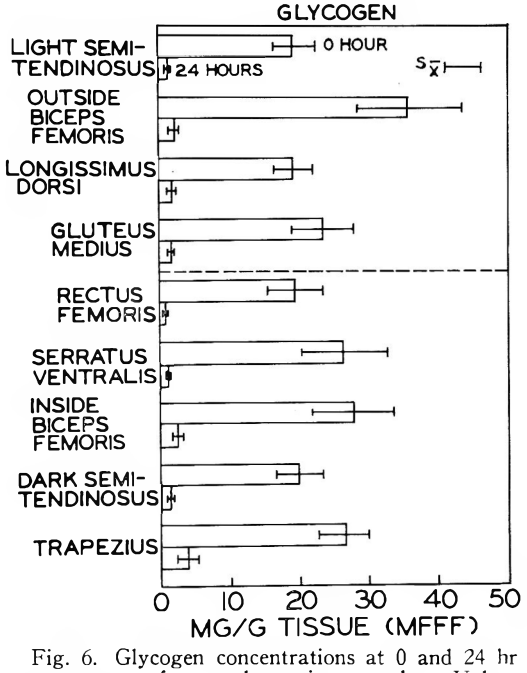

Fig. 6. Glycogen concentrations at 0 and 24 hr post-mortem of several porcine muscles. Values are expressed as mg glycogen per g of moisture-free, fat-free tissue. Dotted line divides muscles into red and white muscle groups.

hr) glycogen values were similar in all muscles studied (Fig. 6). These observations suggest that the extent of post-mortem glycolysis was similar in both white and red muscles. Percent red fibers, myoglobin concentrations, and succinic dehydrogenase activities were not highly associated with initial (0-hr) or ultimate (24-hr) glycogen values. Beecher *et al.* (1965) noted higher initial glycogen concentrations in light portions than in dark portions of the same porcine muscle (semitendinosus). However, these workers also noted that the semitendinosus light portion was more sensitive (faster rate of glycolysis) to reactions and struggle associated with death than the semitendinosus dark portion.

Lactic acid concentrations at the time of death were similar in all muscles, whereas ultimate (24-hr) lactic acid values tended to be slightly higher in white muscles (Fig. 7). Van Wijhe *et al.* (1964) reported that white muscle fibers contained a predominance of lactic dehydrogenase isozyme V, which is not inhibited by pyruvate, whereas red muscle fibers contained lactic dehydrogenase isozyme I, which is inhibited by pyruvate. Domonkos and Latzkovits (1961) reported that when pyruvate is used as a substrate, tonic (red) muscles consume greater volumes of oxygen and produce less lactic acid than tetanic (white) muscles. From observations in this study it can be inferred that the pyruvate produced in red muscles during post-mortem glycolysis either inhibited the production of lactic acid and/or a portion of the pyruvate produced was metabolized by the Krebs cycle post-mortem.

Generally, the post-rigor sarcomere length was slightly longer in red muscles than in white muscles (Fig. 8). However, specific red muscles (semitendinosus dark portion and trapezius) had significantly ($P < .05$) longer post-rigor sarcomeres than specific white muscles (gluteus medius and longissimus dorsi).

Post-rigor sarcomere length was significantly ($P < .01$) associated with the percent red fibers ($r = .45$), succinic dehydrogenase ($r = .53$), and myoglobin content ($r = .66$).

Rigor mortis (*in situ*) develops earlier post-mortem in white muscles than in red muscles (Millo and Schilling, 1964). Sink

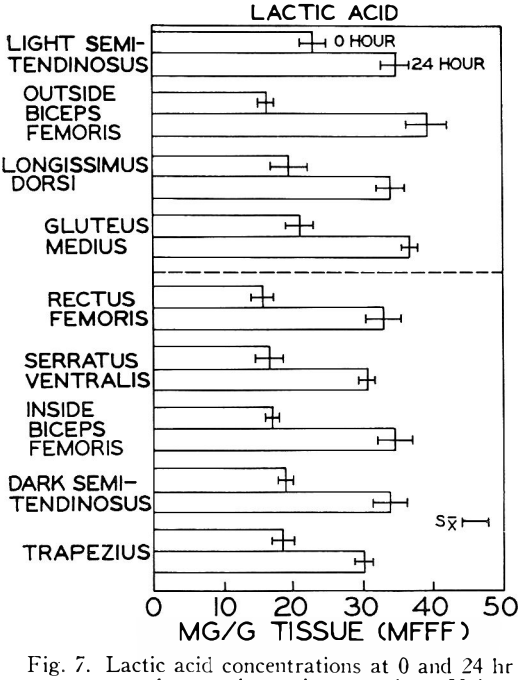

Fig. 7. Lactic acid concentrations at 0 and 24 hr post-mortem of several porcine muscles. Values are expressed as mg lactic acid per g of moisture-free, fat-free tissue. Dotted line divides muscles into red and white muscle groups.

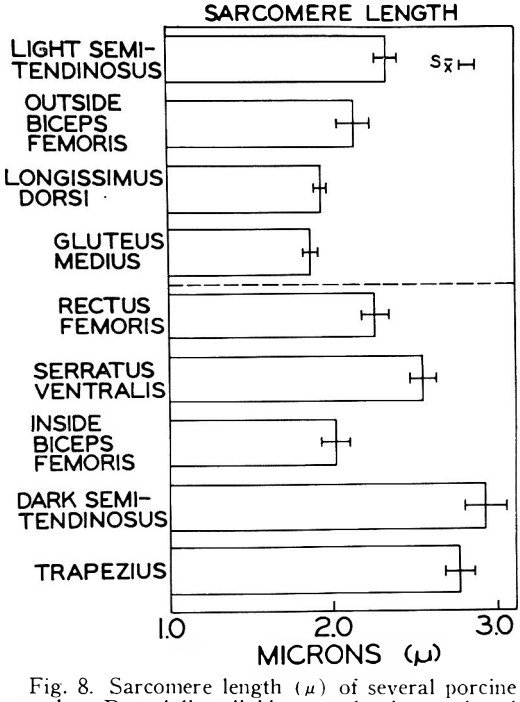

Fig. 8. Sarcomere length (μ) of several porcine muscles. Dotted line divides muscles into red and white muscle groups.

et al. (1965) noted a significant ($P < .01$) relationship ($r = .90$) between post-rigor sarcomere length and delay phase of rigor mortis in the porcine longissimus dorsi. These observations are in agreement with the present results and suggest that sarcomere length is altered by the extent of contraction of the muscle during rigor mortis. Herring *et al.* (1965) indicated that the sarcomere lengths of several bovine muscles were altered when carcass orientation was changed (suspended vertically compared to placed horizontally) prior to development of rigor-mortis.

It should be pointed out that red muscles are much more resistant than white muscles to the development of pale, soft, exudative musculature (Briskey, 1964). The longer sarcomere in the red muscle may be an essential feature contributing to its greater resistance to post-mortem alteration. Additionally the longer sarcomere, of the red muscles, may either contribute to or result from the slower post-mortem glycolytic rate (Infantee *et al.*, 1964). Further work is required to establish the physiological resting length of sarcomeres and to determine the collective effect of post-mortem changes on post-rigor sarcomere lengths.

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SAMUEL KON[a] and JOHN R. WHITAKER
Department of Food Science and Technology
University of California, Davis, California 95616

Separation and Partial Characterization of the Peroxidases of Ficus glabrata Latex

SUMMARY

Three protein components with peroxidase activity were separated from *Ficus glabrata* latex by chromatography on diethylaminoethyl cellulose at pH 7.0. Purification was aided by carboxymethyl cellulose adsorption and ammonium sulfate precipitation. These three components were identical to horse-radish peroxidase II with respect to molecular weights, absorption spectra, and activity-pH relationships. They differed markedly from horse-radish peroxidase II with respect to isoelectric points [at pH 4.25–4.45 (acetate buffer), vs. pH 7.2]. They were more heat-stable than horse-radish peroxidase II. While one of the *F. glabrata* peroxidases had V_{max} values similar to those of horse-radish peroxidase II on hydrogen peroxide and guaiacol, the other two *F. glabrata* peroxidases had quite different kinetic parameters. The three *F. glabrata* peroxidases differed in chromatographic, electrophoretic, heat stability, and kinetic properties (components *B* and *C*, vs. *A*), but all other properties measured were identical or similar for the three peroxidases.

INTRODUCTION

Although peroxidase activity has been found in all plant cells, the biological functions of this group of enzymes are not yet known. It is not certain whether they take part in one of the major respiratory pathways or serve only for the removal of hydrogen peroxide that is formed in a number of biological reactions. Paul (1963) has indicated that this group of enzymes probably perform a variety of biological functions.

Horse-radish peroxidase (HRPO II) has received the most attention (Paul, 1963). However, peroxidases from other sources have also been purified and partially characterized. Other plant peroxidases which have been investigated include those from turnips (Yamazaki *et al.*, 1956), Japanese radish (Morita and Kondo, 1954; Morita and Kameda, 1957), sweet potato tubers (Kondo and Morita, 1952), broad bean leaves (Morita, 1954), corn leaf sheath (McCune, 1961), wheat germ (Tagawa and Shin, 1959), and fig latex (Sumner and Howell, 1936). Myeloperoxidase from leucocytes (Agner, 1941, 1958), cytochrome *C* peroxidase from baker's yeast (Altschul *et al.*, 1940; Abrams *et al.*, 1942), and lactoperoxidase from milk (Theorell and Åkesson, 1943) have also been investigated.

Although peroxidase from fig latex was partially purified in 1936 (Sumner and Howell, 1936) and was found to be present in large amount, it has not received further study. In our work on the proteolytic enzymes of fig latex (Kramer and Whitaker, 1964; Sgarbieri *et al.*, 1964), particularly with regard to their biological function, it appeared necessary to study also the properties of the peroxidase activity present. The presence of this activity should also be considered in practical usage of ficin. This paper describes the purification of three protein components which have peroxidase activity, and presents some of the physicochemical and enzymatic properties of these three components. These properties are compared with those of HRPO II.

EXPERIMENTAL

Materials. *Enzymes.* Dried fig latex collected from the species *Ficus glabrata* was obtained from Merck and Company, Rahway, New Jersey, as a yellow powder. It was stored at −25°. Carefully preserved liquid fig latex, also from the species *F. glabrata*, was obtained from South America through the courtesy of Merck and Company. Acetic acid and chloroform had been added as a preservative. The gum was removed as described previously (Sgarbieri *et al.*, 1964), and the clear, straw colored liquid was stored at −25°.

Horse-radish peroxidase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, and stored at 4°.

Reagents. Carboxymethyl cellulose (CM-cellulose) and diethylaminoethyl cellulose (DEAE-cellulose) were obtained from Whatman, W. and R. Balston, Ltd., England. The CM-cellulose con-

[a] Present address: Western Regional Research Laboratory, United States Department of Agriculture, Albany, California 94710.

tained 0.50 meq of carboxyl groups per gram dry weight of resin, and DEAE-cellulose contained 0.80 meq of nitrogen per gram dry weight of resin. The DEAE-cellulose was sieved dry to obtain 100-mesh material before use.

Guaiacol was from K and K Laboratories, Jamaica, New York, and hydrogen peroxide (30%) was from Merck and Company, Rahway, New Jersey. All other compounds were reagent-grade.

Methods. *Protein determination.* The protein content of fractions from columns was determined at 280 mμ in a Beckman DU spectrophotometer. Protein content of the purified fractions was determined by the method of Lowry *et al.* (1951) against crystallized bovine plasma albumin as a standard protein. Absorbance at 403 mμ was also determined on all fractions.

Enzymatic activity. Activity was determined essentially as described by Maehly and Chance (1954) and Chance and Maehly (1955). The 3-ml reaction mixture consisted of appropriate buffer and appropriate concentrations of guaiacol and hydrogen peroxide equilibrated at 30.0° in a thermostated cell compartment of a Bausch and Lomb colorimeter. The reaction was started by rapid addition and mixing of 0.05 ml of properly diluted enzyme, and change in absorbance at 420 mμ was read at 15-sec intervals for 2 min.

Zone electrophoresis. Electrophoresis was carried out on cellulose acetate strips (1.25 × 16.0 cm; Gelman Instrument Company, Chelsea, Michigan) which were suspended horizontally. All runs were for 2 hr at 300 volts and room temperature. At completion of the run the protein was denatured with methanol and dyed with 0.1% Amido Black 10B in a 50:50:10 solution of water–methanol–acetic acid. Excess dye was removed with the same solvent system. Conductivities of the buffer solutions were determined in a Fisher conductivity cell using a conductivity bridge, Model RC-1, Industrial Instruments.

Molecular weights. Molecular weights were determined on a Sephadex G-100 column (1.4 × 197 cm) as described by Whitaker (1963). The buffer was 0.1*M* acetate–0.4*M* sodium chloride, pH 6.0, and flow rate was 0.1 ml/min/cm^2. γ-Globulin, used to determine V_0, was determined by the Lowry *et al.* method (1951). Peroxidase was determined by activity on guaiacol.

Purification. All operations were performed at room temperature except dialysis and chromatography, which were performed at 4°. The enzymes were found to be stable under these conditions.

Step A. A 20% aqueous solution of dried fig latex was centrifuged for 30 min at 0° in a Spinco ultracentrifuge at 105,000 × G. The clear, brown supernatant liquid was dialyzed against deionized water for 24 hr and centrifuged at 26,000 × G, and the clear supernatant liquid was used in Step B.

Step B. The basic proteins, largely proteolytic enzymes, were adsorbed onto CM-cellulose at pH 4.9 as previously described (Sgarbieri *et al.*, 1964). After filtration, the resin was washed with the same buffer (0.005*M* citric acid–0.010*M* sodium phosphate, pH 4.9) and the two filtrates combined. This removed all the proteolytic enzyme activity.

Step C. Solid ammonium sulfate was added gradually, with mechanical stirring, to the filtrate of Step B to 50% of saturation. After standing at room temperature for 1 hr the solution was centrifuged. Additional solid ammonium sulfate was added slowly to the clear supernatant liquid to 80% of saturation. The solution was again centrifuged, the supernatant liquid discarded, and the precipitate dissolved in a minimum volume of deionized water. The solution was dialyzed against five changes of deionized water for a total of 24 hr.

Step D. The peroxidase components were adsorbed to DEAE-cellulose which had been equilibrated with 0.001*M* pH 7.0 phosphate buffer. One gram (dry weight) of resin was used for each 40 ml of solution. The resin-enzyme was washed twice with 500 ml 0.01*M* sodium phosphate buffer, pH 7.0. The enzymes were then eluted from the resin with 0.08*M* sodium chloride in 0.01*M* phosphate buffer, pH 7.0. Solid ammonium sulfate was added slowly to the solution, with mechanical stirring, to 80% of saturation. After 1 hr the solution was centrifuged, and the precipitate was dissolved in a minimum volume of deionized water and dialyzed as in Step C.

Step E. The enzyme solution prepared in Step D was chromatographed on a DEAE-cellulose column. Before use, the resin was washed with 0.25*M* sodium chloride in 0.25*M* sodium hydroxide, with deionized water until free of chloride, and then five times with 500-ml portions of 0.001*M* sodium phosphate buffer, pH 7.0. The pH was initially adjusted to pH 7.0 when suspended in the buffer, and was checked at the end of the equilibration with buffer. Columns (2.5 × 40 cm) were poured at room temperature and then moved to a cold room (4°). Before use, the columns were washed in the cold room with about 1000 ml of the 0.001*M* sodium phosphate buffer, pH 7.0.

The enzyme components were eluted from the column by a stepwise elution procedure. All sodium chloride solutions were made up in 0.01*M*

sodium phosphate buffer, pH 7.0. Typical chromatograms are shown in Fig. 1.

Step F. The three peroxidase components obtained from Step E were rechromatographed on DEAE-cellulose columns under the conditions described in Step E. Each component was first dialyzed against deionized water and then placed directly on the column. The enzyme was adsorbed to the upper 3 cm of the resin. Elution was the same as in Step E. The rechromatographed components were dialyzed against deionized water, and concentrated by adsorption on a small DEAE-cellulose column (1.2 × 9.0 cm, resin prepared as in step E), cutting off the upper part of the resin column, and eluting the enzyme with a minimum volume of 1*M* sodium chloride. The sodium chloride eluates were dialyzed as in Step C.

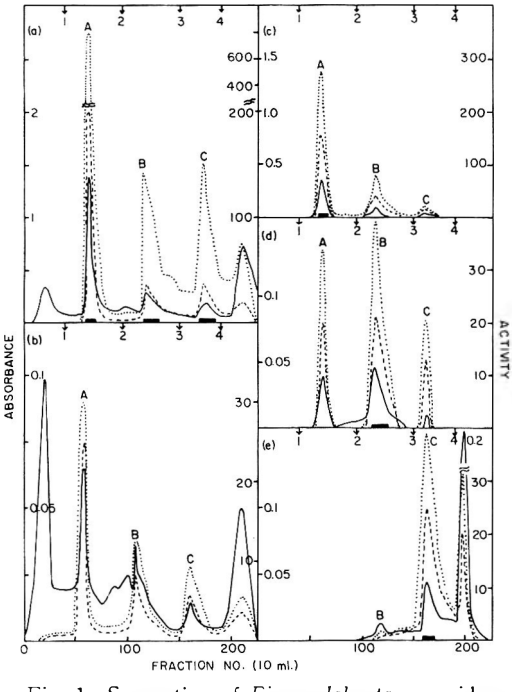

Fig. 1. Separation of *Ficus glabrata* peroxidases on DEAE-cellulose. Chromatography was carried out on 2.5×40-cm columns in 0.001*M* sodium phosphate buffer, pH 7.0, with stepwise increases in sodium chloride concentration as indicated by the arrows 1, 0.04*M*; 2, 0.06*M*; 3, 0.08*M*; and 4, 1.0*M*. ——— absorbance at 280 mμ; - - - - - - absorbance at 403 mμ; ..... peroxidase activity at 30.0° and pH 6.0 in 0.1*M* sodium phosphate–1*M* sodium chloride buffer using 1.8 m*M* guaiacol and 5.0 m*M* hydrogen peroxide. The bars at the bottom of the graph indicate the portion of a component combined and saved for further investigation. *a*) dried *Ficus glabrata* latex purified through Step D (see Table I); *b*) liquid *Ficus glabrata* latex purified through Step C (see Table I); *c, d, e*) respectively rechromatography of components *A, B,* and *C* obtained from *a*.

The purification procedure is summarized in Table 1. Components *A, B,* and *C* of Step E represent only the part of each component which was combined for rechromatography, and components *A, B,* and *C* of Step F represent only the part of each rechromatographed component which was used for further characterization of the peroxidases. This is the reason for the low percentage of the recoveries.

RESULTS

Fig. 1a shows the results of chromatography of material purified through Step D. On rechromatography of the three peroxidase components (Fig. 1c,d,e) some contamination of each component with the other components was found. This was caused by incomplete separation during the first chromatography and does not appear to involve an equilibrium transformation of one component to another during the chromatography. By cellulose acetate zone electrophoresis (see below), the components were free of each other after rechromatography.

Chromatography of a carefully preserved sample of liquid fig latex under conditions identical to those used for the dried latex, gave results essentially the same as obtained with the dried latex (compare Fig. 1a and Fig. 1b). (Step D was omitted with the liquid latex.) The three peroxidase components were present in approximately the same proportion in both the liquid latex and the dried material (28, 36, and 36%, vs. 41, 28, and 31% for components *A, B,* and *C* for liquid and dried latex, respectively). This would appear to eliminate the possibility that one or more of the three components found in dried latex are artifacts produced during drying. The dried latex was found to contain 0.3–0.5% peroxidase by weight.

The remainder of the work reported below was performed using the rechromatographed enzymes. The fraction of each component saved from the rechromatography and used in these studies is indicated by the bars in Figs. 1c, 1d, and 1e. Each component was electrophoretically pure as determined on cellulose acetate at several pH values.

Physicochemical properties. *Molecular weights.* The molecular weights, determined by the Sephadex gel filtration method (Whitaker, 1963), were 52,500 for all three *Ficus glabrata* peroxidases as well as for HRPO II. Since the molecular weight of HRPO II has been determined by sedimentation velocity to be 40,200 (Maehly, 1955), the molecular weights of the three *F. glabrata* peroxidases must also be about 40,000. Proteins which contain appreciable amounts of carbohydrates give high values for molecular

Table 1. Purification of *Ficus glabrata* peroxidases.[a]

Purification step	RZ	mg Protein (Lowry)	Total activity (10^{-3})	Specific activity	Recovery (%)	Purification (fold)
Step A						
1. Original solution		200,000	500	2.50	100.0	1.0
2. After dialysis		117,000	490	4.18	98.0	1.7
Step B						
1. After CM-cellulose adsorption		50,700	443	8.76	88.5	3.5
Step C						
1. Supernate from 50% $(NH_4)_2SO_4$ precipitation		36,400	376	10.3	75.2	4.1
2. Redissolved 80% $(NH_4)_2SO_4$ precipitate	0.16	5,000	318	63.6	63.6	25.3
Step D						
1. After elution from DEAE-cellulose	0.27	1,200	141	118	28.3	47.1
2. Redissolved 80% $(NH_4)_2SO_4$ precipitate	0.73	408	140	343	27.9	137
Step E—chromatography on DEAE-cellulose						
1. Component A[c]	1.32	110	41.6	378	8.3[b]	151
2. Component B[c]	1.06	32	14.3	446	2.9[b]	179
3. Component C[c]	1.39	32	14.5	453	2.9[b]	181
Step F—rechromatography on DEAE-cellulose						
1. Component A	1.55	40.4	15.8	391	3.2[b]	156
2. Component B	1.09	9.2	4.40	478	0.9[b]	191
3. Component C	1.51	10.1	4.14	410	0.8[b]	164

[a] Activity was determined at 30.0° and pH 6.0 in 0.1*M* sodium phosphate—1.0*M* sodium chloride using 1.8m*M* guaiacol and 5.0m*M* hydrogen peroxide. Unit of activity is defined as change in absorbance at 420 mμ per minute. Specific activity is activity per mg protein (Lowry *et al.*, 1951).

[b] Based on original activity.

[c] This represents only the fraction used for further investigation.

weights by the gel filtration method (Whitaker, 1963).

Electrophoretic properties. The three *F. glabrata* peroxidases have slightly different isoelectric points, which are dependent to some extent on the nature of the buffer (Fig. 2, Table 2) and which are quite different from that of HRPO II. When electrophoresis was performed in 0.01*M* sodium phosphate buffer, pH 7.0 (buffer used for chromatography on DEAE-cellulose), the three *Ficus glabrata* peroxidases moved to the anode (*A*, 1.50 cm; *B*, 1.62 cm; *C*, 1.77 cm) whereas HRPO II moved to the cathode (1.40 cm). The isoelectric point of HRPO II has been reported to be at pH 7.2 (Theorell and Åkesson, 1943). A single protein band for each component was found under all conditions used.

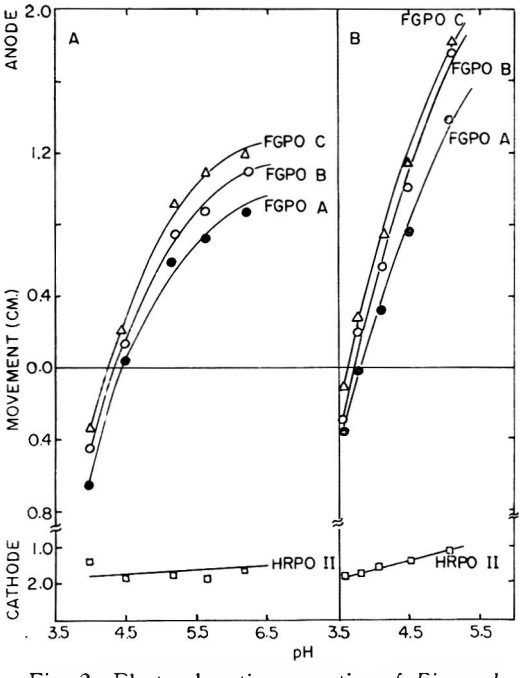

Fig. 2. Electrophoretic properties of *Ficus glabrata* peroxidases *A*, *B*, and *C* and horse radish peroxidase II on cellulose acetate strips in 0.05*M* sodium acetate–sodium chloride buffers (A) and in aniline–acetic acid buffers (B). Electrophoresis was for 2 hr at 300 volts at room temperature. Conductivities of the buffer solutions were maintained reasonably constant at all pH's by the use of sodium chloride, and were 3.23–3.54 × 10^{-3}ohm^{-1} cm^{-1} for the acetate buffers and 0.98–1.56 × 10^{-3} ohm^{-1}cm^{-1} for the aniline-acetic acid buffers.

Heat stability. There is a marked difference in heat stability between the two groups of enzymes, with the *Ficus glabrata* peroxidases being much more stable to heat than is HRPO II (Fig. 3). There are also differences among the *F. glabrata* peroxidaseses (FGPO), with FGPO *B* being the most heat-stable, followed by FGPO *C* and *A*.

Absorption spectra. The absorption spectra of the three *F. glabrata* peroxidases and HRPO II were determined on a Beckman DK-2 spectrophotometer. The spectra in the visible and Soret region were identical for all four components (for spectra of HRPO II, see Saunders *et al.*, 1964). From the hemin content, the molecular weights, and the abosorption spectra, there would appear to be one hemin group per molecule. This hemin group appears to be identical to that of HRPO II, which is the ferric complex of protoporhyrin IX.

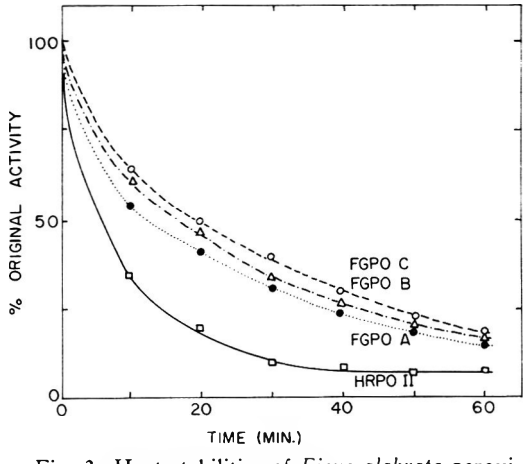

Fig. 3. Heat stabilities of *Ficus glabrata* peroxidases *A*, *B*, and *C* and horse radish peroxidase II at 74.0°. Protein concentrations were 8 μg/ml. Incubation was carried out in 0.02*M* sodium phosphate buffer at pH 7.0.

Table 2. Comparison of physicochemical properties of *Ficus glabrata* peroxidases *A*, *B*, and *C* and horse radish peroxidase II.

	Ficus glabrata peroxidase			HRPO II
	A	B	C	
Molecular weights[a]	40,200	40,200	40,200	40,200
Heat stability ($t_{1/2}$ (min) at 74°)	13.5	21.5	17.0	6.5
Isoelectric point				
Acetate buffer	4.4_5	4.3_5	4.2_5	
Aniline-acetic acid buffer	3.8_0	3.7_0	3.6_0	

[a] Assuming molecular weight of horse radish peroxidase II to be 40,200 (Maehly, 1955). The actual values obtained by gel filtration were 52,500.

Enzymatic properties. The effects of pH on the activity of the three *F. glabrata* peroxidases and of HRPO II are very similar (Fig. 4). The pH optima are at pH 6.3–6.4, and the half-maximal activities (designated as apparent pK_1 and apparent pK_2) are at pH 4.26–4.49 and pH 8.23–8.40 (Table 3). It would appear that the same groups are involved in the activity of both the three *F. glabrata* peroxidases and HRPO II.

Effect of guaiacol and hydrogen peroxide concentrations on the observed activities of FGPO *A*, *B*, *C* and HRPO II is shown in Fig. 5, where the data are plotted according to the method of Lineweaver and Burk (1934), and in Table 3. Under

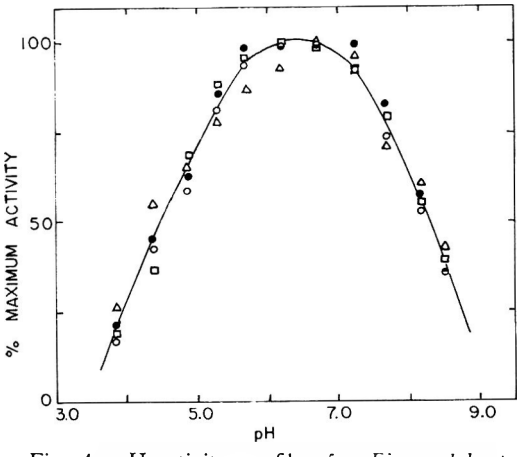

Fig. 4. pH-activity profiles for *Ficus glabrata* peroxidases A (●), B (○) and C(□) and horse radish peroxidase II (△). The reactions were carried out in 0.1*M* Tris-acetate buffers containing 1.0*M* sodium chloride at 30.0° using 30.0m*M* guaiacol and 5.0m*M* hydrogen peroxide.

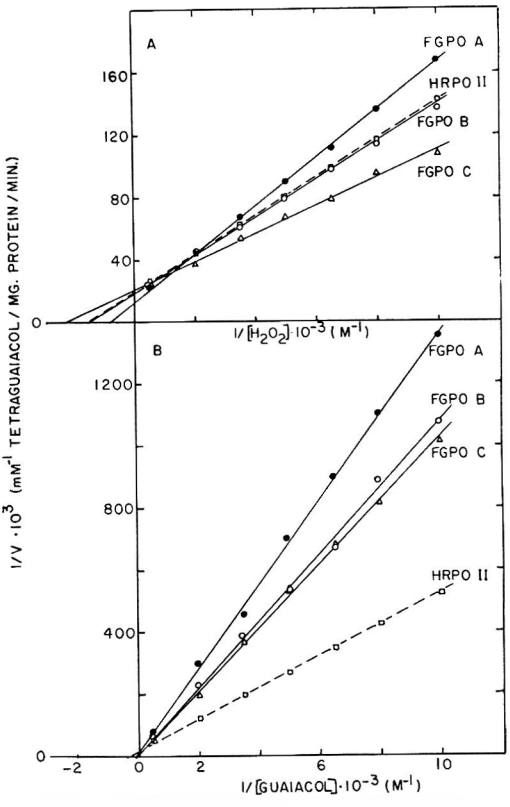

Fig. 5. Substrate-activity and acceptor-activity relationships for *Ficus glabrata* peroxidases *A*, *B*, and *C* and horse radish peroxidase II. A) reciprocal plot for hydrogen peroxide-activity relationship at 30.0m*M* guaiacol; B) reciprocal plot for guaiacol-activity relationship at 5.0m*M* hydrogen peroxide. All reactions were carried out at 30.0° and pH 6.5 in 0.1*M* sodium phosphate–1.0*M* sodium chloride buffer.

Table 3. Comparison of enzymatic properties of *Ficus glabrata* peroxidases *A*, *B*, and *C* and horse radish peroxidase II.

	Ficus glabrata peroxidase			HRPO II
	A	B	C	
pH optimum	6.32	6.37	6.39	6.33
Apparent pK_1[a]	4.42	4.49	4.48	4.26
Apparent pK_2[a]	8.23	8.25	8.30	8.40
Guaiacol[b]				
Apparent K_m (m*M*)	7.71	13.8	14.3	2.20
V_{max} (moles tetraguaiacol formed/ mole enzyme[c]/sec) $\times 10^{-5}$	0.885	1.44	1.64	0.784
Hydrogen peroxide[b]				
Apparent K_m (m*M*)	1.07	0.650	0.420	0.640
V_{max} (moles hydrogen peroxide utilized/ mole enzyme[c]/sec) $\times 10^{-5}$	4.32	2.42	2.17	3.73

[a] Determined by least-squares analysis of the data according to the method employed by Hammond and Gutfreund (1959).
[b] Determined by least-squares analysis of the data shown in Fig. 5.
[c] Based on molecular weight of 40,200 (see Table 2) and a hemin content of 1.36% (Maehly, 1955).

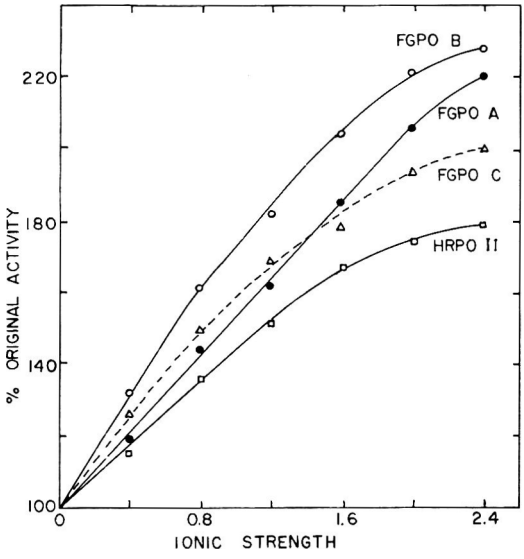

Fig. 6. Effect of ionic strength on activities of *Ficus glabrata* peroxidases A, B, and C and horse radish peroxidase II. Reactions were carried out at 30.0° and pH 6.5 in 0.01*M* sodium phosphate buffer using 5.0m*M* hydrogen peroxide and 1.8m*M* guaiacol. Sodium chloride was used to vary ionic strength. "Zero" ionic strength on the graph is that of 0.01*M* phosphate buffer at pH 6.5 ($\mu = 0.016$).

the conditions used, FGPO *B* and *C* are quite similar with respect to K_m (app) and V_{max} for guaiacol and for hydrogen peroxide but differ significantly from FGPO *A* and HRPO II. FGPO *A* is quite similar to HRPO II with respect to V_{max} for both hydrogen peroxide and guaiacol, but they are quite different with respect to K_m (app) for these compounds.

The effect on the activities of the peroxidases of varying the ionic strength of the reaction medium is shown in Fig. 6. All four enzymes exhibit essentially a linear relationship between increase in activity and ionic strength up to approximately 1.6 ionic strength. This is in agreement with Whitaker and Tappel (1962), who found the same effect on HRPO II when different salts were used. The data indicate 2.2-, 2.3-, 2.0-, and 1.8-fold increases in activity for FGPO *A*, *B*, *C* and HRPO II, respectively, in the presence of 2.4*M* sodium chloride.

DISCUSSION

The three peroxidases isolated from dried latex of *Ficus glabrata* do not appear to be artifacts produced by the drying process or by the separation procedure. The three enzymes were found in carefully preserved liquid latex in essentially the same amounts and with identical chromatographic properties as those in the dried latex. Upon rechromatography the enzymes behaved as individual entities. Multiple components of horse-radish peroxidase with identical spectra and activities but separable by salt precipitation, chromatography, and paper electrophoresis have been reported (Theorell, 1943; Jermyn and Thomas, 1954; Paul, 1958; Tagawa and Shin, 1959). Paul (1958) separated horse-radish peroxidase activity into four components by chromatography on CM-cellulose. Hosoya (1960a) has separated three peroxidases from turnips, McCune (1961) has demonstrated the presence of six peroxidase-active components in cornleaf sheath preparations by starch electrophoresis, and Kondo and Morita (1952) have reported two forms of sweet potato peroxidase. On the other hand, only one peroxidase was found in latex from *Ficus macrophylla* Roxb. (Jermyn and Thomas, 1954).

The molecular weights, prosthetic groups, ionic strength effects, pH optima, and pH-activity dependencies appear to be the same or very similar for FGPO *A*, *B*, and *C* and for HRPO II. pH-activity dependencies of turnip peroxidases A_1, A_2, and D (Hosoya, 1960b) and Japanese radish peroxidase (Tonomura, 1953) similar to those found in this investigation have been reported.

FGPO *A*, *B*, and *C* differed markedly from HRPO II in electrophoretic properties. While the isolectric points of FGPO *A*, *B*, and *C* were respectively found to be at pH 4.4_5, 4.3_5, and 4.2_5, (in acetate buffer), the isoelectric point of HRPO II is at pH 7.2 (Theorell and Åkesson, 1943). However, Jermyn and Thomas (1954) reported that horse-radish juice contained two major peroxidase components which were negatively charged at pH 5.0. The peroxidase of *F. macrophylla* was also found to be negatively charged at pH 5.0 (Jermyn and Thomas, 1954). Turnip peroxidases A_1 and A_2 were reported to have isoelectric points below pH 7.0 (Hosoya, 1960a).

Differences were found among the components with respect to kinetic parameters measured using hydrogen peroxide and guaiacol. FGPO *B* and *C* appeared to be

very similar but differed from FGPO *A.* Hosoya (1960c) reported that the three peroxidase components of turnips had different kinetic parameters with guaiacol as the hydrogen donor.

The three *F. glabrata* peroxidases were also distinguishable on the basis of heat stability. They were more heat-stable than HRPO II.

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ELAINE NELSON McINTOSH[a]

Departments of Home Economics Research and Animal Science
Iowa Agricultural and Home Economics Experiment Station
Iowa State University, Ames, Iowa 50010

Isolation of Mucoprotein in Bovine Skeletal Muscle

SUMMARY

A mild method for the isolation of mucoprotein from bovine skeletal muscle is described. Minced skeletal muscle was extracted twice with neutral 0.6*M* KCl. The washed stromal residue was extracted twice with acetone, air-dried, and pulverized. The stromal residue powder was extracted twice with 10% $CaCl_2$. The pooled $CaCl_2$ extracts were added to 1.5 volumes ice-cold ethanol and refrigerated. The crude mucoprotein precipitate was obtained by centrifugation, and suspended in 5% sodium acetate. After extraction for 24 hr at 4°C, the mucoprotein suspension was filtered through Celite and purified. The final dried product was pulverized to a light tan powder. Dissolved in phosphate buffer, it gave a viscous milky-white solution. Quantitative hexosamine and nitrogen values obtained at each major step of the isolation procedure are given, and their significance is discussed. Less than 10% of the total stromal residue hexosamine is extractable by $CaCl_2$. The $CaCl_2$-extractable fraction was isolated and purified and designated the "soluble mucoprotein fraction." Hydrolysates of this fraction were analyzed by paper chromatography. The consistent observation of galactosamine and glucuronic acid indicated that chondroitin sulfate was present. Glucosamine was also noted consistently, along with galactose, but their presence was not easily explained.

