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CONTENTS

The Role of Free and Bound Water in Irradiation Preservation: Free Radical Damage as a Function of the Physical State of Water[a]

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SUMMARY

English sole fillets previously equilibrated with aqueous 0.1% cysteine were dehydrated by three methods to moisture levels ranging from 2 to 72%. Model systems using cellulose to replace the fish tissue were also used. The samples were irradiated at 1 Mrad in an air, nitrogen, or oxygen atmosphere. The destruction of -SH groups was measured and related to the amount and physical state of the tissue water. As free water was removed, destruction steadily increased, reaching a maximum at about 20% moisture. Destruction decreased markedly at moisture levels below 10%, and calorimetric measurements confirmed that 10% moisture was about the level of bound water in this species. These data suggest that dehydration favors the reaction of solute molecules with free radicals formed in the free water of muscle cells. At moisture levels greater than about 20%, simple free radical recombination is more likely than reaction with solute molecules, while below 20% moisture the reverse is true. The calculated α values support this conclusion, as do the results from model systems using cellulose.

INTRODUCTION

Water, which accounts for 60 to 85% of the total weight of many animal tissues, is one of the dominant and most important constituents of food. It is generally considered to exist both "bound" and "free," bound water existing as a sheath of hydration held by physical forces to macromolecules and free water acting as the dispersing phase in the protoplasm of cells, as water adsorbed on cell membranes, in capillary spaces, etc. There is, however, no clear boundary between bound and free water in the cell (Kuprianoff, 1958).

The radiation sensitivity within dried cells is about the same as the sensitivity in dried extracts for many enzymes (Pollard *et al.*, 1950). In this case only energy deposition (Compton collisions) by gamma photons directly in macromolecules is effective in producing tissue alterations. In the case of cells in hydrated tissue, the molecules are subjected to an additional form of attack by free radicals and peroxides generated in the cell water. Thus, in ordinary muscle tissue, radiation damage to molecules can occur both by direct action and from oxidative chemical attack by the radicals produced in a surrounding volume of water (indirect action). The radius of the effective volume is determined by how far the free radicals can diffuse from their site of formation. The maximum diffusion distance has been variously estimated to be between 30 and 100 Å (Hutchinson, 1957). This corresponds to a distance of about 10 to 30 water molecules. However, there is evidence that much of the water of hydration of protein molecules (bound water) is highly ordered and may exist in a crystalline form (Klotz, 1948; Wood and Rosenberg, 1957). This would tend to make the sensitive volume larger, since a gamma photon could transfer its energy directly to the macromolecule if it interacted with this layer of bound water (Bacq and Alexander, 1961).

The dependence of irradiation damage on the concentration of aqueous solutions of

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proteins, etc., has been known for many years and has been used as a measure of the relative importance of the direct and indirect effects. In intact tissues, however, little information is available. Sparrman *et al.* (1959) irradiated seeds of *Agrostis stolonifera* having water contents ranging from 5 to 20% and reported that a minimum amount of damage occurred at about 15% moisture. More damage occurred at both greater and lesser amounts of water.

Considerations such as these indicate that in irradiation pasteurization both the amount and physical state of water in muscle tissue (or in the cells of microorganisms) are likely to be important, and that the process will probably be more successful if the role of water in its different forms is more completely understood. The present study is aimed at elucidating the role of bound and free water as sources of and vehicles for free radicals in the irradiation pasteurization of fisheries products.

METHODS

English sole (*Parophyrus vetulus*) fillets were cut into pieces about 1 cm^3 and dehydrated to various residual moistures in the range of about 2 to 70% by three methods: 1) the wet-freeze drying method of Mueller and Szent-Gyorgy (1957) at −50°C; 2) the use of commercial freeze-drying equipment; and 3) ethanol-water mixtures in the range of 5 to 70% water. An attempt was made to use changes in water-holding capacity as measured by the press method or by the ATP-induced swelling of myofibrils as an index of irradiation damage (Lawrie *et al.*, 1961). However, under pasteurizing conditions, these effects were too small to be useful indices. The destruction of —SH groups was chosen as a measure of irradiation damage for two reasons: First, because compounds of sulfur are implicated as important components of irradiation odors and flavors (Hedin *et al.*, 1960; Colby *et al.*, 1961; Martin *et al.*, 1962) and, second, because the radiolysis of —SH compounds in cells is well established (Gordy *et al.*, 1955). Because the free —SH content in this species of fish is low, it was necessary to add —SH groups to the tissue, in the form of cysteine prior to irradiation. This was done by either: 1) equilibrating the fresh tissue with a 0.5% aqueous cysteine solution containing a few drops of chloroform for 24 hr at 0°C under a nitrogen atmosphere; or 2) in the case of the alcohol dehydration procedure, saturating the dehydrating solution with cysteine prior to the equilibration. This resulted in a cysteine concentration in the tissue of 10^{-4} to 10^{-3} molar. The residual acetone from method one and alcohol from method two were removed by holding the tissue in light petroleum ether at 0°C for 24 hr, decanting the petroleum, and then evaporating the residual solvent, using a stream of nitrogen or a vacuum desiccator. The samples were analyzed for —SH content both before and after irradiation, using both a modification of the nitroprusside method of Batzer and Dotty (1955) and, in later cases, the *N*-ethylmaleimide method of Siggia (1962). The irradiation was carried out using a dose of one megarad from a Co^{60} source and either an oxygen, an air, or a nitrogen atmosphere.

In addition, model systems using cellulose in place of muscle tissues were used. These were made up with water contents ranging from 80 to 9%, with 10^{-4} moles of cysteine present in each sample. In certain models 0.1% protein (as papain) was added. Bound water was determined in both the model systems and the fish tissue, using a calorimetric method similar to that used by Wood and Rosenberg (1957).

Specific heat determinations were made, using the method of Robinson (1931).

Moisture determinations, total nitrogen, and ash determinations were performed, using AOAC procedures.

RESULTS AND DISCUSSION

The data relating water content of fish muscle tissue to irradiation damage, as measured by —SH oxidation, are presented in Fig. 1. It can be seen (curve A) that as

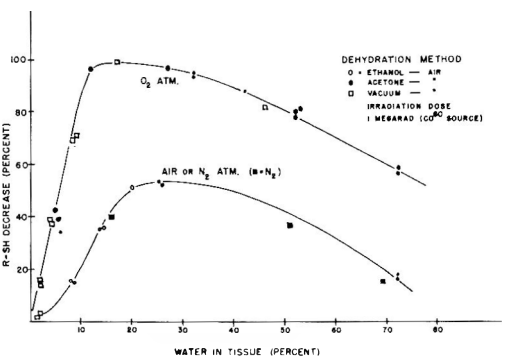

Fig. 1. —SH destruction as a function of the water content of fish muscle tissue.

the water content of the tissue decreases, there is a progressive increase in -SH destruction, which reaches a maximum of

greater than 95% at the 20% moisture level. It is also evident that below about 12% moisture there is a sharp decrease in damage, and below about 6% moisture an actual protection occurs (compared to fresh tissue). A reasonable explanation of the shape of this experimental curve from about 12 to 70% moisture may be the following:

The radiolysis of water is considered to be represented by:

$$H_2O \overset{\gamma}{\leftrightarrows} H \cdot + OH \cdot \overset{\text{solute}}{\longrightarrow} \text{oxidized products.}$$

As more and more water is removed from the tissue, solute concentrations increase correspondingly and the probability of simple free radical recombination becomes progressively smaller. This leads to increased oxidation of solute, even though the absolute number of free radicals formed is probably smaller. Supporting evidence for this explanation is given in Fig. 2.

The equation $D_c = \frac{D_o}{1 + \frac{\alpha}{c}}$, where D_c is the D_{37} dose at solute concentration c, D_o is the D_{37} dose for direct action, and α is the ratio of energy for inactivation by indirect action to the energy for inactivation by direct action, can be used to express irradiation effects (Bacq and Alexander, 1961). Fig. 2 presents a plot of α vs. the concentration of water in the muscle tissue. It is seen that the energy for inactivation by the indirect effect reaches about ten times that for the direct effect, as water is removed to the 30% level, implying that free radical recombination is indeed becoming less and less likely. At the bound water level, α becomes zero, which implies that little free radical attack is occurring, *i.e.* the direct effect is predominant. These conclusions are consistent with those of Samuel and Magee (1953), who calculated that H. and OH. free radicals, when formed, are separated by as much as 150 Å. Hence, free radical recombination would be an infrequent event, even in dilute solutions.

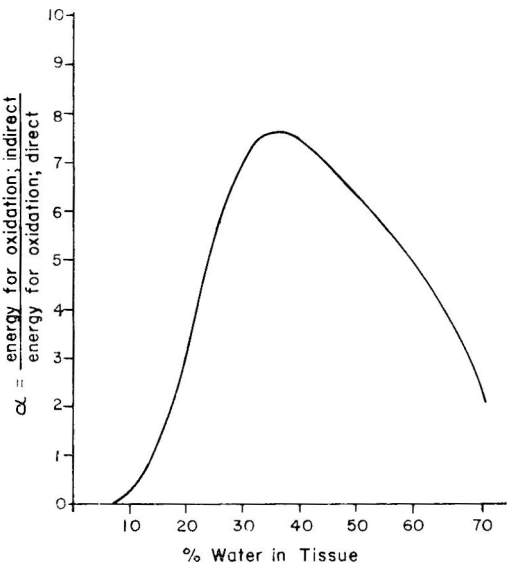

Fig. 2. Calculated values of α as a function of the water content of fish muscle.

$$D_c = \frac{D_o}{1 + \frac{\alpha}{c}}$$

Curve B is similar except that a much lower level of damage occurs, probably due to the presence of fewer $.OH_2$ free radicals and O_2^- ions, and the decrease in –SH oxidation begins at about 20% moisture instead of at 10%. Substantial protection does not occur (compared to fresh tissue) until the tissue is dehydrated to about the 10% moisture level. A few samples were irradiated, using a nitrogen atmosphere. As expected, a still lower level of destruction occurred.

A consideration of the remaining part of the curves (0 to 10% moisture) reveals that the destruction of –SH groups in this region can be due to: 1) "Direct hits"; 2) water-generated free radicals and peroxides; and 3) direct energy transfer from macromolecules. Extrapolation of the curves to 0% moisture indicates that only about 2% of the –SH oxidation occurring is due to direct action. Hence it appears that most of the destruction occurring in the 0 to 10% moisture region of the samples is due to energy transfer from macromolecules, water-generated free radicals and peroxides. The amount of bound water in the muscle tissue of this species was measured, using the calorimetric method previously referred to. About 10% of the total water (0.57 g H_2O/g protein) was found to be bound. This indicates that the irradiation damage occurring in the below 10% moisture region is due

to bound-water-generated free radicals and peroxides and to energy transfer from macromolecules. Although energy transfer has been shown to occur from proteins to added thiol compounds (Norman and Ginoza, 1958), there is no quantitative information available concerning the magnitude of this effect *in vivo*.

These results suggest that both energy transfer and bound-water-generated free radicals are of relatively minor importance in fresh tissue. Only the free water of the fish muscle appears to be of major importance as a free radical and peroxide source, although bound water perhaps participates to a limited extent.

These findings also imply that dehydration prior to irradiation will only increase irradiation damage unless very low moisture levels are obtained, and may explain the findings of Schultz *et al.* (1956), who reported that dehydration to about 6% moisture, followed by irradiation, resulted in a product indistinguishable from undehydrated controls.

The data from the model systems are presented in Figs. 3 and 4. Fig. 4 shows that

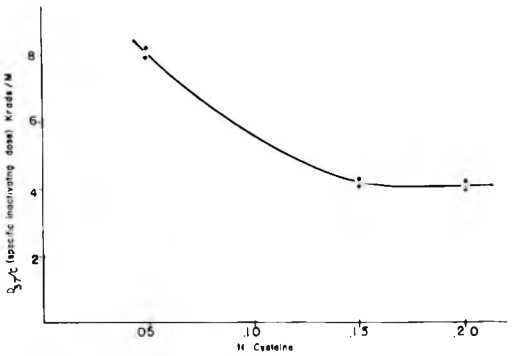

Fig. 3. Oxidation of cysteine in water saturated with air.

the specific inactivating dose (i.e. $D_{37}/_c$) rises sharply as the percent water increases past about 16%. This indicates that free radical recombination is occurring more and more as water is added, a conclusion in full agreement with the previous statements. At less than 16% water, free radical recombination has become unimportant. Using the calorimetric method, the bound water in the

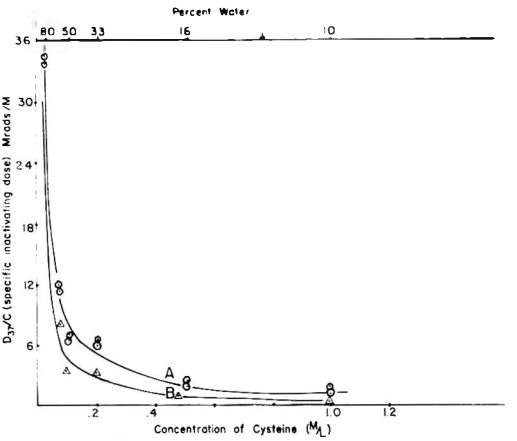

Fig. 4. Oxidation of cysteine in a water-cellulose system containing 10^{-4} moles cysteine. *A*, no added protein; *B*, 0.1% added protein (as papain).

model system was measured. About 12% of the water was bound. Curve *B* shows that in the presence of added protein, the specific inactivating dose is somewhat less than before, perhaps due to energy transfer effects.

Fig. 3 shows that free radical recombination in aqueous cysteine systems begins at about 2×10^{-4} molar cysteine or less.

In summary, the model systems appear to support the data obtained using fish muscle tissue.

The existence of maximum –SH oxidation at about 20% moisture has possible implications in terms of replacing the chemical oxidizers now used as "improvers" in the flour industry with irradiation processing. It is thought that the action of such improvers is due to –SH oxidation (Hird and Yates, 1961; Frater *et al.*, 1961). However, it should be noted that not all varieties of wheat show strengthening of gluten structure upon irradiation (Cornwell, 1959). Milner (1963) has emphasized that not only is very little known of changes in the sulfhydryl groups in irradiated wheat proteins, but also the action of chemical oxidizers may change the gelation of polysaccharides, as well as proteins.

It should be noted that, contrary to appearances, the slopes of these curves do not indicate that indirect free radical attack is responsible for the –SH destruction at less than 20% moisture (Fig. 1). The dilution tests commonly performed to distinguish

direct from indirect effects are done by varying the solute concentration while keeping the total amount of water constant. This cannot be done in muscle tissue.

Dehydration by freezing should also offer protection, but conflicting information has been reported (Schultz *et al.*, 1956; Coleby *et al.*, 1961). Further studies are under way to clarify the role of water at low temperature in irradiation pasteurization. These will be carried out in terms of the amount of water frozen as a function of temperature in fish muscle tissue and its intra- or intercellular location, the influence of these factors in free radical production, and the destructive potential of frozen free radicals, both as a function of time and their sites of formation in intra- or intercellular ice.

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Autoxidation of Turkey Lipids[a]

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SUMMARY

Autoxidation of lipids in raw, ground turkey tissues was studied with the 2-thiobarbituric acid (TBA) method. Samples prepared immediately post-mortem and held at 4°C for approximately one week showed significant differences between the rates of autoxidation for certain of these tissues, notably red and white muscle. Failure to detect other differences, as between skin and white muscle, reflects variation in both lipid and fatty acid composition. A significant delay of autoxidation was achieved by the addition of 1% egg albumen solids (w/w), 0.04% butylated hyldroxyanisole, or 0.35% phosphates to the ground tissue; 1% gelatin exhibited no beneficial effect. Possible mechanisms by which these materials delay oxidative changes are discussed.

INTRODUCTION

Rapid expansion in turkey production, coupled with seasonal consumption of that meat, makes it necessary to store frozen turkeys for periods up to one year. Of the biochemical changes occurring during storage, a prominent one involves autoxidation of lipids which may be detected as rancid flavor in the cooked product. Keskinel *et al.* (1964) studied oxidative changes in turkey, beef, lamb, and pork. Those workers showed that after one week of refrigerated storage, turkey had undergone greater oxidative change than the other meats. Rapid autoxidation observed in turkey can be explained in part by fatty acid composition (Lea, 1938, p. 212), and the presence of low levels of natural antioxidants (Mecchi *et al.*, 1956a,b) in the tissue.

The present investigation was undertaken to establish the rate at which autoxidation occurs in raw turkey tissues and to measure the "antioxidant" properties of egg albumen solids, butylated hydroxyanisole (BHA), gelatin, and phosphates when added to tissue.

EXPERIMENTAL

Materials. Adult Broad Breasted Bronze male turkeys were used in these studies. Muscle samples were taken 1 hr post-mortem, freed of skin and adhering fat, and passed once through a food grinder (4-mm perforations). The ground tissue and any additive such as albumen solids were blended in a closed plastic container from which samples of approximately 10 g were taken. The samples were overwrapped with MSAD-80 cellophane, heat-sealed, and stored at 4°C for periods up to 10 days.

Analytical. The 2-thiobarbituric (TBA) test as described by Tarladgis *et al.* (1960) was used to follow the rate of oxidative change in samples. Duplicate samples of meat were removed from storage daily for TBA determination. Following the distillation step, duplicate 5-ml quantities of each distillate were taken for color development.

Total lipids were measured by Goldfisch extraction using a solvent mixture of diethyl ether, Skellysolve B, and ethanol (5:5:1 v/v).

Statistical. A regression analysis was applied to the data (Snedecor, 1956) in which "TBA number" was regressed on storage time (days).

RESULTS AND DISCUSSION

Red vs. white muscle. Fig. 1 shows the extent of lipid oxidation in red and white muscle during refrigerated storage. The difference, which is highly significant ($P < 0.01$), confirms the results of Keskinel *et al.* (1964), except those authors reported a curvilinear relationship between mg of malonaldehyde per 1000 g of sample (TBA number) and storage time. Based on the variation in total lipids in samples of white and red muscle (1.59 and 3.64%, respectively), a positive relationship exists between amount of total lipid and oxidation rate. Moreover, it is likely that heme catal-

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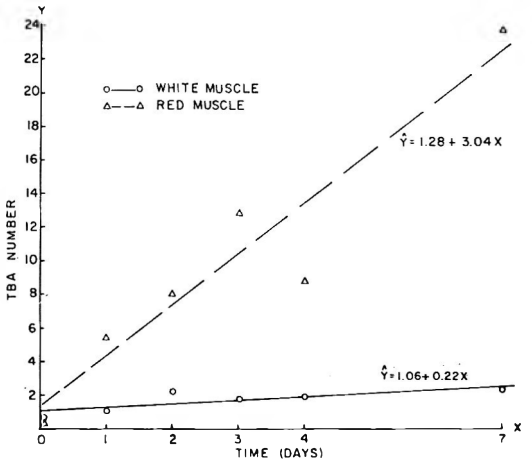

Fig. 1. Autoxidation of lipids in red and white turkey muscle at 4°C.

ysis of oxidation (Barron and Lyman, 1938; Tappel, 1953) is more active in red muscle, which contains a larger amount of myoglobin than does white muscle. Lawrie (1950) reported a ninefold difference in the amount of myoglobin between chicken gastrocnemius and pectoralis superficialis (0.045 and 0.005%, respectively).

Varied proportions of red and white muscle. Ground, raw muscle was studied in the following proportions: 1) 100% white muscle (WM); 2) 75% WM and 25% red muscle (RM); 3) 50% WM and 50% RM; 4) 25% WM and 75% RM; 5) 100% RM. The statistical analysis showed that a significant increase ($P<0.05$) occurred in oxidation rate with each increment of red muscle to the mixture up to the combination of 75% RM and 25% WM; no difference was found between this combination and red muscle alone (see Fig. 2). From these results it is apparent that in a coarsely ground mixture of red and white muscle, the effect of the former is merely additive. There is little chance for hematin compounds and lipids from red muscle to be introduced into white muscle to catalyze oxidative changes.

Comparison of various tissues. Fig. 3 presents the combined results of two experiments on lipid oxidation in six tissues. As before, the difference between white and red muscle was statistically highly significant ($P<0.01$). There was no significant change in TBA numbers for liver over the test

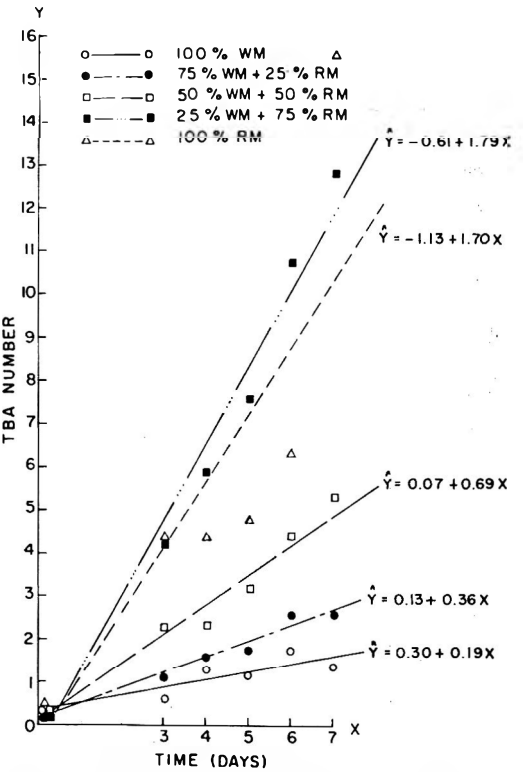

Fig. 2. Autoxidation of lipids in mixtures of red and white muscle at 4°C.

period. Skin, high in total lipids, and gizzard, which is composed of highly pigmented muscle, exhibited relatively low TBA numbers; these patterns did not differ from that for white muscle. From these results it can be inferred that heme catalysis, total lipids and/or fatty acids are important in lipid oxidation. Moreover, the TBA method may be limited in its use on tissues that vary widely with respect to lipid groups and fatty acids. Other work in our laboratory shows that in the 16-week-old turkey male, phospholipids comprise about 60% of total lipid in red or white muscle, and as high as 90% in heart muscle. More research is needed on the quantitative involvement of these complex lipids in oxidation.

"Antioxidant" properties of phosphates. When phosphates (added as "Kena," which contains mostly sodium tripolyphosphate, with lesser amounts of tetrasodium pyrophosphate and disodium dihydrogen pyrophosphate) were added to red muscle at 0, 0.175, 0.35, or 0.70% levels, the response was as shown in Fig. 4. A statistical com-

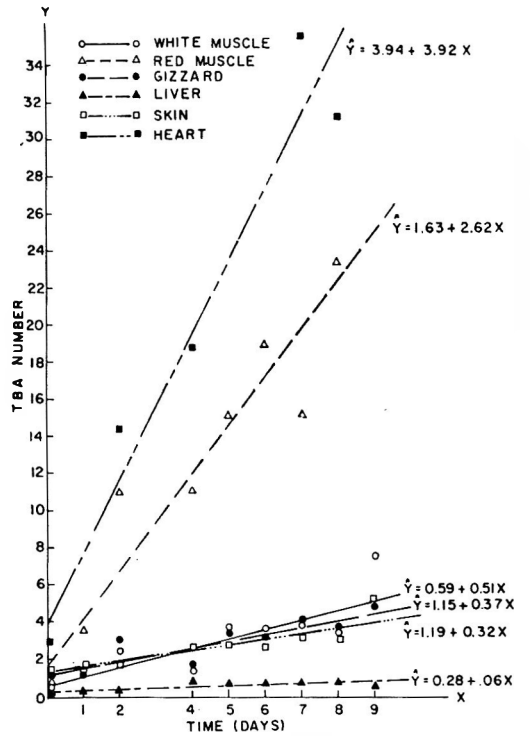

Fig. 3. Autoxidation of lipids in various turkey tissues at 4°C.

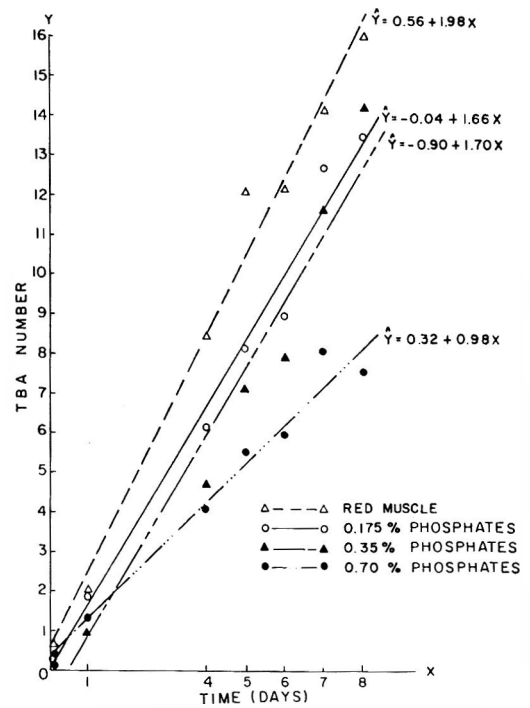

Fig. 4. The influence of tripolyphosphates (see text for composition) on lipid oxidation in red muscle at 4°C.

parison of the regression (b) values indicated that the 0.175%- and 0.35%-treated samples did not differ significantly in rate of lipid oxidation, but both differed from the control and 0.70%-treated samples ($P < 0.05$). There was a slightly different treatment response observed in white muscle compared with red. The lowest level of phosphates had no influence on oxidation rate, whereas the 0.35% level reduced oxidation rate significantly, and the 0.70% level exhibited an even greater effect.

"Antioxidant" properties of different materials. Fig. 5 shows the average response to adding 1.0% egg albumen solids, 0.04% butylated hydroxyanisole (BHA), 1.0% gelatin, or 0.35% phosphates to red and white muscle. Each of the materials, except gelatin, significantly delayed oxidative changes. These results agree with those of Chang *et al.* (1961), who reported that tripolyphosphate delayed oxidation in beef lipids. Further, Bishov *et al.* (1960, 1961) and Togashi *et al.* (1961) reported that albumen solids and gelatin reduce oxidation rate of fat films in a model system. Similarly, Klose *et al.* (1952) have shown that BHA combined with citric acid is effective in delaying rancidification in frozen turkey steaks and half-turkeys.

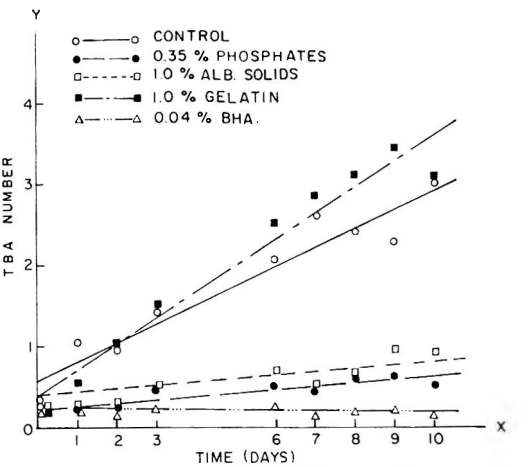

Fig. 5. The average response of adding tripolyphosphate, albumen solids, gelatin, and butylated hydroxyanisole (BHA) on lipid oxidation in white and red muscle at 4°C.

Mechanisms of antioxidant activity. Considerable effort is needed to elucidate prop-

erly the mechanisms of antioxidant activity reported here. Whereas with BHA a proposed mechanism involving the transfer of hydrogen or electrons is tenable, it is probable that albumen solids are active in sequestering cations that would otherwise be available for catalysis. Conalbumin is suspected as the active constituent. However, during repeated experiments in which albumen from the same originally lyophilized batch was used, there was an apparent disappearance of the "antioxidant" characteristic. The exact time required for its disappearance has not been established, but appears to be about 6–8 weeks. Nevertheless, two pertinent questions arise: What biochemical property of albumen is active, and what are the reasons for this loss of activity? Bacterial growth leading to the ultimate saturation of conalbumin with iron is excluded as a possibility since the albumen solids were held in a desiccated ($CaCl_2$) atmosphere.

The significant protection afforded frozen turkey steaks by a 0.2–0.4-mm layer of gelatin as reported by Klose *et al.* (1952) is explained largely by a physical protection of the steak's surface whereas gelatin added to ground tissues at a 1.0% rate has less chance of protecting exposed surfaces.

Compositional differences that exist between albumen and gelatin may contribute to an explanation of the difference in activity. Gelatin contains no tryptophan and a relatively small amount (0.7%) of histidine (Tristram, 1953) both of which have been shown to possess antioxidant activity (Tappel, 1954). Finally, the sulfur-containing amino acids of albumen (Evans *et al.*, 1949) may contribute to its activity, since sulfhydryl groups are generally active in this respect.

Less can be proposed on the mechanism of phosphate activity. In these studies, pH increased by about 0.5 unit when phosphates were added to meat. Such a small change in pH *per se* does not explain the beneficial action imparted to lipids (Chang *et al.*, 1961) since this change can be achieved with different bases, which in turn do not inhibit lipid oxidation. A more tenable speculation is that the phosphates act by forming complexes with fatty acids or their derivatives. It is known that phosphoric acid will complex peroxides (Privett and Quackenbush, 1954).

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Measurement of Discoloration in Fresh Beef[a]

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SUMMARY

A method is described whereby precise measurement of discoloration in fresh red meats can be achieved. The method utilizes *a* values obtained from Gardner automatic color-difference meter readings and depends upon careful control of storage temperature, control of sample area from which readings are taken, and consistent orientation of sample during subsequent readings.

Results obtained with the method indicate considerable variation in discoloration of samples from the same slice of beef round stored at 6°C; a substantial decrease in discoloration with lowered storage temperature; a decrease in discoloration resulting from increased exposure of the sample to air; and the probable importance of respiration by the meat rather than bacterial contamination in determining discoloration at low temperatures (−2°C).

INTRODUCTION

Fresh red meats, cut and packaged for self-service retailing, undergo a discoloration during storage. The surface of the meat turns brown as a result of oxidation of the bright-red oxymyoglobin to brown metmyoglobin.

Early studies on discoloration of red meat were made by Brooks (1929), who observed that methemoglobin forms first beneath the surface of the meat. The experimental system used by Brooks consisted of a piece of meat compressed between two pieces of glass so that the edges of the meat sample were exposed to air. The depth of penetration of oxygen and the formation of methemoglobin were observed spectroscopically. The finding that methemoglobin forms initially beneath the surface of the meat was given meaning by studies of Brooks (1935), on hemoglobin, and George and Stratmann (1952), on myoglobin, showing that both heme pigments autoxidize most rapidly at partial pressures of oxygen considerably below that present in air.

In most studies subsequent to those of Brooks, emphasis has been placed on measurement of color at the surface of the meat.

Kraft and Ayres (1954) and Pirko and Ayres (1957) used the reflectance attachment of the Beckman DU spectrophotometer for measuring color changes in red meats. Butler *et al.* (1953) and Robach and Costilow (1961) used the Munsell spinning disc, and Dean and Ball (1960) used the Gardner color-difference meter. In addition, Mangel (1951), Broumand *et al.* (1958), and Butler *et al.* (1953) used a technique by which the percent metmyoglobin in aqueous extracts of meat can be determined spectrophotometrically. Dean and Ball (1960) developed a method, based on reflectance spectrophotometry, designed to give quantitative data on metmyoglobin, oxymyoglobin, and myoglobin at the meat surface.

Data from previous studies show that meat discolors because of metmyoglobin formation, and that decreased partial pressure of oxygen, inoculation of meat with bacteria, and increases in temperature are factors that increase the rate of discoloration. Available techniques are not sufficiently precise to give a clear understanding of the rate at which meat discolors. For example, if bacteria are an important factor in meat discoloration, the rate of discoloration should increase as numbers of

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bacteria increase. In contrast, if the discoloration were due to autoxidation of myoglobin, the initial rate would decrease as less myoglobin becomes available for autoxidation.

This study was undertaken to determine if a method could be devised whereby color measurements would be sufficiently precise to give information on the causes of metmyoglobin formation. A technique is described that shows promise in measuring rates of discoloration of fresh red meats. Results obtained with the technique are discussed.

EXPERIMENTAL

The Gardner automatic color-difference meter, model AC-1, was used in making color measurements. See Hunter (1958) for a description of the instrument and scales. Slices of beef round were purchased in a local market, and in each instance the slices were freshly cut from an intact round (U. S. Choice). Samples for experiments were cut to fit open, rigid, plastic containers 2 × 1½ × ½ inches.

For preparing meat samples in which the myoglobin was predominantly MbO_2 (oxymyoglobin), Mb (reduced myoglobin), or Mb^+ (metmyoglobin), the meat was overwrapped first with an O_2-impermeable film (Saran) taking precautions to minimize oxygenation of the myoglobin. The samples were stored at room temperature and when *Rd*, *a*, and *b* values no longer were changing rapidly (about 4 hrs.), the myoglobin was considered to be in the reduced deoxygenated state, and *Rd*, *a*, and *b* values were obtained. The Saran film was then removed, replaced with 195-MSAD-80 cellophane, and the samples stored at 0°C for 24 hr. After this time the myoglobin at the surface was predominantly oxygenated, and again *Rd*, *a*, and *b* values were obtained. For production of metmyoglobin, several drops of 0.2% potassium ferricyanide were spread over the surface of the samples at hourly intervals. After three applications the samples were rewrapped and stored for 4 hr at room temperature. Then *Rd*, *a*, and *b* values were again measured. The procedures outlined above, for altering samples so that they contain predominantly Mb, MbO_2 or Mb^+ at the surface, have also been monitored by obtaining reflectance spectra of the samples using a Beckman DK-2A spectroreflectometer. These spectra confirm that the procedures produce the desired derivative.

In the storage experiments, samples were allowed to "bloom" for approximately 1 hr at the storage temperature prior to wrapping with 195-MSAD-80. Initial color measurements were made soon after wrapping, and at approximately 8-hr intervals thereafter until the meat was grossly discolored. Samples were stored in the dark in a freezer chest to obtain temperature stability of ±1°C. Defrost cycles did not interrupt the temperature control in the freezer chest, and by using a closed box, atmospheric conditions had little effect on the storage temperature.

Prior to the measurement of color, the Gardner color-difference meter was allowed to warm up for 15 minutes and was standardized with a light-red color standard, $Rd = 33.8$, $a = 34.4$, $b = 12.9$. The transparent plastic spacer was masked with black tape so that the sample exposure area was kept constant and no external light could reach the photocells. The sample exposure area was equivalent to the area of the sample containers. To make the Gardner readings as consistent as possible, samples were oriented in the same direction each time readings were made. Rotating the samples 180° was found to change the *a* value by 1 unit for some samples. The samples were thick enough so that changes in background had no effect on the *Rd*, *a*, or *b* values.

RESULTS AND DISCUSSION

The major changes one would expect to find in the forms of myoglobin present in meat are first an oxygenation of Mb to yield MbO_2 and secondly an oxidation yielding Mb^+. To determine how *Rd*, *a*, and *b* values would be affected by oxygenation and oxidation of myoglobin, meat samples were cut and packaged such that Mb, MbO_2, or Mb^+ would predominate. Results of color measurements are shown in Table 1. These results show that *a* values change appreciably in relation to the other two parameters of color. Furthermore, in changing from MbO_2 or Mb to Mb^+ *a* values decrease. Using *a* values only, it would be difficult to determine whether the myoglobin in a meat sample was oxidizing to Mb^+ or deoxygenating to Mb, since both changes represent a decrease in *a* value. However, the two types

Table 1. Average *Rd*, *a*, and *b* values for 18 meat samples stored such that myoglobin, oxymyoglobin, and metmyoglobin predominate.

	Rd	*a*	*b*	*a/b*
MbO_2	8.0±1.0[a]	28.6±1.3	13.8±0.6	2.1
Mb	8.0±1.2	13.7±0.7	7.6±0.6	1.8
Mb^+	8.7±1.2	4.1±0.3	10.6±0.6	0.4

[a] Standard deviation.

of change can be distinguished by considering the a/b ratio. For MbO_2 and Mb, which are both fresh meat colors, the a/b ratio does not change appreciably, but for a conversion of MbO_2 or Mb to Mb^+ the a/b ratio decreases considerably. The meat discolorations discussed in this paper were due to changes in myoglobin of the type MbO_2 or Mb going to Mb^+. In this type of discoloration there is considerable change in a value but relatively little change in Rd or b values (Table 1). Although the change in a value is considered to be the most sensitive indicator of color changes in fresh beef, the a/b ratio has also been plotted in Figs. 3 and 4 to indicate that the color change is due to Mb or MbO_2 being oxidized to Mb^+.

Fig. 1 shows a values plotted against time for three samples stored at 6°C. The data of Fig. 1 show that there is a fairly regular decrease in a values for any one sample which results in a smooth curve, a values begin to decrease immediately upon storage, and there is considerable heterogeneity within one slice of round steak.

The heterogeneity shown in Fig. 1 is of interest because experiments are frequently designed to test relations between color deterioration in meat samples and some other factor such as bacterial numbers or percent oxymyoglobin. With considerable differences in the color of the meat samples and in the rates at which they change color, it would be difficult to interpret the results of experiments attempting to correlate color changes with other factors.

The data of Fig. 1 were chosen to show the extreme heterogeneity which can and does occur occasionally. However, the results shown in Fig. 2 are more typical of those obtained with meat samples stored at 6°C. As shown in Fig. 2 samples from the semitendinosus had higher a values initially than did samples from the other muscles in the round. This difference was noted quite often when samples were stored at 6°C but was not so apparent at lower storage temperatures.

To investigate the effect of temperature on color deterioration, samples from beef rounds were stored at 2°C and −2°C, with all other conditions kept the same. Fig. 3

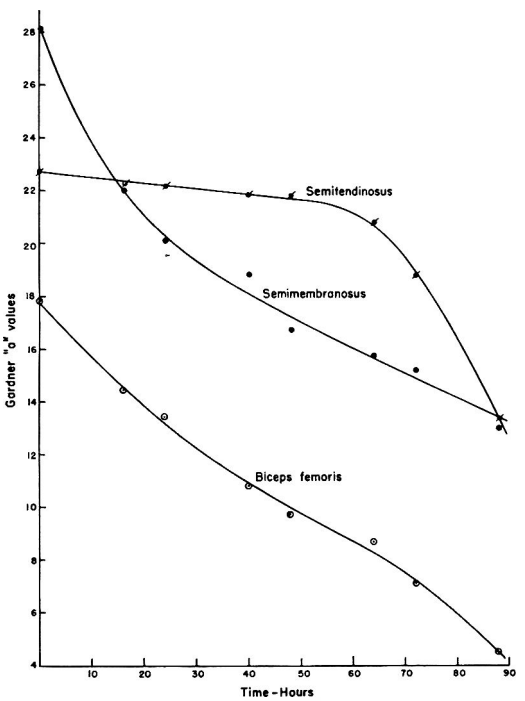

Fig. 1. Color deterioration curves for three samples of beef round all taken from the same slice of beef round. Storage temperature 6°C.

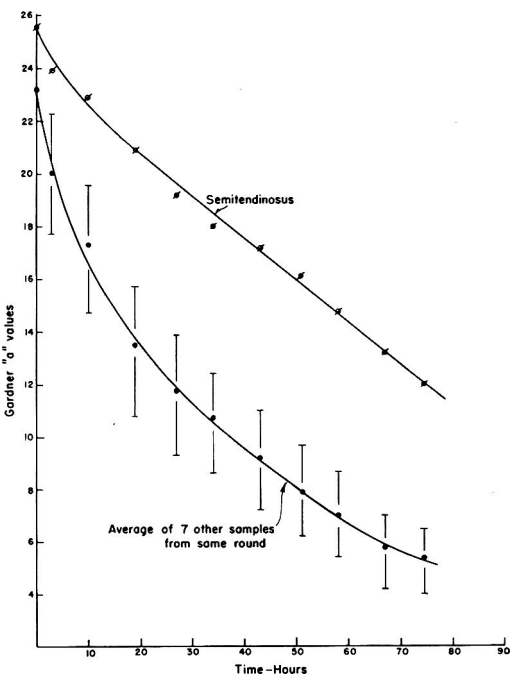

Fig. 2. Color deterioration of meat samples taken from a single slice of beef round. Storage temperature 6°C.

shows the effect of 6, 2, and −2°C temperatures on the discoloration of the beef samples. Two effects of temperature are apparent in Fig. 3. At −2°C, initial *a* values are considerably higher than at 2°C or 6°C. The increase in *a* values with lowering temperature has been noted consistently in these studies. The second effect is a decrease in

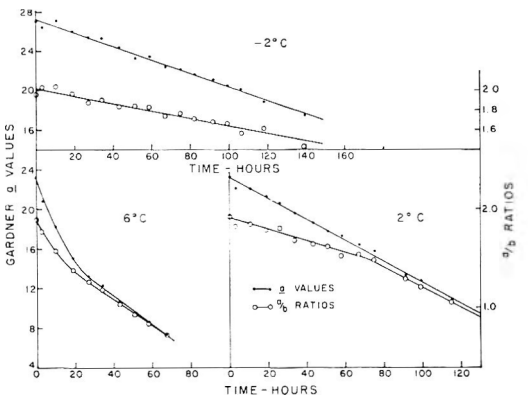

Fig. 3. Effect of temperature on the color deterioration of fresh beef. Each curve represents the average for 16 samples taken from two adjacent slices of beef round.

rate of discoloration with decrease in temperature. Although the departure from linearity prevents calculation of rates for most of the curves in Fig. 3, it can be seen that *a* values decrease more rapidly at 6°C than at 2°C. For the two curves at 2°C and −2°C which are linear, the calculated rate of decrease for *a* values is 0.13 *a*/hr at 2°C and 0.064 *a*/hr at −2°C.

As recently shown by Robach and Costilow (1961), deterioration of meat color is highly dependent upon the partial pressure of oxygen in the meat. Several factors have an influence on the partial pressure of oxygen in stored meat. Bacterial growth can deprive the meat of oxygen; the bacteria utilize oxygen for respiration and thereby decrease the amount available for diffusion into muscle tissue. Respiration by the meat is significant in decreasing the partial pressure of oxygen. As Brooks (1929) pointed out, when meat discolors, the metmyoglobin appears first in a layer a few millimeters below the surface, indicating an area in which the partial pressure of oxygen has been lowered because of respiration by the meat. Also, the amount of oxygen in solution in the meat is influenced by the storage temperature.

Lowering the storage temperature has a favorable effect on all of the factors listed above with regard to maintaining a high partial pressure of oxygen in the meat. A lowered temperature may decrease the residual bacterial flora. A lowered temperature decreases the respiration of the meat itself, thereby increasing the depth to which oxygen may penetrate and decreasing any heat production due to respiration. A lowered temperature increases the amount of oxygen dissolved in fluids of the meat. A lowered temperature shifts the equilibrium, $Mb + O_2 \rightleftarrows MbO_2$, to the right. Finally, a lowered temperature decreases the rate of autoxidation of myoglobin to metmyoglobin. Hence, decreasing the storage temperature has multiple beneficial effects for maintaining oxymyoglobin in fresh meats.

It should be quite simple to experimentally increase the partial pressure of oxygen in meat samples and test the importance of this factor on the discoloration. Preliminary experiments with meat stored in the presence of an atmosphere of oxygen indicated that discoloration was slower than with samples stored in an atmosphere of air. However, it is clumsy to provide a storage atmosphere of oxygen and still remove samples periodically for color determinations, therefore samples were packaged and stored so that all surfaces of the meat sample were exposed to air. This treatment effectively increased the partial pressure of oxygen in the sample and the results of such a treatment are shown in Fig. 4. The rate of

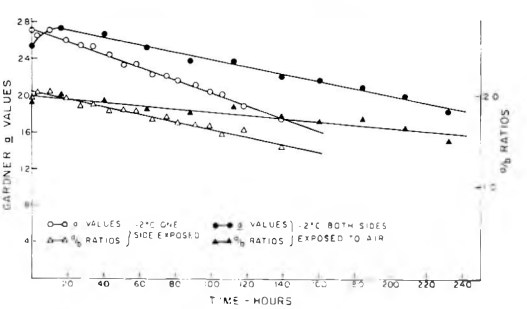

Fig. 4. Effect of increased exposure to air on the color deterioration of fresh beef. Each curve represents the average for 16 samples taken from two adjacent slices of beef round.

discoloration (rate of decrease in *a* value) calculated for the straight-line portion of the curve representing samples with two sides exposed was 0.040 *a*/hr. For comparison, the color deterioration curves of beef samples with only one side exposed and stored at the same temperature (−2°C) are included in Fig. 4.

A technique of packaging and storing meat with maximum surface exposed to air may be of some limited usefulness in storing and retailing fresh meats. The important point, however, is that meat discoloration was retarded by increasing partial pressure of oxygen. From Fig. 4 it can be seen that the major difference between samples with one side and two sides exposed to air is in the rate of change of *a* value rather than a large difference in initial *a* value. The latter result might be the one expected from the technique used, but the fact is that the rate of discoloration was decreased as a result of increased partial pressure of oxygen in the samples. The decrease in rate of discoloration due to exposure of both sides of samples was found at −2°C but not at higher temperatures. Presumably, a volume of meat remains in the reduced state in the interior of samples stored at higher temperatures. Consequently, exposure of more surface area does not change the partial pressure of oxygen at the point where Mb^+ is forming.

The findings that meat samples discolor at −2°C and that the discoloration starts immediately tend to discount the role of bacteria at this temperature. Probably, a more important factor at low temperatures is respiration of the meat. The decreased rate of discoloration noted in samples packaged with both sides exposed to air, as compared with samples packaged in rigid plastic containers, indicates the importance of oxygen utilization by the meat in determining the subsequent rate of discoloration.

The color life of fresh red meats can be extended appreciably by keeping the storage temperature low and by providing for maximum contact of the meat surfaces with air. Any further decrease in discoloration rates appears to depend upon decreasing the respiratory activity of the meat.

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Physicochemical Changes in Some Frozen Foods

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SUMMARY

The pH of peas and of poultry meat frozen pre- and post-rigor was measured during frozen storage at -10°C for up to 6 months. In peas it decreased sharply from 6.7 to as low as 6.0 during the first 3 days of storage, increased to 7.0 during the next 2–3 weeks, decreased to 6.4 in another 3 weeks, and remained there with only small fluctuations during the rest of the storage time. Breast and leg meat of poultry resembled each other in pH changes after freezing: increases and decreases of about 0.2–0.3 unit occurred in all samples at about the same time. Meat frozen pre-rigor differed from meat frozen post-rigor, however, the latter increasing 0.2–0.3 pH unit during freezing, and the former changing little or decreasing slightly under these conditions. Differences in pH between samples at a given time were related to differences in initial pH.

Studies with salt solutions as similar as possible in composition to the foods tested, and with gelatin solutions, showed that pH changes in frozen foods were caused mainly by increasing concentration of food components, including proteins, in the unfrozen phase, by precipitation of salts, by interaction of proteins with ionic substances, and by enzymatic activity (e.g. lactic acid formation) during frozen storage.

INTRODUCTION

Extensive information has been published in recent years on quality deterioration during the freezing and frozen storage of foods, particularly in connection with time-temperature tolerance studies (see Van Arsdel, 1957). This information has provided practical knowledge about the proper methods of processing, storage, and handling of many frozen foods. In several instances, the quality deterioration can be attributed to enzymatic and chemical reactions. In many others, however, the exact nature of the deteriorative reaction cannot be determined with certainty, nor can the factors causing or affecting these reactions. One basic difficulty appears to have been the limited information available on the mechanical, chemical, and physicochemical properties of frozen foods, particularly of the unfrozen phase. The formation of the ice phase as affected by factors such as type of material, rate of cooling, and ultimate temperature, as well as the effect of ice precipitation on the macroscopic and microscopic appearance and structure of the frozen material, has been studied in some detail, but these studies have thrown little light on the processes occurring simultaneously with, or subsequent to, the removal of water by ice formation.

Recent studies in these laboratories have dealt with one of the processes consequent on freezing, namely, the crystallization of salts and its effect on the pH and composition of the unfrozen phase. It was found that ice formation increased the concentration of several salts and other soluble materials above the saturation point. These compounds consequently precipitated during freezing or after a period of frozen storage, causing further ice precipitation and increases in concentration of the remaining dissolved materials with subsequent precipitation of some of these materials. The rates of precipitation were slow, some precipitation still taking place after several months of storage. Precipitation of salts markedly affected the pH and composition of the unfrozen phase, but the effect of these changes on quality deterioration is not known at present.

This paper presents further results on pH and composition changes in frozen foods. Extensive measurements were made with frozen peas and with chicken meat frozen

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post- and pre-rigor, as well as with salt solutions of a composition resembling as closely as possible the salt composition of the foods tested.

EXPERIMENTAL

Fresh peas from the local market were shelled, blanched in boiling water, and put through a very coarse meat grinder, which left most of the cotyledons intact. The peas were put in one-quart tin cans and frozen in five hours in an air blast at −20°C. Processing was completed and the cans were stored at −10°C within twelve hours of harvesting.

Poultry meat was obtained from 5- to 8-month-old commercially-grown chickens which were killed by cutting the jugular vein and carotid arteries and bleeding for about 2 min, scalded 2 min at 54°C, plucked by hand, and eviscerated. Pre-rigor meat was removed from the carcass immediately, whereas post-rigor meat was removed after the carcass was aged 24 hr in drained ice. Both breast and thigh meat was used, and the meat was frozen in an alcohol-dry ice mixture prior to storage at −10°C. In one experiment, the meat was cut in approximately half-inch cubes before freezing, packed in one-quart tin cans, and completely frozen within 75 min of removal from the carcass. In a second experiment, the meat was frozen in one piece within 45 min of removal from the carcass.

Samples for pH measurement were obtained by crushing part of the frozen material and discarding the exposed layer because of possible surface desiccation. The measurements were made at −10°C with glass and calomel electrodes modified for use at this temperature (van den Berg, 1960). Phosphate buffers of pH 6.0 and 7.0 containing glycerol were used for standardization. A small amount (10–20 g) of the sample was packed tightly around the electrodes to ensure good contact with both glass and calomel electrode. All measurements were made in duplicate on each sample and mostly with two samples of the same material, and were continued for up to 6 months. Reproducibility was better than ±0.1 pH unit.

Precipitation of phosphate salts presumed to occur in peas and poultry meat during freezing and frozen storage, was studied in salt solutions comparable to those present in the materials used. Each solution was cooled to a predetermined temperature below the freezing point, seeded frequently with a small amount of the solution which had been frozen at −30°C, and a portion of the liquid phase then removed and analyzed. Next, a new solution similar in composition to that of the analyzed solution was prepared, and the freezing process repeated, this time to a lower temperature. These freezing studies covered the range −2 to −12°C, and the type of salt precipitating was deducted from the change in composition of solutions as freezing progressed.

Tests were also made to study the effect of freezing on the pH of simple protein gels. Gels containing 15% gelatin (USP) and adjusted to pH 5.5–7.0 were frozen and the pH measured at −10°C.

The sodium, potassium, calcium, magnesium, phosphate, chloride, and lactate content of peas, meat, and solutions was determined by methods described previously (van den Berg, 1961). For meat and peas, analyses were made in trichloroacetic acid extracts. To determine the lactic acid content of meat frozen pre-rigor, finely crushed frozen material was suspended in 40% trichloroacetic acid at −10°C and left overnight at this temperature to prevent further glycolysis during thawing. The suspension was then diluted to the proper trichloroacetic acid concentration and treated in the usual way.

RESULTS

Changes in pH in peas during storage at −10°C are shown in Fig. 1. The pH dropped rather sharply immediately after freezing, from about 6.7 to as low as 6.0, and increased rapidly thereafter to pH 7.0. After about 2 months, and to the end of the test, the pH remained at 6.4 with only small fluctuations.

The magnitude and rate of pH changes were smaller in chicken meat than in peas (Figs. 2, 3). In addition, the pH of meat passed through one more cycle of increase and decrease than the pH of peas during the storage time studied (4 months). pH changes were similar in meat (both leg and breast) frozen post- and pre-rigor. During freezing, however, the pH of meat frozen post-rigor always increased 0.3 to 0.5 pH units, whereas the pH of meat frozen pre-rigor either remained constant or decreased slightly. The differences in pH between the meats at a given moment during

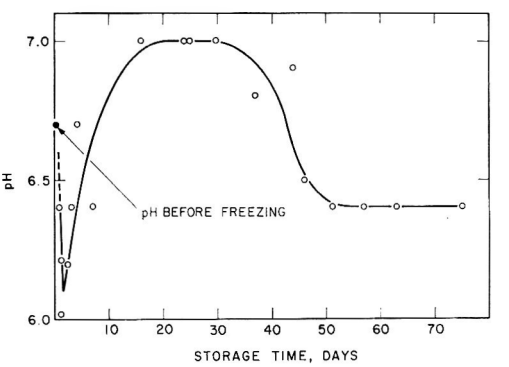

Fig. 1. Changes in pH of peas stored at −10°C.

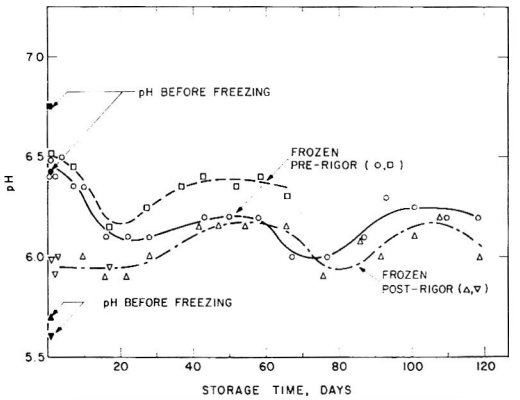

Fig. 2. Changes in pH of poultry breast meat frozen pre- and post-rigor and stored at −10°C (two series of tests).

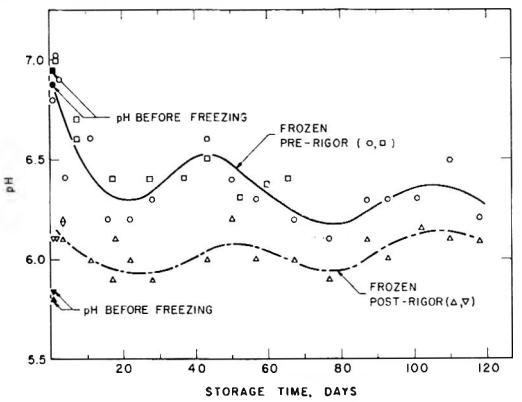

Fig. 3. Changes in pH of poultry leg meat frozen pre- and post-rigor and stored at −10°C (two series of tests).

storage appeared to be largely dependent on differences in initial pH; the differences in pH between meats generally decreased with storage time.

The results obtained with salt solutions showed that disodium, monopotassium, and alkaline calcium and magnesium phosphates would precipitate in most of the foods studied, the amounts depending on the initial composition of the product (Table 1). Disodium phosphate may not precipitate in peas because of the low sodium concentration. The presence of soluble compounds (e.g. sugars) will reduce the amount of salts precipitated, but it is likely that the sugars present in peas are not fully soluble at −10°C, and hence may precipitate at some time during storage, causing further ice formation, salt precipitation, and pH changes. During freezing of test solutions, disodium phosphate usually precipitated first, followed by monopotassium phosphate. Calcium and magnesium phosphate did not precipitate in significant amounts until the temperature reached −9°C. The solubility of calcium and magnesium phosphates was much smaller in these solutions than was indicated by the original composition of the food product, indicating that these minerals are largely protein bound. The solubilities of sodium and potassium phosphates at below freezing temperatures was not noticeably affected by the relatively small amounts of calcium and magnesium soluble (less than 0.015 mmole/ml, varying widely with small differences in pH and salt composition of solutions) and were similar to those observed in an earlier study of complex salt solutions (van den Berg, 1959).

Freezing increased the pH of gelatin gels by 0.5–0.8 unit for all initial pH values tested. The presence of phosphates (0.05*M*) reduced the pH increase of gelatin gels during freezing to 0.2 to 0.3 pH unit.

The lactic acid content of meat frozen pre-rigor increased at a rate of approximately 0.1% per month in breast meat and 0.05% per month in leg meat. This increase in lactic acid content during storage explains the decrease in pH differences between meats mentioned before.

DISCUSSION

The results presented in this paper confirm earlier conclusions (van den Berg, 1961) that pH changes in frozen foods are caused mainly by increasing concentrations

Table 1. Composition of foods used in pH study (% [of fresh weight] soluble in 15% trichloroacetic acid).

Food product	K	Na	Ca	Mg	P	Cl	Lactic acid
Peas [a]	0.31	0.003	0.027	0.006	0.045	0.03	—
Breast meat							
Pre-rigor	0.39	0.045	0.009	0.004	0.13	0.15	0.6–0.8
Post-rigor	0.39	0.045	0.009	0.004	0.13	0.15	1.1
Leg meat							
Pre-rigor	0.35	0.085	0.007	0.005	0.10	0.16	0.2–0.3
Post-rigor	0.35	0.085	0.007	0.005	0.10	0.16	0.8

[a] McCance and Widdowson (1960) report that peas contain about 4% reducing sugars.

of food components in the unfrozen phase, by precipitation of salts from the unfrozen phase, and by interaction of proteins with ionic substances. In addition, it was found that enzymatic activity could also contribute to pH changes in frozen meat.

The increase in pH of gelatin gels during freezing is similar to the pH increase in poultry meat and beef frozen post-rigor (van den Berg, 1961) and is apparently caused by the increase in protein concentration. This increase in pH seems to counteract partly or completely the decrease in pH noted during freezing of buffer salt solutions as a result of increasing concentration (van den Berg and Rose, 1959; van den Berg, 1959) and which may have caused the decrease in pH in peas and meat frozen pre-rigor, during freezing.

The results obtained with the salt solutions indicate that the sequence of precipitation of phosphate salts is usually: disodium phosphate, monopotassium phosphate, and calcuim and magnesium phosphate. This sequence would explain the general shape of pH-storage time curves obtained for most products during the first 2 to 3 months of storage. When the sodium content of a product is low compared to the calcium and magnesium content, as it is in peas, the sequence of precipitation may be reversed with relatively little effect on the sequence of pH changes. In poultry meat, and also in beef, which contains appreciable amounts of carbonate, citrate, and other buffer salts, further pH changes are likely produced by precipitation of some of these salts.

The extent of pH decreases in peas and chicken meat during storage indicates that more calcium and magnesium phosphate precipitates from solution than is present in soluble form originally. Calcium and magnesium are usually associated with proteins in some form or another, and it seems likely that the bound ions go into solution when the concentration in solution decreases from precipitation of calcium and magnesium phosphate. This will lead to further precipitation until equilibrium is obtained. The removal of calcium and magnesium ions from the proteins may affect the stability of the proteins, but no definite information is available at present.

The magnitude of pH changes would depend not only on the amount of salt precipitated but also on the buffering capacity, which in turn depends largely on the protein content. This dependence on protein content would explain why pH changes were smaller in peas than in vegetables previously studied (van den Berg, 1961) although the salt composition is not appreciably different. This protein effect would also indicate that pH changes in a product such as green beans might be different in the shells from in the seeds, since the protein contents of shells and seeds differ markedly (McCance and Widdowson, 1960).

Since enzymatic activity is not eliminated by freezing, enzymatic changes which result in the production of acid or base, or which affect the buffering capacity of the system, may affect pH noticeably. This is apparent in the results obtained in meat frozen pre-rigor, in which lactic acid, and presumably also carbon dioxide, are produced slowly during storage. Proteolytic or lipolytic activity in frozen foods may also affect pH changes.

ACKNOWLEDGMENT

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Progressive Changes in Starch Gel Electrophoretic Patterns of Chicken Muscle Proteins During "Aging" Post-Mortem [a]

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SUMMARY

Proteins extracted from chicken muscle during post-mortem aging in the cold were examined by starch gel electrophoresis. Myofibrillar proteins, extracted and analyzed in concentrated urea, revealed no detectable, consistent change during the two-day aging period. Myogen proteins, extracted by vigorous homogenization in dilute buffer, also remained unchanged in white muscles, but an additional electrophoretic component, possibly derived from myglobin, slowly appeared in red muscle extracts; the delay in its development suggests a secondary relation with tenderizing processes. Important constituents of myogen were lacking in "sacroplasmic" proteins extracted from breast muscle, pre-rigor or in-rigor, with gentle homogenization in 0.44M sucrose solution. Such "sarcoplasmic" preparations could not be obtained effectively by the same method from red muscle with higher content of stroma. Some of the myogen components absent from "sarcoplasm" gradually reappeared as tenderization proceeded; the time required to achieve rigor and to complete tenderization varied with the bird, but the observed changes were consistent. Yields of total and protein nitrogen from fresh, rigor, and aged breast muscle were in agreement with the electrophoretic data.

It is suggested that the additional components obtained in "sarcoplasm" of tenderized muscle reflect soluble proteins escaping into the extract because of the breakdown of intracellular barriers or subcellular particles. These components may include enzymes instrumental in initiating changes of the myofibril, ultimately evident in tenderization.

INTRODUCTION

In recent years, poultry muscle has been recognized as a convenient vehicle for studies of the changes that meats undergo during the "aging" or "tenderization" period after slaughter (Weinberg and Rose, 1960; Bandack-Yuri and Rose, 1961; Fischer, 1963). Fowl, like other warm-blooded animals of a similar size, pass into rigor as intramuscular adenosine triphosphate falls below a threshold level some 2–4 hr after death (deFremery and Pool, 1960), but, in contrast to the slow tenderization of mammalian tissue, chicken muscle recovers a large measure of pliability and "tenderness" within a few hours post-mortem (May *et al.*, 1962; Pool *et al.*, 1959; van den Berg *et al.*, 1964). This rapid "aging" permits study of changes in muscle protein fractions unhampered by microbiological contamination.

Changing distributions of nitrogen among the classical myofibrillar protein fractions of chicken muscle extracts were reported by Weinberg and Rose (1960), who suggested that these changes arose from increasing release of actin and possibly other proteins from the myofilaments. Cytolysis and gross proteolytic activity was inadequate to account for the rapid and complete tenderization during the short period under study. Electrophoretic studies of mammalian meats revealed only gradual obliteration of the fine features of electrophoretic patterns of myogen during the first few days of storage, followed slowly by the appearance of degradative products (Zender *et al.*, 1958; Kronman and Winterbottom, 1960). Initial tenderization-linked changes in the protein must

[a] Issued as N.R.C. No. 8094.

be of a more specific and limited nature (Whitaker, 1959), perhaps detectable only by recently evolved methods of protein resolution. Starch gel electrophoresis, one of the most effective tools for detecting subtle differences and changes in protein mixtures, was used to examine extracts of chicken muscle undergoing rigor and tenderization. These extracts were selected to represent myofibrillar protein, myogen, and sarcoplasmic protein.