INTRODUCTION

The homogeneous ground substance of skeletal muscle, in which the collagen and elastin fibers are embedded (Gersh and Catchpole, 1949) is a mucosubstance. Mucoprotein has long been recognized as a connective-tissue component, and has been the subject of numerous reviews and symposia (Dorfman, 1962; Hoffman and Meyer, 1962; Muir, 1964; New York Heart Assoc., 1964). However, little specific information has been reported regarding its occurrence and character in skeletal muscle. Study of this component of skeletal muscle was initiated in this laboratory by Miller and Kastelic (1956), who reported on a fractionation study of bovine skeletal muscle.

McIntosh (1961) later developed a method for determining relative mucoprotein content in skeletal muscle. She found that, within muscles, mucoprotein hexosamine content paralleled elastin content very closely. There appeared to be a negative correlation between the amount of the three connective-tissue components (collagen, elastin, and mucoprotein) and the relative tenderness of representative muscles within an animal. The results supported the hypothesis of Miller and Kastelic (1956) that mucoprotein should be considered along with collagen and elastin in assessment of the relation of connective tissue to meat tenderness.

In an attempt to delineate this component of connective tissue, efforts were directed toward isolation and characterization of the mucoprotein fraction. A valid characterization of this fraction appeared to be dependent upon a method of isolation that would result in a fair yield with a minimum of degradation. The final procedure developed for the isolation and purification of mucoprotein from skeletal muscle is presented here.

EXPERIMENTAL METHODS

Chemicals. The biochemicals used were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Aminoacridine hydrochloride was obtained from the California Corporation for Biochemical Research, Los Angeles, California.

Preparation of meat samples. Two semitendinosus muscles were excised immediately after slaughter from two 18-month-old Hereford steers of the same genetic backgound which had been fed a normal diet. After the muscles were freed of fat and ground in a Hobart silent cutter, the pooled meat sample (usually 3.5 kg) was subjected to the isolation procedure developed in preliminary trials.

Paper chromatography. Descending paper chromatography was used for separation and preliminary identification of the various sugar components present in the mucoprotein hydrolysates. The sample to be analyzed (10 mg or less) was hydrolyzed for 2 hr in 1*N* HCl in an autoclave at 15 lb pressure. After cooling, chloride ions were removed

[a] Present address: 2842 East Alta Vista Street, Tucson, Arizona.

by adding a stoichiometric amount of silver carbonate (Fischer and Nebel, 1955). The resultant silver chloride precipitate was removed by centrifugation. Suitable amounts of the supernatant were then applied with micropipettes to strips of Whatman No. 1 filter paper, 4¼ × 16 inches, along with appropriate reference sugars. The strips were placed in cylindrical jars, 6 inches in diameter and 18 inches high, and irrigated with butanol-pyridine-water (Partridge, 1948). After 20–24 hr of migration with this solvent at 30°C, the strips were air-dried. The spots were developed by pulling the strips quickly through baths of silver nitrate in acetone and 0.5*N* NaOH in ethyl alcohol (Trevelyan *et al.*, 1950). Following maximum development of the spots, the excess silver hydroxide was removed by passing the strips through a third bath of 5*N* NH_4OH, followed by thorough washing with water. After air-drying, the chromatograms could be preserved for an indefinite period.

Hexosamine. Total hexosamine was determined quantitatively as described earlier by McIntosh (1961).

Nitrogen. Nitrogen was determined by using a modification of the micro-Kjeldahl method of Johnson (1941).

ISOLATION AND PURIFICATION

The first isolates were obtained by using enzymatic and alkali liquefaction techniques. Numerous modifications of methods reported by Meyer and co-workers (Meyer and Chaffee, 1941; Meyer *et al.*, 1953) were explored. Products were obtained by these methods in fair yield, the analyses of which afforded preliminary information as to the monosaccharide constituents of the mucoprotein fraction of bovine skeletal muscle. Because of their drastic nature, however, procedures involving either alkali or enzymatic liquefaction were ultimately abandoned in favor of an isolation method which would involve minimum artifacts. Mild extraction appeared less likely to be accompanied by degradation. Of the various extractants explored, 10% $CaCl_2$ proved to be the most convenient and satisfactory extractant for skeletal muscle mucoprotein.

Preliminary tests showed that skeletal muscle mucoprotein was extractable over a rather broad pH range from pH 5 up. Ease of extraction increased as the pH became progressively more alkaline (Einbinder and Schubert, 1950). To simulate the physiological conditions of muscle tissue, in which the pH ranges from 7.4 to 7.6 *in vivo* (Bate-Smith, 1948), the extractions described here were performed at this pH, and this pH was maintained in subsequent purification steps.

A preliminary extraction with neutral 0.6*M* KCl buffer (Miller and Kastelic, 1956) was adopted for removing intracellular proteins, i.e., the albumins and globulins. Because of their hexosamine content, removal of these foreign proteins was essential (Boas, 1955; McIntosh, 1961). Exhaustive tests showed that a concentration of 0.6*M* KCl, within the pH range 5.5–7.4, does not remove mucoprotein from wet skeletal muscle tissue.

Mild extraction procedure. The pooled meat sample (3.35 kg) was placed in a large 10-L enamel pail, suspended in 7 L of neutral KCl buffer (McIntosh, 1961), and extracted for 24 hr at 4°C with occasional stirring. The stromal residue was separated from the viscous supernatant by straining through a kitchen sieve and washing with ice-cold water. The washed stromal residue was then resuspended in 7 L of extracting solution to remove the remaining muscle protein. After 24 hr, the supernatant was removed again by straining through a sieve. The stromal residue was thoroughly washed with water and suspended in acetone. It was extracted twice with acetone, 48 hr each time, at room temperature, squeezed through cheesecloth, and air-dried. The moisture-free fat-free fibers were finally ground to a powder in a Wiley Mill.

The stromal residue powder was extracted twice with 3 L of 10% $CaCl_2$, pH 7.6, at 4°C with occasional stirring, first for 48 hr, and then for 24 hr. Performing more than two extractions was impractical, since approximately 90% of the extractable mucoprotein was obtained in the first two extractions. The $CaCl_2$ extracts were removed by gently squeezing the residue with several layers of cheesecloth and were added to 1.5 volumes ice-cold ethanol and refrigerated overnight.

The crude mucoprotein precipitate was obtained by centrifugation at approximately $10^3 \times G$. The precipitate was homogeneously suspended in 5% sodium acetate, pH 7.6, by using a Waring blender for 1 min, and was

extracted for 24 hr at 4°C. The suspension was warmed to room temperature, filtered through Celite, added to 4 volumes ethanol, and refrigerated overnight. The precipitate was collected by centrifugation at 8,000 × G, suspended in 400 ml water, and purified by two precipitations with aminoacridine, essentially as described by Muir (1958). The purified mucoprotein was isolated as the sodium salt (Shatton and Schubert, 1954), dried in a desiccator over NaOH pellets for several days at room temperature, and pulverized to a light tan powder. The analysis of a typical product is given elsewhere [(Ia) in Table 1 (McIntosh, 1966)]. The yield was 437 mg. The product dissolved in phosphate buffer (Shatton and Schubert, 1954) gave a viscous, milky-white solution.

Table 1 shows roughly quantitative hexosamine and nitrogen values, as well as nitrogen-hexosamine ratios, at each major step of the isolation procedure. About 50% of the original hexosamine in fresh meat tissue has as its origin the plasma proteins extracted by the potassium chloride buffer (McIntosh, 1961). The remaining hexosamine, which is contained in the stromal protein or connective tissue residue, provides an index for total mucoprotein content (McIntosh, 1961). Less than 10% of the stromal residue hexosamine is extractable by $CaCl_2$. It is the $CaCl_2$-extractable fraction of bovine skeletal muscle that has been purified and characterized. Unless otherwise stated, the results reported refer to this purified "soluble mucoprotein fraction." The crude mucoprotein fractions have a much lower nitrogen-hexosamine ratio than the connective-tissue residue; this ratio decreases further upon subsequent purification (Fraction 4).

Table 2 shows the typical distribution of sugars in the soluble mucoprotein fraction isolated by the preceding extraction procedure, as well as mucoprotein products obtained by other methods. Consistent observation of galactosamine and glucuronic acid in the hydrolysates indicated that chondroitin sulfate was present. Therefore, the sugar components of a commercial chondroitin sulfate product isolated from bovine tracheal cartilage were also studied. For comparison, the sugar components theoretically present in chondroitin sulfate are also given. Regardless of the isolation technique employed, the hydrolyzed products were surprisingly consistent with respect to sugar components. The relative amounts of the sugars varied slightly, however, depending on the method used. Ribose occurred erratically. The presence of glucosamine in both the commercial chondroitin sulfate and the skeletal muscle mucoprotein preparation was initially puzzling. Glucosamine associated with chondroitin sulfate had been reported by Partridge and Davis (1958) and others (Kent and Whitehouse, 1955, pg. 78) working with chondroitin sulfate preparations. However, its presence was unexplained. The presence of ribose could be the result of an artifact—that, under the conditions employed here for mucoprotein isolation, ribonucleic acid was also extracted. Another possibility is the existence of a ribonucleic acid–polysaccharide complex, similar to that reported by Webb (1958). Sherrat and Thomas (1953) found a similar complex of desoxy-

Table 1. Hexosamine and nitrogen analyses of principal skeletal muscle fractions during mucoprotein isolation.[a]

Fraction	Total Hexosamine (mg)	Total nitrogen (mg)	Nitrogen-hexosamine ratio
1. Whole tissue, 3350 g	654	102,400	156.7
2. Stromal residue, 500 g	333	54,612	164.0
3. Alcohol precipitate of calcium chloride extract[b]	22.5	1,825	81.1
4. Purified mucoprotein, 437 mg	10.3	52.6	5.1
5. Residue[c], 511 g[d]	331	49,445	149.4

[a] Using mild extraction procedure described in text.
[b] Suspended in 500 ml distilled water.
[c] After mucoprotein extraction.
[d] The slight increase in weight of final residue over that of original stromal residue is attributed to calcium chloride which was retained after extraction.

Table 2. General distribution of sugars in skeletal muscle mucoprotein products isolated by different methods, and in commercial chondroitin sulfate.

Product	Galactosamine	Glucosamine	Uronate[a]	Galactose	Ribose	Mannose
I. Skeletal muscle mucoprotein						
A. Liquefaction						
1. Enzyme	+++	++++	++++	+	++	+
2. Alkali	+++	++	++	(+)	.....[b]	+
B. Extraction						
1. 5% sodium acetate	++	++++	++	(+)	.....[b]	+
2. 30% potassium chloride + 1% potassium carbonate	++	+	+++	(±)	+	—
3. 10% calcium chloride	+	+	+	—	—	—
4. 10% calcium chloride[c]	+	++	+++	(+)	+	Trace
II. Commercial chondroitin sulfate (bovine cartilage)	++++++	+	+++	+	—	Trace

[a] Also present as lactone.
[b] Not analyzed.
[c] Mild extraction procedure described in text.

ribonucleic acid carbohydrate in a strain of *Sufaecolis*.

The presence of keratosulfate (Meyer *et al.*, 1953) was suggested by the observation that galactose was present in addition to glucosamine.

Small amounts of mannose were frequently observed, particularly in the crude products before purification. Since albumin contains equimolar amounts of glucosamine and mannose, these sugars were first thought to indicate an albumin contaminant. If the mannose and glucosamine had the same origin, one would expect them to disappear to an equal degree following purification. However, while mannose was virtually absent from the purified end product (McIntosh, 1966; Table 1), glucosamine stubbornly remained. This observation suggested that, while mannose was probably of albumin origin, the glucosamine was not.

The precipitation reactions of the mucoprotein products obtained from skeletal muscle were compared with those of known glucosamine-containing mucopolysaccharides, in a preliminary attempt to identify the source of the glucosamine component. The presence of hyaluronic acid seemed unlikely in view of the contrast in precipitation reactions of this mucopolysaccharide vs. the mucoprotein products (Table 3). Heparin seemed to be eliminated also because of its different precipitation reactions.

Table 3. Precipitation reactions of skeletal muscle mucoprotein and other mucopolysaccharide-containing products.

	Skeletal muscle mucoprotein	Commercial chondroitin sulfate	Heparin	Hyaluronic acid	Gastric mucin
Heat	—	—	—	—	—
Saturated ammonium sulfate	—	—	—	—	+
Acid alcohol	+	+	+	+	+
Trichloracetic acid, 2%	+	—	—	—	—
Sulfosalicylic acid	—	—	—	—	—
Picric acid	—	—	—	—	—
Phosphotungstic acid	+	+[a]	—	—	+
Cobaltic luteochloride	+	+	+	—	—
Aminoacridine hydrochloridine	+	+	+	—	—

[a] Small amount of commercial chondroitin sulfate is phosphotungstic-acid-soluble, probably free chondroitin sulfate or chondroitin.

Sialic acid did not appear to be present in the soluble mucoprotein fraction. Results were consistently negative when the mucoprotein products were tested for the "direct Ehrlich reaction" with *p*-dimethylaminobenzaldehyde (Kent and Whitehouse, 1955, pg. 283–284). Red colorations could be obtained only by the Elson-Morgan reaction, following preliminary acid hydrolysis (McIntosh, 1961). Sialic acids have been described as very water-soluble (Zilliken and Whitehouse, 1958), whereas the products obtained here were soluble in water with difficulty. Moreover, the ready humin formation on warming with acid usually exhibited by sialic acids was not observed.

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K. YOSHIKAWA,[a] L. M. LIBBEY, W. Y. COBB, and E. A. DAY
Department of Food Science and Technology
Oregon State University, Corvallis, Oregon 97331

1-Pyrroline: The Odor Component of Strecker-Degraded Proline and Ornithine

SUMMARY

The odor component of proline and ornithine degraded by periodate, isatin, or ninhydrin was identified as 1-pyrroline by means of gas chromatography on three columns, mass spectra, and infrared analysis. Free γ-aminobutanal also yields the same odor component via formation of an internal Schiff base.

INTRODUCTION

The possible role of Strecker amino acid degradation in flavor development in a variety of foods is frequently discussed. The reaction results in the degradation of an amino acid to an aldehyde of one less carbon than the amino acid:

$$RCCR + R'CHNH_2COOH \longrightarrow R'CH + CO_2 + RCHNH_2CR$$

The products and odor properties of many of the amino acids are known. Strecker-degraded proline yields an interesting odor that has been described as mushroom-like (Keeney and Day, 1957) and "like burnt protein" and "bakery aroma" (Herz and Shallenberger, 1960). The compound(s) responsible for the odor have not been reported in the literature. This odor compound was therefore investigated. Since ornithine gives the same odor, it was studied also.

EXPERIMENTAL

Degradation of proline. Degradation of proline with sodium metaperiodate, isatin, and ninhydrin gave the same odor, and the odor fractions from the three reactions behaved identically in gas chromatography. For expediency, periodate was used to obtain large yields of the odor material, and in subsequent experiments the proline degradation products from reaction with isatin and ninhydrin were checked to ascertain that the odor component from the three reactions was the same.

Five grams of L-proline was reacted for 4 hr at 80°C with 8 g of sodium metaperiodate in 150 ml of water. During the reaction, a stream of nitrogen was bubbled through the reaction vessel. The emergent gas stream was passed through two traps cooled by ice followed by two traps cooled with ethanol–dry ice. The second ethanol–dry ice trap contained diethyl ether. Upon completion of the reaction, the contents of the first three traps were saturated with sodium chloride and extracted with peroxide-free ethyl ether. The ether extract was combined with the solvent in the second ethanol–dry ice trap. The total ether extract was dehydrated by means of the ethanol–dry ice coolant. This extract was then concentrated to about 0.5 ml and used for analysis. The odor from the sample was generally described as amine or "corn-like."

In the latter stages of the study, the proline odor component was isolated via an alternate technique. An aqueous solution of one gram of proline was added to an equimolar quantity of sodium metaperiodate solution, stirred, and refrigerated for 2.5 hr at 5°C. The solution was then adjusted to pH 8.0 with K_2CO_3, followed by saturation with NaCl. The mixture was extracted with 100 ml of purified ethyl ether, and the extract was designated POC-1. Subsequently, a 0.1-molar quantity of additional periodate was added to the reaction mixture, and the mixture was stirred for 10 min at room temperature. The solution was then extracted twice with 100-ml quantities of ether, and the two ether portions were combined (POC-2). Both the POC-1 and POC-2 extracts were dried over anhydrous sodium sulfate, filtered, and reduced to about 0.5 ml without heating.

When subjected to gas chromatography (GC) on the base-treated Apiezon L packed column, POC-1 showed two peaks other than ether, whereas POC-2 yielded only the ether peak and a second peak, which possessed the odor of interest.

Preparation of 1-pyrroline. This compound was prepared from ornithine by reaction with ninhydrin (Jakoby and Fredericks, 1959). 1-Pyrroline was isolated from the reaction mixture as follows: The supernatant was treated with Dowex-1 resin and the resulting colorless solution was saturated with NaCl and the pH was adjusted to 7.0 with Na_2CO_3. The solution was extracted with ethyl ether, and the extract was dried and the volume reduced as described above.

Gas chromatography. The Barber-Colman gas

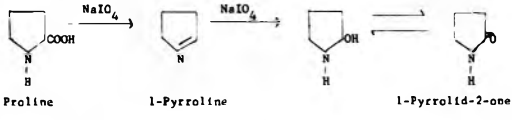
[a] On leave from the Faculty of Engineering, Osaka University, Japan.

chromatograph, model 20, equipped with an on-column injection port and a β-ionization detector, was used to obtain relative retention (t_R/t_R) data. The effluent from the column was split; a portion was passed to the detector and the remainder was vented through a heated exit port where odor characteristics could be observed. The effluent was split in such a manner that a component reached the detector and the exit port simultaneously. Three 10-ft × ⅛-in. GC columns were employed to obtain retention data on the odor component and related compounds: 20% Ucon nonpolar, 20% Carbowax 1000, and 20% Apiezon L. All stationary phases were coated onto acid-alkali-washed Celite (120–140-mesh) which had been treated with 5% NaOH (w/w) in methanol. Following evaporation of the methanol, coating of the support proceeded in the usual manner. Columns were conditioned for 24 hr at 140°C, and subsequently operated at 105–110°C.

Gas-chromatographic mass-spectrometric analysis. After suitable operating parameters had been established on the Barber-Colman gas chromatograph and the odor-bearing peak from the ether extract of degraded proline had been located and relative retention data obtained, the odorous material was then submitted to gas chromatographic analysis in conjunction with rapid-scan mass spectrometry. Used for the analysis was an Aerograph Hy-Fi gas chromatograph, model 600, in combination with an Atlas CH-4 mass spectrometer equipped with an EC-1 gas inlet valve. The Apiezon L chromatographic column described above was operated at 110°C with a helium flow rate of 20 ml/min. The effluent from the column was split: 10% of the stream went to the flame detector, and 90% was directed to the EC-1 gas inlet to the mass spectrometer. The EC-1 inlet contains an adjustable valve for control of the gas flow into the mass spectrometer. Less than 5% of the gas chromatography effluent stream passing to the EC-1 inlet was actually admitted to the mass spectrometer; the remainder was passed to the atmosphere and was observed for odor properties. The pressure in the ion source was maintained at 2×10^{-7} Torr. The lengths of the splitting capillaries were adjusted so that when a compound in the GC effluent reached the GC detector, it was also in the ion source of the mass spectrometer. By this arrangement it was possible to observe the GC strip-chart recording and take mass spectra at the desired points on a gas chromatogram. The mass spectrometer was operated at an acceleration voltage of 3000 V, an ionizing voltage of 35 eV, and an ionizing current of 60 μamp. Ryhage (1964) reported no significant differences in cracking patterns over a range of 9 to 70 eV. Since 35 eV gave the highest total ion current on our mass spectrometer, it was used rather than the more common 70 eV. This improved sensitivity without seriously affecting the cracking patterns. Mass spectra were obtained by magnetic scanning; the scanning speed was such that it took 4.5 sec to cover the range m/e 12 to 100 (m/e is the mass-to-charge ratio). The spectra were recorded on an oscillograph (Visicorder, model 1508).

RESULTS AND DISCUSSION

Fig. 1. is a typical gas chromatogram of the ether extract prepared from proline via the first technique. By smelling the effluent splitter of the GC it was determined that peak 4 exhibited the typical odor of degraded proline. Most of the people in our laboratory described the odor as "corn-like." Peak 5, which was identified by its mass spectrum as pyrrole, did not occur when proline was degraded with isatin or ninhydrin. Bragg and Hough (1958) reported that the reaction of proline with periodate occurred in two stages:



If the reaction was carried out on a mole-per-mole basis, 1-pyrroline was the major product. If two moles of periodate were used per mole of proline, 1-pyrrolid-2-one predominated. In our work, peak 4 of Fig. 1 was almost completely absent when the latter reaction conditions were employed.

In Table 1, the relative retention data for a number of authentic compounds are compared with the proline odor compound (POC). It will be noted that the t_R/t_R of 1-pyrroline and the POC are identical.

The mass spectra for 1-pyrroline, the POC and 3-pyrroline are presented as spectra A, B, and C, respectively, in Fig. 2. Background spectra were not deducted in the spectra as presented. Qualitatively, all three spectra are very similar but differences are evident in peak ratios when spectra A and B are compared with spectrum C. The base peak is m/e 41 and the parent peak is 69 in the three spectra; the small peak at m/e 70 is due to isotopic contribution, for example, from C^{13} and/or N^{15}. The parent peak of

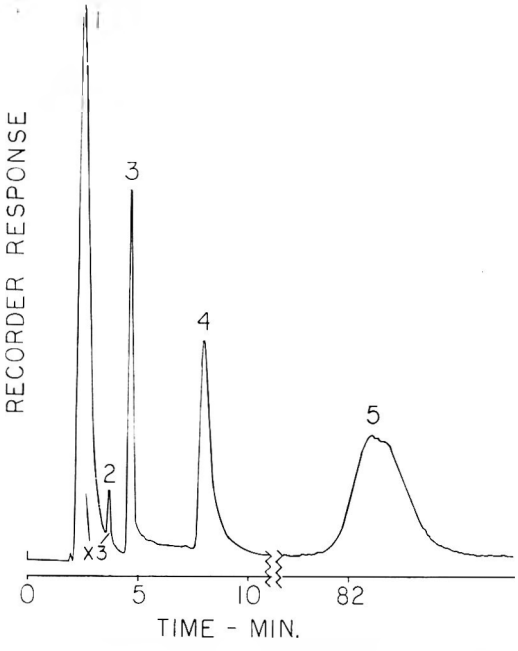


Fig. 1. Gas chromatogram of the ether extract of proline degraded with periodate 20% Ucon nonpolar liquid phase on alkali-treated Celite, column operated at 105°C.

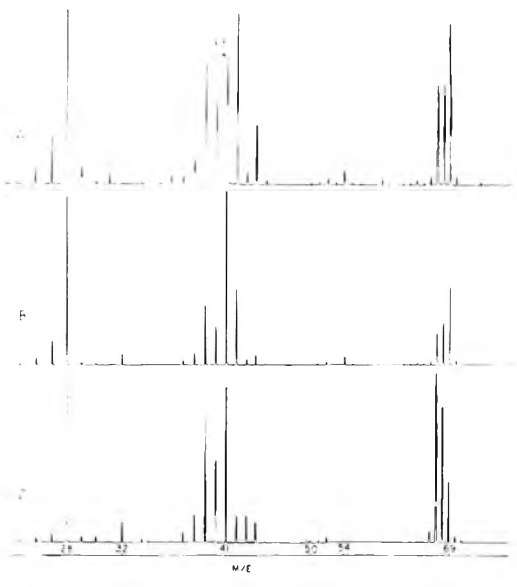


Fig. 2. Rapid-scan mass spectra of gas chromatographic effluent. A = 1-pyrroline, B = proline odor component (POC), C = 3-pyrroline. The spectra are not normalized for background.

m/e 69 indicates an odd number of nitrogen atoms in the compounds. With a low-molecular-weight compound a single nitrogen is much more probable than three nitrogens. Comparison of the POC spectra in Fig. 2 with spectra A and C and with published spectra (ASTM Index, 1963) clearly rules out nitriles. Compounds having a parent peak of 69 with a single nitrogen have the empirical formula C_4H_7N. That the POC is 1-pyrroline is suggested by comparing spectra A and B of Fig. 2. The spectra of the POC and 1-pyrroline are in very close agreement while 3-pyrroline (spectrum C) has a

Table 1. Comparisons of the relative retention values of the proline odor compound (POC) obtained from the Strecker degradation of proline with some known compounds.[a]

Compounds	20% Ucon non-polar[b] at 105°C	20% Carbowax[b] 1000 at 110°C	20% Apiezon[b] at 110°C
Ethylamine	0.298	0.436	
Propanal	0.443		
n-Propylamine	0.503	0.674	
Iso-Butanal	0.562	0.612	
n-Butanal	0.735	0.806	
Tetrahydrofurfural	0.847	0.778	
n-Butylamine	1.000	1.000	1.000
Pyrrolidine	1.431	1.691	
1-Pyrroline	1.459	1.744	1.767
3-Pyrroline	1.626	2.166	
Pyrrole	11.363	17.322	
Pyrroline odor compound (POC)	1.459	1.744	1.767
Mixture of 1-pyrroline and POC	1.459	1.744	1.767

[a] 1/16-inch ID × 10-ft stainless-steel column. The Celite 545 was washed with acid and alkali, and then treated with 5% sodium hydroxide, w/w, prior to coating; on-column injection.
[b] t_R/t_R = relative retention time calculated on the basis t_R/t_R of *n*-butylamine = 1.00.

markedly different spectrum. The most striking difference is at *m/e* 67,68,69. Slight variations are noted in this region for spectra A and B but this is accountable by statistical fluctuations inherent in fast-scan mass spectrometry (McFadden and Day, 1964).

2-Pyrroline was not available for mass spectral and GC analysis. It was ruled out, however, by infrared analysis (IR) of the POC-2 fraction. The POC-2 fraction was used because it contained only the proline odor component and ether. The ether was evaporated from the POC-2 fraction, and the residue was dissolved in 20 μl of chloroform. The IR spectrum was taken in a micro liquid cell by a Beckman IR-5 spectrometer. The spectrum showed a strong band at 1684 cm^{-1}, and a moderate, broad band at 3440 cm^{-1}. The 1-pyrroline would be expected to exhibit a strong C = N stretching vibration in the region of 1690–1640 cm^{-1} (Bellamy, 1962). 2-Pyrroline would be expected to exhibit two characteristic absorption bands. The strongest band, due to the −NH stretching vibration, would appear in the 3400-3200 cm^{-1} region. In this respect, Heacock and Marion (1956) observed a range of 3380–3205 cm^{-1} for the −NH vibration of pyrrolidine and piperazine-type bases. The second expected absorption, a moderate to weak band in the vicinity of 1600–1590 cm^{-1}, would be characteristic of C = C in a cyclopentene-type molecule (Bellamy, 1962) and it should occur in the spectrum of 2-pyrroline. No band was observed in this region in the POC-2 fraction.

The broad band observed at 3440 cm^{-1} in the POC-2 fraction was more characteristic of −OH absorption than of the distinctively sharp absorption usually found with −NH groups. This band may have been due to water in the sample.

On the basis of the gas chromatographic and mass spectral data in conjunction with the IR-analysis, it is concluded that the odor component from the Strecker degradation of proline is 1-pyrroline.

In the initial stages of the investigation it was thought that γ-aminobutanal was the responsible odor component. In fact, hydrolysis of γ-aminobutanal diethylacetal followed by adjustment of the pH to slightly alkaline conditions invariably gave the typical POC odor. Gas-chromatographic mass-spectrometric analysis revealed 1-pyrroline as the odor component. The free aldehyde apparently forms the internal Schiff base, which is 1-pyrroline. This also accounts for 1-pyrroline as the product of Strecker-degraded ornithine.

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RODNEY M. SMITH [a] and B. S. LUH

Department of Food Science and Technology
University of California, Davis, California 95616

Anthocyanin Pigments in the Hybrid Grape Variety Rubired

SUMMARY

The anthocyanins in Rubired grapes were extracted with 0.2% methanolic HCl, partially purified by adsorption on a Dowex-50 W × 4 cation-exchange column, eluted from the washed resin with acidified methanol, and the individual pigments separated by paper chromatography in two dimensions with n-butanol–acetic acid–water (BAW 4:1:5 v/v) and acetic acid–water–conc HCl (AWH, 15:82:3) as solvents. The purified pigments were identified by their R_f values, the sugar moiety, partial acid hydrolysis, alkaline degradation products of the aglycone, fluorescence under ultraviolet radiation, and absorption spectra. The pigments, in decreasing order of photodensitometer readings, were malvidin 3,5-diglucoside, peonidin 3,5-diglucoside, malvidin 3-monoglucoside, peonidin 3-monoglucoside, delphinidin 3-monoglucoside, petunidin 3-monoglucoside, petunidin 3,5-diglucoside, malvidin 3,5-diglucoside acylated with *p*-coumaric acid, malvidin 3-monoglucoside acylated with *p*-coumaric acid, peonidin 3-monoglucoside acylated with *p*-coumaric acid, and delphinidin 3,5-diglucoside.

INTRODUCTION

Grapes are used as dessert fruits and for raisins, wines, and juices. The new California hybrid grape variety Rubired shows promise for blending with other grapes to make red wines, for making standard-quality unblended port wines, and for producing grape juices and concentrates. Because of its high acid content, especially in early pickings, Rubired is well suited for blends with present port-type varieties which are too low in acid (Olmo and Koyama, 1962).

Anthocyanins are the principal constituents of the phenolic substances of red wines and are partly responsible for wine astringency—an important quality factor (Ribéreau-Gayon, 1959). Knowledge of the pigments in grapes is of particular value to the geneticist in developing new grape varieties of desirable color characteristics (Rankine *et al.*, 1958). In a study of the pigments in the Isabella grape, a hybrid of *Vitis vinifera* and *V. labrusca*, Anderson and Nabenhauer (1926) unequivocally established that the structure of the anthocyanidin resulting from the acid hydrolysis of oenin matched the structure of malvidin. Levy *et al.* (1931) compared the principal pigments from Fogarina (*V. vinifera*) grapes with synthetic malvidin 3-monoglucoside. He concluded that malvidin 3-monoglucoside was the main pigment, with traces of delphinidin and petunidin present after acid hydrolysis. Bockian *et al.* (1955) and Somaatmadja and Powers (1963) reported on anthocyanin pigments of Cabernet Sauvignon grapes. Ingalsbe *et al.* (1963) reported on anthocyanin pigments in Concord grapes. Paper chromatographic techniques were applied to the study of *V. vinifera* grape anthocyanins by Ribéreau-Gayon and Ribéreau-Gayon (1958). They reported that anthocyanins in *V. vinifera* grapes are monoglucosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin. Cyanidin was present as a leucoanthocyanin, since it was found only after acid hydrolysis (Ribéreau-Gayon and Ribéreau-Gayon, 1958). Fouassin (1956) reported on pigment patterns in five vinifera varieties.

This work describes the extraction, purification, and identification of five major and six minor anthocyanins in Rubired grapes.

MATERIALS AND METHODS

Grapes. Forty pounds of Rubired grapes were harvested at peak maturity from eight-year-old vines in the University vineyard. After storage for 24 hr at 0°C, the grapes were washed twice with tap water, stemmed, packed in 9 × 3 × 18-inch cellophane bags, and then frozen at −18°C (0°F). Analysis of the mature Rubired grapes revealed the following characteristics: weight per 100 berries, 116.5 g; Brix at 20°C, 23.3°C; pH, 3.75; total acidity, 0.99% as tartaric acid.

[a] Present address: Hunt Foods and Industries, Fullerton, California.

Extraction and purification of anthocyanins. Two hundred grams of frozen grapes were mixed with 600 ml of 0.2% methanolic HCl, and then blended for 5 min in a Waring blender under a nitrogen gas atmosphere. The mixture was filtered through two layers of Whatman No. 1 filter paper in a Buchner funnel under suction. The filtrate was mixed with approximately 200 g of Dowex 50 W × 4 cation-exchange resin (100–200-mesh, Dow Chemical Company, Midland, Michigan) in the hydrogen form. After 1–2 hr the resin was placed in a Buchner funnel containing two layers of Whatman No. 1 filter paper, and was thoroughly washed with pure methanol to remove organic residues and then with distilled water to remove free sugars. The pigment was eluted from the resin by successive extractions with 0.1%, 0.5%, and 1% methanolic HCl. The combined extract was evaporated in a flash evaporator almost to dryness and then redissolved in 10 ml of 0.01% methanolic HCl. The pigment mixture was stored at −26°C (−16°F) in the dark under a nitrogen atmosphere.

Paper chromatography. The solvent systems are shown in Table 1. The direction of solvent flow—either ascending (*A*) or descending (*D*)—and the time in hours were designated, e.g., as follows: BAW:*D*:18 means that the solvent BAW was descending (*D*) for 18 hr.

Two-dimensional technique. Two-dimensional chromatograms of the aforementioned extracts on both Whatman No. 1 and 3MM papers, using BAW:*D*:18 in dimension 1 and AWH:*D*:6 in dimension 2 gave satisfactory separation of most of the pigments. Whatman No. 1 paper was used primarily for determining the number of pigments present and for testing the purity of isolated pigments. When pure pigments were desired for either absorption spectra measurement or for calculation of R_f values, 30 Whatman 3MM papers were used. The pigment mixture was applied with a micropipette in a spot 0.5 cm in diameter. After the chromatograms were air-dried, like spots were cut from the papers and combined. The pigments were eluted 4 times with 0.01% methanolic HCl. The corresponding eluates were combined, evaporated in a flash evaporator to dryness, redissolved in 1 ml of 0.01% methanolic HCl, and transferred via pipette to a small vial, where they were vacuum-dried in a light-proof desiccator.

Bar technique. For partial acid hydrolysis and alkaline-degradation studies, 10–20 mg of pure pigment was obtained by the bar technique. The methanolic HCl extract previously described was applied as streaks 35 cm long on 40 Whatman 3MM papers. A 50-lambda micropipette was used to apply the pigment extract. After the pigment

Table 1. Solvent systems for paper chromatography.

Solvent	Abbreviation	Composition	Proportions (v/v)	Layer used	Solvent for direction[a]		Time (hr)
1	1%HCl	Conc. HCl-water	1:99	miscible	anthocyanins	D	4
2	AWH	Acetic acid-water-conc. HCl	15:82:3	miscible	anthocyanins	D	6,12
					aglycones	D	6
3	BAW[b]	n-Butanol-acetic acid-water	4:1:5	upper	anthocyanins	D,A	18,24
					aglycones	D,A	18,
					sugars	D	18,36
					acids	D	18
4	Forestal	Acetic acid-water-conc. HCl	30:10:3	miscible	aglycones	D	15
5	FHW	Formic acid-conc. HCl-water	5:2:3	miscible	aglycones	D	6
6	EAW	Ethyl acetate-acetic acid-water	3:1:3	upper	sugars	D	9
7	BuPyW	*n*-Butanol-pyridine-water	6:3:1	miscible	sugars	D	18
8	BenHOAcW[c]	Benzene-acetic acid-water	2:2:1	upper	acids	A	18
9	2%HOAc	Glac. acetic acid-water	2:98	miscible	acids	D	4

[a] D = descending; A = ascending.
[b] For R_f values the mixture was allowed to equilibrate 3 days before use.
[c] Because of the high volatility of benzene, the solvent was made anew each time used.

was developed in AWH:*D*:12 and the papers were air-dried, 6 well defined bands, numbered from 1 to 6 in increasing distance from the origin, were cut from the papers. Corresponding bands from all 40 papers were combined and cut in half with pinking shears and then set in groups of 10 paper strips each in chromatographic trays so that 0.01% methanolic HCl in the trays would flow down the strips into receiving beakers. Each band eluate was flash-evaporated to a small volume and then re-streaked on Whatman 3MM papers for development in freshly prepared BAW:*D*:24. Each band thus obtained, representing a pure pigment, was cut, eluted, and flash-evaporated as before. The bands were lettered in increasing distance from the origin—*a*, *b*, etc. Because of some overlapping of bands in AWH purification and because of the presence of acylated anthocyanins, a two-dimensional chromatogram was developed to aid in numbering and identifying the pigments (see Fig. 1).

Sugar moiety. *Hydrolysis with 1N HCl.* Three to 5 mg of the purified pigment was refluxed for 30 min in a boiling brine bath with 2 ml 1*N* HCl in a micro-flask fitted with a cold-finger condenser (Luh *et al.*, 1965; Hsia *et al.*, 1965). The hydrolysate was cooled and mixed with Dowex 50 cation-exchange resin in the hydrogen form and Dowex 1 anion-exchange resin in the acetate form. Sixty lambda of clear supernatant was spotted on Whatman No. 1 chromatography paper. Forty μg each of authentic rhamnose, xylose, arabinose, glucose, maltose, galactose, and rutinose were spotted separately on the chromatogram for comparison. The chromatograms, irrigated with BAW:*D*:36, EAW:*D*:15, and BuPyW:*D*:36, were allowed to run off the paper for determination of R_g values. R_f values were obtained for the sugars with BAW:*D*:18 as solvent. The sugar spots were visualized by spraying with aniline hydrogen phthalate reagent (0.93 g aniline, 1.66 g phthalic acid, and 100 ml water-saturated *n*-butanol) followed by heating for 10 min at 105°C in a ventilated oven as described by Partridge (1949).

Hydrolysis with 10% acetic acid. Four to 5 mg of pigments 6*a* and 6*b*, as well as 10 mg of pigment mixture, were refluxed for 1½ hr with 2 ml of 10% acetic acid at 100°C in a boiling brine bath (Chandler and Harper, 1961). The hydrolysates were mixed with Dowex 50 and Dowex 1 ion-exchange resins and tested for sugars as described above.