METHODS

Slaughter and dissection. White Leghorn or Rhode Island Red roosters, ranging in age from 3 to 12 months and in weight from 3 to 8 lb, were slaughtered by bleeding from neck veins with wings under restraint. After some 2–4 min, depending on the period of reflex spasm, the bird was scalded 2 min at 52°C, plucked by hand, decapitated, and eviscerated. The carcass was transferred to a cold room at 4°C, where all subsequent operations except electrophoresis took place. When several pre-rigor samples were required, only the breast was coarsely plucked, and evisceration was omitted.

The muscles included in this study were selected because they showed slight but significant differences in starch gel electrophoretic patterns, typical of myogens from different muscles (Neelin, 1963b). According to the nomenclature of Chamberlain (1943), these muscles were pectoralis superficialis (often called pectoralis major), biceps brachii, biceps femoris, semimembranosus (by some authorities termed semitendinosus) (Berger, 1960), sartorius, and gastrocnemius. The first muscle was the most amenable to study, and therefore received the most attention.

Muscle was stored as a fine mince in a closed vessel, as a piece of excised tissue wrapped in moisture-proof film (Saran Wrap, Dow Chemical of Canada), or (most commonly) *in situ* on the carcass. All tissue was freed of obvious fat and connective tissue before mincing or grinding.

Extraction. The three classes of muscle extract are herein termed myofibrillar, myogen, and "sarcoplasmic" protein preparations. The first usually contained myogen as a secondary component, and myogen always included sarcoplasmic proteins. Of critical importance in the preparation of these extracts were method of homogenization, proportion of muscle to extractant, and the composition of extractant solution. Residue was removed from all extracts by centrifugation for 30 min at $10^4 \times G$ in a Servall centrifuge Model SS-1; to clarify some veronal extracts, especially from red muscle, centrifugation was repeated. Protein solutions were stored at 4°C, but never for longer than two weeks after extraction.

Myofibrillar proteins were prepared by slicing or by mincing a block of tissue in a Latapie mincer, grinding a weighed sample with an equal part of washed sea sand, and grinding again after adding five parts of extractant solution. Three extractants were used: 7*M* urea (in one experiment, 8*M* urea buffered in dilute sodium phosphate, pH 7); 0.20*M* sodium pyrophosphate adjusted to pH 7 with hydrochloric acid; 0.40*M* potassium chloride, 0.009*M* sodium dihydrogen phosphate, 0.047*M* disodium hydrogen phosphate. Fractions of myofibrillar proteins were obtained by dilution of concentrated urea extract to 3*M* urea, or by dialysis of "KCl-phosphate" extract (μ 0.55) against dilute "KCl-phosphate" solutions (μ 0.225 and 0.05). For starch gel electrophoresis, fractions and extracts were dialyzed against 6.3*M* urea, buffered in sodium veronal, pH 8, μ 0.02; urea extracts were frequently used without dialysis, since they tended to gel with this treatment. Extracts and fractions were rather dilute, but the tendency to gelation in concentrated urea limited the workable proportion of muscle to extractant.

Myogen extracts were prepared by grinding minced or chopped muscle with an equal weight of sand, and again with two parts of buffer, μ 0.05. Buffers used were sodium veronal adjusted to pH 8 with hydrochloric acid, or sodium cacodylate, pH 7. Veronal was preferred, because it avoided ambiguous electrophoretic migration of a major component which was isoelectric near neutrality; cacodylate obviated a correction in nitrogen analyses, but interfered with phosphorus determination.

Sarcoplasmic preparations were made from tissue freshly minced in a Latapie mill. One part of mince was suspended in nine parts of 0.44*M* sucrose in a manually operated glass piston-type homogenizer (loose-fitting piston) (Dounce *et al.*, 1955). The piston was worked through eight complete strokes, each requiring 1–5 min, depending on the toughness of the tissue; during withdrawal of the piston the crushed tissue was subjected to substantial vacuum, which contributed to cytolysis.

Before electrophoresis, myogen and "sarcoplasmic" extracts were dialyzed against veronal buffer, pH 8, ionic strength 0.05. Dialysis of sucrose extracts, already relatively dilute, increased volume by some two-thirds; therefore, after overnight equilibration against veronal, pH 8, μ 0.02, "sarcoplasmic" preparations were concentrated at least fivefold and then dialyzed again against veronal, ionic strength 0.05. Concentration was effected by pervaporation at 4°C, by flash evaporation at 10–20°C, by dialysis at 4°C against 30% polyvinylpyrrolidone or concentrated Carbowax solution, or

by ultrafiltration at 18–20°C (Tessier and Rose, 1958). The evaporation and dialysis methods produced some denaturation, contamination with absorbent, or marked change in pH and ionic constitution. In contrast, ultrafiltration removed some 30 ml from 35–40 ml of dialyzed extract in about 8 hr under air pressure of 25 lb per sq in. without evident denaturation of protein.

Starch gel electrophoresis. Except for preliminary surveys, the vertical apparatus described by Smithies (1959) was used, with details of operation as specified previously (Neelin, 1963a; Neelin, 1964). Gels consisted of 16 g of starch (Connaught Medical Research Laboratories, Toronto) added to each 100 ml of buffer soultion. Buffer systems are described in the form, gel solutes/bridge solutes, as before (Neelin, 1963a). Electrophoretic conditions for illustrated examples are summarized in Table 1.

Myogen and "sarcoplasmic" protein preparations were analyzed in three buffer systems, two gradient systems found especially suitable for myogen (Neelin, 1963a), and one discontinuous buffer system (Poulik, 1957), with the following compositions:

System A—gel, 0.010*M* sodium cacodylate, 0.001*M* cacodylic acid/bridge, 0.010*M* sodium chloride

System B—gel, 0.010*M* sodium diethylbarbiturate, 0.005M hydrochloric acid/bridge, 0.10*M* sodium chloride

System C—gel, 0.076*M* tris(hydroxymethyl)-aminomethane, 0.005*M* citric acid/bridge, 0.06*M* sodium hydroxide, 0.30*M* boric acid.

Myofibrillar proteins were analyzed in gels containing concentrated urea. For each 100 ml of buffer solution, 42 g of finely powdered urea (later amended to 36 g to avoid excessive gel fragility) were added, with brisk swirling, to the hot starch solution before evacuation. The addition of starch to buffer increased the volume by 9%, addition of urea increased it a further 32–33%, and evacuation removed 5–6% of the original buffer volume (in urea-free gels only 1% of total volume was lost on evacuation). Thus, the ultimate urea concentration would approximate 5*M*, although distribution of water and solutes may not be uniform in a gel. The manipulation of gels containing urea differed from the usual gels in the following details: several minutes were allowed for dissolution of the urea and cooling of the starch solution before the gel tray was sealed; several hours, usually overnight, were required to completely "set" the gel; after slicing, the fragile gel halves were supported by the plastic cutting template and cover (Smithies, 1959) during initial staining; more time was required for destaining the protein-free gel.

Conditions of electrophoresis with these buffer systems are summarized in Table 1. Buffer compositions were as follows:

System D—gel, 600 ml of buffer as in B, plus 252 g urea/bridge, as in B

System E—gel, 600 ml of 0.038*M* Tris, 0.005*M* citric acid, plus 216 g urea/bridge, as in C

Miscellaneous analyses. Total nitrogen of extracts, before and after dialysis, was determined by micro-Kjeldahl and corrected for nitrogen of buffer solutes; urea extracts were not analyzed for nitrogen, and marked volume changes of dialyzed sucrose solutions increased variability of these samples. Phosphorus was determined by the method of King (1932). Ultraviolet absorption spectra were obtained with a Cary Model II recording spectrophotometer.

No direct measurements of muscle tenderness were undertaken, since published studies with both organoleptic and shear-press methods (deFremery and Pool, 1960; May *et al.*, 1962) provided an ade-

Table 1. Electrophoretic conditions for vertical SGE of chicken muscle extracts (Figs. 2–8); (buffer compositions are given in *methods*; where current or voltage-drift change is indicated as initial→final measurement).

Buffer system	Gel buffer/ bridge solution	Gel pH	Lower electrode	Current density (ma/cm²)	Field strength (v/cm)	Duration of run (hr)
A	Na cacodylate/ Na chloride	6.8–6.9	cathode	4	5–7→2	16–17
B	Na veronal (NaCl)/ Na chloride	7.4–7.5	cathode	4	5→3	16–18
C	Tris citrate/ Na borate	8.6	anode	4→1	5	13
D	Na veronal (urea)/ Na chloride	7.5–7.8	anode	4	4–5→4	6–7
E	Tris citrate (urea)/ Na borate	7.8	anode	3→1	3→7	7

quate guide to the time course of tenderization. However, relative changes in toughness of tissue were obvious to the operator during the first few strokes of the manual homogenizer. These changes in consistency, reminiscent of the cell-fragility method of determining tissue quality (Love and Mackay, 1962), were in accord with more rigorous tenderness measurements.

RESULTS

Myofibrillar protein. *Selection of electrophoretic conditions.* Of the preparations considered, urea extracts more closely approached complete solubilization of muscle proteins in a form suitable for analysis by high-resolution methods; therefore optimal conditions for resolution of these protein mixtures by starch gel electrophoresis were sought with the hope of detecting subtle changes correlated with the aging phenomena. The effect of pH on resolution was surveyed with horizontal or vertical gels in constant and gradient buffer systems, all containing concentrated urea (Fig. 1); diagrams only are presented in Figs. 1 and 2 because many patterns were too weak for photographic reproduction.

At acid pH (Fig. 1,*a,b*) most of the protein was immobile or cationic, and few zones were resolved. From pH 7.2 to 9.1 in a variety of buffers (Fig. 1, *c–g*) electrophoretic patterns were relatively uniform—some 2 to 4 zones remained cationic and 5 to 7 anionic zones were more or less resolved. A gel buffered at pH 7.5 in the system sodium veronal (urea)/sodium chloride (Fig. 1,*e*) gave optimal resolution of some 10 zones, numbered in order of decreasing anionic mobility (Fig. 2a).

With organic salts in the bridge solution in place of sodium chloride, and with gels of sodium veronal (e.g., sodium veronal [urea]/sodium veronal or sodium borate), zone *ii* was resolved into two clear zones, zone *i* occasionally revealed a hint of heterogeneity, and the breadth of zone *iii* suggested polydispersity. In all continuous buffer systems, zones *v* to *vii* were frequently diffuse and ragged. However, addition of 0.1–0.2% (v/v) mercaptoethanol to extractant solution, dialyzing solution, and the hot urea-gel medium after evacuation, improved resolution of all anionic zones. Discontinuous SGE in Tris citrate (urea)/sodium borate produced an array of close-packed, highly anionic bands (Fig. 3, system E).

Identity of zones. The nature of the protein components extracted with urea can be surmised by comparison with extracts of myofibrillar proteins obtained by conventional methods. Similar electrophoretic patterns were observed with extracts obtained in buffered or unbuffered concentrated urea (Fig. 2,*a*), in pyrophosphate (Fig. 2,*d*), or in phosphate-buffered potassium chloride (Fig. 2, *c*). The cationic zones *viii* to *x* were evidently myogen components, since chiefly anionic protein was recovered by urea extraction of the washed tissue residue (Fig. 2,*c*) remaining after removal of myogen with dilute veronal buffer.

Dilution of myofibrillar protein extracts assisted in identification of some zones. A flocculent precipitate formed in urea extracts diluted to less than 3.5*M* urea; ultraviolet absorption spectra indicated that some, but not all, protein precipitated, while nucleotide remained soluble. Anionic zones *ii* to *iv* and some cationic material (myogen) were redissolved from the precipitate with buffered 6.3*M*

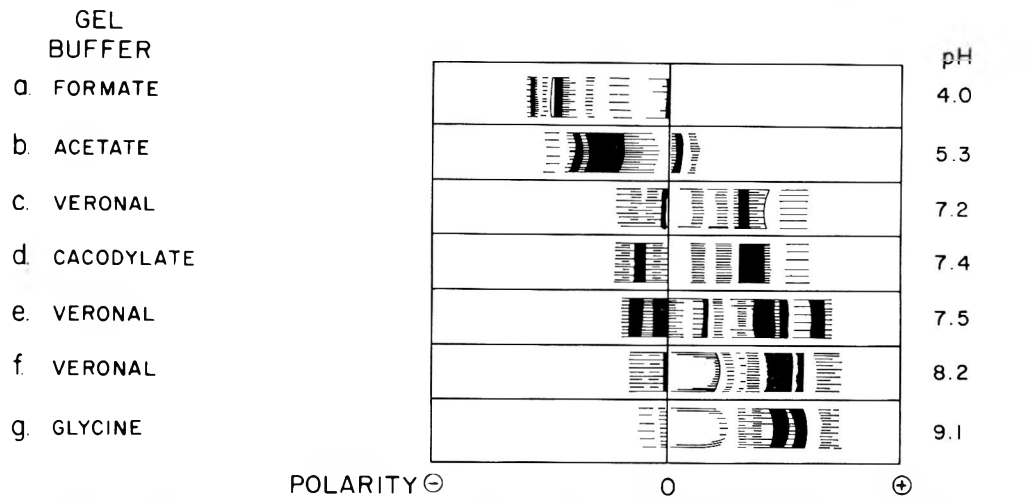

Fig. 1. Starch gel electrophoresis of chicken breast myofibrillar protein in concentrated urea, pH 4.0–9.1. Gel buffers consisted of sodium salts (ionic strength *ca.* 0.02); bridge salts were sodium formate (*a*) or sodium chloride (*b–g*). Electrophoretic conditions were similar to those in Table 1, system *D*.

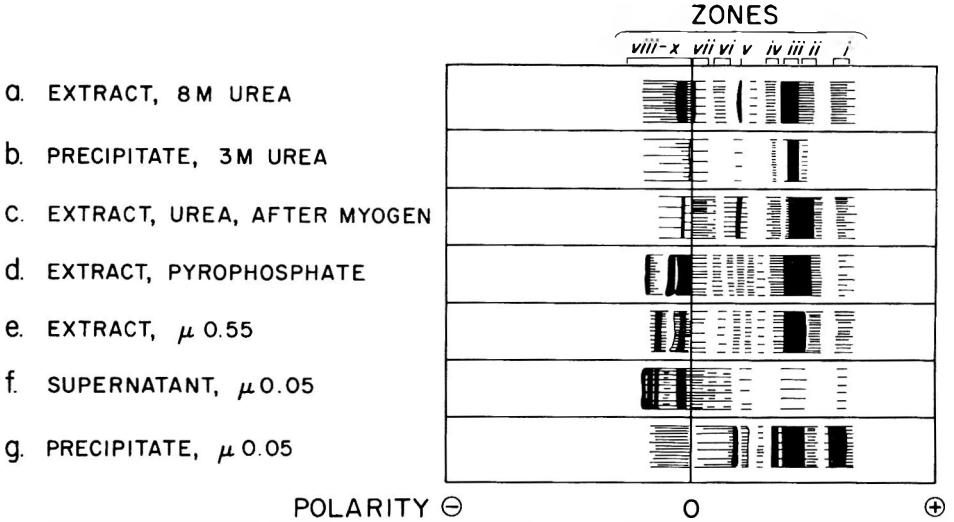

Fig. 2. Starch gel electrophoresis (in concentrated urea, pH 7.5) of myofibrillar protein extracts and fractions from breast muscle pre-rigor.

a) Extract in 8*M* urea (buffered with phosphate, pH 7).
b) Precipitate by dilution of (*a*) to 3*M* urea.
c) Extract (in 7*M* urea) of tissue residue, after myogen extraction.
d) Extract in sodium pyrophosphate-sodium chloride, μ 1.4, pH 7.
e) Extract in potassium chloride, sodium phosphate, μ 0.55, pH 7.
f) Supernatant, and g) precipitate after dialysis of (*e*) to μ 0.05.

Zones are numbered arbitrarily *i* to *x*. Extractions and fractionations are described in *Methods*. Buffer system *D* (*Methods* and Table 1).

urea (Fig. 2,*b*). Dilution by dialysis of potassium chloride-sodium phosphate extracts from ionic strength 0.55 to 0.225 (Weinberg and Rose, 1960) did not result in significant fractionation of components evident in starch gel patterns, despite substantial precipitation of protein. The supernatant or myogen solution remaining after dilution of this extract to ionic strength 0.05 (Weinberg and Rose, 1960) contained chiefly zones *vii* to *x* (Fig. 2, *f*), while the protein recovered from the precipitate was enriched in zones *i* to *vi* (Fig. 2,*g*) attributed to myofibrillar proteins.

Aging. Although the resolution of muscle proteins was unspectacular in starch gel systems containing urea, patterns were reproducible, even with extracts from birds of various sizes, and at least some myofibrillar proteins are represented by the zones observed. Therefore, the effect of aging of freshly slaughtered fowl was studied by analysis of urea extracts of four representative muscles, one white breast muscle and three red leg muscles (Fig. 3). All four muscles showed the same distribution of components, with quantitative differences only in the cationic or myogen proteins. These patterns were not changed significantly by aging for one day (Fig. 3, system D). In the illustrated example, zone *iv* appeared intensified in the aged extracts, but this was not consistently observed in replicate experiments. More often, post-rigor extracts were uniformly more dilute in all components, but relative intensities of zones remained comparable to those of pre-rigor extracts. The similarity of myofibrillar proteins of these four disparate muscles, whether fresh or aged, was corroborated by starch gel electrophoresis in discontinuous Tris citrate (urea)/sodium borate (Fig. 3, system E). In sodium formate (urea)/sodium formate, red muscle (biceps femoris) displayed an additional, fast cationic component, but there was no evident change in this or in breast muscle after aging.

Pyrophosphate extracts, and buffered potassium chloride extracts of fresh and aged breast muscle, also failed to reveal changes detectable by starch gel electrophoresis in buffer system D.

Myogen protein. Myogen extracts of chicken breast muscle are readily reproducible and reasonably stable in the cold (Neelin, 1963a). Although turbidity may develop after several days of storage in solution, the only observed effect on electrophoresis was decreased clarity of definition. Starch-gel patterns of myogen were not responsive to differences in breed or size of the fowl, and were not qualitatively affected by minor changes in post-mortem handling—omission of evisceration, abbreviated plucking, rapid cooling of the dressed carcass in ice water, or storage of the muscle as a mince rather than as an intact section (Fig. 4).

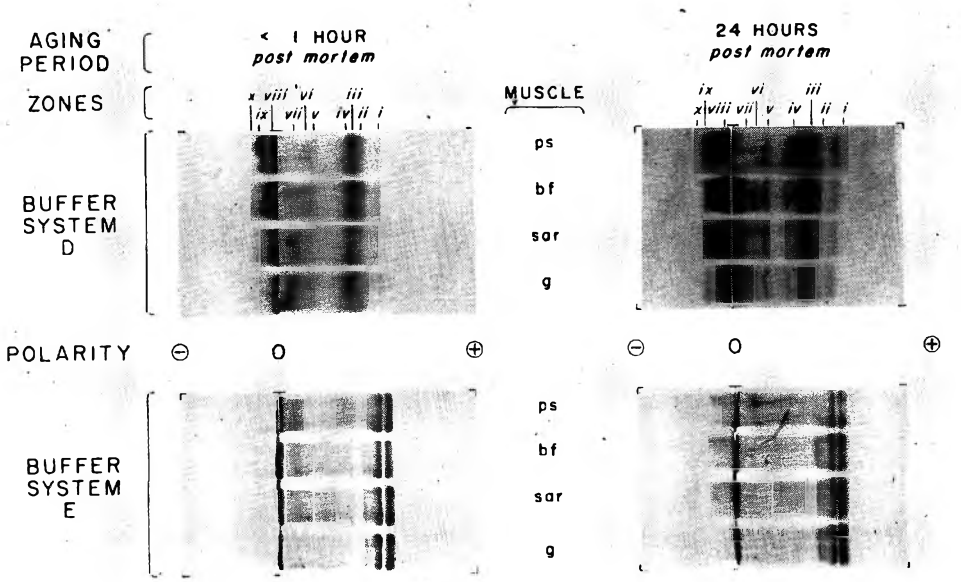

Fig. 3. Vertical starch gel electrophoresis of myofibrillar proteins extracted with urea before and after aging, of white breast muscle, pectoralis superficialis (ps), and three red leg muscles, biceps femoris (bf), sartorius (sar), and gastrocnemius (*g*). Buffer systems *D* and *E* (*Methods* and Table 1).

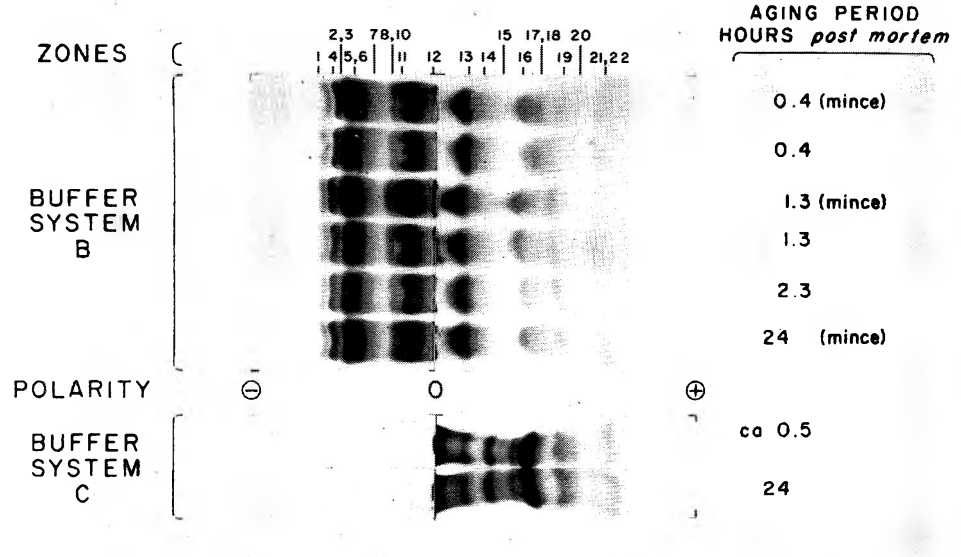

Fig. 4. Vertical starch gel electrophoresis of breast myogen extracted during post-mortem aging. Buffer systems *B* and *C* (*Methods* and Table 1). Zones are numbered according to previously described sequences for myogen (Neelin, 1963a).

The composition of myogen extracts was similarly independent of the period of post-mortem aging. Samples of breast muscle extracted at intervals from less than ½ hr to 24 hr after slaughter did not differ significantly in sodium veronal/sodium chlroide (Fig. 4, system B), in Tris citrate/sodium borate (Fig. 4, system C), or in sodium cacodylate/sodium chloride (Fig. 5, *ps*).

Starch gel electrophoresis of other muscles, both red and white, showed only minor variations during aging. After one day, no significant change was evident in electropherograms of myogen of wing muscle (biceps brachii) or leg muscles (biceps femoris, semimembranosus, and gastrocnemius) in

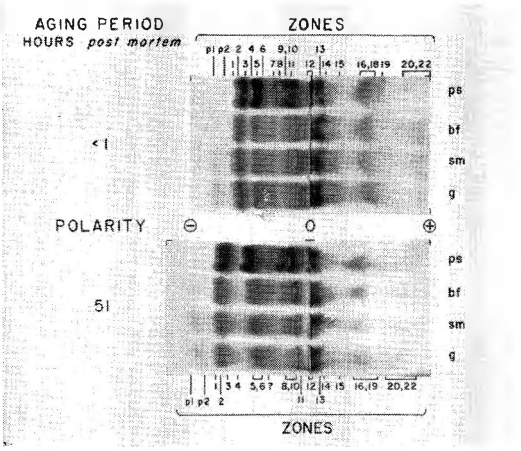

Fig. 5. Vertical starch gel electrophoresis of myogen extracted before and after aging of muscle from breast (*ps*), thigh (*bf* and *sm*), and drumstick (*g*). Abbreviations *ps*, *bf*, and *g* as in Fig. 3; *sm*, semimembranosus. Pigmented zones *p*1 and *p*2 are indicated, as well as the usual components. Buffer system *A* (*Methods* and Table 1).

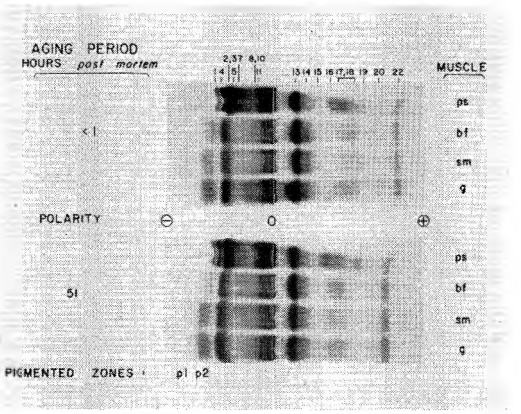

Fig. 6. Vertical starch gel electrophoresis of myogen extracted before and after aging of four muscles (abbreviations as in Fig. 5). Buffer system *B* (*Methods* and Table 1).

buffer systems A, B, or C. After two days, the only appreciable change in buffer system A, which best resolves the cationic components, was a greater diffusion of zones in red muscle myogen (Fig. 5, *bf*, *sm*, *g*). However, buffer system B resolved the pigmented components of red muscle more clearly (Fig. 6, zones *p*1 and *p*2), and revealed an additional fast, cationic zone in samples from red muscle after two days' aging (Fig. 6, *bf* and especially *sm* and *g*). Although this zone was not pigmented, its occurrence was related to the content of myoglobin, from which it may have been derived as a secondary consequence of tenderization.

Sarcoplasmic protein. *Starch gel electrophoresis.* "Sarcoplasmic" extracts differed considerably from myogen extracts in electrophoretic patterns in buffer systems A, B, and C. "Sarcoplasm" from pre-rigor breast muscle was lacking or poor in zones 5, 6, and 11, which stained strongly in electropherograms of myogen (Fig. 7, *ps*), and similar extracts of leg muscle were virtually devoid of many prominent components (Fig. 7,*bf*). Although all buffer systems revealed changes in breast muscle sarcoplasm after aging, system A (Fig. 7) most clearly indicated that zone 11 remained relatively weak, while zones 5 and 6 recovered a large measure of the prominence displayed in myogen. No effect of tenderization could be observed in these meager extracts of any chicken red muscles (e.g., Fig. 7,*bf*); these tissues appear to resist the mild methods of homogenization critical to the selective "sarcoplasmic" preparation.

Since "sarcoplasmic" extracts of chicken breast muscle revealed significant changes during the

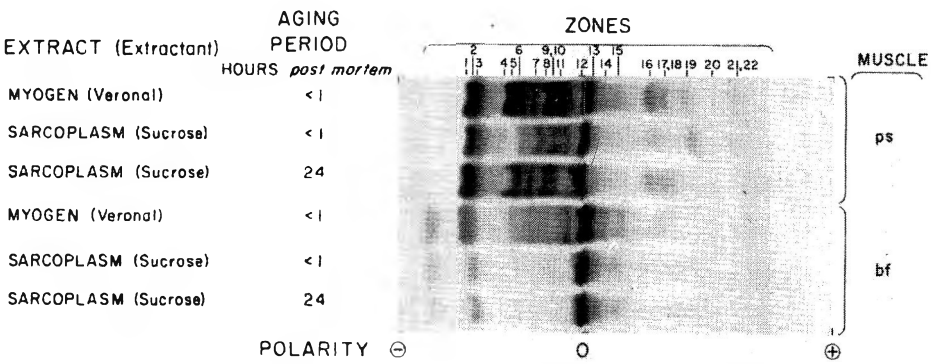

Fig. 7. Vertical starch gel electrophoresis of myogen and "sarcoplasm" of breast muscle (*ps*) and thigh muscle (*bf*) before and after aging. Buffer system *A* (*Methods* and Table 1).

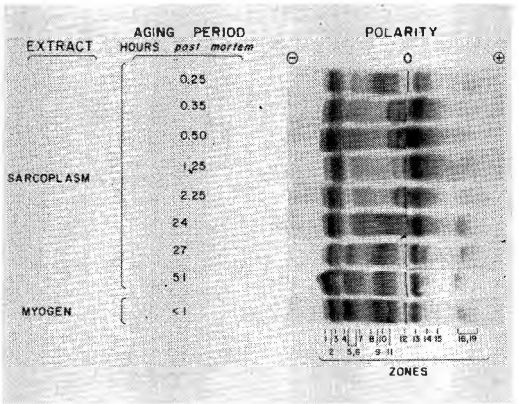

Fig. 8. Vertical starch gel electrophoresis of "sarcoplasmic" proteins extracted from breast muscle during post-mortem aging, up to two days post-rigor. Buffer system *A* (*Methods* and Table 1).

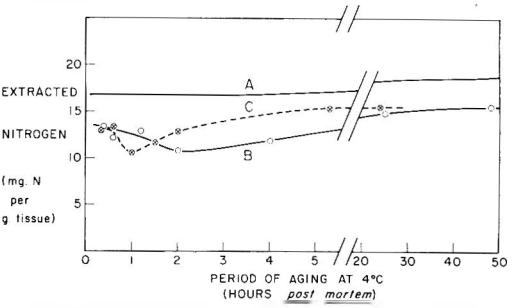

Fig. 9. Changes in total extracted nitrogen during post-mortem aging of chicken breast muscle at 4°. Curve *A*, myogen from muscle aged *in situ* or in mince; curve *B*, "sarcoplasm" from muscle aged *in situ*; curve *C*, "sarcoplasm" from muscle aged in mince.

Curve *A* represents the average trend of values obtained from 14 birds sampled. The points ⊙ and ⊗ in curves *B* and *C* are derived from analyses of representative single birds, from 8 individuals sampled.

tenderization period, the course of these changes was followed in more detail. In freshly killed birds, breast muscle "sarcoplasm" was again relatively poor in zones 4–7 and 11–12 (Fig. 8, 0.25 hr). As the muscle approached rigor (Fig. 8, 0.35–1.25 hr), some zones diminished even further, but the vestige of zone 4 remained more or less constant throughout rigor (Fig. 8, 1.25–2.25 hr). Zone 11 remained low in post-rigor "sarcoplasm," but zones 8–10 recovered their original intensity, while zones 5–7 more slowly approached the level attained in myogen extracts. Zones 1–3 and anionic components (zones 13–22) were unchanged throughout this post-mortem period.

Extracted nitrogen and phosphorus. Recoveries of nitrogen in myogen and "sarcoplasmic" preparations of breast muscle were consistent with the qualitative changes. Myogen extracted from birds of various ages and weights contained 14–19 mg nitrogen per g wet weight of tissue (40–44% of the total muscle nitrogen), with about half in dialyzable form. Replicate extracts from a single bird were more reproducible, and the nitrogen content of myogen extracted during aging increased only slightly (Fig. 9, curve A). This increase may have resulted from improved homogenization of tenderized muscle, or from loss of moisture during storage.

In contrast, the total extracted nitrogen in "sarcoplasmic" preparations underwent a systematic sequence of changes during rigor and aging of breast muscle. These extracts of fresh muscle contained 13–14 mg of nitrogen per g of tissue (Fig. 9, curves B, C), appreciably less than "myogen" of the same muscle, although the dialyzable content was comparable. As the muscle passed into rigor about two hours post-mortem (Fig. 9, curve B), nitrogen recovered by sucrose extraction decreased to less than 11 mg per g; thereafter, extracted nitrogen increased to a maximum of 15–16 mg per g of tenderized breast muscle. The initial, minimum, and maximum levels were reproducible, but since the times required to enter rigor and to complete tenderization were variable, curves representing analyses on a single bird are presented. Minimum and maximum yields of nitrogen were realized more rapidly in muscle stored in the form of mince (Fig. 9, curve C) than in muscle aged *in situ* (Fig. 9, curve B). These values correspond to the changes in relative protein concentrations, depletions, and restorations of major components observed by starch gel electrophoresis of "sarcoplasm" extracted from muscle during the course of aging (Fig. 8).

Essentially uniform yields of 2.0±0.2 mg of total phosphorus per g of tissue were recovered in "sarcoplasmic" preparations of fresh or aged minced and whole muscle, but a slight trend to increased yield in myogen extracts with prolonged aging was reminiscent of the drift in total extracted nitrogen (Fig. 9, curve A). This trend was not apparent in "sarcoplasmic" preparations, nor was there any suggestion of a drop in yield during rigor. Virtually all (96–98%) extracted phosphorus was dialyzable.

DISCUSSION

Although it has been suggested (e.g., Locker, 1960; Hill, 1962; Partman, 1963) that the contractile proteins have a major influence in the tenderness of various meats, it has been difficult to demonstrate any change in these proteins during tenderization

by aging. Weinberg and Rose (1960) found that more myofibrillar protein, possibly actin, was readily extracted from aged chicken breast muscle, but Wierbicki *et al.* (1956) found no consistent relation between the "myosin" extracted and the tenderness of beef. Although Baliga *et al.* (1962) found a decrease in nitrogen extracted at high ionic strength from fish muscle during storage on ice, there was a sharp increase in the "actomyosin" component after 1–3 days. These conflicting conclusions may simply reflect the striking differences in tenderizing properties of different meats.

To obtain the low ionic strength required for zone electrophoresis in the present work, urea was used to extract the myofibril and most auxiliary proteins; therefore, accurate determination of the amount of protein extracted was impractical. However, under the conditions chosen, the actomyosin association is disrupted (Barney *et al.*, 1960; Stracher, 1961) and the myosin complex is dissociated (Weber and Stöver, 1933; Tsao, 1953; Szent-Györgyi and Borbiro, 1956). Although complete deformation of myosin requires 10–12*M* urea (Stracher, 1961; Small *et al.*, 1961), viscosity of both myosin and actomyosin is minimal in 5*M* urea, the ultimate concentration in our starch gels. Myosin and heavy meromyosin migrate in starch gel (Mueller and Perry, 1962) in 8*M* urea buffered with borate, pH 9; myosin yields three, possibly four, moderately anionic zones which may be analogous to zones *i* to *iv* (Figs. 2,*a*; 3,*D*). Actin is heterogeneous in starch gel (Krans *et al.*, 1962), giving four slow anionic zones in 7.2*M* urea buffered in glycine, pH 8.5 (Carsten and Mommaerts, 1963); these should be analogous to zones *v* to *vii* (Figs. 2,*a*; 3,*D*).

The assumption that zones *i* to *vii* represent myofibrillar proteins is supported by several facts: the preponderance of these zones in the sample extracted with urea after prior extraction of myogen (Fig. 2,*c*), the virtual absence of zones *i* to *iv* and depletion of zones *v* to *vii* in extract dialyzed to μ 0.05 (Fig. 2, *f*), and precipitation of zones *i* to *iv* upon dilution of 8*M* urea extract to 3*M* (Fig. 2, *b*). However, there is less certainty that all the myofibrillar protein, particularly macromolecular complexes, can enter the starch gel to be resolved into visible bands. Certainly if myofibrillar proteins are altered during early aging, any changes escape observation or are obliterated by the disruption of secondary and tertiary conformations by urea. It is worth noting that no specific starch gel components precipitate between μ 0.55 and μ 0.225, and that extracts of aged muscle tend to give less intensely stained zones than urea extracts of fresh muscle. This could reflect either less efficient extraction or an increase in actomyosin complex (Weinberg and Rose, 1960), failing to penetrate the gel, even in urea.

The amount of myogen protein extracted from chicken breast muscle remained essentially constant throughout a two-day aging period, even though tenderization of this muscle would be complete within this period under the conditions used (van den Berg *et al.*, 1964; deFremery and Pool, 1960; Sharp, 1963). Changes in solubility, enzyme activity, and electrophoretic components (Kronman and Winterbottom, 1960; Sharp, 1963; Deatherage, 1963; Zender *et al.*, 1958; Scopes and Lawrie, 1963) may reflect denaturative or degradative losses during the prolonged aging period required for mammalian muscles, or species differences may be indicated.

The delayed appearance of an extra cationic zone in "myogen" extracts of red muscle during aging appears to be related to the content of myoglobin, although the new zone is not pigmented. Perhaps it arises from loss of heme; its relation to tenderization seems, at best, only secondary.

The relatively gentle homogenization of tissue in hypertonic sucrose solutions should protect fragile intracellular structures (Dounce *et al.*, 1955). Thus, these extracts, which should approach the freely soluble sarcoplasm, are relatively free of some components (Fig. 7) prominent in less selective myogen preparations. The temporary loss of other cationic components (zones 8 to 10) from extracts of muscle in rigor may reflect only the resistance of rigor tisue to homogenization; however, the recovery of zones 5 to 10 (Fig. 8) and of total extracted nitrogen (Fig. 9) from aged post-rigor muscle strongly suggests release of protein by increased disruption of subcellular structure.

The drastic homogenization and the substantial ionic concentration of the myogen preparation achieves this disintegration, even in rigor muscles. A major myogen component (zone 11) remains deficient in "sarcoplasm" throughout tenderization, and may arise from intracellular structure which is not affected by aging but is susceptible to conditions of homogenization.

The cationic proteins of zones 5 to 7 may be analogous to the chromatographic fraction observed by Fischer (1963) to increase in water extracts of this muscle during tenderization under similar conditions. Since this chromatographic peak was eluted easily at high pH from a cationic adsorbent, it would also be relatively cationic. These components may be initial products of degradation or they may be enzymes that have become free in the sarcoplasm, and thus gain access to structural substrates, weakening their contribution to toughness without releasing soluble products. The higher stroma protein content of red muscle (Khan, 1962) apparently increases its resistance to gentle homogenization and prevents preparation of useful sarcoplasmic extracts; more vigorous homogenization disintegrates cell structures and produces a myogen-type preparation.

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After-Cooking Discoloration of Potatoes Role of the Organic Acids

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SUMMARY

Stem- and bud-end tissue from 41 samples of potatoes representing various degrees of after-cooking discoloration were analyzed for organic acids content. The individual acids determined were glutamic, aspartic, pyroglutamic, malic, citric, orthophosphoric, oxalic, and one unidentified acid. The stem-end tissue contained a lower concentration of all the acids except the unidentified one. The difference between the stem- and bud-end was very large in some cases, notably for malic acid and citric acid.

Examination of the data indicated a strong tendency for degree of blackening to be associated with low organic acid content. A statistical analysis of the data showed highly significant correlation of low citric acid, orthophosphoric acid, and oxalic acid content with blackening. Citric acid exhibited the highest degree of correlation, having an r value of 0.768.

The significant correlation between low citric acid content and after-cooking blackening was maintained in all but one case when subgroups of the samples were formed according to variety, crop year, and location grown. Of the three varieties studied statistically, Ontario and Katahdin showed correlation, whereas Kennebec did not.

The interrelationships of iron, citric acid, and potassium contents were studied. Since there is always a large excess of citric acid over iron, it must be assumed that something prevents the citric acid from chelating the iron in blackening potatoes. The data indicate that potassium may be the main constituent playing this role. In the final analysis, the primary factor in the blackening phenomenon is probably the amount of free organic acid present.

INTRODUCTION AND LITERATURE REVIEW

Several previous publications (Yanovsky, 1955; Hunter *et al.*, 1957; Heisler *et al.*, 1962, 1963) from this laboratory have described the problem of after-cooking discoloration of potatoes. Briefly stated, this is a discoloration that occurs only after cooking and is not observed in the raw tissue. It appears at the surface shortly after cooking, during cooling, and is generally more intense at the stem end than at the bud end of the tuber. It is now generally accepted that the discoloration is due to the formation of a dark-colored complex of iron and chlorogenic acid, but it is also recognized that the presence or absence of other constituents plays a major role in the blackening mechanism.

The role of citric acid, because of its known ability to chelate iron, has been given prime consideration. From the practical standpoint, most of the proposed methods of treating potatoes to alleviate the after-cooking discoloration are based on the chelating principle (Greig and Smith, 1955; Hawkins *et al.*, 1959; Hunsader and Henning, 1958; Smith and Davis, 1962).

Juul (1949) showed that the discoloration was influenced by citric acid. However, he attributed this influence to a pH effect. Mulder (1949) and Bate-Smith *et al.* (1958) recognized the citric acid action as a chelating effect and stated that the distribution of blackening in individual cooked tubers is governed mainly by the competition between chlorogenic acid and citric acid for iron. Hughes and Swain (1962b) studied

the effect of citric, orthophosphoric, and malic acid on the color of various phenol-iron complexes *(in vitro)* and concluded that citric acid was the most important of these factors in reducing the intensity of color of the chlorogenic acid-iron complex.

In spite of the apparent importance of citric acid and perhaps other organic acids, the literature reports no general and intensive study of the organic acid content of potatoes and their relationship to stem end blackening. Hughes and Swain (1962a) studied the citric and phosphoric acid content of four individual tubers and reported correlation of blackening with the ratio of citric acid to chlorogenic acid. While this was sufficient for their purpose of establishing the importance of citric acid in relation to blackening, we felt that a more general analytical study of a large number of samples would be of value.

Thus, in an attempt to gain more information on the organic acids content of potatoes and their possible effect on after-cooking discoloration, we have determined the organic acids content of the stem and bud ends of a large number of samples representing a wide range of discoloration and have made a statistical study of the data.

EXPERIMENTAL METHODS

Potatoes. The 41 samples of potatoes used in these experiments were sent to us as blackening samples from various parts of the country over a three-year period. Most were received from Wisconsin (19 samples) and Michigan (15), with the remainder coming from Pennsylvania (3), Maine (3), and Long Island (1).

The experimental details for obtaining the sample and determining the degree of discoloration have been presented (Heisler *et al.*, 1962). For convenience, a brief outline is given here.

Potato sampling. Plugs were taken from the stem- and bud-end sections of thoroughly washed and scrubbed potatoes using a no. 15 cork borer. The plugs were cut to a length of approximately 1 in. and then cut in half lengthwise. One half was used for the organic acids determination and the other for measurement of degree of discoloration by a reflectance test. The half cylinders used for the organic acids determination were adjusted in length so that 26 pieces totaled 100.00 g. Those used for the reflectance measurement were adjusted to total weight of 65.00 g.

Determination of degree of discoloration. The reflectance obtained from a smooth surface (under glass) of cooked mashed potato was taken as a measure of discoloration. The reflectance attachment to the Beckman Model B spectrophotometer (no endorsement implied) was used, with $MgCO_3$ as standard. In this study, "degree of discoloration" was arbitrarily taken as $(R_B - R_S)/R_S$, where R_B is the reflectance of mash from the bud end, and R_S represents that for the stem end. The more discoloration in the potato, the greater the difference between R_B and R_S and the lower the value of R_S. Thus, these factors reinforce each other to amplify the value for degree of discoloration, making the system more sensitive in differentiating samples.

Preparation of extract. The 100.00-g sample of potato tissue (26 half-plugs from stem or bud end) was ground for 2 min in 300 ml of iron-free water (redistilled in all glass apparatus and checked with reagent) in a Waring blender. The slurry was filtered through Whatman no. 12 paper. The filtrate was immersed in a boiling water bath for 5 min to coagulate the protein and then filtered, while hot, through Whatman no. 12 paper. Loss of vapor during heating and filtering was kept to a minimum by stoppering the flask with a ground-glass stopper and by covering the funnel with a watch glass. The filtrate obtained in this way contained about 1% solids. The individual organic acids of the deproteinized extract were determined.

The resulting samples of deproteinized extracts, though prepared by the same procedure, varied slightly in solids content because of differences inherent in the various lots of potatoes. The exact solids content of each extract was determined by loss of weight after drying. The individual organic acids were determined as the quantity present in the deproteinized extract, but it can be related, percentagewise, to the fresh potato weight or to the solids content of the juice.

While it is recognized that the above procedure is not an exhaustive extraction it was believed sufficient for the purpose of determining differences in the organic acids content of various samples and between the stem and bud end of the same sample.

Determination of the individual organic acids. The method of Schwartz *et al.* (1962) was used. This method utilizes an anion-exchange resin to adsorb the acids, and a gradient-elution technique to elute and fractionate the acids from the column. The individual fractions were then titrated with base.

RESULTS AND DISCUSSION

Forty-one samples, representing a wide range of after-cooking discoloration, were

analyzed for organic acids content. The stem- and bud-end samples of deproteinized extracts were studied. Table 1 lists the 41 samples in order of decreasing degree of discoloration, giving the organic acids content of the stem- and bud-end tissue. Table 2 shows the range of values and the average value for the stem and bud ends and for the difference between the bud and the stem ends. The orthophosphoric and oxalic acids were treated as one because separation of these two acids was not complete in every case. In general, the stem end contained a lower concentration of all the acids except the unknown. The difference between the stem and bud end in some cases is very large, notably for malic and citric acids. The stem-end values have a wider range for all the acids except glutamic and the unknown, the most variation occurring with malic acid.

Examination of all data for the 41 samples shows a strong tendency for degree of blackening to be associated with low organic acid content of the stem end, especially in the case of citric and malic acids. Considering the fact that the stem end blackens and the bud end does not, the bud end of each sample can be considered a control and the organic acid value for the difference between the stem and bud ends should be related to the tendency to blacken. As expected, this value does show a direct relationship to blackening, that is, the bud-stem difference value increases with tendency to blacken. Thus, low organic acid content in the stem end and a large difference in the organic acid content of the stem and bud ends tends to be associated with blackening. By combining these two factors in the ratio $(OA_B - OA_S)/OA_S$ (where OA_B is the organic acid content of the bud end, and OA_S that of the stem end), similarly to that done for reflectance values, one arrives at an expression for organic acid content that should give the highest degree of correlation with blackening. Figs. 1 and 2 are plots of these four functions (stem, bud, bud minus stem, bud minus stem/stem) for the citric acid data and the total organic acid data against degree of blackening. It can be observed that the value of (bud minus stem)/stem gives a much better distribution of points than the other values, especially in the case of the total organic acid content.

A statistical study was made of all the organic acid data by the linear regression method, and an analysis of variance was made. Table 3 gives the F and the r values obtained, indicating the extent of correlation. As stated before, since only the stem end blackens, correlation between the degree of discoloration and organic acid content should be obtained for the values of stem, bud minus stem, and (bud minus stem)/stem, but not for the bud value. This theoretically ideal situation is obtained only with the values for citric acid and for orthophosphoric + oxalic acid. The malic acid results display an anomaly in that the bud-end value shows significance whereas the bud-minus-stem difference value does not. Citric acid shows the highest degree of correlation. The highest r value obtained was 0.768, for the (bud minus stem)/stem (citric acid data). This would give an r^2 of 0.591, indicating that 59% of the variability of blackening is due to this ratio. This is still too low for prediction purposes, so no attempt was made to determine confidence levels.

The significant correlation between low citric acid content and degree of discoloration was maintained in all but one case when subgroups of the samples were formed according to location grown, crop year, and variety. Table 4 summarizes the results of this statistical analysis of the subgroups. Of the three varieties studied, two (Ontario and the Katahdin) showed significant correlation of low citric acid content and blackening. The high r values obtained for the (bud minus stem)/stem function of these two varieties indicate that this value could perhaps be used to predict whether a particular sample will discolor. The Kennebec variety failed to exhibit correlation. When the potato samples from Wisconsin and Michigan were treated separately, both groups showed highly significant correlation of citric acid content and blackening. Also, considering the samples on a yearly basis, a significant correlation was obtained for the three years over which the study extended.

Table 1. Organic acid values and their relation to after-cooking discoloration.

Sample	Variety	$\frac{Rn-Rs}{Rs}$	Mg per 100 ml										Meq per 100 ml					
			Glutamic		Aspartic		Pyroglutamic		Malic		Citric		O-Phosphoric + Oxalic		Unknown		Totals	
			Stem	Bud	Stem	Bud	Stem	Bud	Stem	Bud	Stem	Bud	Stem	Bud	Stem	Bud	Stem	Bud
59–23	Wisc. Antigo	0.697	14.7	12.9	15.7	20.5	5.3	15.4	2.2	19.4	23.0	167.6	.410	.692	.528	.338	1.58	4.25
59–24	Wisc. Red Lasoda	0.695	15.9	15.9	18.5	16.3	6.6	6.7	2.4	11.7	26.1	140.7	.468	.760	.403	.345	.160	3.72
59-19	Wisc. Katahdin	0.623	15.6	16.6	18.7	17.6	6.6	9.7	2.5	16.7	33.8	147.9	.389	.760	.619	.528	1.86	4.13
59–20	Wisc. Kennebec	0.572	16.3	16.5	20.8	24.7	5.4	11.3	2.2	15.8	29.6	154.8	.410	.736	.407	.266	1.61	3.99
59–25	Wisc. Red Lasoda	0.522	16.9	16.4	18.5	18.9	5.7	6.8	2.6	14.9	41.0	184.3	.536	.793	.486	.432	1.99	4.57
59–15	Wisc. Early Gem	0.519	14.4	15.1	18.4	24.3	7.1	14.1	3.5	24.0	32.5	144.0	.303	1.029	.396	.310	1.54	4.01
61– 1	Mich. Ontario	0.516	8.3	10.8	17.5	15.9	5.5	9.7	2.0	20.5	14.0	91.2	.413	.509	.522	.347	1.41	2.82
61– 3	Mich. Huron	0.503	5.5	7.2	12.9	16.2	4.4	6.8	2.5	26.3	21.1	141.6	.383	.654	.421	.329	1.33	3.90
59–22	Wisc. Antigo	0.497	14.5	14.4	18.2	19.7	6.0	10.1	3.2	22.3	34.6	160.6	.365	.677	.515	.304	1.89	4.10
59–18	Wisc. Katahdin	0.475	15.1	15.3	19.0	17.1	8.4	7.7	2.7	16.2	33.5	140.8	.326	.620	.656	.462	1.85	3.77
60– 1	Pa. Merrimack	0.469	8.0	10.5	22.2	21.4	6.7	8.8	3.4	23.2	26.5	128.7	.477	.757	.684	.448	1.72	3.82
60– 2	Pa. Merrimack	0.449	7.8	19.7	19.5	22.2	10.4	13.7	3.3	39.3	20.8	131.2	.440	.675	.663	.438	1.76	4.11
61– 2	Mich. Ontario	0.434	9.1	10.1	18.8	18.0	5.4	9.7	2.4	19.0	26.3	112.6	.365	.689	.484	.341	.153	3.32
60– 3	Pa. Merrimack	0.421	9.5	12.0	17.1	15.0	7.8	9.3	5.2	20.8	33.2	149.0	.501	.771	.663	.382	2.00	4.01
60– 5	Wisc. Antigo	0.405	13.9	13.6	18.4	18.2	5.6	5.3	8.0	29.3	43.4	119.8	.429	.586	.429	.299	1.92	3.42
61– 5	Wisc. Kennebec	0.396	7.9	5.9	19.7	20.1	12.3	12.1	16.7	33.9	87.1	173.6	.593	.740	.646	.414	3.12	4.60
60– 4	Me. Katahdin	0.332	9.5	10.5	22.9	20.6	7.1	8.1	4.4	24.2	69.0	185.8	.468	.718	.610	.390	2.49	4.59
60– 7	Wisc. Katahdin	0.332	10.2	14.1	18.1	15.0	6.2	6.6	7.7	30.5	62.1	144.0	.486	.716	.540	.420	2.35	4.04
59–16	Wisc. Early Gem	0.322	11.1	12.6	19.3	24.6	4.3	12.2	2.5	24.8	82.4	182.5	.316	.832	.405	.300	2.27	4.66

59–13 Mich. Huron	0.306	11.8	11.4	18.5	18.0	8.7	13.2	18.0	29.8	94.8	176.0	.656	.826	.383	.282	3.01	4.42
61– 6 Wisc. Katahdin	0.269	7.2	10.0	21.0	19.3	9.5	9.1	19.2	40.6	94.7	179.8	.663	.776	.586	.372	3.26	4.78
59–17 Wisc. Ontario	0.259	11.3	8.5	17.1	21.3	6.6	11.1	8.7	26.4	58.8	153.4	.394	.736	.622	.466	2.28	4.22
61– 7 Me. Kennebec	0.256	6.4	7.1	24.3	21.1	8.9	8.2	14.9	36.0	76.7	141.0	.602	.657	.541	.382	2.83	3.99
60– 6 Wisc. Red Lasoda	0.239	10.9	14.3	20.7	21.6	7.2	7.9	7.8	21.6	90.4	183.3	.577	.767	.690	.459	3.05	4.67
60–16 Mich. Huron	0.235	4.7	4.9	17.9	20.0	5.7	7.4	10.0	35.0	60.1	174.8	.481	.664	.561	.342	2.32	4.43
60–11 Mich. Ontario	0.204	6.6	6.2	19.1	19.6	5.3	6.7	4.3	30.5	26.9	93.6	.366	.561	.481	.378	1.55	3.05
60–12 Mich. Ontario	0.183	7.5	7.9	19.4	18.3	5.6	7.4	16.4	30.9	65.4	112.6	.565	.620	.427	.351	2.47	3.39
61– 4 L. I. Katahdin	0.162	11.0	12.7	17.7	18.1	12.3	9.3	6.2	24.6	96.6	203.0	.532	.947	.622	.310	3.03	4.90
59–14 Mich. Cherokee	0.150	17.2	13.6	21.4	17.8	10.4	8.9	8.3	20.8	82.2	154.8	.629	.697	.381	.196	2.75	3.87
60– 9 Wisc. Kennebec	0.134	11.1	11.4	19.6	19.4	8.3	9.3	21.8	38.8	92.6	170.4	.557	.798	.665	.446	3.23	4.72
60–10 Wisc. Ontario	0.128	11.1	10.3	20.0	18.2	8.8	10.0	20.6	35.5	76.9	126.4	.541	.752	.676	.500	3.03	3.99
60–13 Mich. Ontario	0.124	6.2	7.3	21.8	21.2	5.2	7.1	7.7	28.3	60.0	133.8	.477	.629	.465	.353	2.22	3.70
60–19 Mich. Ontario	0.095	12.7	12.0	23.6	20.8	7.3	8.8	10.7	30.2	60.5	145.4	.462	.663	.574	.383	2.26	4.02
59–12 Mich. Manota	0.069	0.7	5.1	22.9	24.7	10.4	12.0	15.3	22.2	103.6	158.0	.598	.798	.362	.300	3.13	5.00
60– 8 Wisc. Early Gem	0.052	12.1	11.7	22.1	17.2	13.5	12.5	21.4	31.8	63.5	149.8	.433	.812	.640	.444	2.77	4.33
61– 8 Me. Katahdin	0.052	8.1	10.3	20.5	21.3	4.9	8.2	14.7	37.6	72.0	143.4	.568	.740	.624	.494	2.75	4.27
60–14 Mich.?	0.015	5.1	4.9	21.5	23.7	4.1	6.7	11.4	36.4	75.7	159.6	.549	.746	.554	.407	2.65	4.39
60–17 Mich. Cherokee	0.010	12.0	11.9	18.3	20.4	4.8	7.3	5.3	17.9	55.6	178.4	.445	.648	.423	.325	2.65	4.26
60–18 Mich. Russet Rural	0	9.9	12.5	19.3	20.6	6.7	5.5	16.9	31.4	48.8	151.4	.495	.645	.425	.438	2.17	4.09
59–21 Wisc. Kennebec	0	13.3	13.7	20.0	23.0	7.6	14.7	8.8	26.9	77.9	216.0	.596	.862	.431	.340	2.65	5.29
60–15 Mich. Russet Rural	0	9.8	10.8	15.3	16.9	7.2	7.4	22.7	41.7	93.3	154.5	.409	.604	.352	.330	2.76	4.17

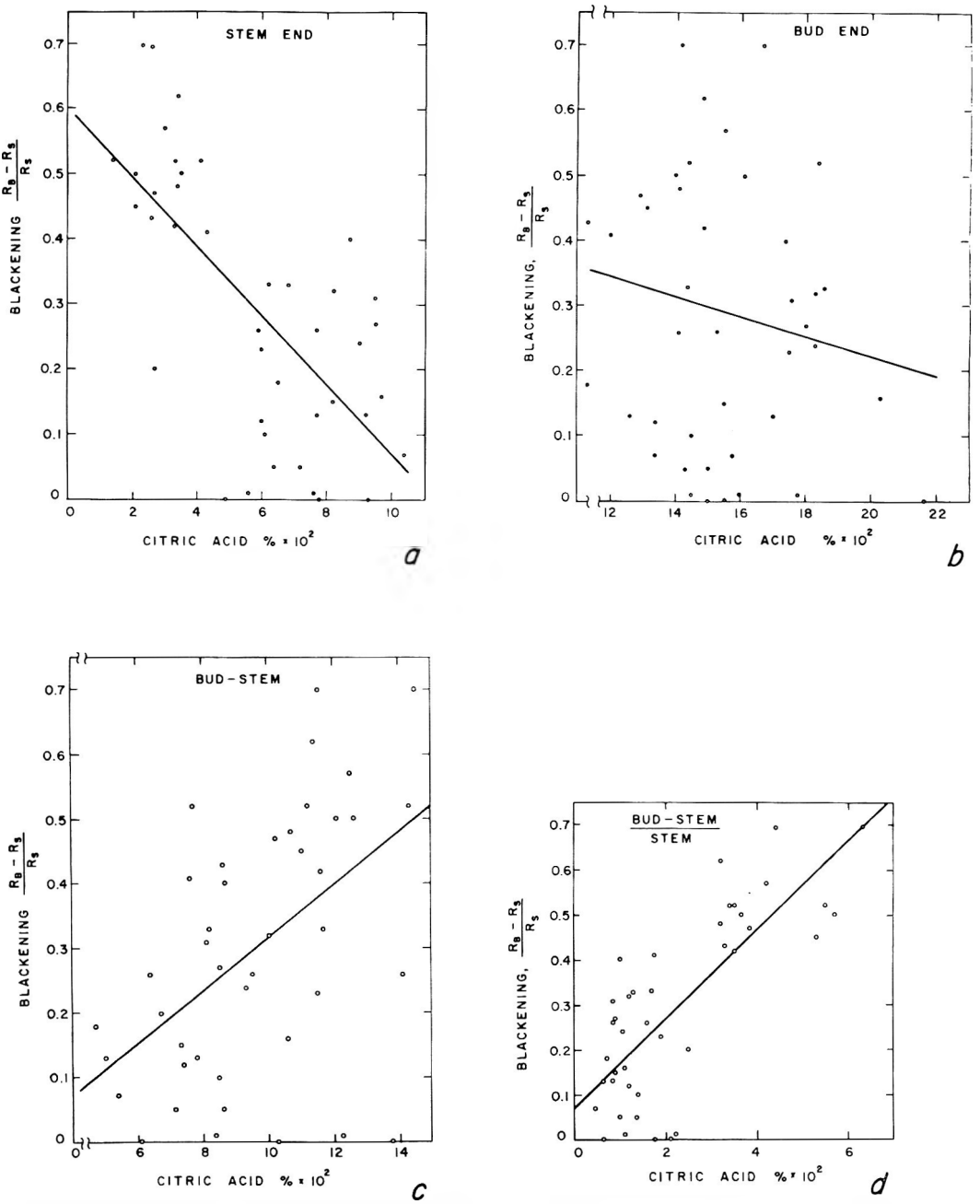

Fig. 1. Variation of degree of discoloration with the citric acid content. (a) Stem end, (b) bud end, (c) bud minus stem, (d) ratio of bud minus stem to stem.

The interrelationships of some of the constituents studied in this and previous publications from this laboratory (Heisler *et al.*, 1962, 1963) were investigated. It was found that the ratio of iron/citric acid on a molar basis gave a slightly higher degree of correlation with blackening than either iron or citric acid alone (see Fig. 3 and Table 5). The r value for the stem-end data was increased from 0.680 to 0.777 (r^2 =

Table 2. Range of organic acid values for 41 samples.

	Stem			Bud			Bud-stem
	High	Low	Av.	High	Low	Av.	Av.
Mg/100 ml							
Glutamic	17.2	04.7	10.7	19.7	04.9	11.4	0.7
Aspartic	24.3	12.9	19.4	24.7	15.0	19.7	0.3
Pyroglutamic	13.5	04.1	07.2	15.4	05.3	09.4	2.2
Malic	22.7	02.0	09.0	41.7	11.7	27.0	18.0
Citric	93.3	14.0	57.7	216.0	91.2	152.8	95.1
Meq/100 ml							
o-Phosphoric + oxalic	0.66	0.30	0.48	1.03	0.51	0.72	.24
Unknown	0.69	0.32	0.53	0.53	0.20	0.38	−.15
Total	3.26	1.33	2.29	5.29	2.82	4.14	1.85

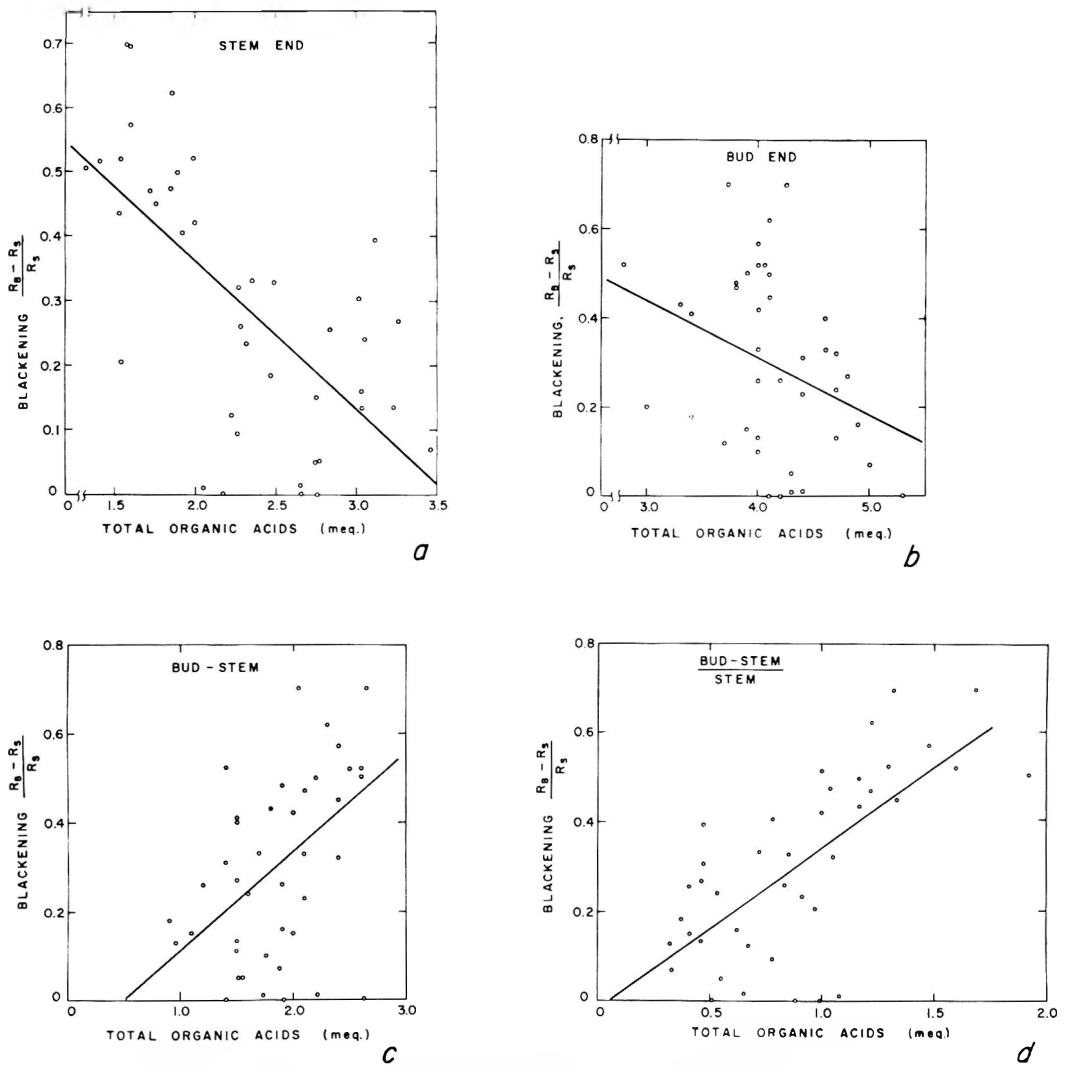

Fig. 2. Variation of degree of discoloration with the total organic acids content. (a) Stem end, (b) bud end, (c) bud minus stem, (d) ratio of bud minus stem to stem.