Studies of the aglycones. The aglycone from acid hydrolysis of the anthocyanin in 1*N* HCl was adsorbed on Dowex 50 W X 4 cation-exchange resin, washed with 100 ml each of distilled water and methanol, and then eluted from the resin with 250 ml of warm 0.1% methanolic HCl. The eluate was flash-evaporated to a small volume and kept under nitrogen gas in the dark at −29°C.

Rf values of the aglycones. Each concentrate containing the aglycone was spotted on four Whatman no. 1 papers. The papers were irrigated respectively with BAW:*D*:18, Forestal:*D*:18, FHW:*D*:6, and AWH:*D*:6 solvents and then air-dried in the dark. It was necessary to spray the BAW-developed paper with 1*N* HCl after drying to prevent the aglycone from fading.

Alkaline degradation of the aglycones. The remaining aglycone concentrate was refluxed for 30 min with 5 ml of 15% $Ba(OH)_2$ solution in a micro-flask fitted with a cold-finger condenser, a nitrogen atmosphere being maintained to prevent oxidation (Lynn and Luh, 1964). The mixture was cooled in an ice-water bath, and acidified with conc. HCl before stopping the nitrogen flow.

Pigments 1*a*, 2*a*, 2*b*, 3*a*, 5*a*, and 5*b* were available in small quantities. They were degraded directly with $Ba(OH)_2$ without acid hydrolysis, thus avoiding the loss incurred in the intermediate steps.

The alkaline degradation products were acidified, extracted three times with 2 ml each of diethyl ether, concentrated, and then spotted on three Whatman no. 1 papers. The ether extracts containing both the acyl acids and the degradation products of the aglycones were purified and spotted on the papers. References were 40 μg each of authentic gallic, protocatechuic, vanillic, syringic, 3-*o*-methyl gallic, caffeic, *p*-coumaric acids, and phloroglucinol. The chromatograms were irrigated with BAW:*D*:18, BenHOAcW:*A*:18, and 2% HOAc:*D*:4, respectively. The air-dried chromatograms were observed under ultraviolet radiation emitted from a G.E. tube with 360-mμ unit output (model 1910, Burton Medi-Quip Co., Van Nuys, California) in the presence of NH_3 vapor. The acids were visualized by spraying with a freshly prepared diazonium salt solution made from 10 ml of 0.3% *p*-nitroaniline in 2*N* HCl, 1 ml of aqueous 5% $NaNO_2$, and 30 ml of aqueous 20% CH_3COONa (Lynn and Luh, 1964). The color change before and after exposure of the sprayed papers to NH_3 vapor was noted.

Partial acid hydrolysis. Partial acid hydrolysis of the purified anthocyanins was done by the method of Abe and Hayashi (1956). Eight to 10 mg of pigment was refluxed with 2 ml of 1*N* HCl in a boiling brine bath. Aliquots of 0.1 ml were taken through a side arm at intervals of 0, 2, 5, 10, 20, 40, and 60 min into vials cooled in ice water. Thirty lambda of each hydrolysate was spotted in sequence on three Whatman no. 1 chromatographic papers. The papers were irrigated with BAW:*A*:15, AWH:*D*:6, and Forestal:*D*:15 solvent systems, respectively.

Properties of anthocyanins. *Paper chromatography.* Purified pigments 1*a*, 1*b*, 2*a*, 2*b*, 3*a*, 3*c*, 4*a*, 5*a*, 5*b*, 6*a*, and 6*b*, obtained by the two-dimensional purification procedure described previously, were dissolved in 0.01% methanolic HCL and spotted on three Whatman no. 1 papers. The papers were irrigated in BAW:*D*:18, 1% HCl:*D*:4, and AWH:*D*:6 solvent systems. The average *Rf* value of each pigment from 2 or more papers was reported. The colors of the anthocyanins were noted under visible and ultraviolet lights.

Reaction of the anthocyanins with $AlCl_3$. The chromatograms developed with BAW, 1% HCl, and AWH were sprayed with saturated $AlCl_3$ in 95% ethanol—a modification of the method described by Lynn and Luh (1964).

Absorption spectra in the visible region. The absorption spectra of the pigments in 0.01% methanolic HCl were recorded with a Beckman DK-2 recording spectrophotometer. The shift in absorption peak was recorded 1 min after adding 3 drops of 5% $AlCl_3$ in 95% ethanol.

Photodensitometric measurement. Photodensitometric measurement of all pigments except 6*c* on Whatman 3MM paper strips were recorded with a recording Photovolt densitometer, model 525 (Photovolt Corporation, 1115 Broadway, New York 10, New York). The slit was 1 × 20 mm. A green filter was used.

RESULTS AND DISCUSSION

Paper chromatography of the anthocyanins. The pigments were separated by either the two-dimensional technique or the bar technique. In separating the pigments by two-dimensional chromatography, it was desirable to use the BAW in the first direction and the AWH solvent in the second direction. The pigments separated best with a freshly purified pigment extract and with freshly prepared BAW solvent.

Two-dimensional chromatographic separation of the pigment mixture indicated the presence of at least 14 pigments. Each pigment was numbered according to the band number from the original when chromatographed with the AWH solvent. It was then given a letter according to the band it would have appeared in if chromatographed with BAW when no contaminating bands were present (Fig. 1).

Determinations of the sugar moiety by acid hydrolysis. The sugar moiety in grape anthocyanins was determined by paper chromatography of the product after hydrolysis of the purified pigments with 1*N* HCl and 10% acetic acid.

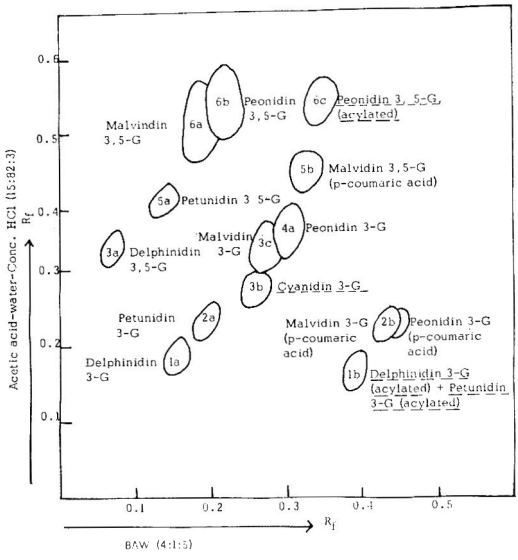


Fig. 1. Two-dimensional paper chromatography of anthoycanins in Rubired grapes. Those pigments underlined were not identified positively.

Table 2 shows the R_f values of sugar moieties of the anthocyanins and those of authentic sugars with BAW, BuPyW, and EAW as solvents. By HCl hydrolysis, the R_f and R_g values of the sugar moieties from all the pigments (1*a*, 2*a*, 3*a*, 3*c*, 4*a*, 5*a*, 5*b*, 6*a* and 6*b*) were identical or close to that of glucose. The same result was obtained when a mixture of the pigments was hydrolyzed.

As a result of hydrolysis of pigments 6*a*, and 6*b*, and a pigment mixture with 10% acetic acid, a single sugar moiety with R_g value identical to that of glucose was detected.

The positions at which sugars may be attached to hydroxyl groups in the aglycone of the anthocyanin molecule are restricted. Sugar residues are normally found only in the 3-position or in the 3- and 5-positions (Harborne, 1958b). Since only glucose was found as the sugar moiety, the pigments are most likely either 3-monoglucosides or 3,5-diglucosides.

A second sugar spot with R_f and R_g values identical to that of arabinose was detected in the HCl hydrolysate of all pigments purified by paper chromatography. It was

Table 2. R_f and R_g values of the sugar moiety in Rubired grape anthocyanins compared with those of authentic sugar samples by paper chromatography.

	BAW (4:1:5)				BuPy-water (6:3:1)		EtOAc-HOAc-H_2O (3:1:3)				Identification
	D:18 R_f		D:36 R_g		D:36 R_g		D:9 R_f		D:15 R_g		
	Found	Ref.	Found	Ref.	Found	Ref.	Found	Ref.	Found	Ref.	
A) 1*N* HCl hydrolysis:											
Pigment											
Mixture	.....	.....	.99	.....	.98	.....	.....	.....	.98	.....	glucose
1*a*	.19	.....	.95	.....	.96	.....	.....	.....	.94	.....	glucose
2*a*	.17	.....	.....	.....	.94	.....	.....	.....	.....	.....	glucose
3*a*	.17	.....	1.03	.....	.....	.....	.....	.....	.99	.....	glucose
3*c*	.18	.....	1.00	.....	.....	.....	.....	.....	.....	.....	glucose
4*a*	.18	.....	1.00	.....	1.00	.....	.27	.....	1.00	.....	glucose
5*a*	.15	.....	.....	.....	1.00	.....	.....	.....	.....	.....	glucose
5*b*	.16	.....	.....	.....	1.01	.....	.....	.....	.....	.....	glucose
6*a*	.16	.....	.98	.....	.98	.....	.....	.....	1.01	.....	glucose
6*b*	.16	.....	.99	.....	1.00	.....	.....	.....	.99	.....	glucose
B) 10% acetic acid hydrolysis:											
Pigment											
Mixture	.....	.....	.94	.....	.98	.....	.....	.....	.95	.....	glucose
6*a*	.16	.....	.....	.....	.....	.....	.....	.....	.....	.....	glucose
6*b*	.16	.....	.....	.....	.....	.....	.....	.....	.....	.....	glucose
C) Authentic sugars:		[a]		[b]				[c]			
Glucose	.18	.18	1.00	1.00	1.00	.....	.27	.17	1.00	1.00 [b]	
Galactose	.15	.16	.88	.90	.81	.....	.....	.14	.87	.94 [b]	
Maltose	.09	.....	.47	.....	.50	.....	.13	.....	.37	.....	
Rutinose [d]	.13	.....	.61	.60	.59	.....	.18	.....	.61	.55 [b]	
Rhamnose	.36	.37	1.72	1.61	2.32	.....	.40	.34	2.16	1.96 [c]	
Arabinose	.21	.21	1.18	1.17	1.27	.....	.....	.22	1.23	1.43 [c]	
Xylose	.....	.28	1.30	1.27	1.56	.....	.....	.27	1.49	1.77 [c]	

[a] Partridge, 1948.
[b] Lynn and Luh, 1964.
[c] Jermyn and Isherwood, 1949.
[d] Obtained by 10% aqueous acetic acid hydrolysis of rutin 1½ hr.

not detected as a sugar moiety in the purified pigment extract.

Studies of the aglycone. *R_f values.* Table 3 shows the R_f values of the aglycone moieties of the anthocyanins and those of anthocyanidins reported in the literature with BAW, Forestal, FHW and AWH as solvents. R_f values of the anthocyanidin from pigment 1*a* are close to those for delphinidin, those from pigments 3*c*, 5*b*, and 6*a* are close to those for malvidin, and those from pigments 4*a* and 6*b* are close to those of peonidin. The R_f values of anthocyanidin from pigment 2*a* indicate the presence of petunidin. Pigments 3*a* and 5*a*, present in small amount, faded after 30 min of hydrolysis. Pigments 1*a* and 2*a* faded partially during paper chromatography.

Anthocyanidins are less stable than anthocyanins to pH change and light, and tend to fade during chromatography in nonacidic solvents (Harborne, 1958b). It was found that, if an acidic extract was chromatographed with BAW as solvent, the aglycone color can be recovered and retained by spraying the still-damp paper after chromatography with aqueous 1*N* HCl.

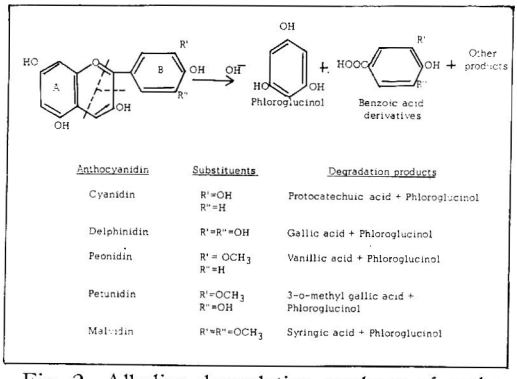
Alkaline degradation. The aglycone of the anthocyanin when refluxed with 15% $Ba(OH)_2$ is hydrolytically cleaved to yield a benzoic acid derivative, phloroglucinol, and other products (Fig 2). Each anthocyanidin yields a distinct benzoic acid derivative which can be identified by comparison of its R_f values and color reactions with those of an authentic sample.

Anthocyanidins from acid hydrolysis of pigments 3*c*, 4*a*, 6*a*, and 6*b*, unhydrolyzed anthocyanins 1*a*, 2*a*, 2*b*, 3*a*, 5*a*, and 5*b*, and a mixture of anthocyanins were degraded with $Ba(OH)_2$, acidified and extracted with ether. The phenolic compounds were separated by paper chromatography. Phloroglucinol and the acyl moiety, *p*-coumaric

Table 3. R_f values of Rubired anthocyanidins by descending chromatography.

	R_f values of anthocyanidins				
Pigment	BAW (4:1:5)[c] *D*:18	Forestal HOAc-water-HCl (30:10:3) *D*:15	Formic acid-HCl-water (FHW) (5:2:3) *D*:15	HOAc-water HCL (AWH) (15:82:3) *D*:6	Identification
1*a*	.38	.30	.....	.....	Delphinidin
1*b*[a]	.....	.....	.....	.....	.....
2*a*	.48	.42	.....	.....	Petunidin
2*b*[a]	.....	.....	.....	.....	.....
3*a*[b]	.....	.....	.....	.....	.....
3*b*[a]	.....	.....	.....	.....	.....
3*c*	.57	.57	.26	.07	Malvidin
4*a*	.62	.63	.32	.11	Peonidin
5*a*[b]	.....	.....	.....	.....	.....
5*b*	.57	.58	.33	.....	Malvidin
6*a*	.56	.60	.34	.13	Malvidin
6*b*	.68	.67	.36	.19	Peonidin
6*c*	.....	.....	.....	.....	.....
Reported[d]	[e]				
Cyanidin	.68	.49	.22		
Delphinidin	.42	.32	.13		
Peonidin	.71	.63	.30		
Petunidin	.52	.46	.20		
Malvidin	.58	.60	.27		

[a] Insufficient quantity of pure pigment for hydrolysis.
[b] Color disappeared after 20 minutes hydrolysis in 1*N* HCl at 100°C.
[c] All BAW papers sprayed with 1*N* HCl after chromatography to prevent aglycone from fading.
[d] Harborne, 1958b.
[e] Run on acid washed paper.



Anthocyanidin	Substituents	Degradation products
Cyanidin	R'=OH R''=H	Protocatechuic acid + Phloroglucinol
Delphinidin	R'=R''=OH	Gallic acid + Phloroglucinol
Peonidin	R' = OCH_3 R''=H	Vanillic acid + Phloroglucinol
Petunidin	R'=OCH_3 R''=OH	3-o-methyl gallic acid + Phloroglucinol
Malvidin	R'=R''=OCH_3	Syringic acid + Phloroglucinol

Fig. 2. Alkaline degradation products of anthocyanidins.

acid, were detected by their characteristic blue fluorescence under ultraviolet radiation in the presence of ammonia vapor. All pigments contained phloroglucinol. Pigments *2b* and *5b* contained *p*-coumaric acid, indicating that these pigments are acylated. The phenolic compounds were visualized by spraying with the diazotized *p*-nitroaniline reagent (pNA).

Comparison of R_f values and color reactions of the phenolic compounds for each degraded anthocyanin with authentic compounds (Table 4) indicates that pigment *1a* is a glucoside of delphinidin, pigments *2a* and *5a* are glucosides of petunidin, pigments *3c* and *6a* are glucosides of malvidin, pigments *4a* and *6b* are glucosides of peonidin, pigment *5b* is an acylated glucoside of mal-

Table 4. Chromatographic comparison of alkaline degradation products of Rubired grape anthocyanins with phenolic compounds.

		R_f values			Color[a]		
Compound	Identification	BAW: (4:1:5) *D*:18	Ben-HOAc-W (2:2:1) *A*:18	2% HOAc *D*:4	UV + NH_3	pNA[b]	pNA + NH_3
1a	Gallic acid	.60	.....	.45	.....	Y	TG
	Phloroglucinol	.70	.....	.62	BF	Or	Or
2a	3-*o*-Methyl gallic acid	.....	.27	.....	.....	RT	P
	Phloroglucinol	.71	.....	.....	BF	Or	Or
2b	Vanillic acid	.85	.97	.58	.....	Y	M
	Syringic acid	.83	.95	.53	.....	Or	BBk
	Phloroglucinol	.69	.....	.62	BF	Or	Or
	p-Coumaric acid	.87	.68	.44	BF	YOr	P
3c	Syringic acid	.84	.94	.52	.....	Or	BBk
	Phloroglucinol	.70	.....	.62	BF	Or	Or
4a	Vanillic acid	.86	.96	.56	.....	Y	M
	Phloroglucinol	.69	.....	.....	BF	Or	Or
5a	3-*o*-Methyl gallic acid	.....	.27	.....	.....	RT	P
	Phloroglucinol	.70	.....	.....	BF	Or	Or
5b	Syringic acid	.83	.95	.53	.....	Or	BBk
	Phloroglucinol	.69	.....	.63	BF	Or	Or
	p-Coumaric acid	.86	.68	.44	BF	YOr	P
6a	Syringic acid	.83	.96	.52	.....	Or	BBk
	Phloroglucinol	.70	.....	.63	BF	Or	Or
6b	Vanillic acid	.87	.97	.57	.....	Y	M
	Phloroglucinol	.70	.....	.63	BF	Or	Or
Mixture	Phloroglucinol	.70	.....	.62	BF	Or	Or
	Syringic acid	.83	.95	.52	.....	Or	BBk
	Vanillic acid	.85	.98	.57	.....	Y	M
	3-o-Methyl gallic acid	.....	.28	.....	.....	RT	P
	Protocatechuic	.....	.15	.....	.....	T	P
	p-Coumaric acid	.86	.69	.46	BF	YOr	P
	Gallic acid	.61	.....	.....	.....	Y	TG
Authentic compounds:							
Vanillic acid		.86	.97	.57	.....	Y	M
Gallic acid		.60	.02	.47	.....	Y	TG
Protocatechuic acid		.80	.16	.55	.....	T	P
Syringic acid		.83	.95	.52	.....	Or	BBk
3-*o*-Methyl gallic acid		.81	.27	.50	.....	RT	P
Phloroglucinol		.70	.04	.63	BF	Or	Or
p-Coumaric acid		.87	.68	.45	BF	YOr	P
Caffeic acid		.81	.08	.32	BF	T	T

[a] (B) blue, (F) fluorescence, (Y) yellow, (Or) orange, (T) tan, (P) purple, (M) maroon, (RT) reddish-tan, (BBk) blue-black, (TG) tan-gray.

[b] Chromatogram sprayed with diazotized *p*-nitroaniline reagent (pNA).

Table 5. Partial acid hydrolysis of anthocyanins from Rubired grapes.

Pigment no.	Structure	No. of simpler products	Components	R_f values: BAW (4:1:5) *A*:15	HOAc-water-HCl (AWH) (15:82:3) *D*:6	Forestal HOAc-water-H (30:10:3) *D*:15
3*c*	Malvidin-3-monoglucoside	1	Original pigment	0.40	.34	.81
			Aglycone (malvidin)	0.62	0.11	0.62
4*a*	Peonidin-3-monoglucoside	1	Original pigment	0.33	0.38	0.83
			Aglycone (peonidin)	0.79	0.14	0.73
6*a*	Malvidin-3,5-diglucoside	3	Original pigment	0.25	0.58	0.87
			Intermediate (malvidin 3-Monoglucoside)	0.34	0.38	0.64
			Intermediate (malvidin 5-Monoglucoside)	0.47	0.35	0.66
			Aglycone (malvidin)	0.55	0.13	0.58
6*b*	Peonidin-3,5-diglucoside	3	Original pigment	0.25	0.57	0.86
			Intermediate (peonidin 3-Monoglucoside)	0.37	0.43	0.71
			Intermediate (peonidin 5-Monoglucoside)	0.42	0.39	0.72
			Aglycone (peonidin)	0.68	0.19	0.60

vidin, and pigment 2*b* is a mixture of an acylated glucoside of malvidin and one of peonidin. Pigments 1*b*, 3*a*, 3*b*, and 6*c* were not available in sufficient quantity for study of their degradation products.

Partial acid hydrolysis. Pigments 3*c*, 4*a*, 6*a*, and 6*b* were available in sufficient quantity for partial acid hydrolysis with aqueous 1*N* HCl. The results are shown in Table 5. Pigments 3*c* and 4*a* each gave 1 simpler product, indicating that they are monoglucosides (Table 5). Pigments 6*a* and 6*b* each gave three simpler products, indicating that they are 3,5-diglucosides. Under ultraviolet radiation the two glucosides can be distinguished by the fact that the 5-monoglucoside fluoresces whereas the 3-monoglucoside does not.

Properties of anthocyanins. *R_f values.* The R_f values of purified anthocyanins from Rubired grapes are summarized in Table 6.

The relationships between R_f value and the structure of anthocyanins have been noted by Abe and Hayashi (1956), Bate-Smith and Westall (1950), and Harborne (1958b). With the use of graphic applications of these relationships, fluorescence behavior under ultraviolet radiation of anthocyanins with a glycoside in the 5-position, and chemical and physical characteristics it is possible to identify the chemical structure of the pigments.

Both pigments 6*a* and 6*b* fluoresced under ultraviolet radiation, indicating 5-glycosidation. The portion of pigment 5*b* deacylated during chromatography fluoresced, indicating 5-glycosidation. It appears that acylated anthocyanins do not fluoresce. That pigments 5*b*, 6*a*, and 6*b* are 3,5-diglucosides is further supported by their fluorescent behavior. By proximal location, pigments 3*a* and 5*a* respectively appear to be the 3,5-diglucosides of delphinidin and petunidin.

Determination of aglycone structure by $AlCl_3$ spray. Pigments 1*a*, 1*b*, 2*a*, 3*a*, and 5*a* changed to blue when sprayed with $AlCl_3$, indicating that they contain two hydroxyl groups at ortho positions to each other in the B-ring. Pigments 2*b*, 3*c*, 4*a*, 5*b*, 6*a*, 6*b*, and 6*c* did not change color when sprayed with $AlCl_3$, indicating that they have only one hydroxyl group, or that one of the adjacent hydroxyl groups was methylated.

Absorption spectra in the visible region. A record was made of the absorption spectra of pigments 1*a*, 1*b*, 2*a*, 2*b*, 3*a*, 3*c*, 4*a*, 5*a*, 5*b*, 6*a*, and 6*b*, and of the bathochromic

Table 6. R_f values of Rubired grape anthocyanins.

Pigment	Identification	R_f values of anthocyanins					
		BAW (4:1:5) *D*:18			1% HCl *D*:4	HOAc-water-HCl (AWH) (15:82:3)	
Found:							
1*a*	Delphinidin 3G		.18		.03	.20	
1*b*	.....		.42		.02	.18	
2*a*	Petunidin 3G		.22		.04	.24	
2*b*	Malvidin 3G—*p*-coumaric acid + peonidin 3G—*p*-coumaric acid		.47		.04[d]	.23	
3*a*	Delphinidin 3,5G		.08		.08	.34	
3*b*	.....		.28		.....	.30	
3*c*	Malvidin 3G		.39		.09	.34	
4*a*	Peonidin 3G		.46		.10	.37	
5*a*	Petunidin 3,5G		.14		.08	.40	
5*b*	Malvidin 3,5G—*p*-Coumaric acid		.34		.09[d]	.45	
6*a*	Malvidin 3,5G		.25		.19	.51	
6*b*	Peonidin 3,5G		.23		.21	.49	
6*c*	.....		.34		.....	.53	
Reported:							
Peonidin		[a]	[b]	[c]	[e]	[a]	[e]
3-Monoglucoside		.41	.47	.....	.09	.33	.....
3,5-Diglucoside		.31	.26	.....	.17	.44	.....
Malvidin							
3-Monoglucoside		.38	.40	.30	.06	.29	.32
3,5-Diglucoside		.31	.22	.25	.13	.42	.51
3-Monoglucoside—*p*-coumaric acid		.....	.....	.....	.....	.....	.....
Petunidin							
3-Monoglucoside		.35	.....	.20	.04	.22	.25
3,5-Diglucoside		.24	.....	.....	.08	.32	.....
Cyanidin							
3-Monoglucoside		.38	.33	.....	.07	.26	.....
3,5-Diglucoside		.28	.16	.....	.16	.40	.....
Delphinidin							
3-Monoglucoside		.26	.....	.15	.03	.18	.20
3,5-Diglucoside		.15	.11	.....	.08	.32	.....

[a] Harborne, 1958b.
[b] Bate-Smith, 1950.
[c] Somaatmadja and Powers, 1963.
[d] Two spots were present each time the pigment was chromatographed.
[e] Harborne, 1958b (12N HCl-water, 3:97, v/v).

shift of pigments 1*a*, 1*b*, 2*a*, 3*a*, and 5*a* with $AlCl_3$. The results are presented in Table 7. Comparison of the absorption maximum and the absorbance ratio ($\lambda_{440}/\lambda_{max}$) with those reported in the literature (Harborne, 1958a) further indicates that pigment 2*a* is petunidin 3-monoglucoside, pigment 3*a* is delphinidin 3,5-diglucoside, pigment 3*c* is malvidin 3-monoglucoside, pigment 4*a* is peonidin 3-monoglucoside, pigment 5*a* is petunidin 3,5-diglucoside, pigment 6*a* is malvidin 3,5-diglucoside, and pigment 6*b* is peonidin 3,5-diglucoside.

Photodensitometric measurement. The relative quantities of anthocyanin pigments isolated from Rubired grapes were investigated. Anthocyanins present in decreasing order of photodensitometer readings in Rubired grapes are malvidin 3,5-diglucoside, peonidin 3,5-diglucoside, malvidin 3-mono-

Table 7. Absorption peak and characteristics of anthocyanins isolated from Rubired grapes.

Rubired grape anthocyanins				Properties of anthocyanins found in the literature[c]		
Pigment[a]	λ_{max}[b] (mμ)	$\frac{\lambda_{440}[d]}{\lambda_{max}}$ (%)	$AlCl_3$ shift (mμ)	Anthocyanins	λ_{max} (mμ)	$\frac{\lambda_{440}}{\lambda_{max}}$ (%)
1*a*	540	16	33	Delphinidin 3-glucoside	535	18
1*b*	545	....	29			
2*a*	536	18	44	Petunidin 3-glucoside	535	18
2*b*	532	....	0			
3*a*	535	12	39	Delphinidin 3,5-diglucoside	534	11
3*c*	533	20	0	Malvidin 3-glucoside	535	18
4*a*	523	26	0	Peonidin 3-glucoside	523	26
5*a*	533	11	44	Petunidin 3,5-diglucoside	533	10
5*b*	533	16	0			
6*a*	532	11	0	Malvidin 3,5-diglucoside	533	12
6*b*	520	13	0	Peonidin 3,5-diglucoside	523	13

[a] Each pigment was dissolved in 0.01% methanolic HCl.
[b] Pure 0.01% methanolic HCl was used as a blank.
[c] Harborne, 1958a.
[d] Absorption maximum values were corrected to zero at 700 mμ before the ratio was calculated.

glucoside, peonidin 3-monoglucoside, delphinidin 3-monoglucoside, petunidin 3-monoglucoside, pigment 3*b*, petunidin 3,5-diglucoside, malvidin 3,5-diglucoside acylated with *p*-coumaric acid, pigment 1*b*, malvidin 3-monoglucoside acylated with *p*-coumaric acid, and delphinidin 3,5-diglucoside. The diglucoside character of a hybrid grape variety is dominant (Ribéreau-Gayon and Ribéreau-Gayon, 1958). In the majority of *Vitis vinifera* varieties, malvidin 3-monoglucoside and, sometimes, peonidin 3-monoglucoside are present in the largest quantity (Albach *et al.*, 1959, 1963; Rankine *et al.*, 1958). Rankine *et al.* (1958) found Alicante Bouschet to be highest in malvidin 3-monoglucoside and second-highest in peonidin 3-monoglucoside.

Pigments 1b, 3b, and 6c. The material available was insufficient for conclusive identification of the minor pigments 1*b*, 3*b*, and 6*c*. However, the possible pigment structures may be deduced from available data and the graphic relationships (Abe and Hayashi, 1956; Bate-Smith and Westall, 1950). Pigment 1*b* may be acylated 3-monoglucosides of delphinidin and/or petunidin. The absorption maximum and chelation with $AlCl_3$ support the presence of delphinidin and/or petunidin as the aglycone of pigment 1*b*. By relative location pigment, 3*b* may be cyanidin 3-monoglucoside. Cyanidin was found present in the anthocyanin mixture after alkaline degradation, but was not shown present as one of the anthocyanidins. Pigment 6*c* may be peonidin 3,5-diglucoside (acylated). Further work is needed for positive identification of these minor pigments.

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Research Note

K. L. STEVENS, A. LEE, W. H. McFADDEN, and R. TERANISHI

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture Albany, California 94710

Volatiles from Grapes
I. Some Volatiles from Concord Essence

SUMMARY

Concord grape essence was examined by GLC-mass spectrometry and sixteen volatile components identified. Aside from ethyl acetate and ethanol, the most abundant was 2-methyl-3-buten-2-ol. Preliminary information on several other components has been obtained.

Power and Chesnut (1921) found that methyl anthranilate is an essential component of grapes of the labrusca (*Vitis labrusca*). It has long been recognized, however, that other constituents are necessary to reproduce the natural flavor of Concord grape juice. Holley *et al.* (1955) recognized this, and, using classical methods, identified ethanol, methanol, ethyl acetate, methyl acetate, acetone, acetaldehyde, methyl anthranilate, and acetic acid from Concord grape juice. To elucidate the chemical make-up of Concord essence further, we investigated the low-boiling constituents.

Concord grape juice essence (100-fold, 7.70 L), Welch Grape Juice Co., was saturated with sodium chloride and extracted with three 500-ml portions of freshly distilled ether. The ether extracts were combined, dried over anhydrous sodium sulfate, and filtered. Removal of the ether with a ten-plate Oldershaw column and the final traces with a rotary evaporator gave 6.1 g of oil. The oil (5 g) was then distilled through a 4-in. Vigreaux column at a pressure of 8 mm Hg. The head temperature rose continuously from 35 to 45° during the distillation. Only a small amount of very pungent material collected in the first receiver, which was held at room temperature. The bulk of the material (about 4 g) was collected in a dry-ice-acetone receiver placed in series after the first receiver. This latter material, which had a pleasant Concord grape aroma, was used in the present investigation. Approximately 0.8 g of higher-boiling material, containing mainly methyl anthranilate, remained undistilled.

The grape oil was analyzed by combined capillary GLC and rapid-scan mass spectrometry as described by McFadden *et al.* (1963). The GLC column was a 200-ft × 0.01-in. ID stainless-steel tube coated with Tween 20 (Atlas Powder Co.). Helium linear flow rate was set at 15.3 cm/sec, and the column was programmed from 30 to 200° over a period of about 70 min.

Most of the components were identified by direct comparison of the mass spectrum with that of an authentic sample. When a positive identification could be made, the compound was added to a sample of the oil and chromatographed under the same conditions. An increase in area of the expected peak confirmed the assignment. For some of the components, the mass spectrum could place the compound in a general class or type of compound but could not establish its exact structure.

Fig. 1 is a chromatogram of grape oil obtained with a hydrogen flame ionization

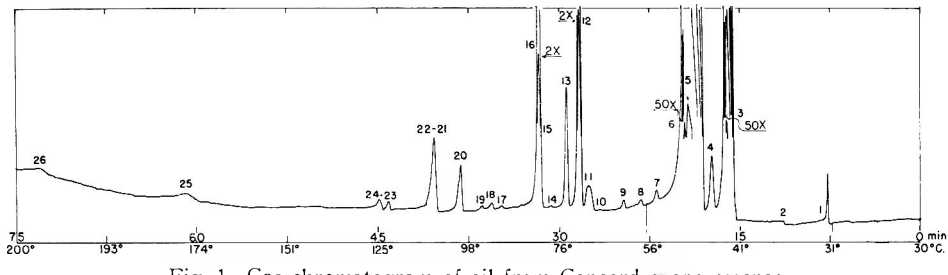

Fig. 1. Gas chromatogram of oil from Concord grape essence.

Table 1. Components of Concord essence.

Peak no.	Compound	Peak no.	Compound
3	Ethyl acetate	12	2-methyl-3-buten-2-ol
4	Isopropyl acetate	13	Ethyl *n*-butyrate
5	Ethanol	16	Isobutanol
6	Isopropanol	18	*n*-butanol
7	Ethyl propionate	21	2-methyl-1-butanol
8	*n*-propyl acetate	22	3-methyl-1-butanol
9	Chloroform	23	Styrene
11	*n*-propanol	24	Ethyl hexanoate

detector. The numbers above the peaks are for identification. The compounds identified are described in Table 1.

The two largest peaks, ethyl acetate and ethanol, have been reported previously. The remaining compounds have not previously been reported in Concord essence. Chloroform (peak No. 9) and styrene (peak No. 23) probably arise from contamination, although the source has not been established.

The third-most intense peak (No. 12) is dimethylvinylcarbinol, which, when pure, has an aroma similar to that of the cresols.

A mass spectrum of the small peak at No. 1 was not obtained; however, when acetaldehyde was added to the grape oil it had a retention time identical to that of peak No. 1. The weak mass spectrum obtained for peak No. 2 suggested either an acetate or acetone. Enrichment of the oil with methyl acetate and acetone suggested that the peak was methyl acetate. However, since the retention times of those two compounds are almost identical under these conditions, identification of peak No. 2 as methyl acetate must still be tentative.

Peak No. 10 (very weak) is possibly a C_5 aldehyde. On the upward slope of isobutanol (No. 16) an acetate (No. 15) appears to be present. By changing the program rate to 0.75°/min, it is possible to separate No. 15 from isobutanol. The spectra of components 17 and 19 were not obtained.

Peak No. 20 (relatively intense) appears to be a C_7 unsaturated alcohol; it shows rather intense peaks at m/e 69, 41, and 99. Although several structures would fit the data now available, it would be quite risky to assign a specific structure without having an authentic sample on hand for direct comparison.

The mass spectra of the last two peaks, 25 and 26, were quite weak but appear to be C_6 alcohols.

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Reference to a company or product does not imply recommendation.

V. J. FILIPIC, J. C. UNDERWOOD, and C. O. WILLITS
Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division
Agricultural Research Service, U. S. Department of Agriculture
600 East Mermaid Lane, Philadelphia, Pennsylvania 19118

The Identification of Methylcyclopentenolone and Other Compounds in Maple Sirup Flavor Extract

SUMMARY

A number of compounds in the flavor-containing chloroform extract of maple sirup have been isolated and identified. A major constituent not previously isolated by gas chromatography was acetol. Other constituents present in lesser concentrations which have not previously been reported were acetoin, ethyl vanillate, syringoyl methyl ketone, and methylcyclopentenolone.

In a previous study utilizing gas chromatography (Underwood and Filipic, 1963) the authors reported the presence of vanillin, syringaldehyde, and dihydroconiferyl alcohol in a chloroform flavor extract of maple sirup. Since these compounds were the major fractions separated by the gas chromatographic techniques used, sufficient materials for infrared identification were easily obtained. By using a much larger amount of the chloroform extract for gas chromatography, quantities of several other compounds sufficient for identification were obtained. One of these was identified as methylcylopentenolone, which is reported to impart a maple flavor to simple sirup (Dow Chemical Company, 1956). This paper is a report on the components identified in the chloroform extract and of the methods by which they were isolated and characterized.

EXPERIMENTAL PROCEDURE AND RESULTS

Twenty gallons of commercial maple sirup, U. S. Grade A, were extracted twice with 20-gallon volumes of chloroform according to the procedure described by Underwood *et al.* (1961). The combined extracts were concentrated to 200 ml by evaporation at atmospheric pressure. This concentrate was then shaken with 800 ml of ether to precipitate the ligneous material. The supernatant liquid was concentrated to 40 ml, thus resulting in a 2,000:1 concentration of the chloroform-ether-soluble components in the maple sirup.

A 500-μl portion of the final concentrate was injected into an F & M chromatograph, model 720, using the instrumental conditions shown in Table 1. The chromatogram obtained is shown in Fig. 1. Portions of this chromatogram are shown in greater detail in Figs. 2 and 3. Except for the chloroform solvent, the entire eluate from 70 such injections was collected in 24 traps inserted in the exit port at the retention times indicated by the dashed lines in Figs. 2 and 3. The traps were 4-mm OD glass U tubes immersed in a dry ice-acetone bath.

The decision as to the retention time at which the collection of a new fraction should begin was based on the consideration that all fractions would be rechromatographed. Therefore, eluates showing incompletely resolved peaks were collected in one trap for greater efficiency. For the same reason, eluates represented by a succession of peaks of low

Table 1. Gas chromatographic operating conditions.

Columns	Dual, stainless steel, ¼-inch OD, 4 ft long
Packing	20% Carbowax 20M on 60-80-mesh acid-washed Chromosorb W
Detector	Thermal conductivity
Temperatures	Injector port, 145°C.
	Column Oven, 4 min at 50°C.
	56 min from 50°C to 240°C (approx. 3.5°/min)
	40 min at 250°C
	Detector 250°C
Flow rate	50 ml He/min
Attenuation	4X

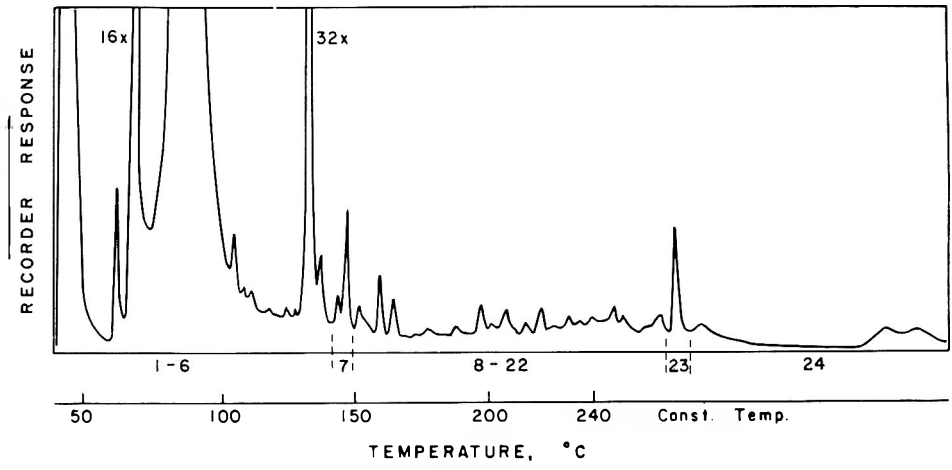

Fig. 1. A gas chromatogram of the chloroform extract of maple sirup (on Carbowax 20M).

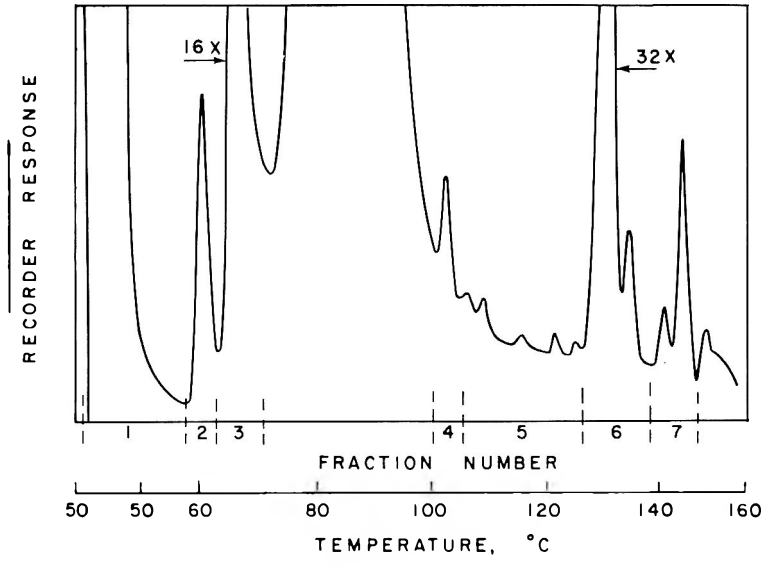

Fig. 2. Fractions 1–7, illustration of temperature range of collection.

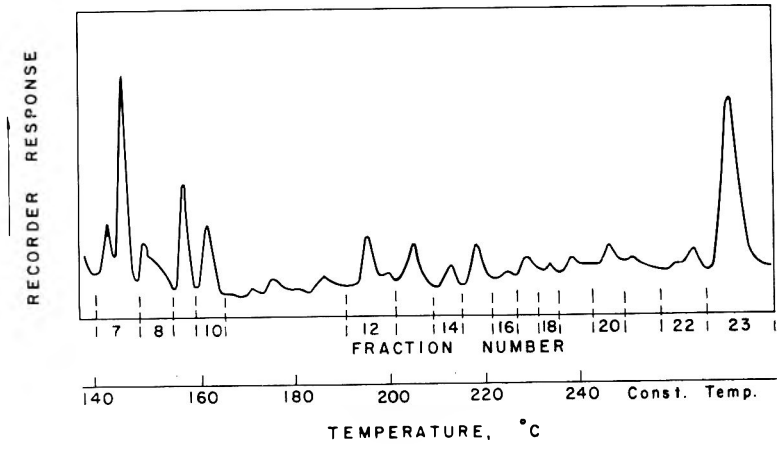

Fig. 3. Fractions 7–23, illustration of temperature range collection.

intensity were also collected in another single trap. Since the individual components of the higher-boiling fractions had been efficiently condensed in the past, they were collected in individual traps, no matter how small the peaks representing them. Since the highest-boiling fractions had been identified previously, all materials eluting at temperatures above those for vanillin were collected in one trap.

The eluate collected in each trap was removed by washing with ether and chloroform. The first seven of the resulting 24 solutions were then rechromatographed on the same Carbowax column to retain the better resolution afforded by a polar substrate for the oxygenated materials present in maple sirup extract. The remaining 17 fractions were rechromatographed on an SE-30 column to reduce the contamination of these higher-boiling eluates with column bleed. The various components of all fractions were collected in 2 × 100-mm capillary tubes cooled with powdered dry ice. As soon as each eluate was obtained, the tubes were sealed with a micro torch.

Identifications. Each identification of the component collected was based on comparison of the infrared spectra and of retention times on both 20M and SE-30 columns with standards. The IR spectra of the most volatile compounds were obtained in sealed micro cells, those of intermediate volatility in micro mull plates, and the remainder in micro KBr discs. The discs were prepared by rinsing the contents of the capillary collection tube onto the surface of a micro agate mortar. After evaporation of the chloroform solvent, the thin film of solute was taken up in 15 mg of powdered KBr by grinding for about 2 min. Pellets of 3 mm diameter were made with a Beckman No. 16585 die. These pellets were placed in a Perkin-Elmer 4× beam condenser mounted in a Beckman IR-8 spectrophotometer. To obtain ultraviolet spectra, the discs were simply dissolved in sufficient water.

Since the first 11 fractions were liquid, sealed cells were used to obtain the spectra of the isolated components. The very large peak of fraction 1 was due to the ether added to the chloroform concentrate. Fraction 2 was identified as ethyl acetate, and fraction 3 as ethyl alcohol. The eluate represented by the large peak between fractions 3 and 4 was due to the chloroform solvent and was not collected. Fraction 4 was water. None of the components in fraction 5 were identified.

There was a shoulder on the ascending slope of the major peak in fraction 6 that was more clearly evident on rechromatographing, as shown between the dashed lines in Fig. 4. This shoulder, fraction 6B, was shown to result from the presence of acetoin. The main constituent, fraction 6C, was shown to be acetol. The infrared spectrum of the

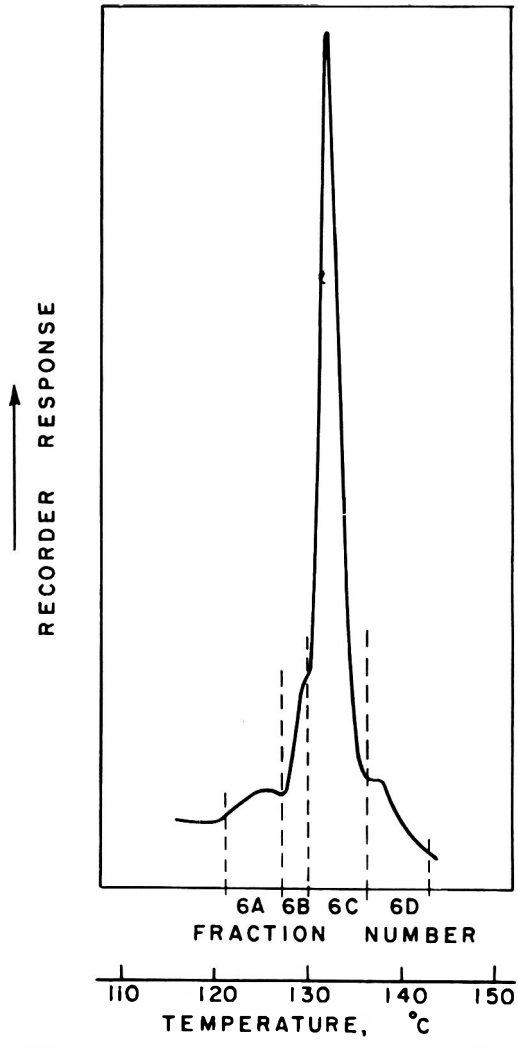

Fig. 4. Rechromatography of fraction 6 on Carbowax 20M.

sample (fraction 6C) is compared with that of acetol in Fig. 6. In this case sufficient material was available to obtain a nuclear magnetic resonance spectrum using a micro cell. This spectrum (Fig. 7) is consistent with the structure of acetol. It has peaks resulting from methyl, hydroxyl, and methylene protons in the peak area ratio of 3:1:2. The lack of coupling indicates that protons are not present on adjacent carbon atoms.

The material recovered in fractions 7 through 11 was insufficient to permit identification. The remaining fractions (12–24) consisted of materials so low in volatility that KBr discs were used to obtain their infrared spectra.

Fraction 12 yielded a crystalline material as the major component on rechromatography. In the process of making a micro KBr disc from it a dis-

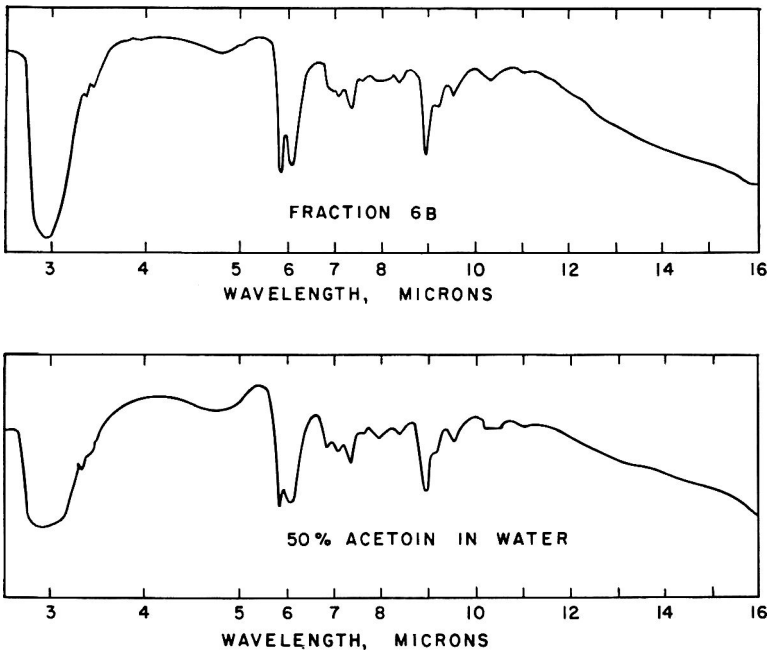

Fig. 5. A comparison of the infrared spectra of fraction 6B and acetoin.

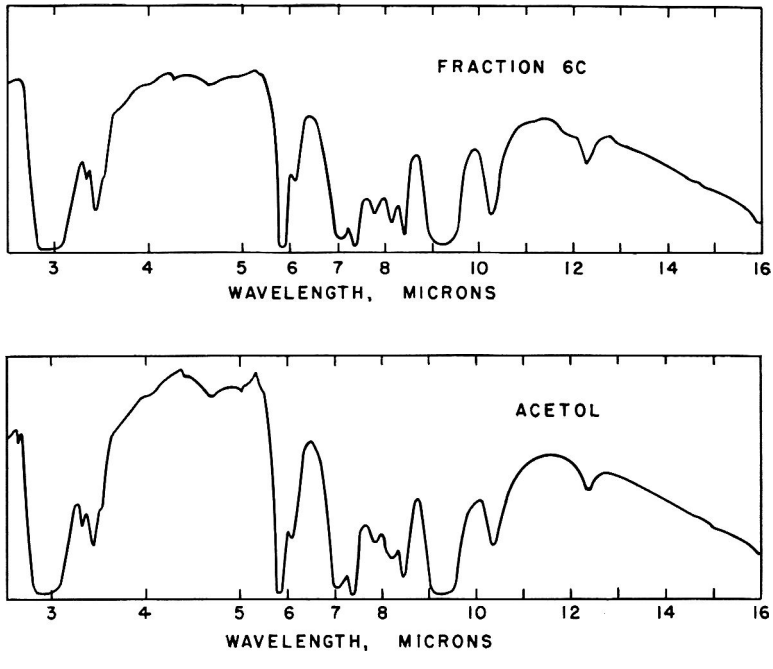

Fig. 6. A comparison of the infrared spectra of fraction 6C and acetol.

tinct maple-like aroma was noted. The infrared spectrum of this component is compared in Fig. 8 with that of 3-methylcyclopent-2-en-2-ol-1-one. A published spectrum of methylcyclopentenolone (Gianturco *et al.*, 1963) aided in establishing the identity of fraction 12. The material also exhibited the same ultraviolet maximum as did the known.

Fraction 13 was identified as butylated hydroxytoluene (BHT) by both infrared and mass spectrometry.

Ultraviolet data confirmed the infrared identification of fraction 14 as phenol. Fig. 9 shows the result of rechromatographing fraction 14 on an SE-30 column. The largest peaks, occurring at 50°C, are due to the solvents used, the next-largest peak, at about 123°C, is due to phenol, and the

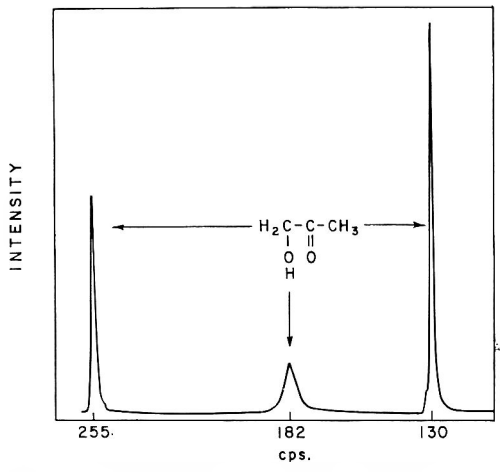

Fig. 7. The nuclear magnetic resonance spectrum of fraction 6C.

final peak, which eluted above 200°C, is due to dihydroconiferyl alcohol. The presence of high-boiling constituents in this fraction probably resulted from a cold spot in the discharge end of the detector, causing condensation of eluates in the exit port. Then, as the temperature of the carrier gas increased during programming, these components would volatilize, causing all subsequent fractions to contain materials of a wide boiling range. They were, however, richer in those components which were eluting from the column at that time.

Fraction 15 was indicated by its infrared spectrum to be a long-chain fatty acid (about C_8), and the infrared spectrum of 16 closely resembled that of the glyceride, diacetin. In fractions 17 through 22 the ratio of component to contaminants even after being rechromatographed was so low that identification by infrared was not possible. This contamination was probably a combination of column bleed and other decomposition products.

Fraction 23 contained vanillin, and the last two peaks in fraction 24 represent syringaldehyde and dihydroconiferyl alcohol, all compounds previously identified.

Fraction 24 was rechromatographed at a much higher injection temperature (280°C) to completely volatilize the higher-boiling components. Based on infrared spectra, guaiacol, 2,6-dimethoxy phenol, ethyl vanillate, and syringoyl methyl ketone were identified. Identification of the syringoyl methyl ketone, a dicarbonyl of yellow color, was based on a comparison with the spectrum given by Pearl (1959). The maximum column temperature was not maintained long enough to elute this diketone in a single chromatogram. Its presence was detected only because of the long series of sequential runs.

Identifications of the compounds in the different eluted fractions are summarized in Table 2.

DISCUSSION

Three of the major constituents of the concentrated chloroform extract of maple sirup (vanillin, syringaldehyde, and dihydroconiferyl alcohol) have been identified previously. Underwood *et al.* (1961) found vanillin and syringaldehyde in column chromatography. In 1963, using gas chromatography, they found dihydroconiferyl alcohol. Acetol, which was tentatively identified by Underwood *et al.* (1956) in the form of a dinitrophenylhydrazine derivative, was not observed in the gas chromatographic procedure used in 1961, no doubt because it decomposes at 146°C and was destroyed by the high injection temperatures then used. This current study, using lower injection temperature, resulted in identification of

Table 2. Compounds identified in GLC studies.

Fraction No.[a]	Compounds[b]
1	Ether
2	Ethyl acetate
3	Ethyl alcohol
4	Water
6B	Acetoin
6C	Acetol
12	Methylcyclopentenolone
13	Butylated hydroxytoluene (BHT)
14	Phenol
15	A long-chain fatty acid
16	Diacetin
23	Vanillin
24	Ethyl vanillate
	Syringaldehyde
	Dihydroconiferyl alcohol
	Syringoyl methyl ketone
	Guaiacol[c]
	2,6-dimethoxyphenol[c]

[a] From original chromatogram on 20M column.

[b] Compounds were identified after rechromatography of each fraction by infrared spectrophotometry and comparison of retention times on Carbowax 20M and SE-30. Other means of identification were ultraviolet spectrophotometry for methylcyclopentenolone and phenol, mass spectrometry for butylated hydroxytoluene, and nuclear magnetic resonance spectrometry for acetol.

[c] Probable degradation products resulting from use of a higher injection temperature. Retention times indicate that these compounds should have been isolated in fractions 13 to 16 if they were initially present in the extract.

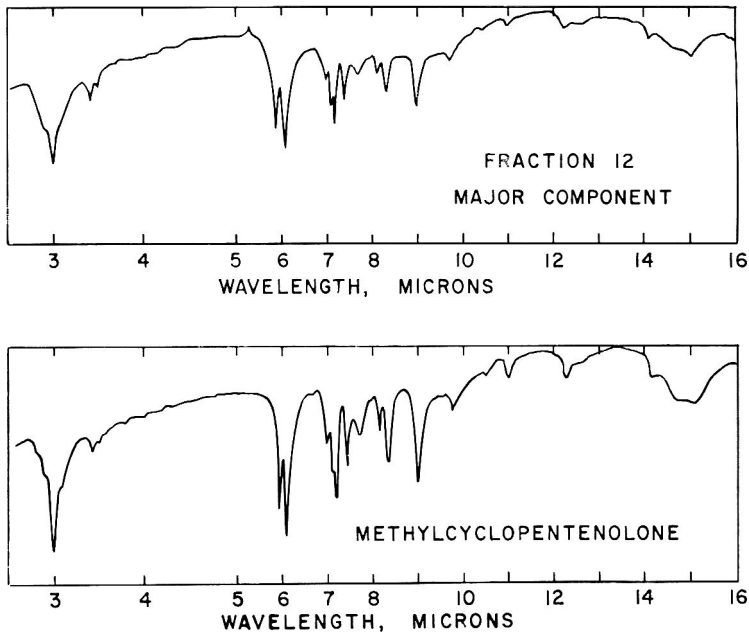

Fig. 8. A comparison of the infrared spectra of fraction 12 and methylcyclopentenolone.

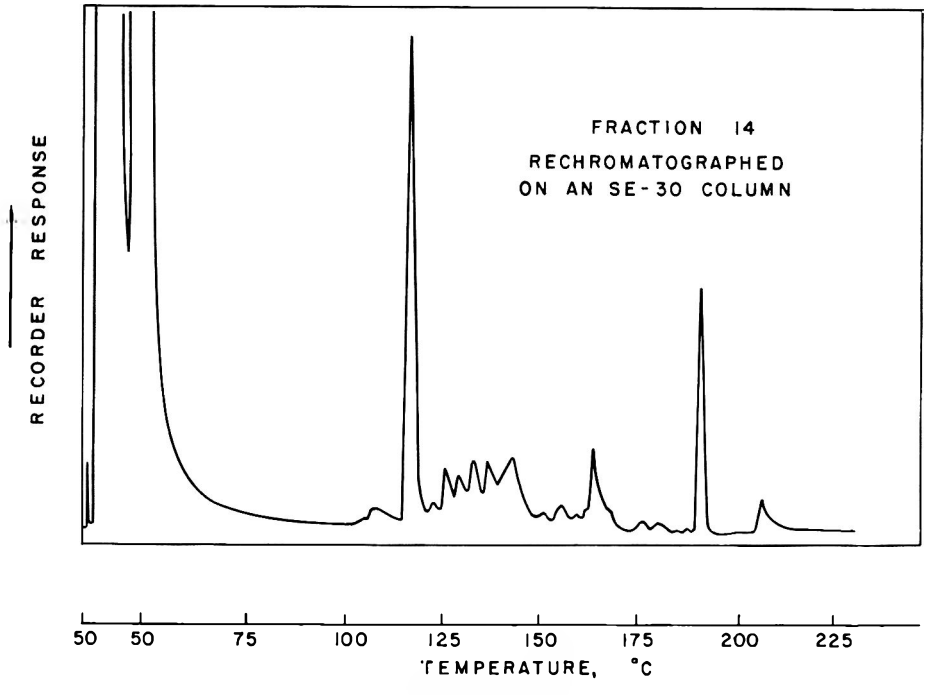

Fig. 9. Rechromatography of fraction 14 on an SE-30 column.

acetol as a major constituent and confirmed Underwood's previous observation.

In this study, a number of minor constituents have been identified in addition to the above four major components. This resulted from: the use of a more efficient trapping procedure; the accumulative collection of fractions from 70 GLC fractionations; and the use of more sensitive infrared techniques.

Among these minor constituents, one compound, the one that may contribute to the

characteristic flavor of maple sirup, is 3-methylcyclopent-2-en-2-ol-1-one. Fig. 10 shows the three possible tautomeric forms of this compound. Bredenberg (1959) has shown that this compound occurs as structure 1, both in the solid state and in solution. Methylcyclopentenolone is sold commercially under the trademark "Cyclotene." It is used in compounding flavors and is said to improve the taste of maple, walnut, or pecan mixtures.

Methylcyclopentenolone can be formed by boiling wood at 100°C in caustic solution (Enkvist, 1954; Linberg and Enkvist, 1953) and also by refluxing glucose and acetone in an aqueous alkaline medium (Fray, 1961). Maple sap is a dilute sugar solution containing among its organic constituents small amounts of soluble lignins. As the sap is converted into sirup by boiling, the solution passes through an alkaline phase (Hayward and Pederson, 1946; Willits *et al.*, 1952), being at a maximum pH of about 9 for some time. Thus, conditions exist under which this cyclic ketol can be formed.

The presence of acetoin and ethyl alcohol also raised the possibility that a fermentation may have occurred in the sap from which this sirup was made. Syringoyl methyl ketone can also arise from the ethanolysis or fermentation of lignin (Brauns and Brauns, 1960).

The ethyl alcohol, in turn, could account for the presence of the esters ethyl acetate and ethyl vanillate, since maple sirup is known to contain acetic acid (Nelson, 1928) and vanillic acid (Risi and Labrie, 1935). On the other hand, hydrolysis of such esters could account for the presence of ethyl alcohol and the acids. Some of the compounds isolated—ethyl acetate, ethyl alcohol, and phenol—are ubiquitous materials. These three components could be contaminants or artifacts introduced during the processing of the maple sirup or in the analytical procedures.

METHYLCYCLOPENTENOLONE

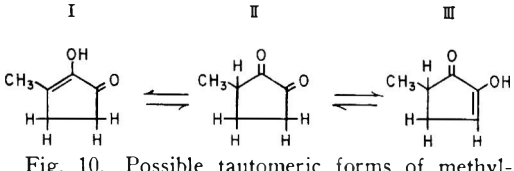

Fig. 10. Possible tautomeric forms of methylcyclopentenolone.

The compounds guaiacol, 2,6-dimethoxyphenol, and syringoyl methyl ketone were isolated from a single analysis made on the SE-30 column in which the injection temperature was raised to 280°C (compared to 145°C, used previously). It is quite possible that they are the products of thermal degradation of higher-molecular-weight materials. Guaiacol, however, has been previously reported as a constituent of maple sirup (Risi and Labrie, 1935). It, as well as vanillin and syringaldehyde, could result from the alkaline hydrolysis of lignin (Brauns and Brauns, 1960).

Commercial fats and oils are frequently used by maple sirup producers as antifoaming agents. Isolation of the fatty acid, glyceride, and antioxidant (BHT) is clear evidence that such an agent was present in the batch of maple sirup analyzed in this study. The ability to isolate and identify a trace constituent, the antioxidant, added in trace amounts to the sirup as an antifoaming agent, illustrates the sensitivity that can be obtained in combining gas chromatography with infrared spectrophotometry.

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A. S. EL-SAYED[a] and B. S. LUH
Department of Food Science and Technology
University of California, Davis, California 95616

Polyphenolic Compounds in Canned Apricots

SUMMARY

Polyphenolic compounds were extracted with ethyl acetate from aqueous infusions of canned Blenheim apricots (*Prunus armenica* L.). The compounds were separated by two-dimensional paper chromatography of the extract with *n*-butanol–acetic acid–water (4:1:5) and 2% acetic acid. The individual compounds were identified by R_f values, fluorescent behavior, absorption spectra, and degradation products. Shown to be present in the extract were three chlorogenic acid isomers, two *p*-coumaric acid derivatives, rutin, isoquercitrin, quercetin, an unidentified quercetin glucoside, catechin, and epicatechin. The predominant polyphenolic compounds were chlorogenic acids and *p*-coumaric acid derivatives.

INTRODUCTION

Polyphenolic compounds are important constituents related to taste and enzymic browning in foods (Swain, 1962). Samisch and Cruess (1934) isolated from apricots a tannin-like substance which served as a substrate for the polyphenol oxidase and caused darkening of dried apricots. Williams and Wender (1953) isolated quercetin and isoquercitrin from apricots (*Prunus armenica* L.). Herrmann (1956, 1958) detected catechins, chlorogenic acids, and *p*-coumaric and ferulic acid esters in apricots by paper chromatographic methods. Chlorogenic acids in various foods have been reported by Barnes *et al.* (1950), Corse (1953), Cartwright *et al.* (1955) and Swain (1962). The biochemistry of phenolic compounds has been reviewed by Harborne (1964).

In the present work, Blenheim apricots were canned as unpeeled halves in sucrose syrup. The water-soluble polyphenolic compounds in the canned product were extracted with ethyl acetate, separated by two-dimensional paper chromatography, and then identified by their R_f values, fluorescent behavior, absorption spectra, and degradation products.

[a] Present address: Department of Agriculture, Section of Horticulture, Khartoum, Republic of Sudan.

MATERIALS AND METHODS

Apricots. Two hundred pounds of Blenheim apricots were harvested at canning maturity from a commercial orchard at Hollister, California. The fruits were sorted, washed, and then halved and pitted in a Filice and Perrelli apricot pitter. Nineteen and one-half ounces of apricot halves and 10.5 ounces of 50° Brix sucrose syrup were filled into each No. 2½ (401 × 411) can, made of differential electrolytic tinplate. The cans were sealed in a double seamer under a vacuum of 16 in. Hg, heat-processed for 19 min at 212°F in an Anderson-Barngrover rotary cooker, and cooled in a rotary water-spray cooler. The canned product was stored for 6 months at 70°F.

Extraction of polyphenols with ethyl acetate. Two cans of apricots were drained on a screen. Five hundred grams of drained apricot halves were blended under nitrogen for 5 min with 1,500 ml of distilled water. The mixture was centrifuged, and the liquid set aside as the first extract. The residue was extracted in the same way with 1,500 ml of distilled water. The extracts were combined, saturated with sodium chloride, and treated with an equal volume of ethyl acetate. The flasks containing the mixture were flushed with nitrogen, covered, and shaken on a mechanical shaker. The ethyl acetate layer was separated in a separatory funnel and stored under nitrogen at 0°C. The aqueous layer was extracted twice with ethyl acetate as described above. The ethyl acetate extracts were combined, treated with anhydrous sodium sulfate, and stored overnight at 0°C in stoppered flasks after flushing with nitrogen. For chromatographic examination the ethyl acetate solution was filtered through a sintered-glass funnel, and the filtrate was concentrated to 5 ml in a flash evaporator.

Paper chromatography. Two-dimensional descending paper chromatography was used to separate the polyphenolic compounds. Fifty to 100 μl of the extract were spotted on Whatman No. 1 papers (46 × 57 cm). The chromatograms were developed in two dimensions at 22±1.5°C for 18 hr with *n*-butanol–acetic acid–water (BAW 4:1:5 v/v) as the first solvent and for 3.5 hr with 2% acetic acid as the second solvent. The air-dried chromatograms were examined for spots under ultraviolet light before and after exposure to ammonia vapor. Some of the chromatograms were sprayed with a freshly prepared chromogenic reagent made of equal volumes of 0.5% $FeCl_3$ and 0.5% $K_3Fe(CN)_6$ (Keppler, 1957).

The chromatograms were rinsed with 0.24 *N* HCl and then washed with distilled water. The polyphenolic compounds were detected as blue spots. Like spots were cut from unsprayed papers and eluted with 99% ethanol.

Catechins and epicatechins were identified by spraying the chromatogram with a mixture of four volumes of a saturated ethanolic solution of vanillin to one volume of concentrated HCl (Swain and Hillis, 1959; Craft, 1961). They appeared as red spots. Identification of ferulic acid, chlorogenic acid, quercetin, caffeic acid, catechin, and epicatechin was confirmed by cochromatography with authentic samples.

Absorption spectra. The absorption spectrum of each purified polyphenolic compound in ethanol was taken in a Beckman DK-2 recording spectrophotometer with 1-cm silica cuvettes and compared with that of an authentic sample.

Hydrolysis of p-coumaric acid derivatives. Spots 6 and 7 were eluted separately from the paper chromatogram (Fig. 1) with ethanol. The eluate was flash-evaporated under vacuum to almost dryness, and then refluxed for 1 hr with 6*N* HCl at 100°C. After cooling, the hydrolysate was extracted with ether. The extract was spotted on Whatman No. 1 filter paper, and irrigated for 18 hr with the upper layer of BAW (4:1:5) solvent. A similar paper was irrigated with 2% acetic acid as solvent. Caffeic and *p*-coumaric acids were used as reference compounds. The R_f values of fluorescent spots under ultraviolet light in the presence of NH_3 vapor were recorded (Table 1).

Hydrolysis of quercetin glycosides. The sugar moieties of spots 9 (rutin), 10 (isoquercitrin), and 11 (quercetin glucoside) were determined by hydrolysis for 1 hr with 1*N* HCl at 100°C. The hydrolysate was concentrated under vacuum, dissolved in 70% ethanol, and then spotted on two Whatman No. 1 filter papers. The papers were irrigated separately for 18 hr in a descending direction with the upper layers of BAW (4:1:5) and ethyl acetate-acetic acid–water (3:1:3), dried in air, and sprayed with a solution made by mixing 0.5 ml of saturated silver nitrate with 1 ml of water and 99 ml of acetone. The chromatogram was then sprayed with an ethanolic sodium hydroxide solution made by mixing 5 ml concentrated NaOH with 95 ml of 95% ethanol.

Table 1. R_f values of polyphenolic compounds in canned apricots.

Spot no.	Identification	R_f values		Color under ultraviolet light	Color under ultraviolet light + NH_3
		BAW (4:1:5)	2% acetic acid		
1	Unknown	.82	.00	blue	blue
2	Ferulic acid	.85	.38	blue	blue
3	Chlorogenic acid	.60	.56	blue	green
4	Chlorogenic acid isomer[a]	.64	.68	blue	green
5	Chlorogenic acid isomer[a]	.54	.65	blue	green
6	*p*-Coumaric acid derivative	.84	.83	blue	green
7	*p*-Coumaric acid derivative	.70	.80	blue	green
8	Unknown	.50	.76	blue	blue
9	Rutin	.45	.34	purple	yellow brown
10	Isoquercitrin	.58	.14	purple	yellow brown
11	Quercetin glucoside	.64	.20	purple	yellow brown
12	Quercetin	.68	.00	yellow	yellow brown
13	Caffeic acid	.76	.21	blue	blue
14	Catechin	.63	.37	none	dark
15	Epicatechin	.53	.31	none	dark
16	Unknown	.78	.47	none	blue
Authentic compounds					
	Chlorogenic acid	.60	.55	blue	green
	Rutin	.46	.33	purple	yellow brown
	Quercitrin	.70	.22	purple	yellow brown
	Quercetin	.67	.00	yellow	yellow brown
	Catechin	.64	.39	none	dark
	Caffeic acid	.80	.23	blue	blue
	Ferulic acid	.86	.40	blue	blue
	Epicatechin	.52	.30	none	dark
	p-Coumaric acid	.87	.45	none	blue

[a] Spots 4 and 5 were not positively identified.

and finally sprayed with glacial acetic acid (Swain, 1953). Authentic samples of rutinose, glucose, rhamnose, galactose, arabinose, and quercetin were used as references. Reducing sugars appeared as brown black spots. The aglycone appeared as yellow spots.

Spot 9 was hydrolyzed at 100°C for 60 min with 10% acetic acid (Chandler and Harper, 1961). Rutinose, glucose, and rhamnose in the hydrolysate were identified as described above.

Relative amounts of polyphenolic compounds. Fifty μl of the ethyl acetate extract was spotted on each of six Whatman No. 1 papers and chromatographed in a descending direction by the two-dimensional method as described previously. The spots were located under ultraviolet light, cut, and extracted for 12 hr with 35 ml of methanol on a mechanical shaker. The eluates were evaporated to dryness on a hot-water bath. Seven ml of distilled water, 0.5 ml of Folin-Denis reagent, 1.0 ml of saturated Na_2CO_3 solution, and 1.5 ml of distilled water were added to the mixture. The mixture was kept in the dark for 1 hr, and centrifuged, and the absorbance of the blue solution was measured in a Klett-Summerson photoelectric colorimeter with a No. 66 red filter (Swain and Hillis, 1959).

RESULTS AND DISCUSSION

Paper chromatography. The water-soluble polyphenolic compounds in canned apricots were extracted with ethyl acetate, concentrated, and then spotted on Whatman No. 1 paper for two-dimensional paper chromatography. Fig. 1 presents the pattern of a two-dimensional chromatogram and shows the position of 16 spots detected with the $FeCl_3$—$K_3Fe(CN)_6$ reagent. The color of the spots varied from light to deep blue, depending on the quantity of polyphenols present.

Table 1 shows the R_f values and the color under ultraviolet light, with and without ammonia vapor, of polyphenolic compounds present in the ethyl acetate extract of canned apricots. They were identified as ferulic acid (spot 2), chlorogenic acids (spots 3, 4 and 5), rutin (spot 9), isoquercitrin (spot 10), quercetin glucoside (spot 11), quercetin (spot 12), caffeic acid (spot 13), catechin (spot 14), and epicatechin (spot 15). Some of these compounds were further characterized by their spectral properties in the ultraviolet region, chelation with $AlCl_3$ reagent, and acid-hydrolysis products. Spots 1, 6, and 18 were not identified.

Cinnamic acids. Spot 3 was chlorogenic acid. It was present in larger amounts than its isomers (spots 4 and 5). The absorption spectrum of spot 3 is shown in Fig. 2. It has a maximum absorption peak at 325 mμ which showed a bathochromic shift on addition of aluminum chloride. This was due to the presence of an adjacent pair of hydroxyl groups in the caffeic acid moiety. In comparison with the results of Cartwright *et al.* (1955), spot 3 was chlorogenic acid (quinic acid 3-caffeate) and spots 4 and 5 were probably its isomers.

Spot 2 was identified as ferulic acid, and spot 13 as caffeic acid by comparison of their R_f values and color properties with those

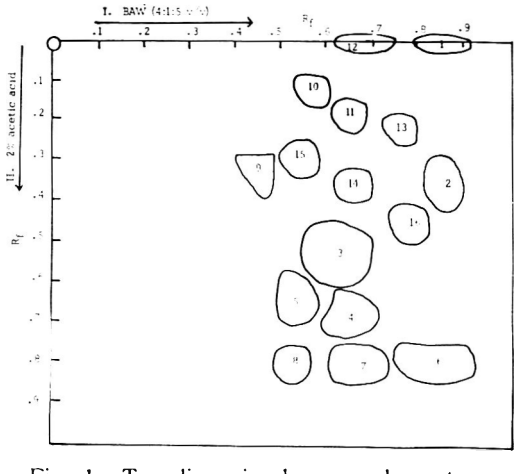

Fig. 1. Two-dimensional paper chromatogram of polyphenolic compounds extracted from aqueous infusion of canned apricots with ethyl acetate.

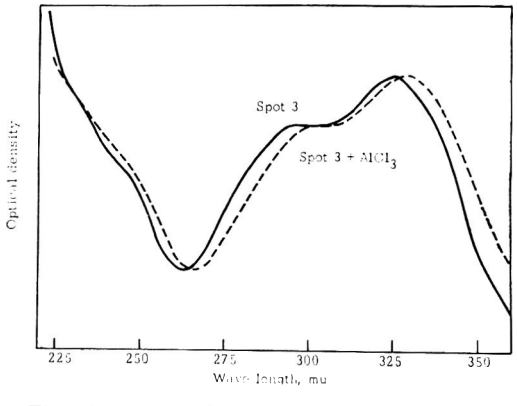

Fig. 2. Absorption spectrum of chlorogenic acid (spot 3) in ethanol. The dotted curve shows the bathochromic shift with $AlCl_3$.

of authentic compounds (Table 1). Both showed blue fluorescence under ultraviolet light and remained unchanged when exposed to NH_3 vapor.

p-Coumaric acid derivatives. Spots 6 and 7 were identified as *p*-coumaric acid derivatives by examination of the R_f values and color properties of their acid hydrolysis products. The R_f values were comparable to those reported for *p*-coumaryl quinic acids found in tea, apples, and pears (Cartwright *et al.*, 1955). *p*-Coumaric acid was derived from spots 6 and 7 after hydrolysis with 6*N* HCl. Table 1 shows the R_f values and color characteristics under ultraviolet light of *p*-coumaric acid.

Catechin and epicatechin. After examination of their chromatographic behavior (Table 1), spots 14 and 15 were respectively identified as catechin and epicatechin. They did not fluoresce under ultraviolet light with or without ammonia. When the paper chromatogram was sprayed with $FeCl_3$—$K_3Fe(CN)_6$ reagent, they appeared as blue spots. When a separate chromatogram was sprayed with the vanillin-HCl reagent (Swain, 1953), characteristic red spots appeared, indicating catechins. This was further confirmed by cochromatography with authentic catechin and epicatechin, with BAW (4:1:5) and 2% acetic acid as solvents.

The identities of spots 14 and 15 were confirmed by their absorption spectra in the ultraviolet region. The maximum absorption peaks of spots 14 and 15 in ethanol appeared at 278–280 mμ. Jurd (1962) reported a maximum absorption peak at 280 mμ for catechin and epicatechin in ethanol.