Table 3. Summary of statistical study of organic acid data.

Acid	Stem		Bud		Bud-stem		Bud-stem/stem	
	F	r	F	r	F	r	F	r
Glutamic	5.55*	.352*	9.34**	.438**	< 1	.095	.....	.....
Aspartic	5.65*	.356*	1.58	.198	< 1	.114	.....	.....
Pyroglutamic	≅ 1	.192	≅ 1	.184	.....	.331**	.....	.....
Malic	31.38**	.667**	18.46**	.568**	< 1	.....	19.86**	.580**
Citric	33.58**	.680**	≅ 1	.203	12.90**	.498**	56.59**	.768**
o-Phosphoric + oxalic	8.77**	.427**	≅ 1	.....	4.73*	.329*	4.18*	.312*
Unknown	< 1	.055	< 1	.045	< 1	.127	.....	.....
Total acids	28.36**	.649**	4.41*	.320*	11.76**	.480**	37.23**	.699**

* Significant at 5% level.
** Significant at 1% level.

0.604). This indicates that 60.4% of the variability of blackening is due to the ratio of iron to citric acid. Again, this is on the low side for prediction purposes. It is readily apparent that even in blackening potatoes there is always a large excess of citric acid over iron. What, therefore, prevents the citric acid from chelating the iron in blackening potatoes? Hughes and Swain (1962b) discussed this situation and suggested that a mixed complex of iron, chlorogenic acid, and citric acid is perhaps formed. They also recognized the fact that perhaps not all the citric acid in the tuber is free for complex formation, that some may be bound by calcium or other substance.

A possible solution to the problem is suggested by studying the ratio of potassium/citric acid on an equivalent basis. This ratio (stem-end values) is plotted against blackening in Fig. 4, and the F values obtained by statistical analysis are presented in Table

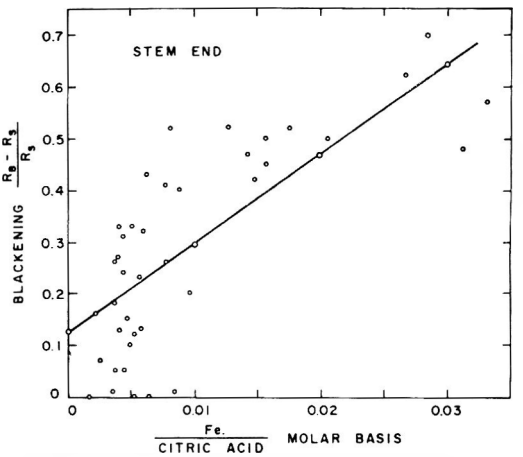

Fig. 3. Variation of degree of discoloration with the ratio of iron content to citric acid content, stem-end values.

Table 4. Summary of statistical study of subgroups (citric acid data).

Subgroups	No. of samples	Stem		Bud		Bud-stem		Bud-stem/stem	
		F	r	F	r	F	r	F	r
Varieties									
Ontario	8	12.80*	.825*	2.59	.549	< 1	.346	32.33**	.919**
Katahdin	7	7.92*	.782	< 1	.339	3.34	.635	20.33**	.896**
Kennebec	5	2.66	.685	2.08	.640	< 1	0	1.71	.602
State grown									
Wisconsin	19	28.56**	.792**	1.70	.302	6.40*	.524*	36.94**	.828**
Michigan	15	8.19*	.621*	4.25	.490	< 1	.158	19.49**	.774**
Crop year									
1959	14	43.33**	.885**	3.84	.500	5.22*	.549*	37.59**	.874**
1960	19	8.32*	.572*	< 1	.230	1.65	.298	11.34**	.632**
1961	8	6.84*	.730*	2.54	.543	< 1	.303	9.84*	.788*

* Significant, 5% level.
** Significant, 1% level.

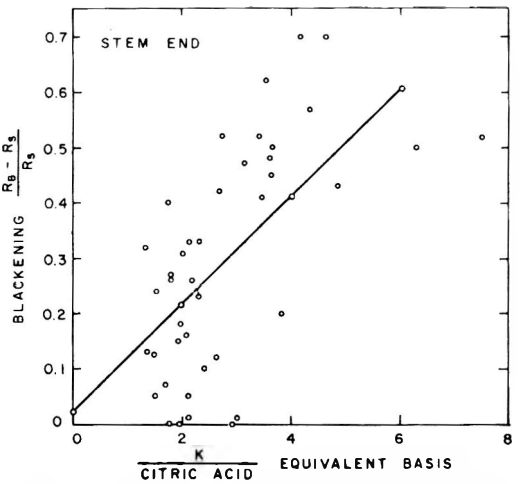

Fig. 4. Variation of degree of discoloration with the ratio of potassium content to citric acid content. stem-end values.

5. The high F values indicate that a highly significant correlation exists between this ratio and degree of discoloration, the larger ratio tending to be associated with the discoloration. Thus, blackening is associated with a large excess of potassium over citric acid. In this role, potassium would act as a blackening inducer. These new data change the role of potassium in the blackening picture from that which was indicated by a previous analytical study (Heisler *et al.*, 1962) in which it was found that the stem end always contained less potassium than the bud end. The study of the ratio of potassium/citric acid on an equivalent basis, however, shows that the citric acid content of the stem end is lower relative to the bud end, than the potassium content. This accounts for the fact that the stem-end ratio is always higher than the bud-end ratio. The role of potassium was further investigated by some *in vitro* experiments. Working with a model system of iron (.030 mmoles) and chlorogenic acid (.045 mmoles) brought to a pH of 6.0 with .050 mmoles of NaOH (total volume 15 ml), it can be demonstrated that although citric acid (as little as .007 mmoles) completely decolorized the dark blue-green iron-chlorogenic acid solution, an equal amount of potassium citrate did not, and a large excess darkened the solution further. The pH changed from 6.0 to 4.9 on addition of citric acid, and to 6.3 on addition of potassium citrate. Generally the same result was obtained in a system containing 10 ml of 1% aqueous potato extract plus all other reagents mentioned above except NaOH (total volume again = 15 ml). In this case, however, .020 mmole of citric acid was required to effect decolorization, probably because of the buffer effect of the extract. Here the pH was reduced from 4.9 to 4.5 by the citric acid, and raised to 5.2 by the potassium citrate. It appears that potassium may act in two ways. From the fact that potassium citrate does not decolorize the iron-chlorogenic acid solution it follows that if the concentration of potassium and other conditions are such that the citric acid-salt equilibrium is shifted to the salt side, then potassium would act as a blackening inducer by lessening the effect of citric acid. Potassium citrate may also act to enhance the formation of the iron-chlorogenic acid complex by raising the pH. The above work also indicates that the citric acid effect is at least partly due to its lowering of the pH. It should be emphasized, however, that an iron-chlorogenic acid solution is normally blue-green at a pH above 4.0, and that citric acid decolorized the solution at a pH higher than 4.0. Also, the pH of the potato is fairly constant, varying only slightly from sample to sample, normally from pH 5.9 to 6.1.

Even though potassium appears to be an important factor in the blackening mechanism, it is not likely a controlling factor, since there is probably an excess of potas-

Table 5. Summary of statistical study of interrelationships of constituents.

	Stem		Bud		Stem-bud	
	F	r	F	r	F	r
Fe/citric acid molar basis	59.01**	.777**	9.71**	.446**	61.25**	.782**
K/citric acid equiv. basis	25.02**	.625**	7.18*	.394*	24.40**	.621**

* Significant, 5% level.
** Significant, 1% level.

sium available to the potato tuber. The prime consideration as to whether a potato blackens or not is probably the amount of free organic acid (citric, oxalic, phosphoric, malic) present, or potentially present, and this in turn is dependent on the equilibrium constants of the possible reactions involved.

ACKNOWLEDGMENT

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Sesquiterpenes.
I. Nootkatone, A New Grapefruit Flavor Constituent

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SUMMARY

The bicyclic conjugated sesquiterpene ketone, nootkatone, has been found in grapefruit peel oil and in peel-oil-free grapefruit juice. The flavor intensity of grapefruit oil appears to be related to the relative abundance of nootkatone. A combination of gas-liquid chromatography and thin-layer chromatography revealed the presence of this ketone, which was identified by its infrared spectrum and the melting point of its 2,4-dinitrophenylhydrazone derivative. Traces of nootkatone were found also in bergamot, lemon, lime, orange, and tangerine oils.

INTRODUCTION

A desire for an understanding of grapefruit (*Citrus paradisi* Macfayden) aroma or flavor has prompted a number of investigations into the volatile composition of grapefruit peel oil and juice. Guenther (1949) listed several early chemical characterizations of grapefruit oil. Kirchner *et al.* (1953) and Kirchner and Miller (1953) reported an extensive study of the volatiles in grapefruit juice. More recently, Stanley *et al.* (1961) and Ikeda *et al.* (1962a,b) reinvestigated the terpene hydrocarbons and aldehydes of grapefruit oil by gas-liquid chromatography (GLC), and Hunter and Brogden (1964) combined GLC with mass spectroscopy to determine the sesquiterpene hydrocarbon composition of the oil.

Despite these studies, the unique flavor-aroma essence of grapefruit cannot be ascribed solely to the constituents thus far identified, nor has any single compound been found to dominate the essence, as citral does in lemon. The finding of nootkatone in grapefruit oil and juice adds a significant compound to the list of grapefruit flavor constituents.

The relation of this grapefruit oil constituent to grapefruit flavor intensity was first pointed out by Beisel *et al.* (1963) of Sunkist Growers. They observed in gas chromatograms of California cold-pressed grapefruit oil that after the passage of sesquiterpene hydrocarbons an unidentified peak appeared which was prominent in the more highly flavored oils but less apparent in oils of mediocre or weak flavor.

EXPERIMENTAL

Gas-liquid chromatography. GLC operations were conducted with an Aerograph Autoprep thermal-conductivity detection chromatograph. Preparative GLC samples were obtained from a ⅜-in. × 10-ft aluminum column packed with 30% w/w SE-30 on 60/80-mesh Chromosorb P. When the column was operated at 200°C under a helium flow rate of 200 ml/min, the retention volume for nootkatone was 2100–2300 ml. Automatic sample injection and peak collection were used for samples having a well defined nootkatone peak; otherwise the ketonic sample was injected and collected manually with collection based on the ketone retention volume.

The GLC survey of citrus oils was carried out with the same chromatograph using a ¼-in. × 10-ft stainless-steel column containing 20% w/w SE–30 on 60/80-mesh Chromosorb P. At 215°C and helium flow of 120 ml/min the retention volume for nootkatone was 2400–2500 ml. Effluent vapor corresponding to the ketone retention volume was collected on a TLC plate as described below.

Thin-layer chromatography. Silica gel TLC plates containing $CaSO_4$ binder were prepared on Brinkmann-Desaga apparatus and dried for 1 hr at 115°C. The sample to be analyzed was applied

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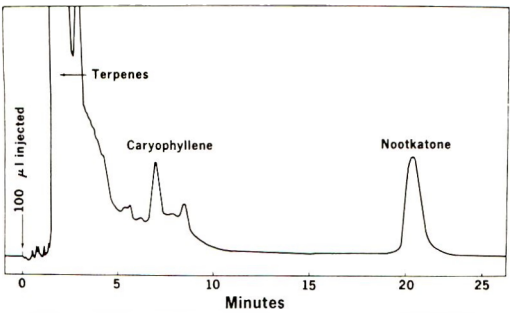

Fig. 1. Gas chromatogram of high-quality grapefruit oil on the ¼-inch column at 215°C.

to the plate by directing the effluent GLC vapor onto the sample origin position on the plate, as described by Nigam *et al.* (1963). Sample application was synchronized with the nootkatone emergence from the GLC column, as determined by its retention volume. A control sample of nootkatone was also applied to the plate, in parallel, for R_f comparison. The plate was developed by ascending elution with 20% v/v ethyl acetate in heptane, dried, and sprayed with a 0.3% w/v solution of 2,4-dinitrophenylhydrazine in methanol containing 3% v/v of 38% w/w hydrochloric acid. Collected GLC samples containing nootkatone develop an orange-colored spot at the same R_f as did the control sample. Warming the plate accelerates color development.

Infrared measurements. Infrared spectral identifications were obtained in carbon tetrachloride solution on a Perkin-Elmer 521 spectrophotometer.

Melting points. All melting points were taken on a Kofler hotstage microscope and were corrected.

Nootkatone in grapefruit oil. The constituent corresponding to an unidentified GLC peak in better-quality California cold-pressed grapefruit oil was isolated and identified in the following manner. A 500-g sample of oil was rapidly distilled up to 180°C/1 mm Hg in a short-path apparatus. Automatic preparative GLC of the distillate provided 2.4 g of the unknown constituent, which was shown to be a ketone by infrared and TLC analyses. Chromatography on 60 g of Merck acid-washed alumina, eluting with 30% v/v ether in petroleum ether, afforded 2.1 g of the ketone. Little change, except for increased definition, was noted in the infrared spectrum. A 2,4-dinitrophenylhydrazone derivative, mp 157–158°C, was prepared in the usual manner. When the derivative was mixed with authentic nootkatone 2,4-dinitrophenylhydrazone (Erdtman and Hirose, 1962), no depression in the melting point resulted. The chromatographed ketone was further purified by fractional distillation in a 6-mm × 10-cm Vigreaux column at 128–130°C/1 mm Hg. Overall yield was 1.7 g, or 0.3%. A center cut, bp 129°C/1 mm Hg, crystallized upon refrigeration. Recrystallization twice from petroleum ether at −70°C and twice from methanol at −15°C gave pure nootkatone, mp 36–37°C, identical in infrared spectrum to authentic nootkatone.

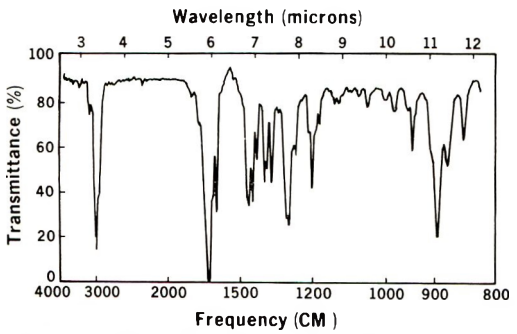

Fig. 2. Infrared spectrum of nootkatone, 10% w/v in CCl_4.

Nootkatone in other citrus oils. Commercially cold-pressed domestic lemon, lime, orange, and tangerine oils and imported bergamot oil were surveyed for nootkatone by the analytical GLC-TLC combination. Oil samples of 0.2 ml were injected into the analytical GLC column. Occasional column backflushing at 350°C was required to minimize accumulation of residue. All samples analyzed gave TLC spots comparable to nootkatone.

To confirm the presence of nootkatone, a 200-g portion of each oil was concentrated 4-fold on a 6-mm × 18-in. Nester/Faust spinning band distillation column at 70–90°C/30–25 mm Hg. The pressure was gradually reduced to 2 mm Hg, and a fraction, bp 125–160°C, was collected. Unwanted lactones and esters were saponified by boiling the fraction for 30 min in 50 ml of 10% w/v ethanolic KOH. The mixture was diluted with 10% w/v aqueous KOH and extracted with petroleum ether. The extract after solvent evaporation was chromatographed on 30 g of Merck acid-washed alumina with 30% v/v ether in petroleum ether. Finally, micro short-path distillation up to 150°C/1 mm Hg afforded 50-200 mg of an amber-colored oil. Manual preparative GLC collection combined with infrared spectral identification confirmed the presence (less than 0.01%) of nootkatone in these samples.

Nootkatone in grapefruit juice. Fourteen ripe, Arizona, Marsh seedless grapefruit were selected. The outer peel, i.e., the flavedo and part of the albedo, was manually removed in the draft of an efficient hood to sweep away any peel mist generated. Immediately the partially peeled fruit and the operator's hands were immersed several times

in two successive 1000-ml baths of petroleum ether, then the fruit was set aside to dry. The remainder of the albedo was then carefully stripped by hand, and the washing procedure repeated with fresh solvent. The fruit were macerated in a Waring blender to furnish 1 gal. of juice and rag, which was extracted with 1000 ml of ether. Then 1000 ml of petroleum ether was added and the phases were separated. The extraction was repeated. The extract was evaporated to about 2 g of residue, which was chromatographed on 60 g of Merck acid-washed alumina. Fractions eluted with 30% v/v ether in petroleum ether amounted to 77 mg of crude ketone. Preparative GLC of the principal peak gave a few mg of nootkatone, which was identified by its GLC retention volume, TLC R_f, and infrared spectrum.

Commercial waxes. Coumarone-indene resin, turpentine resin, carnauba wax and paraffin, waxes frequently applied to the exterior of citrus fruit as a preservative, were analyzed for nootkatone. Wax samples were dissolved or slurried in a volatile solvent, such as methylene chloride, and applied to a TLC plate. Development in the manner described above failed to reveal an orange spot comparable to the R_f of nootkatone.

Taste studies. A 5% v/v solution of nootkatone, mp 30–31°, in 100 μl of 190-proof ethanol and a 32° Baumé solution of sucrose in 500 ml of distilled water were prepared. Ten taste-panel members evaluated samples of 5 ml of sugar solution in 10-ml beakers to which various amounts of nootkatone solution had been added and well mixed with individual glass rods. The order of evaluation was odor first, then taste, in samples containing increasing increments of nootkatone. Continual reference was made to an alcohol-sugar-water blank.

Stability studies. Separate samples of nootkatone were allowed to stand in ambient air 30 days, heated 1 hr at 300°C under nitrogen, refluxed 30 min in 10% w/v ethanolic KOH, and heated overnight at 100°C in glacial acetic acid. The recovered samples were analyzed by IR.

RESULTS AND DISCUSSION

The constituent corresponding to an unidentified GLC peak prominent in highly flavored grapefruit oil was isolated. It is shown to be the sesquiterpene ketone, nootkatone, by its melting point, infrared spectrum, and melting point of its 2,4-dinitrophenylhydrazone, underpressed when mixed with the authentic nootkatone derivative (Erdtman and Hirose, 1962).

In pure form, nootkatone is a colorless, low-melting, crystalline solid having an astringent taste and having a musty odor somewhat reminiscent of certain green teas. Numerous experienced citrus tasters have indicated that the taste and odor of nootkatone is definitely recognizable as contributing to grapefruit flavor. Taste-panel studies were conducted with ten ordinary participants selected at random to determine the threshold of flavor detection. Typical threshold values of taste detection in sugar solution are 20–40 parts per million (ppm). At 100 ppm the taste of nootkatone is distinct. The odor of the ketone is detectable below 10 ppm. The abundance of nootkatone in grapefruit peel oil ranges as high as 3,000 ppm (0.3%). However, its presence in the grapefruit juice analyzed appeared to be around the threshold of taste detection when extensive precautions were taken to prevent juice contamination with peel oil. Since most means of eating the fruit or obtaining the juice ensure the mixing of some peel oil flavor with the juice, the concentration of nootkatone in grapefruit juice ordinarily encountered is probably above the threshold of odor and taste detection.

Table 1. Nootkatone taste detection in 32° Baumé aqueous sucrose by a ten-member panel.

Ketone (ppm)	Tasters noting difference from control
10	1
20	5
40	9
100	10

Kirchner and Miller (1953) reported the first indication of an unknown sesquiterpene ketone in grapefruit juice. Our investigation of peel-oil-free, oily extract from grapefruit juice suggests that the sesquiterpene ketone they isolated was probably nootkatone. Traces (<0.01%) of the ketone were found in the cold-pressed peel oils of lemon, lime, orange, tangerine, and bergamot. Teranishi (1963) also reports its presence in the oily condensate obtained from the commercial concentration of Florida orange juice.

As a flavor constituent nootkatone is a reasonably stable compound largely unaffected by prolonged exposure to ambient air or elevated temperatures in inert atmos-

phere. Heating in ethanolic KOH or glacial acetic likewise causes little alteration.

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

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Some Volatile Constituents of Passion Fruit Wine

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SUMMARY

A dry table wine prepared from *Passiflora edulis* var. *flavicarpa* was found to contain the following volatile substances: methyl alcohol, ethyl alcohol, isobutyl alcohol, isoamyl alcohol, active amyl alcohol, *n*-hexyl alcohol, 2-phenethyl alcohol, acetaldehyde, diethyl acetal, 2-butanone, 2,3-butanedione, ethyl acetate, ethyl isovalerate, ethyl caproate, ethyl heptanoate, ethyl caprylate, ethyl caprate, ethyl laurate, isobutyl isobutyrate and isoamyl acetate. Probably present are: *n*-butanol, ethyl isobutyrate, and *n*-hexyl caproate. Gas chromatographic retention times are given for a number of unidentified components of the passion fruit wine volatiles. The compounds responsible for the typical passion fruit wine aroma remain unidentified.

INTRODUCTION

Of the over 300 species of the genus *Passiflora*, two (*Passiflora edulis* Sims and the yellow passion fruit, *Passiflora edulis*, var. *flavicarpa*) are grown commercially for human consumption. The *flavicarpa* variety, with its yellow rind at maturity, produces generous amounts of juice or pulp and has more acid at maturity than does the purple Sims variety.

Both the purple and yellow passion fruit varieties have intense pleasant aromas and flavor at maturity. Because of the intense aroma there has been interest in California in preparing wines from passion fruit, both as 100% passion fruit wine and as blends with more neutral vinifera grape wines.

Little had been known about the flavor constituents of passion fruit juice, and even less about the flavor constituents of wine made with passion fruit juice. Investigations had been limited to analyses of an elementary nature on the juice pulp and whole fruit until recently, when Hiu and Scheuer (1961) reported the use of classical and gas chromatographic techniques to identify four esters, ethyl butyrate, ethyl caproate, *n*-hexyl butyrate, and *n*-hexyl caproate as the major constituents of passion fruit juice. Identification of a number of the volatile aroma materials from passion fruit dry table wine is the subject of this report.

EXPERIMENTAL

Passion fruit juice. Forty-eight No. 10 cans of single-strength frozen juice of *Passiflora edulis* var. *flavicarpa* were most generously made available by the Food Processing Laboratory of the University of Hawaii. Upon melting and mixing, the sample was found to consist of 299 lb of juice with a density of 17.7° Brix and a pH of 2.82.

Wine preparation. To the passion fruit juice, contained in a 200-gallon stainless-steel tank equipped with a stirrer, was added 25 lb of sucrose in order to raise the Brix degree to 23.0. The sweetened juice was inoculated with 1 L of a specially prepared starter of *Saccharomyces cerevisae* var. *ellipsoideus*, trained to grow at a pH of 2.8 on especially acidified Thompson Seedless grape juice. Within 24 hr the juice was fermenting actively and the temperature had risen from 20°C to 30°C. At this time the fermenting juice was transferred to a smaller stainless-steel tank and placed in a cold room at 4°C. At the completion of fermentation (when all the sugar has been converted to ethanol and carbon dioxide), the wine was transferred into four 12-gallon Pyrex bottles and allowed to settle. Three layers were formed. The upper layer contained floating pulp fragments, the lower layer was predominantly yeast sediment, and the center layer consisted of the clear wine. After separation of the layers, the upper and lower layers were washed by agitation with distilled water and decantation after a period to permit settling. The clear wine combined with the washings was stored in completely filled bottles at 4°C.

[a] Taken from the M.S. thesis of Carlos J. Muller, University of California, Davis, 1962.

Flash evaporator. The volatiles were stripped from the passion fruit wine by passing it through a stainless-steel-and-glass flash evaporator, described previously (Kepner and Webb, 1956). The wine was fed into the evaporator at a rate of 16 L/hr. Under operation at a pressure of 20 mm Hg, approximately 60% of the feed material was flash-distilled into the volatiles collectors. The nonvolatilized portion from the first flash evaporation was diluted with an equal quantity of water and repassed through the flash evaporator. This sequence of operation was repeated again, after which the nonvolatile portion was discarded since it no longer smelled of passion fruit. The three volatile fractions obtained were blended and stored at 4°C until they could be extracted.

Extraction. The flash-evaporated volatiles from the passion fruit wine were extracted in Kutscher-Steudel-type extractors of 12-L capacity. A battery of four of these extractors was available. The boilers used were of 100-ml capacity. Redistilled normal pentane (Phillips Petroleum Co.) of boiling range 35–36.5°C was used for extraction of the wine. Because of the small size of the boiler and the large amount of extractable materials present in the wine it was necessary to empty and recharge the boiler every 6 hr for the first 24 hr of operation. Daily changes of the pentane in the boiler thereafter sufficed. Extractions were continued for a total of 190 hr, at which time there was no significant temperature rise in the boiling point of the contents of the boiler. The pentane extract collected totaled 1.2 L. These extracts were stored over anhydrous magnesium sulfate at −10°C. The aqueous alcoholic residues from the extraction were mixed and stored at 4°C.

About 80% of the normal pentane was stripped from the pentane extracts of the wine by a single passage through the flash evaporator. This stripped pentane fraction was shown by gas chromatography on the Pluronic and Squalene columns to be identical in composition to the pentane used for the extraction. The pentane was further stripped from the extract with a Wheeler center-rod column provided with a dry-ice condenser to reflux some of the pentane. The pressure of the distillation was 150 mm Hg. One hundred and ten ml of pentane were distilled from the extract by this technique. Gas chromatographic examination of this distillate showed it to be essentially the normal pentane-extracting solvent. The approximately 50 ml residue from this distillation (labeled PEX) was stored at −10° until used for gas chromatographic analyses.

Separation of ethanol from extraction residue. The Oldershaw distillation setup, adapted for continuous operation as previously described (Webb and Kepner, 1956), was used to separate the ethanol from the other non-pentane-soluble substances in the extraction residues. The feed rate to the column was 500 ml/hr. The overhead take-off rate was 100 ml/hr, and the bottom take-off rate was 400 ml/hr. Forty-one and one-half L of ethanol boiling between 77.0 and 79.0°C and labeled ALC were collected.

The essentially dealcoholized underflow fraction from the column was rerun at a feed rate of 600 ml/hr with an overhead take-off rate of 10 ml/hr and an underflow take-off rate of 590 to 600 ml/hr. The underflow material had no odor and was discarded. The overhead distillates were saturated with sodium chloride and extracted with diethyl ether in the continuous Kutscher-Steudel type extractors. Three and one half liters of ether extract were obtained. After drying over magnesium sulfate, the ether was stripped from this fraction with the Wheeler center-rod column used at atmospheric pressure. Gas chromatograms of this stripped fraction showed it to be ether plus trace amounts of ethanol. The stripped residue was labeled ETX.

The aqueous residue from the ether extraction was steam distilled at atmospheric pressure to yield 1.6 L of distillate which was labeled EWR.

Gas liquid partition chromatograph. The instrument used in this research, constructed in this laboratory, has been described more fully elsewhere (Webb and Kepner, 1961). The various columns used in the anlyses were prepared in this laboratory from the 40–60-mesh fractions of Johns-Manville C-22 firebrick. All tubing columns were of ¼-inch outside diameter.

Pluronic column. Five grams of pluronic F-68 on 18 g of firebrick was packed into a 6-ft length of stainless-steel tubing.

Squalane column. Three-and-one-half grams of squalane (2,6,10,15,19,23-hexamethyl-tetracosane) on 12 g of firebrick was packed into a 6-ft length of stainless-steel tubing. A 3-inch section of untreated firebrick was added at the exit end of the column.

Dinonyl phthalate. Six grams of dinonyl phthalate was mixed with 18 g of the firebrick and packed into a 9.5-ft length of stainless-steel tubing.

Reoplex. Five grams of Reoplex 400 was mixed with 12 g of firebrick and packed into a 6-ft length of stainless-steel tubing. Three inches of untreated firebrick was packed at the exit end.

Carbowax. Twelve grams of Carbowax 400 was mixed with 20 g of firebrick and packed in an 8-ft length of stainless-steel tubing. Three inches of untreated firebrick was packed at the exit end of the column.

UCON LB 385. Eight grams of UCON material was mixed with 25 g of firebrick and packed

in a 78-in. length of stainless-steel tubing. Three inches of untreated firebrick was packed into the exit end of the column.

Glycerol. Nineteen grams of glycerol was mixed with 72 g of firebrick and packed into a 25-ft length of copper tubing.

Diglycerol. Three grams of diglycerol was mixed with 27 g of firebrick and packed into a 10-ft length of copper tubing.

DEGS. Four and one-half grams of polydiethyleneglycolsuccinate was mixed with 40 g of the firebrick and packed in a 13-ft length of copper tubing.

Dimer acid. Three grams of Emery Industries "Empol 1022" dimer acid was mixed with 27 g of firebrick and packed in a 68-in. length of stainless-steel tubing.

Analyses of the different fractions. Each of the various fractions obtained by extraction and distillation was analyzed for halogens, nitrogen, and sulfur by the sodium fusion test. Functional group tests were run as follows: Schiff's test for aldehydes, Tollen's test, 2,4-dinitrophenylhydrazine test, periodic acid test, and test for unsaturation using bromine in carbon tetrachloride. The chromotropic acid test for methanol was performed on three of the four fractions.

Solid derivatives were prepared for identification of certain of the components of each of the passion fruit wine fractions. The 2,4-dinitrophenylhydrazones of carbonyl compounds were prepared and separated on columns of bentonite-celite according to the technique of White (1948). Alcohols in the mixtures were characterized as the 3,5-dinitrobenzoates. These derivatives, prepared according to the technique of Webb *et al.* (1952), were analyzed by column chromatography by the method of White and Dryden (1948).

Esters in the pentane-extract fraction of the passion fruit wine aroma material were determined by the technique described by Kepner and Webb (1961). The ester components were identified gas chromatographically in this case.

The pentane extract and the ether extract of the passion fruit wine were examined in some detail gas chromatographically. A number of different columns were employed.

RESULTS AND DISCUSSION

Four fractions of the volatile-aroma materials from the passion fruit wine were available for analysis. These were the pentane extract of the flash-evaporated volatiles, labeled PEX; the principally ethanol-containing fraction obtained by distillation of the residue from the pentane extraction, labeled ALC; the ether extract of the underflow fraction from the Oldershaw distillation, labeled ETX; and the distillate from the aqueous residue of the ether extraction, labeled EWR. The processing steps used in isolation of the four fractions of the volatile aroma material are indicated in a schematic flow sheet (Fig. 1). Table 1 presents the results of sodium fusion tests and the functional group analyses on these 4 fractions.

Table 1. Results of sodium fusion and functional group tests on the fractions of passion fruit wine volatiles.

Test	Fraction			
	PEX	ETX	ALC	EWR
Sodium fusion; halogens, N, S	−	−	−	−
Schiff's aldehyde	++	++	+	+
Tollen's	++	−	−	−
2,4-Dinitrophenylhydrazine	++	+	+	+
Periodic acid	+	+	+	−
Bromine in CCl_4	+	−	−	−
Chromotropic acid	−	−	+	

Fraction EWR, being essentially water, could not be investigated gas chromatographically with the thermal-conductivity detector in the equipment available. The fact that fraction EWR showed positive Schiff's and 2,4-dinitrophenylhydrazine tests with a negative Tollen's test indicates that traces of acetals remained with the water. This fraction was not investigated further.

Fraction ETX was analyzed gas chromatographically and by the preparation of carbonyl 2,4-dinitrophenylhydrazones as well as by the functional group analyses listed in Table 1. As shown in Table 2, the ETX fraction contained acetaldehyde and 2,3-butanedione as carbonyl components. The 2,3-butanedione was a very minor component. The acetaldehyde detected undoubtedly results from the hydrolysis of acetal by the strongly acid 2,4-dinitrophenylhydrazine derivatizing reagent. The compounds detected by gas chromatography—ethanol, 2,3-butanedione, diethylacetal, isobutyl alcohol, *n*-butyl alcohol, isoamyl and active amyl alcohols, and *n*-hexyl alcohol—were all present in the PEX fraction as well. Their presence in the ETX fraction means simply that pro-

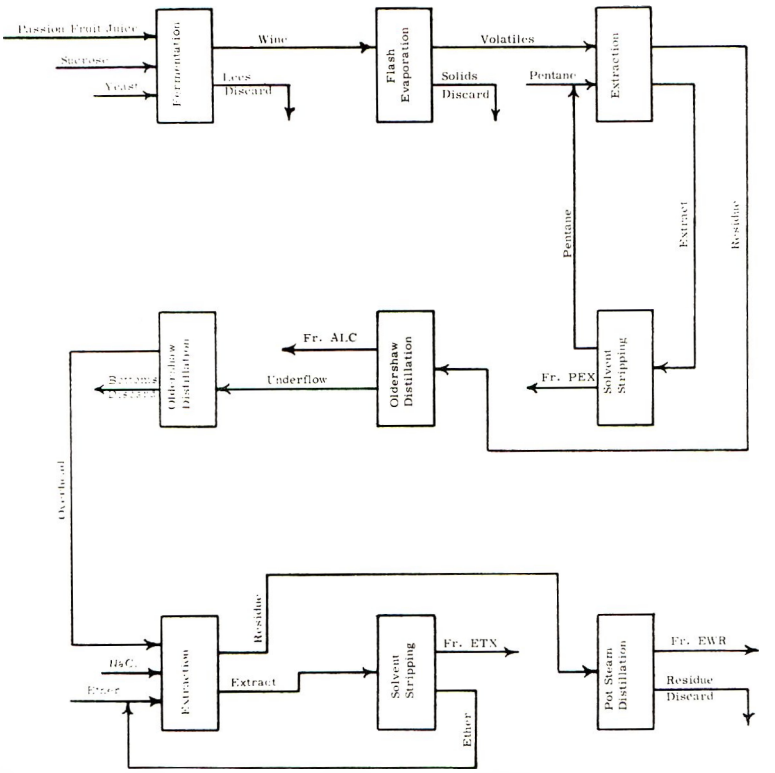

Fig. 1. Schematic flow sheet for volatile aroma isolation from passion fruit wine.

longed extraction does not remove these substances completely from an alcoholic aqueous solution.

Fraction ALC was analyzed gas chromatographically and by the preparation of carbonyl 2,4-dinitrophenylhydrazones as well as by the functional group tests listed in Table 1. The gas chromatograms showed the presence of methanol, ethanol, diethylacetal, 2,3-butanedione, ethyl acetate, and isobutyl alcohol, all of which were also identified in the PEX fraction. Table 2

Table 2. Carbonyl 2,4-dinitrophenylhydrazones from the various fractions of the volatiles from passion fruit wine.

Fraction and band no. in chromatogram [c]	2,4-DNH deriv., m.p. (°C)	2,4-DNH of:	m.p. of mixed known and unknown 2,4-DNH's (°C)
PEX—1	115.2–116.3	2-Butanone	115.5–116.0
—2	oil		
—3	141–149	Acetaldehyde	141–151 [a]
—4	165–166.3	Acetaldehyde	165–166.5 [b]
—5	313.8–315.0	2,3-Butanedione	314–315
ALC—1	115.5–116.2	2-Butanone	115.5–116.2
—2	trace [a]		
—3	165.2–165.9	Acetaldehyde	165.2–165.9 [b]
—4	314.2–315.0	2,3-Butanedione	314–315
ETX—1	143–148	Acetaldehyde	141–151 [a]
—2	165.1–165.7	Acetaldehyde	165–166.5 [b]
—3	314–314.9	2,3-Butanedione	314–315

[a] Unstable isomer of acetaldehyde-2,4-dinitrophenylhydrazone.
[b] Stable isomer recrystallized from ethanol-water-dioxane.
[c] Column chromatography on 3:1 bentonite-Celite, Ref. White (1948).

presents the carbonyl compounds identified in the ALC fraction. The large quantities of acetaldehyde found in this fraction undoubtedly result from the acid hydrolysis of diethylacetal, for the Tollen's test on this fraction was negative. Saponification of a portion of the ALC fraction and preparation of the *p*-phenylazophenacyl derivatives of the acids from esters showed that an acetate ester and traces of some other esters were present. To be present in the ALC fraction, components would have to be either lower-boiling than ethanol or form more complicated azeotropes with ethanol and water since this fraction was obtained by distillation in a 40-plate column under conditions of high reflux. The conditions are easily met by all of the components identified with the possible exception of the isobutyl alcohol. Its presence in the ALC fraction may be taken as tentative evidence of isobutyl alcohol's being a component of some complex azeotrope with a boiling point not too different from that of the ethanol water azeotrope.

Fraction PEX was the most interesting of the four fractions investigated in that it contained the largest number of chemical substances and also in that it was the only fraction which smelled distinctly like the passion fruit. The sodium fusion test indicated that there were no halogens, nitrogen, or sulfur-containing compounds in this fraction. Free carbonyls were indicated present, a positive periodic acid test was obtained, and the presence of some unsaturation was indicated by the bromine absorption test. The chromotropic acid test showed that there was no methyl alcohol present in this fraction. As indicated in Table 2, three carbonyl compounds were identified—2-butanone, acetaldehyde, and 2,3-butanedione. When an aliquot of fraction PEX was saponified and the acids from esters analyzed according to the technique of Kepner and Webb (1961), the acids as ester components listed in Table 3 were identified.

Table 3. Acids from esters in fraction PEX of passion fruit wine volatiles—gas chromatographic analyses.

Free acids, Dimer acid col.	Low-boiling methyl-esters,[a] DiGly col.	High-boiling methyl esters, DEGS col.
Acetic	Acetate	
Isobutyric	Isobutyrate	
n-Butyric	*n*-Butyrate	
Isovaleric	Isovalerate	Isovalerate
Caproic		Caproate
Caprylic		Heptanoate
Capric		Caprylate
		Caprate
		Unidentified [b]
		Laurate

[a] Reference: Kepner and Webb (1961).
[b] Retention time relative to methyl caprylate on DEGS, 180°C, 2.04.

With the exception of the *n*-butyrate and the laurate, it was possible, as described later, to identify an ester by gas chromatographic techniques for each of the acids from esters listed in Table 3.

An aliquot of the PEX fraction was reacted with 3,5-dinitrobenzoyl chloride according to the technique of Webb *et al.* (1952) to yield the solid alcohol 3,5-dinitrobenzoates, which were separated by column chromatography on a 2:1 mixture of silicic acid and Celite by the technique of White and Dryden (1948). Table 4 lists the alcohols identified by this method. Isobutyl and *n*-butyl alcohol were not identified by the column chromatographic method although

Table 4. Alcohol 3,5-dinitrobenzoates from the PEX fraction of the volatiles from passion fruit wine.

Fraction and band no. on chromatogram[a]	3,5-DNB deriv., m.p. (°C)	3,5-DNB of alcohol	m.p. of mixed known and unknown 3,5-DNB's (°C)
PEX—1A1	57.2–58.0	*n*-Hexyl	57.5–58.0
—1B	49–58	act. and iso-Amyl	50–58
—2	92.1–93.0	Ethyl	92.5–93.0
—3	106.5–107.4	2-Phenethyl	106.5–107.5
—4	106.5–107.1	Methyl	106.5–107.1

[a] Column chromatography on 2:1 silicic acid-Celite-Rhodamine 6G, Ref. White and Dryden (1948).

Table 5. Retention times of fraction PEX components of passion fruit wine volatiles relative to that of isoamyl alcohol on various gas chromatographic columns.

Component	UCON LB-385	Glycerol	DEGS
Methyl alcohol	0.077	....	....
Ethyl alcohol	.106	0.590	....
Isobutyl alcohol	.388	.620	.746
n-Butyl alcohol	.481	....	....
act.-Amyl alcohol	1.00	.910	1.00
Isoamyl alcohol	1.00	1.00	1.00
n-Hexyl alcohol	....	1.88	1.51
2-Phenethyl alcohol	....	....	16.2
2,3-Butanedione	.149	.266	....
2-Butanone	.117	....	....
Acetal	.226	.079	....
Ethyl acetate	.127	.079	....
Isoamyl acetate	....	.204	.895
Ethyl isobutyrate	....	....	.642
Isobutyl isobutyrate	....	....	.851
Ethyl isovalerate	....	....	.791
Ethyl caproate	....	....	1.21
Hexyl caproate	....	....	4.46
Ethyl heptanoate	....	....	1.63
Ethyl caprylate	....	....	2.30
Ethyl caprate	....	....	4.78

they were found by gas chromatographic techniques described later. Even though the quantity of methyl alcohol present in fraction PEX was too small to give a positive test with chromotropic acid, the 3,5-dinitrobenzoate was obtained and separated by column chromatography, and the methyl alcohol was found by gas chromatography.

Table 5 lists the retention times of the various components identified in the PEX fraction from the passion fruit wine volatiles. These retention times, calculated with respect to isoamyl alcohol as unity, are determined on three different columns. Among the alcohols listed in Table 5, all are identified both by gas chromatography and the preparation of derivatives with the exception of normal and isobutyl alcohols. In these cases no solid derivatives were obtainable. There was coincidence of the retention times for known and unknown *n*-butyl alcohol only on one gas chromatographic column. For isobutyl alcohol there was coincidence of retention times on three columns. The carbonyl derivatives are well identified both gas chromatographically and through the preparation of the 2,4-dinitrophenylhydrazone derivatives. The esters listed in Table 5, while identified by gas chromatography only on the DEGS column, contain as their acid portions those acids which were identified in the saponified aliquot of the PEX fraction. These esters also have been previously identified in other fermented beverages.

None of the substances listed in Table 5 contain unsaturation. Thus the compound or compounds responsible for the absorption of bromine must be among those substances unidentified.

Table 6 lists the retention times relative to that of isoamyl alcohol on 3 gas chromatographic columns for a number of unidentified substances in the passion fruit wine volatiles. Work on their identification continues in this laboratory. While a mixture of the substances identified and listed in Table 5 has a generally fruity and perfumed aroma, it does not resemble the aroma of passion fruit or a passion fruit wine. The compounds responsible for this distinctive aroma remain unidentified.

Hiu and Scheuer (1961), in their investigation of the volatile oil from distillation of a puree of passion fruit, found that 95% of the oil consisted of four esters—*n*-hexyl caproate, *n*-hexyl butyrate, ethyl caproate, and ethyl butyrate. About 70% of the vola-

Table 6. Retention times of unidentified fraction PEX components of passion fruit wine volatiles relative to that of isoamyl alcohol on various gas chromatographic columns.

UCON LB-385	Glycerol	DEGS
0.051	0.059	0.403
.053	.097	.463
.061	.107	.552
.088	.129	1.39
.097	.366	1.85
.162	.425	2.05
.183	.811	2.64
2.23		2.90
2.86		3.04
		3.34
		3.85
		5.97
		6.86
		9.20
		10.7
		11.5
		13.7

tile passion fruit essence was found to be *n*-hexyl caproate. The passion fruit *wine* prepared from the same variety of passion fruit studied by Hiu and Scheuer was found in this laboratory to contain, at best, only trace quantities of *n*-hexyl caproate. Further investigations are under way to determine if the hexyl caproate is hydrolyzed by the low-pH medium of the passion fruit juice or whether the hydrolysis occurs during fermentation of the passion fruit juice.

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The Mechanism of Formation of *p*,α-Dimethylstyrene in the Essential Oil of Distilled Lime (Citrus Aurantifolia)

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(Manuscript received January 24, 1964)

SUMMARY

The terpene *p*,α-dimethylstyrene was isolated and identified in the essential oil of distilled Mexican lime (*Citrus aurantifolia*, Swingle) but could not be demonstrated in oil expressed from *Citrus aurantifolia*. Experimental evidence is presented supporting a nonbiogenetic mechanism of formation of *p*,α-dimethylstyrene in distilled lime oil from the acid-catalyzed cyclization of citral. The mechanism involves novel reactions of citral and its reaction products that are not consistent with the classically accepted acid-catalyzed reactions of citral and seem to provide an explanation for the appearance of *p*,α-dimethylstyrene in other citral-containing essential oils of the Rutaceae citrus plants.

INTRODUCTION

Recent investigations of the chemical composition of volatiles from orange juice (Teranishi and Buttery, 1962) with gas chromatography and mass spectrometry have resulted in the identification of *p*,α-dimethylstyrene as one of the constituents. However, specific details of this identification were not given. A later, more comprehensive study on orange volatiles (Teranishi *et al.*, 1963) indicates the presence of a compound $C_{10}H_{12}$ that was tentatively assigned the structure of *p*,α-dimethylstyrene based on mass spectroscopic fragmentation pattern and gas chromatographic retention time. It is interesting to note that the author reports finding this compound in that portion of the oil that appeared to be predominantly aldehydes.

Investigations of the essential oils of distilled lime (Kovats, 1963) and mandarin (Kugler and Kovats, 1963) have also resulted in identification of this material.

Other studies of the composition of the essential oils of distilled and expressed lime (Slater, 1961a,b), and orange juice (Wolford *et al.*, 1963) have not resulted in identification of this compound, although there are peaks in the reported chromatograms that could possibly be *p*,α-dimethylstyrene.

Studies of the chemical composition of citrus essential oils have shown striking qualitative similarities among the various oils of this genus (Stanley, 1958); yet *p*,α-dimethylstyrene does not always seem to occur. This seems unusual in view of the obvious structural relationship that exists between this compound and those already commonly found in citrus essential oils.

The literature of terpene chemistry (Simonsen, 1947; Pinder, 1960; de Mayo, 1959; Klouwen and ter Heide, 1962) has not included *p*-α-dimethylstyrene as one of the terpene hydrocarbons. Indeed, other than in recent reports by Teranishi *et al.* (1963) and Kovàts (1963), the literature does not indicate its identification in any of the essential oils (Guenther, 1947a; Gildemeister, 1929; Ikeda *et al.*, 1962); yet, in accordance with the classically accepted definition of "terpenes" (Haagen-Smit, 1948), there is no reason not to consider it as a member of this class of compounds. Studies of the biogenesis of terpenes (Nicholas, 1963) seem to present ample possibilities for the formation of the *p*,α-dimethylstyrene structure, yet the biogenetic existence of this compound does not seem to have been predicted or observed.

These conflicting observations raise a number of questions concerning this compound's appearance in natural products, such as its biogenetic or nonbiogenetic ori-

gin, its precursors and mechanism of formation, an explanation of its appearance in predominantly aldehyde fractions, and its appearance in some varieties of citrus oils and absence in others.

These considerations prompted us to publish our recent investigations of the formation of *p*,α-dimethylstyrene from citral in distilled *Citrus aurantifolia* essential oil.

EXPERIMENTAL

Infrared spectroscopy. Infrared spectra were obtained on a Perkin-Elmer M-21 infrared spectrophotometer equipped with a rock-salt prism. All runs were made in a 25-μ cell and recorded from 2 to 15μ at standard conditions. The instrument was calibrated against water vapor immediately before use.

Ultraviolet spectroscopy. Spectra were obtained on a Beckman Model DU spectrophotometer equipped with a quartz prism and Process Instruments recorder. Runs were made at a 0.001% dilution in ethyl alcohol with a 1-cm quartz cell.

Vapor-phase chromatography. Vapor chromatograms were obtained on an Aerograph Model VPC unit equipped with a thermal conductivity detector and ¼-inch × 10-ft 20% LAC 728 on 80–100-mesh firebrick column at 145°C and an F & M Model No. 609 VPC unit equipped with a flame ionization detector and automatic temperature programming. A ¼-inch × 10-ft 20% LAC 728 on 80–100-mesh firebrick column was used with programming from 50 to 130°C. Direct on-column injections were made to avoid possible artifact formation in the normal injection system.

Distillation. Commercial distilled Mexican lime oil (2.6 kg) was deaerated under vacuum at room temperature and saturated with helium. It was then distilled at 0.2–0.4 mm Hg under helium at a liquid temperature range of 60–110°C and vapor temperature range of 25–50°C, using a 2-inch × 3-ft vacuum jacketed Goodloe packed column with 5:1 reflux ratio. Fraction No. 30 (bp 29°C at .25 mm Hg), representing the fraction distilling between 78.3 and 82.4% of the distillation, was used for the isolation of *p*,α-dimethylstyrene. Results were similar with a ¾-inch × 2-ft insulated Vigreux column with a 5:1 reflux ratio and vacuum of 5 mm Hg. The fraction distilling between 78 and 82% of the distillation gave results similar to those obtained in the careful fractionation. Similar distillation of the expressed oil did not give indications of *p*,α-dimethylstyrene by VPC, infrared, or ultraviolet examination of the fractions.

Citrus aurantifolia used for isolation

Distilled Mexican:	
Specific gravity 15/4	0.860
Optical rotation	+40°
n_D^{20}	1.4750
Peroxide value	0–1
Distilled West Indian (Haitian):	
Specific gravity 15/4	0.862
Optical rotation	+42°25′
n_D^{20}	1.477
Peroxide value	0–1
Expressed Persian type (Florida):	
Specific gravity 15/4	0.875
Optical rotation	+38°
n_D^{20}	1.484
Peroxide value	0–1
Expressed Mexican:	
Specific gravity 15/4	0.999
Optical rotation	+36°30′
n_D^{20}	1.4867
Peroxide value	0–1

Preparative-scale VPC. A unit constructed in these laboratories was used. This was equipped with thermal-conductivity detector and a 50:1 split-ratio detector bypass. Column was six ½-inch × 4-ft lengths connected by ¼-inch tubing and packed with 20% LAC 728 on firebrick and was operated at 140°C.

Purification of p,α-dimethylstyrene. *p*,α-dimethylstyrene obtained from Hercules Powder Company (n_D^{20} 1.525) was purified by preparative-scale VPC to give physical constants n_D^{20} 1.535, specific gravity 20/20 .900, boiling point 185°C, and infrared and ultraviolet spectra that were identical with literature values.

Steam-distillation of expressed Citrus aurantifolia under acid conditions. One hundred grams of cold-pressed "Persian" Lime Oil was steam-distilled in the presence of 5.2% citric acid (Winton and Winton, 1935) under conditions approximating the commercial steam-distillation of lime oil (Guenther, 1947). This experiment was also conducted with one hundred grams of cold-pressed Mexican Lime Oil, and again with one hundred grams of citral.

Acid-catalyzed cyclization of citral. Treatment of 88.0 grams of citral, n_D^{20} 1.4875 sp. gr. 15/4 0.8922 with 100 ml of 4.8 normal aqueous hydrochloric acid for 5 hr at 25°C under vigorous agitation gives 90% conversion of citral to a mixture consisting of 71% terpene hydrocarbons and 19% unidentified alcohols. When the terpenes were isolated by preparative VPC at 100°C using

a 20% LAC 728 substrate on 80–100-mesh firebrick column, they were found to consist of 9.2% unidentified hydrocarbon of questionable purity and the following:

Compound	%	Structure	bp	n_D^{20}	UV (mμ)
p-Cymene	29.4	IV	176°	1.4947	Max. 273
p-α-Dimethylstyrene	21.8	V	185°	1.5352	Max. 247
1,8(9)-*p*-menthadiene	5.9	VII	176°	1.4730	Max. 220
1,3-*p*-Menthadiene	4.7	VI	180°	1.4850	Max. 265

Each of these compounds gave spectra identical to those reported in the literature (Barnes *et al.*, 1943; O'Connor and Goldblatt, 1954).

RESULTS AND DISCUSSION

Gas chromatographic examinations of the untreated steam-distilled oil of *Citrus aurantifolia* Mexican yields a peak occurring after *p*-cymene (Fig. 1) calculated as 0.1% and having a relative retention time different from that of any of the reported constituents of lime oil (Guenther, 1947a; Guenther and Langenau, 1943; Slater, 1961a). The concentration of this component was enriched by vacuum-fractionation, and it was then isolated by preparative-scale gas chromatography yielding a material with an n_D^{20} of 1.535, specific gravity 20/20 0.900, and bp 760 mm 185°C. This material gave an infrared spectrum (Fig. 2) and ultraviolet max (247 mμ) which were identical with those of purified commercial *p*,α-dimethylstyrene (Max 247μ) as well as spectral data given in the literature (Barnes *et al.*, 1943; Elliott and Cook, 1944). The VPC relative retention time (Ambrose, 1958) was identical to that of the unknown peak in the intact oil under two different conditions of temperature and flow rate.

Examination of steam-distilled Haitian oil of *Citrus aurantifolia* indicated the presence of this compound also. However, expressed Mexican and "Persian-type" *Citrus aurantifolia* did not show the *p*,α-dimethylstyrene peak by VPC. (The varieties of the Persian-type *Citrus aurantifolia* seem to differ from the true Mexican type (Webber and Batchelor, 1943) and are suspected of

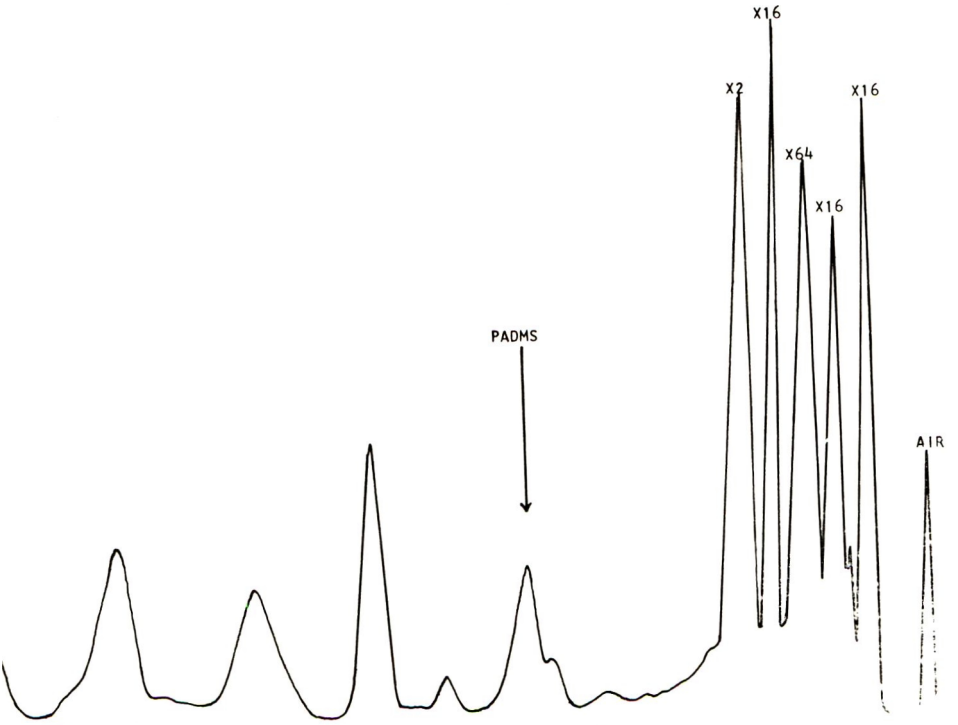

Fig. 1. A portion of the vapor phase chromatogram of commercial steam-distilled *Citrus aurantifolia*.

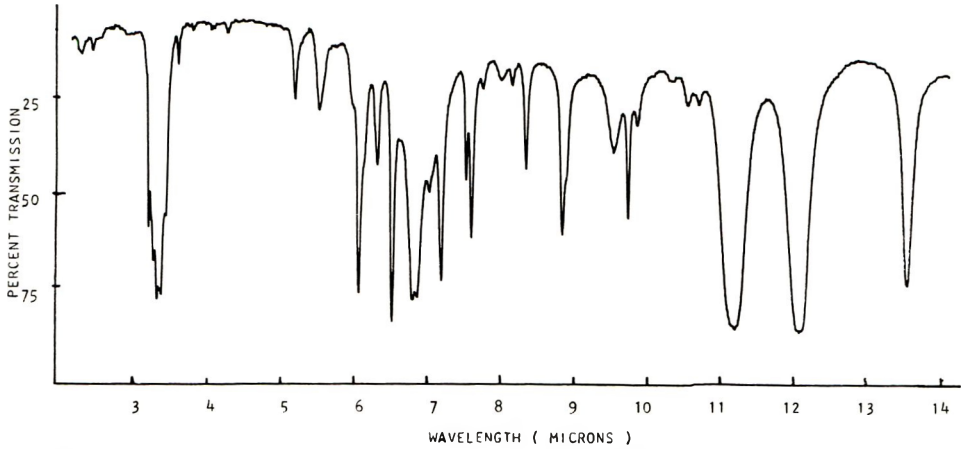

Fig. 2. Infrared spectrum of p,α-dimethylstyrene isolated from commercial steam-distilled *Citrus aurantifolia*.

being the result of hybridization). Attempted enrichment of expressed oils by fractional distillation did not give any fractions which showed the presence of the compound by infrared and ultraviolet spectroscopy or by gas chromatography which under identical conditions gave positive results with the steam-distilled oils. This was considered to be ample evidence that the presence of *p*-α-dimethylstyrene in the steam-distilled oils was not due to the methods of isolation and identification used.

Since the major difference between the steam-distilled oils and the cold-pressed oils is steam-distillation in the presence of natural fruit acids, and since acid conditions are known to alter the properties of the citrus oils (Guenther and Langenau, 1943; Guenther, 1943; Guenther, 1947b), it is reasonable to postulate that acid-catalyzed reactions of lime constituents may result in the formation of *p*,α-dimethylstyrene.

Steam distillation of expressed Mexican and Persian-type lime oil in the presence of citric acid at concentrations found in the fruit, conducted under conditions approximating the commercial process (Guenther, 1947b), gave the characteristic peak of *p*,α-dimethylstyrene by VPC. This was further verified by ultraviolet spectroscopic examination of the oil before and after steam distillation by the appearance of a band at 247 mμ. Another major change noted in the chromatogram of the treated oil was the lowering of the concentration of the citral peak, indicating involvement of citral in formation of the *p*,α-dimethylstyrene. Citral was then steam distilled under acid conditions and was found to give *p*,α-dimethylstyrene as well as other terpenes and oxygenated terpenes.

It has been generally reported that citral (I), upon treatment with acids, cyclizes to give *p*-cymene (IV) (Simonsen, 1947; Pinder, 1960). Isolation of the intermediate 1-methyl 4-isopropylidene-1-cyclohexene-3-ol (II) gave some indications as to a possible mechanism (Bedoukian, 1951) (Fig. 3).

Later, isolation of the intermediate compound 2,4,(8)6-paramenthatriene (III) (Okuda, 1940), which upon further acid

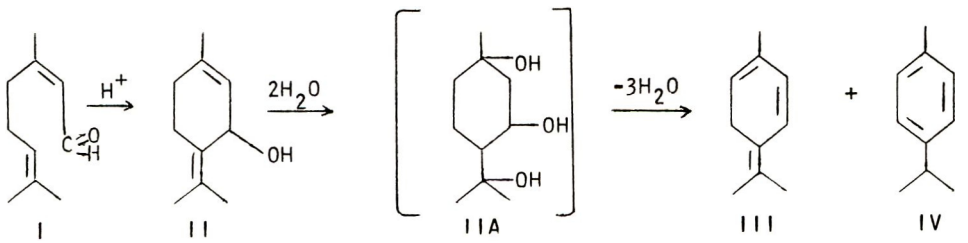

Fig. 3. The classically accepted reaction course for the acid-catalyzed cyclization of citral.

treatment was found to give *p*-cymene, seemed to provide final verification of the postulated mechanism. Our experimental evidence is not consistent with the mechanism.

The treatment of citral with acids was found to give a mixture of hydrocarbons (see the table) of which *p*,α-dimethylstyrene (V) was a major component. By monitoring the reaction by vapor phase chromatography and infrared spectroscopy, it was possible to terminate the conversion at a point that gave maximum yields of intermediate products which were found to be a mixture of alcohols by IR spectroscopy.

When these alcohols were isolated by preparative scale VPC they were found to have a molecular weight of approximately 152 (by Rast Method). These alcohols were found to have a different VPC retention time than those obtained by the thermal isomerization of citral (Ohloff, 1960). Quantitative esterification indicated a single hydroxyl group.

These alcohols upon further acid treatment were found to give *p*-cymene and *p*,α-dimethylstyrene as the major products and the monocyclic terpene dienes as the minor products.

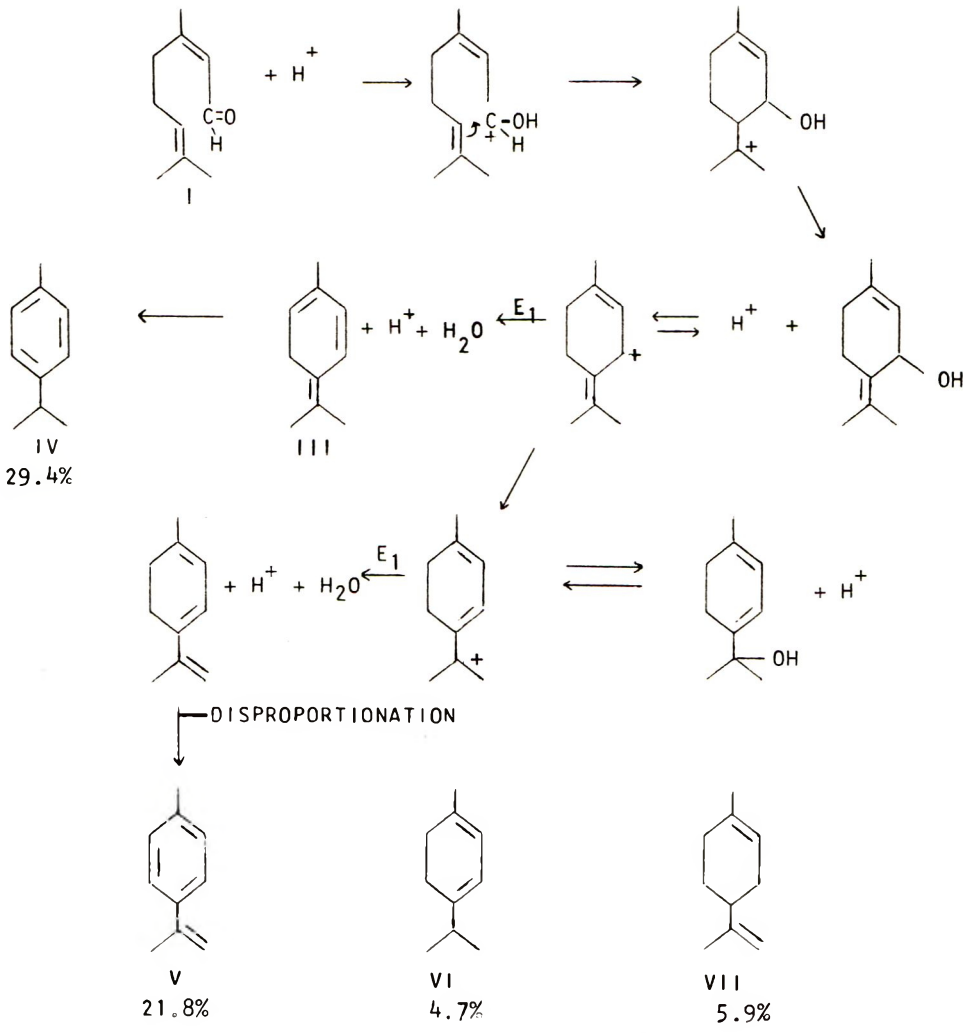

Fig. 4. Postulated course of reaction of the formation of p,α-dimethylstyrene in *Citrus aurantifolia*.

Since the terpene dienes 1,8(9)-*p*-menthadiene (VII) and 1,3-*p*-menthadiene (VI) could be reduction products of a monocyclic terpene triene of the type obtained by Okuda from the cyclization of citral, and since acid-catalyzed disproportionation of monocyclic terpene dienes is known to occur (Hunter and Brogden, 1963), the possibility of disproportionation reactions of the triene becomes evident (Fig. 4).

It is postulated that citral (I), upon attack of a hydrogen ion on the carbonyl group, produces a carbonium ion which upon reaction with the isopropylidene double bond yields monocyclic terpene diene alcohols. These, in turn, give the monocyclic terpene trienes by unimolecular elimination under the acid conditions. Finally, disproportionation and rearrangement reactions yield the observed hydrocarbons. It should be noted that heat treatment alone of citral at high temperatures for extended periods does not produce *p*,α-dimethylstyrene (Ohloff, 1960).

These observations suggest that the presence of *p*,α-dimethylstyrene in citrus oils is the result of commercial processing rather than biogenesis, although they do not preclude the presence of small and as yet undetected amounts of biogenetically formed *p*,α-dimethylstyrene, since the requirements essential for the reaction are present in whole fruit.

It also seems reasonable to expect *p*,α-dimethylstyrene to be a constituent of other citral-containing essential oils.

Structure elucidation of the reaction intermediates from the acid catalyzed cyclization of citral is currently being investigated.

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Cheddar Cheese Flavor: Gas Chromatographic and Mass Spectral Analyses of the Neutral Components of the Aroma Fraction[a]

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SUMMARY

The aroma fraction from the fat of high-quality raw-milk Cheddar cheese was isolated by centrifugation of the intact cheese and passing the recovered fat through a molecular still at 40°C. The aroma fraction was separated by gas chromatography on packed columns containing polar and nonpolar phases and by programmed-temperature capillary-column gas chromatography. The effluent from the latter column was admitted directly to the inlet of a rapid-scan mass spectrometer enabling concurrent recordings of mass spectra for each chromatographic peak. The aroma fraction was separated into approximately 130 components by the capillary column. By correlation of gas chromatography and mass spectral data, most of the major neutral components were characterized. These included aldehydes, methyl ketones, primary and secondary alcohols, esters of the primary and secondary alcohols and fatty acids, δ-lactones, and the isomeric lactides of lactic acid.

The current status of published research on the chemistry of Cheddar cheese flavor is given in recent reviews by Mabbitt (1961) and Marth (1963). Although the list of compounds isolated in the aroma fraction has become extensive, no complete description has been reported that is satisfactory to most researchers. Endless failures in relating the aroma to a few discrete compounds have prompted the theory of balanced components where it is proposed that the aroma is due to a combination of many compounds which, when in proper quantitative balance, give rise to the typical aroma. Apparently, however, certain compounds are more important than others (Day *et al.*, 1960; Walker, 1960; Patton, 1963), though there is disagreement on this point.

The isolation of a representative sample that can be manipulated without artifacts is a major problem in studying Cheddar aroma and could account for the limited success in characterizing it. The procedure recently published by Libbey *et al.* (1963) eliminates most of the known problems of sample isolation and gives a typical Cheddar aroma concentrate. Application of the procedure in conjunction with programmed-temperature capillary-column gas chromatography revealed approximately 130 components in the aroma fraction from Cheddar cheese. The findings reported herein deal with the major neutral components identified by correlation of gas chromatography and mass spectral data.

EXPERIMENTAL

The fat from 8.4 kg of high-quality raw-milk 20-month-old Cheddar cheese was isolated by the centrifugation procedure of Libbey *et al.* (1963). The yield was approximately 67%, and the aroma of the fat was considered by expert opinion to be typical of the intact cheese. The volatiles were isolated from 1700 g of the fat by the molecular distillation apparatus described by Libbey *et al.* (1963) except that trap 1 was eliminated; hence only two traps were used, a trap packed with 3-mm glass beads and a safety trap. The molecular still was operated at 40°C and a feed rate of 3 ml/min. Upon completion of the distillation the glass bead trap, containing the Cheddar aroma concentrate, was filled with ethyl chloride while still at liquid nitrogen temperature. Afterward the trap was allowed to warm to 5°C and the liquid contents

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transferred to the pot of the distillation apparatus described by Bills *et al.* (1963), where the bulk of the ethyl chloride was removed. The pot residue, which contained most of the Cheddar aroma, was submitted to analysis by gas chromatography in conjunction with rapid-scanning mass spectrometry.

A Perkin-Elmer Model 800 gas chromatograph equipped with a hydrogen flame detector and a 300-ft by 0.01-in. ID stainless-steel capillary coated with polypropylene glycol was used. The capillary was operated isothermally at 73°C for 8 min, then temperature-programmed at 25°/min to 174°C and operated at 174° until the separation was complete. Retention data were obtained for both the cheese volatiles and authentic compounds under these conditions. The same conditions were employed for the analysis in conjunction with rapid-scan mass spectrometry. These analyses were conducted as described by McFadden *et al.* (1963) except that the positive ions occurring at m/e 43 (m/e is the mass to charge ratio) were monitored by one gate of the electron multiplier to obtain a concurrent strip-chart recording of the gas chromatogram. The other electron multiplier gate was used to scan m/e 12 to 250 in 2 or 6 seconds, and the spectra were recorded by an oscillograph. The spectra were observed concurrently on an oscilloscope, which provided an important means of monitoring the effluent from the capillary column.

Additional gas chromatographic data were obtained by variation of the temperature program for the capillary column and by using packed polar (20% diethylene glycol succinate on 80–100-mesh Celite 545) and nonpolar (20% Apiezon M on 80–100-mesh Celite 545) columns operated isothermally at 70 and 100°C. The columns were ⅛-inch OD × 11-ft stainless steel. A β-ionization detector was employed, and the retention data were compared with those of authentic compounds.

RESULTS AND DISCUSSION

Fig. 1 is a gas chromatogram showing separation of the first 92 components of the Cheddar aroma fraction. A total of 130 components were observed in the chromatogram, but peak 92 was the last that gave a usable mass spectrum. Relative retention values for peaks from the gas chromatogram obtained by monitoring m/e 43 in the mass spectrometer agreed with those given in Table 1. As reported previously (Buttery *et al.*, 1963; McFadden and Teranishi, 1963) the two types of detectors vary in response, but this does not affect the correlation of retention data. Evidence used in assigning identities to the numbered peaks of Fig. 1 is presented in Table 1. In some cases the quality of the mass spectra was not adequate to enable positive identification; hence, even though gas chromatography data suggested particular compounds, positive mass spectral evidence was considered essential for identification. The quality of mass spectra of the unidentified components should be improved by prefractionation of the Cheddar aroma for the purpose of concentrating minor components and by varying the chromatographic parameters.

The type of data obtained by the combination of gas chromatography with rapid-scan mass spectrometry has been demonstrated elsewhere (McFadden *et al.*, 1963; Teranishi *et al.*, 1963). Application of the technique made it possible to characterize most of the major components appearing in Fig. 1. The ketones and aldehydes have been observed in earlier work (Day *et al.*,

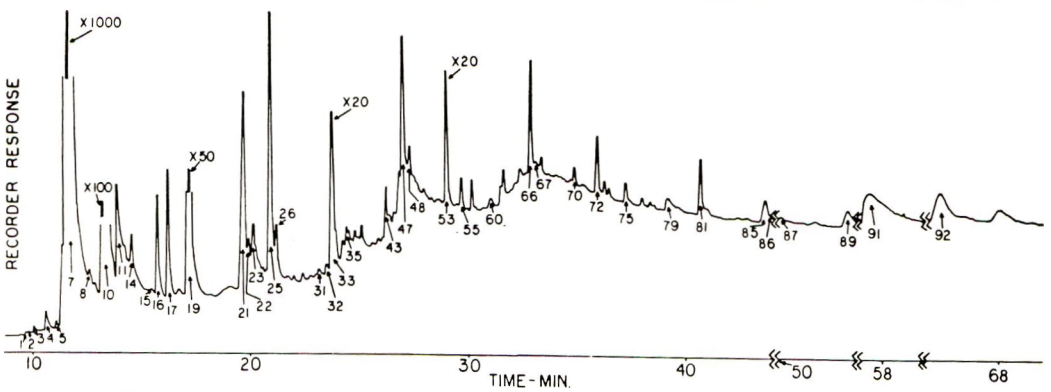


Fig. 1. Gas chromatogram of the neutral components of Cheddar cheese aroma. Capillary column 300-ft × 0.01-inch ID, coated with polypropylene glycol; isothermal at 73°C for 8 min, then temperature-programmed at 25°/min to 174°C.

Table 1. Gas chromatographic and mass spectral identification of components in the Cheddar cheese aroma fraction.

G. C. Peak No.	Identity	$t_R/t_R{}^a$ Cheddar	$t_R/t_R{}^a$ Authentic	Confirmed by packed column G. C.	Mass spectral identification	Mass spectral reference
1	Ethyl chloride	.462	.....	.....	Positive	API [b]
2	Propyl chloride	.470	.....	.....	Positive	API [b]
3	Ethanal	.481	.481	yes	Positive	API [b]
4	Acetone	.508	.496	yes	Positive	API [b]
5	C=C—N—C or C=C—C—NH_2	.530	.....	.....	Tentative	
6	Ethyl acetate	.539	.545	.....	—	API [b]
7	Butanone	.554	.545	yes	Positive	API [b]
7	Ethanol	.554	.547	yes	Positive	API [b]
7	Diacetyl	.554	.544	yes	Positive	API [b]
8	Benzene	.602	.600	.....	Positive	API [b]
10	2-Pentanone	.633	.628	yes	Positive	Sharkey *et al.* (1956)
11	2-Butanol	.662	.....	yes	Positive	Friedel *et al.* (1956)
14	Methyl benzene	.695	.....	.....	Positive	API [b]
15	2-Pentanol	.741	.737	.....	Positive	Friedel *et al.* (1956)
16	2-Butyl acetate	.752	.....	yes	Tentative	Beynon *et al.* (1961)
17	Ethyl butanoate	.774	.771	yes	Positive	Sharkey *et al.* (1959)
19	Lactide of lactic acid	.821	.....	.....	Tentative	
21	Lactide of lactic acid	.943	.....	.....	Tentative	
22	Lactide of lactic acid	.951	.....	.....	Tentative	
22	Phenyl C_2	.951	.....	.....	Tentative	API [b]
23	2-Butyl butanoate	.964	.....	.....	Tentative	This work
25	2-Heptanone	1.000	1.000	yes	Positive	Sharkey *et al.* (1956)
26	Methyl hexanoate	1.020	1.020	yes	Positive	Sharkey *et al.* (1956)
31	Phenyl C_2	1.17	.....	.....	Positive	API [b]
32	*n*-Pentanol	1.12	1.004	yes	Positive	Friedel *et al.* (1956)
33	Ethyl hexanoate	1.14	1.12	.....	Positive	ASTM [c]
35	Phenyl C_3	1.11	.....	.....	Positive	API [b]
43	2-Butyl hexanoate	1.25	.....	.....	Tentative	
47	2-Nonanone	1.28	1.28	.....	Positive	Sharkey *et al.* (1956)
48	Methyl octanoate	1.30	1.30	.....	Positive	ASTM [c]
53	Ethyl octanoate	1.37	1.38	.....	Positive	ASTM [c]
55	*n*-Decanal	1.42	1.41	.....	Positive	Gilpin & McLafferty (1957)
60	2-Nonanol	1.48	.....	.....	Positive	This work
66	2-Undecanone	1.56	1.57	.....	Positive	Sharkey *et al.* (1956)
67	Methyl decanoate	1.58	1.58	.....	Positive	ASTM [c]

Table 1 (continued)

G. C. Peak No.	Identity	t_R/t_R [a] Cheddar	t_R/t_R [a] Authentic	Confirmed by packed column G. C.	Mass spectral identification	Mass spectral reference
70	Aldehyde?	1.66	.....	.....	Tentative	
72	Ethyl decanoate	1.71	1.69	.....	Positive	ASTM [c]
75	*n*-Dodecanal	1.77	1.76	.....	Positive	Gilpin & McLafferty (1957)
79	δ-Octalactone	1.86	1.85	.....	Tentative	This work
81	Sesquiterpene	1.94	.....	.....	Tentative	
85	2-Tridecanone	2.08	2.10	.....	Positive	This work
86	Methyl dodecanoate	2.09	2.12	.....	Tentative	ASTM [c]
87	Ethyl dodecanoate	2.39	2.35	.....	Tentative	ASTM [c]
89	Methyl phthalate	2.53	.....	.....	Positive	Emery (1960)
91	δ-Decalactone	2.74	2.70	.....	Tentative	This work
92	2-Pentadecanone	3.12	.....	.....	Tentative	This work

[a] t_R/t_R = relative retention time calculated for 2-heptanone = 1.000. Stainless steel capillary, 300 ft × 0.01 in. ID coated with polypropylene glycol; operated at 73°C for 8 min, then at 25°/min to 174°C.

[b] Am. Petroleum Inst. Catalog of Mass Spectral Data. 1948.

[c] Am. Soc. Testing Materials, Uncertified Mass Spectra File.

1960; Walker and Harvey, 1959). This is the first conclusive evidence, however, for an extensive number of esters. Esters were suggested as early as 1910 (Suzuki *et al.*), and Dacre (1955) reported ethyl acetate and ethyl butanoate.

It will be noted that the chromatographic data for peaks 79 and 91 give good agreement with the authentic δ-lactones. The mass spectra were only suggestive, and the peak assignments are therefore tentative. The presence of lactones in Cheddar cheese was suggested in earlier work (Day *et al.*, 1960).

Based on our knowledge of the cheese ripening process, it is difficult to rationalize the presence of *n*-pentanol and the alkyl-substituted benzenes. Peaks 8, 22, 31, and 35 all contained aromatics. Exact assignment of structure for the alkyl-substituted benzenes was not possible with the mass spectral data at hand. Hence no attempt was made to characterize the isomers. Aromatic compounds have been found in heated milk (Cobb and Patton, 1963) and in silage (Morgan and Pereira, 1962). The spectrum for peak 70 was indicative of an aldehyde. The pattern did not fit a saturated aldehyde, nor did the chromatographic data. Similarly the mass spectrum of peak 81 was typical of sesquiterpenes but the exact structure could not be assigned. The sesquiterpene might result from the degradation of β-carotene or similar structures common to milk. The halogenated compounds were contributed by the ethyl chloride used as the solvent.

Of particular interest, because of their unique mass spectra, are chromatographic peaks 19 and 21, shown in Fig. 1. The mass spectral charts obtained for these peaks are presented in Fig. 2 as chart sequence *a–f*. Chart *a* gives the background following elution of ethyl butanoate (peak 17); charts *b* and *c* were taken near the center and on the downward slope of peak 19; chart *d* shows the return to background; chart *e* gives the mass spectrum for peak 21; chart *f* shows almost complete elution of peak 21 and the existence of a trace amount of a dimethyl- or ethyl-substituted benzene.

It is immediately obvious that mass spec-

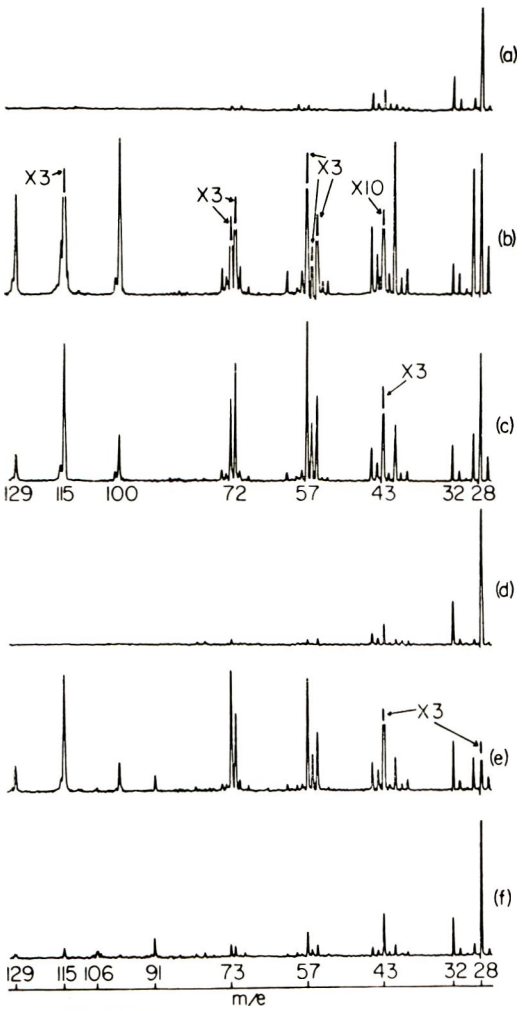
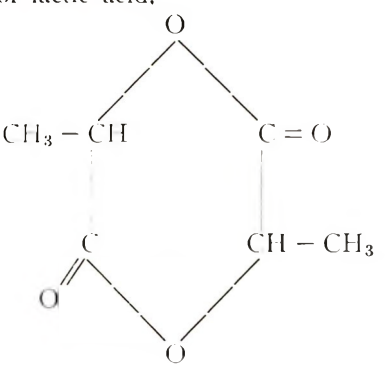


Fig. 2. Mass spectral charts for chromatographic peaks 19 and 21. Chart a = background; charts b and c = chromatographic peak 19; chart d = background after peak 19; chart e = chromatographic peak 21; chart f = almost complete elution of peak 21.

tral charts b and e are very similar, with a modest difference in the ratio of the ionization, due to masses 72 and 73. This indicates molecules of very similar structure, probably geometric isomers. However, it was not possible to find a mass spectrum in the literature that compared favorably (use was made of a card catalog containing API mass spectral data, ASTM uncertified mass spectral data, and most of the mass spectra published in chemistry journals). Interpretation of these mass spectra required establishing whether the mass 129 peak was a parent ion or a fragment. Because odd-number molecular weights can occur only for those compounds containing an odd number of nitrogens, consideration was given to the amines (Gohlke and McLafferty, 1962) or other nitrogen compounds of molecular weight 129 that might elute at this point. None fit the observed spectra. Furthermore, since the mass 115 ion appears in both of the mass spectra (charts b and e), it seems improbable that it would be due to a second component of the effluent, and loss of 14 mass units from a parent ion is not a normally encountered mode of ion decomposition. Thus, the mass 129 peak was concluded to be a fragment ion.

The next logical consideration is that the mass 129 ion is formed by loss of CH_3 from a parent ion of mass 144. The mass 115 ion would then be due to loss of 29 mass units (CHO or C_2H_5), and the mass 100 due to loss of 44 (CO_2> probable than C_2H_4O> probable than C_3H_8). Ions at mass 129, 115, 73, etc., normally indicate the existence of one ether-type oxygen, but the system could also contain two ether-type oxygens and a double bond or ring. The absence of ionization in the region of masses 71 or 85 indicates extreme improbability that there are five or six carbons in an aliphatic chain. This would mean that, for a compound of molecular weight 144 (or larger), there will probably be one or more carbonyl oxygens.

From such arguments, a variety of possible structures were considered. The different ether-carbonyl, ester-carbonyl, or alcohol-carbonyl combinations that were proposed did not seem logical from consideration of the known or expected characteristics of their mass spectra. However, the lactide of lactic acid,



would be expected to have a mass spectrum similar to those observed in charts *b* and *e*. Thus, loss of CH_3 would be very likely and would lead to the mass 129 ion. Rearrangement of the molecule and subsequent loss of CHO is logically expected and would lead to mass 115. The mass 100 ion would be expected by loss of CO_2. It might also occur as loss of CH_3CHO. The ion at mass 72 is reasonably attributed to the ion

$$\left[CH_3CH\overset{\overset{O}{\|}}{C}-O\right]^+$$

and that at 73 due to a rearrangement in which the same fragment ion contains an additional hydrogen. The ionization at masses 57, 56, 55 could arise from the CH_3CHCO fragment with possible loss or addition of hydrogen. The ionization at masses 43 and 45 could occur from rearrangement of the $CH_3 - CHO$ group.

Such considerations point strongly to the possibility that peaks 19 and 21 in Fig. 1 are the *cis* and *trans* isomers of the lactide of lactic acid. Unfortunately, a proof is constituted only by an actual comparison with the authentic compounds, preferably with both mass spectra and retention data. Attempts to obtain authentic samples of lactide have failed, because of the known instability of this compound.

The sensory properties of the Cheddar aroma were most easily observed by smelling the effluent from the injection splitter of the gas chromatograph when injecting a sample. It was apparent that the concentrate contained free fatty acids but these did not elute from the capillary column during the period of the analysis. This point was confirmed by injection of authentic acids. Similar observations were made for other highly polar compounds such as amines. The aroma, after removal of the ethyl chloride, appeared to lack the H_2S and mercaptan notes that were evident immediately after distillation of the cheese fat. These compounds could have been lost during solvent removal, and work is in progress using the direct-transfer techniques of Libbey *et al.* (1963) to characterize these and similar low-boiling components. Because of the aforementioned points, Fig. 1 does not represent the complete pattern of Cheddar aroma. Since the unidentified compounds in low concentration may also have significant aroma-contributing qualities, conclusions on the significance of various compounds are forbidden at this point.

Lawrence (1963) recently reported work in which it is suggested that odd-numbered methyl ketones found in cheese were largely heat-induced during distillation procedures for aroma isolation. It will be noted in Fig. 1 and Table 1 of this manuscript that substantial quantities of odd-numbered methyl ketones C_5–C_{15} were observed. The maximum heat treatment received by the cheese fat was about 40°C. The molecular still was operated at 40°C and the time required for fat to pass through the heated portion was short. Control experiments in earlier work (Day *et al.*, 1960) also indicated that substantial amounts of ketones exist in cured cheese. Hence, while it is recognized that the ketones can be heat-generated from milk fat, some also appear to be produced during the normal cheese curing process.

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Post-Mortem Changes in Physical and Chemical Properties of Bovine Muscle[a]

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SUMMARY

Post-mortem changes in tenderness and protein solubility were studied in bovine semitendinosus muscles. Muscles excised immediately post-mortem were compared with muscles left attached to the skeleton. Post-mortem times of 0, 6, 12, 24, 72, and 312 hr were studied. Sarcoplasmic protein solubility was highest immediately after slaughter and lowest in muscles left attached to the skeleton. Myofibrillar protein solubility was decreased in muscles left attached. Protein solubility changed during the first 6 hr post-mortem but not during the 6- to 312-hr aging period. Muscles left attached to the skeleton were least tender immediately after death and gradually increased in tenderness during post-mortem aging. Excised muscles were least tender 6–12 hr post-mortem and became progressively more tender thereafter. Even after 312 hr of aging, excised muscles were less tender than muscles still attached to the skeleton. Protein solubility did not appear to be related to tenderness. Possible relationships of muscle contraction to tenderness were discussed.

INTRODUCTION

Since the early report of Moran and Smith (1929), rigor mortis has been known to be associated with large differences in meat tenderness. The chemical changes associated with rigor mortis and the aging of meat have been summarized by Whitaker (1959). Ramsbottom and Strandine (1949) reported that beef was more tender at two hr post-mortem than at two days post-mortem. Numerous other workers have observed increased tenderness during aging (ripening) of beef carcasses (Deatherage and Harsham, 1947; Deatherage and Reiman, 1946; Hoagland *et al.*, 1917). It was generally accepted among early workers that this increased tenderness was due to autolysis of the muscle proteins (Bate-Smith, 1948; McCarthy and King, 1942), presumably by the muscle cathepsins (Smorodintsev and Nikolaeva, 1936, 1942). Husaini *et al.* (1950a,b), however, failed to find any increase in the non-protein or proteose-peptone nitrogen fractions during aging. This led Wierbicki *et al.* (1954) to suggest that post-mortem tenderization may be caused by a dissociation of actomyosin to actin and myosin or by a redistribution of ions within the muscle, causing increased hydration and tenderness. Wierbicki *et al.* (1956) later reported that they could find no evidence for the post-mortem dissociation of actomyosin.

Locker (1960b) found no increase in N-terminal amino groups after either 3 or 16 days post-mortem aging at 2°C, although small increases were observed in nonprotein nitrogen and free amino acids. Locker concluded that proteolysis was not important in normal post-mortem tenderization. Weinberg and Rose (1960) suggested that specific cleavage of an actin association responsible for the maintenance of the muscle matrix, and not merely random autolysis, was responsible for the increased tenderness of chicken breast muscle aged 24 hr. Partmann (1963) stated that muscle contraction and rigor mortis have the same mechanism and that post-mortem tenderization probably occurs as a result of dissociation of the actomyosin complex. Davies (1963), in discussing a new molecular theory of muscle contraction, postulated that rigor mortis is caused by a strong attraction of positive binding sites in H-meromyosin to the nega-

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tive charge of the bound nucleotide of actin. Elucidation of the molecular structure of muscle (Huxley, 1957, 1963) and investigations of changes in modulus of elasticity during contraction (Barany and Jaisle, 1960) suggest that rigor mortis is probably caused by an irreversible combination of thick and thin filaments (myosin and actin). The molecular changes responsible for post-mortem tenderization, however, remain a mystery.

Solubility of different fractions of muscle proteins have recently been associated with tenderness of beef (Hegarty *et al.*, 1963) and fish (Love, 1962). Sayre and Briskey (1963) and Scopes and Lawrie (1963) have reported that muscle protein solubility was also influenced by post-mortem conditions in the muscle. An experiment was made to: 1) study post-mortem changes in tenderness and protein solubility of bovine muscle; and 2) relate changes in protein solubility to differences in tenderness. Since Love (1962) and Lowe (1948) have reported a substantial decrease in tenderness following excision of a muscle immediately after death, the post-mortem changes of muscles left attached to the skeleton were compared with those of muscles removed as soon after death as possible.

MATERIALS AND METHODS

The muscle used was the semitendinosus from 15 steers of known age and genetic background, raised on identical rations. The animals ranged from 16 to 19 months in age. Nine different sire groups were used in the tenderness determinations, and five of these groups were represented in the protein solubility measurements. Animals from the same sire group were sampled at different times post-mortem. The animals yielded carcasses of Good and Choice grades.

As soon after death as possible, the semitendinosus muscle was taken from the left side of each carcass, and a steak 1½ inches thick was removed at a point approximately 7 inches from the distal attachment of the muscle for a Warner-Bratzler shear test of tenderness. An additional sample for protein solubility measurements was taken from the center of the muscle just distal to the point of removal of the steak. This sampling was completed 15–20 min after the death of the animal, and the remainder of the muscle was stored at 4°C until further sampling. Post-mortem sampling times of 6, 12, 24, 72, and 312 hr were used. At an appropriate post-mortem time for each animal, the semitendinosus muscle was excised from the right side of the carcass, and steaks 1½ inches thick were removed both from this muscle and from its companion from the left side, excised immediately post-mortem. These steaks were removed at a point approximately 9 inches from the distal attachment of the muscle. Protein solubility measurements were conducted on samples from both the left and right muscles. These samples were taken from the center of the muscles at a point just proximal to removal of the steak. Thus, the left semitendinosus muscle of each animal had two steaks removed for Warner-Bratzler shear tests of tenderness and two samples taken for measurements of protein solubility. The corresponding muscle from the right side was excised from the carcass at a certain time post-mortem and had one steak removed for a Warner-Bratzler shear test of tenderness and one sample taken for measurements of protein solubility.

Tenderness determinations. Steaks 1½ inches thick were cooked in deep fat (temperature, 135°C) to an internal temperature of 60°C. Cooking time varied among the different sampling times. Steaks taken immediately post-mortem required only 5–6 min to reach 60°C whereas those removed at later post-mortem times required 10–15 min to reach this same internal temperature. The steaks were cooled at room temperature for 5 min, and four ½-inch cores were removed from each steak, one core being removed from each quadrant of the steak. Two shears were recorded for each core.

Protein solubility. Samples taken for protein solubility were minced, and 4–6 g portions, weighed accurately, were used for analysis. Three different extracting solutions were used for each sample: Solution I, a 0.03*M* potassium phosphate buffer (pH 7.4) to extract the sarcoplasmic protein fraction; Solution II, 1.1*M* KI in 0.1*M* potassium phosphate (pH 7.4) to extract total soluble protein; Solution III, an unbuffered 5% NaCl solution. The 4–6-g samples were homogenized with solvent-to-sample ratios of exactly 10:1 (v/w) using precooled solutions. All extractions were conducted at 4°C according to the method of Helander (1957). The extracted protein was separated by centrifugation at 30,000 × G for 15 min at 4°C. Protein analyses were conducted by the biuret method (Gornall *et al.*, 1949), and myofibrillar protein was calculated as the difference between the total soluble protein and the sarcoplasmic protein. The pH of each muscle was measured by using a Beckman Zeromatic pH meter on 10-g samples immediately after homogenization.

RESULTS AND DISCUSSION

Table 1 shows post-mortem changes in

Table 1. Changes in Warner-Bratzler shear-force values and pH of bovine semitendinosus muscle during post-mortem aging.

Animal no.	Sire group	At time of death [a] Shear values	pH	Time post-mortem (hr)	Muscle left in carcass [b] Shear values	pH	Muscle removed from carcass [c] Shear values	pH
623	3	15.66	5.98	6	12.94	5.35	18.19	5.90
618	6	12.18	6.15	6	11.97	5.55	17.72	6.30
600	7	14.55	6.35	6	14.00	5.49	17.94	6.51
601	8	13.81	6.54	12	11.78	5.35	21.69	5.80
624	3	13.06	6.60	12	9.81	6.22	18.00	5.51
612	5	12.25	6.80	12	12.25	5.10	19.83	6.45
605	2	14.95	6.40	24	9.28	5.30	20.32	5.40
615	6	11.86	6.60	24	9.00	5.10	17.50	5.29
622	4	13.50	6.55	24	9.28	5.40	19.31	5.62
619	5	13.09	6.48	72	7.97	5.24	15.16	4.91
631	1	21.25	6.31	72	9.31	5.10	13.56	4.70
602	2	13.13	6.65	72	8.41	5.43	11.66	5.58
607	8	18.47	6.30	312	7.00	5.38	11.41	5.13
610	7	12.44	6.45	312	8.00	5.35	7.63	5.52
606	9	12.03	6.44	312	7.34	5.47	6.78	5.52

[a] Muscle was removed as soon after death as possible. Actual time of sampling was 15–20 min post-mortem.

[b] Muscle was left in carcass until the appropriate time post-mortem when it was excised and shear values obtained.

[c] Muscle was excised as soon after death as possible and a steak removed to obtain shear values at this time. The remaining portion of the muscle was stored at 4°C until the appropriate time post-mortem, when a second steak was removed and shear values obtained.

pH and tenderness for each of the 15 animals in the study. Shear-force values immediately after death varied substantially among individual animals. With one exception, muscles sampled immediately post-mortem had pH values above 6.0. Table 2 shows the average shear-force values at each of the five post-mortem times studied. The results indicate that semitendinosus muscles left attached to the skeleton were least tender immediately after death and became progressively more tender with post-mortem aging. Muscles excised at the time of death became progressively less tender during the first 12 hr of post-mortem aging, and then gradually increased in tenderness. However, even after 312 hr aging, muscles excised from the skeleton immediately post-mortem were still less tender than muscles that remained with the carcass. These results are summarized in Fig. 1, which shows average shear-force values for each post-mortem time. Fig. 2 shows the average change in shear-force values during post-mortem aging. The tenderness changes in muscles excised from the skeleton resemble those reported by Paul *et al.* (1952), who found that semitendinosus steaks decreased in tenderness during the

Table 2. Average changes in Warner-Bratzler shear-force values of bovine semitendinosus muscle during post-mortem aging.

Shear values at time of death [a]	Time post-mortem (hr)	Shear value of muscle left in carcass [b]	Shear value of muscle removed from carcass [c]
14.28 ± 0.62 [d]	6	12.97 ± 0.94	17.78 ± 1.06
13.04 ± 0.37	12	11.28 ± 0.54	19.74 ± 0.72
13.49 ± 0.56	24	9.19 ± 0.49	19.06 ± 0.80
15.82 ± 0.90	72	8.56 ± 0.91	13.46 ± 0.58
15.14 ± 0.85	312	7.45 ± 0.27	8.60 ± 0.73

[a] Muscle was removed as soon after death as possible. Actual time of sampling was 15–20 min post-mortem.

[b] Muscle was left in carcass until the appropriate time post-mortem, when it was excised and shear values obtained.

[c] Muscle was excised as soon after death as possible and a steak removed to obtain shear values at this time. The remaining portion of the muscle was stored at 4°C until the appropriate time post-mortem, when a second steak was removed and shear values obtained.

[d] Figures are means plus or minus the standard errors of 24 observations.

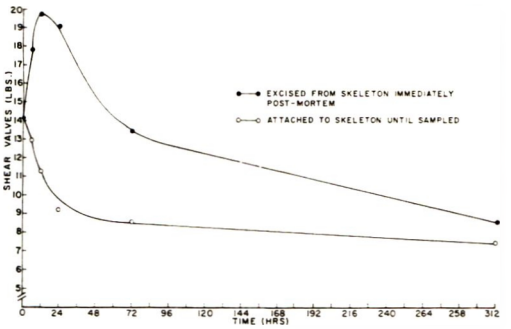

Fig. 1. Effect of post-mortem aging on Warner-Bratzler shear values of semitendinosus muscle.

first 24 hr post-mortem and then returned approximately to their original tenderness after 144–149 hr. The muscles used in Paul's study had been removed from the skeleton approximately 1 hr post-mortem. The tenderness changes in the muscles left attached to the carcass appear similar to those reported by Love (1962) in fish muscle which was allowed to pass into and through rigor mortis as whole fish. Love (1962) also found that fish muscle removed immediately post-mortem and allowed to pass into rigor as fillets remained less tender, even 150 hr after death, than muscle which had passed through rigor as whole fish.

The tenderness measurements in this study are in agreement with findings of Ramsbottom and Strandine (1949), who observed decreased tenderness in muscles excised from sides of beef before they were chilled. Tenderness differences between muscles left intact in the carcass and those removed from the carcass immediately post-mortem lessened with aging time, but even after 12 days post-mortem, muscles left attached to the skeleton were more tender.

Although the rate of chilling in this study was not measured for every muscle, those measurements which were taken indicated that the temperature of muscles excised from the carcass was lower for the first 24 hr post-mortem than the temperature of muscles left with the carcass. Temperature differences between the two muscles were a maximum at 6 hr post-mortem. This is in accord with the results of Ramsbottom and Strandine (1949).

Table 3 shows changes in protein solubility of bovine semitendinosus muscle dur-

Table 3. Changes in protein solubility of bovine semitendinosus muscle during post-mortem aging.[a]

Animal no.	Sire group	At time of death[b]			Time post-mortem (hr)	Muscle left in carcass[c]			Muscle removed from carcass[d]		
		Sarcoplasmic protein	Myofibrillar protein	Total soluble in 5% NaCl		Sarcoplasmic protein	Myofibrillar protein	Total soluble in 5% NaCl	Sarcoplasmic protein	Myofibrillar protein	Total soluble in 5% NaCl
618	6	69	84	123	6	51	78	81	63	84	105
600	7	84	93	117	6	75	33	81	78	72	123
601	8	69	75	105	12	81	51	87	63	87	93
612	5	69	81	105	12	45	66	75	57	72	93
605	2	72	84	135	24	63	111	114	69	105	111
615	6	57	60	69	24	57	54	78	63	69	99
619	5	69	72	81	72	57	60	99	63	57	108
602	2	75	110	111	72	66	63	108	72	84	114
607	8	63	72	81	312	51	69	87	57	69	108
610	7	78	81	108	312	60	69	105	69	69	111
Av.		70	81	104	.....	61	65	92	65	77	106

[a] All protein figures are expressed as mg protein per g of fresh tissue.
[b] Actual time of sampling was 15–20 min post-mortem.
[c] Muscle left in right side of carcass until sampled at appropriate time post-mortem.
[d] Muscle removed from left side of carcass 15–20 min post-mortem and stored at 4°C until sampled at appropriate time post-mortem.

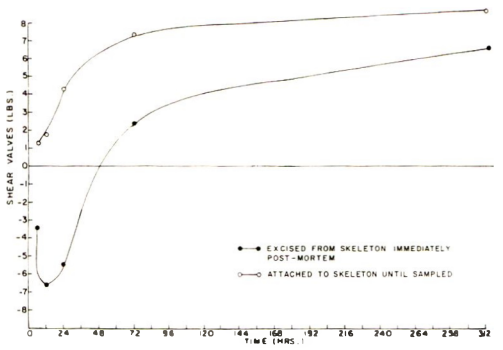

Fig. 2. Effect of post-mortem aging on the change in Warner-Bratzler shear values from the shear values taken immediately after death.

ing post-mortem aging. Protein solubility measurements were conducted on only 10 of the 15 animals used. Thus, measurements were conducted on only two animals for each of the five post-mortem periods. Least squares analysis showed that significantly greater amounts of protein were extracted from muscles which had been excised immediately post-mortem than from muscles left attached to the skeleton. Since the excised muscles were the least tender, these findings are in contradiction to those of Hegarty *et al.* (1963), who found a positive relation between myofibrillar protein solubility and tenderness. Although it is possible that changes in the excised muscle are occurring which tend to mask or negate the relationship between protein solubility and tenderness, it appears more probable that tenderness and protein solubility are related only casually, perhaps because of a common relationship to some factor as yet unknown. Correlation coefficients between Warner-Bratzler shear values and protein solubility measurements were calculated for muscles removed immediately after death, muscles left attached to the skeleton, and muscles excised from the skeleton (Tables 4, 5, 6). None of these correlations were significant, and even those which were nonsignificant fail to show any pattern of relationship between protein solubility and tenderness. For example, the correlation between Warner-Bratzler shear and myofibrillar protein solubility is negative for muscles excised from the skeleton but positive for muscles left attached to the skeleton.

The method of Helander (1957) extracted about 6.6% of the muscle as sarcoplasmic protein and 7.4% as myofibrillar protein. These figures correspond closely to those of Hegarty *et al.* (1963), who reported that 6.7% of the muscle was extracted as sarcoplasmic protein and 7.6% was extracted as myofibrillar protein. Statistical analyses found no significant differences among the amounts of sarcoplasmic or myofibrillar protein extracted at the five different post-mortem times. However, in accord with the findings of Sayre and Briskey (1963), the amount of sarcoplasmic protein extracted at any of the post-mortem times was less than the amount extracted immediately after death. For muscles left attached to the skeleton, least squares analysis showed that the amount of myofibrillar protein extracted at any of the five post-mortem times was less than the amount extracted immediately post-mortem. Myofibrillar protein solubility of the excised muscles, however, did not change appreciably during the post-mortem period studied in this experiment. Since excised muscles cooled faster, it is possible that the myofibrillar protein in these muscles were not "denatured" by post-mortem changes in pH and other factors. Thus, the results provide support for the conclusions of Sayre

Table 4. Correlations among measurements taken on bovine semitendinosus muscle at the time of death.

Variable	Warner-Bratzler shear-force values	pH	Sarcoplasmic protein solubility	Myofibrillar protein solubility	Total protein soluble in 5% NaCl
pH	−0.39				
Sarcoplasmic protein solubility	−0.03	−0.13			
Myofibrillar protein solubility	−0.03	0.04	0.73*		
Total protein soluble in 5% NaCl	−0.06	−0.26	0.67*	0.66*	
Ratio of myofibrillar/sarcoplasmic protein	0.17	−0.01	0.17	0.55	0.65*

* $P < 0.05$, 0.63 required for significance at this level.

Table 5. Correlations among measurements taken on bovine semitendinosus muscle which had remained with the carcass from time of death until time of measurement.

Variable	Warner-Bratzler shear-force values	pH	Sarcoplasmic protein solubility	Myofibrillar protein solubility	Total protein soluble in 5% NaCl
pH	0.27				
Sarcoplasmic protein solubility	0.25	0.35			
Myofibrillar protein solubility	−0.32	−0.04	−0.38		
Total protein soluble in 5% NaCl	−0.57	0.05	0.23	0.55	
Ratio of myofibrillar/sarcoplasmic protein	−0.25	−0.17	−0.74*	−0.18	0.23

* $P < 0.05$, 0.63 required for significance at this level.

and Briskey (1963) that myofibrillar protein solubility is affected by post-mortem physiological conditions in the muscle, although the protein solubility changes reported in the present study are small compared with the large changes observed by altering post-mortem conditions in pig muscle. The total protein extracted by unbuffered 5% NaCl solutions followed the same solubility pattern as myofibrillar protein. The NaCl extraction in this experiment was conducted to afford some comparison with Love's studies on the solubility of fish muscle protein.

Locker (1960a) found that contracted muscles were less tender than muscles in the relaxed state and suggested that degree of muscular contraction may be an important factor contributing to variations in meat tenderness. The excised muscles in the present study had shortened noticeably by 6 hr post-mortem. This shortening is likely a manifestation of the cold-shortening effect recently reported by Locker and Hagyard (1963). These workers state that observed tenderness is largely a measure of shortening. However, no visible lengthening occurred at the longer post-mortem aging times, even though the muscles became more tender. While loss of the ability of the muscle to contract upon heating would account for the tenderness changes of the attached muscles, this does not explain the decreased tenderness of the excised muscles after aging 6 hr. It is possible that contractility and the cold-shortening effect are acting together to influence tenderness of the excised muscles.

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Table 6. Correlations among measurements taken on bovine semitendinosus muscle excised from the carcass at time of death.

Variable	Warner-Bratzler shear-force values	pH	Sarcoplasmic protein solubility	Myofibrillar protein solubility	Total protein soluble in 5% NaCl
pH	0.42				
Sarcoplasmic protein solubility	−0.12	0.22			
Myofibrillar protein solubility	0.44	0.20	0.24		
Total protein soluble in 5% NaCl	−0.44	−0.02	0.79**	0.00	
Ratio of myofibrillar/sarcoplasmic protein	0.59	−0.15	−0.53	0.35	−0.50

** $P < 0.01$, 0.76 required for significance at this level.

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Some Protein Changes During Post-Mortem Tenderization in Poultry Meat

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SUMMARY

In both breast and leg muscle from 6-, 9-, and 12-month-old chickens, held for aging at 0°C, the buffer-extractable nitrogen rapidly decreased after death during the onset of rigor and gradually increased to a maximum value during post-rigor aging. Changes in extractable nitrogen occurred mainly as a result of changes in the solubility of myofibrillar proteins. Changes in sarcoplasmic and stroma protein fractions were small. In the nonprotein-nitrogen fraction, some of the amino-acid-containing polymers were removed during the onset of rigor mortis as a result of their interaction or aggregation with proteins. During the post-rigor tenderization period, amino acids and peptide increased in meat as a result of proteolysis. Different rates of post-mortem tenderization in breast and leg muscle appear to be related to the differences in stroma protein content of muscle. Proteolysis seems to weaken or break the bonds which bind myofibrils to the matrix of the muscle and causes protein changes that are responsible for post-rigor tenderization.

INTRODUCTION

During a study of the effect of fresh and frozen storage on chicken muscle proteins (Khan *et al.*, 1963; Khan and van den Berg, 1964), a wide variation in the results on protein extractability was noted in leg muscle from old birds which were aged for 24 hr in drained crushed ice, whereas the breast muscle from the same birds gave consistent results. Since more recent work using taste-panel and shear-press tests has shown that tenderization occurs more slowly in leg muscle than in breast muscle (van den Berg *et al.*, 1964), the inconsistency in the results on leg muscles may be attributed to the degree of completion of post-rigor tenderization changes in leg muscle at 24 hr post-mortem. Changes introduced by incomplete tenderization could interfere seriously with the study of protein changes caused by freezing, frozen storage, and thawing; therefore, tests were made to compare the effect of aging time on the extractability of protein in breast and leg muscle of birds from 6 to 12 months old. Changes in muscle protein fractions and in the nonprotein nitrogenous constituents of chicken meat that occur from the moment of death to the time tenderness changes were complete were also studied.

EXPERIMENTAL

Materials and procedure. Tests were made with meat from 6-, 9-, and 12-month-old broiler-type chickens (male). Four birds in each age group were obtained from a single flock. They were killed in the laboratory by cutting the jugular vein and carotid arteries, bled for 2–3 min, scalded for 2 min at 53–54°C, plucked by hand, eviscerated, and washed. To avoid changes introduced by microbial action during aging, each carcass was rinsed in chlortetracycline solution (10 ppm, 15 sec) and placed in a sterile plastic bag. The bagged meat was then placed in crushed drained ice for aging. The onset of rigor mortis and post-rigor relaxation were followed by the technique of judging the firmness and elasticity of flesh manually. To reduce the effect of bird-to-bird variability, comparisons were made between four samples from breast or leg meat of one bird aged for different periods. The analyses were made at suitable intervals beginning 15–30 min after the death of the bird.

Protein extraction and fractionation. Preparation of sample, method for the determination of extractable nitrogen, and protein fractionation techniques have been described earlier (Khan, 1962). In brief, protein extractability was determined in KCl-borate or KCl-phosphate buffer (ionic strength

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[$\Gamma/2$] = 1.0 and pH 7.4). The extractable nitrogen was fractionated into myofibrillar proteins, soluble at $\Gamma/2 = 0.5$ and insoluble at $\Gamma/2 = 0.08$, and sarcoplasmic proteins, soluble at $\Gamma/2 = 0.08$. The nonprotein-nitrogen fraction was obtained by precipitating protein from the extractable-nitrogen fraction with trichloroacetic acid and the stroma-protein nitrogen was determined in the unextractable-nitrogen fraction according to Lowry *et al.* (1941). All tests were made at 0°C.

Nonprotein-nitrogen fraction analysis. Protein-breakdown products were estimated in the nonprotein-nitrogen fraction by ninhydrin (Rosen, 1957) and by phenol reagents (Folin and Ciocalteu, 1927), both before and after the hydrolysis of peptides, and are expressed in terms of tyrosine equivalents. Hydrolysis was carried out by boiling the nonprotein-nitrogen fraction in the presence of 6*N* HCl for 20 hr under reflux. The hydrolysate was concentrated to dryness and the residue taken up in water to original volume. The increase in ninhydrin-positive or phenol-reagent-positive materials that occurred after hydrolysis is referred to as ninhydrin-positive polymers or phenol reagent-positive polymers.

RESULTS

Changes in protein nitrogen extractability. The amount of nitrogen extractable in KCl-borate buffer from chicken breast and leg muscle rapidly decreased after death to a minimum value in about 4–8 hr, and gradually approached a maximum value thereafter (Figs. 1 and 2). The onset of rigor mortis corresponded with the decrease in the extractable nitrogen. When the amount of extractable nitrogen approached a maximum, the muscles became soft and relaxed. Most of the soluble nitrogen (total nitrogen minus stroma nitrogen) was extractable from breast muscle after about 1–1½ days of aging and from leg muscle after about 4–6 days of aging, about the same time required to complete tenderization (van den Berg *et al.*, 1964). The post-mortem changes in protein solubility were more pronounced in buffer containing borate than in buffer containing phosphate (Fig. 3).

Post-mortem changes in extractable nitrogen occurred mainly as a result of changes in the solubility of myofibrillar proteins (Table 1). The

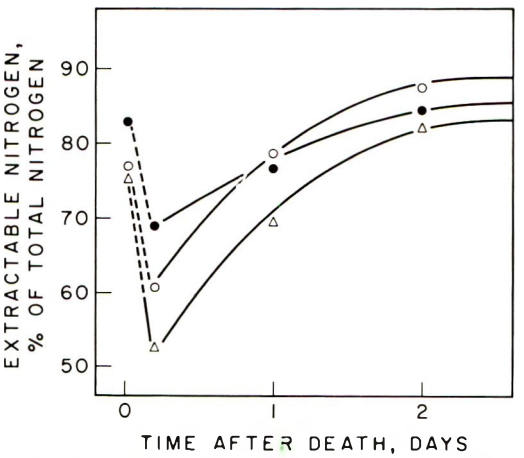

Fig. 1. Changes in extractable nitrogen (KCl-borate buffer) in chicken breast muscle during post-mortem aging period. ○, 6-month-old birds; ●, 9-month-old birds; △, 12-month-old birds; values plotted are averages of results for muscle from three birds.

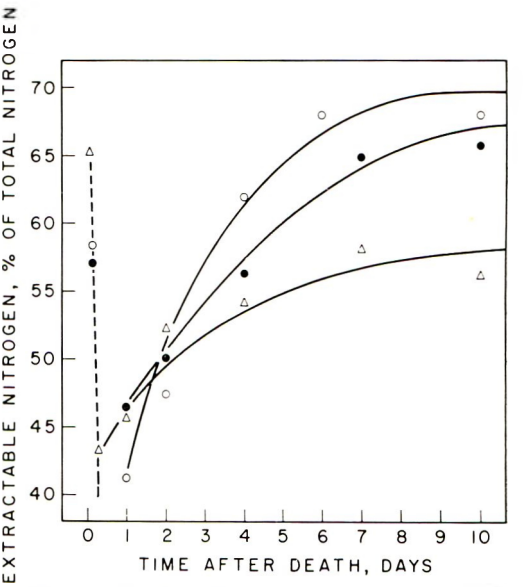

Fig. 2. Changes in extractable nitrogen (KCl-borate buffer) in chicken leg muscle during post-mortem aging period. ○, 6-month-old birds; ●, 9-month-old birds; △, 12-month-old birds; values plotted are averages of results for muscle from three birds.

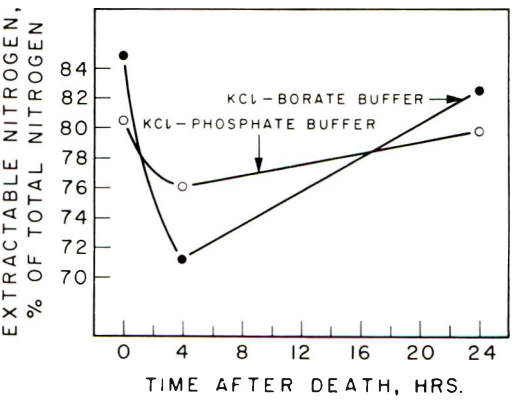

Fig. 3. Comparison of protein extractability in KCl-borate and KCl-phosphate buffers during post-mortem aging of chicken breast muscle.

Table 1. Post-mortem changes in the nitrogen distribution in chicken breast and leg muscle from 9-month-old birds.

Time after death	Nitrogen, % of total N				
	Stroma	Extractable	Myofibrillar	Sarcoplasmic	Nonprotein
Breast muscle					
30 min	11	85	37	32	16
2 hr	11	78	32	30	16
4	11	71	24	31	16
24	11	82	32	33	17
48	11	87	37	33	17
Leg muscle					
30 min	28	57	26	21	10
1 day	28	46	15	21	10
2	28	50	18	20	11
4	28	56	23	21	12
6	28	65	32	21	12

solubility of myofibrillar fraction decreased with the onset of rigor and increased during post-rigor tenderization, whereas quantitative changes in stroma and sarcoplasmic fractions were small. No attempt was made to determine if the onset of rigor mortis and the decrease in solubility of myofibrillar proteins were a result of actomyosin formation (Whitaker, 1959), and if post-rigor tenderization and increase in solubility occurred as a result of an increase in the solubility of actin (Weinberg and Rose, 1960).

Changes in the nonprotein-nitrogen fraction. Tables 2 and 3 show changes in the nonprotein-nitrogen fraction that accompany post-mortem tenderization in breast and in leg muscle. The nonprotein-nitrogen content decreased slightly during the onset of rigor mortis and increased during the post-rigor aging period. The decrease in the non-

Table 3. Post-mortem changes in ninhydrin- and phenol-reagent-positive materials and polymers in chicken breast and leg muscle from 9-month-old birds.

Time after death	Phenol-reagent-positive materials as μg tyrosine/g muscle		Ninhydrin-positive materials as mg tyrosine/g muscle	
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
Breast				
30 min	192	768	3.6	18.3
1 day	252	600	4.2	17.7
2	348	744	4.8	20.4
Leg				
30 min	216	696	3.6	11.7
1 day	240	504	4.2	10.5
4	276	696	4.8	10.8
6	368	744	6.1	12.0

Table 2. Post-mortem changes in nonprotein nitrogen and ninhydrin- and phenol-reagent-positive materials in breast and leg muscle of chickens of 3 ages.

Time after death	Nonprotein nitrogen, mg/g of muscle			Phenol-reagent-positive materials, as μg tyrosine/g muscle			Ninhydrin-positive materials, as mg tyrosine/g muscle		
	6 mo	9 mo	12 mo	6 mo	9 mo	12 mo	6 mo	9 mo	12 mo
Breast									
30 min	5.7	5.5	6.3	240	288	125	4.7	5.0	7.2
6 hr	....	5.4	5.9	.....	.....	125	....	....	7.2
1 day	5.5	....	....	264	.....	.....	5.0	....	....
2	5.6	5.7	6.2	288	313	187	5.0	9.4	7.8
3	5.7	5.9	6.4	336	425	287	5.6	10.9	8.5
Leg									
30 min	3.8	3.7	3.3	216	113	125	5.1	5.4	6.5
1 day	3.5	3.5	3.2	300	138	162	5.0	5.6	7.2
2	....	....	3.3	.....	.....	187	....	....	....
3	3.8	3.7	....	324	156	.....	5.2	9.7	....
4	....	....	3.4	.....	.....	262	....	....	8.7
6	3.8	3.9	....	366	225	.....	5.5	10.0	....

protein nitrogen during the onset of rigor occurred as a result of loss of phenol-reagent- and ninhydrin-reagent-positive polymers. Since the decrease in these polymers was more than twice the simultaneous increase in the ninhydrin- and phenol-reagent-positive materials estimated before hydrolysis (Table 3), these polymers appear to be removed from this fraction and not broken down. The removal of these polymers may have occurred as a result of their interaction or aggregation with proteins. The amount of both ninhydrin- and phenol-reagent-positive materials and polymers gradually increased in both kinds of meat during post-rigor tenderization.

DISCUSSION

A comparison between the state of myofibrils as indicated by the protein extractability, and the results of taste-panel tests (van den Berg *et al.*, 1964), shows a close relation between tenderness and the state of contraction of the muscle. Similar results have been shown earlier for chicken breast muscle (Weinberg and Rose, 1960), for beef (Locker, 1960), and for fish (Otake and Yamamoto, 1954). The increase in tenderness in leg muscle during the first day of aging as shown by both taste-panel and shear-press tests on cooked meat (van den Berg *et al.*, 1964) indicates that the meat cooked in the primary stage of post-rigor aging is more tender than the meat cooked during the onset of rigor mortis.

Results show the formation of protein-breakdown products during post-rigor tenderization of both breast and leg muscle. Niewiarowiez (1956), Leinati (1957), and Wierbicki *et al.* (1956) have also reported similar increases in the nonprotein nitrogen, amino acids, and peptides during aging of beef and pork. The view that the post-rigor tenderization process is proteolytic has been suggested earlier (Whitaker, 1959; American Meat Institute, 1960), but the results have been inconclusive for beef because changes introduced by the action of microbial enzymes could not be avoided during 14–17 days of aging at 0°C. The treatment of chicken muscle with chlortetracycline rules out the possibility of microbial action, particularly in breast muscle where post-rigor tenderization was complete within 1–1½ days. The small increase in protein-breakdown products suggests the possibility of limited proteolysis, and the increase in tenderness may occur as a result of this specific attack. Wang *et al.* (1957) have shown a correlation between the observed microscopic changes in the tissue and increase in tenderness, whether as a result of natural aging or by the action of proteolytic enzymes; however, the mode of action of these enzymes is not known.

Since the stroma protein content of muscle plays an important role in determining the tenderness of meat (Callow, 1957; Hiner *et al.*, 1955), the difference in post-mortem tenderization rates in breast and leg muscle is considered on the basis of the difference in their stroma-protein fractions. Although leg muscle contains more than twice as much stroma as breast muscle, no post-mortem changes in stroma content of the muscle were detected. On the other hand, collagenase has been used for the isolation of myofibrils (Perry, 1951) although this enzyme does not attack myofibrils or sarcolemma (Barer, 1948). The point of action of this enzyme is not clear, but the function of collagenase treatment is to break down the intrafibrillar links, possibly the Z bands which extend transversely across the myofibrils, binding them together and to the sarcolemma (Draper and Hodge, 1949). Other proteolytic enzymes, including trypsin, cause similar changes (Perry, 1951). The increase in the extractability of myofibrillar proteins during post-rigor tenderization may therefore be a result of a similar proteolytic action which weakens or breaks the bonds between some of these proteins and the matrix of the muscle (Rose, 1963), and a larger stroma-protein content may influence the stroma-myofibril relation and the rate of post-rigor tenderization.

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The Activity of Partially Purified Bovine Catheptic Enzymes on Various Natural and Synthetic Substrates[a]

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SUMMARY

A partially purified fraction of bovine muscle cathepsins prepared from a crude extract by precipitation with ammonium sulfate between 45 and 55% saturation was assayed for activity on 18 peptides and other synthetic substrates, and on preparations of four natural substrates isolated from beef. Three of the synthetic substrates were partially hydrolyzed by the enzyme preparation. The hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine indicated endopeptidase activity. Dipeptidase and/or endopeptidase activity was evidenced by the hydrolysis of glycyl-L-tyrosine and L-leucyl-L-tyrosine. When using either of these two peptides as the substrate, a chromatographically distinct compound was detected which was absent in the enzyme and substrate blanks.

The fraction had no detectable enzymatic action on crude preparations of actin, myosin, and actomyosin. Commercially purified serum albumin was hydrolyzed, but at a much slower rate than denatured hemoglobin, which has been widely used for assaying catheptic activity. Sarcoplasmic proteins indigenous to the crude extract appeared to be readily hydrolyzed by the cathepsins.

INTRODUCTION

On fractionation of the cathepsins from beef muscle, Bodwell and Pearson (1963) observed that the fraction precipitated between 45 and 55% saturation with ammonium sulfate showed the greatest activity on a denatured hemoglobin substrate. Hydrolysis of hemoglobin, though widely used as an indication of catheptic enzyme activity (Sliwinski *et al.*, 1959; Snoke and Neurath, 1950; Bandack-Yuri and Rose, 1961), fails to give information as to the specificity of the enzyme or enzymes involved. In order to characterize the type of proteolytic activity in the fraction precipitated by 45–55% ammonium sulfate saturation, it was assayed for activity on various peptides and other synthetic proteolytic enzyme substrates. The activity was also determined using crude preparations of myosin, acin and actomyosin, commercially purified serum albumin, and crude sarcoplasmic proteins indigenous to beef muscle.

EXPERIMENTAL

Extraction and partial purification. Muscle samples removed from beef carcasses 48 hr after slaughter were used for preparation of the enzyme solutions. Extraction and fractionation procedures were identical to those reported previously by Bodwell and Pearson (1963). The fraction precipitated between 45 and 55% ammonium sulfate saturation was used in all assays in the current investigation, unless otherwise indicated. The activity of this fraction was approximately six times that of crude extracts when using denatured hemoglobin as a substrate.

Preparation of natural substrates. Actin was extracted and prepared as an acetone powder according to the procedure of Tsao and Bailey (1953). For use as a substrate, the actin powder was brought into solution with cold H_2O containing 0.2 mg ATP (adenosine triphosphate)/ml, which yielded a solution containing 1.5–3.1 mg

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protein/ml solution. Myosin was extracted according to the method of Mommaerts and Parrish (1951), which resulted in a 0.3*M* KCl solution containing 3.2–4.4 mg protein/ml. Actomyosin solutions containing 2.6–3.1 mg protein/ml in 0.3*M* KCl were prepared as described by Szent-Gyorgyi (1951). In all cases, the enzyme blank for each of the three substrates was adjusted to contain amounts of ATP or KCl equivalent to that of the reaction mixtures or substrate blanks.

Calf longissimus dorsi muscle was used as the source of the actin, myosin and actomyosin preparations. Isolation and analysis are described elsewhere (Hegarty *et al.*, 1963). The albumin substrate consisted of a 4% solution of powdered bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.). A similar solution of denatured hemoglobin (Armour Pharmaceutical Co.) was used as a reference for all of the natural substrates. The synthetic substrates were obtained from either Nutritional Biochemical Co. (Cleveland), Sigma Chemical Co. (St. Louis) or Mann Research Laboratories (N.Y.)

Assay of proteolytic action on natural substrates. For estimation of proteolytic activity on actin and bovine serum albumin, reaction mixtures contained equal volumes of substrate solution, enzyme solution, 0.2*M* sodium acetate buffer at pH 4.4, and 0.02*M* $FeCl_2$. For enzyme or substrate blanks, 1 volume of H_2O or ATP solution (actin substrate blank) was included instead of enzyme or substrate. Assay procedures were otherwise identical to those previously reported (Bodwell and Pearson, 1963) except that the trichloroacetic acid (TCA) precipitated aliquots were filtered through Whatman No. 1 paper rather than being centrifuged.

Under assay conditions, myosin tended to aggregate while actomyosin eventually formed a somewhat gelatinous solution. Consequently, 2 ml reaction mixtures were used, which were identical to those described above except that 0.4*M* sodium acetate buffer was used. Four replicates of each of the three mixtures (reaction mixture, enzyme blank, substrate blank) were prepared and placed in a 38°C water bath. Upon reaching 38°C, 4 ml of 10% TCA were pipetted into 2 replicates of each mixture. After 4 hr, TCA was pipetted into the 2 remaining replicates. After standing for at least 1 hr at room temperature, the sloutions were filtered through Whatman No. 1 paper and absorbance readings of the filtrate were taken at 280 mμ with a Beckman DU spectrophotometer.