Quercetin group. Spots 9, 10, 11, and 12 contained quercetin as the common aglycone, identified by cochromatography of the HCl hydrolysate with an authentic sample, using the upper layer of BAW (4:1:5) as the irrigating solvent. Spot 9 was shown to be rutin (quercetin 3-rutinoside), spot 10 isoquercitrin (quercetin 3-glucoside), spot 11 a quercetin-glucoside, and spot 12 quercetin. Table 2 shows the R_f values of the sugar moieties of spots 9 to 12 after refluxing for 60 min with 1*N* HCl.

When 10% acetic acid was used for hydrolysis, spot 9 gave a sugar with an R_f value of 0.17 in BAW (4:1:5) which is identical to that of authentic rutinose. Rhamnose and glucose were products of hydrolysis of spot 9 when 1*N* HCl was used.

The above results prove that spot 9 was rutin (quercetin 3-rutinoside). Williams and Wender (1953) reported quercetin and isoquercitrin in apricots. They did not make positive identification of rutin in apricots.

The absorption peaks of spots 9, 10, 11, and 12 in ethanol are respectively in agreement with data reported by Jurd (1962) for rutin, isoquercitrin, quercetin glucoside, and quercetin.

Relative amounts of polyphenols. Based on the color reaction of the various polyphenolic compounds with the Folin-Denis reagent, it was observed that chlorogenic acids and *p*-coumaric acid derivatives were the predominant water-soluble polyphenolic compounds in canned apricots. The

Table 2. R_f values of sugar moieties of spots 9, 10, 11, and 12 obtained by hydrolysis with 1*N* HCl.

Spot no.	Identification	R_f values of sugar moiety	
		EtOAc-acetic acid-water (3:1:3)	*n*-Butanol–acetic acid–water (4:1:5)
9	Rutin (quercetin 3-rutinoside)	0.15 (glucose) 0.32 (rhamnose)	0.20 (glucose) 0.41 (rhamnose) 0.17 (rutinose)[a]
10	Isoquercitrin (quercetin 3-glucoside)	0.15 (glucose)	0.20 (glucose)
11	Quercetin-glucoside	0.15 (glucose)	0.20 (glucose)
12	Quercetin	None	None

[a] Obtained by hydrolysis with 10% acetic acid.

other polyphenols were present in relatively smaller quantities.

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EUGENE F. JANSEN, KENNETH J. PALMER, and ROBERT E. LUNDIN
Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture Albany, California 94710

The Isolation of Asym-Monoethylcitrate from Avocado Fruit

SUMMARY

***Asym*-monoethylcitrate was isolated from avocado fruit. Its identity was established by analysis, by its proton magnetic resonance, and by comparison with the synthetic ester, including X-ray diffraction patterns. Approximately 6% of the citric acid of avocado fruit exists as its *asym*-monoethyl ester. The ester exists in two crystalline forms of different melting points and different X-ray diffraction patterns.**

The nonvolatile acids contained in 70% methanolic extracts of mature green avocados were separated. One of the acids isolated was found to be *asym*-monoethylcitrate. The clue to its identity came from analysis of its proton magnetic resonance (pmr) spectrum. The pmr spectrum established the presence of one ethyl ester group, at least one carboxyl group, and two slightly different methylene groups bonded to proton-free carbon. The identity of the ester was established by analysis and by a comparison with synthetic *asym*-monoethylcitrate, including X-ray diffraction patterns. An interesting property of the ester is its ability to crystallize into two forms with different X-ray diffraction patterns and melting points. Either form can be prepared at will.

The only previous report on the occurrence of this ester is by Wolfrum and Pinnow (1915), who isolated it from lemon juice which contained considerable ethanol. The occurrence of the ethanol was unexplained, so the origin of the ester is uncertain.

MATERIALS AND METHODS

Ester analyses were performed according to the procedure of Hestrin (1949). Equivalent weights were determined from the titration curves obtained with standard 0.1*N* NaOH titrations, using the Di-Functional Recording Titrator manufactured by International Instrument Co., Canyon, California.

The flesh and skin (1000 g) of mature green avocado fruit (Haas variety) was ground in a large Waring blender with 2.08 L of methanol. A total of 5000 g of tissue was so treated. The extraction was allowed to proceed for 2 days at room temperature, after which the extract was removed from the pulp by filtration. The extract was divided into four equal fractions and stored at −30°C.

Each quarter of the extract was treated as follows:

The solution was removed from storage and evaporated in vacuum at a maximum temperature of 35°, collecting the distillate in a dry-ice trap. The residue was dissolved in 500 ml of water, and the fat was removed therefrom by filtration through Celite analytical filter aid. The solution was passed through Dowex-50 cation-exchange resin (column 15 cm high and 3.5 cm in diameter), and the effluent and washings therefrom were passed through a Dowex-1 anion-exchange resin in the formate form (column 31.5 cm high and 3.5 cm in diameter). After water washing, the Dowex-1 column was eluted with 1200 ml of 2*N* formic acid, followed by 750 ml of 4*N* formic acid. The first 700 ml of eluate, collected separately, constituted the first fraction, and the remaining eluate constituted the second fraction.

After removal of the formic acid solution in vacuum under the aforementioned conditions, analyses of aliquots of the respective residues revealed that over 90% of the acid-esters were present in the second fraction. Accordingly, esters were isolated only from this fraction. (The first fraction was composed primarily of fumaric, succinic, pyrrolidonecarboxylic, and malic acids; unpublished results.)

RESULTS

Isolation of *asym*-monoethylcitrate. The acids and acidic esters in the second acid fraction from the Dowex-1 column from each quarter of the methanol extracts were separated by the method of Bulen *et al.* (1952) except that the butanol-chloroform mixtures were *not* equilibrated with 0.5*N* H_2SO_4 prior to use as eluants of the acids on the silicic acid column. The column, 1.5 cm ID × 45 cm, consisted of 35 g of silicic acid to which was added 20 ml H_2O. The column was formed through the use of $CHCl_3$ as a vehicle. The acids were dissolved in 2 ml H_2O, 10 g of silicic acid was added, and the mixture was applied to the top of the column suspended in $CHCl_3$. The eluates were collected in 17-ml fractions, and 0.5-ml portions of each fraction were assayed for their ester content (Hestrin, 1949). One ester was eluted with water-saturated 5% butanol in $CHCl_3$, two esters with 15% butanol, and one ester with 25%

butanol. The identity of the esters other than *asym*-monoethylcitrate is under investigation.

The fractions that eluted with 15% butanol in $CHCl_3$ from all four separations were combined. After removal of the solvent by evaporation in vacuum, the material was rechromatographed on a silicic acid column double the length used previously, using 250 ml of 5% butanol in chloroform followed by 15% butanol in chloroform for elution, and fractions collected as before. The latter solvent eluted first an unknown ester and then *asym*-monoethylcitrate. The fractions composing the latter were combined, and the solvent was removed by evaporation in vacuum. The residue contained a total of approximately one mmole of ester and consisted of a mixture of crystals and some oily material. The crystals were harvested and washed several times with $CHCl_3$. The crystalline substance melted at 108.5–110°, and its X-ray diffraction pattern was one which was later shown to be identical with the high-melting crystalline form of synthetic *asym*-monoethylcitrate (see Fig. 2). After the pmr data were obtained, it was found that the melting point was unaltered by mixing with the high-melting form of synthetic *asym*-monoethylcitrate (see below).

The oily residue from the crystalline material was treated with 25 ml of chloroform, and the chloroform was extracted three times with 30 ml H_2O. Evaporation of the H_2O solution yielded additional ester. The two crops were combined and dissolved in 5 ml of warm ethylpropionate. A small amount of insoluble material was removed by filtration, and the ethylpropionate was evaporated under a stream of N_2 at ambient temperature. The ester was recrystallized after dissolving it in hot ethylpropionate (0.6 ml). A yield of 77 mg of pure ester was obtained. The material possessed a melting point of 102.5–103°, considerably lower than that of the first crop of crystals. However, after the melted sample was allowed to crystallize, it always possessed a higher melting point. This observation was the clue to the two crystalline forms of the ester. Furthermore, the X-ray diffraction pattern of the ethylpropionate-recrystallized ester was one which was later shown to be identical with the low-melting crystalline form of synthetic *asym*-monoethylcitrate. The ester, after drying for 16 hr in vacuum at 60°, analyzed as follows: C, 43.8%; H, 5.51%; OC_2H_5, 20.4%; equiv. wt., 110. (An inflection in the titration curve was noted at the point of half-titration.) Theory for *asym*-monoethylcitrate: C, 43.6%; H, 5.49%; OC_2H_5, 20.44%; equiv. wt., 110. At a concentration of 15 mg/ml of ethanol no optical rotation was found at the sodium D-line, or at 250 mμ. Hence the ester is optically inactive.

Pmr spectrum. Fig. 1 shows the 60*M* cps pmr spectrum of the isolated ester in deuterium oxide. The quartet centered at $\tau' = 5.82$ with a relative area of two, and the triplet at 8.74 with an area of three, are completely typical of the spin-spin multiplets of an ethyl group. (Separation in ppm from the methyl peak of internal sodium trimethylsilyl-propanesulfonate with the methyl peak assigned a value of 10.00. τ' values increase with increasing magnetic fields.) The quartet structure of the methylene resonance arises from spin-spin coupling of the two methylene protons to the three equivalent protons on the adjacent methyl carbon. In similar fashion, spin-spin coupling to the two adjacent methylene protons produces the triplet structure of the methyl resonance. The position of the quartet strongly suggests that the methylene carbon is attached to ester oxygen (Varian Associates, 1963), thereby indicating that the isolated compound is an ethyl ester.

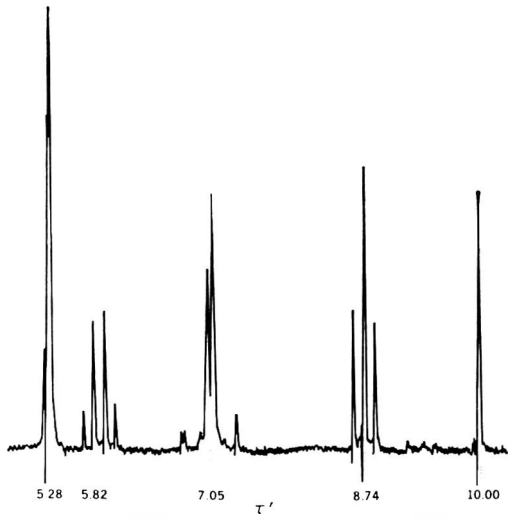

Fig. 1. 60*M* cps pmr spectrum of a 2.5% solution of isolated *asym*-monoethylcitrate in deuterium oxide. Methyl peak of sodium trimethylsilylpropanesulfonate ($\tau' = 10.00$ ppm) used as internal standard.

The multiplet centered at $\tau' = 7.05$ with its weak outer components separated from the adjacent strong lines by 15 cps is typical of the spin coupling pattern obtained from the two protons of a paraffinic methylene group when it is adjacent to an asymmetric carbon atom. The absence of additional splitting of all lines shows that the methylene group must be separated by at least two bonds from any other proton-bearing group. The great intensity difference of the two lines constituting each spin-spin doublet arises because of the small difference in magnetic shielding of the two protons caused by the adjacent asymmetric center. Since this band has a relative area of four (after correcting for the overlapping —CH_2SOO—

band from the internal reference), it must represent two methylene groups. The partial splitting of the low-field doublet shows that the two groups have very slightly different chemical environments. The position of this band suggests that the methylenes are adjacent both to carboxyl carbons and to a weakly polar group. The published spectrum of diethyl acetyl succinate displays the same spectral features (Varian Associates, 1963).

The sharp singlet at $\tau' = 5.28$ arises from the residual hydroxyl protons of the solvent and approximately four additional oxygen-bonded protons in the sample. This number is only an upper limit, because moisture may have been picked up during transfer of sample and solvent. Because of the rapid proton exchange between these species in this solvent they always give rise to a single line without fine structure whose position represents the weighted mean of the positions of the various hydroxyl types in the absence of exchange.

The very weak broad peak centered at $\tau' = 8.21$ and the weak multiplet in the region of $\tau' = 9.2$–9.5 arise from the methylene protons of the reference compound. The third reference peak is concealed by the multiplet at $\tau' = 7.04$.

The only apparent compound that satisfies all conditions imposed both by the pmr data and the elemental analysis appeared to be *asym*-monoethylcitrate. The pmr spectrum of an authentic sample of this material was identical to that of the unknown ester, thus proving the identity of the isolated compound.

To establish definitely that the splitting of the low-field doublet in the $\tau' = 7.05$ methylene band arises because of the asymmetric ethyl group, the spectrum of the *sym*-monoethylcitrate was also obtained. [The symmetric ester was prepared by the method of Wolfrum and Pinnow (1918).] As would be predicted on the basis of the argument given above, the spectrum of the symmetric ester is identical to that of the asymmetric ester except that the low-field methylene doublet displays no trace of additional splitting.

Synthesis of *asym*-monoethylcitrate. *Asym*-monoethylcitrate was prepared from citric acid by a modification of the procedure of Wolfrum and Pinnow (1918). A mixture of 100 g of dry citric acid and 500 ml of absolute C_2H_5OH were refluxed for 7 hr, which caused a gradual solution of the citric acid. The alcohol was evaporated from the reaction mixture, and the resulting syrup was dissolved in 400 ml H_2O; the aqueous solution was extracted twice with 450 ml of ether. Evaporation of the ether solution left a syrup. Titration of aliquots of this syrup showed it to be a mixture of the mono- and diester of citric acid. The mixture was separated on a silicic acid column by the procedure described above; the diester was eluted with 5% butanol in $CHCl_3$, and the monoester with 15% butanol in $CHCl_3$. The fractions composing the monoester were combined, and the solvent was removed by evaporation in vacuum. The product was dissolved in 18 ml of hot ethylpropionate, and the solution was allowed to cool without agitation during crystallization. The yield was 1.64 g of the recrystallized ester. The ester melted at 102.5–103°; remelt, 109°; reported, 108.5–109.5° (Wolfrum and Pinnow, 1915). The results suggested the possibility that the synthetic ester could crystallize in two different forms.

Preparation and interconversion of two crystalline forms of *asym*-monoethylcitrate. To 200 mg of the synthetic ester (mp 102.5–103°) was added 1.0 ml of ethylpropionate. The solvent was heated to dissolve the ester, and the solution was mildly and continuously agitated. After cooling slightly and before crystallization began, the solution was inoculated with crystals obtained by melting the ester and allowing the melt to crystallize. On further cooling and with agitation, the ester crystallized—mp 109–110°. A 100-mg portion of this high-melting *asym*-monoethylcitrate was recrystallized from 0.5 ml of ethylpropionate, avoiding agitation during crystallization; the product melted at 102–103°. This low-melting form was converted to the high-melting form by recrystallization from ethylpropionate under the agitation conditions described above, which was in turn converted to the low-melting form under nonagitation conditions. Thus, *asym*-monoethylcitrate crystallizes in two crystalline forms.

X-ray diffraction patterns of two crystalline forms of *asym*-monoethylcitrate. The X-ray powder patterns of the two polymorphs are shown in Fig. 2. The pattern of the high-melting form

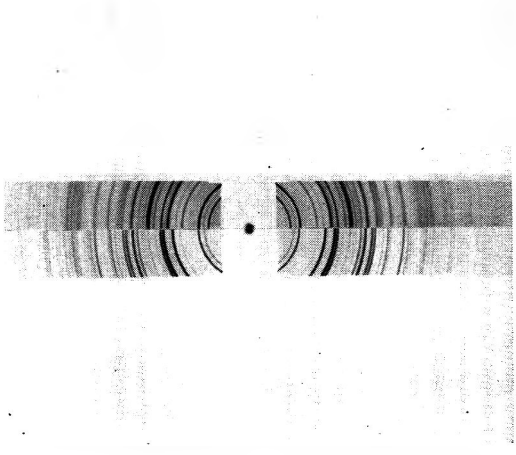

Fig. 2. X-ray powder patterns of the two polymorphs of synthetic *asym*-monoethylcitrate. (Upper: high-melting form; lower: low-melting form.)

is on top, and that of the low-melting form on the bottom. Table 1 shows the interplanar spacings and visually estimated intensities.

DISCUSSION

Wolfrum and Pinnow (1915) isolated *asym*-monoethylcitrate from lemon juice which contained considerable ethanol. Ethanol does not occur in fresh lemon juice in the amounts they reported. Accordingly, the ester may have in part had its origin by virtue of esterification of citric acid by the contaminating ethanol. In the present case, the ester was isolated from avocados in the absence of ethanol and was identified by melting point and X-ray diffraction pattern prior to treatment with an ethyl ester (which might result in transesterification). Hence, *asym*-monoethylcitrate is indeed a constituent of avocado fruit. The possibility exists that the ester might also occur in other fruit.

Table 1. X-ray diffraction data. Interplanar spacings, d ($CuK_\alpha = 1.5418$ A), and visually estimated intensities for low- and high-melting *asym*-monoethylcitrate.

High melting		Low melting	
I	d	I	d
56	10.14	11	9.34
100	8.18	31	8.27
29	6.16	5	6.05
85	5.09	71	5.45
57	4.71	2	5.22
22	4.35	2	5.03
48	4.13	100	4.87
34	4.05	44	4.70
21	3.92	3	4.44
12	3.82	7	4.13
19	3.73	2	3.98
44	3.53	6	3.90
18	3.30	21	3.74
10	3.21	42	3.58
10	3.15	31	3.39
15	2.95	28	3.32
14	2.705	4	3.21
4	2.534	12	3.12
6	2.474	15	3.02
7	2.365	5	2.96
7	2.312	3	2.840
5	2.156	22	2.719
5	2.088	10	2.630
3	2.006	4	2.594
3	1.965	9	2.381
4	1.928	6	2.336
2	1.844	7	2.284
		4	2.150
		9	2.103
		6	2.073
		5	1.868

The same amount of flesh and skin of avocado fruit used here contains approximately 15 mmoles of free citric acid (unpublished results). Accordingly, approximately 6% of the citric acid of avocado fruit is present as its *asym*-monoethyl ester. Since the ester is optically inactive, it is possibly formed from citric acid rather than from acetyl-CoA and the half-ester of oxaloacetic acid. The biochemical function of the ester is unknown. Perhaps isolation and identification of the other esters present might elucidate their function.

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BOBBY J. GOUGH and JOHN A. ALFORD
Eastern Utilization Research and Development Division
Agricultural Research Service, United States Department of Agriculture
Beltsville, Maryland 20705

Effect of Curing Agents on the Growth and Survival of Food-Poisoning Strains of Clostridium perfringens

SUMMARY

The effects of different combinations of curing salts (NaCl, $NaNO_3$, $NaNO_2$) and temperature on the growth, survival, and heat resistance of several strains of *Clostridium perfringens* were investigated. These strains were shown to survive and grow in concentrations of curing salts considerably higher than those used in normal curing operations. When used in conjunction with heat, the concentrations of curing salts required to affect cell survival were still well above those usually employed. Hams were pumped with curing brines inoculated with low concentrations of spores of a food-poisoning strain of *C. perfringens*, then cured and smoked according to routine procedures. *C. perfringens* could still be isolated from the hams after cooling.

A simple procedure for preparing reproducible inocula of aged spores is described. These aged spores showed much greater resistance to heat after storage in meat-brine mixtures than did spores from young sporulating cultures.

INTRODUCTION

The role of *Clostridium perfringens* as a causative agent of food poisoning has received increasing attention in the last few years, particularly in the United States and England (Hobbs, 1962; Angelotti *et al.*, 1962). Hall *et al.* (1963), in a study of 83 strains of *C. perfringens* associated with cases of food poisoning in both countries, reported that the spores from the English strains were considerably more heat-resistant. It was suggested that cases of *C. perfringens* food poisoning in England came primarily from foods contaminated prior to cooking, and in the United States from food contaminated after cooking. Barnes *et al.* (1963) reported that there is a greater possibility of *C. perfringens* food poisoning if meat is contaminated prior to cooking.

Studies on the resistance of anaerobic bacteria to curing agents have indicated that *C. botulinum* and putrefactive anaerobes will survive and sometimes grow in the presence of appreciable concentrations of curing agents (Silliker, 1959). An outbreak of food poisoning from salted beef contaminated with *C. perfringens* was reported by Hobbs (1962). She also reported that unpublished data from her laboratory had indicated that vegetative cells of *C. perfringens* would survive six days on raw meat immersed in 22% NaCl brine and that spores would germinate in 5% NaCl. No details of this work were presented, however, and no heat-resistance determinations were made. Studies by Strong *et al.* (1963) have shown a rather high incidence of contamination of fresh meats with *C. perfringens*. Hall and Angelotti (1965) have also shown a high incidence of fresh meat contamination with *C. perfringens*, but found only a small percentage of these to produce heat-resistant spores. These studies, however, do not give any data on the survival of *C. perfringens* in hams.

With increased interest in *C. perfringens* as a cause of food poisoning, the present investigation was undertaken to obtain more definitive data regarding the effects of curing salts on the growth and heat resistance of this microorganism as well as on its survival in a normal curing process.

EXPERIMENTAL PROCEDURES

Preparation of vegetable cell and spore suspensions. When only vegetative cells of *C. perfringens* were required, a culture in the log phase of growth (3–4 hr) from fluid thioglycollate medium (Difco) was used. When a sporulating culture was needed, a 24-hr SEC culture, as described by Angelotti *et al.* (1962), was used. Although the freshly sporulated SEC cultures were adequate for the studies on heat resistance and survival in curing salts, a more stable preparation, primarily containing spores, was desired for inoculation into hams. Spore inocula prepared by heating a culture at 80°C for 5 min were unsatisfactory because this treatment decreased their heat resistance, probably through activation of the spores (Barnes *et al.*, 1963). Attempts failed to separate spores from vegetative cells by the procedures of Sacks

and Alderton (1961), Stewart and Halvorson (1953), and Long and Williams (1958). Strong and Canada (1964) used cultures stored on sterile soil for their spore inocula, and the procedure employed in our study was similar except that filter-paper discs were substituted for soil. Onto each sterile filter-paper disc (Schleicher and Schuell Co., No. 740-E) was placed 0.05 ml of a sporulating culture grown in SEC medium; these were dried over $CaSO_4$ in a desiccator at room temperature. As indicated in Fig. 1, the unheated count (i.e., vegetative cells and any spores not requiring heat shock for germination) of the inoculated discs decreased rapidly during the first day after the culture was added to the disc, then slowly declined. The spore count (heated 80°C, 5 min), however, increased rapidly during the first day, decreased somewhat the second day, and declined very slowly thereafter. If all cells appearing in the unheated count are considered vegetative cells, this means that over 99% of the vegetative cells have lost their viability by the end of two days. Even if they are considered spores not requiring heat shock, they represent less than 5% of the total spore count. Therefore, use of a count on the spore discs after heat shock is a valid indication of total spore population. The discs afforded an easy way of obtaining spore inocula of approximately known concentrations without having to weigh the sample, as is necessary in using soil stocks. To use the discs, one or more were placed in a tube of sterile distilled water and agitated vigorously. The discs disintegrated completely, and the filter-paper pulp could be removed by filtration through sterile cheesecloth with no decrease in spore count.

Plate counts and incubation. Plate counts were made on SPS agar, on which strains of *C. perfringens* associated with food poisoning produce black colonies (Angelotti *et al.*, 1962). All plates and tubes (including thioglycollate tubes) were incubated at 37°C in an anaerobic incubator (National Appliance Co.) flushed four times with a mixture of 90% N_2 and 10% CO_2. All plates were incubated for 48 hr.

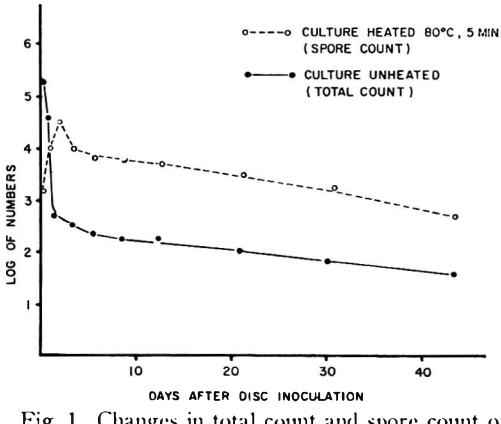

Fig. 1. Changes in total count and spore count of *C. perfringens* No. 15 on filter-paper discs.

Effect of curing salts on growth of C. perfringens in an artificial medium. Fluid thioglycollate medium was prepared containing various concentrations of curing salts. Castellani and Niven (1955) have shown that sterilization of glucose and nitrite together considerably reduced the apparent concentration of nitrite required to inhibit anaerobic growth of *Staphylococcus aureus*. Results were similar in our study with *C. perfringens*. Therefore, the nitrite solutions were sterilized by filtration through membrane filters and added aseptically to the fluid thioglycollate medium. Duplicate thioglycollate tubes containing each of the salt concentrations were inoculated with 0.1 ml of a vegetative cell culture. After 24 hr of incubation, the tubes were examined visually for extent of growth. Table 1 shows that 6% (w/v) NaCl, 10,000 ppm $NaNO_3$, or 400 ppm $NaNO_2$ are required to inhibit the growth of *C. perfringens* significantly.

Survival of C. perfringens in solutions of curing salts. Four solutions were prepared containing the following range of concentrations of curing salts: from 7.5% (w/v) NaCl, 3,700 ppm $NaNO_3$, and 370 ppm $NaNO_2$ to 17% NaCl, 23,000 ppm $NaNO_3$, and 2,300 ppm $NaNO_2$. They were sterilized by filtration, and 15 ml were placed in sterile screw-cap tubes (15 × 150 mm). One-half milliliter of a 24-hr SEC spore culture of each of five strains of *C. perfringens* was inoculated in triplicate into these solutions, and the inoculated solutions were stored at 3°C. (This approximates the temperature at which meats are held while being cured.) One-half-milliliter samples were withdrawn periodically from these tubes and inoculated into fluid thioglycollate medium. Four of the five strains tested survived at least 48 days in all of the stored brine solutions. In a subsequent trial, a spore disc culture of one of the most heat-resistant strains was used. Fifty percent of the spores (original inoculum 30/ml) remained viable for at least 35 days in a brine containing 21.5% NaCl, 1,800 ppm $NaNO_3$, and 1,200 ppm $NaNO_2$. This brine is similar in concentration to that pumped into hams in routine curing operations (Fields and Dunker, 1952).

Additional tests were carried out to determine the effect of curing salts on the heat resistance. In these tests the cultures survived 100°C for at least 30 min in the presence of 6% NaCl, 30,000 ppm $NaNO_3$, or 2,000 ppm $NaNO_2$, used individually. The cells also survived 80°C for at least

Table 1. Growth of 18 strains of *C. perfringens* in various concentrations of NaCl, $NaNO_3$, and $NaNO_2$ contained in thioglycollate medium.

Extent of growth	NaCl (w/v%)			$NaNO_3$ (ppm)			$NaNO_2$ (ppm)		
	4	6	8	8,000	10,000	12,000	300	400	500
Good	14[a]	1	0	18	0	0	18	0	0
Slight	4	8	1	0	11	0	0	13	0
None	0	9	17	0	7	18	0	5	18

[a] Number of strains.

6 hr in a combination of 10% NaCl, 3,000 ppm $NaNO_3$, and 1,000 ppm $NaNO_2$, a combination of heat and curing salts well above that normally encountered in cured meats.

Survival of C. perfringens under simulated curing conditions in the laboratory. Fresh meat was aseptically removed from hams and ground in a sterile grinder, and sterile concentrated solutions of the curing salts were added to give a final concentration of 3% NaCl, 500 ppm $NaNO_3$, and 200 ppm $NaNO_2$. These represent the maximum allowable concentration of $NaNO_2$ as well as the usual maximums of NaCl and $NaNO_3$ found in cured meats. A brine solution without meat was also prepared and placed in several tubes. Spore suspensions, treated as described in Table 2, were inoculated into these meat-brine and brine samples. The inoculated tubes were held at 3°C for 3 days and then heated at 80°C for 60 min. Plate counts were made, and the number of cells of *C. perfringens* type was determined after 48 hr of incubation. The uninoculated meat had an aerobic count of < 50 per gram; no anaerobic bacteria were encountered.

As seen in Table 2, both heated and unheated spores from SEC cultures survive in stored brine solutions better than in meat-brine mixtures, where nutrients are available. It is also evident that spores maintained on filter-paper discs have a much higher percentage of survival than unaged spores from regular SEC cultures. Results were similar with four other strains.

Table 2. Survival of spores of *C. perfringens* in simulated curing procedures.

Inoculum[a]	Substrate[b]	Spore count[c]	% survival[d]
Unheated SEC	Meat + brine	4,500	<0.1
	Brine	2,280	30.0
Unheated disc	Meat + brine	70	39.0
	Brine	850	64.0
Heated SEC	Meat + brine	4,500	<0.1
	Brine	1,800	4.0
Heated disc	Meat + brine	850	0.4
	Brine	850	3.0

[a] Spore culture unheated or heated 80°C, 5 min.
[b] Final concentrations in the ground ham and brine without meat were 3% NaCl, 0.05% $NaNO_3$, and 0.02% $NaNO_2$.
[c] Number of spores added per gram of meat or ml of brine.
[d] After inoculation, held 3 days at 3°C, heated 60 min at 80°C.

Survival of C. perfringens in cured hams. Whole hams were cured and smoked by a procedure similar to Type I of Fields and Dunker (1952). Hams were spray-pumped with a brine containing 17.9% (w/v) NaCl, 1,500 ppm $NaNO_3$, and 980 ppm $NaNO_2$. They were then cured for seven days at 3°C in a cover pickle containing 15.8% NaCl and 1,300 ppm each of $NaNO_3$ and $NaNO_2$. The hams were smoked for 19–20 hr, during which time the internal temperature reached 61°C and remained there for about 1 hr. Twelve hams were pumped to 10% net weight increase with brine inoculated with a low concentration (20/ml) of *C. perfringens* spores prepared from paper discs. At various stages of the curing and smoking process, two hams were removed and a count was made of typical *C.-perfringens*-type colonies. Samples were cut from three locations in the center and bone areas of the hams, and plate counts were made from the meat taken from these areas and from the fluid that drained into the cavity remaining after removal of the meat sample.

As indicated by Table 3, *C. perfringens* consistently survived both curing and smoking processes. The fluid probably contained exudate from the tissue as well as brine remaining in the intermuscular spaces. This fluid consistently had a higher count than the meat samples. In hams in which a heavier inoculum was used (40/ml brine), it was possible to isolate *C. perfringens* from the meat as well as from the fluid after smoking and cooling.

DISCUSSION

Barnes *et al.* (1963) showed that heat-shocking 48-hr spore cultures caused them to lose their heat resistance when subsequently cooled to 10°C within 3 hr in a meat medium. Curran and Pallansch (1963) have also demonstrated incipient germination of *Bacillus* spores at sub-minimal growth temperatures. Our investigation showed a similar loss of resistance on storage at 3°C for 3 days for the heat-shocked cells from both

Table 3. Survival of *C. perfringens* during routine curing procedures.

Phase of process[a]	Treatment of sample			
	Unheated		Heated[b]	
	Fluid[c]	Meat[c]	Fluid[c]	Meat[c]
After pumping	+	+	+	+
After curing	+	+	+	+
Smoked to 30°C[d]	+	−	+	−
Smoked to 45°C[e]	+	±	+	±
After smoking (not cooled)	+	+	+	+
After smoking and cooling	+	+	+	±
Uninoculated controls				
Fresh ham	−	−	−	−
After smoking and curing	−	−	−	−

[a] Two hams were removed for examination at each phase of the process.
[b] Samples heated 5 min at 80°C.
[c] Source of sample.
[d] Required 6 hr in smokehouse to reach 30°C.
[e] Required 13 hr in smokehouse to reach 45°C.

SEC cultures and spore disc cultures inoculated into a meat-brine substrate. However, for the spores that were stored 3 days at 3°C without prior heat shock < 0.1% of the spores from the SEC cultures retained their heat resistance whereas 39% of the spores from the discs were resistant. These data suggest that, while the young spores in the SEC culture had developed heat resistance, their immaturity permitted much more germination at sub-minimal growth temperatures than occurred in the mature spores. Since a mature spore is the type more likely to be encountered as a contaminant from the environment, it would appear that aged spores should be used in studies relating to the survival of *C. perfringens* in foods.

Although there are definite differences in the heat tolerance of different strains of *C. perfringens*, there was little difference in the effect of curing salts on the various strains even at levels well above that found in commercial curing operations. Not only will they survive, but they may actually grow if the suspending medium and temperature are favorable for growth. This ability to survive in concentrated brines indicates the need for proper sanitary precautions in storing commercial brines to be used for pumping hams and cover pickle.

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HOMER W. WALKER and JACK R. MATCHES [a]

Department of Dairy and Food Industry
Iowa State University of Science and Technology, Ames, Iowa 50010

Release of Cellular Constituents During Heat Inactivation of Endospores of Aerobic Bacilli

SUMMARY

Release of dipicolinic acid from spores of some species of *Bacillus* during heating at 100°C was determined. The total amount and rate of release of dipicolinic acid from these spores varied between species and strains, the most resistant spores releasing the least. Studies were made on *Bacillus polymyxa* and *B. megaterium* to determine the influence of buffers and pH on the liberation of dipicolinic acid, carbohydrate, and nitrogen. Release of these materials was usually least at pH 7.0 and greatest under conditions that favored thermal inactivation. Some of the amino acids and carbohydrates released were identified.

INTRODUCTION

When a suspension of bacterial spores is subjected to thermal inactivation, there is a progressive loss of dipicolinic acid (DPA), proteins, and other constituents from the cell (Levinson and Hyatt, 1960; Hunnell and Ordal, 1961; Walker *et al.*, 1961b; El-Bisi *et al.*, 1962). Rode and Foster (1960) reported that a minimal temperature of 70°C was necessary for release of DPA. Levinson and Hyatt (1960) found that pH and constitution of the heating medium influenced the loss of DPA from spores. El-Bisi *et al.* (1962) reported, as did Rode and Foster (1960), that the death of spores progressed at a more rapid rate than release of DPA. The exact relationship between heat inactivation of spores and release of cellular materials remains to be explained.

These studies were undertaken to investigate some of the changes that occur during the thermal destruction of bacterial spores with respect to release of DPA, carbohydrate, and nitrogenous material and also to investigate the influence of suspending buffer and pH on the release of these cell constituents.

MATERIALS AND METHODS

The following organisms were used: *Bacillus polymyxa* IA39; *B. megaterium* IA28, IA34, and IA47; *B. cereus* IA31 and IA54; and *B. subtilis* IA52. These cultures were obtained from the Department of Bacteriology, Iowa State University, Ames. Spores were produced on "G" medium (Stewart and Halvorson, 1953) and on tomato juice agar (T) (Difco). Spores were harvested and cleaned as described by Walker *et al.* (1961a).

Buffered suspensions of spores containing approximately 5.0×10^7 to 1.0×10^8 spores per ml were heated at 100°C. Samples were taken at 3-min intervals for determination of survivors and estimation of the amounts of DPA, carbohydrate, and nitrogen released. Buffers used included McIlvaine's buffer, consisting of disodium phosphate and citric acid, and a buffer consisting of monopotassium phosphate and sodium hydroxide. Both buffer systems were adjusted, as needed, to pH values of 5.5, 7.0, and 8.0. The buffers were 0.05*M* with respect to phosphate ion concentration.

Surviving organisms were determined by plating on a medium consisting of 5 g trypticase, 5 g yeast extract, 5 g sodium chloride, and 20 g agar per liter (Halvorson, 1957). Nitrogen was determined by the micro-Kjeldahl method and carbohydrate by the anthrone method with glucose as a standard (Umbreit *et al.*, 1957). DPA was determined by the method of Janssen *et al.* (1958), and calcium by an adaptation of an EDTA titration method developed by Bird *et al.* (1961). Dry weight of spores was determined by drying a measured quantity of spore suspension to a constant weight at 55°C. A procedure described by Mizell and Simpson (1961) was used for qualitative two-dimensional paper chromatographic analysis for amino acids. A mixture of amino acids was spotted on Whatman No. 1 filter paper and subjected to descending development in the first dimension with *n*-butanol–acetic acid–water (25:6:25 by vol.) and in the second dimension with *n*-butanol-methyl ethlyl ketone–water (2:2:1 by vol.). A beaker of cyclohexylamine (1 ml for every 25 ml of solvent mixture) was placed in the chamber to provide cyclohexylamine vapors. Some of the carbohydrate constituents were identified tentatively by paper chromatographic techniques with butanol-pyridine-water (1:1:1) used as the solvent system (Hough, 1959). After separation of the components, the papers were dipped in 0.5% triphenyltetrazolium chloride dissolved in chloroform. After drying, the papers were sprayed with 0.5*N* NaOH

[a] Present address: College of Fisheries, University of Washington, Seattle.