Since the sarcoplasmic proteins were extracted simultaneously with the cathepsins, it was impossible to demonstrate the hydrolysis of sarcoplasmic protein spectrophotometrically. Thus, paper chromatography was subsequently used. Two volumes of a crude undialyzed extract containing the cathepsins and other sarcoplasmic proteins were incubated at 38°C with a volume of both 0.02*M* $FeCl_2$ and 0.4*M* sodium acetate buffer (pH 4.4). Five-ml aliquots were withdrawn at 0, 60, 180 and 240 min and mixed with 10 ml of absolute ethanol. The precipitate was removed by filtering through Whatman No. 1 paper, and 0.2-ml aliquots of the filtrate were chromatographed according to the procedure of Koszalka and Miller (1960).

Assay for hydrolysis of synthetic substrates. Assays of the substrates DL-leucine amide HCl, benzoyl-L-tyrosyl-glycinamide and benzoyl-L-arginineamide HCl were carried out by a modified Conway micro-diffusion method (Davis and Smith, 1955). Grassman and Heyde's alcoholic KOH titrimetric method (Davis and Smith, 1955) was used to detect any hydrolysis of glycyl-L-asparagine, *N*-carbobenzoxy-L-leucylglycine, L-leucyl-glycyl-glycine, glycyl-L-tryptophan, glycylglycine, glycyl-glycylglycine. glycylglycylglycylglycine, glycyl-L-proline, glycyl-L-leucine, glycyl-L-alanine, or benzoyl-glycylglycine.

With substrates having terminal tyrosine residues (carbobenzoxy-L-glutamyl-L-tyrosine, glycyl-L-tyrosine, L-leucyl-L-tyrosine, and *N*-phthalyl-glycyl-L-tyrosine), neither of the above methods was satisfactory (Davis and Smith, 1955). Therefore, paper chromatography was used to detect possible hydrolysis of these compounds qualitatively. Duplicate 2.5 ml reaction mixtures (0.5 ml enzyme extract; 1.0 ml 0.125*M* substrate; 0.5 ml 0.4*M* sodium acetate buffer, pH 4.4; and 0.5 ml of either 0.025*M* $FeCl_2$, 0.02*M* $MnCl_2$, 0.02*M* L-cysteine, or H_2O) were prepared, and 5 ml of absolute ethanol were added to one of each reaction mixture pair at zero time or a subsequent time interval (8 to 10 hr). The mixtures were filtered through Whatman No. 1 paper after standing at least 1 hr at room temperature. Enzyme and substrate blanks were treated similarly. Aliquots (0.1 ml) of the filtrates were spotted on Whatman No. 1 paper and chromatographed by ascending paper chromatography carried out in butanol–acetic acid–water (4:1:1) or the organic layer of butanol-ethanol-water (4:1:5). Chromatograms were sprayed with a 0.5% solution of ninhydrin in 95% ethanol.

RESULTS AND DISCUSSION

Activity on natural substrates. Table 1 shows the changes in absorbance at 280 mμ with natural substrates. It has been previously reported (Bodwell and Pearson, 1963) that the activity of different enzyme preparations from tissues derived from the same animal or from different animals varied con-

Table 1. Activity of catheptic enzymes using natural substrates as indicated by change in absorbance.[a]

Substrate	Absorbance change[b]
Hemoglobin	0.192 (0.125/ 0.268)
Serum albumin	0.025 (0.019/ 0.036)
Myosin	— .014 (— .011/— .042)
Actin	— .052 (— .013/— .087)
Actomyosin	0.012 (0.003/ 0.025)

[a] Reaction mixtures consisted of equal volumes of substrate solution, 0.02*M* $FeCl_2$, 0.4*M* sodium acetate buffer (pH 4.4), and enzyme solution (11.6 to 13.1 mg protein/ml); 4-hr assay at 38°C.

[b] Average change in absorbance at 280 mμ (corrected for changes in absorbance of enzyme and substrate blanks) of at least three duplicate assays using enzyme extracts from two or more different animals and (in the case of actin, myosin and actomyosin) substrate preparations from three different animals. Values in parentheses denote range of values.

siderably when denatured hemoglobin was the substrate. The variation found with other natural substrates in the current study was even more pronounced. Consequently, the values in Table 1 are averages of at least three duplicate assays with enzyme extracts from two or more different animals.

When serum albumin was the substrate, the change in absorbance was much less relative to changes occurring with the hemoglobin substrate. It was concluded that serum albumin was hydrolyzed by the enzyme fraction, but much more slowly than hemoglobin. This is in agreement with data for rat skeletal muscle cathepsins reported by Koszalka and Miller (1960).

The myosin and actin substrate reaction mixtures both gave consistently negative changes in absorbance, while actomyosin substrate reaction mixtures resulted in small but consistently positive changes. The actin substrate blanks were always much higher than the same values for myosin and actomyosin, indicating that actin was apparently more labile under assay conditions. Actin and myosin were not hydrolyzed by the enzyme preparation, and the proteolytic action on actomyosin was negligible. While these results suggest that the enzyme fraction caused no major changes in these proteins, minor structural changes were not excluded, since they are difficult to detect spectrophotometrically. As suggested by Whitaker (1959), such minor structural changes in these proteins could have an important effect on the physical properties of the particular protein involved.

Hydrolysis of sarcoplasmic proteins. Fig. 1 gives the results of chromatographing the alcohol-precipitated aliquots of the crude sarcoplasmic extract. Spectrophotometric assay data were in agreement with those previously reported for similar crude extracts (Bodwell and Pearson, 1963). No

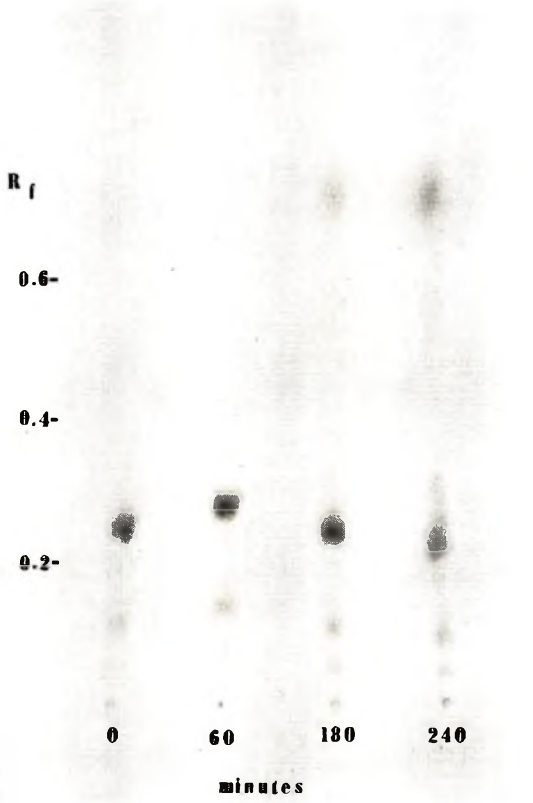

Fig. 1. Results of paper chromatography of aliquots from alcohol-precipitated reaction mixture of crude enzyme extract containing noncatheptic sarcoplasmic protein. Time intervals refer to reaction time prior to treatment with alcohol.

spots were detectable after 240 min that were not present at 0 time, but the intensity of the faster-moving spots was greatly increased. On the original chromatogram, a slight increase in intensity was apparent for the slower-moving spots. Considerable hydrolysis has occurred during the 4-hr assay, suggesting that the sarcoplasmic proteins provided a good natural substrate. This is in agreement with the recent report of

Sharp (1963), in which he concluded that the sarcoplasmic proteins are the major substrate for the natural proteolytic enzymes in muscle.

Hydrolysis of synthetic substrates. None of the substrates enzymatically treated and assayed by Conway's micro-diffusion method or the alcoholic KOH titration technique resulted in detectable hydrolysis. Smith (1948a) reported hydrolysis of L-leucylglycine, glycylglycine, L-leucylglycine, glycylglycylglycine, and L-leucylglycylglycine by crude extracts of either rat or rabbit skeletal muscle. Likewise, Kies and Schwimmer (1942) reported activity of similar extracts from beef on glycyl-L-leucine, glycylglycine, and DL-leucylglycylglycine. However, in all these studies muscle samples were removed immediately post-mortem, whereas in the present study samples were not removed until 48 hr after death. In the current study, it is possible that the enzymes hydrolyzing these peptides were inactivated prior to removal by physiological conditions, or that the purification procedures either inactivated these enzymes or excluded them from the fraction. The general instability of some of the peptidases has been discussed by Smith (1948b) and Davis (1961).

Benzoyl-L-arginineamide HCl (a substrate specific for cathepsin B) and benzoyl-L-tyrosyl-glycinamide (a substrate specific for cathepsin C) were not hydrolyzed. The presence of 0.004*M* L-cysteine, with or without ferrous ion, produced no activation of the enzyme toward either substrate. This is in contrast to work reported by Sliwinski *et al.* (1961), who assayed a crude enzyme fraction of bovine cathepsins with synthetic substrates and concluded that the enzymes were similar to cathepsins B and C though not identical. No details were given by these authors as to substrate used or as to the particular enzyme fraction hydrolyzing the substrates for cathepsin B and C. The particular fraction used by these authors probably differed from that used in the current study.

The enzyme fraction was active upon three of the four substrates in which the carboxyl residue was provided by tyrosine. The common product on hydrolysis of these substrates was tyrosine. This may have been expected since Dahl (1962) reported an increase in free tyrosine from 0.6 to 2.5% of the total tyrosine on aging beef muscle 16 days post-mortem. Figs 2, 3, and 4 show

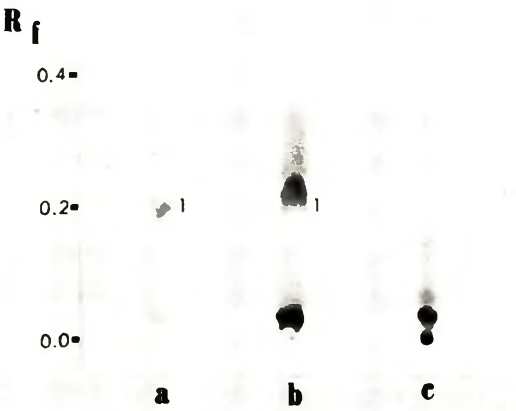

Fig. 2. Results from paper chromatography showing a) substrate blank, b) reaction mixture, and c) enzyme blank when using carbobenzoxy-L-glutamyl-L-tyrosine as substrate. Spots located at a-1 and b-1 are tyrosine resulting from autolysis of substrate and hydrolysis of substrate, respectively. R_f values for unhydrolyzed substrate = 0.75.

photographs of paper chromatograms of the substrate blank, enzyme blank, and reaction mixture, when using each of these substrates.

When using carbobenzoxy-L-glutamyl-L-tyrosine as a substrate (Fig. 2), the spot a-1 was identified as tyrosine liberated as a result of autolysis. The corresponding spot (b-1) for the reaction mixture aliquot shows that a considerable amount of tyrosine had been liberated by enzymic hydrolysis in addition to that resulting from the substrate blank. The enzyme blank aliquot did not yield a tyrosine spot. Thus, enzymic activity toward the substrate was evident. Under assay conditions, carbobenzoxy-L-glutamyl-L-tyrosine is a specific substrate for the endopeptidases, cathepsin A, and pepsin. Therefore, it was concluded that the enzyme fraction contained endopeptidase activity similar to that of cathepsin A.

The results with glycyl-L-tyrosine as a substrate are shown in Fig. 3. Spots a-1 and b-2 were glycine, one of the expected products of hydrolysis. The greater inten-

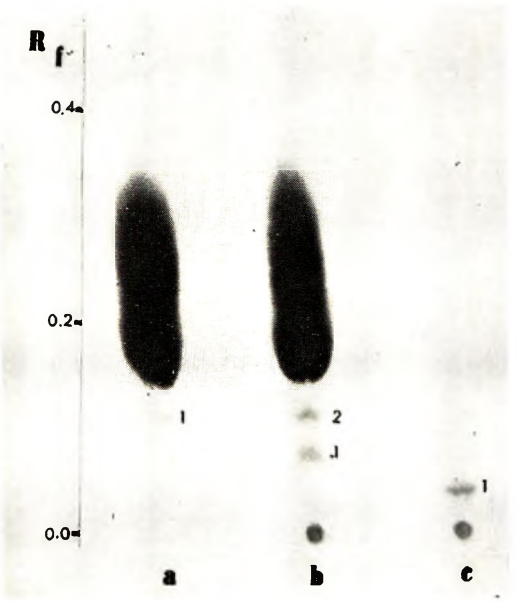

Fig. 3. Results from paper chromatography showing a) substrate blank, b) reaction mixture, and c) enzyme blank when using glycyl-L-tyrosine as substrate. Spots located at a-1 and b-2 are glycine resulting from autolysis and hydrolysis of substrate. Spot located at b-1 is absent from *a* and *c*. R_f values for tyrosine = 0.27 and glycine = 0.11.

sity of spot b-2 than of a-1 suggests that hydrolysis of the substrate occurred in addition to that produced by substrate autolysis. A compound resulting from the reaction mixture (spot b-1) is absent from both substrate and enzyme blanks. Apparently this compound was formed from one of the products of the hydrolyzed substrate (glycine or tyrosine) and some product of autolysis or hydrolysis of the enzyme fraction. The compound formed, not identified, may possibly be a low-molecular-weight peptide.

Results were similar with L-leucyl-L-tyrosine used as substrate (Fig. 4). Glycine (spots b-2 and c-1) and tyrosine (spots b-3 and c-2) were evident in both the substrate blank and to a slightly greater extent in the reaction mixture. A detectable compound (spot b-1) from the reaction mixture was absent in both the enzyme and substrate blanks. Again, it is suggested that peptide formation may have occurred. Any enzymic hydrolysis of L-leucyl-L-tyrosine of glycyl-L-tyrosine must be attributed to endopeptidase or dipeptidase action since the particular bonds involved are not susceptible to hydrolysis by aminopeptidases or carboxypeptidases. Hydrolysis followed by transpeptidation could be the result of dipeptidase activity alone. Alternately, the formation of the new compounds by transpeptidation followed by hydrolysis could be carried out entirely by endopeptidases. Without further information it is impossible to determine which of these possibilities (or a combination thereof) is more feasible.

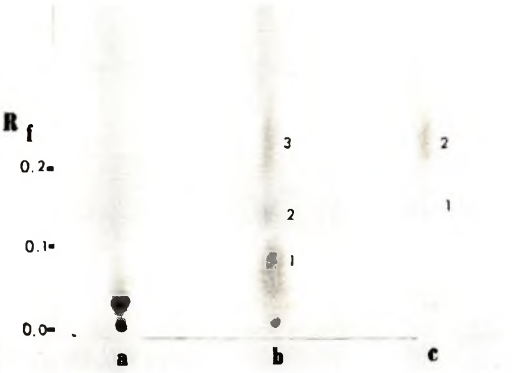

Fig. 4. Results from paper chromatography when using leucyl-L-tyrosine as substrate showing a) enzyme blank, b) reaction mixture, and c) substrate blank. Spots b-2 and c-1 are tyrosine resulting from hydrolysis + autolysis and autolysis, respectively. Similarly, b-3 and c-2 are leucine. R_f value for unhydrolyzed substrate = 0.58.

The results reported here tentatively suggest that peptide synthesis or transpeptidation may result from the action of some of the catheptic enzymes found in beef muscle. It has been previously established that proteolytic enzymes from various sources are capable of transpeptidation and peptide elongation (Durell and Fruton, 1954; Fruton *et al.*, 1951). If such reactions were to occur to any extent in intact muscle of beef carcasses, an analysis of free amino acids and of free end groups would not necessarily be indicative of the amount of proteolysis occurring within the muscle.

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Age-Associated Changes in Bovine Muscle Connective Tissue. I. Rate of Hydrolysis by Collagenase[a]

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(Manuscript received January 27, 1964)

SUMMARY

Age-associated structural changes in collagenous residues isolated from the loose connective-tissue network found within bovine biceps femoris muscle were studied with collagenase used as a structural probe. Rate of enzymatic digestion was measured by following the release of soluble protein, hydroxyproline, and ninhydrin-positive material into the medium surrounding the fibrous residues. Under the conditions of the experiment, 8–11% of the protein and 8–21% of the hydroxyproline in the residues were solubilized after 12 hr of incubation with collagenase. Samples from four age groups were studied: Group I, veal, 40–49 days old; Group II, steers, 403–495 days old; Group III, cows, 4 years, 8 months to 5 years, 5 months old; Group IV, aged cows, 10 years, 2 months and 10 years, 5 months old. The groups ranked I, III, IV, and II, from fastest to slowest, in rate of hydrolysis by collagenase. However, it was postulated that the larger amounts of lipid associated with the Group II samples acted to shield collagenase-labile bonds and cause a slow rate of hydrolysis. Making this assumption, it appears that susceptibility to collagenase digestion of loose connective tissue follows an age pattern similar to that reported for skin and tendon collagen.

INTRODUCTION

Structural changes in collagen have been implicated as playing an important role in the "collagen diseases" and in the aging process. It has also been suggested that structural alterations in collagen may cause differences in meat tenderness (Goll *et al.*, 1963). These phenomena may be linked through common changes in the state of the collagen fibers.

Several investigators have suggested that mature collagen is more highly cross-linked than collagen from younger subjects (Harrington and von Hippel, 1961; Gross, 1958; Veis *et al.*, 1960). Jackson and Bentley (1960) proposed that a continuous spectrum of molecular aggregates exists in native connective tissue. Increasing age connotes increased cross-linking and consequent decreasing solubility of the collagen fibers. New collagen fibrils are formed on the fibers laid down earlier, and the latter are made insoluble by further cross-linking, possibly involving covalent bonds (Veis and Anesey, 1961; Gustavson, 1962) to give thicker fibers (Gross, 1950; Banfield, 1955). As Scheraga (1961) pointed out, increased cross-linking of protein molecules should result in slower observed rate of proteolytic hydrolysis.

The several reports of age-associated changes in the rate of hydrolysis of fibrous collagen by collagenase have substantiated these conclusions. Keech (1954a,b; 1955) found that the collagenase susceptibility of biopsy samples of human dermal collagen decreased with increasing age of the donor. Collagen from young patients was readily and completely hydrolyzed, but collagen samples from older patients were only partially hydrolyzed, leaving behind a residue which Keech termed "moth-eaten fibers" because of their appearance under the electron

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microscope. Diseased patients possessed a more collagenase-resistant tissue than healthy ones. Kohn and Rollerson (1960) have also reported a decrease in collagenase susceptibility of human tendon collagen with increasing age.

Harrington, von Hippel, and their coworkers have proposed the use of proteolytic enzymes as probes of the secondary structure of fibrous proteins (Harrington *et al.*, 1959; Mihalyi and Harrington, 1959; von Hippel *et al.*, 1960; von Hippel and Harrington, 1959). By heating soluble collagen to temperatures above the collagen→gelatin transition temperature, those investigators were able to conclude that the differences observed in the kinetics of proteolysis by collagenase were, at least for the soluble collagen-collagenase-gelatin system, entirely configurational in origin. Although caution should be used in extrapolating results with soluble collagen to the main part of the collagen fiber, it seems likely that extensive cross-linking within collagen fibers may also restrict the availability of susceptible bonds to enzymic attack.

This study was conducted to investigate age-associated structural changes in collagenous residues obtained from the loose connective-tissue network found within bovine biceps femoris muscle with collagenase used as a structural probe. If such changes occur, they may be, in part, responsible for the decreased tenderness of meat from older animals. Jackson (1958) also has pointed out the need for studies of connective tissue other than the conventional skin, tendon, and granulation tissue in order to establish the pattern of *in vivo* fibrogenesis.

METHODS

Eleven animals, varying in genetic and nutritional background, were divided into four age groups: Group I, three Holstein calves, 40–49 days old; Group II, three Hereford steers, 403–495 days old; Group III, three cows (one Holstein, one Hereford, and one Jersey) ranging from 4 years, 8 months to 5 years, 5 months old; Group IV, two Holstein cows, 10 years, 2 months and 10 years, 5 months old. The entire biceps femoris muscle was removed from the right side of each animal within 20 min of death, immediately chilled on dry ice, freed of adhering fat and connective tissue, and minced five min with dry ice in a Buffalo silent cutter, Model 23. The minced tissue was extracted at 0–4°C with precooled 1.1*M* KI buffered at pH 7.4 with 0.1*M* potassium phosphate, and the connective tissue residue from this extraction was then freed of lipid according to the procedure described by Goll *et al.* (1963). The residue left after lipid extraction was lyophilized and powdered in a small Wiley mill at 0°C. Seventy-five-mg samples of the lyophilized, powdered connective-tissue residues were weighed into 50-ml Erlenmeyer flasks. Three samples were weighed for each animal. A small piece of glass wool, 20.5 ml of deionized water, 1.5 ml of 1.0*M* $CaCl_2$, and 3.0 ml of 1.0*M* Tris [tris(hydroxymethyl)aminomethane] buffer (pH 7.8) were added to each flask. The flasks were placed in a Dubnoff Incu-Shaker, Model 61, water bath for 1 hr at 37°C, and then 5 ml of a prewarmed 0.1% collagenase solution were added. A blank flask containing 5 ml of deionized water in place of the enzyme was included in each run for every animal.

The 0.1% collagenase solution was prepared by dissolving 250 mg of crude collagenase powder obtained from Nutritional Biochemicals Corporation in 125 ml deionized water. This solution was centrifuged at 17,000 × G for 2 hr at 2°C and the supernatant made up to 200 ml. The enzyme was dialyzed for 24 hr against two changes of Ringer's solution, and the dialysate was made up to 250 ml.

Immediately after addition of the enzyme to initiate the reaction, two 1-ml samples were removed from each flask. To stop the enzymatic reaction, one of these was placed in a centrifuge tube containing 1 ml of 5% Na_2CO_3 and the other in a centrifuge tube containing 1 ml of a saturated acetate buffer (pH 4.7) prepared according to the method of Rosen (1957). These tubes were centrifuged for 15 min, and 0.5-ml samples were removed in duplicate from the supernatant and made up to 1 ml with deionized water for analysis. Samples from the tubes containing the 5% Na_2CO_3 were used for protein analysis by a modified Folin-Lowry (Lowry *et al.*, 1951) procedure, and samples from the tubes containing the acetate buffer were used for ninhydrin analysis of free amino groups according to the method of Rosen (1957). Duplicate 1-ml samples were removed from each flask and handled according to the procedure described at 0, 1, 3, 5, 8, and 12 hr after addition of the enzyme.

The Folin-Lowry protein analysis (Lowry *et al.*, 1951) was modified to give greater sensitivity. Just prior to an analysis, 1 ml of 0.05*M* $CuSO_4$ was mixed with 1.5 ml of 2.5% sodium tartrate, and 2 ml of this mixture were then diluted to 25 ml with an 8% Na_2CO_3 solution in 0.2*N* NaOH. One ml of this reagent was mixed with 1 ml of

sample and allowed to stand at room temperature for 10 min or longer. Then 0.2 ml of the diluted Folin-Ciocalteu reagent (Hawk *et al.*, 1949) was added and mixed immediately. After the mixture sat at room temperature for 45 min, the absorbancies were read on a Zeiss spectrophotometer at 7500 Å. This modified analysis gave a color which was stable for at least 4 hr and permitted determination of as little as 10 μg of gelatin per ml. The standard curve, made from gelatin purchased from Distillation Products Industries, Division of Eastman Kodak Company, was linear through the origin up to 50 μg of gelatin per ml. Above this concentration, the slope decreased. The upper limit of the analysis was approximately 250 μg per ml when gelatin was the standard protein used.

Rosen's ninhydrin analysis was also modified slightly, using 1 ml of the acetate-cyanide buffer and 1 ml of the 3% ninhydrin reagent on 1-ml samples. Leucine was used as a standard, and all ninhydrin values are expressed in terms of leucine equivalents.

A separate collagenase digestion was used to measure the rate of release of soluble hydroxyproline-containing material under the same conditions described above. One-ml samples of the collagenase digests were removed and placed in 0.5 ml of 6*N* HCl. After centrifugation, 1-ml samples were removed from the supernatant of each tube and placed in 15-ml calibrated glass tubes. One ml of 6*N* HCl was added to each tube, and the tubes were autoclaved 6 hr at 19 lb pressure (126°C), previous trials having shown that the yield of hydroxyproline was greater at 6 hr than at 3, 4, or 8 hr under these conditions. After removal from the autoclave and cooling, the samples were neutralized to a phenolphthalein endpoint with NaOH and made up to 6 ml with deionized water. One-half-ml portions were removed in duplicate from each tube and made up to 2 ml with deionized water for hydroxyproline analysis by the modified Woessner method described by Goll *et al.* (1963).

The results of these three analyses were expressed on the basis of the concentrations of soluble protein, ninhydrin-positive material, and hydroxyproline in the Erlenmeyer flasks at the various sampling times after correction for the zero-time values. Since variation was expected among animals of the same age group and among different samples of the same lyophilized connective-tissue preparation, all results were subjected to detailed statistical analysis according to the methods presented by Snedecor (1956).

RESULTS

Release of soluble protein. Table 1 shows the release of soluble protein from the lyophilized connective-tissue samples of the four age groups. The values in the table represent the average concentrations of soluble protein in the incubation medium after subtraction of zero-time soluble protein concentrations. The concentrations of soluble protein in the flasks containing deionized water in place of the enzyme did not change over the 12-hr incubation period, remaining close to zero.

Analyses of the lyophilized residues revealed that not all residues had the same chemical composition (Goll *et al.*, 1963). The samples from Group I averaged 49% collagen as measured by hydroxyproline analysis, whereas those from the other three groups averaged from 58–68% collagen. Since collagenase is specific for collagen or gelatin (Mandl, 1961), the amount of collagen in the samples would be expected to influence their rate of digestion by collagenase. Therefore, the release of soluble protein was adjusted to put all samples on an equal collagen-content basis. This adjustment was done by multiplying the concentrations of soluble protein in the flasks by a ratio calculated from the hydroxyproline contents of the lyophilized samples. The ratio was determined for each animal by dividing the hydroxyproline content of that animal's connective-tissue samples into the lowest hydroxyproline content among the eleven samples. Thus, the ratios for the eleven animals ranged from 0.57 to 1.00. Although it is not claimed that these ratios represent exact adjustments for the different amounts of collagen in the samples, it does appear necessary to make some correction for the varying amounts of substrate present. Therefore, Table 1 shows both adjusted and unadjusted data for the release of soluble protein by collagenase. The adjusted data indicate that soluble protein was released more rapidly from the Group I (veal) samples and more slowly from the Group II (steer) samples.

The age-time interaction was the most interesting source of variation in the analyses of variance since it indicated the significance of the differences existing among the age groups in the time course of hydrolysis. This interaction was highly significant ($p<0.005$) in analyses of both the adjusted and the unadjusted data, showing that collagenase digestion of the connective-tissue residues followed a different kinetic pattern for each of the four age groups. It is evident from Table 1 that the samples from Group III (cows) had a rapid initial release of protein compared to the samples from the other three groups. A more detailed kinetic analysis was not attempted, since the insoluble substrate limits the interpretation of kinetic results.

Release of soluble hydroxyproline. Table 1 also shows unadjusted and adjusted data for the release of hydroxyproline from the connective-tissue

residues. The concentration of hydroxyproline in the flask containing deionized water in place of enzyme remained near zero throughout the hydrolysis. These data closely parallel those showing the release of soluble protein, although the differences among the four groups are more pronounced in the case of the release of soluble hydroxyproline. Also, analyses of variance of the adjusted and unadjusted data revealed that the age-time interaction was highly significant for both sets of data. Again, the samples from Group III (cows) released soluble material more rapidly during the initial stages of hydrolysis than the samples from the other three groups.

Release of soluble ninhydrin-positive material. Table 1 shows the release of soluble ninhydrin-positive material from the insoluble connective-tissue residues. The figures represent mμmoles of soluble leucine-equivalent material per ml of incubation medium in the flasks after correction for the zero-time values. Although the flasks containing deionized water in place of enzyme showed some fluctuations in content of ninhydrin-positive material during hydrolysis, no consistent increase or decrease was noted, and therefore no correction for these fluctuations was attempted.

The results of the ninhydrin analyses closely parallel those of the protein and hydroxyproline

Table 1. Release of soluble protein, hydroxyproline, and ninhydrin-positive material from bovine biceps femoris connective tissue by collagenase.[a]

Incubation time (hr)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows) (10 years old)
Protein (gelatin standard)				
1	54.8±2.0	26.0±2.5	68.8±2.2	44.8±4.3
	(65.7±2.8)[b]	(40.9±3.6)	(113.1±3.0)	(67.3±5.0)
3	118.9±4.1	60.5±4.5	114.4±3.9	92.6±7.7
	(142.2±2.8)	(95.3±5.8)	(188.0±5.6)	(139.7±8.6)
5	154.0±5.5	75.2±5.1	124.7±2.9	127.9±9.7
	(183.7±2.7)	(118.7±6.4)	(205.0±3.7)	(193.2±10.4)
8	196.3±7.3	97.8±7.1	144.7±3.2	153.2±10.9
	(233.8±2.9)	(153.8±9.0)	(237.8±9.0)	(231.7±11.4)
12	230.7±9.2	122.7±8.5	160.2±4.2	172.8±12.4
	(274.4±3.6)	(193.6±10.8)	(278.0±5.2)	(261.3±13.0)
Hydroxyproline				
1	8.6±1.0	2.8±0.2	7.8±0.4	4.4±0.2
	(10.8±1.4)[b]	(4.0±0.2)	(13.1±0.5)	(6.5±0.3)
3	15.9±0.9	5.4±0.4	10.7±0.5	6.9±0.2
	(19.3±1.4)	(7.9±0.4)	(17.9±0.8)	(10.1±0.4)
5	17.2±1.2	8.9±0.3	13.8±0.6	8.8±0.2
	(21.2±1.9)	(13.0±0.4)	(23.0±0.8)	(12.9±0.4)
8	24.0±1.3	9.4±0.3	16.3±0.6	11.0±0.3
	(29.5±2.2)	(13.8±0.4)	(27.3±0.8)	(16.1±0.4)
12	28.8±0.9	11.3±0.2	18.2±0.6	13.5±0.1
	(35.1±2.0)	(16.6±0.5)	(30.4±0.8)	(19.8±0.4)
Ninhydrin-positive material (leucine equiv.)				
1	84±6	34±3	88±4	44±4
	(104±11)[b]	(56.5)	(145±6)	(69±7)
3	216±10	97±8	159±6	130±11
	(264±17)	(155±12)	(262±8)	(198±14)
5	296±14	119±5	203±7	163±9
	(333±24)	(189±6)	(334±11)	(249±11)
8	361±11	140±7	253±8	180±11
	(441±24)	(225±12)	(415±10)	(274±12)
12	456±10	148±7	270±7	207±8
	(556±25)	(236±10)	(444±10)	(318±12)

[a] Figures represent average μg (mμmoles for ninhydrin-positive material) per ml of incubation medium (plus or minus the standard error) of 18 determinations for all groups except Group IV, where an average of 12 determinations is represented.

[b] Figures enclosed by parentheses represent the data which are not adjusted for the hydroxyproline content of the samples. The figures not enclosed are the adjusted data.

analyses. Group III (cows) samples released soluble ninhydrin-positive material very rapidly when compared to samples from the other three age groups during the first hour of incubation, but this rate of release decreased after 3 hr.

Molecular weight of the soluble protein. Since the protein and ninhydrin analyses were conducted on samples taken from the same flask at the same time, it was possible to calculate an average molecular weight of the soluble protein in the flasks. Any effect due to the initial addition of the enzyme and to the small amounts of soluble protein originally present in the flasks was removed by correction for the zero-time values of protein and ninhydrin-positive material in the flasks. In calculating molecular weight in this manner, it is necessary to assume that the ninhydrin values are measuring only the concentration of N-terminal amino groups in the reaction medium. Although about 2.5% of the amino acid residues of collagen are lysine, this amino acid appears to be concentrated in the non-collagenase-susceptible regions of the molecule (Nishigai *et al.*, 1960; Nordwig *et al.*, 1961). Therefore, it seems likely that the largest part of the ninhydrin value is due to the N-terminal amino groups.

Table 2 shows the approximate molecular weight of the soluble protein in the collagenase digests after the various incubation periods. There was some decrease in the average molecular weight of the soluble protein after the first hour of incubation. This may indicate some hydrolysis of the soluble peptides after their release from the insoluble fiber, either by collangenase itself or by contaminating proteases. No significant difference was noted among the four age groups in the molecular weight of the soluble protein released by the enzyme.

DISCUSSION

Twelve hours of incubation with collagenase under the conditions described under Methods resulted in the solubilization of 11% of the total protein in the Group I (veal) samples, 8% of the protein in the Group II (steer) samples, 11% of the protein in the Group III (cows) samples, and 10% of the protein in the Group IV (aged cows) samples. The same conditions caused the solubilization of 21% of the total hydroxyproline in the Group I samples, 8% in the Group II samples, 13% in the Group III samples, and 10% in the Group IV samples.

The protein, hydroxyproline, and ninhydrin analyses all indicated that samples from Group I (veal) were hydrolyzed most rapidly during a 12-hr incubation with collagenase. Group III (cows), Group IV (aged cows), and Group II (steers) followed Group I in order from fastest to slowest in rate of digestion by collagenase. Unless Group II is omitted, these data do not entirely support the conclusions of Keech (1954a,b; 1955) and Kohn and Rollerson (1960) regarding the effect of age upon the rate of digestion of collagen by collagenase. Chemical analyses of the lyophilized connective-tissue residues of Group II revealed that they contained nearly 2.5% lipid in spite of the rigorous fat-extracting methods used in their isolation. Samples from the other three groups all possessed less than 1% lipid. The bound lipid content of Group II samples may suggest the presence of a large amount of reticulin which has a high bound lipid content (Robb-Smith, 1958). Since reticulin may be resistant to collagenase, this may explain the slow rate of hydrolysis of Group II samples. It is also possible that the large amount of lipid associated with the samples from Group II may act to shield collagenase-labile bonds from the enzyme or to restrict diffusion of the enzyme to the

Table 2. Approximate molecular weight of soluble protein in collagenase digests of bovine biceps femoris connective tissue.[a]

Incubation time (hr)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows) (10 years old)
1	766±55	870±118	795±31	1123±172
3	592±15	639±43	721±15	710±43
5	647±49	622±21	621±20	774±37
8	580±22	699±46	574±8	848±33
12	533±23	824±41	629±13	832±56

[a] Figures represent average g/mole of soluble protein (plus or minus the standard error) of 18 determinations for all groups except Group IV, where an average of 12 determinations is represented.

insoluble substrate. Data presented in the following papers tend to confirm this possibility (Goll *et al.*, 1964a,b). There is no apparent reason why lipid should have remained bound to the samples from Group II and not to the samples from the other three groups unless a sex effect is involved. Groups I, III, and IV were all females, whereas Group II was castrated males.

Analyses of variance revealed that enzymic hydrolysis of the lyophilized samples followed a different kinetic pattern for the different age groups. The protein, hydroxyproline, and ninhydrin analyses all indicated that the samples from Group III (cows) were hydrolyzed very rapidly compared to samples from the other three groups during the first hour of incubation. A possible explanation is that the residues from Group III were partially denatured during their isolation. The enzyme would then rapidly hydrolyze the denatured collagen, releasing it as soluble material. Such degradation must be slight, since hydrolysis slows considerably after the first hour, before 3% of the protein has been solubilized.

The values of 600–800 g/mole for the average molecular weight of the soluble protein released by collagenase is in good agreement with the values of 500–600 g/mole reported by Seifter *et al.* (1959), who used the same method to estimate molecular weight.

The differences observed in the rate of collagenase digestion of the samples may be due either to differences in the availability of substrate to the enzyme or to the type of limited proteolysis reaction discussed by Scheraga (1961). Both of these effects would be caused by increased cross-linking within or among tropocollagen molecules. Although change in the rate of enzymic digestion provides presumptive evidence for the existence of more frequent or stronger cross-linkages in more mature collagen, it does not, by itself, rule out the effect in insoluble substrates of shielding of collagenase-susceptible bonds by elastin, lipid, or any other non-collagenase-labile substances in the samples. While it might be assumed that these shielding effects remain the same among similarly treated samples from different age groups, the data presented here indicate that it may be unwise to do so. Analyses showing the protein, lipid, and collagen composition of the samples should be presented when collagenase is used as a structural probe of fibrous collagen. Even with these data, additional information obtained by other techniques would appear necessary before any definite conclusions can be reached regarding the nature of the cross-linking in fibrous collagen. Following papers will present two techniques which should prove valuable for the study of fibrous collagen.

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Age-Associated Changes in Bovine Muscle Connective Tissue. II. Exposure to Increasing Temperature[a]

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SUMMARY

Changes in collagen structure during maturation were studied with collagenous residues obtained from the loose connective-tissue network within bovine biceps femoris muscle from animals of different ages. A new technique for studying thermal shrinkage and collagen structure of powdered collagenous residues was described. Samples from four age groups were studied: Group I, three calves, 40–49 days old; Group II, three steers, 403–495 days old; Group III, three cows, 4 years, 8 months to 5 years, 5 months old; Group IV, two cows, 10 years, 2 months and 10 years, 5 months old.

The release of soluble protein, ninhydrin-positive material, and hydroxyproline from collagenous residues into a phosphate-buffered medium (pH 7.0) upon incubation at gradually increasing increments of temperature from 25 to 70°C was measured. Differences between various age groups were marked, and at 60°C and above, the groups ranked I, II, III, and IV, from highest to lowest, in amounts of soluble material released. At the final temperature of 70°C, Group I samples had released 42% of their hydroxyproline in a soluble form, compared to only 2% from Group IV. The thermal shrinkage temperature, which was taken as that temperature at which a sudden release of soluble hydroxyproline occurred, increased with advancing age, from near 55°C for Group I to 70°C or above for Group IV, while the average molecular weight of the soluble protein released was greater for the younger animals. The results were discussed in relation to experiments involving the rate of collagenase digestion of the same samples. The findings indicate stronger or more extensive cross-linkages in the collagen from older animals.

INTRODUCTION

Thermal shrinkage has been recognized as one of the characteristic properties of collagen for many years (Ewald, 1919; Gustavson, 1956). This phenomenon may be observed simply by subjecting a bundle of collagen fibers to slow heating. At a temperature, T_s, which is characteristic of the species from which the collagen was obtained, the fibers suddenly contract to 1/3 of their original length. According to Gustavson (1955a,b), this is caused by a rupture of the interchain cross-linkages of collagen. Strengthening or increasing the number of these cross-linkages leads to an increase in T_s, but the addition of agents which interfere with cross-linkages by direct competition for valency will decrease T_s. Gustavson (1957) has also stated that the principal type of cross-linkage broken during the thermal contraction of collagen is the hydrogen bond, and he postulated that the hydrogen bonds formed by the hydroxyl group of hydroxyproline are among the principal sources of stabilization of the collagen fiber against thermal shrinkage. However, recent evidence indicates that the influence of pyrrolidine residues in locking the amino acid residues of collagen into a poly-L-proline II-type configuration may be a more important source of stabilization of

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the collagen fiber (Burge and Hynes, 1959; Piez and Gross, 1960).

The thermal shrinkage of collagen fibers has been related to the thermal denaturation of soluble collagen molecules (Esipova *et al.*, 1958; Doty and Nishihara, 1958). If the optical rotation or viscosity of a tropocollagen solution is followed as the temperature is slowly increased, large changes in these two properties will occur over a narrow temperature range. The midpoint of this temperature range may be called T_D. For all classes of collagen studied to date, the difference between T_s and T_D has been found to be 27 ± 3°C. This indicates that thermal shrinkage may be a manifestation of the collapse of the triple-helical collagen molecule. Fiber shrinkage occurs at a higher temperature than the denaturation of soluble collagen because both the intramolecular forces within the triple-stranded molecule and the crystallization energy must be overcome. When interpreted in view of this evidence, differences in the thermal-shrinkage temperature of collagen fibers appear to depend upon intra- rather than intermolecular interactions (Harrington and von Hippel, 1961).

Verzar and his co-workers (Brocas and Verzar, 1961; Verzar, 1957; Verzar and Freydberg-Lucas, 1958; Verar and Huber, 1958) have reported a close correlation between the isometric tension developed during thermal contraction and age as well as between the weight required to prevent shortening of rat-tail tendons and age. Those workers stated that the temperature of thermal shrinkage, T_s, was not related to age. This is in accord with results of Hall and Reed (1957). Other workers, however, have reported an increase in T_s with advancing age (Brown and Consden, 1958; Brown *et al.*, 1958; Joseph and Bose, 1962).

The difficulty in unequivocally interpreting the results of collagenase digestion of insoluble collagen samples from animals of different ages has been described in a previous paper (Goll *et al.*, 1964b). The thermal shrinkage of collagen fibers appeared to offer a method for obtaining additional information regarding changes in collagen structure upon maturation and the relation of these changes to meat tenderness. Verzar and his associates (Meyer and Verzar, 1959; Verzar, 1960; Verzar and Meyer, 1961; Verzar and Thoenen, 1960) recently reported that soluble substances are released from collagen fibers during thermal shrinkage. These substances contain ninhydrin-positive material and hydroxyproline. The amount of soluble substance released appeared to decrease with advancing age in Verzar's studies. This paper describes a new method for measuring thermal shrinkage of insoluble collagen isolated from the loose connective-tissue network within bovine biceps femoris muscle. Use of this method demonstrates wide differences between collagen samples from animals of different ages and dispenses with the necessity of using fibers to measure thermal shrinkage.

METHODS

Connective-tissue samples were obtained from the loose connective-tissue network with the biceps femoris muscle as already described (Goll *et al.*, 1964b). Four age groups were represented: Group I, three Holstein veal calves, 40–49 days old; Group II, three Hereford steers, 403–495 days old; Group III, three cows (Holstein, Hereford, and Guernsey breeding), 4 years, 8 months to 5 years, 5 months old; Group IV, two Holstein cows, 10 years, 2 months and 10 years, 5 months old.

Triplicate 150-mg samples of the lyophilized connective-tissue residues were weighed into 50-ml Erlenmeyer flasks. Forty ml of 0.1*M* sodium phosphate buffer, pH 7.0, were added to each flask. The flasks were incubated in a Dubnoff Incu-shaker, Model 61, water bath at 25°C for 1 hr, and 4-ml samples were then removed from each flask. The samples were placed in glass centrifuge tubes, allowed to cool, and centrifuged for 15 min. Following removal of the sample, the temperature of the water bath was raised to 45°C. After remaining at this temperature for 15 min, another 4-ml sample was removed from each of the flasks and placed in cold centrifuge tubes. The temperature of the water bath was then raised to 55°C. This procedure was repeated at 55, 65, and 70°, holding the flasks at each of these temperatures for 15 min before sampling and moving on to the next-higher temperature. Temperatures higher than 70°C were not used, because the temperature of the water bath was difficult to control accurately.

Samples (0.5 ml) were withdrawn in duplicate from the supernatant in the centrifuge tubes for protein analysis by the modified Folin-Lowry

method already described (Goll *et al.*, 1964b). Two additional 0.5-ml portions were removed for ninhydrin analysis (Goll *et al.*, 1964b). Lastly, a 1-ml sample was taken from the supernatant and placed with 1 ml of 6*N* HCl in a graduated tube. This tube was autoclaved for 6 hr at 19 lb pressure (126°C). The samples were neutralized to a phenolphthalein end-point with NaOH, made up to 6 ml with deionized water, and 0.5-ml portions were removed in duplicate and placed in test tubes. These were made up to 2 ml with deionized water and analyzed for hydroxyproline by a modification (Goll *et al.*, 1963) of the procedure described by Woessner (1961).

The results of the three analyses were expressed on the basis of the concentration of soluble protein, leucine-equivalent ninhydrin-positive material, and hydroxyproline in the flasks at each temperature. Since all three analyses were conducted on the same samples, an estimate could also be obtained of the molecular weight and the hydroxyproline content of the soluble protein. All results were analyzed according to the procedures outlined by Snedecor (1956).

RESULTS AND DISCUSSION

Incubation of the insoluble connective-tissue residues under the conditions described in Methods resulted in solubilization of 23% of the total protein and 42% of the total hydroxyproline in the Group I (veal) samples, 11% of the protein and 17% of the hydroxyproline in the Group II (steer) samples, 4% of the protein and 5% of the hydroxyproline in the Group III (cow) samples, and only 2% of the protein and 2% of the hydroxyproline in the Group IV (aged cow) samples. These figures show that a higher percentage of hydroxyproline than of protein was solubilized. Thus, collagen was probably the primary protein affected in this experiment although the samples probably contained elastin and possibly reticulin as well as collagen.

The protein, ninhydrin, and hydroxyproline analyses showed marked age-associated differences among the four groups in release of soluble material with increasing temperature (Table 1). In each of these analyses, the samples from the younger animals released more soluble material and released it at a lower temperature. Previous analyses (Goll *et al.*, 1963) had shown that significant differences existed in the hydroxyproline content of the samples from the four groups, so the data for this release of hydroxyproline were adjusted to put all samples on an equal hydroxyproline-content basis (*cf.* Goll *et al.*, 1946b). This appeared necessary since the samples from Group I contained only 49% collagen by hydroxyproline content whereas the samples from the other three groups ranged from 59 to 68% collagen. The adjusted data for the release of soluble hydroxyproline appear in Table 1.

By assuming that all of the hydroxyproline released by the increasing temperatures was in peptide-linkage, the hydroxyproline content of the soluble protein could be calculated. Since the ratio of hydroxyproline to protein in the insoluble residues was not the same among the four groups, the data were adjusted to put all samples on an equal hydroxyproline-to-protein ratio. This was done by calculating the mg of hydroxyproline per g of protein (Kjeldahl N multiplied by 6.25) in the samples from each animal. The animal with the lowest ratio of hydroxyproline to protein in the insoluble residues was selected as a base for the adjustment, and the hydroxyproline-to-protein ratios of all the other animals were divided into this base to give a correction factor. The hydroxyproline content of the soluble protein released from each animal's samples was then multiplied by that animal's correction factor. The eleven correction factors calculated in this manner ranged from 0.54 to 1.00. This method of correcting the data resulted in a decrease of all values for the hydroxyproline content of the soluble protein. Adjusting the data in this fashion recognized the fact that the samples from different animals possessed varying amounts of noncollagenous protein and that some of this protein may be solubilized by the conditions employed in this experiment. Both the adjusted and unadjusted data appear in Table 2.

The hydroxyproline content of collagen has been reported to be 13.3% on a weight basis (Eastoe and Leach, 1958). This compares closely with hydroxyproline contents of 12.4 and 13.0% for the soluble protein released by Groups I and II, respectively, indicating that most of this protein originates from the collagen in the connective-tissue

Table 1. Release of soluble protein, ninhydrin-positive material, and hydroxyproline from bovine biceps femoris connective tissue with increasing temperature.[a]

Temp. (°C)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows (10 years old)
Protein (gelatin standard)				
25°	22±3	11±1	26±2	13±1
45°	54±5	19±1	39±2	18±1
55°	225±17	53±2	59±2	26±1
60°	544±29	168±10	82±1	38±1
65°	726±32	310±21	122±2	60±1
70°	863±34	409±26	171±4	82±1
Ninhydrin-positive material (leucine equiv.)				
25°	29±1	3±1	17±1	4±1
45°	38±2	4±1	24±1	6±1
55°	73±4	10±2	33±2	8±1
60°	130±4	48±4	30±1	9±1
65°	170±6	90±5	66±3	14±2
70°	214±8	129±11	92±2	33±2
Hydroxyproline				
25°	0	0	0.1±0.1	0
	0	0	(0.2±0.1)	0
45°	1.2±0.4	0	0.6±0.1	0
	(1.6±0.5)[b]	0	(1.1±0.2)	0
55°	16.8±1.3	0.8±0.1	1.1±0.1	0
	(20.9±2.0)	(1.1±0.2)	(1.8±0.2)	0
60°	51.5±2.0	12.2±0.9	3.0±0.2	0.1±0.1
	(63.2±4.0)	(18.0±1.4)	(5.0±0.4)	(0.1±0.1)
65°	73.2±2.5	25.1±2.0	6.7±0.2	1.8±0.1
	(89.5±5.2)	(37.1±3.3)	(11.2±0.3)	(2.6±0.2)
70°	88.3±2.9	36.2±2.2	10.3±0.3	3.9±0.1
	(107.1±5.5)	(53.6±4.1)	(17.2±0.5)	(5.7±0.2)

[a] Figures represent average μg (mμmoles for the ninhydrin-positive material) per ml of incubation medium (plus or minus the standard error) of 18 determinations for all groups except Group IV, where the figures are averages of 12 determinations. Samples were maintained at each of the designated temperatures for 15 min before elevating the temperature to the next level.

[b] Figures enclosed by parentheses represent the data which are not adjusted for the hydroxyproline content of the samples. The figures not enclosed are the adjusted data.

samples. It appears that most of the noncollagenic protein which was released by the increasing temperature was solubilized at the lower temperatures. The sudden increase in hydroxyproline content of the soluble protein should be a good indication of melting temperature of the secondary structure of collagen in the samples from the four age groups. This melting temperature for the four groups appears to be: Group I, 55°C; Group II, 55–60°C; Group III, 60–65°C; Group IV, over 70°C. The melting temperature of the secondary structure of collagen may be closely related to the thermal shrinkage temperature. The melting temperatures reported here for Groups II and III correspond closely to the thermal shrinkage temperatures reported by Gustavson (1956) for bovine tendon collagen. Joseph and Bose (1962), Brown and Consden (1958), and Brown *et al.* (1958) have also reported collagen shrinkage temperature to increase with advancing age. If maturation of collagen is accompanied by the formation of stronger cross-linkages, possibly covalent in nature, within the tropocollagen molecule (Gustavson, 1962), and not merely by an increase in the number of cross-linkages, an increase in the thermal-shrinkage temperature should be expected.

By making the assumption that the ninhydrin values were due entirely to N-terminal amino groups, it was also possible to

Table 2. Hydroxyproline content of soluble protein released from bovine biceps femoris connective tissue with increasing temperature.[a]

Temp. (°C)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows) (10 years old)
25°	0	0	0.5±0.2	0
	0	0	(0.3±0.1)	0
45°	2.1±0.5	0	2.4±0.5	0
	(1.5±0.5)[b]	0	(1.5±0.3)	0
55°	9.0±0.4	1.9±0.4	3.0±0.2	0
	(7.5±0.2)	(1.2±0.2)	(1.8±0.2)	0
60°	11.5±0.1	10.5±0.3	5.9±0.4	0.3±0.1
	(9.6±0.2)	(6.5±0.1)	(3.6±0.2)	(0.2±0.1)
65°	12.2±0.2	11.7±0.4	9.2±0.1	4.4±0.3
	(10.1±0.2)	(7.2±0.2)	(5.6±0.1)	(2.9±0.2)
70°	12.4±0.4	13.0±0.2	10.1±0.1	6.9±0.2
	(10.5±0.4)	(8.1±0.1)	(6.1±0.1)	(4.5±0.2)

[a] Figures represent average % hydroxyproline content of the soluble protein on a weight basis (plus or minus the standard error) of 18 determinations for all groups except Group IV, where the figures are averages of 12 determinations. Samples were maintained at each of the designated temperatures for 15 min before elevating the temperature to the next level.

[b] Figures enclosed by parentheses represent the data which are adjusted for the hydroxyproline content of the samples. The figures not enclosed are the unadjusted data.

calculate an average molecular weight for the soluble protein released from the insoluble residues for each of the four age groups. Since some of the ninhydrin values at the lower temperatures were zero, the molecular-weight computation could not be made for every temperature.

The mean values, with their standard errors, for the average molecular weight of the soluble protein in the flasks at 70°C were: Group I, 4030±100; Group II, 3290±50; Group III, 1860±100; Group IV, 2560±50. These figures may be low, since no attempt was made to correct the ninhydrin values for lysine. About 2.5% of the amino acid residues of collagen are lysine, and, in contrast to the collagenase experiment, there is no reason to believe that lysine would not be included in the soluble material released. Therefore, if the soluble protein is assumed to be primarily collagenous, the calculated molecular-weight values may be 2–3 times too small. However, the figures presented do offer a relative comparison of the average molecular weight of the soluble protein released by the four age groups.

Analyses of variance indicated the presence of a significant age-temperature interaction in the data for the release of soluble protein, hydroxyproline, and ninhydrin-positive material. Significance of this interaction may be interpreted as differences among four age groups in the time-course of their release of soluble material. The samples from Group II (steers) appeared remarkably inert below 55°C, whereas the Group III (cow) samples appeared remarkably soluble below this same temperature. A preceding paper (Goll *et al.*, 1964b) has already pointed out some peculiarities of the samples from these two groups in their rate of collagenase digestion. It was postulated that a large amount of lipid associated with the Group II samples was acting to shield them from enzymic attack. If that is so, then in the experiments described in this paper, the lipid coating on the Group II samples would act to prevent any surface erosion of the collagen fibrils in the samples with the consequent liberation of a small amount of soluble material into the surrounding medium until the thermal shrinkage temperature was reached. The sudden contraction of the collagen fibrils at T_s would then break the bonds between the lipid and the protein, and large amounts of collagenous material would be rapidly released into solution. It seems unlikely that the bonds between the lipid and the protein are covalent in nature, since the lipid could also be released upon denaturation of the protein by chloroform extraction of the samples at room temperature. It is possible that

the association is a physical one due to the interaction of charged groups. This may explain the resistance of the lipid to the chloroform extraction at −21°C used to isolate connective-tissue residues from the biceps femoris muscle (Goll *et al.*, 1963).

The rapid initial solubilization of the samples from Group III (cows) which was noted in the previous paper was also evident in the experiments described in this paper. It appears quite likely that both of these results may be explained by the presence of some small, soluble, hydroxyproline-containing peptides in the Group III samples, possibly resulting from partial degradation of the samples during their preparation. This hypothesis is, in part, supported by the low average molecular weight of the soluble protein released from the Group III samples. Such degradation must not have been very extensive, since the samples from this group appeared to undergo thermal shrinkage in the range 60–65°C, which was given by Gustavson (1956) for bovine collagen. This conclusion is also supported by results given in the other two papers of this series (Goll *et al.*, 1964a,b). There is no apparent explanation for the partial degradation of the Group III samples or the lipid in the Group II samples, since all samples were subjected to the same conditions of isolation.

The release of larger-molecular-weight fragments from the samples from the younger age groups confirms the hypothesis that collagen from older animals possesses more extensive or stronger cross-linkages. The presence of several cross-linkages could act to tie a peptide strand onto the body of the fiber even after it had been freed at both ends. Portions of this peptide could then be released only by cleavages along the strand. Younger animals, with fewer cross-linkages, would tend to release larger peptides since the average distance between cross-linkages would be larger.

The evidence obtained with the technique described in this paper appears to be a valuable complement to information gained from collagenase digestion. They both indicate that collagen from more mature animals possesses more frequent or stronger cross-linkages. Since it does not require intact fibers, which do not exist in the loose connective-tissue network within a muscle, the technique described in this paper is particularly applicable to a study of the role of collagen in meat tenderness. A following paper will describe a technique which offers additional information on the composition and structure of insoluble collagenous residues.

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Age-Associated Changes in Bovine Muscle Connective Tissue. III. Rate of Solubilization at 100°C[a]

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SUMMARY

The rate of solubilization of insoluble connective-tissue residues obtained from the loose connective-tissue network within bovine biceps femoris muscle was studied in phosphate-buffered (pH 7.0) aqueous medium at 100°C. Eleven animals from four age groups were used: Group I, three veal calves, 40–49 days old; Group II, three steers, 403–495 days old; Group III, three cows, 4 years, 8 months to 5 years, 5 months old; Group IV, two cows, 10 years, 2 months and 10 years, 5 months old. The conditions under which the samples were heated resulted in the solubilization of 95–100% of the hydroxyproline and 72–94% of the protein in the samples within 240 min. The release of soluble protein and soluble hydroxyproline indicated that collagen from the younger animals was solubilized (i.e. converted to gelatin) much more rapidly than collagen from the older animals. The low hydroxyproline content of the soluble protein released from the Group I samples was discussed, and it was suggested that these samples contained some reticulin. The average molecular weight of the soluble protein decreased with increasing age. The findings were discussed with relation to the results reported in two previous papers and the extent and strength of cross-linkages in collagen from animals of different ages. It was suggested that the number and strength of cross-linkages in collagen may play an important role in meat tenderness.

INTRODUCTION

The thermal or chemical degradation of collagen leads to soluble products called gelatin. If native collagen is placed in an alkaline solution for a prolonged period and then extracted at neutral pH and 60–65°C, the soluble gelatins appear as single-chain polypeptide units (Boedtker and Doty, 1954; Gouinlock *et al.*, 1955) with an isoelectric point near pH 5.0 (Kenchington and Ward, 1954) and a weight-average molecular weight of 95,000 (Williams, 1958). However, native collagen which is first equilibrated for a short period at room temperature with an acid buffer and then extracted at 60–65°C breaks down to form acid-precursor gelatins, which have markedly different molecular parameters than the alkali-precursor gelatins (Harrington and von Hippel, 1961; Veis and Cohen, 1956, 1957; Veis *et al.*, 1955).

Veis and co-workers (Veis and Anesey, 1961; Veis *et al.*, 1958, 1960, 1962) have conducted an extensive study of the acid-precursor gelatins and have isolated, from the soluble products released from collagen fibers by 60–65°C temperatures at pH 2, four types of gelatin components, each possessing a discrete molecular-weight distribution. One of these components had a molecular weight larger than that of the tropocollagen molecule. This fact, plus the findings that the two largest-molecular-weight fractions could be readily precipitated in the form of collagen fibers, led Veis to conclude that: 1) strong intra-tropocollagen bonds exist which are undoubtedly covalent in nature; and 2) similar, strong inter-tropocollagen covalent linkages exist in ma-

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ture fibers. Gallop and his group (Gallop *et al.*, 1959; Seifter *et al.*, 1960) have also obtained evidence showing the presence of covalent cross-linkages within the tropocollagen molecule.

A previous paper (Goll *et al.*, 1964b) discussed the release of soluble products from skeletal muscle collagen from animals of different ages by exposure to increments of temperature from 25 to 70°C. If covalent cross-linkages exist among tropocollagen molecules in the collagen fiber, it appears possible that these cross-linkages may increase during maturation and affect the rate and extent of transformation of the fiber into gelatin. Fysh (1958) has stated that collagen from young animals is more readily converted to gelatin than collagen from older animals of the same species. Therefore, a study of the rate of solubilization of insoluble bovine-muscle connective-tissue residues at the temperature of a boiling-water bath (hereinafter referred to as 100°C for convenience) was undertaken to provide additional information on the nature and strength of the cross-linkages existing in the collagen fibers from animals of different ages.

METHODS

Samples from the loose connective-tissue network within bovine biceps femoris muscle were obtained according to a procedure previously described (Goll *et al.*, 1964a). The eleven bovine animals in the study were divided into four age groups: Group I, three Holstein veal calves, 40–49 days old; Group II, three Hereford steers, 403–495 days old; Group III, three cows (Hereford, Holstein, and Guernsey breeding), 4 years, 8 months to 5 years, 5 months old; Group IV, two Holstein cows, 10 years, 2 months and 10 years, 5 months old.

Triplicate 50-mg samples of lyophilized, powdered connective-tissue residue were weighed into large test tubes. Fifty ml of 0.1*M* sodium phosphate buffer (pH 7.0) were added to each tube and the tubes allowed to equilibrate at room temperature for 1 hr. A 6-ml sample was then removed from each and placed in a centrifuge tube. The large test tubes were capped with marbles and put into a boiling-water bath. Three-ml samples were removed 15, 30, 60, 120, and 240 min after introduction into the water bath. These samples were each placed in centrifuge tubes containing 3 ml of deionized water and sitting in crushed ice. After chilling, the tubes were centrifuged and four 0.5-ml samples of the supernatant were removed for analysis in duplicate by the Rosen ninhydrin procedure for free amino groups and the modified Folin-Lowry procedure for protein as previously described (Goll *et al.*, 1964a). A 1-ml sample was also removed, placed with 1 ml of 6*N* HCl, and autoclaved for 6 hr at 19 lb pressure (126°C). These samples were neutralized with NaOH to a phenolphthalein end-point and made up to 6 ml with deionized water, and 0.5-ml samples were analyzed in duplicate by modification (Goll *et al.*, 1963) of the procedure described by Woessner (1961).

The results were expressed as concentration of protein, hydroxyproline, and ninhydrin-positive material per ml of incubation medium in the tubes at each of the five sampling times after correction for zero-time values. The average molecular weight and average hydroxyproline content of the soluble protein were calculated, and these results are also presented.

RESULTS AND DISCUSSION

Incubation of the connective-tissue residues for 240 min in a boiling-water bath under the conditions described in Methods resulted in complete solubilization of hydroxyproline in the samples of all groups except Group I. In this group, 95% of the hydroxyproline was released in the soluble form. The same conditions resulted in solubilization of 75% of the protein in Group I samples, 72% of the Group II protein, 94% of the Group III protein, and 81% of the protein in Group IV samples. These figures indicate that, for Groups II, III, and IV, solubilization of collagen in the samples was complete in 240 min of heating.

Table 1 shows the release of soluble protein, hydroxyproline, and ninhydrin-positive material from the insoluble residues at 100°C. Samples from the younger groups exhibited a more rapid initial release of soluble protein and and hydroxyproline than samples from the older groups. After 240 min in the boiling-water bath, however, more soluble protein was found in the tubes containing samples from Groups III (cows) and IV (aged cows) than in those containing samples from Group I (veal). The soluble-protein concentrations in the tubes after 240 min may reflect differences in the elastin content of the samples, since elastin would not be solubilized at 100°C.

Table 1. Release of soluble protein, hydroxyproline, and ninhydrin-positive material from bovine biceps femoris connective tissue at 100°C.[a]

Heating time (min)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows) (10 years old)
Protein (gelatin standard)				
15	230±10	116±6	82±4	33±3
30	363±9	197±14	156±5	95±6
60	523±9	334±16	326±9	158±14
120	691±12	583±28	604±11	365±18
240	754±8	726±24	942±17	811±31
Hydroxyproline				
15	13.2±1.5	7.4±0.6	1.0±0.3	0
	(15.9±1.9)[b]	(10.8±1.0)	(1.6±0.5)	0
30	25.7±2.2	14.9±1.2	6.2±0.5	1.5±0.4
	(31.2±2.9)	(21.7±1.9)	(10.4±0.8)	(2.2±0.6)
60	37.4±2.5	27.3±1.8	17.0±0.8	10.1±1.6
	(44.9±3.3)	(39.7±2.5)	(28.5±1.3)	(14.7±2.2)
120	44.2±3.4	52.6±2.4	42.5±1.0	28.8±1.4
	(54.6±5.2)	(76.6±3.3)	(71.2±1.4)	(42.5±2.6)
240	53.9±4.3	60.6±1.9	64.2±1.1	64.3±1.8
	(63.6±4.1)	(88.7±3.5)	(107.2±2.0)	(94.6±3.7)
Ninhydrin-positive material (leucine equiv.)				
15	20±5	4±2	46±3	0
30	52±4	48±6	84±2	10±4
60	136±6	95±6	138±4	66±10
120	107±2	164±8	235±6	124±8
240	167±5	209±12	313±7	256±8

[a] Figures represent average μg (mμmoles for ninhydrin-positive material) per ml of incubation medium (plus or minus the standard error) of 18 determinations for all groups except Group IV, where an average of 12 determinations is represented.