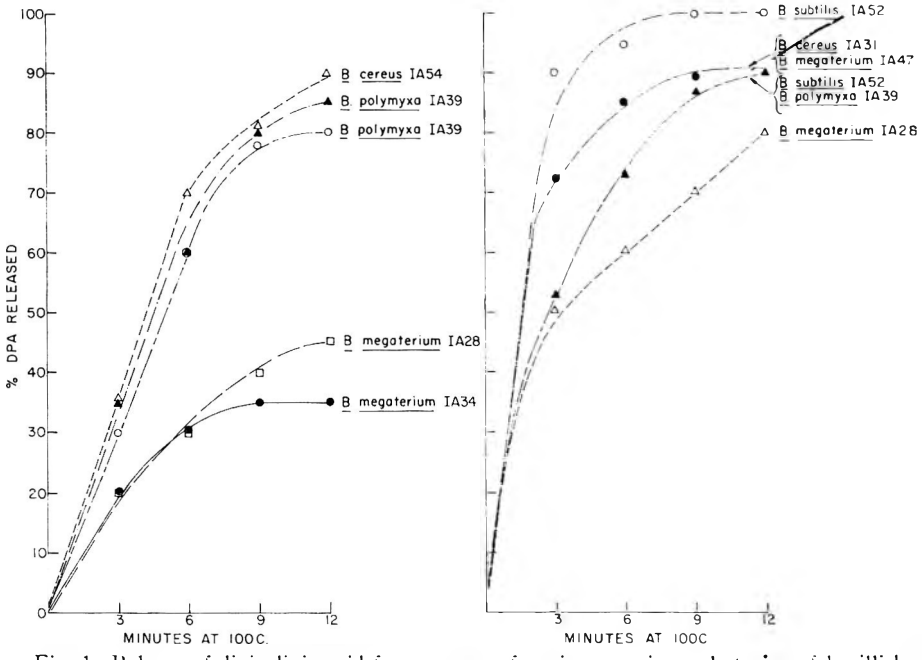

Fig. 1. Release of dipicolinic acid from spores of various species and strains of bacilli heated at 100°C in KH_2PO_4-NaOH buffer, pH 7.0.

and heated 5 min at 105°C. An ammoniacal solution of silver nitrate was used as the spray reagent for some samples. Samples were hydrolyzed by autoclaving 2 hr at 121°C in 1*N* HCl.

RESULTS AND DISCUSSION

The rate of release of dipicolinic acid (DPA) from spores of several species and strains of *Bacillus* upon heating at 100°C is shown in Fig. 1. Those instances in which the same strain is shown twice indicate different batches of spores. The ratio of DPA to calcium, the release of DPA and the relative heat resistance of these same batches of spores are shown in Table 1. The heat resistance of the various crops of spores was compared by the *D* or *Z* value as calculated by Stumbo (1948). This value was derived by the following formula: $D = U/(\log a - \log b)$. In this equation, *U* is the time of heating in minutes; *a*, the initial number of spores; *b*, the number of cells remaining after heating time *U*; and *D*, the number of minutes required for the rate of destruction curve to traverse one log cycle. The calculations were made for the period between 3 min and 15 min because the thermal destruction curves were essentially straight lines during these 12 min.

Sometimes during the first 3 to 6 min, an initial drop in the surviving population was observed which theoretically could be attributed to the destruction of spores relatively more sensitive to heat than the major portion of the population either because of their inherent properties or because of germination. Little or no detectable germination had occurred in the suspensions before heating since the spores appeared refractile when examined under the dark-phase microscope and did not stain with methylene blue. Also, in some samples, this initial period was characterized by a plateau or shoulder when plotted. This type of reaction could be attributed to a period during which heat activation occurred as well as thermal destruction or to the breaking up of clumps of spores. Little clumping was observed microscopically. These variations can be attributed, in part, to population heterogeneity. The majority of the population, however, was represented by a straight line between 3 and 15 min and the calculated *D* values would better represent the population than if the zero time values were included. Examples of the types of curves that were obtained can be seen in Figs. 2 and 3.

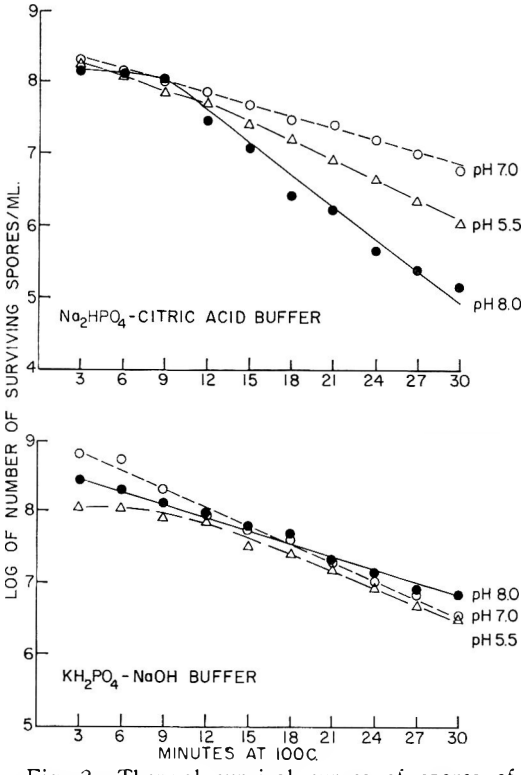

Fig. 2. Thermal survival curves of spores of *Bacillus megaterium* IA28 in buffers adjusted to pH values of 5.5, 7.0, and 8.0.

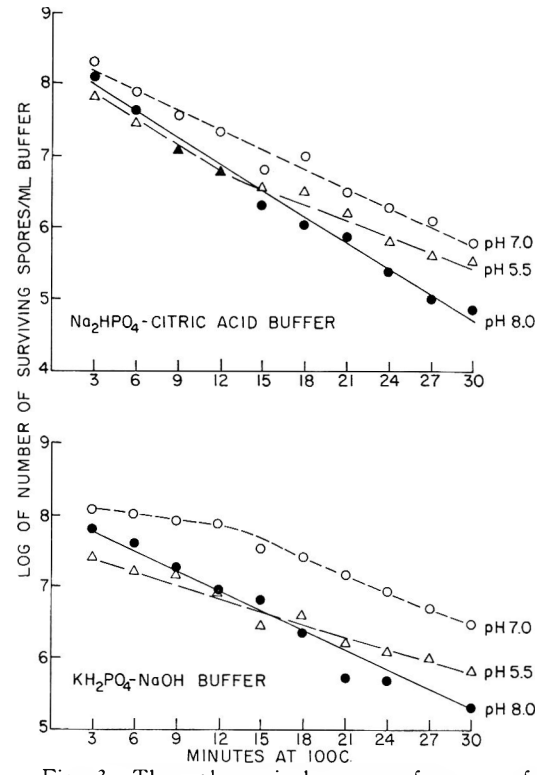

Fig. 3. Thermal survival curves of spores of *Bacillus polymyxa* IA30 in buffers adjusted to pH values of 5.5, 7.0, and 8.0.

The calcium-DPA molar ratios reported in Table 1 tend to support the findings of Fleming and Ordal (1964) that a calcium-DPA molar ratio above one is indicative of an elevated heat resistance and that a ratio of less than one is characteristic of low resistance. On the other hand, however, Murrell and Warth (1965) have shown that *Bacillus stearothermophilus* and *B. coagulans* have very high *D* values and also have calcium-DPA ratios of less than one, which would not support this generalization. Perhaps, comparisons cannot or should not be made between those organisms with a moderate range in heat resistance and thermophilic, highly heat resistant organisms such as *B. stearothermophilus*. A study of these relationships in some of the thermophilic sporeformers might demonstrate that other factors have equal or greater influence in these organisms than the apparent relationship between calcium-DPA ratio and heat resistance.

A comparison of the *D* values found in our work with those reported by Murrell and Warth (1965) show some large differences. Some of the variations can be explained on the basis of strain of organism, temperature of incubation, and medium for sporulation. In our work, similar variations were observed but none of the values were as low or as high as those reported by them. However, Table 1 shows that when spores of *B. megaterium* IA28 were produced on "G" medium, on two different occasions there was a three-fold difference in resistance. With *B. polymyxa*, on three occasions *D* values of 8.2, 6.4, and 3.9 were obtained. No satisfactory explanation can be proposed since, theoretically, the conditions were identical in all instances. On the basis of these observations, the unsubstantiated conclusion can be made that uncontrolled and unrecognized fluctuations in environment and trace nutrients during sporulation are responsible for these modifications in resistance.

Table 1. Comparison of relative heat resistance, dipicolinic acid and calcium content, and release of dipicolinic acid from bacterial spores heated at 100°C in monopotassium phosphate-sodium hydroxide buffer, pH 7.0.

Organism		Sporulation[a] medium	D[b]	DPA (mM/100 mg)[c]	Ca	Ratio Ca/DPA	% total DPA released 3 min	12 min
B. megaterium	IA34	G	12.9	.079	.093	1.19	20	35
B. megaterium	IA28	G	12.9	.054	.060	1.11	20	45
B. polymyxa	IA39	G	8.2	.054	.060	1.11	30	80
B. cereus	IA54	T	7.2	.058	.065	1.12	35	90
B. polymyxa	IA39	G	6.4	.048	.048	1.00	35	85
B. megaterium	IA47	T	5.8	.061	.040	0.66	70	90
B. cereus	IA31	T	4.8	.061	.058	0.95	75	90
B. subtilis	IA52	T	4.4	.056	.045	0.80	50	100
B. megaterium	IA28	G	4.3	.056	.048	0.86	50	80
B. subtilis	IA52	T	4.1	.058	.054	0.93	50	95
B. polymyxa	IA39	G	3.9	.076	.060	0.79	55	90

[a] See Materials and Methods for identification.
[b] D is the number of minutes required for the heat survival curve to traverse one log cycle; see text.
[c] These data are based on dry weight of the spores.

Table 1 and Fig. 1 show that spores with a high D value and a calcium-DPA ratio of one or more seem to release DPA more slowly than cells with a low D value and calcium-DPA ratio of less than one. Fig. 1 represents the variation in rate of release of DPA from the different crops of spores. Although certain levels of calcium and DPA are necessary to give heat stability to the spore, the ratios in which calcium, DPA, and magnesium occur seem to reflect more closely variations in heat resistance than the percentage of these compounds in the spore in terms of dry weight (Walker *et al.*, 1961a). Murrell and Warth (1965) similarly concluded that the magnesium-calcium ratio was very significantly related to heat resistance.

On the basis of these observations, further studies were made to determine the influence of buffers and pH on liberation of DPA, proteinaceous material, and carbohydrate. The spores for these experiments were produced in "G" medium but were different crops from those discussed in Fig. 1 and Table 1. This may partly explain the D value of approximately 30 for *B. polymyxa* spores in KH_2PO_4-NaOH buffer (Fig. 3), which is about 2½ times as high as the highest values shown in Table 1. These differences again emphasize the influence of undetected alteration in environmental and nutritional factors on heat resistance of spores. Figs. 2 and 3 show the rate of kill of spores of these organisms in KH_2PO_4-NaOH buffer and in Na_2HPO_4-citric acid buffer; destruction was usually greater in the buffer containing citric acid.

Fig. 4 shows the rate of release of DPA from *B. megaterium* and *B. polymyxa*. A comparison of these curves with the survival curves (Figs. 2, 3) suggests that the freeing of DPA and other material lagged behind inactivation of the spore. This conclusion is based on the observation that the percentage of spores killed in a given time was greater than the percentage of total DPA, carbohydrate, or nitrogen released in the same period. These data support the contention of Rode and Foster (1960) and El Bisi *et al.* (1962) that loss of viability usually exceeds the rate of release of DPA indicating that cells die first. The release of DPA was least at pH 7.0, which was reflected in the survival rate at this pH. In addition, the liberation of DPA was usually greatest in the presence of citric acid, which has been associated with reduced thermal survival of bacterial spores (Walker, 1964). Wooley and Collier (1965) observed that during the germination process of *Clostridium roseum*, thermoresistance decreased rap-

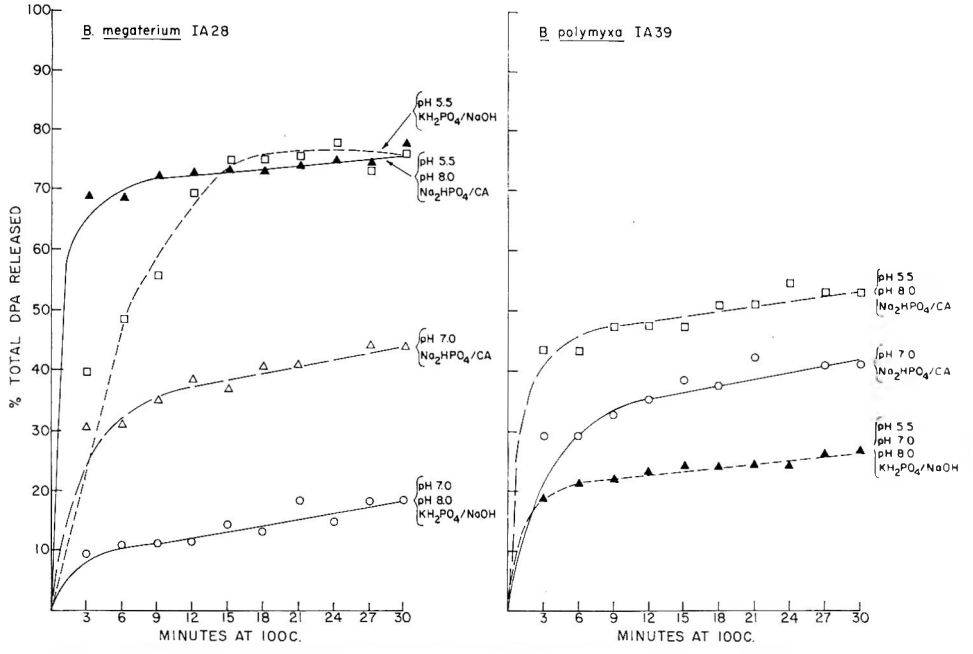

Fig. 4. Release of dipicolinic acid from spores of *Bacillus megaterium* IA28 and *B. polymyxa* IA39 heated in buffers adjusted to pH values of 5.5, 7.0 and 8.0. CA refers to citric acid.

idly after heat activation of the spores and that this decrease preceded the loss of both calcium and DPA. They suggested that upon heat activation and germination, cellular organization is altered in some manner with the subsequent and almost immediate loss of thermoresistance followed by the release of the calcium and DPA. Apparently heat inactivation parallels at least to some degree, the process that occurs during germination and loss of heat resistance; as was pointed out previously, heating first causes death of the spore and then causes release of DPA. On the basis of these observations and those of Rode and Foster (1960), El Bisi *et al.* (1962), and Riemann (1957), it seems likely that the release of DPA is a secondary reaction reflecting a change in cellular organization which was primarily responsible for inactivation of the spore.

Figs. 5 and 6 show a rapid initial release of carbohydrate and nitrogenous materials. The extent and, to some degree, the rate of release of these materials were dependent on pH and buffer system used. As with DPA, release of nitrogenous materials from the spore was usually lower at pH 7.0 than at either pH 5.5 or 8.0. Similar comparisons for carbohydrate release (Fig. 6) showed that the amount of carbohydrate freed was

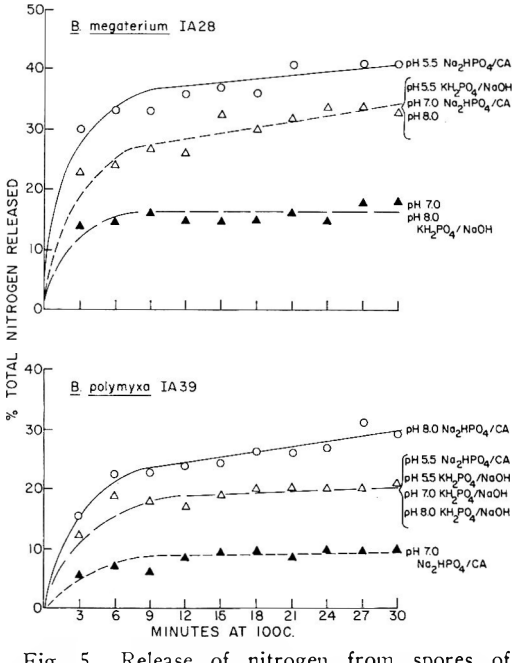

Fig. 5. Release of nitrogen from spores of *Bacillus megaterium* IA28 and *B. polymyxa* IA39 heated in buffers adjusted to pH values of 5.5, 7.0, and 8.0. CA refers to citric acid.

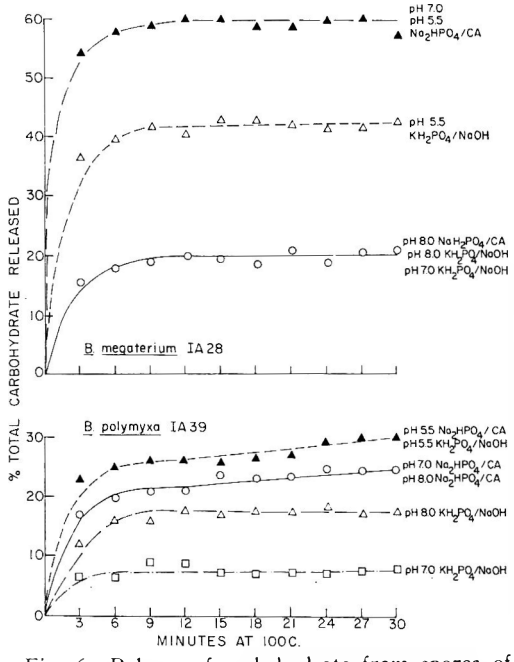

Fig. 6. Release of carbohydrate from spores of *Bacillus megaterium* IA28 and *B. polymyxa* IA39 heated in buffers adjusted to pH values of 5.5, 7.0, and 8.0. CA refers to citric acid.

usually greater at pH 5.5 than at pH 7.0 or pH 8.0. The type of protein or carbohydrate released might be of more significance than the total amount. In future studies, it would be useful to identify as well as quantify these compounds as they are released. The use of several temperatures, including both lethal and non-lethal, might reveal some of those compounds which can or cannot be released without damage to the stable structure of the spore and resistance of the cell.

Some chromatographic analyses were made of the carbohydrate and nitrogenous constituents liberated from the spore during heating. Chromatograms were made from five spore preparations; both unhydrolyzed and hydrolyzed samples were examined. Only a few amino acids were detected in the unhydrolyzed samples, suggesting that these nitrogenous compounds occurred mainly as peptides. Strange and Powell (1954) and Powell (1957) found that exudates of germinating spores contained peptides as well as amino acids and hexosamines. In both *B. megaterium* and *B. polymyxa*, in the unhydrolyzed samples, lysine, arginine, asparagine and alpha-alanine were found. In the acid-hydrolyzed samples these same amino compounds were found plus glycine, aspartic acid, proline, phenylalanine, serine, tyrosine, methionine and leucine. Other nitrogenous materials were probably present but no analyses and identification were made of several spots that appeared near the origin of the chromatograms. The spot corresponding to serine was detected on one occasion when an unusually large sample was applied; serine is partially destroyed by acid hydrolysis and was probably present in only very small amounts in the sample added to the paper. In every case, large quantities of aspartic acid were detected. Young (1959) found arginine, glutamine, serine, alanine, aspartic acid, and glutamic acid when she broke open the spores mechanically and extracted in boiling water for 15 min. Apparently mechanical damage to the spore enables release of additional amino compounds, which supports the observation that a change in spore structure affects the lability of spore constituents. Young suggested that the DPA found in the spore might combine with the amino acids or peptides in a manner which could provide protective stabilization for essential proteins and/or nucleic acids.

The following carbohydrate compounds were tentatively identified: ribose, galactose, glucose, galactosamine, and glucosamine. Other spots were observed but did not correspond to any of the standards used. Murrell and Warth (1965) could find no correlation between hexosamine content and heat resistance. Previously, Berger and Marr (1960) had found hexosamine located in the exosporium. They reported that spores which had been stripped of their exosporia are still viable and heat resistant. In our work, no evidence is presented to show a relationship of carbohydrate to heat resistance other than that release is least under conditions which favor survival of the spore. This release of carbohydrate could reflect a change in permeability of the spore structure which precedes the death of the spore.

When spores are subjected to lethal heat treatment, several stages of breakdown prob-

ably occur. For example, Hunnell and Ordal (1961) observed that in a suspension of spores heated almost to the sterilization point, some spores apparently were unaffected by the treatment, others seemingly lost their cortical material, and others appeared as empty spore coats. The carbohydrate, nitrogen, and DPA released during the initial stages of heating could easily have originated in the spore coat and the structures associated with it and in the cortex; however, more knowledge is needed of the chemical composition of various structures of the spore before any reliable conclusions can be drawn. Spore coats are known to contain amino acids and carbohydrate (Salton and Marshall, 1959; Hunnell and Ordal, 1961). The distribution of DPA and the occurrence of DPA conjugates in the spore have yet to be substantiated, but it is possible that several forms or states of DPA exist in the spore and are located in the cytoplasm or cortex rather than in the spore coat or exosporium (Halvorson and Howitt, 1961). Bailey *et al.* (1965) have presented ultraviolet absorption spectra of dry bacterial spores that suggest that DPA does exist largely as a calcium chelate in the spore. Windle and Sacks (1963) have found evidence for bonding of copper with protein and for two different types of bonding for manganese in spores, which could lead to speculation on the role of various metallic cations on structure and stability of the spore. The presence, even in small quantities, or absence of such cations might also explain some of the variation observed in different batches of spores. Knowledge of the distribution of these materials in the spore and the order of release of materials from the spore during heating would enable formulation of some ideas concerning the sequence of degradative changes which cause inactivation of the spore.

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F. WESLEY, B. ROURKE, and O. DARBISHIRE
Crosse & Blackwell Ltd., London, England

The Formation of Persistent Toxic Chlorohydrins in Foodstuffs by Fumigation with Ethylene Oxide and with Propylene Oxide

SUMMARY

Under conditions for effective fumigation with ethylene oxide or propylene oxide these reagents can combine not only with moisture but also with chlorine from the natural inorganic chloride content of foodstuffs, whereby the corresponding chlorohydrins are formed. Concentrations of ethylene chlorohydrin up to about 1000 ppm were found in whole spices and ground spice mixtures after commercial fumigation with ethylene oxide. These chlorohydrins are very toxic substances by all accounts; they also are sufficiently involatile and unreactive chemically to be persistent under food processing conditions.

INTRODUCTION

Fumigation with ethylene oxide is now being used extensively for sterilizing materials ranging from medical instruments to foodstuffs, such as spices, cocoa, flour, dried egg powder, desiccated coconut, dried fruits, and dehydrated vegetables. Propylene oxide also has recently been advocated for this purpose. These epoxides, besides being capable of eliminating or drastically diminishing the numbers of viable organisms present, possess other properties that have commended them for the fumigation of foodstuffs. The most advantageous of these is that neither epoxide, appropriately used, imparts any after-odor or flavor taint to foods by direct effect or as a consequence of the residues which result.

Residues are produced by two reactions of epoxides, i.e., their slow chemical combination with the elements of water to form the corresponding glycols, and their combination with the elements of hydrochloric acid to form the corresponding chlorohydrins. In published descriptions of these fumigation processes, e.g., Rauscher *et al.* (1957), only glycols have been reported to result as residues in foodstuffs, and we have found no statement that chlorohydrins are formed by such treatments. It is apparent that glycol formation will occur unavoidably, because all nominally dry materials contain moisture. Moreover, without the presence of some moisture, sterilization cannot be effected in this way. Such residues of glycols are, however, regarded as harmless or tolerable. It has not been apparent that chlorohydrins could be formed during fumigation in materials which do not contain free hydrochloric acid. Our object is therefore to show, by reference to established epoxide chemistry and the results of our investigations, that, if chloride is present, this reaction can and will occur, creating in the fumigated foodstuffs objectionable residues that are persistent under food processing conditions.

LITERATURE REVIEW

More than 30 years ago, Cotton and Roark (1928) found that ethylene oxide was effective as a fumigant against insects infesting furniture and foodstuffs. Later, Cotton and Young (1929) found that added carbon dioxide could increase its insecticidal efficiency. In the next ten years came recognition that ethylene oxide was also effective for destroying bacteria, and toward the end of that period patents were granted to Gross and Dixon (1937) and to Griffith and Hall (1938) concerning the use of ethylene oxide in processes for sterilizing foodstuffs. At that time such ideas apparently attracted little interest, but since then its effectiveness has been amply demonstrated by many investigators and its use in this connection is now being fairly widely exploited in industrial fumigation processes.

Regarding the relevant chemical reactions of epoxides, it has long been known that ethylene oxide possessed pseudo-basic activity, i.e., although not alkaline in solution it could effectively neutralize the free acid produced by hydrolysis of ferric chloride, causing ferric hydroxide to precipitate.

The first to study the physicochemical aspects of this reaction between ethylene oxide and hydrochloric acid were Hantzsch and Hibbert (1907), who reported that it went with considerable velocity initially, but stopped before all acid was removed. Henry (1907) also showed that oxides such as ethylene oxide, in the presence of acids, reacted with water to form glycols. Little more than this was known about either reaction until Brönsted *et al.* (1929) reported on the kinetics and reaction mechanisms of some simple epoxides, in-

cluding ethylene oxide, toward both water and the ions of various acids. Their work has shown that, with water alone, combination occurs only at a very slow rate, but, because this process is accelerated by hydrogen-ion catalysis, some acid must be present for glycol formation to proceed rapidly. Contrasting with this, they have shown that pseudo-basic activity is not dependent on the presence of free acid produced by the hydrolysis of a salt and can occur even in solutions that are somewhat alkaline. Thus, ethylene oxide can withdraw some HCl from a neutral solution of KCl and at a rate initially many orders of magnitude faster than would be possible if combination were occurring by a simple addition process, dependent on concentrations of the respective cations and anions available. As HCl is removed in this way the solution becomes correspondingly alkaline and the process slows and finally stops because the products, ethylene chlorohydrin and potassium hydroxide, can react mutually and reversibly. However, if the alkalinity is limited in some way to a value not higher than about pH 8.5, this state of equilibrium will not be reached and the reaction can go forward to completion.

Regarding available information on the toxicology of the chlorohydrins, it is apparent that although these substances are not officially scheduled poisons, they are nevertheless very toxic. Ethylene chlorohydrin in particular has caused several industrial fatalities. Two such cases were reported by Koelsch (1927), from Germany, one in a paper factory and the other in a linoleum factory. Middleton (1930) has reported on a similar case that occurred in England at a dye works. Turning to more recent information on ethylene chlorohydrin, the following is abstracted from a Royal Institute of Chemistry (1960) publication: "2-chloroethanol . . . Extremely harmful vapour . . . Harmful by skin absorption . . . Avoid breathing vapour. Prevent contact with skin and eyes . . . The vapour causes nausea, headaches, vomiting, stupefaction and unconsciousness. It irritates the mucous membranes. The liquid is rapidly absorbed by the skin, producing similar effects to inhalation. Assumed to be extremely poisonous if taken by mouth." The maximum allowable concentration (MAC), defined as . . . "the concentration of vapour expressed in parts per million of air per eight-hour day of continuous exposure," is given as 5 ppm.

Browning (1953) presented similar information, in much greater detail and from a variety of sources, concerning the nervous and metabolic effects of ethylene chlorohydrin poisoning, citing Goldblatt and Chiesman (1944) for evidence of possible cumulative action and their suggestion of a still lower MAC, i.e., 2 ppm. The lethal dose, LD_{50}, for animals by oral administration is given as 7.2 mg per 100 g. The Merck Index (1960) summarizes similar information, but is even more cautious about the MAC, which is given as probably below 1 ppm.

Very little information is available on the respective toxicities of the two isomeric propylene chlorohydrins. The Merck Index (1960) gives for 2-chloropropyl alcohol . . . "Caution: May be fatal if inhaled, swallowed, or absorbed through the skin." For 1-chloroisopropyl alcohol the same caution is implied.

The relatively high toxicities of these chlorohydrins contrast with the corresponding glycols, which, as already mentioned, are harmless or tolerable as residues. Spector (1955) gives 8348 mg/kg as the lethal dose, LD_{50}, of ethylene glycol for mice by oral administration. On this evidence, ethylene chlorohydrin may be regarded as at least 100 times as toxic as ethylene glycol.

Regarding the stability of chlorohydrins at elevated temperature, Browning (1953) cited information given by Koelsch (1927) that ethylene chlorohydrin decomposes into ethylene glycol and acetaldehyde when heated to 100°C with water. From this it might be assumed that, simply by cooking foodstuffs sufficiently, the residues of chlorohydrins resulting from fumigation would be converted to unobjectionable decomposition products. Such an assumption is contraindicated by the evidence of investigations we have made in this connection.

EXPERIMENTAL METHODS

Samples were fumigated with ethylene oxide or propylene oxide on a small scale in the laboratory by placing them in a closed vessel from which the air could be evacuated and exchanged for vapor of the appropriate epoxide at known pressure, as shown on a manometer. Alternatively, certain materials were simply suspended centrally within a 2-L conical flask containing about 10 g of precooled liquid ethylene oxide or propylene oxide, whereby exposure to action of the vapor occurred as the liquid evaporated. Unless otherwise stated, all samples remained in these vessels overnight.

Commercial fumigation of samples on a larger scale was undertaken by an independent specialist company using a mixture of 10% carbon dioxide and 90% ethylene oxide. Initially the treatments given were the following: 750 ml per cubic meter for 5 hr, 250 ml per cubic meter for 18 hr, 1000 ml per cubic meter for 5 hr, and 600 ml per cubic meter for 18 hr. Subsequently, increases were made to 1500 ml per cubic meter for 5 hr, and 1200 ml per cubic meter for 16 hr.

Investigation concerning the loss of chlorohydrins that might occur by volatilization and decomposition at elevated temperatures was conducted on the following lines: In one experiment, a curry powder known to have been commercially fumigated with ethylene oxide and found to contain 900 ppm of ethylene chlorohydrin, was used in preparing a curry sauce composed of curry powder, 12.5 g; flour, 20 g; and water, 467.5 ml. The flour was mixed to a slurry with a little of the water, the curry powder was mixed with the remaining water and heated to boiling, whereupon the flour slurry was added to thicken it and the whole was then brought again to boiling, which was continued for a further 10 min. A sample of this sauce was subsequently analyzed for residual ethylene chlorohydrin. In another experiment, some of this curry powder was used in making a trial batch of meat curry soup, which was sealed into 10-oz cans and then autoclaved for 70 min at 246°F and finally analyzed for residual ethylene chlorohydrin. This latter experiment was also repeated, using a soup containing the two propylene chlorohydrins, totaling approximately the same initial concentration.

Samples were examined for chlorohydrin residues both chemically and by gas chromatography. The chemical method used was as follows: A weight of sample, generally between 20 and 100 g according to the probable content of chlorohydrin, was mixed to a slurry with water and steam-distilled until 150 ml of distillate was collected. The distillate was made alkaline by the addition of approximately 5 ml of 20% sodium hydroxide solution and heated 30 min on a steam bath to hydrolyze the chlorohydrin. The solution was then acidified with nitric acid and the chloride determined by the Volhard method, using 5 ml of .05*N* silver nitrate and back-titrating with .05*N* thiocyanate. Blank determinations were made on the reagents and on a small aliquot of the distillate before hydrolysis.

For materials with a content of chlorohydrin below 10 ppm the chemical method of estimation, although applicable with modification, is inconvenient because of the larger weight of sample required and the need for evaporating the distillate after alkaline hydrolysis to a volume suitable for the titration. Gas chromatography is altogether preferable in these circumstances and we have relied on it for all estimates ranging from 1 to 10 ppm. The apparatus used was essentially similar to that described by Smith (1960). The column had a bore of ⅛ in. and a length of 5 ft. The packing was Celite impregnated with 10% polyethylene glycol 1540. Operation was isothermal at 100°C. The carrier gas was hydrogen containing 25% nitrogen, moistened to saturated humidity at room temperature and used at a flow rate of 30 ml per min. Detection was by flame ionization. Volatiles were steam-distilled from the samples and injected as aqueous solutions ranging from 0.3 to 5 μl.

RESULTS AND DISCUSSION

Our first realization that another substance besides ethylene glycol could be formed during this commercial fumigation came as a consequence of using gas chromatography to compare the volatiles of ground mixed spice samples taken before and after treatment. Initially, the comparison was made only to ascertain whether ethylene oxide could be detected in these materials after fumigation. None was found, but instead there was an extra peak, due to some new component intermediate in volatility between ethylene oxide and ethylene glycol, which appeared in the chromatograms of all treated materials. In every other respect, chromatograms of samples before and after treatment were essentially similar. From this observation it was deduced that the extra component was not a reaction product of the fumigant with some volatile constituent of these spices. Further batches of ground mixed spices were then fumigated, the combinations of fumigant concentration and exposure time being 1500 ml per cubic meter for 5 hr, and 1200 ml per cubic meter for 16 hr, as previously mentioned. The same additional peak, much increased in area, continued to appear in the chromatograms of all treated material, but at this stage it was noticed also that the color of these spice mixtures was perceptibly different after being fumigated, although odor and flavor remained unaffected. Investigation showed that a change was occurring in pH from 6.05 initially to 6.30 after treatment, which could account for the color difference. Thus, interest became centered on the nature of chemical change resulting from fumigation and the problem of identifying this extra component.

After finding that the unknown substance, at very low concentration but devoid of other volatile matter, could be detected in a sample of cotton wool fumigated concurrently with a batch of ground mixed spices, a sample of the fumigant was next obtained and examined for impurities. However, nothing was found in it corresponding to the un-

known substance. Finally, by having a sufficient quantity of this cotton wool commercially fumigated, it was possible to extract and concentrate enough of the unknown substance for its provisional recognition as ethylene chlorohydrin on evidence of chemical tests and gas chromatography, e.g., it gave a peak congruent with that obtained from ethylene chlorohydrin, and when made alkaline with caustic soda a distillate could be obtained from it which gave a peak congruent with that of ethylene oxide, leaving a residue which then gave the reactions for chloride. From this basis a tentative estimate could be made of ethylene chlorohydrin concentration in these fumigated materials, both by the chemical method described and by gas chromatography, using prepared standards for comparison. Further investigation and reference to the chemical reactions of epoxides then put the manner of its formation and identity beyond reasonable doubt.

It was realized that, for ethylene chlorohydrin to be formed under these conditions in a nominally dry material, the hydrogen could be derived from moisture present as in glycol formation, while the chlorine would come from trace amounts or more of naturally occuring chlorides. Analysis showed that in a sample of this cotton wool before fumigation there was the equivalent of 50 ppm of sodium chloride, all of which had reacted during the treatment. Additional evidence on the readiness and extent of chlorohydrin formation in a nominally dry inert material containing more than a trace of salt was obtained from universal pH-indicating test papers, dipped to half length in a dilute salt solution and then dried. These, upon exposure to the vapor of ethylene oxide or propylene oxide, showed within about 15 min a color change, indicating development of alkalinity confined to the chloride-containing part of the paper. Similarly, filter papers were weighed, then soaked in a dilute solution of salt, dried and reweighed. These prepared papers were exposed to vapor of the appropriate epoxide for 3 hr. Estimation of their chlorohydrin content, by the chemical method described, showed 380 ppm present when ethylene oxide was used, and a total of 550 ppm for the two possible isomers when propylene oxide was used. From the weight of salt known to be present in these papers initially, it was found that only 0.15 and 0.19%, respectively, had reacted. The reaction, under these conditions, cannot go to completion, and stops in equilibrium because of the alkalinity which results.

Although in commercial processes these fumigants would be applied at lower partial pressures than probably obtained in these experiments, the concentration of ethylene chlorohydrin formed in foodstuffs can reach much higher levels, as our analyses of materials thus treated have shown. One difference, theoretically capable of contributing some effect in this direction, is that in commercial practice ethylene oxide is not used alone, but admixed with carbon dioxide. The latter, by its ability to convert strongly alkaline hydroxides to the milder carbonated compounds, could shift this equilibrium in favor of chlorohydrin formation. More important, probably, is the fact that foodstuffs are not inert materials, like paper. They contain proteins, amino acids, salts of organic and inorganic acids, etc., which by buffer action could effectively neutralize much of this alkalinity as produced, thereby enabling the reaction to proceed more readily and extensively. Salt is also invariably present, and its concentration will seldom be so low that chloride availability would limit the amount of chlorohydrin that could be formed in these circumstances. Consequently, other factors, such as duration of exposure to the fumigant, its concentration, the permeability of materials to ethylene oxide, their moisture content and temperature, determine the extent to which this reaction can go.

In a series of commercially fumigated ground spice mixtures, treated as previously mentioned, ethylene chlorohydrin concentrations have been found ranging from 490 ppm, among the earlier samples, to 1030 ppm, found subsequently. The average of 34 such samples tested was 805 ppm. In two samples of whole spices, i.e., cumin seeds from India and from Iran, we have found ethylene chlorohydrin, its concentration in one being as high as 980 ppm. Both samples of cumin seeds came from stocks fumigated abroad,

the particulars of treatment not being known. None of the materials in this series had more than their natural chloride content, which for our ground spice mixtures and for cumin seed can be taken as equivalent to 0.5% and 0.9% NaCl, respectively.

Since finding this chlorohydrin at such high concentrations in spices, we have been looking for it in other foodstuffs, including some fumigated expressly for this investigation. The following results were obtained from a range of materials commercially fumigated with ethylene oxide for 5 hr at a concentration of 750 ml per cubic meter.

Description of sample	Chlorohydrin found (ppm)	Initial Cl in sample (% NaCl)
Flour	260	0.24
Desiccated coconut	42	0.52
Currants	4	0.57
Spray-dried albumen	310	2.10
Sliced freeze-dried French beans	8	0.39
Whole air-dried green peas	1	0.14
Ground air-dried green peas	36	0.14

Stability and persistence of chlorohydrins in foodstuffs. Regarding our investigations concerning the prospects for eliminating residues of chlorohydrins from foodstuffs by decomposition at elevated temperatures under food processing conditions and by volatilization during cooking, the following summarizes the results obtained: Canned soup with 6.8 ppm concentration of ethylene chlorohydrin initially present, still contained 5.1 ppm after 70 min of autoclaving at 246°F. Results were similar with soup in which a mixture of the two propylene chlorohydrins, totaling approximately the same concentration, was present initially. Thus, under conditions precluding volatilization, little or no loss of chlorohydrins occurs through decomposition, despite a drastic combination of time and temperature. There remains the possibility of effecting some elimination when foodstuffs are cooked under conditions which allow loss of chlorohydrins by volatilization, as in preparing the curry sauce previously mentioned. Nevertheless, after a much longer period of boiling than culinary needs would dictate, the final concentration of ethylene chlorohydrin was 11 ppm, this being about half the amount present initially.

To conclude, it seems appropriate to compare the circumstances now obtaining as a consequence of our findings in this connection, with those which earlier followed the discovery that an objectionable substance was formed in flour by treatment with nitrogen trichloride. It will be recalled that the treatment of flour with Agene was widely practiced until it became known that a wholesome and beneficial amino acid constituent was being converted to a substance exceedingly toxic to dogs. Thereafter the process was discontinued and flour so treated was regarded as potentially harmful to humans, even though it was being consumed by them without any apparent harmful effects. Similarly, the fumigation of foodstuffs with these epoxides is now fairly widely practiced, and this too results in the formation of known toxic substances from another natural and wholesome food constituent. It may well be that these chlorohydrins are not demonstrably harmful to humans in the quantities that might be ingested with a normal dietary intake of such fumigated foodstuffs. It now becomes a matter for the proponents of this treatment and others to show whether this is so and establish tolerance levels for chlorohydrins in foods. Meanwhile, it would seem that a case exists for proposing that fumigation of foodstuffs with these agents should be discontinued forthwith.

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B. R. STILLINGS and L. R. HACKLER

Department of Food Science and Technology, New York State Agricultural Experiment Station Cornell University, Geneva, New York 14456

Amino Acid Studies on the Effect of Fermentation Time and Heat-Processing of Tempeh

SUMMARY

In general, amino acids as analyzed by column chromatography declined slightly in tempeh fermented up to 72 hr, and these declines were partially attributable to amino acid deficiencies in the fermentative mold. Tryptophan declined in the dehulled lactic-acid-soaked soybeans, increased in 24-hr-fermented tempeh, and decreased slightly as the fermentation continued to 72 hr. Free amino acids and ammonia increased during fermentation. Glucosamine was isolated from the mold and subsequently identified. An increase in glucosamine in tempeh during fermentation reflected large amounts in the mold. Deep-fat frying of tempeh caused some amino acids to decrease after 5 min, while most declined after 7 min. Lysine and cystine were most susceptible to heat destruction. Steaming of tempeh for 2 hr or less had no effect on the amino acids. Changes in amino acid indexes during fermentation and heat-processing were related and, in some instances, significantly correlated with animal data.

INTRODUCTION

Soybeans provide an important source of protein for people in many parts of the world. Before being consumed, the protein in soybeans may be subjected to a variety of processing conditions which may ultimately affect nutritional value. The process of fermentation has been used to produce numerous products from soybeans (Platt, 1964), and one product which serves as an important source of protein, especially in certain Asian diets, is tempeh. The effects of fermentation and subsequent cooking procedures on nutritional value deserve special attention, since these products often constitute the principal source of dietary protein. The nutritive value of tempeh has been found to decline with increased fermentation time (Smith *et al.*, 1964; Hackler *et al.*, 1964). Hackler *et al.* (1964) also evaluated two cooking procedures and reported that extensive deep-fat frying of tempeh reduced the nutritional value, while steaming had no effect.

For further evaluation of the effect of fermentation and heat-processing of soybeans on their nutritional value, amino acid composition studies were conducted. This evaluation was undertaken in an attempt to relate the amino acid composition of soybean products to the nutritional data obtained with rats. It was anticipated that these studies might result in evidence which would relate to and partially explain results from animal studies.

EXPERIMENTAL METHODS

Materials. The detailed procedure followed in preparing and fermenting tempeh has been described by Hackler *et al.* (1964). To examine the effect of length of fermentation, raw soybeans were dehulled and then soaked overnight in 0.85% lactic acid. After steam sterilization for 90 min at 100°C, the beans were cooled, inoculated with *Rhizopus oligosporus* Saito, and fermented for 0, 12, 24, 36, or 72 hr. Tempeh produced by fermenting soybeans for 18 hr was deep-fat-fried in corn oil at 196°C. At the end of 1, 3, 5, and 7 min, the respective temperatures of the oil were 147, 152, 167, and 190°C.

After soaking overnight in 0.85% lactic acid and prior to fermentation, the dehulled soybeans were steamed for 90 min at 100°C. After fermentation, the effect of additional steaming for 15, 30, 60, and 120 min was investigated.

To examine the amino acid composition of the *Rhizopus oligosporus* Saito, a 10% aqueous slurry of soybeans was inoculated and incubated for 13 days at 37°C. The mold pellicle was removed, washed thoroughly to remove the substrate, ground, and prepared for analysis.

Analytical methods. Samples containing approximately 35 mg protein were hydrolyzed for 22 hr at 110°C in sealed evacuated test tubes, each containing 10 ml of 6*N* HCl. The hydrolysate was filtered, and 1 ml dried in a vacuum desiccator under NaOH. Five ml of pH 2.2 sodium citrate buffer were added, and the samples were stored at −10°C until analyzed. Amino acid compositions were determined with a Beckman Spinco amino acid analyzer, Model 120B. Cystine was analyzed by conversion to cysteic acid, as described by Moore (1963), and then quantitatively chromatographed on the amino acid analyzer. Tryptophan was determined by procedure *N* of the method of

Spies and Chambers (1949). Prior to tryptophan analysis, the samples were extracted for 20 hr with a methanol-chloroform mixture (v/v) to remove interfering substances (Rackis *et al.*, 1961). A subsequent extraction with 80% ethyl alcohol was not necessary to reduce high blank values.

Correlation coefficients were calculated as outlined by Steel and Torrie (1960).

RESULTS AND DISCUSSION

During amino acid studies, we have noted a 14% loss in serine when added to biological samples during hydrolysis. Therefore, serine values have been increased 14% to correct for this loss. We have also found no difference in the amino acid content of hydrolyzed samples whether refrigerated in sodium citrate buffer, pH 2.2, at 2°C or frozen at −10°C for up to 6 months. However, in samples refrigerated 2 months at 2°C in 6*N* HCl, 50% of the methionine was converted to methionine sulfoxide.

Effect of fermentation. The amino acid compositions of soybeans fermented 0–72 hr are shown in Table 1. For comparative purposes, compositions are also given for soybean oil meal and raw soybeans. In general, most amino acids either declined slightly or were unchanged as fermentation progressed. A notable exception was tryptophan, which was significantly higher in tempeh fermented for 24 hr but declined thereafter. Tryptophan values in the 24- through 72-hr tempeh samples were similar to those found in soybean oil meal and raw soybeans. The tryptophan content of the soybeans fermented 0 and 12 hr was considerably lower, indicating its loss during overnight soaking in 0.85% lactic acid. During fermentation, ammonia increased significantly, possibly from amino acid deamination.

Steinkraus *et al.* (1961) reported that lysine and methionine decreased with increasing fermentation time. The results reported here support and extend the previous findings. On the other hand, Smith *et al.* (1964) found no significant change in the amino acid composition of tempeh fermented up to 30 hr. In contrast to our findings of increased tryptophan levels in the fermented

Table 1. Amino acid compositions (g/16 g/N) and nutritive indexes of processed and fermented soybeans and fermentative mold.

Item	Soybean oil meal	Raw soybeans	Length of soybean fermentation (hr)					Mycelium of *R. oligosporus*
			0	12	24	36	72	
Lysine	6.24	6.08	5.92	5.88	5.79	5.51	5.54	4.07
Histidine	2.63	2.50	2.53	2.46	2.47	2.27	2.46	1.52
Arginine	7.53	7.13	7.07	6.73	6.57	6.14	6.17	2.47
Aspartic	11.0	11.3	11.1	10.8	10.6	10.6	10.5	4.82
Threonine	3.71	3.76	3.82	3.76	3.73	3.71	3.62	2.56
Serine	5.86	5.67	5.92	5.80	5.55	5.81	5.55	2.95
Glutamic	17.6	16.9	17.1	16.4	15.7	16.1	15.8	4.72
Proline	5.03	4.86	5.10	4.87	4.80	4.54	4.52	1.96
Glycine	4.11	4.01	4.07	3.97	3.91	3.92	3.86	2.68
Alanine	4.23	4.23	4.30	4.20	4.27	4.34	4.29	2.77
Valine	4.43	4.59	4.81	4.58	4.51	4.48	4.43	3.41
Cystine	1.61	1.70	1.70	1.61	1.56	1.61	1.55	1.03
Methionine	1.15	1.22	1.30	1.35	1.34	1.28	1.25	0.89
Isoleucine	4.47	4.62	4.83	4.78	4.65	4.65	4.59	3.22
Leucine	7.64	7.72	8.07	7.98	7.69	7.78	7.65	4.61
Tyrosine	3.38	3.39	3.50	3.56	3.63	3.58	3.48	1.85
Phenylalanine	4.78	4.84	5.02	4.96	4.96	4.92	5.06	2.89
Tryptophan	1.23	1.24	1.03	1.03	1.32	1.29	1.23	0.498
Ammonia	1.86	1.85	1.87	2.00	2.22	2.04	2.68	4.37
Glucosamine	0.216	0.187	0.199	0.385	0.646	0.881	1.23	27.3
Essential amino acid index	73.3	74.4	74.4	73.6	74.6	73.5	72.8	46.2
Requirement index	83.0	84.0	84.4	83.3	83.2	81.8	81.9	53.9

soybeans, Smith *et al.* (1964) found no change in tryptophan values. Their procedure differed slightly from ours in that their soybeans were not dehulled prior to hydration, and their hydration was accomplished with tap water rather than 0.85% lactic acid. These differences may have contributed to the discrepancies in the amino acid values. Additional changes in amino acid composition during fermentation have been reported by Sano (1961), who found increases in essential amino acids in natto, prepared by fermenting soybeans with *Bacillus natto* (*subtilis*). Radhakrishna Rao (1960) reported a 20% increase in methionine in idli, prepared by fermenting black gram and rice.

The amino acid composition of the mold, as shown in Table 1, was considerably lower than that of the soybean substrates. This would, in part, account for the decreases in amino acids in the tempeh. It is difficult to explain the increase in tryptophan during fermentation, since the mold contained relatively little.

During analysis of the tempeh and mold, a ninhydrin-positive compound was observed which emerged 38 min prior to lysine on the 15-cm column, when operated at 50°C with sodium citrate buffer, pH 5.28, flowing at 30 ml/hr. This compound was isolated from the 50-cm preparative column operated at 50°C with pH 5.28 buffer. It was subsequently identified as glucosamine by co-chromatographing it with D-glucosamine hydrochloride at 50°C on the 15-cm column with pH 5.28 buffer, on the 50-cm column with pH 5.28 buffer, and on the 150-cm column with pH 3.28 and 4.25 buffer. On the 150-cm column glucosamine emerged just prior to and partially under tyrosine. Therefore, tyrosine values were corrected for glucosamine present.

As shown in Table 1, glucosamine in tempeh increased from 0.20 to 1.23 g/16 g N as fermentation progressed from 0 to 72 hr. The increase reflected mold growth, since the mold contained 27.3 g/16 g N. This is in comparison to a total of 48.5 g/16 g N for the amino acids. Glucosamine therefore provides a convenient indicator for measuring the extent of mold growth.

The effect of fermentation on the free amino acids in soybeans was determined. Lactic-acid-soaked soybeans and 72-hr-fermented soybeans were refluxed with water at 110°C for 22 hr, and the extract was analyzed for amino acids. As shown in Table 2, most of the water-extractable amino acids increased markedly in the fermented product. Generally, these extracted amino acids accounted for only a small portion of the total amount present; however, 15.1% of methionine, 10.1% of histidine, and 9.87% of alanine were found in the water extract. As is evident, the water-

Table 2. Effect of fermentation on free amino acids in soybeans.

Amino acid	Hours fermented	
	0	72
	% of total	
Lysine	0.38	3.81
Histidine	0.00	10.1
Arginine	0.97	1.93
Aspartic	3.65	3.27
Threonine	0.24	4.82
Serine	1.24	6.22
Glutamic	0.34	3.96
Proline	0.00	0.00
Glycine	0.75	4.88
Alanine	0.73	9.87
Cystine	0.00	0.00
Valine	0.00	1.41
Methionine	4.59	15.1
Isoleucine	0.00	1.89
Leucine	0.49	2.36
Tyrosine	0.00	3.22
Phenylalanine	0.00	4.09
Ammonia	30.3	58.8
Glucosamine	0.00	0.00

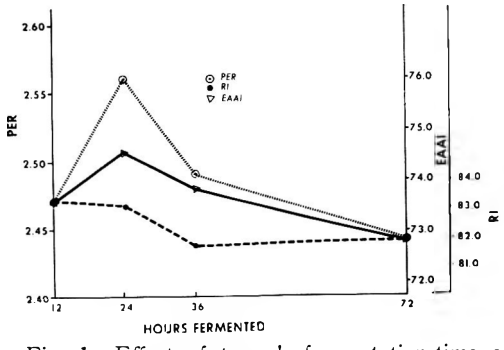

Fig. 1. Effect of tempeh fermentation-time on the protein efficiency ratio (PER), essential amino acid index (EAAI), and requirement index (RI).

Table 3. Correlation coefficients of essential amino acid index (EAAI) and requirement index (RI) with protein efficiency ratio for fermented and heat-processed soybeans.

Treatment	EAAI	RI
12 to 72 hours fermentation	0.97*	0.51
0 to 7 minutes deep-fat frying	0.96**	0.99**
15 to 120 minutes steaming	0.40	0.49

* $P < .05$.
** $P < .01$.

soluble amino acids would be lost from the product during any processing condition involving an aqueous medium.

To relate the amino acid composition of the soybean products to their nutritional value, the essential acid index (EAAI) (Mitchell, 1954) and the requirement index (RI) (Rama Rao *et al.*, 1964) were calculated. The calculated values are shown in Table 1, and Fig. 1 reveals the graphic relationship between these indices and protein efficiency ratios (PER) as given by Hackler *et al.* (1964). Although the changes in these criteria during fermentation were slight, a significant ($P < 0.05$) correlation of EAAI with PER was found (Table 3). An increase in tryptophan was the major reason for the slight increase in EAAI at the 24-hr fermentation time, while a general decline in most of the essential amino acids were responsible for the decrease in EAAI after 24 hr.

Effect of deep-fat frying. The effect on amino acid composition of deep-fat frying tempeh for 1, 3, 5, and 7 min is shown in Table 4. No effect was noted until 5 min, when slight decreases were found in some amino acids, especially lysine and cystine. After 7 min of deep-fat frying, declines were noted in most amino acids. Lysine and cystine were exceptionally heat labile and were destroyed in excess of 20%. Histidine and arginine were less sensitive to heat but showed 7–8% destruction. Ammonia values declined as the time of deep-fat frying increased.

Losses of lysine and cystine in soybeans autoclaved 60 min at 130°C have been reported by Evans and McGinnis (1948). Beuk *et al.* (1949) found that 44% of cystine was destroyed in pork autoclaved 25 hr at 112°C. By enzyme hydrolysis, 35–73% of the other amino acids examined

Table 4. Amino acid composition (g/16 g/N) and nutritive indexes of deep-fat-fried tempeh.

Item	Minutes fried					Casein
	0	1	3	5	7	
Lysine	5.21	5.14	5.36	4.85	3.95	8.32
Histidine	2.32	2.33	2.36	2.31	2.14	2.99
Arginine	6.32	6.09	6.30	6.27	5.79	3.90
Aspartic	10.7	11.0	11.0	10.9	10.4	7.53
Threonine	3.68	3.61	3.63	3.67	3.51	4.37
Serine	5.55	5.31	5.51	5.35	5.20	6.97
Glutamic	16.7	16.5	17.0	17.2	16.8	23.8
Proline	4.77	4.63	4.76	4.92	4.74	11.2
Glycine	3.99	3.99	4.06	4.02	3.93	1.96
Alanine	4.57	4.59	4.78	4.52	4.63	3.31
Valine	4.71	4.63	4.69	4.69	4.53	6.91
Cystine	1.54	1.48	1.54	1.46	1.19	0.49
Methionine	1.28	1.24	1.27	1.24	1.22	2.97
Isoleucine	4.76	4.76	4.81	4.80	4.59	5.80
Leucine	7.67	7.74	7.79	7.92	7.59	10.1
Tyrosine	3.82	3.16	3.30	3.61	3.09	6.07
Phenylalanine	4.84	4.84	4.88	4.86	4.66	5.28
Tryptophan	1.34	1.24	1.34	1.39	1.33	1.57
Ammonia	2.29	2.11	2.16	2.07	1.93	1.90
Essential amino acid index	73.9	72.0	73.8	73.3	67.9	89.1
Requirement index	81.9	81.1	82.2	80.8	76.3	93.7

were unavailable, but all were recoverable in the acid hydrolysates. More recently, Rios Triarte and Barnes (1965) noted cystine destruction in heat-treated soybeans.

The destruction of amino acids was reflected in the EAAI and RI, both of which decreased significantly at 7 min. As shown in Fig. 2, a close relation was found between these indexes and PER values, as evidenced by the highly significant ($P < 0.01$) correlations (Table 2). EAAI and RI values for all soybean products were considerably lower than those found for casein.

Since extensive heat processing is known to decrease amino acid availability (Carpenter *et al.*, 1957; Donoso *et al.*, 1962) prior to chemical destruction, it was unexpected to find the biological and chemical data so highly correlated (Fig. 2, Table 3).

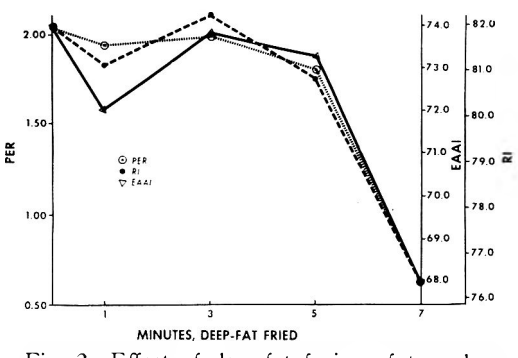

Fig. 2. Effect of deep-fat frying of tempeh on the protein efficiency ratio (PER), essential amino acid index (EAAI), and requirement index (RI).

It was expected that the biological data would reflect the unavailability of amino acids (brought about by heat treatment) prior to actual chemical destruction. Such was not the case, and, although in most instances the magnitude of amino acid destruction was not great, an excellent relationship was found between the biological and amino acid data.

Effect of steaming. As shown in Table 5, additional steaming for 120 min at 100°C had little effect upon the amino acid composition of tempeh. None of the amino acids were affected significantly, and no significant trends were noted in EAAI or RI. These indices tend to be related; however, correlation coefficients were not significant, which was not unexpected, since no significant differences were found between treatments.

Table 5. Amino acid compositions (g/16 g/N) and nutritive indexes of tempeh steamed at 100°C.

Item	Minutes steamed			
	15	30	60	120
Lysine	5.53	5.50	5.60	5.56
Histidine	2.36	2.34	2.44	2.40
Arginine	6.41	6.34	6.54	6.44
Aspartic	10.7	10.7	10.9	10.9
Threonine	3.69	3.69	3.74	3.74
Serine	5.59	5.58	5.74	5.77
Glutamic	16.2	16.5	16.8	17.0
Proline	4.73	4.64	4.80	4.87
Glycine	3.96	4.01	4.08	4.08
Alanine	4.32	4.40	4.65	4.62
Valine	4.76	4.78	4.77	4.70
Cystine	1.56	1.54	1.57	1.55
Methionine	1.41	1.38	1.39	1.38
Isoleucine	4.85	4.84	4.88	4.80
Leucine	7.80	7.86	7.97	7.90
Tyrosine	4.02	3.88	4.16	4.19
Phenylalanine	4.82	4.82	4.99	4.91
Tryptophan	1.59	1.53	1.61	1.58
Ammonia	2.35	2.30	2.34	2.36
Essential amino acid index	76.5	76.2	77.4	76.8
Requirement index	83.3	83.1	83.9	83.4

In these studies on the effect of fermentation and heat processing of soybeans, close relationships were found between amino acid compositions and protein efficiency ratios obtained with weanling rats. Undoubtedly, other nutrients or food components are also affected by processing and contribute to the over-all changes in nutritional value. In the studies presented, however, knowledge of only the amino acid composition of the processed soybeans provided a good estimate of the nutritional quality of the soy protein.

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H. K. HERRING, R. G. CASSENS, and E. J. BRISKEY
University of Wisconsin, Madison, Wisconsin 53706

Further Studies on Bovine Muscle Tenderness as Influenced by Carcass Position, Sarcomere Length, and Fiber Diameter

SUMMARY

Interrelationships of fiber diameter, sarcomere length, and tenderness were studied in 12 bovine muscles of horizontally placed and vertically suspended carcass sides. In comparison with the horizontally placed sides, the vertically suspended sides had greater sarcomere lengths in the psoas major, latissimus dorsi, and rectus femoris muscles. Conversely, vertical suspension permitted the longissimus dorsi, gluteus medius, adductor, biceps femoris, and semitendinosus muscles to shorten in sarcomere length. In general the differences in sarcomere lengths of muscles (between sides) were associated ($r = -.82$ $P < .01$) with differences in fiber diameter. Differences in fiber diameter (between sides) were highly related to differences in shear force ($r = .73$, $P < .01$), as were differences in sarcomere length ($r = -.80$, $P < .01$). When muscles shortened, there were corresponding decreases in sarcomere length, increases in fiber diameter, and decreases in tenderness.

INTRODUCTION

Locker (1960) postulated that some of the variations in sarcomere lengths among muscles may be due to strains induced in the muscles during vertical suspension. Previous work in this laboratory (Herring *et al.*, 1965) showed that sarcomere lengths, when altered, in different portions of the excised semitendinosus and psoas major, by treatment, were associated with tenderness. Preliminary observations also indicated that as a fiber shortened, its diameter increased.

The present study was made to: 1) determine the effect of carcass position on the sarcomere length and fiber diameter of various muscles; and 2) relate these differences to changes in tenderness.

EXPERIMENTAL

Twelve bovine muscles (Fig. 1) were used from two pairs of similarly treated fraternal twins (325–350 kg). All animals were stunned with a captive-bolt pistol, exsanguinated, eviscerated, and separated at the midline. The carcasses were suspended vertically for 30–40 min during the foregoing process. All right sides were suspended vertically according to normal procedure. The left sides were placed horizontally, bone down, on a flat surface; the flexible flank was supported with metal supports to facilitate cooling; and the limbs were oriented and fixed perpendicular to the long axis of the sides (Fig. 2). Carcasses were chilled 48 hr at 4°C, and samples from all 12 muscles were taken for sarcomere length and fiber diameter determinations; 2.54-cm-thick portions were removed, frozen, and held at −18°C for subsequent tenderness evaluation.

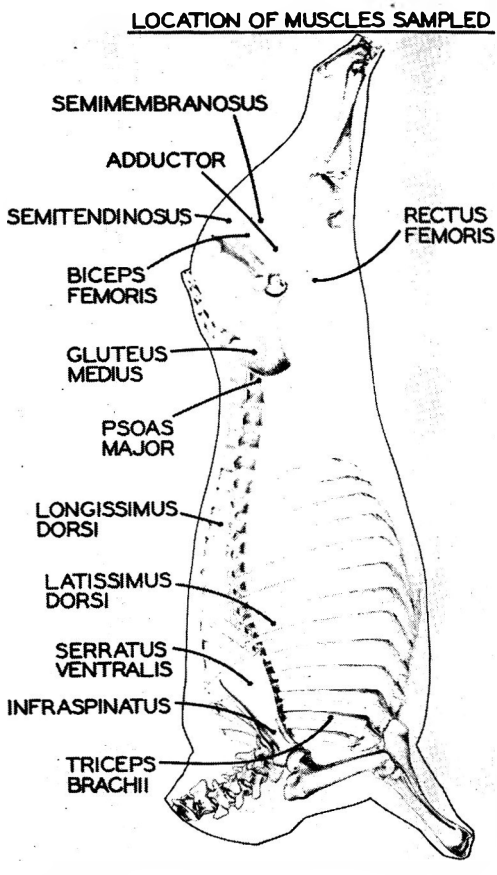

Fig. 1. Names and locations of muscles which were used in this study.

Sarcomere length determinations. An unfixed sample of each muscle (48 hr post-mortem) was homogenized for one minute in 0.08*M* KCl in a chilled Lourde's blender (Locker, 1960). The suspension of myofibrils was examined directly in an A. O. Spencer phase-contrast microscope equipped

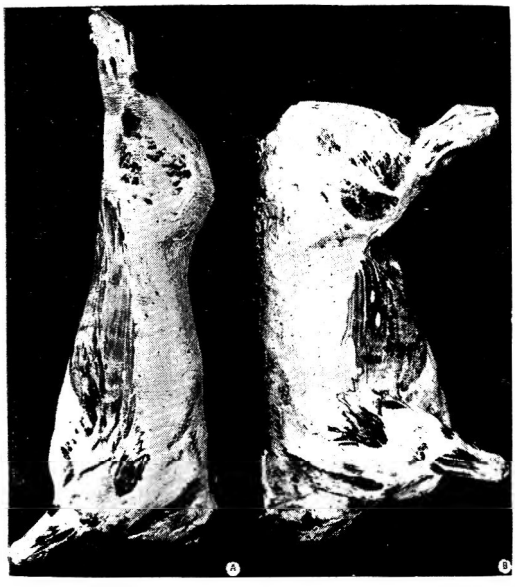

Fig. 2. Effect of position on shape of carcass sides. A) Vertically suspended according to normal practice. B) Horizontally placed, bone down, with limbs perpendicular to long axis of side.

with an ocular micrometer, and sarcomere length was determined as an average of 25 myofibrils.

Fiber diameter determinations. Samples of each muscle (48 hr post-mortem) were fixed in 10% formalin for at least 48 hr, and cross sections (12 μ) were then cut in a cryostat.

A secondary bundle was selected at random, and all fibers in the bundle were measured with an ocular micrometer. The average of all fibers in the bundle was used as the fiber diameter of the muscle.

Tenderness determinations. Muscle portions were thawed at 5°C and prepared for evaluation by roasting to an internal temperature of 66°C in a 177°C oven. The portions were cooled to room temperature and placed in a 5°C cooler for 24 hr. Three 2-cm cores, of parallel fibers, were removed from each portion, and two shears were made on each core with a Warner-Bratzler shear device.

RESULTS AND DISCUSSION

Effect of carcass position on sarcomere length of various muscles. The sarcomere length of muscles from carcasses suspended vertically (Fig. 3) varied from 3.6 μ to 1.8 μ, a range of 1.8 μ, while muscles from the opposite side which had been placed horizontally varied from 2.7 μ to 2.0 μ, a range of only 0.7 μ. While the actual *in vivo* range in sarcomeres is not known, it may be considerably smaller than shown for the vertically suspended sides because of the smaller range in horizontally placed carcasses, which more nearly approximates the physiological position of the animal. It would appear that the long sarcomeres, particularly in the psoas major, are due to a stretching which results from vertical suspension, whereas the shortest sarcomeres may be reduced in length as a result of tension release. In general, muscles which lengthened in vertically suspended sides, in comparison to horizontally placed sides, were the psoas major, latissimus dorsi, and rectus femoris. Conversely, muscles which shortened because of suspension were the longissimus dorsi, gluteus medius, adductor, biceps femoris, and semitendinosus. Statistically nonsignificant ($p > .05$) differences due to carcass position were observed in sarcomere length of the infraspinatus, triceps brachii, serratus ventralis, and semimembranosus.

Locker (1960) postulated that in muscles attached to the carcass the variation in sarcomere length may be due, in part, to the strains induced during vertical suspension. Carcass suspension forced extreme extension of the hind limb, releasing tension on certain muscles of the hind limb and back, permitting them to shorten, part of which may be coincident with the onset of rigor mortis (Marsh, 1954; Sink *et al.*, 1965). However,

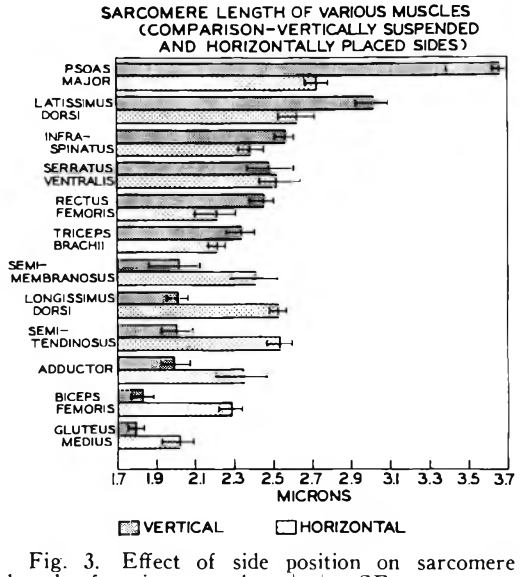

Fig. 3. Effect of side position on sarcomere length of various muscles. |—| = SE.

when sides were placed horizontally, these muscles, which otherwise shortened, were retained with longer sarcomeres, probably from sufficient tension to prevent shortening during the process of rigor mortis. The psoas major appears to be stretched in the suspended side, and shortened in the horizontally placed side. In studies involving the measurement of sarcomeres in eight porcine muscles, Beecher *et al.* (1965) also noted that the gluteus medius, in particular, had extremely short post-mortem sarcomeres. Since Sink *et al.* (1965) noted a high relationship between rigor mortis delay phase duration and sarcomere length, and since Beecher *et al.* (1965) also noted that the gluteus medius had a fast rate of glycolysis, which also contributed to a short duration of the delay phase of rigor, these physiological situations may represent some of the important factors, in addition to tension, which contribute to the short sarcomere length in this particular muscle.

Effect of carcass position on fiber diameter. Fiber diameter varied greatly among muscles in vertically suspended sides, with mean diameters varying by 30 μ, ranging from 34.5 to 64.5 μ (Fig. 4). These values are in general agreement with those of Hiner *et al.* (1953) and Tuma *et al.* (1962). However, when sides were placed horizontally, this variation among means was reduced to 7.1 μ. Muscles which had smaller fiber diameters when the sides were suspended vertically than when they were placed horizontally were the psoas major, rectus femoris, and latissimus dorsi. Conversely, muscles in which fiber diameter increased ($P < .05$) were the semitendinosus, biceps femoris, semimembranosus, and triceps brachii. Nonsignificant differences ($P > .05$) between sides for fiber diameter were noted in the serratus ventralis, longissimus dorsi, adductor, gluteus medius, and infraspinatus. It is evident from these data that muscles which had smaller fiber diameters than the corresponding muscles in the opposite sides also had longer sarcomeres. Differences in fiber diameter between sides were apparently due to muscle shortening or lengthening. The psoas major in the horizontally placed sides, with its fiber diameter of 46.8 μ, is no longer the fine-fibered muscle for which it is noted (34.9 μ in suspended sides). Other workers have also reported small fibers in the bovine and porcine psoas (Moran and Smith, 1929; Staun, 1963); however, this small diameter was probably attributed to normal strains imposed in the vertical suspension of the carcass.

A highly significant simple correlation of -0.67 was calculated for sarcomere length and fiber diameter, which substantiates the fact that there is an association between fiber diameter and sarcomere length when muscles are either stretched or contracted. Fig. 5 shows a regression of fiber diameter change versus sarcomere length change of all muscles. This was calculated by obtaining differences between sides for sarcomere length

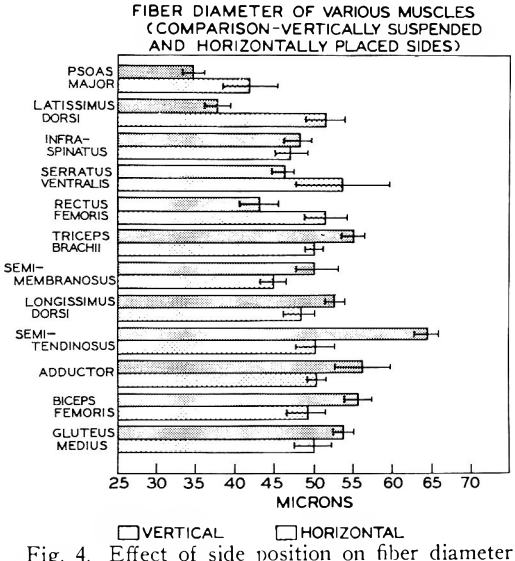

Fig. 4. Effect of side position on fiber diameter of various muscles. |—| = SE.

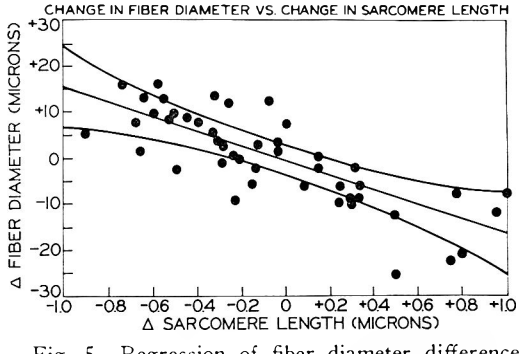

Fig. 5. Regression of fiber diameter difference with sarcomere length difference. 95% confidence limits shown. ($b = -15.98$; $r = -.82$).

and fiber diameter and applying a regression analysis. As sarcomere length increased, there was a corresponding decrease in fiber diameter.

Fig. 6 contains photomicrographs of cross sections of fibers of the psoas major of opposite sides differing only in carcass position. Also shown are the corresponding myofibrils, with their sarcomere lengths. Note that the small, fine fibers from the muscle of the suspended side display a myofibril in a stretched state (3.6 μ). However, the muscle of the horizontally placed side had a larger fiber diameter and a more contracted myofibril (2.7 μ). Ernst (1963), Aronson (1963), and Hanson (1956) have noted a general increase in diameter of isolated myofibrils when the sarcomeres shorten. Ernst (1962) also pointed out that, according to common experience, muscle becomes shorter and broader when active; however, it appears that specific studies are lacking relating state of contraction to fiber diameter. It seems especially pertinent to point out, therefore, that this study is of major importance as to the post-mortem dependence of fiber diameter on the shortening of sarcomeres in muscles of various anatomical positions, sizes, functions, and inherent differences in diameter and tensions. Furthermore, these data emphasize that when studies are conducted to determine the effect of maturity, nutrition, and other factors on fiber diameter, shortening of the fiber post-mortem should be considered.

Effect of variation of sarcomere length and fiber diameter on tenderness. Fig. 7 presents data on tenderness in vertically suspended and horizontally placed sides.

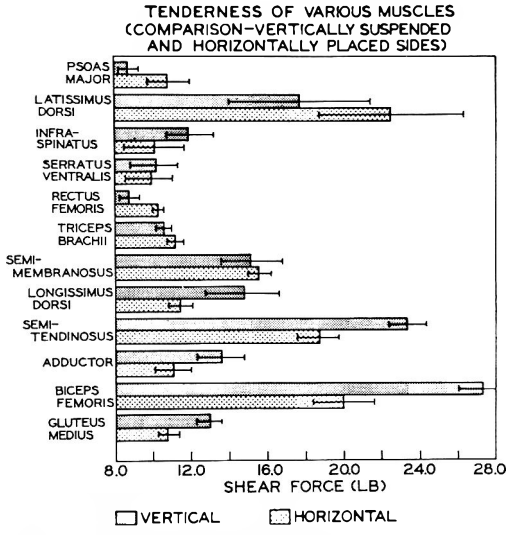

Fig. 7. Effect of side position on tenderness of various muscles. |—| = SE.

The relative differences between muscles in the vertically suspended sides are in reasonable agreement with a report of Ramsbottom *et al.* (1945). Those muscles which increased in sarcomere length and had a corresponding decrease in fiber diameter (resulting from vertical suspension) also tended to show a decrease in shear force. Conversely, shear force increased when muscles shortened and had larger fiber diameters. Rigor shortening of excised muscles has been known to be associated with decreased tenderness (Marsh, 1963; de Fremery and Pool, 1960); however, the shortening was not as marked in the present experiment, since all muscles were attached to the carcass when they went into rigor mortis.

A simple correlation of -0.28 ($P < .01$) was found between sarcomere length and

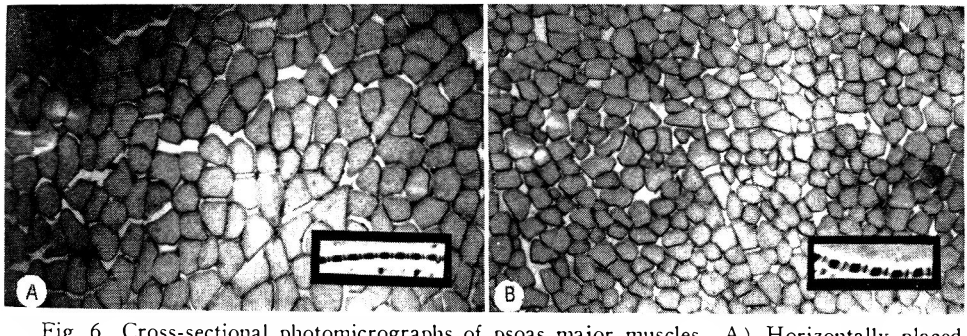

Fig. 6. Cross-sectional photomicrographs of psoas major muscles. A) Horizontally placed side (fiber diameter 46.8 μ, sarcomere length 2.7 μ). B) Vertically suspended side (fiber diameter 34.9 μ, sarcomere length 3.6 μ).

tenderness as measured by Warner-Bratzler shear force. When the effect of between-muscle variation was removed, the correlation was −0.46 ($P < .01$), indicating that sarcomere length is a factor affecting shear force. Fiber diameter was also related to shear force ($r = 0.34$, $P < .01$); however, removing the effect of between-muscle variation gave essentially no change in correlation (0.35. $P < .01$). A multiple correlation of 0.35 ($P < .01$) was found for fiber diameter and sarcomere length with tenderness, indicating that these factors accounted for about 12% of the variation in tenderness. This would be expected, however, since 12 different muscles, containing different inherent properties, are involved in these studies. It thus appears that an area of greater relevance to the objectives of this study might be exposed by viewing how a change in fiber diameter (shortening or stretching as a result of position) is related to a change in tenderness.