[b] Figures enclosed by parentheses represent the data which are not adjusted for the hydroxyproline content of the samples. The figures not enclosed are the adjusted data.

Because the samples from the four groups ranged from 49 to 68% collagen by hydroxyproline analysis (Goll *et al.*, 1963), the results were adjusted to put all samples on an equal collagen-content basis (cf. Goll *et al.*, 1964a). The adjusted data are presented in Table 1. These data show that, after 240 min in the 100°C bath, the concentration of hydroxyproline released by the four groups approached the same value. This would be expected if the collagen in each of the samples was entirely solubilized.

The release of soluble, ninhydrin-positive material does not show consistent age-associated differences (Table 1). After 240 min of incubation, Group III (cow) samples had released large amounts of ninhydrin-positive material compared to the samples from Groups I (veal) or II (steers). The initial rate of release of soluble ninhydrin-positive material from Group III (cow) samples also was large compared to the initial release of material from Group II (steer) and Group IV (aged cow) samples. After 60 min, the release of free amino groups from the Group I (veal) samples decreased and appeared to stop. Although these data do not show age-associated differences, the ninhydrin values are not necessarily indicative of the amount of soluble material released, since any hydrolysis of peptide bonds in the soluble protein would also be reflected as an increase in the concentration of soluble ninhydrin-positive material. Thus, although the protein concentration in the tubes containing Group II (steer) samples didn't quite double during the 15–30-min interval of incubation, the concentration of ninhydrin-positive material in these same tubes increased 12-fold during this time interval. Similar, although less pronounced, results occurred at lower temperatures in the previous study (Goll *et al.*, 1964b).

Assuming that all the soluble hydroxyproline was in peptide linkage, it was possible to calculate an average hydroxyproline content of protein released from the connective-tissue residues. Since the residues of the four groups did not have the same hydroxyproline-to-protein ratio (Goll *et al.*, 1963), the data were adjusted to put all samples on an equal hydroxyproline-protein ratio (*cf.* Goll *et al.*, 1964a). The effect of this adjustment was to raise the hydroxyproline content of Group I protein relative to that from the other three groups. Both the adjusted and the unadjusted data appear in Table 2.

The unadjusted figures indicate that small amounts of noncollagenous protein, possibly some muscle protein remaining with the connective-tissue residues after salt extraction, were released during the first part of the incubation period. Such contamination must be small in the samples from Groups II, III, and IV, since the hydroxyproline content of protein released by these samples after 240 min of incubation approached the 13.3% figure reported as the hydroxyproline content of collagen (Eastoe and Leach, 1958). The soluble protein released from the Group I samples, however, contained less hydroxyproline than would be expected if it were entirely collagenous. It is possible that collagen from younger animals possesses less hydroxyproline than that from more mature animals, or that some protein other than collagen is present in the Group I samples. The former possibility appears unlikely, since the soluble protein released from the Group I samples at 70°C possessed the hydroxyproline content of collagen (Goll *et al.*, 1964b). It seems more likely that the Group I samples contained substantial amounts of some protein which is insoluble at 70°C but is solubilized at 100°C. Elastin, which would be expected to be present with collagen in the residues, is not affected by 100°C temperatures (Hall, 1955; Partridge *et al.*, 1955). Reticulin, a protein which resembles collagen but has a lower hydroxyproline content of 7.4% by weight (Bose and Das, 1956) may also remain with the insoluble residues through the salt extractions. Although this protein has not been well characterized, the work of Robb-Smith (1958) and others (Bose *et al.*, 1955; Bowes and Kenton, 1959; Denisova and Zaides, 1957; Zaides *et al.*, 1959) suggests that reticulin may be more resistant to aqueous extraction than collagen. Therefore, it appears quite possible that the Group I samples contain appreciable amounts of reticulin. Boucek *et al.* (1961) have also reported a low hydroxyproline content of human lung collagen isolated from young donors. Those workers suggested that their results could be explained either by a strong conjugation of lipids with the protein, resulting in their ability to resist alcohol-ether extraction, or by the presence of reticulin. In view of the low lipid content and the high Kjeldahl

Table 2. Hydroxyproline content of soluble protein released from bovine biceps femoris connective tissue at 100°C.[a]

Heating time (min)	Group I (veal) (40–49 days old)	Group II (steers) (403–495 days old)	Group III (cows) (5 years old)	Group IV (aged cows) (10 years old)
15	6.8±0.6	9.0±0.4	1.7±0.5	0
	(5.7±0.6)[b]	(5.6±0.2)	(1.1±0.3)	0
30	8.6±0.8	10.8±0.2	6.6±0.4	2.4±0.6
	(7.2±0.7)	(6.8±0.2)	(4.0±0.1)	(1.2±0.4)
60	8.5±0.5	11.7±0.2	8.7±0.2	8.7±0.6
	(7.1±0.4)	(7.3±0.2)	(5.3±0.1)	(5.7±0.4)
120	7.8±0.7	13.2±0.1	11.8±0.2	11.6±0.2
	(6.3±0.4)	(8.2±0.2)	(7.2±0.2)	(7.5±0.2)
240	8.4±0.6	12.2±0.1	11.5±0.1	11.7±0.1
	(7.1±0.6)	(7.6±0.2)	(7.0±0.1)	(7.6±0.2)

[a] Figures represent average % hydroxyproline content of the soluble protein on a weight basis (plus or minus the standard error) of 18 determinations for all groups except Group IV, where the figures are averages of 12 determinations.

[b] Figures enclosed by parentheses represent the data which are adjusted for the hydroxyproline content of the samples. The figures not enclosed are the unadjusted data.

nitrogen content reported for the Group I samples (Goll *et al.*, 1963), the former hypothesis doesn't seem to offer a likely explanation for the results obtained in this study. The possibility that reticulin is associated with the connective-tissue samples of young animals is particularly interesting since reticulin has been postulated to be a form of immature collagen (Irving and Tomlin, 1954; Mandl, 1961).

An average molecular weight of the soluble protein released from the connective-tissue residues may be calculated by assuming that ninhydrin values were due entirely to N-terminal amino groups. Since the ninhydrin values for some tubes after 15 min of incubation were zero, the molecular-weight calculations were carried out only for the 240-min incubation period.

The mean values, with their standard errors, for the average molecular weight of the soluble protein were: Group I, 4540±90; Group II, 3540±80; Group III, 3020±40; Group IV, 3160±60. Since the calculations did not take the lysine content of collagen into consideration, the figures presented above may be 2–3 times too low. However, they should give a fairly accurate representation of the relative differences among the four age groups. In those instances where the calculations could be made, the average molecular weight of the soluble protein appeared to decrease with increasing incubation time. Therefore, temperatures of 100°C appeared to rupture some peptide bonds in the soluble protein under the conditions used in this experiment.

The results of the protein and hydroxyproline analyses clearly indicated that collagen from older animals was converted to gelatin at a slower rate than collagen from younger animals. The lipid which, on the basis of the results of the two previous studies (Goll *et al.*, 1964a,b), had been postulated to be present on the surface of collagen in the Group II (steer) samples was not apparent in this study. The 100°C temperature apparently caused almost immediate thermal shrinkage of the collagen fibers, thereby breaking any bonds between lipid and protein. However, the suggestion that some degradation of collagen in the Group III samples had occurred during their isolation received additional support in this study. The Group III samples released large amounts of ninhydrin-positive material within 15 min of introduction into the 100°C bath. Also, the average molecular weight of the soluble protein released from Group III samples was lower than the average molecular weight of the soluble protein released by any of the other three groups. This situation also existed in the previous study in which connective-tissue residues were heated to 70°C.

As explained in the previous paper (Goll *et al.*, 1964b), the larger average molecular weight of the soluble protein released by samples from the younger animals may indicate the presence of stronger or more frequent cross-linkages in the more mature collagen. The similarity between the average molecular weight of the soluble protein released at 100° in this study and the molecular weight of the protein released at 70° in the previous study suggests that the same bonds were affected in both cases.

The results of three independent physicochemical tests described in this paper, and the two preceding it, all suggest that collagen isolated from the loose connective-tissue network within bovine muscle changes in character with maturation of the animal. Mature collagen appears to contain more extensive or stronger cross-linkages than collagen from young animals. The three tests described may offer an approach to the study of changes in connective tissue during muscular dystrophy and some of the "collagen diseases." The three-pronged approach resulting from these tests has several advantages over the use of any single method, particularly in a study involving an insoluble substrate which may contain several substances in addition to collagen.

The results of these tests may also offer a new explanation for variations in meat tenderness. Although collagen content of muscle and increasing maturity of the animal have been independently associated with decreasing tenderness, several studies have indicated that the collagen content of muscle does not incresae with age (Loyd and Hiner, 1960; Mitchell *et al.*, 1928). Analysis of the biceps femoris muscle used in this study also did not reveal any difference among the four

groups in collagen content of the fresh muscle (Goll *et al.*, 1963). However, some simple tests of tenderness conducted on steaks taken from this same muscle indicated that tenderness decreased with age (Goll *et al.*, 1963). The results described here suggest that the cooking process may have less effect on the connective tissue in meat cuts from older animals, thereby leaving the collagen relatively undegraded, permitting less tenderness.

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RESEARCH NOTE

Conversions of Sugars to Organic Acids in the Strawberry Fruit

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The prevailing theory concerning biogenesis of the organic acids in plants has been that these acids are synthesized primarily in the leaves and later translocated to other organs, including the fruit (Peynaud and Maurié, 1958; Ulrich, 1952; Gatet, 1939). The possibility of *in situ* formation of the fruit acids has been discussed by Ulrich (1952) and experimentally demonstrated by Hale (1962) and Loewus (1961).

The present communication presents evidence for the conversion of glucose and fructose to organic acids in strawberries detached from the plant.

Strawberry fruits of the cultivated varieties Robinson and Everbear were severed from the plant when they were yellow-white, leaving about 1 inch of stem on them. Any green sepals remaining on the detached berries were removed, and the stem was immersed in a solution of ^{14}C-labeled sugars. Water was added as the fruit absorbed the solution at a greenhouse. Twenty-four to 36 hours later, the acids of the berries were extracted with boiling water, purified with lead precipitation, and separated by gradient elution ion-exchange column chromatography, as described previously (Markakis *et al.*, 1963). In some repetitions of the experiment the lead precipitation step was omitted without loss of resolution of the acids.

The fractions of the column chromatographic separation were dried *in vacuo*, titrated with 0.02*N* (or 0.1*N* for the major acids) NaOH, transferred to 30-mm-diameter planchets, dried under infrared lamps, and measured for radioactivity by a gas-flow thin window detector connected with an automatic sample changer, scaler, and printing timer (all manufactured by Nuclear-Chicago Corp.).

Identification of the individual acids was based on the effluent volumes of the column separation and the R_f values of the subsequent paper chromatography of the fractions performed by methods already described (Markakis *et al.*, 1963). The identity of the citric acid was confirmed by subjecting the corresponding peak to silica-gel chromatography with isocitric acid (Zbinovsky and Burris, 1954), since citric and isocitric are not separable by the column and paper chromatographic methods used here. The molybdate test was used for confirmation of the presence of phosphoric acid. The ascorbic acid was detected by the 2,6-dichlorophenolindophenol reaction applied photometrically to all the column chromatographic fractions of an analysis of unlabeled strawberries.

Fig. 1 presents results of a typical experiment in which two Everbear strawberries were fed 8 microcuries of uniformly labeled crystalline D-fructose-^{14}C (sp. activity 2 millicuries/millimole) and analyzed 24 hr later. The correspondence of the radioactivity and titration peaks is very satisfactory. Peak 1 did not display an acid spot upon paper chromatography, but it was weakly ninhydrin-positive; it may correspond to amino acids which had not been completely removed by the Dowex-50 treatment. Peaks 2 and 3 respectively represent glutamic and aspartic acids. Peak 4 has the effluent volume of shikimic acid, but no other evidence was ascertained for the identity of this acid. Peak 5 corresponds to quinic acid. Peak 6 was resolved by paper chromatography to galacturonic, glyceric, and glycollic acids. Peak 7 was also resolved by paper chromatography to succinic and glucuronic acids. Peak 8 represents ascorbic acid. No attempt was made to quan-

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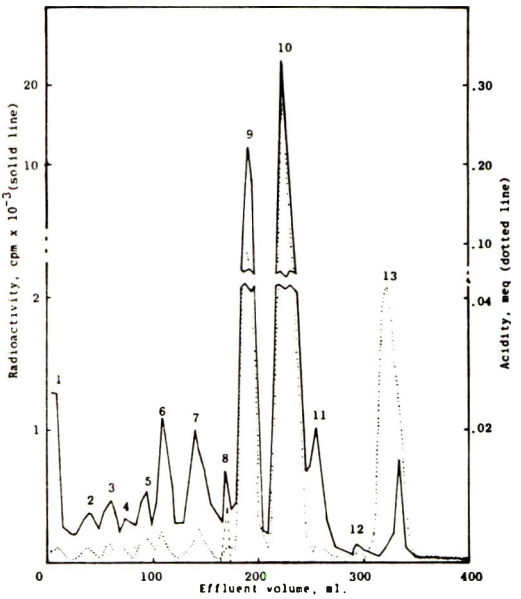

Fig. 1. Radioactivity and acidity of the column chromatography fractions of the acids of detached strawberries fed fructose-^{14}C. Peaks are identified in text.

tify this acid. Peak 9 corresponds to malic acid. Peak 10 was resolved to citric acid and a trace of malonic acid. Peaks 11 and 12 were not identified. Peak 13 represents phosphoric acid and a small quantity of an unidentified organic acid which was separated from phosphoric by paper chromatography. The presence of this organic acid explains the radioactivity of some of the phosphoric acid fractions. Results were similar when D-glucose-UL-^{14}C was administered to detached strawberries. Some of the organic acids of strawberries were previously identified by Hulme and Wooltorton (1958).

It was estimated that 2.5% of the ^{14}C of the radiofructose fed to the strawberries was incorporated in the citric acid of the fruit. This acid represented about 75% of the total equivalent acidity of the strawberries used in this experiment.

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A Pressing Theory with Validating Experiments on Apples

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SUMMARY

A theory of pressing is presented. This takes the form of an integral equation relating all relevant variables. Experiments carried out on the pressing of apples into cider validated this theory.

INTRODUCTION

Filtration theory has been studied by a number of investigators, but there is very little information in the literature on pressing. Some studies have appeared in which investigators attempted to apply filtration theory to pressing, but with little success. The author believes that the theory presented in this paper will prove useful in the design of continuous pressing devices.

THEORY

Fig. 1 presents a diagram of the pressing operation. As pressure is applied on the piston, liquid is forced out of the product, through the crushed mass and filter cloth, into the receiver. It may be assumed that the pressure drop due to resistance of the filter cloth is negligible. Further, the total cross-sectional area perpendicular to the direction of flow may be chosen as unity. Darcy's Law is now applied to the system. The pressure drop (dp) in the liquid as it moves through a given differentially small thickness (dl) of the solid-liquid mixture at a distance (l) from the piston surface at time t may be expressed as follows:

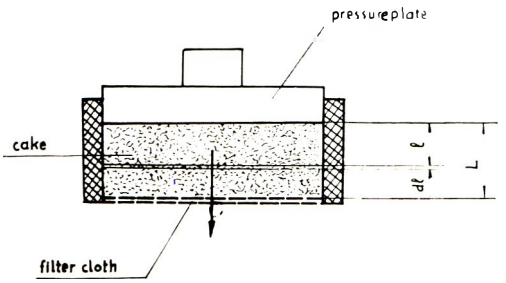

Fig. 1. Schematic diagram of pressing.

$$-\frac{\partial p}{\partial l}\,dl = \alpha_1 \cdot \frac{dV}{dt}\,dl \qquad [1]$$

Dividing by dl gives:

$$-\frac{\partial p}{\partial l} = \alpha_1 \frac{dV}{dt} \qquad [2]$$

α_1 is the pressure drop through a press cake of unit thickness when the flow rate through unit cross-sectional area is one. α_1 will be referred to as the specific flow resistance. In filtration theory, specific resistance relates to a unit-viscosity liquid phase; however, in the present situation, this is omitted for simplicity's sake. dV/dt is the flow rate through thickness dl at time t.

During pressing, the cake becomes thinner, and, accordingly, l becomes smaller. Since the cross-sectional area has been chosen as unity, the flow rate is equal to the rate of decrease in l while the weight of the solid phase remains constant, since only the liquid phase passes through the cross-section determined by l. Thus it is evident that:

$$\frac{dV}{dt} = -\frac{dl}{dt} \qquad [3]$$

The following equation can now be introduced:

$$l = S_m \int_o^s W ds \qquad [4]$$

where the term S_m is the weight of the solid phase in the entire cake, W is the volume of the solid-liquid mixture containing a unit weight of the solid phase (W now to be termed the specific volume of the solid phase). s is the weight ratio of the solid phase from the piston surface to l relative to all the solid phase S_m.

Based on Eq. 4, the following substitutions can be made in Eq. 3:

$$\frac{dV}{dt} = -\frac{dl}{dt} = -S_m \frac{\partial}{\partial t}\int_o^s W ds = -S_m \int_o^s \frac{\partial W}{\partial t}\,ds \qquad [5]$$

As l is decreased such that s remains constant, then:

$$\frac{\partial p}{\partial l} = \frac{\partial p}{\partial s} \cdot \frac{1}{WS_m} \qquad [6]$$

Eq. 2 can now be transformed to:

$$\frac{\partial p}{\partial s} \cdot \frac{1}{WS_m} = \alpha_1 S_m \int_o^s \frac{\partial W}{\partial t}\,ds \qquad [7]$$

The following considerations regarding the pres-

sure distribution are taken from the filtration theory (Carman, 1938). The following symbols and definitions apply: p_o is the total pressure (that is, the total force on the pressed cake divided by the total cross-sectional area); p (as before) is the pressure in the liquid phase (that is, the force transmitted by the liquid phase divided by its cross-sectional area, a part of the total one).

Cake pressure is calculated thus:

$$p_c = p_o - p \qquad [8]$$

Carman (1938), Rietema (1952), and Stjenitzer (1955) have shown that the compression of the solid phase depends on the cake pressure. They found that the specific resistance and cake pressure are both functions of the specific volume:

$$\alpha_l = \alpha_l(W)$$
$$p_c = p_c(W)$$

Both functions characterize the physical properties of the pressed material and can be determined experimentally. In order to simplify Eq. 7 further, α_l is replaced by

$$\alpha_s = \alpha_l W \qquad [9]$$

α_s is the specific flow resistance of a layer which contains a unit weight of solid on unit cross-section.

Substituting p_c from Eq. 8 for p (Eq. 7), we arrive at the final form of the integral equation:

$$\frac{1}{\alpha_s} \cdot \frac{dp_c}{dW} \cdot \frac{\partial W}{\partial_s} = -S^2_m \int_0^s \frac{\partial W}{\partial t} ds \qquad [10]$$

The initial conditions are $t = 0$ and $W = W_t$, which means that, before pressing, the mixture is characterized by its initial specific volume of the solid phase (W_t). The boundary conditions are $s = 1$ and $W = W(p_o) = W_o$ which means that at the filter cloth the pressure in the liquid phase is negligible; the cake pressure is equal to the total pressure. p_o (the total pressure) may be kept constant or varied.

The most important conclusion expressed by Eq. 10 is that the pressing time required to achieve the same percent yield of press fluid is proportional to the square of the initial thickness of the material being pressed (L_{i1} and L_{i2} are two different initial thicknesses). Thus:

$$\frac{t_1}{t_2} = \left(\frac{S_{m1}}{S_{m2}}\right)^2 = \left(\frac{L_{i1}}{L_{i2}}\right)^2 = \left(\frac{G_{i1}}{G_{i2}}\right)^2 \qquad [11]$$

The initial thickness of the material is proportional to the initial weight of the pressed material (G_{i1} and G_{i2}) or to the weight of the solid phase (S_{m1} and S_{m2}), reminding that the total cross-sectional area was chosen as unity.

Eq. 11 was obtained by substituting $t = U S^2_m$ (U is a new variable of time). Since S_m is a constant, in order to get the same W at a constant total pressure it is necessary to have the same U value.

This conclusion is also valid when the initial press cake has different thickness and is subjected to pressure varying in time, assuming that the pressure does not decrease ($dp_o : dt > 0$) and provided that $p_o(U)$ is the same in both cases. This would indicate that the processes with varying pressures may be compared in this special case and the relation $t_1 : S^2_{m1} = t_2 : S^2_{m2}$ holds if the percent yield is the same.

Fig. 2 shows $W = W(s, t)$ function of integral

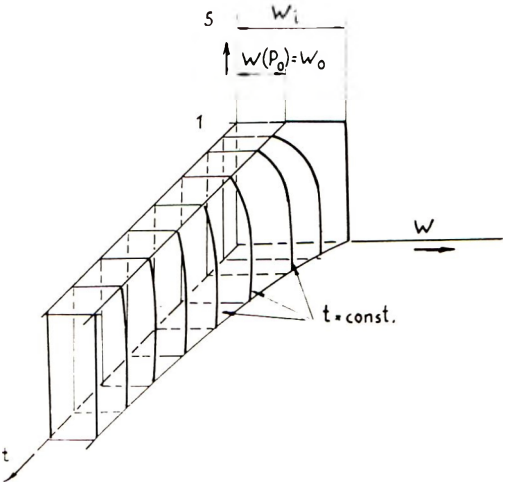

Fig. 2. Surface described by integral equation.

Eq. 10. Initial and boundary conditions are included. This function of three variables is illustrated by geometric surface (Fig. 2).

EXPERIMENTAL

Apparatus. The apparatus used to test the theory is shown in Fig. 3. Air pressure adjustments

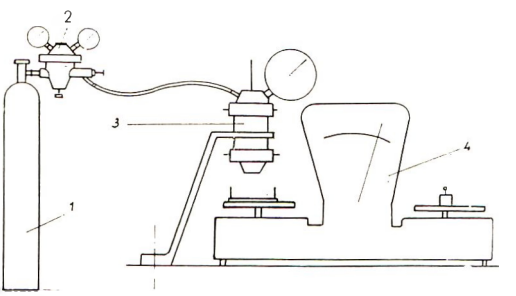

Fig. 3. Testing apparatus. (1) air tank; (2) pressure-reducing valve; (3) pressing assembly; (4) scale.

were made with a pressure-reducing valve. The air supplied the pressure needed to drive the piston

against the material being pressed. The juice from the cake flows through the filter cloth into the receiver placed on a balance. Fig. 4 shows a cross-section of the pressing assembly.

In operation, the material to be pressed is placed

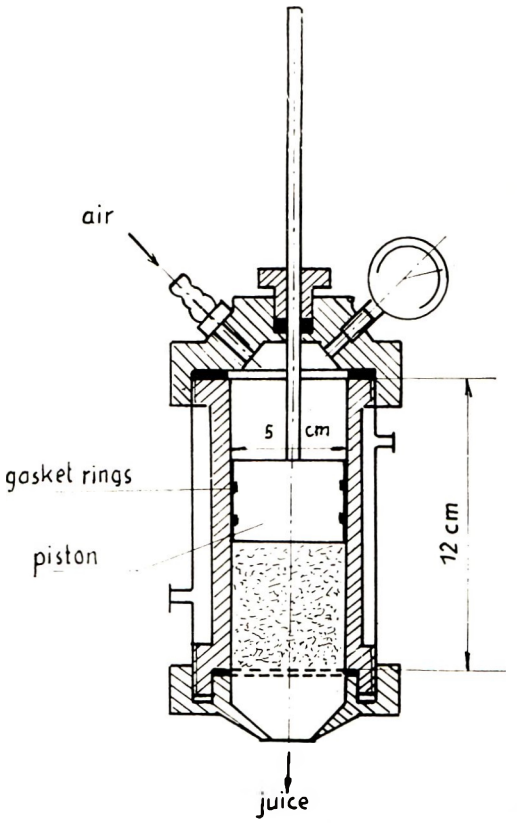

Fig. 4. Cross section of pressing assembly.

on this piston in an upside-down position. The filter cloth was next placed on the material, and a perforated disc and sealing ring added. The latter was fastened to the assembly by a clamp, a part of which had a funnel for directing the juice into the receiver. The whole assembly was then placed in an upright position for the test. Temperature control was maintained by a water jacket with connections to a constant-temperature bath. Pressure on the system was measured by a gauge. Pressure was kept constant by a regulating screw on the reduction valve. The apparatus was operated up to pressures of 30 kg/cm^2 (used by European processors).

The piston rings for the apparatus were made of rubber. The average value of the friction of the piston assembly was found to be 0.17 kg/cm^2. This value was taken into account in the calculations involved.

Experiments. Experimental work was carried out on crushed apples prepared in several ways. In the main the test material was prepared with and without peel and with and without core. Apple pulp, grated apple, a mixture of both, and homogenized apple pulp were used. The quantity of expressed juice was determined at constant pressure as a function of pressing time. Preliminary experiments were made to determine flow resistance of the filter cloth, which was found to be negligible. In the regular experiments the effects of pressure were studied as well as the influence of time lapse between comminution of the apple and pressing. Most of the experiments, however, related to the effect of the initial thickness of the material to be pressed.

RESULTS AND DISCUSSION

If Eq. 11 was found to be valid, then the percent-yield-time curves [$y = f(t)$] for different initial thicknesses, plotted as the ratio of time and the square of initial thickness, should coincide for different thicknesses and result in a single curve [$y = f(t/G_i^2)$]. Satisfactory confirmation of theory was found in experiments carried out on apple pulp and grated apples over a wide range of thicknesses. Fig. 5 shows the curve for apple pulp which included core and peel; Fig. 6 is for apple pulp without core or peel.

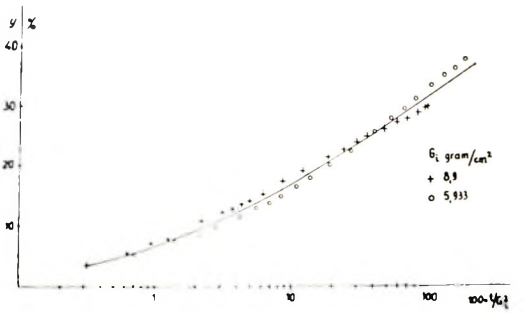

Fig. 5. Yield as a function of time and initial thickness (apple pulp including core and peel).

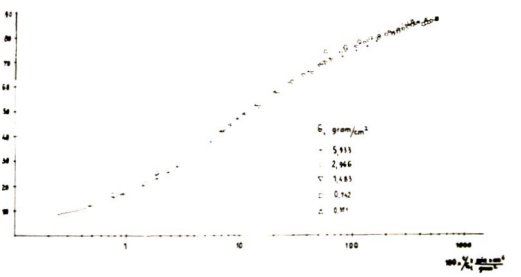

Fig. 6. Yield as a function of time and initial thickness (apple pulp without core or peel).

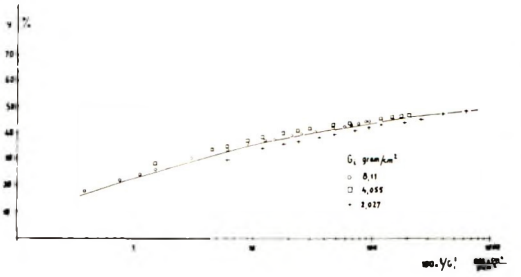

Fig. 7. Yield as a function of time and initial thickness (grated apple including core and peel).

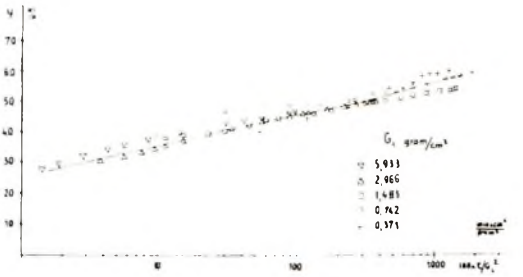

Fig. 8. Yield as a function of time and initial thickness (grated apple without core and peel).

Fig. 7 gives results with grated apple (made up of small, firm particles), including core and peel; Fig. 8 is for grated apple without core or peel. All these experiments were carried out at a pressure of 10 kg/cm^2.

Standard deviations for the curves shown in Figs. 5–8 were respectively ± 0.981, ± 2.24, ± 1.59, and ± 2.04.

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The Effect of Selected Carbohydrates and Plant Extracts on the Heat Activation of Bacillus Stearothermophilus Spores

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SUMMARY

Spores of two suspensions of *Bacillus stearothermophilus* strain M were heat-shocked in monosaccharides, disaccharides, and polysaccharides. The concentration had significant effects. The dominant significant trend was for reduced counts with increased concentrations of carbohydrate. These significant effects are thought to be associated with osmotic pressure. It would appear, therefore, that there was considerable difference in the osmosensitivity of spores in these populations. Spores were activated as well as suppressed when heated in plant extracts. Pea, spinach, and corn extracts tended to suppress spore germination, and green bean extract stimulated spore germination.

INTRODUCTION

Bacillus stearothermophilus is of particular interest to the food processor and food microbiologist because the spore form is extremely heat resistant and the vegetative form, which is capable of growth at 70°C or slightly higher, is responsible for flat sour spoilage of low-acid canned foods. This thermophile has been used routinely in studies involving low-acid food in the canning industry. In such studies, the number of spores that will germinate at the time the experiment is performed, must be known. To increase the number of spores that germinate, the common practice has been to heat (activate) prior to plating and counting. Curran and Evans (1944, 1945) were the first workers to demonstrate systematically that sublethal heat (62–95°C) could induce dormant spores to germinate. It was Brachfeld (1955) and Titus (1957), however, who showed that the spores of *B. stearothermophilus* were activated by heat at temperatures greater than 100°C. Studies in our laboratory confirmed that spores of *B. stearothermophilus* were activated at temperatures greater than 100°C (Finley and Fields, 1962; Fields and Finley, 1962; and Fields, 1963).

Our studies with *B. stearothermophilus* NCA strain 1518 and strain M (Finley and Fields, 1962), showed that spores of *B. stearothermophilus* were susceptible to dormancy as produced by heat during incubation and low heat shock. The concept of "heat-induced dormancy" as introduced by Brachfeld (1955) was therefore confirmed. Also, it has been shown that the presence of rough and smooth variants within a spore population may be a factor in measuring a treatment effect by the standard plate-count method (Fields, 1963). These factors of heat-induced dormancy, and rough and smooth variants, although they are not the main objective of this study, must be considered in interpreting the data. It was originally hoped that spore suspensions could be considered as duplicates, but the above data indicated that each suspension had different spore populations; hence, each must be considered separately.

Extensive studies were performed on two suspensions of of *B. stearothermophilus*. Time-temperature values previously determined for each suspension (Finley and Fields, 1962) were used as the experimental basis for these experiments.

Although *B. stearothermophilus* has long been known to cause flat sour spoilage in canned foods, the effect of common carbohydrates and plant extracts on the heat-activation response of spores at temperatures above 100°C has not been studied.

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It is known that certain components in vegetables influence the death of spores. Some vegetables afforded more protection than others for putrefactive anaerobe N.C.A. 3679 when pH was controlled (Sognefest *et al.*, 1948).

These experiments were instituted to determine the effect of heating spores at their maximum activation temperatures, as determined in distilled water, in selected carbohydrate and plant extracts. The questions that this research poses are as follows: 1) Will an increase in sugar concentration have an effect on the spore during the heat-shock period? 2) Does the type of plant extract have an effect when the spore is heat-shocked in these extracts?

METHODS AND MATERIALS

Preparation of spore suspensions and heat activation. Strain M of *B. stearothermophilus* was used. The strain and procedures followed were as previously reported (Finley and Fields, 1962). Spores of strain M were heated 7 min at 110°C, heat-activation conditions resulting in maximum plate counts.

Preparation of carbohydrate solutions. Glucose, fructose, sucrose, and maltose solutions, having molarities of 0.001, 0.01 and 0.1, were prepared with distilled water. These were sterilized by passing through a Seitz filter. Nine ml of these solutions were pipetted aseptically into sterile test tubes with screw tops. By adding 1 ml of the appropriate spore suspensions, the number of unheated spores per ml was in a countable range (50 to 70) yet allowed for a threefold heat activation increase without subsequent dilutions.

Dextrin and starch solutions were prepared having the following percentages: 0.02, 0.2, and 2.0. These solutions were also sterilized by filtering through a Seitz filter, and were handled like the monosaccharides and disaccharides.

Preparation of plant extracts. In the preparation of green bean, corn, and spinach extracts, fresh products were used. Fresh peas were not immediately available, so the frozen product was substituted. Duplicate 100-g quantities of each were weighed, placed in 800-ml beakers, and covered with 400 ml of distilled water. Extraction was performed by refluxing at the boiling point on a heating apparatus commonly used for crude-fiber extractions (Laboratory Construction Company). Upon reaching the boiling point, one set of the duplicate preparations was extracted for 5 min and the other for 60 min. Extraneous materials were removed from the extracts by filtering them through No. 2 Whatman filter paper. Sterilization was achieved by refiltering the extracts through Seitz filters. The presence of starchy materials made this latter filtration a lengthy process, and in the case of the corn extracts the filter pads had to be replaced 3 or 4 times before sufficient quantities were obtained. Following filtrations, the collected filtrates were coded and stored under refrigeration (4°C) until use, on the next day.

Enumeration of spores. The number of germinated spores was determined by plating 1-ml quantities of the suspending menstrua with dextrose tryptone agar (Difco Laboratories, Inc., Detroit, Mich.). Triplicate plates were poured for each treatment, and all plates were incubated 48 hr at 52°C.

Analysis of data. The data reported are from a series of experiments performed over several weeks. The data are grouped according to suspension. Originally, it was our intention to use suspensions as duplicates, but bacteriological analysis of these suspensions indicated that heat-induced dormancy was greater in the second suspension (Finley and Fields, 1962). Also, there was a greater percentage of rough variants in the second suspension of strain M (Fields, 1963). For these reasons and since the treatments within suspensions were replicated, each statistical analysis is based upon one suspension.

In the design of these experiments the heat activation conditions resulting in maximum plate counts in distilled water were used as the basis for comparisons, and spores of each suspension were heat-shocked in distilled water in each run made.

An analysis of variance was computed on the data of each experiment, and the standard error of the mean was computed. The D value of Tukey, as modified by Snedecor, was calculated for each experiment for determining significance (Snedecor, 1957). Also, for comparisons between sugars and plant extracts, which were not done at the same time, the plate counts were calculated as percent of distilled water control.

Summary tables were prepared to show trends in the data. These tables were prepared by tabulating the numbers of comparisons that were significantly higher, lower, or not significantly different between treatment and distilled-water (control) plate-count means. Each concentration of carbohydrates or plant extract was compared with the distilled-water control plate count of that particular run.

RESULTS

Effect of monosaccharides and disaccharides. Table 1 gives data on the effect of glucose, fruc-

Table 1. Effect of selected monosaccharides and disaccharides on heat activation of *B. stearothermophilus* spores strain M.

Concentration of carbohydrate	Type of carbohydrate							
	Glucose		Fructose		Sucrose		Maltose	
	Plate count[a]	% of control	Plate count[a]	% of control	Plate count[a]	% of control	Plate count[a]	% of control
Suspension 1								
Distilled-water control	215.3	100.0	199.8	100.0	186.3	100.0	186.0	100.0
.001*M*	205.2	95.3	173.0	86.6	183.7	98.6	184.8	99.4
.01*M*	194.8	90.5	145.5	72.8	163.0	87.5	183.2	98.5
.1*M*	160.3	74.5	152.8	76.5	148.7	79.8	184.8	99.4
	D = 19.7[b]		D = 16.8[b]		D = 16.0[b]		D = 13.1[b]	
Suspension 2								
Distilled-water control	233.0	100.0	234.0	100.0	240.0	100.0	248.7	100.0
.001*M*	261.2	112.1	228.2	97.5	240.2	100.1	249.8	100.4
.01*M*	208.2	89.4	192.0	82.1	204.0	85.0	202.5	81.4
.1*M*	177.0	76.0	156.2	66.8	129.3	53.9	205.7	82.7
	D = 33.6[b]		D = 27.5[b]		D = 26.2[b]		D = 23.0[b]	

[a] Mean of six replications.
[b] To be significant at 5% level, difference between means must be greater than D value.

tose, sucrose, and maltose on spores of strain M, suspension No. 1. Counts were significantly lower for the 0.01*M* and 0.1*M* levels of glucose than for the distilled-water control. The number of spores germinating decreased significantly after being heated in fructose at all levels of concentration. The spore counts also decreased with increased concentrations of sucrose in the heating menstrua, with the counts being significantly lower for 0.01*M* and 0.1*M* levels than for the distilled-water control. In maltose, however, there was no significant difference at any sugar level.

Activation responses of spores of strain M, suspension No. 2, are also given in Table 1. Control counts were significantly higher than the counts obtained at 0.01 and 0.1*M* levels of glucose. The plate count of the 0.001*M* solution was higher than the distilled-water control, though not significantly. Plate counts were also significantly lower when spores were heated in fructose, sucrose, and maltose at 0.01 and 0.1*M* levels.

Effect of polysaccharides. The influences of dextrin and starch on spores of suspension No. 1 are shown in Table 2. In dextrin, only the 2% level affected the spore counts significantly. In this case, the counts were significantly lower than the distilled-water control. The starch counts, however, were significantly lower at the 0.02 and 0.2% levels, whereas counts representing the 2.0% level were not significantly different from the distilled-water control.

The responses of spores of suspension No. 2 in starch and dextrin are shown in Table 2. The counts at 0.2 and 2.0% dextrin were significantly lower than the distilled-water control, whereas the plate count at 0.02% was not significantly different from the control. In starch, however, the plate count in the 0.02% starch was significantly higher than the control and the plate count at 2.0% was significantly lower than the control.

Effect of plant extracts. As shown in Table 3, only the spore count in the 5-min bean extract was significantly higher than the distilled-water control when first suspension strain M spores were heated. Plate counts of spores heat-shocked in peas, spinach, and corn extracts were significantly lower. The same relationships were encountered with the 60-min extracts. For suspension No. 2, also shown in Table 3, the response patterns were essentially the same. However, there was no significant difference between the spore counts in the 60-min spinach extract and the distilled-water control.

DISCUSSION

It was shown previously that strain M spores were activated to a high degree in distilled water and that there was little heat-induced dormancy (Finley and Fields, 1962). This fact would suggest that a certain portion of the strain M spores used in these experiments was very responsive to heat. As previously described, 50–70 spores in each run did not need a heat shock to stimulate germination while the heat-activated portion did. The spore population may be divided into two groups based on

Table 2. Effect of selected polysaccharides on heat activation of *B. stearothermophilus* spores of strain M.

Concentration of carbohydrate	Type of carbohydrate: Dextrin — Plate count [a]	Dextrin — % of control	Starch — Plate count [a]	Starch — % of control
Suspension 1				
Distilled-water control	176.7	100.0	188.8	100.0
0.02%	195.7	110.8	132.8	70.3
0.2%	187.5	106.1	119.5	63.3
2.0%	150.8	85.3	170.7	90.4
	D = 23.0 [b]		D = 23.0 [b]	
Suspension 2				
Distilled-water control	248.8	100.0	170.8	100.0
0.02%	268.2	107.8	226.0	132.3
0.2%	181.0	72.7	165.5	96.9
2.0%	110.3	44.3	120.7	70.7
	D = 23.8 [b]		D = 20.9 [b]	

[a] Mean of six replicates.
[b] To be significant at 5% level, difference between means must be greater than D value.

Table 3. Effect of selected plant extracts on heat activation of *B. stearothermophilus* spores of strain M.

Type of water extract	Extraction time: 5 min — Plate count [a]	5 min — % of control	60 min — Plate count [a]	60 min — % of control
Suspension 1				
Distilled-water control	121.3	100.0	121.3	100.0
Green beans	154.2	127.1	156.8	129.3
Peas	82.5	68.0	78.8	65.0
Spinach	74.2	61.2	91.3	75.3
Corn	53.5	44.1	65.8	54.2
Suspension 2				
Distilled-water control	196.6	100.0	196.6	100.0
Green beans	239.5	121.8	244.8	124.5
Spinach	153.8	78.2	201.2	102.3
Peas	116.5	59.2	124.2	63.1
Corn	105.0	53.4	138.0	70.2

[a] Mean of six replicates. To be significant at 5% level, difference between means must be greater than D value (21.4 for suspension 1, and 23.6 for suspension 2).

the need for heat to stimulate germination. The work of Fields (1963) also showed that each suspension was made up of rough and smooth variants. These spore populations may be divided, therefore, into another two fractions. The spore population was, therefore, a heterogeneous population. When the spores of strain M were heated in carbohydrates, counts were usually lower. These relationships are summarized for all experiments at 0.1*M* concentration in pure solutions of carbohydrates in Table 4. Ten of 12 determinations exhibited a significant decline in germination. Since suspension No. 2 was significantly lower for all carbohydrates only two times more than suspension No. 1, the higher concentration of the rough variant apparently did not have an over-all effect.

Table 4. Summary table showing the number of comparisons in which significant results were obtained when compared to distilled-water control.

Suspension	Lower[a]	Higher[a]	Not significantly changed[a]
1	4	0	2
2	6	0	0
Totals	10	0	2

[a] In 0.1*M* mono- and disaccharides and in 2.0% polysaccharides.

How, then, can one explain these results? As a working hypothesis, the decline in spore germination is attributed to osmotic pressure. The effects of osmotic pressure on spores in both homogeneous and heterogeneous populations need more research so as to elucidate this working hypothesis.

As shown in the plant-extracts data in Table 5, more comparisons were significantly lower than with the distilled-water controls. Here again, this is probably due to osmotic pressure. There were also higher counts in the plant extracts. The mechanism involving higher counts is not known.

Bretz and Hartsell (1959) introduced the term "osmosensitive" to describe the reaction of *Escherichia coli* cells (which succumb to dilution in ordinary buffer or distilled water) in remaining capable of producing colonies when suspended in sucrose solutions after having been frozen. The data presented here suggest that the term osmosensitive also applies to spores, but the term, as used here, means that certain levels of sugar, especially the 0.1*M* level, suppressed spore germination.

Apparently, only Curran (1931) has made a study of the effect of osmotic pressure on spores, and he did not study the effect of carbohydrates. Curran found that the germination of spores of *Bacillus mycoides* was the most rapid and complete at relatively low osmotic pressure. The fact that the spores in our study were conditioned to low osmotic pressure would probably explain the decreases in counts when heat-shocked in carbohydrate solutions.

Working with dormant spores of *Bacillus cereus* strain *terminalis*, Black *et al.* (1960) showed that glucose could be taken up by spores at pH 6.3 and 0°C, in 15-min exposure periods. In our studies, the spores were in contact with sugars for 30–40 min (including come-up times) at temperatures of 110°C. Whether the sugars were penetrating the spores or producing an effect upon the spore coat and membranes, is not known. The data reported herein show that spores produced as in this study may be affected significantly when they are heat-shocked in carbohydrate solutions.

Table 5. Summary table showing the number of comparisons in which significant results were obtained when compared to the distilled-water control. All plant extracts.

	5-min extract			60-min extract		
Suspension	Lower	Higher	Not significant	Lower	Higher	Not significant
1	3	1	0	3	1	0
2	3	1	0	2	1	1
Totals	6	2	0	5	2	1

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RESEARCH NOTE

Sensitization of Microorganisms to Radiation by Previous Ultrasonic Treatment

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The spoilage organisms of food and food products, comprising the sporulating as well as nonsporulating bacteria and molds and yeasts, show a wide range in radiation sensitivities (U. S. Army Quartermaster Corps, 1957). In general, the lethal effects of radiation on resistant organisms are observed with a radiation dose in the range of 2×10^6 rads, which causes loss of color and flavor, and destruction of vitamins of the food. Considerable attention is therefore focused on developing processes which minimize the objectionable side reactions arising from radiation sterilization of food products. The use of chemical compounds such as vitamin K[5] or its analogs has been suggested by Silverman, Shehata and Goldblith (1962) to increase the sensitivity of the organisms toward radiation. Kempe (1960) and Licciardello and Nickerson (1962) were able to sensitize spoilage microorganisms to heat by preirradiation. In this laboratory, it was observed that spoilage organisms in preirradiated orange juice became sensitized to heat treatment (Dharkar, 1964). This combination treatment eliminates the defects of sterilization due to high radiation dose (8×10^5 rads) and offers the advantage of sterilizing at a lower radiation dose (4×10^5 rads), thereby minimizing the radiation damage to the product.

This communication presents some observations which demonstrate sensitization of microorganisms by combination of ultrasonic waves and ionizing radiations.

Two organisms, *Micrococus radiodurans* (radiation-resistant) and *Streptococcus faecalis* (relatively radiation-sensitive), were chosen as representatives of organisms which contaminate foods. These were routinely cultured in a complete medium containing tryptone, glucose, beef extract, and casamino acids (required for *M. radiodurans* only). The cultures were grown for 24 hr either on slants for *M. radiodurans* or in shake cultures for *S. faecalis*. The cells were washed twice in *M*/15 phosphate buffer at pH 7.0 and resuspended in the same buffer to give a final concentration of about 5×10^7/ml. Three-ml portions were placed in sealed ampoules and exposed to ultrasonic waves in a G.E. ultrasonic generator at 300 kilocycles. Since it was necessary to separate the lethal from the sensitizing effects, the experiments were performed with low transducer power of 10 watts, which did not affect the viability of the cells. It was observed that under these conditions the organisms were not lysed by longer exposures to ultrasonic waves (up to 60 minutes) or by maintaining the pH of the medium at 4.4 or 7.

Studies on the effect of combination treatment of ultrasonic waves and radiation were carried out as follows. The organisms were first treated for 20 min at 300 kilocycles and 10 watts of transducer power. Both the sonicated and nonsonicated cells (controls) were exposed to gamma radiation in a Co^{60} source at a dose rate of 1.8×10^5 rads/hour. Plating was done on tryptone, glucose, beef extract agar with 0.05% casamino acids for *M. radiodurans* (incubation temp. 30°C), whereas the same medium without casamino acids was used for *S. faecalis* (incubation temp. 37°C). Colonies developed after 72 and 48 hr, respectively. Figs. 1 and 2 depict the sensitizing action of the ultrasonic treatment on *M. radiodurans* and *S. faecalis*, as seen by the change of the slope of the survival curve. The experiments with *M. radiodurans* were repeated ten times on different days, whereas those with *S. faecalis* were done eight times, also on different days. The various points obtained were subjected to a regression analysis. As can be seen, the slopes of the regression lines are significantly different, since the D_0 value (90% mortal-

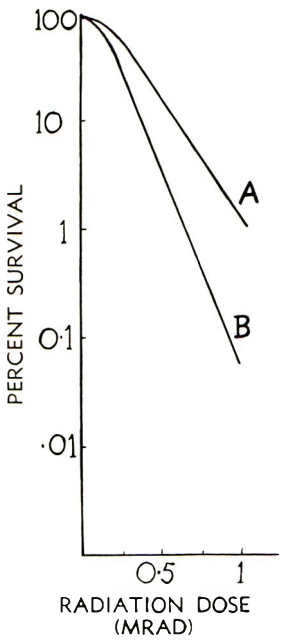

Fig. 1. Radiation sensitivity of *Micrococcus radiodurans*.

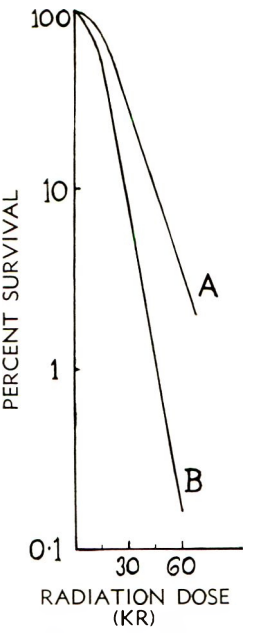

Fig. 2. Radiation sensitivity of *Streptococcus faecalis*.

ity) for *M. radiodurans* has fallen down from 5.8×10^5 rads to 2.9×10^5 rads, whereas that for *S. faecalis* shows a decrease from 37×10^3 to 22×10^3 rads. Tables 1 and 2 show the various points, together with the calculated standard deviation from the mean, which demonstrate that there is no overlapping of the points anywhere.

Application of the sonication-radiation method can be further amplified, by calculating the sterilizing dose, by the following equation (U. S. Army Quartermaster Corps, 1957).

$$D = D_0 (\log N_0 - \log N) + L,$$

where D is the sterilizing dose, D_0 is the 90% mortality dose, N_0 is the initial number of microorganisms per container (for example, 10^6), N is the number of organisms per

Table 1. Radiation sensitization effect of ultrasonic treatment.

Radiation dose	Percent survival without ultrasonic treatment	Standard deviation (±) from the mean	Percent survival with ultrasonic treatment	Standard deviation (±) from the mean
Micrococcus radiodurans (av. of 10 expts.)				
(rads $\times 10^5$)				
1.8	73	11.0	56.5	2.99
3.6	37	5.6	13.1	2.39
4.5	27	1.73	6.4	1.14
5.4	14.5	1.75		
7.2	9.2	1.5	0.82	0.25
10.8	1.55	0.8	0.058	0.013
Streptococcus faccalis (av. of 8 expts.)				
(rads $\times 10^3$)				
15	87	5.66	55.7	7.7
30	30	3.46	12.3	2.4
60	3.64	0.54	0.385	0.84
90			0.0111	0.0028

container after irradiation (for example, 10^{-4}) and L is the lag value (1.08×10^5 rads from Fig. 1):

$$D = (5.8 \times 10^5)\,[6 - (-4)] + (1.08 \times 10^5)$$
$$= 5.91 \times 10^6 \text{ rads.}$$

This would be the sterilizing dose for radiation alone. For the combination treatment the sterilizing dose works out to be 3.01×10^6 rads, which is half of the above value. This marked effect of ultrasonic treatment in enhancing the radiation sensitivity of microorganisms points out its practical value in radiation sterilization of liquid foods such as fruit juices, milk, etc.

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Anthocyanins. V. The Influence of Anthocyanins and Related Compounds on Glucose Oxidation By Bacteria[a]

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SUMMARY

The influence of 11 anthocyanin pigments on glucose oxidation by resting cells of *Escherichia coli* K-12, *Staphylococcus aureus*, *Lactobacillus casei* 7469, *Salmonella enteritidis*, *S. typhosa*, *Proteus vulgaris*, and *Aerobacter aerogenes* was determined with a manometric technique. Malvidin, delphinidin, and the monoglucosides of malvidin, pelargonidin, petunidin, and delphinidin generally inhibited glucose oxidation by the test organisms, but in the absence of glucose the organisms oxidized the pigments. The action of the anthocyanins on respiration varied with pH. Apigeninidin chloride, 5-desoxy-apigeninidin-chloride-4′-methyl-ether, and 5-desoxy-3′-methoxy-apigeninidin-chloride-4′-methyl-ether inhibited glucose oxidation by *S. aureus*. The latter two compounds and 3′-methoxy-apigeninidin-chloride-4′-methyl-ether had a similar effect on *E. coli*. The depressing effect of glycosidic anthocyanins on the respiration of *S. aureus* was reversed by the addition of MgCl$_2$.

INTRODUCTION

Determination of the action of anthocyanin pigments toward bacteria has to date been confined to methods which demonstrate either bactericidal activity or an influence on the ability of microorganisms to reproduce. A study was made of the effect of anthocyanin pigments on the respiration of microorganisms in the presence and absence of glucose.

REVIEW OF LITERATURE

Masquelier and Jensen (1953a,b) were the first to show that an anthocyanin compound may affect microbial activity. They demonstrated that a partially demethoxylated malvidin was bactericidal toward *E. coli* by a procedure which was essentially a Phenol Coefficient method (Jensen, 1954). In this laboratory, Zimmerman (1957), Hartman (1959), Pratt *et al.* (1960), Powers *et al.* (1960), Hamdy *et al.* (1961), and Somaatmadja (1962) have established that various anthocyanin compounds influence multiplication by five genera of bacteria. Turbidimetric, plate count, and sensitivity-disc methods were used—all of which hinge upon the ability of microorganisms to reproduce. The literature relative to the action of anthocyanins toward microorganisms was reviewed by Pratt *et al.* (1960), Powers *et al.* (1960), and Hamdy *et al.* (1961). Among relevant investigations are those of Huddleson *et al.* (1944), who examined plants for antibacterial substance. Gardner (1953) attributed antibacterial activity to components of wine other than alcohol. Remlinger and Bailly (1937) reported that wine was inhibitory toward dysentery-producing organisms.

EXPERIMENTAL PROCEDURE

Natural and synthetic anthocyanin pigments. The natural anthocyanin pigments used were malvidin, malvidin-3-monoglucoside, delphinidin, delphinidin-3-monoglucoside, petunidin-3-monoglucoside, and pelargonidin-3-monoglucoside. Pelargonidin-3-monoglucoside was isolated from strawberries as described by Pratt *et al.* (1960); the other compounds were isolated from Cabernet Sauvignon grapes as described by Powers *et al.* (1960).

Five synthetic apigeninidin compounds, supplied by Hoffman-La Roche, Inc., were used. They were: apigeninidin-chloride, apigeninidin-chloride-4′-methyl-ether, 3′-methoxy-apigeninidin-chloride-4′-methyl-ether, 5-desoxy-apigeninidin-chloride-4′-methyl-ether, and 5-desoxy-3-methoxy-apigeninidin-chloride-4′-methyl-ether.

Bacterial procedures. The test organisms were *Escherichia coli*, *Staphylococcus aureus*, *Lactobacil-*

[a] Contribution from the College Experiment Station, University of Georgia, Athens, Georgia. Approved as Journal paper No. 301. Portions of the data are from a Ph.D. dissertation, University of Georgia, 1962, by the senior author.

lus casei, Salmonella typhosa, S. enteritidis, Proteus vulgaris, and *Aerobacter aerogenes. E. coli, S. aureus,* and *L. casei* cultures were maintained on tryptone-glucose-yeast-extract broth (Difco B2). *P. vulgaris* and *S. typhosa* cultures were maintained on brain-heart-infusion broth (Difco B37), and *S. enteritidis* and *A. aerogenes* were cultured in nutrient broth (Difco B3).

For the manometric studies the test organisms were grown for 18–24 hr. The cells were harvested by centrifugation for 10 min at 10,000 rpm and then washed 3 times with 0.85% saline solution. The washed cells were suspended in saline and their weight per ml was determined gravimetrically. One ml of the cell suspension was pipetted into each of several 5-ml test tubes, the tubes were centrifuged, the supernatant discarded, and the precipitate (the resting cells) immediately frozen at −25°C. The frozen cells were resuspended at a concentration of 1 mg/ml in saline solution at the time of use. Originally, comparisons were made of cells used within a few hours after washing, after freeze-drying, and after freezing as described above. Fresh cells were slightly more active than frozen or freeze-dried cells but also more variable as judged by replicate determinations. Frozen cells were as active as freeze-dried cells and easier to prepare. Provided the temperature of storage did not fluctuate, frozen cells were the most satisfactory in terms of activity and uniformity.

The technique described by Umbreit *et al.* (1957) to measure oxygen uptake was followed. Into the main well of the flask were added 1 ml of potassium phosphate buffer and 1 ml of the cell suspension (1 mg cells). The potassium phosphate buffer was adjusted to pH levels ranging from 3.5 to 7.0. The pigments to be tested (0.1, 0.2, 0.4, or 0.6 ml of the 0.01*M*) were placed in the side well together with the substrate (0.2 ml of 0.01*M* glucose). CO_2 was absorbed with strips of filter paper saturated with KOH. The flasks were incubated in a water bath of 37°C with constant stirring. The oxygen uptake was measured at 10-min intervals.

To study whether the anthocyanins have the ability to chelate metallic ions and thereby stimulate or inhibit glucose oxidation, the pigments were mixed with $MgCl_2$ in different molar ratios at various pH levels. The pigment-magnesium mixtures were allowed to react for 1 hr before they were introduced into the reaction mixtures in the manometric flasks. The oxygen-uptake measurements were conducted as above. Sensitivity-disc measurements were also used to demonstrate the action of $MgCl_2$ and $CaCl_2$ on growth of the test organisms. The sensitivity-disc procedure followed was that described by Powers *et al.* (1960).

RESULTS AND DISCUSSION

Table 1 shows the effects of 4 glycosidic anthocyanins and 5 aglycones on the respiration of *E. coli* and *S. aureus.* All of the compounds depressed respiration below that with substrates containing glucose alone. The action of malvidin-3-monoglucoside and malvidin is depicted in Fig. 1. The monoglucoside depressed respiration moderately; the aglucone was slightly more effective.

When glucose was not present in the substrate, *E. coli* and *S. aureus* had the ability to oxidize the monoglucosides of pelargonidin, petunidin, delphinidin, and malvidin (Table 2). The action of the aglycones was variable. At the 2μM level, *S. aureus* was able to utilize malvidin and the synthetic compound apigeninidin-chloride.

In general, *P. vulgaris, A. aerogenes,* and *L. casei* were affected by malvidin-3-monoglucoside in the same manner as *E. coli* and *S. aureus.* This is shown in Table 3. With these organisms, comparisons were made at pH levels from 4.5 to 7.0. Respiration of *P. vulgaris* and *A. aerogenes* was depressed at the 3 pH levels, but at pH 4.5, *L. casei* consumed more oxygen in the presence of the pigment than in its absence.

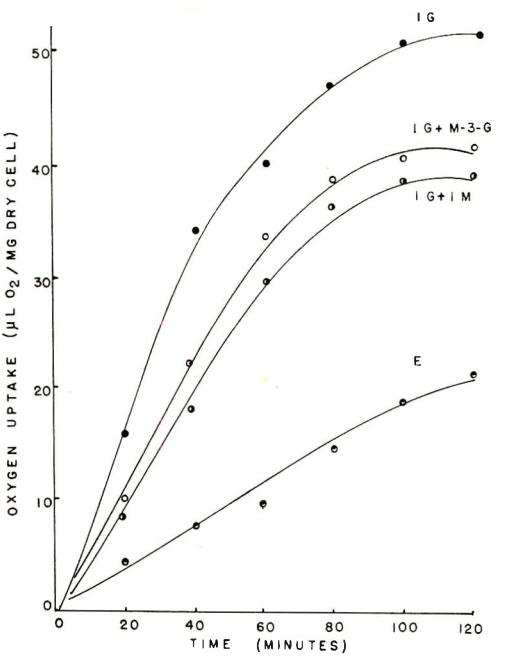

Fig. 1. Influence of malvidin-3-monoglucoside and malvidin on glucose oxidation by *E. coli* at pH 7.0.

Table 1. Influence of anthocyanins and selected synthetic compounds on the oxidation of glucose by 1 mg resting cells of *E. coli* and *S. aureus* at pH 7.0.

	μL O_2 utilized after incubation (min)							
	E. coli				*S. aureus*			
Substrate	30	60	90	120	30	60	90	120
2 μ*M* glucose (G)	4	29	43	56	43	68	79	85
G + 1 μ*M* P-3-G	— 2	— 6	—11	—15	— 9	—12	—13	—17
G + 2 μ*M* P-3-G	— 3	—21	—17	—24	—22	—30	—33	—38
G + 1 μ*M* Pt-3-G	— 7	—13	—21	—20	— 8	— 8	—10	—12
G + 2 μ*M* Pt-3-G	— 9	—20	—29	—32	—16	—27	—31	—35
G + 1 μ*M* D-3-G	— 7	—17	—21	—20	— 1	—18	—21	—23
G + 2 μ*M* D-3-G	—10	—22	—32	—37	—12	—27	—35	—43
G + 4 μ*M* D-3-G	—11	—26	—40	—44	.....	.....	.....	.....
G + 1 μ*M* M-3-G	.....	.....	.....	.....	—22	—60	—60	—69
G + 2 μ*M* M-3-G	.....	.....	.....	.....	—29	—66	—70	—81
G + 1 μ*M* malvidin	.....	.....	.....	.....	—17	—13	—10	—11
G + 2 μ*M* malvidin	.....	.....	.....	.....	—30	—20	—30	—29
G + 1 μ*M* MAM	— 4	— 5	—10	— 8	.....	.....	.....	.....
G + 2 μ*M* MAM	—14	—25	—30	—29	.....	.....	.....	.....
G + 1 μ*M* DAM	— 6	—12	—18	—18	—20	—27	—34	—30
G + 2 μ*M* DAM	—10	—27	—28	—31	—37	—44	—48	—48
G + 1 μ*M* DMAM	—15	—31	—37	—29	—18	—28	—30	—35
G + 2 μ*M* DMAM	—19	—39	—45	—41	—31	—41	—47	—48
G + 1 μ*M* ApC	.....	.....	.....	.....	—34	—43	—50	—48
G + 2 μ*M* ApC	.....	.....	.....	.....	—30	—43	—48	—57

Negative values indicate a decrease in O_2 uptake from that with glucose alone.
P-3-G = pelargonidin-3-monoglucoside.
D-3-G = delphinidin-3-monoglucoside.
MAM = 3′-methoxy-apigeninidin-chloride-4′-methyl-ether.
DAM = 5′-desoxy-apigeninidin-chloride-4′-methyl-ether.
DMAM = 5′-desoxy-3′methoxy-apigeninidin-chloride-4′-methyl-ether.
ApC = apigeninidin-chloride.
Pt-3-G = petunidin-3-monoglucoside.
M-3-G = malvidin-3-monoglucoside.

When *P. vulgaris, A. aerogenes, S. enteritidis, S. typhosa,* and *L. casei* were incubated in substrates containing malvidin-3-monoglucoside but no glucose at pH 4.5, 5.6, and 7.0, the organisms utilized the pigment (Table 4) in the same manner as did *E. coli* and *S. aureus* (Table 2).

Figs. 2 and 3 show the action of the aglucones malvidin and delphinidin toward *S. enteritidis* and *S. typhosa*. In the presence of glucose, 2 and 4μ*M* of malvidin affected respiration only slightly, but at the level of 6μ*M* of malvidin, respiration was depressed. In the absence of glucose, respiration was considerably lower, but the organism was able to derive some energy from 2 and 6μ*M* of malvidin as compared with respiration in the endogenous substrate.

Toward *S. typhosa*, the action of delphinidin was more drastic. At levels of 2 and 4μ*M* of delphinidin respiration was lower than with glucose alone (Fig. 3). However, as was true for *S. enteritidis, S. typhosa* was able to utilize delphinidin in the absence of glucose.