Fig. 8 shows a regression of tenderness differences versus sarcomere length differences of all muscles. This was calculated by obtaining differences between sides for tenderness values (shear force) and sarcomere lengths, and then using these coded values in a regression analysis. As sarcomere length increased, there was a decrease in shear force or an increase in tenderness. However, as sarcomere length decreased, there was an increase in shear force or a decrease in tenderness. The correlation coefficient (r) between difference in sarcomere length and difference in shear force (between sides) was −.80. Both the regression line and the correlation coefficient between differences in these parameters point up an area which may have major practical implications. Previous work of Ramsbottom and Strandine (1949), Locker (1960), Goll *et al.* (1964), and Herring *et al.* (1965) has shown that muscles excised prerigor, free to contract, are less tender than muscles excised postrigor. In addition to post-mortem change and tension (due to position), which are known to influence sarcomere length, the tension differences on muscles in vertically suspended sides may, from a practical standpoint, be influenced by vertebral splitting, external fat and fat firmness, as well as weight distribution on the sides. Clearly, a carcass with an abnormally high percentage of its weight in the forelimbs and forequarter area would alter the tension on several muscles abnormally when suspended vertically. Likewise, in the splitting of the vertebral column a complete severance of the bone attachment at any point may completely alter the subsequent tensions which are produced. Furthermore, post-mortem carcass position, which is herein shown to alter sarcomere length and the subsequent tenderness of muscles, may have practical implications for the improvement of bovine muscle quality.

Fig. 9 shows a regression of tenderness differences (between sides) versus fiber diameter differences (between sides) of all muscles. As fiber diameter increased, tenderness decreased, whereas the opposite was true when fiber diameter decreased. The correlation coefficient (r) between differences in fiber diameter and differences in

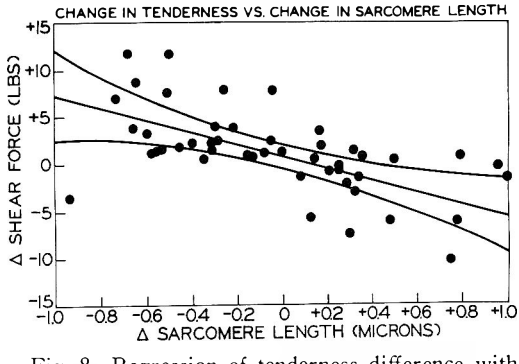

Fig. 8. Regression of tenderness difference with sarcomere length difference. 95% confidence limits shown. ($b = -6.33$, $r = -.80$).

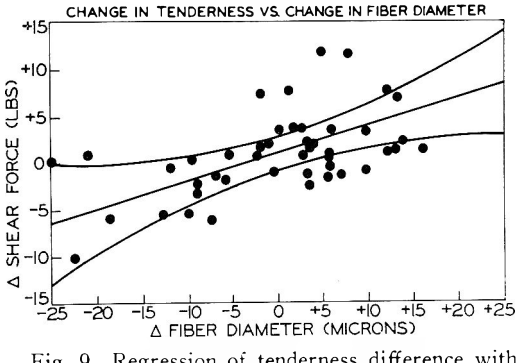

Fig. 9. Regression of tenderness difference with fiber diameter difference. 95% confidence limits shown. ($b = 0.30$; $r = .73$).

tenderness (between sides) was +.73. Since it was shown (above) that a change in fiber diameter was related to a change in sarcomere length (which was also shown to be related to tenderness), the regression shown in Fig. 9 was expected. However, it seems relevant to point out that in previous work (Herring *et al.*, 1965), when a single muscle was separated into two parts, one being stretched and the other permitted to contract, the stretched part was considerably more tender than the contracted part. Since the stretched part also had smaller fiber diameters, it is reasonable to assume that a greater number of fibers per unit area were being severed in these muscles. Theoretically, in those as well as in the stretched muscles of the present experiment, a greater number of fibers per unit should have made these muscles less tender. However, since the opposite effect was true, the thickness of the sarcolemma and associated connective tissue, which Casella (1950) observed to reduce in thickness when fibers were stretched, may represent another important factor, in addition to the contractile elements, which contributes to tenderness.

Additionally, the sarcomere length differences may represent gross molecular alterations and influence the interdigitation of thick and thin filaments and the number of cross bridges within each sarcomere. Postmortem molecular alterations may be of prime importance to the over-all problem of muscle tenderness.

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G. A. BAKER, C. S. OUGH, and M. A. AMERINE
University of California, Davis, California 95616

Scoring vs. Comparative Rating of Sensory Quality of Wines

SUMMARY

One hundred and fifty wines of different types and regions were scored by 11 experienced judges by two methods (scoring and comparative rating). It was thought that the second method (which included a reference wine) would give more stable and consistent results than the standard method. For some categories of wines the standard method resulted in more stable and consistent responses, but for other categories the reverse is true. For some judges and for some categories of wines the two methods were practically identical. The reactions of the judges to the two methods varied greatly.

INTRODUCTION

Reports by Ough and Baker (1961) and Amerine *et al.* (1962) indicated that scoring wines for quality attributes by the use of 20-point score cards was satisfactory. Variations in judge response, however, were quite large and unpredictable for some judges as compared to others. Also, some judges did not respond in the same manner when the same wines were submitted to them over a period of time or when the design of the presentation was modified.

In a study of the effect of stressed-time on subjects response, Ough *et al.* (1964) used a 10-point comparative rating method and were able to show good subject correlation with 10 judges. A comparison wine was available at each end of a quality series of wines in that study. However, response was bimodal and the quantity differences, by choice, were dependent.

With these facts in mind it was decided to test a 20-point comparative rating method using a reference wine for quality evaluation of the varietal wine evaluation studies against the method used routinely in this laboratory—that of 20-point quality scoring method. It was thought that giving a "reference" or "anchor wine" with each wine tasted would tend to stablize the judges and cause them to have more nearly similar quality response patterns.

METHOD

The panel consisted of wine judges (all male) of varying degrees of experience who normally evaluate wines in the laboratory daily about 6–8 months of the year. Table 1 gives the approximate years of experience of each judge. These judges, if the experience was less than 10 years, had most of their experience as part of a panel. Those with experience more than 10 years had previous experience (in addition to about 10 years of taste-panel work) as expert wine judges at state fairs and as individual expert judges for wineries or for research projects. Taster 1 was dropped from the experiment because he failed to complete the tastings.

Table 1. Experience of the judges in evaluating wines.

Judge no.	Years as wine judge
1	8
2	6
3	20
4	30
5	10
6	6
8	6
9	6
10	3
11	½
12	½

The wines used were made from grapes grown in several vineyards under different climatic and soil conditions. This wine is usually evaluated by expert judges beginning in January and continuing until approximately May. The wines were either filtered or had become reasonably clear by settling and racking prior to the tastings, to eliminate visual clues, such as clarity, as a variable. The wines were grouped into nine general main types: cabernet, dry red table wine, dry white table wine (all regions), dry white table wine (region I, a cool grape-growing area), dry white table wine (region IV, a warm grape-growing area), dry white table wine (muscat types), white dessert wine (muscatel type), white dessert wine, and red dessert wine. The cabernet type was tasted at the beginning (set 1) and retasted at the end of the experiment (set 2). Each type with more than 10 wines was divided into sub-groups of about 9; if sub-groups did not

come out even, replicates were included to make the number of daily tastings about 10 since the reference wine was always duplicated. The sub-groupings were made by random selection from the parent type.

One wine from each type was selected as the reference wine and was included in each tasting of the sub-groups. The reference wine was chosen by reviewing earlier tasting scores, made by several experienced judges, and selecting a wine which seemed to be of medium quality with no outstanding features. The panel was instructed that this wine was of medium quality or a standard wine in the comparative rating tastings, but they were allowed to score it as they pleased with only the statement in the instructions to guide them into remaining within the same general range.

The standard system of scoring for quality is fully described by Amerine *et al.* (1959). Briefly, it consists of a 20-point quality score card divided into 10 attributes with points assignable for each. Ranges are delineated for 5 categories of quality. The panel was quite familiar with this system, which has been in use in this laboratory since 1958.

The comparative rating used is similar to other "anchored" rating systems (Helson *et al.* 1954). In this case the panel was asked to use a 20-point rating scale (1 to 20) and to use the same criteria for quality for this scale as were used for the traditional 20-point scoring scale. The panel first received the "anchor," or "reference," wine, to which they individually assigned a quality score. They then received, one at a time, 10 wines to rate in comparison with the "reference" wine, which they kept to compare with each wine. They recorded the rating value as they judged the wine in comparison with the reference. Fig. 1 shows an example of the comparative rating card and the instructions to the judges. Four practice periods were given at the start of the tasting to allow the judges to become familiar with the methodology of the experiment.

Each day, one sub-group of a type was presented to the judges and they were asked either to score or to assign comparative ratings to the wines. The scoring and comparative rating methods were alternated daily. Sub-groups of each type were presented so that the scoring and comparative

Figure 1
Comparative Rating Card

Name _____ Taster No. _____ Date _____

You will receive a "reference" or "anchor" wine that will be more or less of medium quality for the group of wines you will be tasting. You are asked to assign this wine a score based on the standard scoring system used (1 to 20) remembering that: 17 to 20 scores are for wines of outstanding characteristic or no marked defects; 13 to 16 are for standard wines with no outstanding character or defect; 9 to 12 for wines which are of commercial acceptance but with noticeable defects; 5 to 8 for wines below commercial acceptability; and 1 to 4 for spoiled wines. After you have given the reference wine its appropriate score and recorded it on this sheet, you will keep that wine and be given several more wines, one at a time. These will be compared to the reference wine using the quality scoring system scale for the comparative rating. These values will also be recorded on this card in the appropriate space below.

	Score (1–20)
Reference sample	
Comparative Samples	Comparative Rating (1–20)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

rating of an individual sub-group was not done on consecutive days.

Wines for the day were presented in random order, with the "reference" wine of the group included at least twice in the scoring system and as the "reference" in the comparative rating systems and at least once within the 10 samples.

Samples were presented in 9-ounce tulip-shaped wine glasses coded with two digit numbers. Used for the judgings were partitioned booths of standard design in an air-conditioned room (72°F) illuminated with incandescent lighting. Any judge who missed a session made up the test as soon as possible. No restrictions were placed on the judges as to time of testing.

The judges were allowed to review their decoded scores and ratings at the end of each session.

RESULTS AND DISCUSSION

As pointed out by Mrak *et al.* (1959) and many other investigators, judges will respond quite differently to the same set of sensory stimuli. This is again demonstrated in the present study by the variations in the score and comparative rating difference distributions. Table 2 gives the numbers of comparative ratings which were less than the score values for the same wine and those which were more. The χ^2 values, testing the data against the hypothesis that the differences would be evenly distributed about "no difference," show that four of the judges have strong tendencies to assign lower quality values by the comparative rating method. Referring to Table 1 it can be seen that these four judges are among the least experienced, and while the differences are all in the same direction for the rest of the judges (except number 9) their individual differences were not significant. However, as a group they assigned a significant number of lower quality values by method 2 than by method 1.

Similar frequency distributions of the variations between the score and rating values were made for the wine types. Table 3 summarized differences in distributions and χ^2 values. In this instance, four wine types showed significant differences, two of the types again showed strong trends towards lower quality values by method 2, and three types showed no trends at all.

The inexperienced tasters were confused more by some types than by others, and the judgment of quality was affected by the interaction of types of wine with the methods. This is all reflected in unequal response to the two judging methods as shown in Tables 2 and 3.

To determine whether there was a lesser or greater ability to duplicate scores or ratings by either method, and to determine if significant changes occurred in judge response during the testing, the duplicate values for the reference wines for the cabernet type for set 1 and 2 were analyzed by

Table 2. Variation in individual score and rating difference distributions between the tails.

Judge	Number of comparative rating			χ^2 test for heterogeneity to even distribution, 1 df
	More than standard score	Less than standard score	Same as standard score[a]	
1	56	60	32	0.08
2	51	61	36	.72
3	54	61	33	.31
4	43	61	44	2.78
5	47	56	45	.62
6	36	75	37	13.01***
8	55	57	36	.01
9	59	49	40	.75
10	45	68	35	4.28*
11	30	85	33	25.33***
12	43	72	33	6.81**
Total	519	705	404	27.96***

* Significant at the 5% level.
** Significant at the 1% level.
*** Significant at the 0.1% level.
[a] Not used in χ^2 analyses.

Table 3. Variations in wine-type score and rating difference; distributions between the tails.

Wine samples	Number of comparative scores: More than standard score	Less than standard score	Same as standard score[a]	χ^2 test for heterogeneity to even distribution, 1 df
Cabernet (1st set)[b]	55	82	61	4.93*
Cabernet (2nd set)[c]	60	80	58	2.58
Dry red	90	115	103	2.81
Dry white (Region I)	55	88	55	7.16**
Dry white (Region IV)	91	134	105	7.84**
Dry white (Muscat)	39	45	26	.30
White dessert (Muscatel)	31	32	25	.00
White dessert	39	32	17	.51
Red dessert	51	79	68	5.61*

* Significant at the 5% level.
** Significant at the 1% level.
[a] Not used in χ^2 analysis.
[b] Evaluated at beginning of study.
[c] Evaluated at end of study.

"student's" t-test. The mean scores, standard deviations of the differences of the mean scores, and the *t*-values are given in table 4. No significant differences in the mean values of the duplicates were found within sets by scoring or rating for either set. Checking the reference wine against one of the duplicate values for each wine for both sets showed no significant differences. Checking the first-set duplicate against the second-set duplicate also showed no significant difference, indicating that the responses did not vary appreciably between the start and finish of the experiment.

Table 4. Score and rating differences, standard deviations of difference, and *t*-values within and between sets for the cabernet type reference wine.

Comparison	Av. values: Score 1st	Score 2nd	Rating 1st	Rating 2nd	Standard deviation of the differences: Score	Rating	t-values: Score	Rating
Duplicate values within the 1st set	14.55	14.55	14.77	14.95	0.35	0.30	0.00	0.60
Duplicate values within 2nd set	14.27	14.41	14.14	14.14	0.27	0.21	0.53	0.00
			Reference	Duplicate				
Reference versus duplicate[a] for the first set			14.59	15.05		0.28		1.59
Reference versus duplicate[a] for the second set			14.50	14.23		0.21		1.29
			1st set	2nd set				
1st set duplicate[a] versus 2nd set duplicate			15.05	14.23		0.41		2.02

[a] One duplicate chosen randomly for each wine.

Duplicates of each of five white dessert wine types were tested by both methods, and analysis of variance was applied to the sensory values (Table 5). Large judge × method interactions are shown and a high but non-significant wine × meth interaction. No replicate interactions or significances were noted. The replicates were combined and the data partitioned into "scoring" and "rating" parts, and a second analysis was done. This shows that most of the judge × wine interaction was associated with the rating method, and that the scoring method gave much better results allowing wine differences to be detected, whereas with the rating method the judge variance was larger and the wine variance insignificant.

One way to test the inner consistency or reliability of a method of ranking wines is to divide the judges into two groups and correlate the total or mean scores of the wines for the two groups. Of the 11 judges, one (number 2) was consistently aberrant; therefore the other 10 were divided into pairs of sets of 5 in all possible ways—128 in number. The corresponding correlation coefficients were computed, and the means, variances, standard deviations, and ranges of the 128 coefficients for each type and method were calculated (Table 6). In computing the correlations comparing methods, the reference wine was included each time it occurred as a member of a set of 9 or 10. Thus in Tables 6, 7, and 8, two of the 18 wines listed for cabernet Set 1 are the reference wine, once in each of two series of tastings. The duplicates for the reference wine were averaged, and the average was used as the score or rating. Table 7 shows the numbers of times the reference wine was included for each wine type.

The best inner consistency seems to be the dry red wines for method 1, but that with method 2 is practically the same. The reliability for some of the type-method categories was quite low and erratic as to which method had greater inner consistency.

In a wine evaluation program extending over several seasons, if the rankings are the same or the correlation coefficients for the methods are near unity, it does not make any difference which method is used. If the

Table 5. Comparison of scoring and rating methods by analysis of variance of sensory values for white dessert wine.

Source of variation	DF	SS	MS	F
Total	219	522.78		
Judges	10	158.33	15.83	14.93**
Wines	4	66.89	16.72	15.77**
Replicates	1	2.84	2.84	2.67
*Methods	1	1.31	1.31	1.23
Judges × wines	40	78.31	1.95	1.83*
Judges × replicates	10	13.11	1.31	1.23
Judges × methods	10	46.64	4.66	4.39**
Wines × replicates	4	1.64	0.41	0.38
Wines × methods	4	10.26	2.56	2.41
Replicates × methods	1	0.23	0.23	0.21
Error	134	143.22	1.06	
Scoring				
Total	54	364.19		
Judges	10	134.59	13.46	4.78**
Wine	4	115.28	28.82	10.11**
Judges × wine	40	114.32	2.85	
Rating				
Total	54	475.75		
Judges	10	275.35	27.54	6.83**
Wine	4	39.02	9.75	2.41
Judges × wine	40	161.38	4.03	

* Significant at the 5% level.
** Significant at the 1% level.

Table 6. The reliability of each method by wine type as measured by all possible (128) correlations between paired totals of sets of five judges.[a]

Wine type	No.	Method[b]	Mean	Variance	Standard deviation	Range
Cabernet (set 1)	18	1	0.5227	0.01099	0.1048	0.5072
		2	0.6857	0.00753	0.0868	0.3625
Cabernet (set 2)	18	1	0.6961	0.00779	0.0883	0.4725
		2	0.6392	0.01682	0.1297	0.5642
Dry red	28	1	0.9162	0.00059	0.0243	0.1059
		2	0.9185	0.00087	0.0296	0.1712
Dry white	50	1	0.7493	0.00136	0.0369	0.1668
		2	0.6999	0.00407	0.0638	0.3127
Dry white (Region I)	18	1	0.7398	0.00630	0.0794	0.3553
		2	0.6099	0.01771	0.1331	0.6153
Dry white (Region IV)	30	1	0.7454	0.00192	0.0439	0.2444
		2	0.7278	0.00370	0.0609	0.3790
Dry white (Muscat)	10	1	0.5997	0.02232	0.1494	0.6556
		2	0.4033	0.05219	0.2285	1.0750
Dessert (Muscatel)	8	1	0.2249	0.08126	0.2851	1.1040
		2	0.6349	0.03795	0.1948	0.8130
Dessert red	18	1	0.7046	0.01003	0.1002	0.4849
		2	0.7731	0.00539	0.0734	0.3119

[a] One judge was omitted. Thus there were 128 possible pairs of totals of sets of 5 or 128 correlations between totals for 5 judges for each of the 9 categories of wines for each of the two methods.

[b] 1 = standard system of scoring; 2 = comparative rating system of scoring.

correlations are low, then the "best" wines by one method are not the "best" wines by another method, and it becomes necessary to examine the details of each situation to see which method gives a satisfactory ordering of the wines.

If we correlate the total scores for all 11 judges for the wines of the nine categories for the two methods, we get the results given in Table 7. The correlations for dry white wine from region I (0.920) and dry red (0.927) are quite high but some of the others are much lower. In this connection it is well to remember that "predictability" is measured by the square of the correlation coefficient.

Table 7 gives the correlations between the two methods for all 11 judges combined. It is of some interest to give similar coefficients for individuals separately, as shown in Table 8. There were great differences in the correlations between the two methods for the

Table 7. Correlation coefficients of the methods for each wine type with all judges.

Wine type	No. of wines[a]	No. of times reference wine included	Correlation coefficient
Cabernet (1st set)	18	2	0.840
Cabernet (2nd set)	18	2	0.775
Dry red	28	3	0.927
Dry white	50	5	0.875
Dry white (Region I)[b]	18	0	0.920
Dry white (Region IV)	30	5	0.864
Dry white (Muscat)	10	1	0.748
Dessert white (Muscatel)	8	1	0.611
Dessert red	18	2	0.766

[a] Includes wine when used as reference.

[b] After the reference wine was selected from the 50 dry white wines and the tastings made, the white wines for regions I and IV were tabulated separately because of obvious differences. The reference wine happened to be from region IV which accounts for the "0" in the column for "number of times reference wine included" for dry white (Region I).

Table 8. Correlations between Method I and Method II for each type and judge.

Wine type	No.	Judge 1	2	3	4	5	6	8	9	10	11	12
Cabernet (set 1)	18	.474	.682	.411	.518	.772	.779	.613	.264	.130	.315	−.023
Cabernet (set 2)	18	.718	.432	.463	.442	.545	.479	.218	.498	−.054	.471	.270
Dry red	28	.677	.570	.582	.740	.858	.865	.681	.805	.874	.490	.848
Dry white (Region I)	18	.621	.160	.054	.130	.039	.272	.515	.586	.536	.594	.585
Dry white (Region IV)	30	.709	.257	.658	.534	.427	.394	.610	.516	.286	.873	.484
Dry white (Muscat)	10	.411	.183	.704	.282	.467	.538	.790	.506	−.114	−.079	.448
Dessert (Muscatel)	8	.910	.555	.857	.374	.532	.371	−.254	.770	−.134	−.561	.833
Dessert red	18	.765	.173	.602	.703	.657	.582	.320	.321	.451	.258	.686

judges separately. Some gave practically the same results by either method, but others give quite different results, depending on the method used.

Wine "judging" is an extremely complicated combination of science and art and doesn't fall into the nice mathematical treatments such as paired difference testing with its single-component variability. Also, it is extremely subjective and the judge variations are generally larger than the wine variations.

Nor can it be compared with subjective preference tests, which allow that the judges will and should vary. Some arbitrary median definition of quality may be made by a single judge or a group of judges, and this can be used as a basis for further quality evaluations. This was tried with only partial success in this experiment, but as a training methodology for long-term judge-response standardization it might have some value.

In truth, what is desired from an "expert" judge or panel of judges is a multidimensional scaling of the various recognizable attributes and an integrated score or rating composed of these values associated with quality. Stevens (1961) shows that various sensory systems can be related. In their review (Helm *et al.*, 1961), the various psychological models relating discrimination and magnitude estimation scales are discussed from a theoretical view. Work by Baker and Amerine (1935) and Baker (1954) is one of the few successful attempts to relate the various measured chemical componets of a food to quality scores. The interpretation of quality differences can certainly be aided by gas chromatographic analysis (Wynn *et al.*, 1960; Jennings *et al.*, 1962), and should, as the techniques improve, be an increasingly valuable tool. Until more work is done along these lines, understanding of quality scoring and improvements of the methodology will progress slowly.

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JOHN SPINELLI
Bureau of Commercial Fisheries Technological Laboratory
Seattle, Washington 98102

Effect of Hypoxanthine on the Flavor of Fresh and Stored Low-Dose-Irradiated Petrale Sole Fillets

SUMMARY

Dilute aqueous solutions of hypoxanthine were found to be predominantly bitter. Triangle tests showed, however, that the addition of hypoxanthine to fresh and stored low-dose irradiated petrale sole containing less than 10^6 bacteria per gram did not alter flavor. Flavor alteration due to added hypoxanthine was detectable in the irradiated fish only when the total bacterial counts exceeded 10^6.

INTRODUCTION

Research on taste-producing substances has shown that 5′-nucleotides exert a major influence on the flavor of fish (Kuninaka *et al.*, 1964). The most abundant 5′-nucleotide in fish is 5′-inosine monophosphate (IMP), which after the death of the fish quickly appears in its flesh as the result of the degradation of muscle ATP. IMP is then slowly degradated to hypoxanthine via inosine.

Although there is good agreement that IMP imparts a pleasant flavor to the foods in which it is found, there is less unanimity on the flavor sensations produced by the degradation products of nucleotides—that is, by their corresponding nucleosides and purine bases. The major purine base found in aging fish is hypoxanthine. Hashimoto (1964), investigating taste-producing substances in fish, found that many Japanese investigators consider hypoxanthine to be tasteless. Jones (1963), however, believes that hypoxanthine imparts a bitter flavor to cod that has been stored in ice. Dyer *et al.* (1963) suspect that hypoxanthine produces a bitter flavor in porbeagle flesh (*Lamna masus*). Kazeniac (1961) described hypoxanthine as being a bitter component of chicken broth.

The question as to whether hypoxanthine does indeed impart bitter or "off" flavors to fish is an important question to be resolved for radiation-pasteurized fish products. Hypoxanthine in many species of fish reaches peak concentrations after 10–16 days of storage at the temperature of melting ice (Kassemsarn *et al.*, 1963; Spinelli *et al.*, 1964). Since low-dose irradiation does not affect the enzymatic systems regulating the degradation of IMP to hypoxanthine (Guardia and Dollar, 1965), it would be reasonable to expect that irradiated fish that are to be consumed after being stored for two weeks would contain a peak concentration of hypoxanthine. If hypoxanthine does contribute bitter or off flavors, these flavors should then become readily apparent in irradiated fish. We found, however, during our regular panel testing of irradiated petrale sole, that bitter flavors, when noted, generally occurred in samples having bacterial counts in excess of 10^6 (Spinelli *et al.*, 1965). It therefore seemed that hypoxanthine *per se* did not contribute to bitter flavors in irradiated fish with low bacterial counts.

To evaluate more fully whether the hypoxanthine that accumulates in stored low-dose-irradiated fish contributes to any flavor change, we undertook a study to determine:

1) The flavor sensations produced by aqueous solutions of hypoxanthine.

2) Whether hypoxanthine produces a flavor change in fresh fish.

3) Whether hypoxanthine produces a flavor change in stored irradiated fish.

4) Whether there is a relation between bacterial counts, hypoxanthine content, and flavor in irradiated fish.

Flavor sensations produced by aqueous solutions of hypoxanthine. Solutions of hypoxanthine ranging from 0.00005 to 0.1% in distilled water were prepared. Eight of these solutions were presented to the taster in increasing order of concentration. The order or nature, however, of the material tested was unknown to him. He was required to express the flavor sensations and the degree of intensity of these sensations produced by the solutions. Each taster had received previous training concerning the nature and descriptive terminology used in

this type of testing. Between tests, unsalted soda wafers and water were provided to alleviate taste fatigue.

Individual reactions of 10 tasters to the test solutions are given in Table 1. Analysis of the reactions shows that hypoxanthine, even in dilute solutions, produces a variety of flavor sensations and that the predominating ones are bitterness or dryness. Eight of the ten tasters found hypoxanthine to be bitter at a concentration of 0.01%.

Flavor effect of adding hypoxanthine to fresh fish. Commercially landed petrale sole (*Eopsetta jordani*) were used in testing the effect of adding hypoxanthine to fresh fish. The fish had been held in melting ice for four days prior to being landed. To ensure uniformity of sample, we passed 1 kg of the fillets through a meat grinder having a plate with holes ¼ inch in diameter. Analysis of this mixture showed it to contain 2.8 μM of hypoxanthine per gram. One-half of the ground fillet sample was used as the control, and 408 mg of crystalline hypoxanthine (6 μM per gram) was added to the other portion. With this added hypoxanthine, the total content (8.8 μM per gram) of hypoxanthine was now about 50% higher than the 6 μM per gram normally found in petrale sole at peak concentrations. The portion containing the hypoxanthine was thoroughly mixed, reground, and thoroughly mixed again. The control portion was similarly prepared, but contained no added hypoxanthine.

For taste analysis, aliquot portions of the prepared samples were put into 4¼ × 3¼ ×1¼-inch aluminum trays, covered, and steamed for 10 min. After being cooked, the sample in each tray was cut into equal portions for use in the taste analysis. A triangle test was used to determine whether the panel could detect a difference in flavor between the samples. Five to eight tasters participated in each test, and the tests were repeated three times.

Table 2 shows that in the 23 triangle comparisons made, only nine correct responses were obtained. This number of correct responses is not significant. Thus, hypoxanthine, when added to fresh fish, does not produce a consistently detectable change in flavor.

Flavor effect of adding hypoxanthine to irradiated fish. Petrale sole fillets were vacuum packed into No. 2 C-enameled cans, irradiated at 0.2 megarad, and stored 12 days at 33–35°F to allow for maximum accumulation of natural hypoxanthine (about 6 μM per gram) in the relative absence of bacterial growth. Four cans of fillets were composited and prepared for sensory analysis as previously described. The sample was divided into equal portions, and 3 μM per gram of crystalline hypoxanthine was added to one portion of the fish. Triangle tests were conducted on the two sets of samples.

When 23 triangle comparisons were made, 8 correct responses were obtained (Table 2). This again is not a significant difference. It thus indicates that consistently detectable flavor change is not induced when hypoxanthine is added to stored low-dose-irradiated petrale sole fillets.

Interrelation of bacterial counts, hypoxanthine content, and flavor of fish. As previously stated, it was noted that when sensory tests were made on irradiated petrale sole fillets, bitter flavors were not generally recorded by taste panelists until bacterial counts exceeded 10^6 per gram. To determine whether there was a relation between hypoxanthine content, bacterial counts, and flavor, we irradiated petrale sole fillets at 0.2, 0.3, and 0.4 megarad. By irradiating the samples at these different dose levels, we obtained wide variations in bacterial counts in fish that were stored for the same length of time and were of identical initial quality.

After 19, 20, and 21 days of storage at 33–35°F, samples were removed and tested as follows:

1) Triangle tests were made (after the prescribed storage periods) to determine if any difference in flavor could be found among the samples irradiated at the various dose levels.

2) Hypoxanthine was added to all samples (3 μM per gram) and retested by the triangle test.

3) Bacterial counts and hypoxanthine content were determined on the samples used for the sensory tests.

Table 1. Reaction of 10 tasters to aqueous solutions of varying concentration of hypoxanthine.

Concentration of hypoxanthine (%)	Reaction of individual taster number:									
	1	2	3	4	5	6	7	8	9	10
0 (water)	H_2O	H_2O	D±	H_2O,Sa±	H_2O	H_2O	H_2O	H_2O	B(1)	H_2O,S±
0 (water)	H_2O	H_2O	D±	H_2O,S±	H_2O	H_2O	H_2O	H_2O	So(1) B(1)	H_2O
0.00005	H_2O	H_2O	D±	H_2O,S±	H_2O	H_2O	B±	H_2O	So(1) B(1)	H_2O
0.0001	H_2O	H_2O	D±	H_2O,S±	A±	H_2O	D±	H_2O	So(1) B(1)	H_2O S±
0.0005	H_2O	H_2O	B±	Bu± B± S±	B±	H_2O	D,As±	H_2O	So(2) B±	D±
0.001	B(1)	Sat(1)	B,D±	B± S±	B±	H_2O	D±	H_2O	B(1) So(1)	D±
0.005	B(1)	Sat(1)	B,D(1)	Bu± B± Sa±	B(1)	H_2O	As± B± D±	H_2O	So(2) B(2)	D(1) S±
0.01	B(1) B(1)	B(1)	B,D(2)	Bu(1) Sa(1)	B(1)	B±	B±	H_2O	So(2) B(2)	B(1) D(1)
0.05	B(2)	B(2)	B(2)	Sa(2) S(2)	B(1)	B±	Sa± B±	H_2O	So(2) B(3)	B(1) D(2)
0.1	B(3)	B(3)	B(3)	Sa(1) Bu(1)	B(2)	B±	B(1)	B(1)	So(2) B(4)	B± D(2)

A, acid; As, astringent; B, bitter; Bu, bouillon; D, drying; Sa, salty; S, sweet; So, sour; Sat, salty aftertaste.
± threshhold, 1 definite, 2 fairly strong, 3 strong, 4 very strong.

Table 2. Triangle tests showing the difference in relation of sensory reaction to bacterial counts when hypoxanthine is added to petrale sole fillets irradiated at different dose levels and stored at 33°F. (Comparisons were made on sets of samples that had been stored for the same length of time.)

	Sample treatment	Comparisons (no.)	Correct responses (no.)	Significance	Bacterial count (count/g)	Hypoxanthine (μmoles/g)
A	Fresh fillets	23	9	Not signif.	1×10^5	2.8 natural 5. added
B	Irrad. at 0.2 megarad, stored 12 days	23	8	Not signif.	8.5×10^4	5 natural 3 added
C	Irrad. at 0.2 and 0.3 megarad, stored 19 days	20	8	Not signif.	8×10^{3a} 1.6×10^6	5.5 natural
D	Irrad. at 0.2 and 0.3 megarad, stored 19 days	19	7	Not signif.	8×10^{3a} 1×10^6	5.5 natural 3 added
E	Irrad. at 0.2 and 0.3 megarad, stored 21 days	20	8	Not signif.	2×10^{5a} 9×10^6	5.2 natural
F	Irrad. at 0.2 and 0.3 megarad, stored 21 days	20	14	Signif. at 1% level	2×10^{5a} 9×10^6	5.5 natural 3 added
G	Irrad. at 0.2 and 0.4 megarad, stored 22 days	22	10	Not signif.	10^{3b} 1.3×10^7	5.2 natural
H	Irrad. at 0.2 and 0.4 megarad, stored 22 days	20	15	Signif. at 1% level	10^{3b} 1.3×10^7	5.2 natural 3.0 added
I	Irrad. at 0.2 and 0.4 megarad, stored 28 days	8	8	Signif. at 0.1% level	2×10^8 1.2×10^{11b}	5.5 natural 0 added

[a] Bacterial count of 0.3-megarad sample.
[b] Bacterial count of 0.4-megarad sample.

When the bacterial counts of the samples irradiated at the three different dose levels ranged from 1.0×10^3 to 1.3×10^7 per gram, the panel could not distinguish between the samples at the 5% level of significance (Table 2, lines C, E, G). When 3 μM of hypoxanthine per gram of fish was added to all of the samples, however, the panel could distinguish the difference between samples and was able to identify the sample(s) with the high bacterial count as being the odd (Table 2, lines F and H). Not all panelists agreed that the odd sample(s) (high bacteria plus added hypoxanthine were bitter. After 28 days of storage, the 0.2-meg-irradiated samples had bacterial counts of 2.0×10^8 (Table 2, line I) and the panelists were consistent in their ability to identify these samples when they were compared against the 0.4-meg-irradiated samples having bacterial counts of 1.2×10^4 per gram. In all of the triangle tests, no panel member was consistent in his ability to respond correctly to the treated sample, and there was a fairly equal distribution of correct responses among the panel members.

Results from the above experiment suggest that bacterial growth changes the flavor characteristics of hypoxanthine by either utilizing or altering some of the natural constituents of fish that normally render hypoxanthine tasteless in the fish, or by producing metabolic end products that enhance its flavor-producing properties.

CONCLUSIONS

In aqueous solution, hypoxanthine produced a variety of flavor sensations but was predominantly bitter. Six micromoles per gram of hypoxanthine did not produce significantly detectable flavor differences when added to fresh petrale sole. Taste tests on stored low-dose-irradiated petrale sole fillets with 3 μM per gram of added hypoxanthine indicated that the accumulation of hypoxanthine did not produce significantly detectable flavor differences. If hypoxanthine does contribute to flavor change, it does not contribute noticeably until bacterial counts exceed 10^6 per gram.

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HERBERT STONE
Department of Food Sciences, Stanford Research Institute
Menlo Park, California 94025

The Acidulant Properties of L-aspartic Acid

SUMMARY

Preliminary studies suggest L-aspartic acid possesses acidulant properties of potential use in foods. Present costs are not competitive with citric acid, though special application may exist. The concept of using amino acids as flavoring materials is suggested, with a second role in improving nutritional quality.

In the course of investigating the taste interactions of L-amino acids, L-aspartic acid was found to have interesting acidulant properties at concentrations of less than .01*M* (.01-.003*M*) (Stone, 1965). The "mouth-puckering" effects normally associated with organic acids were not evident, and at lower concentrations other taste properties, such as sweetness and saltiness, were evident. Recent studies on acidulants other than citric acid (malic, fumaric, and adipic acids) suggest that these acids have specific food application (Ough, 1963; Pangborn, 1963). Therefore, this study was undertaken to determine whether L-aspartic acid might possess similar properties.

A trained panel of 5 subjects was used to determine the taste properties of L-aspartic acid. All panel members were familiar with psychophysical procedures and were the same panel members who evaluated the taste interactions of L-amino acids. Citric acid was used as the reference because of its common use in the food industry. The paired-comparison method was used to establish which acid was more sour at equimolar concentrations and at equi-pH. The results (Table 1) indicated that citric acid was more sour at equimolar concentrations (0.1 and .005*M*), while L-aspartic acid was rated more sour at the same pH. However, in this latter case, there was a 10-fold difference in molarity. According to the panel, L-aspartic acid at .01*M* and citric acid between .004 and .0045*M* were equally sour. The fact that citric acid is more sour than *L*-aspartic acid at equimolar concentrations is not surprising in view of the additional carboxyl group in citric acid. The pH difference was approximately .6 unit at .01*M*; however, when the pH of citric acid was matched with that of L-aspartic acid, the latter was more sour. These data suggest that sourness depends more on titratable acidity than on pH. However, both are important factors in determining relative sourness. This tentative conclusion concurs with reports by Amerine *et al.* (1959), Pfaffman (1959), Ough (1963), and Pangborn (1963) that titratable acidity is more important than pH, but that neither can be used as the only basis. A more recent report by Amerine *et al.* (1965) suggests that both are equally important in determining the sensory response to sourness. Although the price of aspartic acid ($2.63–3.73 per

Table 1. Results of the paired-comparison tests for sourness of citric and aspartic acids at equimolar and equi-pH concentrations.

Acid	Molarity	pH	More-sour sample of the pair[a]										
Aspartic	.01	3.12	0[b]	19	0			4	17	19	12	31	
	.005	3.22		1			0						
Citric	.01	2.60	20			20							
	.005	2.79			20	0	20						
	.0045	2.80						8					
	.004	2.88							7				
	.003	2.95								5			
	.002	3.08									0		
	.0013	3.13										1	

[a] Each subject evaluated each pair 4–8 times.
[b] The two numbers refer to the number of times the panel rated that sample of the pair more sour.

pound) is greater than that for citric acid ($.30 per pound), a demand might reduce production cost to some economically feasible level.

The possibility should not be overlooked that other amino acids (both D and L forms) also possess flavoring properties. Experience in our laboratory suggests that many of the L-amino acids do possess such properties, but their application has not yet been demonstrated.

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