Table 5 shows the effects of pH and malvidin-3-monoglucoside on respiration of these organisms. At pH 7.0 and 4.5, respiration of *S. enteritidis* was inhibited by 2 and 4μ*M*; at the other pH levels the action of the pigment was variable. Respiration of *S. typhosa* was inhibited in the range pH 4.0–7.0, but at pH 3.5 respiration was greater. It should be pointed out that respiration in the presence of glucose alone was slow at pH 3.5 (as would be expected); consequently, the error involved in measurement was greater, so this apparent increase in respiration should be evaluated with reservations. One interesting observation made throughout the

Table 2. Oxidation of anthocyanins and selected synthetic compounds in the absence of glucose by 1 mg resting cells of *E. coli* and *S. aureus* at pH 7.0.

	μL O_2 utilized after incubation (min)							
	E. coli				*S. aureus*			
Substrate	30	60	90	120	30	60	90	120
Endogenous	4	10	12	15	4	12	16	18
1 μ*M* P-3-G					+ 3	+ 2	+ 2	+ 6
2 μ*M* P-3-G	0	+ 5	+18	+25	+ 4	+ 8	+12	+16
2 μ*M* Pt-3-G					+10	+15	+22	+34
2 μ*M* D-3-G					+14	+19	+27	+34
1 μ*M* M-3-G	+ 6	+18	+20	+17				
2 μ*M* M-3-G					+11	+10	+12	+12
1 μ*M* malvidin					− 2	− 4	− 1	+10
2 μ*M* malvidin					+ 6	+ 8	+16	+30
2 μ*M* DAM	− 3	− 2	0	+ 3				
2 μ*M* ApC					+ 4	+ 5	+ 5	+ 8

P-3-G = pelargonidin-3-monoglucoside.
D-3-G = delphinidin-3-monoglucoside.
DAM = 5′-desoxy-apigeninidin-chloride-4′-methyl-ether.
Pt-3-G = petunidin-3-monoglucoside.
M-3-G = malvidin-3-monoglucoside.
ApC = apigeninidin-chloride.
Positive values indicate oxidation of pigment (values above those of endogenous).
Negative values indicate depression of endogenous by pigment.

investigation was that there was a tendency for the action of the anthocyanin compounds to be at a minimum at pH 5.6 as compared with other pH levels.

Table 6 shows the influence of delphinidin-3-monoglucoside on the respiration of *P. vulgaris*, *A. aerogenes*, *S. enteritidis*, and *S. typhosa* at pH 4.5–7.0. Toward *P. vulgaris*, *A. aerogenes*, and *S. enteritidis*, the anthocyanin was inhibitory. Toward *S. typhosa* the compound stimulated respiration at the

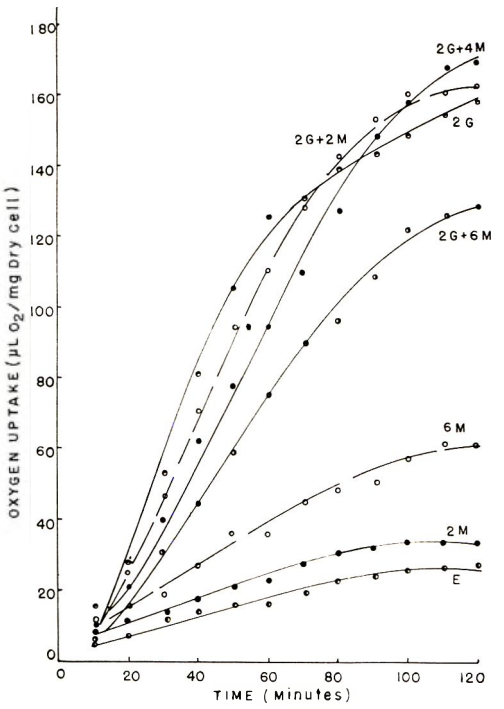

Fig. 2. Influence of malvidin on glucose oxidation by *S. enteritidis* at pH 7.0.

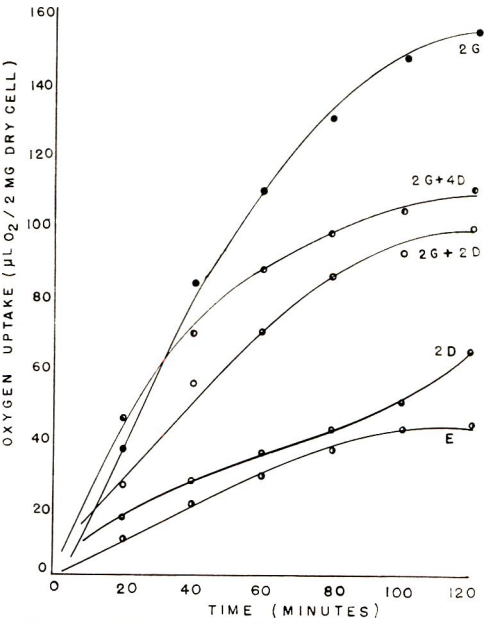

Fig. 3. Influence of delphinidin on glucose oxidation by *S. typhosa* at pH 7.0.

Table 3. Influence of malvidin-3-monoglucoside (M-3-G) on oxidation of glucose by 1 mg resting cells of *P. vulgaris*, *A. aerogenes*, and *L. casei* at different pH levels.

	μL O_2 utilized after incubation (min)											
	30			60			90			120		
Organisms/substrate	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0
P. vulgaris												
2 μ*M* glucose (G)	10	7	17	12	25	36	24	38	58	35	46	88
G + 2 μ*M* M-3-G	− 8	− 5	− 5	− 4	−14	−10	−12	−22	−17	−22	−28	−21
G + 4 μ*M* M-3-G	−12	−12	− 4	−11	−29	− 2	−21	−39	−10	−31	−40	−23
A. aerogenes												
2 μ*M* glucose (G)	26	39	34	64	84	62	97	135	97	118	178	132
G + 2 μ*M* M-3-G	+ 8	−13	−27	+ 4	−27	−38	− 4	−30	−62	− 5	−53	−77
G + 4 μ*M* M-3-G	− 1	−35	−33	−24	−67	−60	−42	−107	−95	−49	−138	−123
L. casei												
2 μ*M* glucose (G)	0	2	13	4	3	23	6	7	27	4	10	35
G + 2 μ*M* M-3-G	+ 5	− 3	−10	+ 4	0	−14	+ 3	− 2	−17	+ 4	− 2	−19
G + 4 μ*M* M-3-G	0	− 2	−12	+ 4	0	−19	+ 3	− 3	−19	+10	− 7	−26

Negative values indicate a decrease in O_2 uptake (inhibition), positive values indicate an increase in O_2 uptake (stimulation) over that with glucose alone.

Table 4. Oxidation of malvidin-3-monoglucoside (M-3-G) in the absence of glucose by 1 mg of resting cells of *P. vulgaris*, *A. aerogenes*, *S. enteritidis*, *L. casei*, and *S. typhosa* at different pH levels.

	μL O_2 utilized after incubation (min)											
	30			60			90			120		
Organisms/substrate	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0
P. vulgaris												
Endogenous	7	9	9	8	10	13	10	12	16	12	16	18
3 μM M-3-G	− 5	− 4	+ 9	− 4	0	+14	− 4	+ 1	+19	− 3	+ 4	+28
A. acrogenes												
Endogenous	4	5	5	10	8	10	15	12	16	17	14	18
2 μM M-3-G	+16	+22	+ 3	+29	+22	+10	+35	+32	+16	+38	+36	+29
4 μM M-3-G	+16	....	....	+20	....	....	+37	....	....	+34	....	....
S. enteritidis												
Endogenous	10	7	7	18	13	13	26	20	19	28	25	20
2 μM M-3-G	+ 5	+33	+28	+17	+65	+54	+20	+96	+89	+23	+123	+105
4 μM M-3-G	+10	+28	+25	+27	+50	+56	+35	+77	+96	+42	+104	+120
L. casei												
Endogenous	4	2	3	5	5	4	7	8	5	9	10	16
2 μM M-3-G	− 3	− 1	+ 4	+ 9	0	+ 9	+ 8	0	+16	+11	− 1	+ 9
4 μM M-3-G	− 1	− 2	− 2	+ 1	+ 5	0	0	+ 7	+ 2	+ 5	+ 8	− 7
S. typhosa												
Endogenous	6	5	5	11	11	13	17	16	16	21	17	17
2 μM M-3-G		+ 7	+ 9	....	+10	+10	....	+15	+15	....	+23	+22
4 μM M-3-G	− 1	+ 2	+ 2	− 1	+ 4	+ 2	− 2	+ 9	+10	− 3	+14	+13

Positive values indicate oxidation of pigment (values above those of endogenous).
Negative values indicate depression of endogenous by pigment.

Table 5. Influence of malvidin-3-monoglucoside (M-3-G) on oxidation of 2 μM glucose by 2 mg resting cells of *S. typhosa* and *S. enteritidis* at various pH levels.

Organisms/substrate	μL O_2 utilized after incubation (min)																			
	30					60					90					120				
	3.5	4.0	4.5	5.6	7.0	3.5	4.0	4.5	5.6	7.0	3.5	4.0	4.5	5.6	7.0	3.5	4.0	4.5	5.6	7.0
S. typhosa																				
2 μM glucose (G)	1	2	5	9	14	3	4	7	16	18	3	7	14	25	29	5	9	21	31	37
2G + 2 μM M-3-G	+5	−1	−5	−2	−7	+5	−3	+2	−2	−4	+5	−3	−5	−4	−8	+2	−3	−5	−6	−8
G + μM M-3-G	−1	−3	−4	−3	−5	+8	−1	0	−2	−15	+3	−5	−2	−5	−16	++3	−6	−9	−4	−17
S. enteritidis																				
2 μM glucose (G)	2	25	28	35	45	12	58	54	68	94	19	84	79	92	120	33	108	97	106	135
G + 2 μM M-3-G	+5	−2	−4	0	−20	+2	+2	−8	+2	−32	+6	−3	−14	+6	−21	0	−5	−10	+17	−18
G + 4 μM M-3-G	+9	+4	−11	−3	−21	+15	+3	−11	−1	−37	+23	+9	−17	+13	−31	+20	+13	−16	+23	−12

Negative values indicate a decrease in O_2 uptake (inhibition), positive values indicate an increase in O_2 uptake (stimulation) over that with glucose alone.

2μM level but had no effect at the 4μM level.

As between *S. enteritidis* and *S. typhosa*, the action of the two compounds, malvidin-3-monoglucoside and delphinidin-3-monoglucoside, was reversed. Delphinidin-3-monoglucoside inhibited the respiration of *S. enteritidis*, whereas malvidin-3-monoglucoside depressed the respiration of *S. typhosa* more.

In the absence of sugar, *P. vulgaris*, *A. aerogenes*, *S. enteritidis*, and *S. typhosa* utilized delphinidin-3-monoglucoside for energy (Table 7), as they did malvidin-3-monoglucoside (Table 4).

To learn whether chelation of metals might be involved in the action observed, a series of sensitivity discs were set up, with pigment added to some discs and pigment plus either $CaCl_2$ or $MgCl_2$ added to other discs. Fig. 4 shows the influence of the Ca

Fig. 4. Influence of addition of calcium and magnesium (1:1 molar ratio with pigment) on the activity of anthocyanin pigments toward the growth of *S. aureus* and *L. casei*.

or Mg salt in reversing the action of the pigment. The monoglucosides of delphinidin, petunidin, and malvidin inhibited growth, as has been reported in a previous publication (Powers *et al.*, 1960). When $CaCl_2$ or $MgCl_2$ was also added, the inhibitory action of the anthocyanin was reversed in whole or in part. As described by Powers *et al.* (1960) and Hamdy *et al.* (1961), there was a tendency for the zone of inhibition immediately surrounding the disc to be surrounded in turn by a zone of greater population density than that at locations well removed from the treatment discs. In prior publications this has been referred to as a zone of stimulation. It appears to the authors of those

Table 6. Influence of delphinidin-3-monoglucoside (D-3-G) on oxidation of glucose by 1 mg resting cells of *P. vulgaris*, *A. aerogenes*, *S. enteritidis*, and *S. typhosa* at various pH levels.

	μL O_2 utilized after incubation (min)											
	30			60			90			120		
Organisms/substrate	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0
P. vulgaris												
2 μ*M* glucose (G)	2	9	16	10	20	35	19	35	54	33	43	80
G + 2 μ*M* D-3-G	+ 4	− 2	− 9	+ 6	− 4	−12	+ 1	− 5	−12	− 1	−13	−17
G + 4 μ*M* D-3-G	+ 4	− 1	− 9	+ 2	− 2	−12	− 8	− 4	−19	−10	−12	−23
A. aerogenes												
2 μ*M* glucose (G)	26	50	43	60	105	83	95	156	122	111	203	151
G + 2 μ*M* D-3-G	− 5	− 9	−39	−10	− 4	−65	−21	+ 4	−89	−13	− 4	−119
G + 4 μ*M* D-3-G	+10	−17	−40	+10	−20	−73	− 1	−15	−107	−13	−18	−134
S. enteritidis												
2 μ*M* glucose	35	32	53	55	43	113	68	57	130	88	69	137
G + 2 μ*M* D-3-G	−18	− 2	−10	−31	0	−39	−42	− 5	−33	−43	− 4	−16
G + 4 μ*M* D-3-G	−23	−15	−23	−38	−19	−57	−53	−29	−35	−77	−33	−28
S. typhosa												
2 μ*M* glucose (G)	3	11	20	6	20	21	12	32	35	20	43	43
G + 2 μ*M* D-3-G	+ 2	+ 7	+ 2	− 1	+ 5	− 7	+ 3	+ 9	+ 1	+ 5	+13	+ 4
G + 4 μ*M* D-3-G	0	0	+ 6	− 2	+ 2	+ 3	+ 1	+ 2	+ 3	− 1	0	0

Negative values indicate a decrease in O_2 uptake (inhibition), positive values indicate an increase in O_2 uptake (stimulation) over that with glucose alone.

Table 7. Oxidation of delphinidin-3-monoglucoside (D-3-G) in the absence of glucose by 1 mg resting cells of *P. vulgaris*, *A. aerogenes*, *S. enteritidis*, and *S. typhosa* at various pH levels.

	μL O_2 utilized after incubation (min)											
	30			60			90			120		
Organisms/substrate	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0	4.5	5.6	7.0
P. vulgaris												
Endogenous	4	1	5	9	2	10	12	4	13	15	4	18
2 μ*M* D-3-G	+ 3	+ 2	+11	+ 6	+11	+23	+ 8	+32	+32	+18	+18	+41
4 μ*M* D-3-G	0	+ 6	+ 7	+ 1	+13	+18	0	+20	+29	+14	+33	+35
A. aerogenes												
Endogenous	3	3	4	8	12	13	16	15	17	19	18	20
2 μ*M* D-3-G	+18	+28	+12	+48	+72	+17	+71	+113	+13	+96	+154	+19
4 μ*M* D-3-G	+24	+34	+ 3	+58	+84	+18	+88	+10	+18	+104	+151	+21
S. enteritidis												
Endogenous	5	6	7	14	14	14	25	20	19	27	23	21
2 μ*M* D-3-G	+21	+23	+30	+24	+29	+53	+24	+37	+81	+22	+52	+105
4 μ*M* D-3-G	+ 8	....	....	+ 8	....	....	+ 6	....	....	+ 2	....	....
S. typhosa												
Endogenous	1	6	9	4	10	12	10	16	17	10	23	23
2 μ*M* D-3-G	+ 2	− 4	0	+ 3	− 5	+ 7	+ 3	− 5	+12	+ 8	−10	+12
4 μ*M* D-3-G	+ 2	0	+ 4	+ 2	+ 2	+12	− 1	+ 7	+18	+ 2	+ 7	+17

Positive values indicate oxidation of pigment (values above those of endogenous).
Negative values indicate depression of endogenous by pigment.

publications that the zone of greater colony density may merely be a zone wherein both the medium nutrients and small amounts of pigment and/or the Mg and Ca salts are being utilized by the organisms. This is borne out by some of the manometric results described above. Small amounts of anthocyanin sometimes increased respiration rather than depressed it, as did larger amounts of pigment. The concentration of anthocyanin falls off rapidly at points only a short distance from the sensitivity disc. The zone previously referred to as a zone of stimulation may merely be an example of the Arndt-Schutz rule (Townsend and Luckey, 1960), wherein stimulation results through utilization of compounds which are inhibitory in higher concentrations.

The sensitivity-disc observations reflect the growth of organisms, not merely respiration. To learn whether added $MgCl_2$ also affected respiration, manometric measurements were likewise made. The results of some of these experiments are shown in Fig. 5. When one mole of Mg per mole of malvidin-3-monoglucoside or of petunidin-3-monoglucoside was added, respiration was increased over that with the pigment alone. When 2 moles of Mg was added, respiration was increased even more. Both by the sensitivity-disc procedure—which reflects growth—and by the manometric method—which reflects respiration—$MgCl_2$ could offset in part the inhibition produced by malvidin-3-monoglucoside or petunidin-3-monoglucoside.

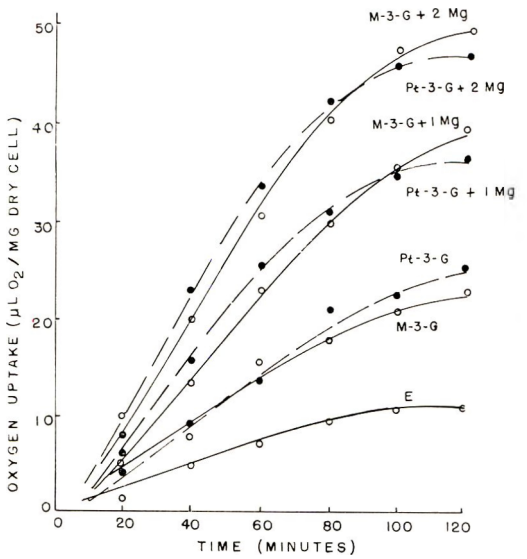

Fig. 5. The reversal of the inhibitory effect of malvidin-3-monoglucoside and petunidin-3-monoglucoside on oxidation of 2 μM glucose by *S. aureus* by magnesium at pH 4.5.

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Anthocyanins. VI. Chelation Studies on Anthocyanins and Other Related Compounds[a]

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SUMMARY

The metal-complexing properties of malvidin-3-monoglucoside, petunidin-3-monoglucoside, and 3′-methoxy-apigeninidin-chloride-4′-methyl-ether were studied polarographically and spectrophotometrieally to complement bacteriological findings previously made. The molar ratios at which malvidin-3-monoglucoside combined with cupric ions depended on the pH of the supporting electrolyte solutions. The molar ratio of cupric ion to malvidin-3-monoglucoside was 2:1 at pH 3.9; the molar ratios were 1:2 at pH 3.5 and 4.5, but these ratios were 1:1 at pH 5.0 and 6.0.

The absorption peaks of malvidin-3-monoglucoside were shifted to shorter wavelengths in the visible range but to longer wavelengths in the UV range upon addition of various concentrations of cupric ions. The absorption peaks of petunidin-3-monoglucoside were shifted to shorter wavelength in both the UV and the visible light ranges upon addition of various concentrations of cupric ions. The infrared absorbancies of 3′-methoxy-apigeninidin-chloride-4′-methyl-ether at 11 to 14.5 μ disappeared upon the addition of Ca and Mg ions.

INTRODUCTION

The capability of the flavone compound quercetin to chelate certain metallic ions has been reported by Clark and Geissman (1949), Heimann *et al.* (1953), and Detty *et al.* (1955). Anthocyanins differ structurally from flavones in that the former do not have a carbonyl group at the fourth carbon of the molecule (Bate-Smith, 1954).

Earlier papers from this laboratory (Pratt *et al.*, 1960; Powers *et al.*, 1960; Hamdy *et al.*, 1961) reported that anthocyanin pigments inhibited the growth of certain bacteria. Somaatmadja (1962) found that anthocyanin pigments as a class inhibited glucose oxidation of certain bacteria but that these pigments were oxidized when no glucose was available. Somaatmadja *et al.* (1964) found that inhibition of the growth of *S. aureus* by malvidin-3-monoglucoside and delphinidin-3-monoglucoside was reversed by the addition of Mg or Ca. The decrease in rate of glucose oxidation by *S. aureus* upon the addition of malvidin-3-monoglucoside and petunidin-3-monoglucoside was reversed by the addition of 1:1 and 1:2 molar ratios of Mg to pigment. The study reported here was made to investigate by polarographic and spectrophotometric means whether anthocyanins chelate with metals.

EXPERIMENTAL PROCEDURE

Anthocyanin pigments. Malvidin-3-monoglucoside and petunidin-3-monoglucoside were isolated from Cabernet Sauvignon grapes as described earlier (Pratt *et al.*, 1960; Powers *et al.*, 1960). Synthetic 3′-methoxy-apigeninidin-chloride-4′-methyl-ether was also used (Powers *et al.*, 1960).

Polarographic analysis. Aqueous solutions of $CuSO_4$ (0.01*M*), pigment (0.01*M*) and mixtures of $CuSO_4$ and pigments in different molar ratios were pipetted into 25 ml of supporting electrolyte solutions (0.2*M* citrate buffer of pH 3.5, 3.9, 4.5, 5.0, and 6.0). The mixtures were deaerated for 10 min with nitrogen gas. A Sargent-Heyrovsky Polarograph instrument, Model XII, was used.

The diminution of copper was measured by the difference in the height or disappearance of its wave. The formation of copper-pigment complex was indicated by the appearance of new polarographic waves with new half-wave potentials.

Spectrophotometric analysis. Aqueous solutions of anthocyanin pigment and $CuSO_4$ were mixed in

[a] Contribution from the College Experiment Station, University of Georgia, Athens, Georgia. Approved as Journal Paper No. 302. Portions of the data were taken from the Ph.D. dissertation, University of Georgia, 1962, of the senior author.

different molar ratios. The mixtures were allowed to stand for 1 hr and then transferred to HCl solution of pH 1.0. The spectral curves were determined with a Beckman DU spectrophotometer. The optical density readings were compared with the blank containing the same amount of $CuSO_4$ with respect to the mixture being investigated.

For infrared analysis, mixtures of $CaCl_2$ or $MgCl_2$ and anthocyanin pigment solutions in 1:1 molar ratios were dried with ground KBr. The KBr-pigment pellets were prepared as described by Powers *et al.* (1960). The infrared absorbancy curves were determined with a Beckman IR 5 spectrophotometer.

RESULTS AND DISCUSSION

Spectrophotometric analysis in the UV and visible spectra showed that the shifts of the absorption peaks of malvidin-3-monoglucoside upon the addition of 1:1 and 1:2 molar ratios of cupric ions to pigment (Fig. 1) were more pronounced in the UV region. A similar situation was observed with petunidin-3-monoglucoside (Fig. 2).

Infrared analysis of 3′-methoxy-apigeninidin-chloride-4′-methyl-ether (Fig. 3) showed that the addition of Ca or Mg resulted in the disappearance of absorbancy peaks at 11 to 14.5 μ. Somaatmadja (1962) found that substituting methoxyl groups into the 3rd and 4th carbon on the side ring and removing the OH at the 5th carbon of various synthetic apigeninidin chloride derivatives increased the number of absorbancy peaks in this region.

Polarographic studies of malvidin-3-monoglucoside established that this pigment produces two distinct waves (Figs. 4, 5, 6, 7). These results are in general agreement

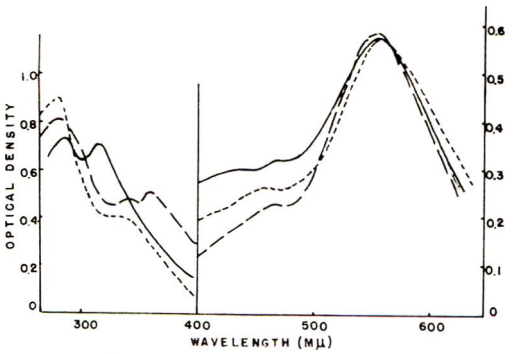


Fig. 1. Influence of copper on absorption characteristics of malvidin-3-monoglucoside (——— P = pigment; —— —— P + Cu (1:1); ..... P + Cu (2:1)).

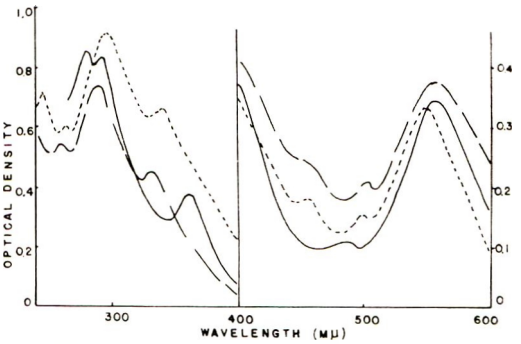


Fig. 2. Influence of copper on absorption characteristics of petunidin-3-monoglucoside (——— P = pigment; —— —— P + Cu (1:1); ..... P + Cu (2:1)).

with those of Zuman (1953). Kolthoff and Lingane (1952) reported that flavones gave two polarographic waves.

When malvidin-3-monoglucoside was mixed with cupric ions in different molar ratios in citrate buffer of pH 3.5, 3.9, 4.5, and 6.0, the diffusion currents of the anthocyanin and copper changed in height and new waves became evident as the end products of the reactions of copper and the pigment. Table 1 shows the values of the half-wave potentials of copper, malvidin-3-monoglucoside, and copper-pigment complex in solutions of different pH. Table 2 gives the values of the diffusion current in microamperes of the residual copper.

At pH 3.5 when one part of copper was polarographed with one part of pigment, one-half of the amount of copper was recovered, as evidenced by the diffusion current of the residual copper of 5.17 μa as compared with the original of 10.58 μa. Corresponding changes in diffusion current of the residual copper were obtained when two parts of copper and three parts of the pigments were polarographed. When one part of copper was polarographed with two parts of the pigment, all the copper was tied up. At this pH, copper and malvidin-3-monoglucoside formed a complex in a 1:2 molar ratio with a half-wave potential of −0.42 V. The polarograms of these reactions are shown in Fig. 4.

At pH 3.9, no diffusion current of the residual copper was found when one part of copper and one part of malvidin-3-monoglucoside were polarographed. Results were

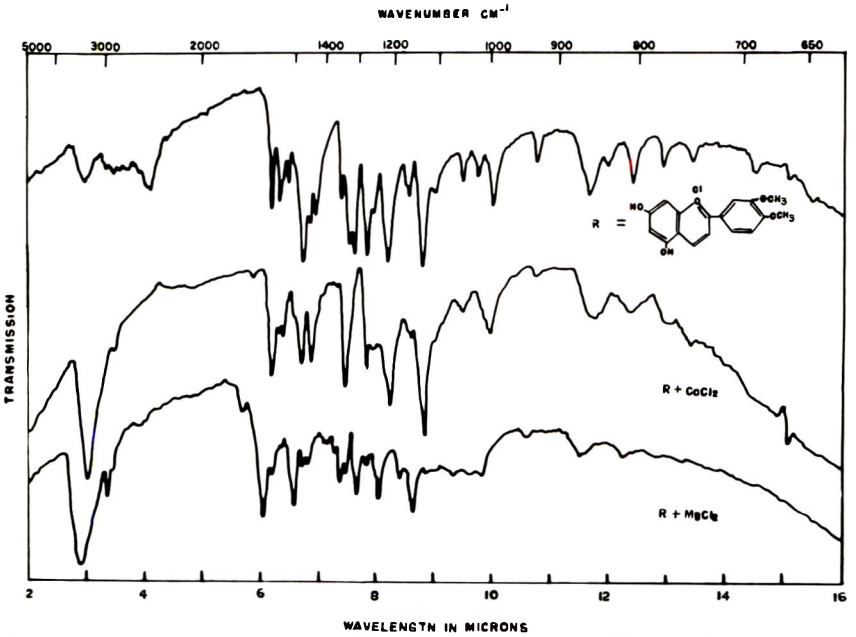


Fig. 3. Influence of calcium and magnesium on the infrared spectral curve of 5-desoxy-3'-methoxy-apigeninidin-chloride-4'-methyl-ether.

similar when two parts of copper were polarographed with one part or three parts of the pigment, indicating that all copper had been tied up into a copper-pigment complex. At pH 3.9, copper and malvidin-3-monoglucoside formed a complex in a 2:1 molar ratio. The half-wave potential of the copper-pigment complex was −0.43 V. The polarograms of the reactions are shown in Fig. 5.

Fig. 6 shows the polarograms of the reactions of copper and malvidin-3-monoglucoside at different molar ratios at pH 4.5. At this pH a diffusion current of 4.23 μa was observed when copper and the pigment were polarographed in a 1:1 molar ratio. When

Table 1. Half-wave potentials (volts) of copper, malvidin-3-monoglucoside, and copper-malvidin-3-monoglucoside complexes at different pH levels.

pH	Cu	M-3-P (P)	Cu:P 2:1	Cu:P 1:1	Cu:P 2:3	Cu:P 1:2
3.5	−0.344	−0.196		−0.197	−0.196	−0.200
		−0.247		−0.256	−0.316	−0.289
		−0.372		−0.336	−0.367	−0.360
				−0.440	−0.436	−0.400
3.9	−0.326	−0.219	−0.209	−0.196	−0.256	−0.261
		−0.330	−0.284	−0.335	−0.354	−0.354
			−0.447	−0.428	−0.423	−0.410
4.5	−0.312	−0.187	−0.229	−0.205	−0.200	−0.177
		−0.252	−0.327	−0.280	−0.322	−0.234
			−0.410	−0.322	−0.428	−0.415
				−0.400		
5.0	−0.302	−0.215	−0.247		−0.191	−0.200
		−0.317	−0.330		−0.293	−0.317
			−0.400		−0.428	−0.420
6.0	−0.279	−0.215	−0.260	−0.219		−0.238
		−0.279		−0.290		−0.272
						−0.320

Table 2. Diffusion current in micro-amperes of the residual copper at different pH levels.

pH	3.5	3.9	4.5	5.0	6.0
Cu	10.58	7.05	7.76	10.11	16.45
Cu : P (2 : 1)	....	none	8.46	11.76	15.67
Cu : P (1 : 1)	5.17	none	4.23	....	none
Cu : P (2 : 3)	4.70	none	4.70	none	....
Cu : P (1 : 2)	none	none	none	none	none

None, no residual copper. —, not determined.

two parts of copper and one part of the pigment were polarographed, the diffusion current of the residual copper was higher than that of the original (7.76 μa). This value indicated that more than one part of copper was still free. On the other hand, when two parts of copper were polarographed with three parts of the pigment the diffusion current of the residual copper was one-half of that of the original copper, indicating that half of the amount of copper was still free. When one part of copper was polarographed with two parts of the pigment no residual copper was recovered. These results indicate that at pH 4.5, copper and malvidin-3-monoglucoside react to form a complex in a molar ratio of 1 : 2; the half-wave potential of the complex at this pH was −0.41 V.

At pH 5.0, one part copper was recovered when two parts of this metal was mixed with one part of the pigment. The diffusion current of the residual copper (11.76 μa) was similar to that of the original (10.11 μa). On the other hand, when two parts of copper were reacted with three parts of pigment no residual copper was recovered. A similar result was observed when one part of copper was polarographed with two parts of pigment. This indicated that the reaction of copper with the pigment to form a copper-pigment complex was at a 1 : 1 molar ratio. The half-wave potential of the complex at this pH was −0.40 V.

Fig. 7 shows the polarograms of copper and the pigment reactions at pH 6.0. No residual copper was recovered when one part of this metal was polarographed with one part of pigment. Results were similar when one part of copper was reacted with two parts of pigment. One part of copper was still free when two parts of this metal were polarographed with one part of pigment. The residual current of copper was 15.67 μa as compared to the original of 16.45 μa. These results indicate that at pH 6.0, copper reacts with malvidin-3-monoglucoside in a 1 : 1 molar ratio to form a complex with a half-wave potential of −0.31 V.

The molar ratios of copper and pigment to react to form complexes varied with the pH of the supporting electrolyte solution. Clark and Geissman (1949) suggested that the chelatogenic sites of flavones are the 3rd and 4th dihydroxyls on the side ring and the 3-hydroxy-4-ketone group. Mehta and Sehadri (1958) suggested that the keto-enol tautomerism of the hydroxyl on the 3rd carbon and the carbonyl on the 4th were the major factors for the chelating action of the molecule of flavones.

Malvidin-3-monoglucoside does not contain a hydroxyl group at the 3rd carbon of

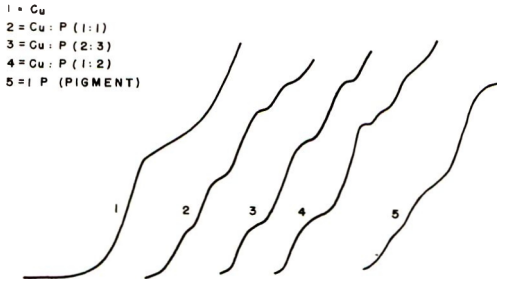


Fig. 4. Polarograms of copper and malvidin-3-monoglucoside at different molar ratios in citrate buffer of pH 3.5.

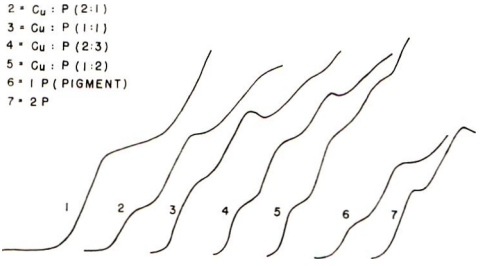


Fig. 5. Polarograms of copper and malvidin-3-monoglucoside at different molar ratios in citrate buffer of pH 3.9.

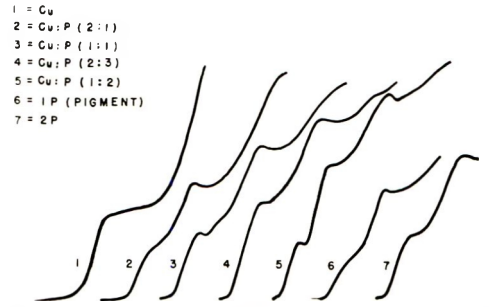
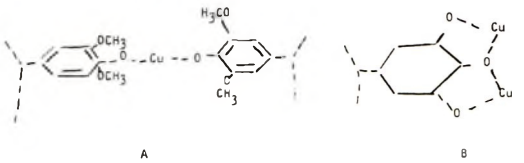


Fig. 6. Polarograms of copper and malvidin-3-monoglucoside at different molar ratios in citrate buffer of pH 4.5.

the side ring, and it does not contain a carbonyl group. It seems from the results of these studies that the reactive site of malvidin-3-monoglucoside to combine with cupric ions is in the methoxyl and the OH groups on the side ring or the OH groups at the 3rd, 5th, or 7th carbon of the molecule.

At pH 3.5 and 4.5 the molar ratios of copper to pigment to form a complex were 1:2. It is possible that the OH group at the 4th carbon of the side ring of one molecule of pigment and that of another react with one Cu, as shown in A. At pH 3.9 the molar ratio of copper to pigment was 2:1; the site of the reaction at this pH may be on the ring side, i.e., 2 Cu ions react with two methoxyls and OH as shown in B.



At pH 5.0 and 6.0, the molar ratios of copper and pigment to form a complex was 1:1. In this case it may be that the methoxyl groups acted as the chelatogenic site of the molecule.

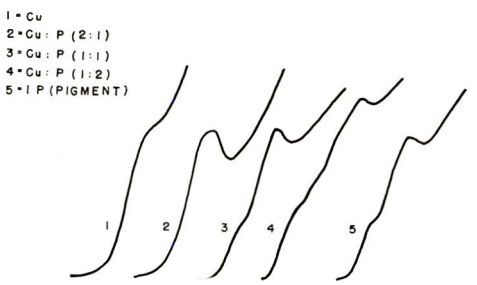


Fig. 7. Polarograms of copper and malvidin-3-monoglucoside at different molar ratios in citrate buffer of pH 6.0.

Methoxyl groups of the pigment molecule as chelating sites have not been previously reported. The results of these investigations indicated that OH groups alone or both methoxyl and OH groups may be the reactive site of malvidin-3-monoglucoside molecule, depending on the pH of the solution. The dependence on pH of the availability of the hydroxyl group to chelate metals was reported by Detty *et al.* (1955).

The chelating ability of malvidin-3-monoglucoside observed in this study may partially explain the mode of action of malvidin derivatives in inhibiting the growth and glucose oxidation of various test organisms.

Masquelier and Jensen (1953a,b) showed that partially-demethoxylated malvidin had bactericidal property. This may be accounted for by the presence of both OH and OCH_3 groups on the side ring of the pigment molecule. The fact that malvidin, malvidin-3-monoglucoside, and 5-desoxy-3′-methoxy-apigeninidin-chloride-4′-methyl-ether were more reactive in inhibiting the test organisms than any other pigments studied (Powers *et al.*, 1960; Somaatmadja *et al.*, 1963) gives further support to the role played by the methoxyl and hydroxyl groups as chelatogenic sites of the pigment molecule.

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The Prediction of Perceptibility of Luminous-Transmittance and Dominant Wave-Length Differences Among Red Wines by Spectrophotometric Measurements

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SUMMARY

A method of prediction of perceptibility of luminous-transmittance and dominant wave-length differences by spectrophotometric measurements is given for a small area of the color continuum with a specific product. Graphs are presented from which the dominant wave-length or luminous-transmittance differences necessary for discrimination are read directly, at various probability levels of success, for any dominant wave length or transmittance over the range of red wines.

INTRODUCTION

Based on the work of Munsell (1933), Lovibond (1890), Adams (1942), MacAdam (1943), and others, a number of methods have been developed for the prediction of perceptibility of color differences over the complete color continuum. These methods are usually used with reflectance measurements. However, in dealing with discrete areas of color under conditions where light is transmitted to the observer through a liquid, these methods do not yield acceptable results.

In preliminary studies (Ough and Berg, 1959) on the effect of different light sources on color differentiation in red wines, the ΔC values obtained from Davidson's (1951) charts correlated rather poorly with panel results. Because of this finding the present study was undertaken to determine if a more reliable prediction method could be established for red wines.

EXPERIMENTAL DETAILS

Test wines. Used as base wines were three wines which covered the gamut of red-wine dominant wave length and transmittance (Table 1). With these wines, blends were made to give a total of 5 reference wines spaced in dominant wave length and transmittance and purity over the red-wine range. Four blends were made daily to give the desired differences in dominant wave length or transmittance from the reference wine under study. With these reference wines of constant pigment composition, independent shifting of dominant wave length, transmittance, and purity by blending was not possible. Previous work on wines indicated that, from an observer viewpoint, transmittance and dominant wave length were the most characteristic attributes of wine. Under these circumstances the blends were studied with major reference to either dominant wave length or transmittance, although purity was calculated and considered in all cases. From 16 to 32 blends were made for each reference wine.

In the investigation of transmittance variation without dominant wave-length change, 3 reference wines covering the transmittance range of red wine were made by blending a concentrated red wine with a decolorized white wine. Since the pigment composition of the blending wine was constant, the blends fell on a line of increasing transmittance and decreasing purity. Since previous work (Ough and Berg, 1959) had shown that the major attribute considered by the observer

Table 1. Color characteristics of the base wines.

Wine type	General color description	x	y	$\%Y$	% purity	Dominant wave length (mμ)
Cabernet	Purple-red, dark	0.5538	0.3470	33.2	39.7	505.0$_c$
Burgundy	Red, dark	0.5397	0.3784	32.5	41.5	611.2
Rosé	Orange-red, light	0.4639	0.4082	87.8	11.7	592.0

was the change in transmittance, purity was not included in the treatment of the data. Twenty blends were used in the study of each reference wine.

All wines were filtered to a high degree of brilliance before use. Blends were made each day just prior to evaluation.

Physical color measurements. Transmittance measurements of the blends and the reference wines were made daily on a Beckman Model DU spectrophotometer. The percent transmittance was measured at 10-mμ intervals over the range of 400 to 700 mμ, through a 2-mm light path with water as the reference liquid. From these measurements the dominant wave length, percent purity, and luminous transmittance (%Y) were determined by means of the weighted ordinate method of Hardy (1948) for illuminant A.

With wines having only transmittance and purity differences, spectrophotometric readings at 420 and 520 mμ were used to determine %Y by the method outlined by Ough *et al.* (1962).

Subjects. Thirty subjects, of both sexes, ranging in age from the early twenties to the early sixties, were selected from the department staff. Some were familiar with judging wine color, others were not. All were tested with the Farnsworth-Munsell 100 Hue Test (1943) and the ISSC color aptitude test described by Dimmick (1956). The results of these tests indicated that the panel was a fair representation of the general population. Two of the male subjects were found to be deuteranoid. Neither case was extreme, and the subjects were retained on the panel.

Test conditions and methodology. Sample comparisons were made by using a viewing box under illuminant A (Holuk Lighting and Manufacturing Co., Model K-6 Color Comparison Grading Light Unit). Illuminant A was used because most wines are viewed and consumed under incandescent light. The light was positioned 37 inches above the samples at the top of the box, which was closed on three sides and the bottom. The box was 40 inches wide, 24 inches deep, and 40 inches high. The interior was painted a dull neutral gray (19% reflectance) to give a neutral, nonspecular surrounding. Illumination of sample area was checked with a photometer to ensure uniformity. Positioning marks for the sample glasses were spaced equally (2 inches) right and left of center in a parallel plane to the open face and an equal distance between back and front of the box. Samples were presented in 8-ounce, matched, tulip-shaped wine glasses. A measured amount (50 ml) of wine was placed in each glass. The subjects were instructed to place the glasses, one pair at a time, on the marks and make the comparisons without moving or tilting the glasses. No restriction was placed on the subject as to his position in reference to the glasses. The effect of extraneous light was minimized by sealing the windows and illuminating the room with subdued indirect incandescent light.

Four pairs of samples were presented to the subjects each day over a period of several months. One glass of each pair contained the reference wine and the other a blend. The presentation was forced choice, with complete randomization of coding, inter-, and intra-pair order. In lightness comparisons the subjects were requested to select the less light of each pair. In hue comparisons they were asked to select the more red, or orange or purple, depending on the dominant wave length area being tested. Lightness and hue comparisons were made on alternate days. The chromaticity diagram (Fig. 1) gives the variations that all the wines viewed in the 5 series would have had if viewed in a layer 2 mm thick.

Analysis of test data. *Transmittance.* Differences between the %Y of the reference wine and the test blends were plotted against the panel response as normal deviates for each reference group. By using the method of least squares, regression lines were determined showing a reasonably linear response by the panel to changes in %Y. Fig. 2 is an example of one of these plots. The slopes of these regression lines were then transformed from deviates /Δ%Y to [Δ log 1/%Y]/deviate in order to give linear functions. This result would be expected since response to changes in transmittance is generally a power function. Also, having the deviate at unity allows further plotting of the slope data with reference to the various constant stimuli. [The transformation could have been made prior to plotting; but, as is pointed out by Guilford (1954), there is little difference in the residual error term between making or not making the transformation before plotting. This was checked on one of the plots by making the transformation prior to plotting. No change was found in final slope value or residual error, indicating that the normal response law was not violated by plotting the non-transformed data.]

These transformed slope values were then plotted on log-linear paper in the form: $x = ab^{\Delta x}$

where $x = \log \frac{1}{\%Y}$

$\Delta x = \Delta \log \frac{1}{\%Y}$ (Δx in this case is the variation required for the panel to respond with a correct decision 84% of the time).

b = slope of the line, and a = the x intercept.

By using the above form, plots were made (Fig. 3) of the transmittance variations with

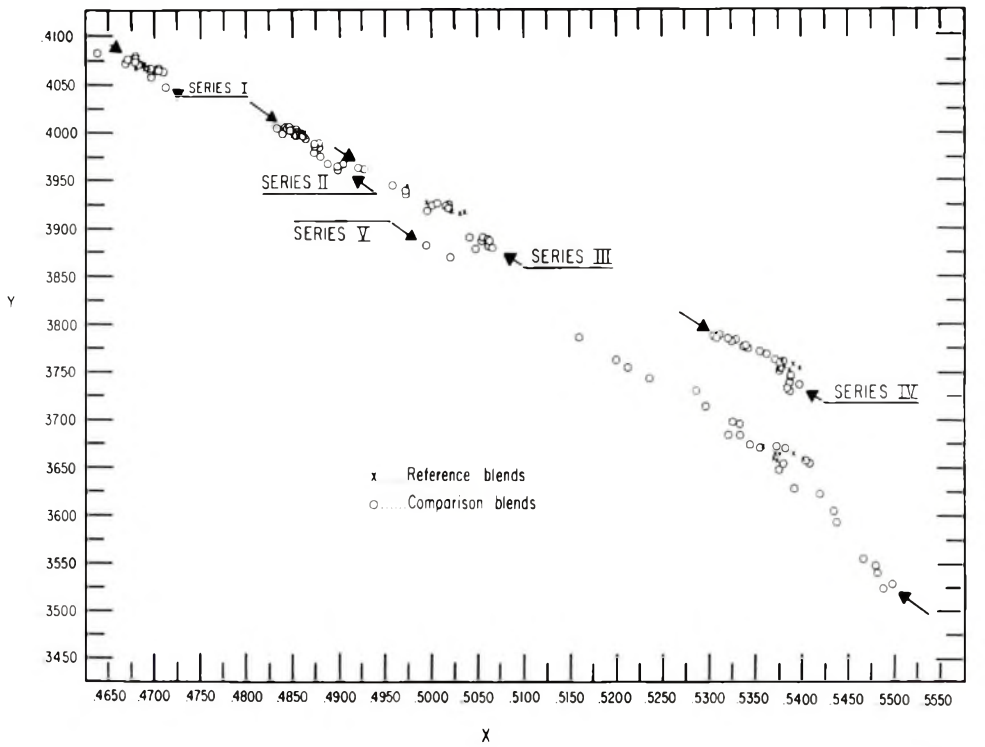

Fig. 1. Chromaticities of the 5 series blends viewed by illuminant A in a layer 2 mm thick.

dominant wave-length variations, and without dominant wave-length variations.

In order to obtain a linear relationship of the transmittance variations where dominant wave length also varied the data were replotted (Fig. 4) in the log-log form $\Delta x = k\ (x - x_t)^n$ where n is the slope of the line. The threshold value, x_t, was taken as zero since it was evident from the data that the value was so low as to be insignificant in the calculations. The degree of expectation of a correct decision is found from the deviate (in the case of a deviate of 1.0 the probability of a correct decision would be 0.84). The other probabilities are readily calculated from the initial regression lines.

Dominant wave length. By trial it was found that the $\sqrt{\Delta\text{dominant wave length}}$ gave the best linear relationship when plotted against the normal deviates of the hue responses of the subjects. Fig. 5 shows a typical example of one of these plots. The slopes of the regression lines were transformed from deviates/$\sqrt{\Delta\text{dominant wave length}}$ to $\sqrt{\Delta\text{dominant wave length}}/\Delta$ deviate, and the transformed slope values plotted (Fig. 6) on log linear paper in the form

$$y = ab^x$$

where $y = \sqrt{\Delta\text{dominant wave length}}$ (Δdominant wave length is the minimum change in wave length which can be detected by a given percentage of the viewing population.)

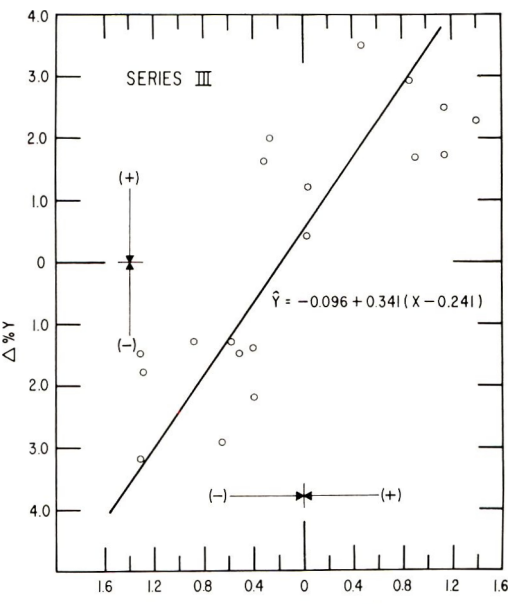

Fig. 2. Panel response to incremental transmittance differences for Series III.

x = the reference dominant wave length
b = the slope of the line
a = the intercept

RESULTS

Fig. 3 shows the effect of dominant-wave-length variations on the relationship of $\Delta\%Y$ to reference $\%Y$ (plotted as $\Delta \log 1/\%Y$ against $\log 1/\%Y$. Where the dominant wave length remains constant, the relationship is linear. This is in contrast to the curvilinear line, which shows the effect of dominant-wave-length variations. In this experiment the determined difference most nearly fits the Munsell value function $V = 10\ R^{1/2}$. The accuracy of the data derived from these observations would not warrant comparisons to the value functions of Godlove (1933) or those of the OSA subcommittee (Newhall *et al.*, 1943). The analysis of the data was made without reference to this function, but independent statistical analysis arrived at this function as the function of best fit.

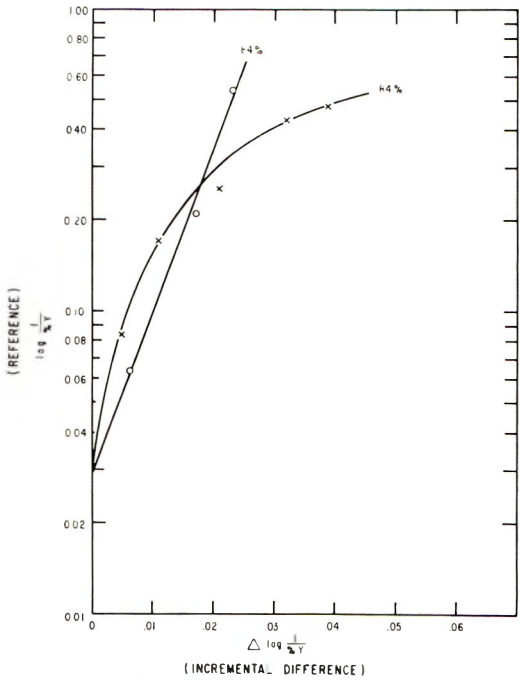

Fig. 3. Transmittance differences required at various transmittances for an 84% correct decision by the panel for constant dominant wave length and for varying dominant wave length. 0 constant dominant wave length, 504$_e$ mμ (refer to Table 2 for the tabulated values). X- covers the range from 594 to 635 mμ (refer to Tables 3 and 4 for the tabulated values).

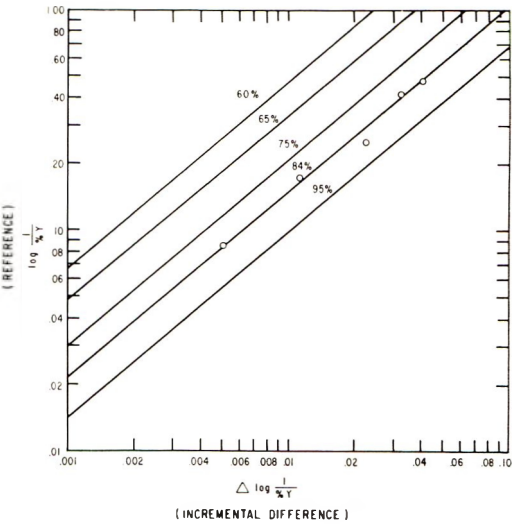

Fig. 4. Transmittance differences required over the range covered by the 5-blend series for various expected probabilities of a correct lightness decision.

Decreasing transmittance, as well as increasing dominant wave length, requires a greater $\Delta\%Y$ for a perceptual difference in lightness between two wines. This is evidenced by the straight line of Fig. 3 and the data of Table 2. As shown in Table 2, the reciprocal of the slope $(1/b)$ increases with increasing $\log 1/\%Y$.

A comparison of the data in Tables 2 and 3

Table 2. Regression equations of normal probability plots of $\Delta\%Y$ versus normal deviates with standard error terms. No dominant-wave-length variations.

Reference					
$\%Y$	$\log 1/\%Y$	Regression line	SE[a] (y)	SE[a] (b)	$1/b$[b]
28.6	0.544	$\hat{y} = 0.141 + 0.676$ $(x + 0.035)$	0.066	0.073	0.023
61.6	0.210	$\hat{y} = 0.103 + 0.395$ $(x - 0.242)$	0.095	0.050	0.017
86.3	0.064	$\hat{y} = 0.006 + 0.768$ $(x - 0.027)$	0.102	0.104	0.006

[a] Standard error.

[b] $1/b$ as $(\Delta \log \frac{1}{\%Y})$/deviate.

Table 3. Regression equations of normal probability plots of $\Delta\%Y$ versus normal deviates with standard error terms for wines of both dominant-wave-length and transmittance variations.

Series	Reference %Y	Reference log 1/%Y	Regression line	SE (y)	SE (b)	1/b[a]
V.	33.60	0.474	$\hat{y} = 0.0098 + 0.483 (x + 0.960)$	0.104	0.061	0.039
IV.	36.44	0.438	$\hat{y} = 0.162 + 0.358 (x + 0.031)$	0.144	0.091	0.032
III.	55.96	0.252	$\hat{y} = -0.096 + 0.341 (x - 0.241)$	0.113	0.057	0.022
II.	67.24	0.171	$\hat{y} = 0.060 + 0.513 (x - 0.230)$	0.118	0.098	0.011
I.	82.30	0.084	$\hat{y} = -0.0509 + 0.9525 (x + 0.439)$	0.157	0.177	0.005

[a] $1/b$ as $(\Delta \log \frac{1}{\%Y})$/deviate.

shows that increasing dominant wave length and decreasing transmittance changes are additive in their effect on $\Delta\%Y$. A $\%Y$ value of 28.6 in Table 2 gives a $1/b$ value of 0.023, whereas a $\%Y$ value of 33.60 in Table 3 gives a $1/b$ value of 0.039. Further evidence for the interaction between dominant wave length and transmittance is given by the plots of Figs. 3 and 4. The straight-line data of Fig. 3 replotted on log-log paper gave a slope value of 0.65, compared to 1.16 for Fig. 4.

Table 4 and Fig. 6 show the striking effect of increasing dominant wave length on the perceptual discriminatory ability of the panel members. As shown in Table 4, the reciprocal of the slope $(1/b)$ increases with increasing dominant wave length, becoming very large at the longer wave lengths. This effect becomes so pronounced in the deep red that the linear relationship shown in Fig. 6 probably should not be extrapolated beyond 635 mμ.

In lightness comparisons toward the purple end of the dominant-wave-length range the phenomenon of lightness reversal appeared. When a reference wine of 635.3 mμ dominant wave length was compared with wines of longer wave length and lesser transmittance, the latter were called lighter. This appears to be a critical area of the color spectrum as wines of lower dominant-wave-length and greater transmittance than this reference wine were correctly judged lighter. This phenomenon was not encountered with dominant-wave-length determination, for the panel's response to the hue question was as expected. This would tend to agree with Sanders and Wyszecki (1958) since in this area of the chromaticity diagram they show the L/Y response to change rapidly.

DISCUSSION

No attempt has been made to combine the brightness and hue observations to formulate a visual color difference prediction curve. This is believed to be unnecessary

Table 4. Regression equations of normal probability plots of $\sqrt{\Delta}$ dominant wave length versus normal deviates with standard error terms for wines of both dominant-wave-length and transmittance variations.

Series	Reference Dom WL (mμ)	Reference Purity (%)	Regression line	SE (y)	SE (b)	1/b[a]
I.	593.9	14.6	$\hat{y} = -0.079 + 0.908 (x - 0.132)$	0.114	0.130	1.101
II.	600.5	21.0	$\hat{y} = 0.194 + 0.707 (x - 0.338)$	0.064	0.072	1.414
III.	605.4	26.4	$\hat{y} = -0.904 + 0.656 (x - 0.501)$	0.120	0.098	1.524
IV.	611.4	40.8	$\hat{y} = -0.118 + 0.313 (x - 0.230)$	0.099	0.094	3.195
V.	635.3	33.8	$\hat{y} = -0.319 + 0.126 (x - 0.017)$	0.107	0.039	7.911

[a] 1/b as $\sqrt{\Delta \text{ dominant wave length}}$/deviate.

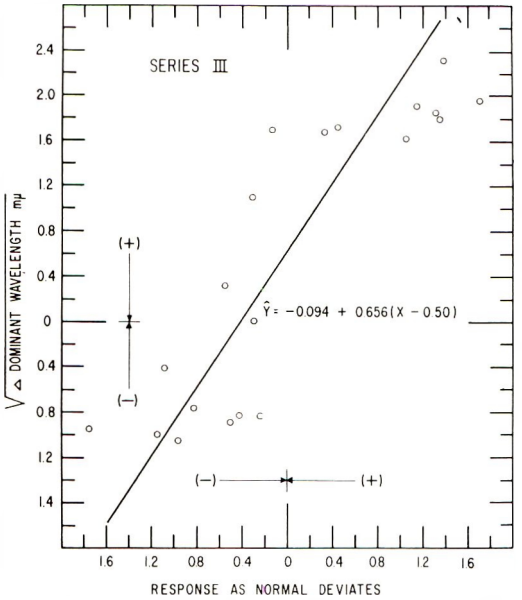

Fig. 5. Panel response to incremental dominant-wave-length differences for Series III.

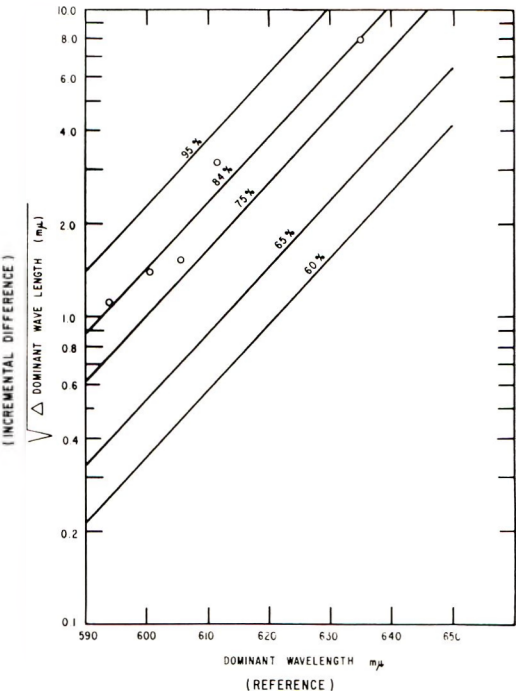

Fig. 6. Dominant-wave-length differences required over the range covered by the 5-blend series for various expected probabilities of a correct hue decision.

since the interaction effects of hue on lightness and lightness on hue are included in the plots of Figs. 4 and 6. Therefore it is felt that these graphs may be used to predict the various probabilities of a perceptual color difference when two wines are compared by spectrophotometric means even though the purity was not taken into account in the calculations. This is because, under the conditions of these experiments, the purity is determined when the dominant wave length and transmittance are fixed. Secondly, the judgments of color differences in wines appear to be largely dependent on the attributes of hue and lightness. Thirdly, for the range covered by red wines, the chromaticity parameters are such that they would preclude major changes in purity without corresponding changes in transmittance.

While only a very small and discrete portion of the color continuum was covered, there is no reason why this approach could not be applied to other areas where critical color evaluation is important.

ACKNOWLEDGMENTS

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Applications of the Modified Triangle Test in Sensory Difference Trials [a]

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SUMMARY

Experimenters involved with sensory difference tests have long used the triangle test as a basic procedure. In some circumstances, they have augmented the basic triangle test through obtaining "degree-of-difference" scores between the supposed odd sample and the pair of like samples. A companion paper gives a method of formally utilizing these scores in the analysis of these modified triangle tests when a computer is available. This paper summarizes the method and gives the results of applications of the modified triangle test to experimental data.

INTRODUCTION

In three earlier references (Bradley, 1958, 1963; Ura, 1960) a mathematical model was formulated that permitted theoretical associations and comparisons among sensory difference tests. This same mathematical model formed the basis for the development of a method of analysis for modified triangle tests by Bradley and Harmon (1964). This paper summarizes the modified triangle test procedure and provides additional illustrations of its use without going into the computational details.

The procedure for the modified triangle test is that the respondent first, in the usual way, selects the odd sample from three samples, one of which is a variant or odd and two of which are standard or like. Then the respondent has the additional assignment of scoring the degree of difference between the sample selected as odd and the remaining pair of samples through use of a scoring scale. Scoring scales may differ but a typical one would be as follows: 0, no difference; 2, very slight difference; 4, slight difference; 6, moderate difference; 8, large difference; 10, very large difference. It is clear that some respondents will be scoring correctly a measure of difference between the odd sample and the like pair, but it is also clear that some respondents will be scoring a difference between one of the like samples and a pair consisting of the odd sample and the other like sample. The modified triangle test procedure utilizes both the information on numbers of correct selections in triangle tests and the information contained in the degree-of-difference scores. While this utilization of the scores should increase test power, it also increases the computational problem so that an electronic computer is necessary.

ASSUMPTIONS

The modified triangle test is based on the assumption of a conceptual, sensory-difference, stimulus-response scale for sensory sensations of respondents in difference testing. It is assumed that responses are normally and independently distributed on the scale with homogeneous variances σ^2. Responses to the standard samples are taken to have mean zero without loss of generality, and responses to the variant samples are taken to have mean μ. Thus, in a single triangle test, responses x_1 and x_2 to the standard samples and response y to the variant sample are received by the respondent. It is assumed that the correct selection of the odd sample will result when x_1 and x_2 are closer together than y is to either x_1 or x_2 and that incorrect selection will result otherwise. When the odd

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sample is correctly selected, the score on degree of difference measures or approximates to $|y - \frac{1}{2}(x_1 + x_2)| = R$ and, when the odd sample is not correctly selected, the score on degree of difference measures either $|x_2 - \frac{1}{2}(y + x_2)| = W$ or $|x_2 - \frac{1}{2}(y + x_1)| = W$. When N triangle tests are conducted, observations $R_1, \ldots, R_m$ $W_1, \ldots, W_n$ are recorded, there being m correct judgments and n incorrect judgments in some order, $m + n = N$; m and n are random variables. Note that in the usual triangle test only m is recorded, and now we have the additional information in the R- and W-values.

On the basis of the assumptions above, a relationship has been developed between p_Δ the probability of correct selection of the odd sample, and $\theta = \mu/\sigma$, the standardized difference in means of odd and like populations. Table 1 briefly summarizes this relationship, taken from Bradley (1963). The table may be used for a preliminary estimate of θ based on a simply estimated value of p_Δ, namely m/N.

Table 1. The relationship between probability of correct selection and the standardized difference in means.

p_Δ	μ/σ	p_Δ	μ/σ	p_Δ	μ/σ
.3333	0	.6048	2.0	.8977	4.0
.3370	.2	.6437	2.2	.9137	4.2
.3478	.4	.6812	2.4	.9276	4.4
.3654	.6	.7169	2.6	.9396	4.6
.3891	.8	.7504	2.8	.9500	4.8
.4181	1.0	.7814	3.0	.9588	5.0
.4512	1.2	.8098	3.2	.9753	5.5
.4875	1.4	.8356	3.4	.9857	6.0
.5258	1.6	.8588	3.6	.9920	6.5
.5653	1.8	.8794	3.8	.9957	7.0

THE MODIFIED TRIANGLE TEST

Although the emphasis in this paper is on applications of the modified triangle test, a brief summary of the method is necessary. To consider applications fully, reference to Bradley and Harmon (1964) is necessary since difficulties may enter unless attention is given to indeterminancies that may arise.

The standard statistical method of maximum likelihood is applied to develop a likelihood-ratio test procedure. Based on the assumptions of the preceding section, appropriate conditional distributions of R and W were derived and included in a likelihood function, the natural logarithm of which is

$$\begin{aligned} lnL = {} & k - Nln\sigma - \left(\sum_1^m R_i^2 + \sum_1^n W_j^2\right)/3\sigma^2 \\ & - (4m + n)\theta^2/12 \\ & + \sum_1^m ln\ \cosh\ (2\theta\ R_i/3\sigma) + \\ & \sum_1^n ln\ \cosh\ (\theta W_j/3\sigma) \\ & + \sum_1^m ln\ I(\sqrt{2}R_i/3\sigma) + \sum_1^n ln\ [I_{+,j} + I_{-,j}] \end{aligned} \quad [1]$$

where

$$I(u) = \frac{1}{\sqrt{2\pi}} \int_0^u e^{-t^2/2}\, dt, \quad [2]$$

$$I_{+,j} = I[(\sqrt{2}W_j/3\sigma) + (\theta/\sqrt{2})], \quad [3]$$

$$I_{-,j} = I[(\sqrt{2}W_j/3\sigma) - (\theta/\sqrt{2})], \quad [4]$$

and

$$k = 2Nln2 - \tfrac{1}{2}Nln(3\pi).$$

The usual null hypothesis of the triangle test is $H_o: p_\Delta = \frac{1}{3}$ versus the alternative hypothesis, $H_a: p_\Delta > \frac{1}{3}$. These hypotheses remain for the modified triangle test but, in view of Table 1, may also be translated into hypotheses on θ. Thus we have

$$H_o: p_\Delta = \tfrac{1}{3},\ \theta = 0 \quad [5]$$

and

$$H_a: p_\Delta > \tfrac{1}{3},\ \theta \neq 0. \quad [6]$$

The appropriate statistic for the modified triangle test is

$$\chi_1^2 = -2ln\lambda = -2[\text{Max } lnL|_{H_o} - \text{Max } lnL|_{H_a}] \quad [7]$$

where Max $lnL|_{H_o}$ is obtained by finding $\bar{\sigma}$, the value of σ that maximizes lnL when $\theta = 0$, and substituting $\bar{\sigma}$ for σ and 0 for θ in Eq. 1. Similarly, Max $lnL|_{H_a}$ is obtained by finding $\hat{\theta}$ and $\hat{\sigma}$, values of θ and σ that maximize lnL in Eq. 1, and substituting these values appropriately in Eq. 1. The chi-square distribution with one degree of freedom applies to $-2\ ln\lambda$ (λ is the usual likelihood-ratio statistic) and this is indicated in Eq. 7; large values of χ_1^2 are significant, the critical

value determined by specification of a significance level α.

The only real difficulty in application of the modified triangle test is in the determination of $\bar{\sigma}$, $\hat{\sigma}$ and $\hat{\theta}$. This is done through an iterative procedure leading to solution of the likelihood equations for the maximization of lnL. These equations are too complicated for desk-calculator solutions but solutions are easily available from electronic calculators of modest size. The applications in this paper are based on IBM 709 calculations at the Florida State University. Details of the equations to be solved are given in Bradley and Harmon (1964), and that reference should be consulted in computer programming.

The method of maximum likelihood leads to an information matrix and thence to asymptotic variances and covariances of maximum-likelihood estimates. Through this theory it is possible to take $\sqrt{N}(\hat{\theta} - \theta)$ to be normal, with zero mean and a variance obtainable from the information matrix. In this way, confidence intervals on θ and thence on p_Δ (through use of Table 1) may be obtained. Again, details are given in Bradley and Harmon (1964), and examples are shown below.

EXPERIMENTAL DATA

Four sets of experimental data are shown in Table 2. The first three experiments involved use of the illustrative degree-of-difference scale of the Introduction. The scale used in the fourth experiment was a "confidence scale": 0, pure guess; 2, very doubtful; 4, somewhat doubtful; 6, almost sure; 8, confident; 10, absolutely confident. Experiment 2 is a comparison of baked goods, and the other experiments all involve control and experimental beverages.

In Table 2, values $\overline{R}$ and $\overline{W}$ are shown as average scores for triangle tests with correct and incorrect selections of the odd sample for each of the four experiments. As one would expect, $\overline{R}$ is larger than $\overline{W}$ in each case, but for the fourth experiment the small difference between $\overline{R}$ and $\overline{W}$ seems somewhat anomalous in view of the relative sizes of m and n. The fourth experiment uses the confidence scale, and this may suggest that this scale is less desirable than the degree-of-difference scale.

Table 2. Modified-triangle-test data from four experiments.

Expt. 1 (beverages)[a]				Expt. 2 (baked goods)[a]		Expt. 3 (beverages)[b]				Expt. 4 (beverages)[b]			
R		W		R	W	R		W		R		W	
2	6	4	4	8	2	4	2	2	2	6	10	8	8
2	4	2	2	4	2	4	4	2	2	2	2	4	4
2	4	4	4	10	4	2	2	6	4	0	0	8	4
4	2	2	2	4	4	6	6	2	2	10	6	2	6
6	4	2	6	4	6	2	2	4	4	6	6	6	4
2	2	4	4	4	4	4	2	8	2	8	4	6	4
4	8	6	6	2	2	4	6	2	6	8	2	2	2
2	8	2	6	4	6	3	2	2	2	8	8	4	2
6	4	2	4		2	4	4	2	2	0	2	8	4
6	0	2	2		2	8	10	2	4	8	4	2	2
2		4	2		8	6	4	6	1	8	6	8	8
2		4	4		6	6	6	0	2	4	2	4	
2		0	0		6	2	4	4	2	4	8	4	
2		2	2			8		4		4	6	4	
4		4				2		2		6	2	8	
$m = 25$ $n = 29$				$m = 8$ $n = 13$		$m = 28$ $n = 28$				$m = 30$ $n = 26$			
$\overline{R} = 3.60$ $\overline{W} = 3.17$				$\overline{R} = 5.00$ $\overline{W} = 4.15$		$\overline{R} = 4.25$ $\overline{W} = 2.96$				$\overline{R} = 5.00$ $\overline{W} = 4.85$			

[a] Data provided through the courtesy of Mr. Charles C. Beazley, Post Division, General Foods Corporation.

[b] Data provided through the courtesy of Mrs. Mavis B. Carroll, Technical Center, General Foods Corporation.

DATA ANALYSIS

To apply the modified triangle test, we obtain the maximum-likelihood estimates of θ and σ under alternative and null hypotheses. These values, $\hat{\theta}$, $\hat{\sigma}$, and $\tilde{\sigma}$, are given in Table 3 for each of the four experiments, together with values of χ_1^2 based on Eqs. 7 and 1. The probability that the observed value of χ_1^2 is exceeded under the null hypothesis is shown in order to assess the significance in the experiment. Note that three of the experiments lead to significance at the classical .05 level of significance but that the small set of data on the baked goods in Experiment 2 does not.

Estimates of p_Δ, the probability of correct choice of the odd sample in a triangle test, are given in Table 3; these values are obtained through use of Table 1 with that table entered with the values $\hat{\theta}$ and interpolations made for the corresponding values of $\hat{p}_\Delta$ ($\theta = \mu/\sigma$).

As indicated above, large-sample maximum-likelihood theory may be used to obtain confidence intervals on θ. This has been done, and the intervals are shown in Table 3. Table 1 may be used to convert the end points of the confidence intervals on θ into end points of the corresponding confidence intervals on p_Δ shown in the last line of Table 3. For these confidence intervals, a confidence coefficient of 0.95 has been used.

Table 4 permits comparison of the modified triangle test with the usual simple triangle test. The normal approximation to the binominal distribution depends on the statistic

$$u = \frac{m - \frac{1}{3}N \pm \frac{1}{2}}{\sqrt{2N/9}}, \qquad [8]$$

wherein the last numerator term is a continuity correction. The statistic u is taken to be standard normal, and large values of u lead to rejection of H_0 in Eq. 5 in favor of H_a in Eq. 6, the normal test being one-sided. Values of u for the four experiments are given in Table 4, together with the cor-

Table 3. Data analysis for the four experiments: modified triangle test.

Experiment	1	2	3	4
$\tilde{\sigma}$	2.3192	3.0179	2.5065	3.3738
$\hat{\theta}$	1.0931	0.8713	1.3261	1.3784
$\hat{\sigma}$	1.9598	2.6950	1.9867	2.6380
χ_1^2	4.0980	0.7307	9.1768	8.4712
Prob. of larger χ_1^2	.043	.393	.0024	.0036
$\hat{p}_\Delta$	.433	.399	.474	.483
.95 conf. int. on θ	(.427, 1.759)	(0, 2.097)[a]	(.767, 1.886)	(.802, 1.954)
.95 conf. int. on p_Δ	(.350, .557)	(.333, .624)[a]	(.385, .582)	(.389, .596)

[a] The lower limit for θ was negative, but the realistic lower limit must thence be zero corresponding to a lower limit of 1/3 for p_Δ.

Table 4. Data analysis for the four experiments: simple triangle test.

Experiment	1	2	3	4
m	25	8	28	30
N	54	21	56	56
u	1.876	0.231	2.504	3.071
Prob. of larger u	.030	.409	.0061	.0011
$\hat{p}_\Delta$	.463	.381	.500	.536
.95 conf. int. on p_Δ	(.333, .596)[a]	(.333, .589)[a]	(.369, .631)	(.405, .666)

[a] The lower limit obtained for p_Δ was less than 1/3, and 1/3 was used.

responding probabilities that the observed values of u be exceeded under H_0. The estimates of p_Δ in Table 4 may be compared with those of Table 3; in Table 4, $\hat{p}_\Delta = m/N$. It is seen that the two sets of estimates of p_Δ in the two tables agree quite closely.

The normal approximation to the binomial may be used to obtain confidence intervals on p_Δ for the simple triangle test. The approximate interval is

$$(\hat{p}_\Delta - 1.96\sqrt{\hat{p}_\Delta\,(1-\hat{p}_\Delta)/N},\ \hat{p}_\Delta + 1.96\sqrt{\hat{p}_\Delta\,(1-\hat{p}_\Delta)/N}) \qquad [9]$$

where a confidence coefficient of .95 is used. We have used Eq. 9 to yield the intervals in the last row of Table 4. It is seen that the confidence intervals in Table 3, based on the modified triangle test, tend to be shorter than those in Table 4, based on the simple triangle test.

DISCUSSION

The modified triangle test has been applied to four samples in this paper, to an additional similar example in Bradley and Harmon (1964), and to a simulated experiment consistent with the assumptions for the method, also in Bradley and Harmon (1964). General indications are that the modified triangle test should yield greater power for the detection of sensory differences and shorter confidence intervals for p_Δ than does the simple triangle test. For the simulated experiment, the modified triangle test appeared to lead adequately to reasonable estimates of the parameters μ and σ, known in that example.

Various questions arise in regard to the modified triangle test, and some of these can be answered only through extensive use. Note that the method may be used as an adjunct to the simple triangle test on a trial basis, as such tests are routinely conducted. It is believed that the procedure should be relatively insensitive to departures from the assumptions made and this conclusion is partially based on Bradley (1963). Usual questions arise in regard to the discreteness of the scoring scale for degree of difference but this should not lead to bias; an approach similar to that in Bradley *et al.* (1962) might be undertaken, although the mathematical problem appears to be difficult. Monte Carlo studies could be considered to check various properties of the modified triangle test, and such studies seem to hold forth the easiest method of further study.

The author would like to receive reports on the use of the modified triangle test. It is believed that many workers have used modified triangle tests before in the sense that degree-of-difference scores have been obtained. However, examples are difficult to find and we note only the paper by Mahoney *et al.* (1957).

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Evaluation of Toughness Differences in Turkeys

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SUMMARY

The reaction of a large untrained panel to turkey meat samples ranging from very tender to very tough was studied. Suitable samples were produced by varying the post-mortem aging time before freezing from 1 to 24 hr. Warner-Bratzler shear-force values were compared with toughness differences detectable by a small trained panel and with toughness complained of by the large panel. The small panel, using a triangle test, distinguished toughness differences in light meat that differed in shear resistance by 4 lb in a 9- to 22-lb range. Complaints about toughness by the large panel increased markedly when the light meat had shear resistance above 25 lb, and to some extent between 19 and 25 lb.

INTRODUCTION

The main purpose of this investigation was to find out at what quantitative level of toughness the consumer becomes dissatisfied with the tenderness of turkey meat. Numerous studies (Koonz *et al.*, 1954; Klose *et al.*, 1959, 1960, 1961a,b; Dodge and Stadelman, 1960) have shown that aging (or chilling) time and variations in processing can influence tenderness as measured by trained taste panels and objective shear measurements, but the results available do not tell us the toughness levels that will make consumers complain.

The problem is complicated by the fact that some birds become tender in the few hours before freezing that elapse in normal processing whereas others require longer aging. We therefore selected birds for test according to shear measurements, since previous results showed that this measurement correlates well with results with trained panels (Klose *et al.*, 1961a). Suitable samples were submitted to a large untrained panel consisting of laboratory personnel and their families.

METHODS

Turkey processing and shear force. One hundred broad white turkey hens 20 weeks old were slaughtered in a commercial plant, chilled in ice slush for 1, 4, 6, 8, or 24 hr, packaged in plastic bags, frozen in a blast freezer, and held at −10°F for 1 to 3 weeks before cooking. The average eviscerated weight (without giblets) was 10.3 lb. The day before cooking they were placed at 0°F. They were roasted from the frozen state at 325°F in a rotating electric oven, breast down, for two hours, then breast up until the temperature in the center of the thigh was 185°F. Total roasting time was 4½–5 hr; weight loss during cooking was 30–32%. After one hour at room temperature, the breast, thigh, and leg meat was removed.

Strips of meat one inch wide, parallel to the longitudinal grain, were removed from both sides of the outer breast muscle (pectoralis superficialis) and the outer left thigh muscle (biceps femoris) and tested on the Warner-Bratzler shear apparatus. The strips from the breast muscles were removed from an area approximately 1 inch from the anterior end. Thickness of the strip was that of the muscle unless thickness exceeded 1 inch. In the latter case the portion exceeding 1 inch was trimmed. The strips from the thigh muscle were the length that could be cut 1 inch wide and the thickness of the muscle, and were centered over the narrow end of the muscle. Six readings were made on each strip, and the remainder of the cooked meat from these parts was frozen in polyethylene bags and held at −10°F.

Description of shear resistance of meat tested. The shear resistance of the selected light meat ranged from 7 to 42 lb, and that of dark meat ranged from 4 to 13 lb. The influence of aging

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time on the distribution of samples within various ranges of shear values (in Table 1) illustrates the usually encountered relation between shear values and aging time (Klose *et al.*, 1959). None of the light meat from birds chilled 24 hr had a shear resistance of 19 or above; 15% of the meat from birds chilled 8 hr was in this range.

Dark meat from only 3 birds had shear resistance of more than 10 lb, and approximately 85% were in the 4- to 7-lb range. The shear values of light and dark meat are not comparable, since the light muscle was considerably thicker than the dark muscle. Since the left wing tips of all birds were clipped to hinder their flying, a comparison of right and left breast muscles was made to determine whether clipping affected shear values. No difference was found.

Procedure used for trained-panel tests. The trained panel compared samples of different shear values to determine differences discernible under typical laboratory conditions. Seven judges tasted samples of the outer and inner breast muscles, pectoralis superficialis and pectoralis secundus. The samples consisted of comparable slices cut perpendicular to the meat fibers either through both breast muscles or through a single muscle. Three coded samples were presented at a time, two samples of approximately equal shear value from different birds and the third from a bird of shear value differing from the other two. There were four replications of each test. The judges were members of a panel that has judged poultry tenderness for a number of years at this laboratory, and their evaluation of tenderness in previous studies had shown good correlation with measurements of shear force. They worked in booths designed to eliminate distracting influence. The statistical significance of the differences in the triangle tests was determined by the method of Davis and Hanson (1954) in order to obtain information from the partially correct as well as completely correct judgments.

Procedure used for untrained panel tests. Requests for volunteers for the untrained panel were sent to all members of the staff except those on the pretest panel and those in the poultry laboratory. The panel was not informed of the specific purpose of the study. The only information they received until after completion of the test was from the mimeographed sheet requesting volunteers to participate: "Each panel member . . . will be asked to give us his opinion about the flavor, moistness and tenderness of turkey. The information that you give will help us in determining ways to improve the quality of turkey meat." Those who agreed to participate completed forms showing their age, sex, and preference for light or dark meat. Of the 355 persons participating, 154 were on the staff. Of those who started the study, 339 completed it. The panel of 175 men and 180 women ranged in age from 16 to over 55. Students working during the summer raised the number in the 16- to 24-year age group (99). In the rest of the panel, 55 people were 25–34, 75 were 35–44, 76 were 45–54, and 50 were over 55. Thirty-nine percent of the panel preferred light meat, 31% preferred dark meat, and 30% had no preference. More women (45%) liked light meat than dark meat (26%). The preference of the men was about equally divided; 33% favored light meat and 35% dark meat. Ninety percent of the members reported that they eat turkey at least once every 6 months, and over half once every 3 months. The 154 regular staff members of the consumer panel were slightly but consistently more critical than the other 200 members, as might be expected (Table 2). However, since the difference is so small a panel of either composition would be expected to lead to essentially the same conclusion.

The meat to be evaluated by the untrained panel was thawed overnight at 35°F, sliced 1/10 in. thick across the grain on a slicing machine, and presented in sandwiches. The sandwiches were made with thin-sliced wheat sandwich bread, 6 g of oleomargarine, and 50 g of turkey meat. Each sandwich was placed in a plastic sandwich bag and sealed with a pressure-sensitive tape on which a code number identifying the sample was typed. Aproximately six sandwiches were made from each side of the breast, and 7 to 9 from the dark meat. The introductory letter, instructions, questionnaire,

Table 1. Shear-resistance values of samples selected for tests.

Aging time (hr)	Birds cooked (no.)	Distribution of samples in shear resistance ranges (%)					
		Shear ranges (lb)					
		Light meat				Dark meat	
		7–12	13–18	19–24	25+	4–6	7+
1	15	3	47	37	13	87	13
4	30	28	47	17	8	43	57
6	20	22	53	10	15	55	45
8	20	45	40	13	2	35	65
24	15	73	27	0	0	93	7

Table 2. Similarity of tenderness ratings by laboratory employees and other panelists.[a]

Shear resistance (lb)	Number of samples tested by:		Tenderness ratings of:	
	Employees	Others	Employees	Others[b]
Light meat				
7–12	91	138	3.2	3.1
13–18	90	162	3.5	3.2
19–24	48	92	3.8	3.4
25+	25	47	4.3	3.6
Dark meat				
4– 6	96	130	3.5	3.0
7–12	52	62	3.8	3.5
	402	631		

[a] "Employees" exclude trained panel and poultry laboratory members; "others" include families of staff members and students employed during vacation.
[b] 1 = extremely tender; 8 = extremely tough.

and sandwiches were pretested on 15 staff members, and changes were made on the basis of their comments.

Each person received three sandwiches, one each week for three weeks. They were randomly divided into nine groups. Three of the nine groups were used as controls to determine whether the general reaction varied from the first to the third samples. They received meat from within a single shear-force range. Nair (1949) reported that consumer panels tended to prefer the sample tasted first, whether foods were consumed at different times or side by side. Hanson *et al.* (1955) reported similar results, but Schwartz and Pratt (1956) reported that in consumer tests of soups compared simultaneously and after 1,- 3,- 7,- and 10-day intervals, panelists consistently preferred the second sample. The latter authors favored use of a series of samples over a single sample in determining consumer reaction.

The other six panel groups received meat of different shear resistance each week, allowing evaluation of the influence of toughness of the preceding sample on the scores for tenderness. The plan for all groups follows, with letters indicating shear range: A, low (7–12 lb); B, intermediate (13–18 lb); C, high (19+ lb).

The sandwiches were taken home to be eaten within 24 hr, with instructions explaining the procedure and a form (Fig. 1) for reporting reactions. Selection of the scales used on the form considered the report of Jones *et al.* (1955) that longer scales up to nine intervals, tend to be more sensitive to differences among foods and that elimination of the neutral category seems to be beneficial.

Plan for distribution of sandwiches

	Constant shear-force groups			Variable shear-force groups					
	1	2	3	4	5	6	7	8	9
1st week	A	B	C	A	A	B	B	C	C
2nd week	A	B	C	B	C	A	C	A	B
3rd week	A	B	C	C	B	C	A	B	A

RESULTS AND DISCUSSION

Shear-resistance differences detectable by trained panel. The trained panel detected differences in the tenderness of single turkey breast muscles differing by shear resistance of approximately 4 lb in the 9– to 22–lb shear range (Table 3). Samples with shear values greater than 22 lb were not tested by this panel. The tenderness difference was detected most readily in the outer breast muscle, pectoralis superficialis, the muscle on which shear-resistance determinations were made. Possible differences in rate of tenderization during aging may have resulted in different relative tenderness in the inner breast muscles, as reported by Koonz *et al.* (1954). Tenderness differences between the inner breast muscles smaller than between outer breast muscles would account for the greater difficulty in detecting tenderness differences between the inner breast muscles or between combinations of inner and outer muscles judged at the same time. Although the trained panel was not asked to comment on toughness, samples of shear resistance of 15 lb and above elicited voluntary toughness comments.

Table 3. Detection of tenderness difference in turkey breast meat of different shear resistance by small, trained panel.

Shear values compared[a] (lb)	Percent of times birds with lower shear resistance were judged more tender[b]		
	Both breast muscles	Pectoralis superficialis	Pectoralis secundus
9 vs. 16	83***		
10 vs. 16		74***	64*
11 vs. 17	55		
9 vs. 13		83***	48
11 vs. 15		81***	74***
14 vs. 18	62		
15 vs. 19 / 18 vs. 22		69**	60*

[a] Shear resistance determined on outer breast muscle, pectoralis superficialis.
[b] Statistical significance of triangle tests, 28 judgments per comparison except that 14 judgments in the two last comparisons were combined for analysis.

Nontenderness quality ratings by the untrained panel. The panel ratings for moistness, flavor, and over-all quality were high, as would be expected since the birds were raised and processed under optimum commercial conditions except for the chill time. The flavor ratings averaged approximately 3, "good," on a 6-point scale ranging from excellent to very poor. The ratings for moistness approximated 4, "slightly moist," on an 8-point scale ranging from "extremely moist" to "extremely dry." The over-all quality ratings for all samples averaged approximately 3, "like moderately," on an 8-point scale ranging from "like extremely" to "dislike extremely." These three ratings were unaffected by shear-resistance values (Table 3).

The tenderness ratings by the untrained panel. Average ratings for light meat ranged from 3.1, for that having shear resistance of 7–12 lb, to 3.8, for shear resistance of more than 25 lb (Table 4). Although these results show the expected increase in toughness with shear force, the average rating, 3.8, for samples with the highest shear force is within the range described as tender on the questionnaire. Ratings of 3 and 4 are respectively described as "moderately tender" and "slightly tender." However, the average rating may be misleading. Thirty-eight percent of the judges rated the samples in categories from "slightly tough" to "very tough."

In this test the panelists liked the first sample more than subsequent samples. This tendency was apparent to some extent in the average ratings given by the control groups who received three similar samples. Control groups rated successive light-meat samples from the same shear range increasingly tougher (Table 5). This was not true of the dark-meat samples. The adverse reaction to the second and third samples was readily apparent, however, in the toughness comments of the whole panel.

Table 5. Tendency for control groups to become increasingly critical of tenderness of light meat with successive samples.[a]

Shear resistance range (lb)	Samples tested per week (no.)	Mean weekly tenderness rating		
		1	2	3
7–12	26	3.2	3.2	3.8
13–18	27	2.8	3.5	3.7
19+	26	3.1	3.3	3.3
Total	79	3.0	3.3	3.6

[a] No trend was found in rating of dark-meat samples.

The frequency of comments on toughness of light and dark meat in the lowest shear ranges, about 15 to 20%, can be considered the usual base level of complaints for this panel. Consumer panel members began to make an increasing number of adverse comments about toughness of samples when the shear resistance values were over twice (greater than 18 lb) the minimum values found for the most tender samples. Approximately half of those who for their second or third samples received light meat of shear resistance greater than 25 lb, and approximately one-third of those receiving dark meat of shear greater than 7, commented that it was tough (Table 6). In addition to the increase in comments on toughness of later samples, a greater percentage of panel members reported that they would avoid the product in the future.

Table 4. Influence of shear resistance on tenderness, with other quality ratings for comparison.

Shear resistance (lb)	Samples (no.)	Tenderness[a]	Average ratings		
			Moistness	Flavor	Quality
Light meat					
7–12	229	3.1	3.7	2.8	2.8
13–18	252	3.3	3.8	2.9	2.9
19–24	140	3.5	3.9	3.1	3.1
25+	72	3.8	3.7	2.8	2.9
Dark meat					
4–6	228	3.2	3.4	2.8	2.7
7–12	112	2.9	3.7	2.9	2.9

[a] 1 = extremely tender; 8 = extremely tough.

Fig. 1. Forms used by untrained panel for reporting reaction to turkey sandwiches.

CODE NO._____ FAMILY NO._____ NAME_____

The following questions regarding the quality of the turkey meat in these sandwiches are to be answered by each member of the family participating in the study. We are interested in your **own** opinion. Please answer the questions before discussing the study with other members of the family.

A. GENERAL OR OVERALL QUALITY (check one)
1. Like extremely well_____
2. Like very much_____
3. Like moderately_____
4. Like slightly_____
5. Dislike slightly_____
6. Dislike moderately_____
7. Dislike very much_____
8. Dislike extremely_____

B. TENDERNESS (check one)
1. Extremely tender_____
2. Very tender_____
3. Moderately tender_____
4. Slightly tender_____
5. Slightly tough_____
6. Moderately tough_____
7. Very tough_____
8. Extremely tough_____

C. MOISTNESS (check one)
1. Extremely moist_____
2. Very moist_____
3. Moderately moist_____
4. Slightly moist_____
5. Slightly dry_____
6. Moderately dry_____
7. Very dry_____
8. Extremely dry_____

D. FLAVOR (check one)
1. Excellent_____
2. Very good_____
3. Good_____
4. Fair_____
5. Poor
6. Very poor_____

E. Assuming this turkey had been purchased under a "brand" name and at the usual market price, you would (check one)
1. Specifically request this brand again_____
2. Specifically avoid this brand_____
3. Have no preference as to brand_____

Why?_____

F. Do you eat turkey (check one)
1. At least once every 3 mos._____
2. At least once every 6 mos._____
3. At least once a year_____
4. Never_____

G. Is turkey served in your home (check one)
1. At least once every 3 mos._____
2. At least once every 6 mos._____
3. At least once a year_____
4. Never_____

H. Please comment briefly on the turkey meat with respect to its over-all quality, tenderness, moistness and flavor.

About 8% percent reported that they would avoid turkey similar to the first samples; about 28% said they would avoid the second and third samples. Since responses indicating that the turkey would be avoided were unrelated to shear range, they are additional evidence that reaction to a single sample may not be typical of the long-term reaction. The percent who said they would request a similar product again decreased from about 42% on the first sample to about 30% on the second and third samples. This further shows the vagaries of single sample tests.

Table 6. Increase in toughness comments on second and third samples in high-shear-resistance groups.

Shear resistance	Total samples tested		Percent toughness comments	
	Sample No.		Sample No.	
	1	2, 3	1	2, 3
Light meat				
7–18	153	328	18	17
19–24	60	80	20	26
25+	24	48	13	53
Dark meat				
4– 6	95	143	15	21
7–12	33	79	12	37

Although a fairly wide variation in degree of tenderness seems to be equally acceptable, birds with values for shear resistance above 20 lb, as determined in this study, would appear to be suboptimal in tenderness. The frequent criticism of toughness on the samples with shear forces above 25 lb, and criticism by one-fourth of the panel on samples in the 19- to 24-lb shear range, indicate that the aging time for birds of this size should be long enough to assure shear resistance of less than 19 lb. Of the 20 birds aged 8 hr, 85% had a shear resistance less than 19. Klose *et al.* (1959) found that 8 hr reduced shear resistance to a range of 12–18 lb for 20-week-old Broad-Breasted Bronze hens of about the same weight (15 lb average live weight) roasted from the frozen state. Thus, aging slightly longer than 8 hr should adequately tenderize such birds. Although aging was the only variable studied in this experiment, tenderness variation may of course occur from excessive scalding, severe picking treatment, size of bird, and perhaps other factors.

Influence of shear force of preceding sample. In addition to a general decrease in tenderness rating with successive samples, the panelists were influenced by the tenderness of the preceding sample. Tender samples seemed more tender when preceded by tough samples, and tough samples seemed tougher when preceded by tender samples. For example, the rating for light meat of 7–12 lb shear resistance was 3.5 when the preceding sample was in the same shear range; when the preceding sample had a high shear value, 19 or more, the sample received a more tender rating—2.8. The rating of samples with a shear value of over 19 lb, when preceded by samples of the same shear value, was 3.3; when preceded by a sample with a lower shear value, the sample received a tougher rating—4.0 The differences between these means are statistically significant. Similar contrast effects have been reported previously (Hanson *et al.*, 1955; Peryam and Pilgrim, 1957).

Relation between tenderness ratings and comments of untrained panel. The untrained panel was more consistent in scoring and commenting on tenderness than on toughness. The average scores of 131 light-meat samples receiving tenderness comments was 2.5, and of 64 dark-meat samples was 2.3. A score of 2 is described as "very tender" and a score of 3 as "moderately tender" on the questionnaire. Although the samples receiving toughness comments had ratings toward the tougher end of the scale, some of the ratings were in the tender range. The average ratings of 139 light-meat samples receiving toughness comments was 4.6, and of 77 dark-meat samples was 4.7. Ratings of 4 and 5 are respectively described on the questionnaire as "slightly tender," and "slightly tough." Thus, some panel members commented on toughness but rated these samples in the tender range. Jones *et al.* (1955) found that the level of preference implied in descriptive phrases may be interpreted differently and cannot always be predicted.

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

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RESEARCH NOTE

Flavor Thresholds of Volatile Fatty Acids

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One method (Patton and Josephson, 1957) of assessing a compound's flavor significance in a food is to determine the quantitative level and the flavor threshold for the compound. If the actual concentration exceeds the threshold concentration, it is reasonable to assume that the compound has a perceptible effect on flavor. In reviewing flavor threshold data for fatty acids, Lea (1963) compared our findings, with milk used as a medium, with those of Feron and Govignon (1961), who used neutral edible oil. The respective threshold values for butyric and caproic acids in milk were 25 and 14 ppm, whereas in edible oil these values were 0.6 and 2.5 ppm. Respective values for the other volatile fatty acids measured in oil were 350, 200 and 700 ppm for caprylic, capric, and lauric acids. Data which follow suggest that the differences are valid and traceable to the media used.

EXPERIMENTAL

Flavor thresholds for acetic, butyric, caproic, caprylic, and capric acids (Distillation Products Industries purest grade; acetic acid, reagent grade, from Fisher Scientific Co.) were determined in distilled water at room temperature as described by Patton and Josephson (1957). The same nine graduate students and staff members (all male) of the department were used for the sessions involving the five acids. After some preliminary delineation of reasonable concentration ranges, four concentrations of each acid were evaluated three times by each of the nine observers. Thus each concentration of an acid was evaluated 27 times. The threshold was defined as that concentration of acid, by plot of the data, corresponding to the 50% level of positive response.

RESULTS AND DISCUSSION

Flavor threshold values for the five acids are shown in Table 1. The values for butyric and caproic acids (6.8 and 5.4 ppm, respectively) are not unreasonable with respect to those obtained earlier (25 and 14 ppm) with milk used as the medium. At normal pH (6.6), milk would exert some buffer action, tending to sequester the acids and raise their thresholds above those with water. However, the data derived with water and extended to the longer-carbon-chain acids clearly confirm an order of values different from those for the same acids in oil as observed by Feron and Govignon. The longer-chain acids have low thresholds in water and high thresholds in oil. This observation is in accord with that of Lea and Swoboda (1958) for aliphatic aldehydes, which also yielded lower thresholds in water than in oils. Reversal of the principle with the more-polar, short-chain acids is somewhat surprising, though not illogical, and may have implications to cheese flavor (Patton, 1963). Volatility of the stimulus is essential for olfactory perception. Bonding to solvent molecules apparently tends to reduce volatilization of

Table 1. Flavor thresholds for volatile fatty acids in water and in oil.

Fatty acid	Parts per million	
	Water	Oil[a]
C_2	54	.....
C_4	6.8	0.6
C_6	5.4	2.5
C_8	5.8	350
C_{10}	3.5	200
C_{12}	.....	700

[a] Data by Feron and Govignon (1).

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The experimental work reported was contributed by graduate students in connection with a graduate seminar.

polar components from water in much the same manner that oils "fix" nonpolar odorants.

Differences in the efficacy of flavor components depending upon their relative distributions between fat and aqueous phases in foods seem to be a significant and complicating factor in the understanding and control of flavor.

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Effect of Coring Methods on Shear Values Determined by Warner-Bratzler Shear [a]

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(Manuscript received February 24, 1964)

SUMMARY

Shear-force values were determined with the Warner-Bratzler shear machine on cores obtained by two methods from steaks from longissimus dorsi (LD) and biceps femoris (BF) muscles cooked to 61 and 80°C. One-half-inch cores were cut either parallel with the muscle fibers or perpendicular to the cut surface of the steak without regard to orientation of the fibers. The LD and BF muscles did not react in the same manner to changes in coring method. Method of coring made a large difference in shear values in LD at 80°C. With one exception shear values of paired steaks from the same muscle with cores cut parallel were more closely related than shear values of cores from within the same steak cut differently.

INTRODUCTION

The evaluation of published reports on meat tenderness is complicated by several factors. Comparison of subjective measurements is difficult because of variation in scoring procedures and in the abilities of judges to distinguish differences between samples. Comparisons of objective measurements are impaired because techniques used in determining tenderness differ among laboratories.

The Warner-Bratzler shearing device is probably the most widely used instrument in measuring tenderness of meat. There are several sources of variation in the technique of using this device. Two sources of variation between investigators are size of the core and method of obtaining the core to be sheared. Both one-half and one-inch cores were found suitable by Paul and Bratzler (1955). Harrison *et al.* (1959) stated that cores are obtained by cutting the meat parallel to the muscle fibers. However, many reports in the literature do not reveal the method used. In personal conversations and oral reports it has been stated that cores were obtained by cutting through the meat without regard to the direction of fibers.

The orientation of muscle fibers within steaks cut from individual muscles is dependent upon the orientation of the fibers in the whole muscle, and upon the way steaks are cut from the muscle. Only when the muscle fibers run parallel to the long dimension of the muscle and the steaks are cut across the muscle will the fibers be perpendicular to the cut surface of steaks. If the fibers do not run parallel to the long dimension of the muscle the orientation of the fibers in the steak will deviate from being perpendicular to the cut surface. In some muscles the fibers do not all run in the same direction. In such cases, steaks cannot be cut that have the fibers orientated in the same way within the entire steak.

One of two methods of obtaining cores for shear determination is likely to be used. Coring with the fibers is done so that the orientation of muscle fibers during shearing will be similar for all cores from a steak and for cores from steaks of different muscles. A second method of obtaining cores is to disregard fiber orientation and simply core perpendicular to the cut surface of the steak. When this method is used, the orientation of muscle fibers may vary in cores from different areas within a steak, as well as in cores from steaks from different muscles.

EXPERIMENTAL

Meat was obtained from 20 animals produced at Texas Agricultural Experiment Station No. 23

[a] Supported in part by the Animal Husbandry Research Division, Agricultural Research Service, U. S. Department of Agriculture.

[b] This paper is a contribution of the Texas Agricultural Experiment Station.

and slaughtered in 1961 and 1962. Details of slaughter and storage were as given by Ritchey *et al.* (1963). The steaks used were one-inch steaks cut from the loin end of the longissimus dorsi (LD) muscle, beginning at the last thoracic vertebra, and from the dorsal end of the biceps femoris (BF) muscle in the round. The ischiatic head was removed from the BF steaks. The steaks were cut as nearly as possible across the two shortest dimensions of the muscle. The steaks were cooked to final temperatures of 61 and 80°C with procedures described by Cover and Hostetler (1960).

Eight one-half-inch cores were obtained from each steak. Four cores were cut parallel with the muscle fibers as described by Cover *et al.* (1962). The other four were obtained by cutting the cores perpendicular to the flat surface of the steak. The division of steaks into quarters was not as straightforward in LD as in BF. For this reason only two locations are considered in LD and four in BF. In LD, four cores (two parallel and two perpendicular) were removed from the medial half (near the vertebra) and four from the lateral half (away from the vertebra) of the steaks (Fig. 1). In BF, two cores (one parallel and one perpendicular) were cut from each quarter of the steak. Quarters one and two were adjacent to the ischiatic head. Quarters one and three were on the outside (fat side) of the muscle.

The analysis of variance in Table 1 was calculated to test the significance of interactions and of differences between muscles, final cooking temperatures, coring methods, and animals. The analyses in Table 2, within LD and BF, also test the significance of interactions and differences between locations in the steaks, as well as in final cooking temperatures, coring methods, and animals. Duncan's multiple-range test (Steel and Torrie, 1960) was used to test differences between individual means. For eight of the animals, simple correlations (Snedecor, 1956) were calculated to determine the repeatability of shear values within steaks with cores cut differently. Intraclass correlations were calculated to determine the relationship between shear values of paired steaks from the same muscle with cores cut parallel with the muscle fibers in both steaks.

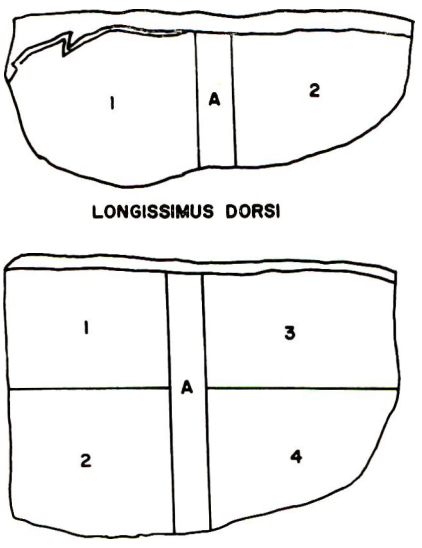

Fig. 1. Division of steaks for analysis of shear variation within steaks. Medial (1) and lateral (2) halves of LD. Quarters (1) through (4) of BF. Taste-panel areas (A).

Table 1. Analysis of variance of shear values (mean of values from four locations in each steak).

Variables[a]	Degrees of freedom	Mean square
Muscles (M)	1	7.779 n.s.
Temperatures (T)	1	63.23 ***
Coring methods (C)	1	12.42 *
Animals (A)	19	14.98 ***
M × T	1	143.3 ***
M × C	1	54.76 ***
M × A	19	6.264 **
T × C	1	4.672 n.s.
T × A	19	6.008 **
M × T × C	1	8.668 n.s.
Error	95	2.528
Total	159	

* p 0.05; ** p 0.01; *** p 0.001.
[a] (M) longissimus dorsi and biceps femoris; (T) 61 and 80°C; (C) perpendicular to the steak surface and parallel with the fibers.

RESULTS AND CONCLUSIONS

The analysis of variance indicated two very highly significant interactions (Table 1). The larger was between muscles and final cooking temperatures. The mean shear value (mean of both methods) of LD increased 3.1 lb between 61 and 80°C, whereas the mean of BF decreased 0.6 lb. When the means in Table 3 were compared individually in LD it was found that the increase in shear values in LD took place regardless of how the cores were cut, but the increase was 4.0 lb when cores were cut parallel with the fibers and only 2.4 lb when cut perpendicular to the surface of the steak. For LD, the difference between shear values at 61 and 80°C was highly significant by both meth-

Table 2. Analyses of variance of shear values.

Variables[c]	L. dorsi[a] Degrees of freedom	L. dorsi[a] Mean square	B. femoris[b] Degrees of freedom	B. femoris[b] Mean square
Temperatures (T)	1	396.9 ***	1	37.81 *
Locations (L)	1	53.79 ***	3	2.318 n.s.
Coring methods (C)	1	119.5 ***	1	35.44 *
Animals (A)	19	26.57 ***	19	31.85 ***
T × L	1	4.904 n.s.	3	70.13 ***
T × C	1	26.01 *	1	1.250 n.s.
T × A	19	10.77 **	19	8.639 n.s.
L × C	1	27.83 **	3	75.57 ***
L × A	19	6.213 n.s.	57	7.148 n.s.
C × A	19	6.917 n.s.	3	6.918 n.s.
T × L × C	1	6.412 n.s.	3	25.85 *
T × C × A	19	7.833 *	19	7.437 n.s.
Error	57	3.693	171	7.576
Total	159		319	

* p 0.05; ** p 0.01; *** p 0.001.
[a] Each observation is a mean of two shear values.
[b] Each observation is a single shear value.
[c] (T) 61 and 80°C; (L) see Fig. 1; (C) perpendicular to the steak surface and parallel with the fibers.

ods. The difference between methods was not significant at 61 but was highly significant at 80°C. In BF, the mean shear values were lower at 80 than at 61°C with both coring methods, but the differences were not significant.

The second interaction was between muscles and coring methods. Cores cut parallel with the fibers sheared 1.7 lb higher in LD and 0.6 lb lower in BF than cores cut perpendicular to the cut surface. Further investigation may reveal that connective tissue has more effect on the shear values of BF when coring is done by one of the methods than by the other.

When cores were cut parallel with the fibers the difference in shear values between LD and BF was 4.0 lb at 80°C but only 0.8 lb at 61°C. This agrees with findings of Cover *et al.* (1962). However, when cores were cut perpendicular to the cut surface, the larger difference (2.2 lb) between the muscles was at 61°C, with only 0.7 lb difference at 80°C. Thus, changing the method changed the relationship between the muscles.

Several workers (Murphy and Carlin, 1961; Urbin *et al.*, 1962; Tuma *et al.*, 1962; Cover *et al.*, 1962) have found differences in shear value between the areas near to (me-

Table 3. Effect of coring method on shear values of steaks from two muscles cooked to two temperatures.

Muscle	Final steak temperature	Method of coring: Parallel with fibers Mean (lb)	Parallel with fibers S. D.	Perpendicular to cut surface of steak Mean (lb)	Perpendicular to cut surface of steak S. D.	Mean of both methods (lb)
Longissimus dorsi	61°C	8.9	1.6	8.0	1.7	8.5
	80°C	12.9	3.7	10.4	3.3	11.6
Mean of both temp.		10.9		9.2		
Biceps femoris	61°C	9.7	1.3	10.2	2.4	9.9
	80°C	8.9	1.7	9.7	1.6	9.3
Mean of both temp.		9.3		9.9		

dial) and away from (lateral) the vertebrae in LD muscles of pork and beef, but do not agree as to the area having the higher shear value. The possible effect of coring method on this problem was investigated. Shear values for cores 1 and 2 (medial) were averaged and compared with the average of cores 3 and 4 (lateral).

The analysis of variance (Table 2) for LD indicates a highly significant interaction between location and coring method. When coring was done parallel with the fibers the difference between shear values of the medial and lateral ends of LD was highly significant. Cores from the medial end sheared 2.0 lb higher than cores from the lateral end. This agrees with work of Cover *et al.* (1962) and Urbin *et al.* (1962). When coring was perpendicular to the cut surface of the steak the cores from the medial end sheared only 0.4 lb higher (not significant) than cores from the lateral end.

The temperature × coring method interaction was also significant. When coring was done parallel with the fibers the medial end sheared 2.0 lb higher (Table 4) than the lateral end at both 61 and 80°C. When coring was done perpendicular to the cut surface, cores from the medial end sheared 1.1 lb higher (not significant) than cores from the lateral end at 61°C, but at 80°C the cores from the lateral end sheared slightly higher than those from the medial end. Thus, coring perpendicular to the cut surface reduced the significance of the difference between the medial and lateral end of LD at both 61 and 80°C. At 80°C this method also reversed the trend.

Cover *et al.* (1962) found considerable variation in shear-force values within steaks from the BF muscle. The analysis of variance (Table 3) for BF indicates that the interaction of temperature × location × coring method is significant and that the temperature × location and location × coring method interactions are very highly significant. At 61°C, cores one, two, and three (Table 4) sheared higher when coring was done perpendicular to the cut surface whereas core four sheared significantly higher when coring was with the muscle fibers. At 80°C, cores one and three, again, sheared significantly higher when coring was perpendicular to cut surface, but core two now sheared significantly higher when coring was done parallel, and the difference between the methods was now not significant in core four.

The significance of differences between locations varied within the temperature-cor-

Table 4. Effect of coring method on shear values in different locations of steaks.

	Final steak temperature	Location in steak[a]	Method of coring: Parallel with fibers, Mean (lb)	Parallel with fibers, S. D.	Perpendicular to cut surface of steaks, Mean (lb)	Perpendicular to cut surface of steaks, S. D.	Mean of both methods (lb)
L. dorsi	61°C	1[b]	9.9	2.3	8.6	2.6	9.2
		2	8.0	1.5	7.5	1.9	7.7
	80°C	1	13.9	4.2	10.2	4.3	12.0
		2	11.9	2.8	10.6	2.9	11.2
B. femoris	61°C	1[c]	9.1	1.6	10.4	4.0	9.8
		2	8.7	2.2	9.8	4.3	9.2
		3	10.1	2.6	12.0	5.5	11.0
		4	10.8	2.9	8.8	4.0	9.8
	80°C	1	8.4	2.7	11.0	2.0	9.7
		2	11.4	2.1	9.2	2.5	10.3
		3	6.0	1.8	9.4	3.0	7.7
		4	9.8	2.1	9.0	1.7	9.4

[a] See Fig. 1.
[b] Each observation is a mean of two shear values.
[c] Each observation is a single shear value.

ing method combinations. At 80°C, when the coring was done parallel with the fibers, all location differences were significant except that locations one and two were not different from four. At 61°C, when coring was done perpendicular to the surface of the steaks, location three was significantly different from two and four but all other differences were not significant. At 61°C, when coring was done parallel with the fibers, location two was significantly different from four. All other differences were not significant. There were no significant differences at 80°C when coring was done perpendicular to the surface of the steak.

All comparisons reported thus far have been for differences between coring methods between and within steaks. For eight of the 20 animals, additional cooked steaks from the same muscles had been sheared using cores cut parallel with the fibers. Thus it was possible to compare shear values of paired steaks from the same muscle with coring done parallel with the fibers, and to compare shear values within the same steaks with coring done by two methods. The correlation coefficients (Table 5) were higher

Table 5. Correlations between shear values from eight animals.

	L. dorsi		B. femoris	
	61°C	80°C	61°C	80°C
Between steaks within a coring method[a]	0.25	0.86**	0.45	0.60
Between coring methods within steaks[b]	0.07	0.41	0.68	0.02

** p 0.01.
[a] Paired steaks from same animal with cores cut parallel with the fibers.
[b] Parallel with the fibers vs. perpendicular to cut surface of the same steak.

between steaks with coring done the same way (parallel with the fibers) except for BF at 61°C.

As a main effect, coring method had the most influence on the shear values of LD. Of greater importance was the interaction of coring methods with muscles and temperatures. LD and BF did not react in the same way to changes in coring method. This might be expected since coring perpendicular to the surface of the steak produced cores with muscle fibers oriented differently for the two muscles. Shear-value relationships between temperatures within muscles were altered by changing the coring methods. Relationships between shear values of cores within muscles were also altered by changing the method.

The data indicate that valid comparisons of shear data cannot be made if the method of coring differs between tests. This is of importance when ranking the progeny of different sires and when comparisons are to be made between variables such as age groups and breed of animals, and between treatments such as aging of carcasses.

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A Dietary Evaluation of Synthetic Amino Acid Amides

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SUMMARY

Growth of mice on a complete synthetic diet has been used to determine the ability of synthetic amino acid derivatives to replace the corresponding essential metabolites in nutrition. In a series of range-finding experiments, it was found that dietary valine, phenylalanine, leucine, methionine, and, to a lesser extent, isoleucine could be effectively replaced by the corresponding DL-amides or amide salts. In addition, DL-valinamide salts could be administered in aqueous solution to supplement a solid valine-free diet. The physical, chemical, and biological properties of amino acid amide salts may make them of special value in protein supplementation.

INTRODUCTION

It is generally recognized that protein resources may be the eventual limiting factor in meeting the food requirements of the world's burgeoning population. Although it has been amply demonstrated on both a practical and a laboratory scale that the component amino acids may be substituted for complete protein or used effectively to supplement low-grade food sources, the high cost of the pure substances makes their use as food supplements economically unfeasible in most instances.

A number of derivatives of *alpha*-amino acids have been tested for their ability to replace corresponding essential nutrients in mammalian diets. For example, substituted 2,5-piperazinediones ("amino acid anhydrides") have been examined by several investigators (see Wretlind, 1953), and a variety of compounds related to tryptophan (see Gilman, 1943) and leucine (Rechcigl and Williams, 1961) have received attention. However, with few exceptions, the proposed substitutes failed to exhibit biological activity.

Recently, a new synthesis of amino acid amides was disclosed (Johnson and Crosby, 1962) by which many of these compounds might be manufactured in quantity at low cost. The present paper shows that the amides of several essential amino acids can replace the parent nutrients in the diets of experimental animals.

EXPERIMENTAL

Weanling male Swiss mice were housed in groups of ten in 10 × 7 × 6-inch stainless-steel cages in an air-conditioned room maintained at 25°C. The animals weighed 12–15 g at the beginning of each experiment and were weighed daily.

Food and water were provided *ad libitum*. Dietary amino acids were supplied in crystalline form, as purchased commercially, in proportions similar to those recommended by Maddy and Elvehjem (1949) (Table 1). In addition to the amino acid mixtures, the diets also contained the following major components (g/kg of diet): salt mix "W" (Wesson, 1932), 40; corn oil, 80; cod liver oil, 20; choline, 2; and inositol, 1. The following minor components (mg/kg) were also included: riboflavin, 6; pyridoxine hydrochloride, 6; thiamine hydrochloride, 6; calcium pantothenate, 30; nicotinic acid 25; menadione, 5; vitamin B_{12}, 0.05; biotin, 0.5; folic acid, 2; and tocopherol, 200. Sucrose was added to bring these total weights to 1 kg, and alphacel (100 g) and $NaHCO_3$ (17 g) were added. All test diets were made isonitrogenous with respect to the complete synthetic diet (CSD) by varying the glutamic acid content to the calculated degree.

The amino acid amide salts employed in these experiments were prepared by the method of Johnson and Crosby (1962). The compounds were recrystallized to a purity of at least 98% as shown by C, H, and NH_4Cl analysis, the only detectable impurity being ammonium chloride.

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Table 1. Amino acid composition of the complete synthetic diet.

Essential acids	
L-Isoleucine (+ D-alloisoleucine)	1.00%
L-Leucine	0.80
DL-Methionine	0.90
DL-Phenylalanine	0.90
DL-Valine	1.40
L-Arginine (HCl)	1.35
L-Histidine (HCl)	0.55
L-Lysine (HCl)	1.37
DL-Threonine (allo-free)	1.00
DL-Tryptophan	0.40
Nonessential acids	
DL-Alanine	0.40
L-Asparagine	1.00
L-Glutamic acid	5.10
Glycine	2.00
DL-Serine	0.50
L-Tyrosine	1.00
Total	19.67%

Experimental diets were varied by removal of a single amino acid component from the CSD and substitution of the corresponding derivative or other replacement in an amount calculated to be equivalent on a molar basis. The degree of utilization of the racemic parent amino acid (Van Pilsum and Berg, 1950) was also taken into consideration.

In one series of experiments, a solid valine-free diet was fed and the necessary valine or its amide salt was dissolved in the drinking water. In this case, equivalent quantities of amide were not employed; both valine and its amide were given *ad libitum* at a concentration of 4% in tap water.

Each test required between 14 and 30 days. During initial experiments, diet consumption averaged 17.5 g/day/mouse, including the portion wasted. In order to reduce loss, the open-cup feeder was replaced with a compartmented box which permitted only entry of the animal's head and which was fitted with wire screen food cover to prevent scattering. With this improvement, the consumption of diet dropped to 6.5 g/day/mouse while weight gains remained identical with those of the cup-fed group. No attempt was made to determine the actual food intake of individual animals, but very little diet waste was noted.

RESULTS AND DISCUSSION

Almost without exception, earlier investigations on nutrient replacement have used rats as experimental animals. However, Skipper and Thomson (1958) have shown that mice are particularly susceptible to amino acid deficiencies. This fact, together with the proportionately large short-term weight changes on both normal and deficient diets and the relatively economical quantities of the synthetic rations which these animals consume, suggested that mice might be well suited to an investigation of the structural parameters governing the "essential" nature of a number of important dietary constituents.

The synthetic diet approached an optimum composition with respect to the amino acids examined in these experiments, as shown by the response of mice receiving a varying amount of DL-valine. The growth rate was maximum near the standard valine level of 1.4%, while higher and lower levels were less satisfactory. At best, the CSD sustained only about 65% of the growth rate obtained when casein was the protein source, perhaps because of the use of mixed isoleucines.

Although, as expected, D-valine failed to support growth (Bauer and Berg, 1943), it did not appear to interfere with utilization of the L-form when a DL-mixture was employed (Table 2). The considerable differ-

Table 2. Effect of valine isomers on mouse growth.

Diet	Av. initial wt[a]	Av. weight gain				Av. daily food consumption[b]
		day 3	day 6	day 9	day 12	
	g			g		g
Control (casein)	10.7	4.3	7.8	11.2	13.5	9.3
Control (CSD)	13.0	1.0	2.8	5.5	9.0	8.4
Valine-free diet (VFD)	12.8	−1.2	−3.0	−3.9	−3.8	7.8
VFD + L-valine	14.3	1.2	2.6	7.2	10.6	8.4
VFD + D-valine	12.5	−1.4	−2.8	−3.6		8.1
VFD + DL-valine	12.8	1.4	2.9	5.6	9.3	7.4

[a] Ten animals.
[b] Includes a small amount of waste.

ence in weight gain between animals fed the CSD and those deprived of a single essential amino acid afforded a clear-cut test of nutritional activity in synthetic compounds.

Valinamide hydrochloride provided typical results. This compound was able to replace completely the corresponding amino acid in the mouse. No toxicity or unusual effects were observed over periods of almost two months; unlike with valine, quantities up to three times the normal level were without apparent deleterious effect on growth or health. The response to the free amide, not described here, did not differ from that obtained with the salt.

No significant difference was observed between the growth rate on diets containing L-leucinamide or the stoichiometric equivalent of DL-leucinamide (Fig. 1). Comparable

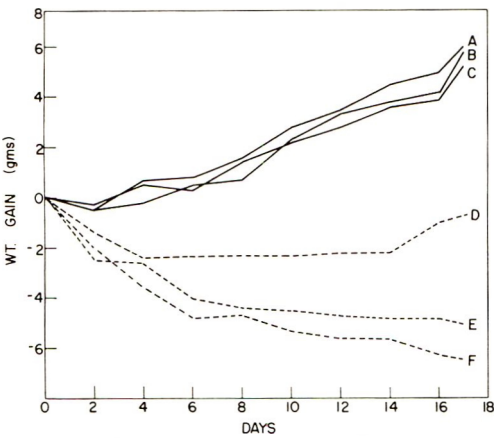

Fig. 1. Mouse growth rate (average of 10 animals). A) complete diet; B) leucine-free diet + L-leucinamide · HCl; C) leucine-free diet + DL-leucinamide · HCl; D) isoleucine-free diet + isoleucinamide · HCl (isomer mixture); E) leucine-free diet; F) isoleucine-free diet.

experiments with synthetic isoleucinamide which contained all four possible optical isomers showed that consumption of even twice the normal (DL) level resulted in severely depressed growth. Thus, it appears that *allo*-isoleucinamide may inhibit utilization of the other forms.

When provided as a 4% solution in drinking water, a DL-valine supplement to the solid valine-free diet produced growth rates which corresponded to those obtained with purified casein as a protein source (Fig. 2). This effect apparently was not due to an

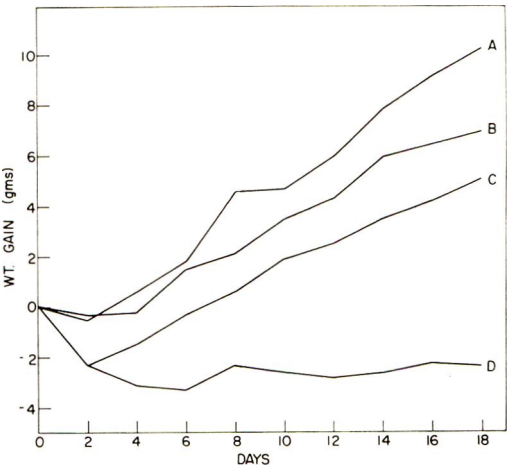

Fig. 2. Mouse growth rate (average of 10 animals). A) Valine-free diet + DL-valine solution; B) complete solid diet; C) valine-free diet + DL-valinamide acetate solution; D) valine-free diet.

unusual intake of the amino acid, since the volume of solution consumed provided approximately normal dietary valine levels. Substitution of an equivalent solution of valinamide hydrochloride for that containing valine afforded a growth rate which was satisfactory but somewhat less than that of the control for the first 14 days. Between days 15 and 23 there occurred a sharp weight loss, after which the animals again gained rapidly. It was noted that a thin layer of the amide salt accumulated on the rim of the drinking tube during the experiment, and presumably the mice were repelled by the taste of this deposit which, to the human tongue, is very saline. The difficulty was circumvented by using the tasteless (to humans) valinamide acetate, and this substance produced weight gains equivalent to those obtained with either DL-valine or DL-valinamide hydrochloride administered in solid diets (Fig. 2).

As shown in Fig. 1 and Table 3, the hydrochlorides of DL-valinamide, DL-leucinamide, L-leucinamide, DL-phenylalaninamide, DL-methioninamide, and probably DL-isoleucinamide replaced completely the corresponding amino acids in the mouse. The development of these amides as protein supplements would present several advantages. The high degree of water solubility permits their use in low-volume liquid nutritional supplements

Table 3. Effects of DL-amino acid amides on mouse growth.

Diet	Av. wt. gain (g)[a]			
	day 7	day 14	day 21	day 28
CSD	1.3	4.5	7.3	7.3
Valine-free diet (VFD)	−4.7(d)[b]	−6.0(d)	−6.5(3d)	−5.0(9d)
VFD + Valinamide · HCl	0.9	3.9	5.6	6.4
CSD	2.1	6.0	6.9	9.1
Phenylalanine-free diet (PFD)	−2.2	−2.0	−1.0	0.3
PFD + phenylalaninamide · HCl	1.5	6.1	9.3	11.0
CSD	2.2	6.0	6.9	9.3
Methionine-free diet (MFD)	−4.1	−5.5	−6.0(d)	−6.0(7d)
MFD + methioninamide · HCl	1.5	4.0	7.9	9.8
CSD	1.3	4.5	6.3	....
Leucine-free diet (LFD)	−3.7	−4.8(d)	−4.8(d)	....
LFD + leucinamide · HCl	1.2	3.6	4.6	....

[a] Ten animals weighing 15.0 ± 0.5 g.
[b] Dead = d.

and under other circumstances where the relative insolubility of most amino acids hinders effective use. Racemic amides have been successfully converted to the corresponding L-amino acids; the use of common and inexpensive proteolytic enzymes such as papain (Tatsuoka and Honjo, 1953a; Smith *et al.*, 1952) as well as strictly chemical methods (Tatsuoka and Honjo, 1953b) could greatly simplify the manufacture of the optically pure compounds. Above all, the substances are prepared with relative ease from inexpensive industrial raw materials, and the physical and chemical characteristics of this type of structure offer advantages in manufacture and handling which could make amino acid amides important in large-scale food supplementation.

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RESEARCH NOTE

Nutrient Content of Morel Mushroom Mycelium: B-Vitamin Composition

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The growth characteristics of morel mushroom mycelium in submerged culture have been reported (Litchfield and Overbeck, 1962; Litchfield *et al.*, 1963a). *Morchella hortensis* was selected as the organism of choice for large-scale production on the basis that it has a greater growth rate and better flavor than other species (Litchfield *et al.*, 1963a). A strain of this organism is now being grown on a commercial scale for food use (Klis, 1963).

Litchfield *et al.* (1963b) presented data on the amino acid composition of morel mushroom mycelia. The results of assays for B-vitamin content of *M. hortensis* mycelium are presented in this communication to complete the characterization of the nutrient content of this organism.

M. hortensis was grown in aerated culture vessels in a glucose–ammonium phosphate–cornsteep liquor–calcium carbonate medium as described previously (Litchfield *et al.*, 1963a,b). Assays for B-vitamin content of dried *M. hortensis* mycelium were performed by the Laboratory of Vitamin Technology, Chicago, Illinois, according to the procedures shown in Table 1.

From the results (Table 1) it appears that *M. hortensis* mycelium is quite similar to *M. esculenta* (Szuecs, 1956) and *Agaricus blazei* (Block *et al.*, 1953) in contents of thiamin, and niacin; higher in Vitamin B_6, pantothenic acid, and folic acid; and lower in riboflavin and biotin. Szuecs (1956) did not specify in his patent the assay procedures used. Some of these differences in assay results might be explainable on the basis of differences in assay procedures and differences in the composition of the growth media.

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Table 1. B-Vitamin composition of mushroom mycelia.

Vitamin	Method used for *M. hortensis*	Composition (mg/100 g dry weight)		
		M. hortensis	*M. esculenta* (Szuecs, 1956)	*Agaricus blazei* (Block *et al.*, 1953)
Thiamin	AOAC, 1960 (fluorometric)	0.518	0.392	0.200
Riboflavin	AOAC, 1960 (fluorometric)	1.31	2.46	3.4
Niacin	AOAC, 1960 (microbiological)	12.4	8.20	14.6
Vitamin B_6 (as pyridoxine hydrochloride)	Atkin *et al.*, 1943	2.62	0.58	....
Pantothenic acid (as D-calcium pantothenate)	AOAC, 1960	12.6	0.87	6.9
Choline	Glick, 1944	461.	....	....
Folic acid	AOAC, 1960	1.09	0.348	....
Inositol	Atkin *et al.*, 1943	178.	....	....
Biotin	Wright and Skeggs, 1944	0.015	0.075	....

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