Journal of FOOD SCIENCE

MARCH-APRIL, 1966 Volume 31, No. 2

CONTENTS

BIOCHEMISTRY AND BIOPHYSICS:

C. L. DAVEY AND K. V. GILBERT
Studies in Meat Tenderness II. Proteolysis and the Aging of Beef 135

S. H. SCHANDERL AND D. Y. C. LYNN
Changes in Chlorophylls and Spectrally Related Pigments During Ripening of Capsicum frutescens 141

M. HAMOSH, R. ATIA, AND B. SHAPIRO
Fatty Acid Uptake and Esterification by Fish Muscle 146

E. D. ABERLE AND R. A. MERKEL
Solubility and Electrophoretic Behavior of Some Proteins of Post-Mortem Aged Bovine Muscle 151

N. I. MONDY, S. B. GEDDE-DAHL, AND E. O. MOBLEY
Relationship of Specific Gravity to the Enzymatic Activity and Phenolic Content of Potatoes 157

F. HILL
The Solubility of Intramuscular Collagen in Meat Animals of Various Ages 161

CHEMISTRY AND PHYSICS:

G. L. K. HUNTER AND M. G. MOSHONAS
Analysis of Alcohols in Essential Oils of Grapefruit, Lemon, Lime, and Tangerine 167

L. W. AURAND, J. A. SINGLETON, T. A. BELL, AND J. L. ETCHELLS
Volatile Components in the Vapors of Natural and Distilled Vinegars 172

B. S. LUH AND K. D. DASTUR
Texture and Pectin Changes in Canned Apricots 178

A. M. PEARSON, B. G. TARLADGIS, M. E. SPOONER, AND J. R. QUINN
The Browning Produced on Heating Fresh Pork. II. The Nature of the Reaction 184

R. ELLIS, A. M. GADDIS, AND G. T. CURRIE
Carbonyls in Oxidizing Fat. IX. Aldehydes Isolated from Autoxidized Methyl Arachidonate 191

(Continued on next page)

An Official Publication of the Institute of Food Technologists
Suite 2120, 221 North LaSalle St., Chicago, Illinois 60601, U.S.A.

D. F. DYER, D. K. CARPENTER, AND J. E. SUNDERLAND
Equilibrium Vapor Pressure of Frozen Bovine Muscle 196

T. A. ROHAN AND T. STEWART
The Precursors of Chocolate Aroma: Changes in the Free Amino Acids During the Roasting of Cocoa Beans 202

T. A. ROHAN AND T. STEWART
The Precursors of Chocolate Aroma: Changes in the Sugars During the Roasting of Cocoa Beans 206

M-D. NUTTING AND R. BECKER
Chlorophylls a and b in Green Vegetables: A Comparison of Procedures 210

R. C. MORGAN
Chemical Studies on Concentrated Pineapple Juice. I. Carotenoid Composition of Fresh Pineapples 213

S. LOVE AND L. J. BRATZLER
Tentative Identification of Carbonyl Compounds in Wood Smoke by Gas Chromatography 218

T. W. KWON AND M. J. NORGAARD
Observations on the Reactivity of 2-Thiobarbituric Acid-Reactive Substances in Dried Whole Egg Powder 223

E. D. DEKAZOS
Anthocyanin in Red Tart Cherries as Related to Anaerobiosis and Scald 226

MICROBIOLOGY AND MICROBIAL TOXICOLOGY:

D. F. SPLITTSTOESSER AND I. GADJO
The Groups of Microorganisms Composing the "Total"-Count Population in Frozen Vegetables 234

J. H. SILLIKER, A. SCHMALL, AND J. Y. CHIU
The Fluorescent Antibody Technique as a Means of Detecting Salmonellae in Foods 240

I. S. PABLO, G. J. SILVERMAN, AND S. A. GOLDBLITH
Microbial Growth Patterns of Rehydrated Freeze-Dried Foods. II. Chicken 245

NUTRITION AND CHEMICAL TOXICOLOGY:

M. F. NELSON, T. A. POULOS, L. E. GONGWER, AND J. C. KIRSCHMAN
Preparations of Carbon-14-Labeled Polyoxyethylene (20) Sorbitan Monolaurate and Their Metabolic Fate in Rats 253

K. W. CHENG AND W. W. KILGORE
Determination of 2,6-dichloro-4-nitroaniline Residues in Fruits by Electron-Capture Gas Chromatography 259

QUALITY EVALUATION AND CONSUMER ACCEPTANCE:

B. B. MARSH, P. R. WOODHAMS, AND N. G. LEET
Studies in Meat Tenderness. I. Sensory and Objective Assessments of Tenderness 262

D. A. KENDALL AND A. J. NEILSON
Sensory and Chromatographic Analysis of Mixtures Formulated from Pure Odorants 268

L. M. BEIDLER
A Physiological Basis of Taste Sensation 275

M. C. BOURNE
Measure of Shear and Compression Components of Puncture Tests 282

C. L. DAVEY and K. V. GILBERT
Meat Industry Research Institute of New Zealand, Hamilton, New Zealand

Studies in Meat Tenderness
II. Proteolysis and the Aging of Beef

SUMMARY

The proteolytic changes which occur in the longissimus dorsi muscles of beef carcasses during 30 days' aging at 2°C give rise to a mean increase of nonprotein nitrogen of 0.045m*M*/g meat, representing a degradation of 2.3% of the meat protein. The tenderizing and proteolysis which occur during aging are not related, for differences in the rates of tenderizing among carcasses are not paralleled by similar differences in the rates of proteolysis. Bacterial action is not responsible for the observed proteolytic and tenderizing changes.

The widely-held view that aging is a consequence of proteolytic action has its origin in the work of Hoagland *et al.* (1917), who showed that, as meat becomes more tender during aging, nonprotein nitrogen accumulates within the tissue. These studies are supported by the findings that muscle tissues contain the cathepsins (Balls, 1938; Kastellic, 1953; Landmann, 1963). These enzymes have been the subject of recent studies designed to determine their site of action within the muscle cell (Bodwell and Pearson, 1964). That proteolysis, independent of bacterial action, occurs in meat during aging is a matter of some dispute. Husaini *et al.* (1950) and Wierbicki *et al.* (1954) could not detect an increase in nonprotein nitrogen indicative of proteolysis in beef aged 15 days at 2°C. Hodgkiss and Jones (1955) and Shewan and Jones (1957) showed that, except for a threefold accumulation of glutamic acid in cod muscle, no other free amino acids or presumably noncoaguable low-molecular-weight peptides are produced during long periods of storage at 5°. Extensive autolytic changes in fish muscle cannot therefore be occurring.

As examples of studies favoring proteolysis during the aging of meat, the following can be mentioned. Niewiarowicz (1956) and Leinati (1957) demonstrated that the nonprotein nitrogen which increases during the aging of beef is due mainly to free alanine, glutamic acid, leucine, and cystine. Zender *et al.* (1958) and Radouco-Thomas *et al.* (1959) showed that autolysis occurring in meat over long periods of storage is accompanied by the degradation of the fine structure of the meat fiber. Sharp (1963) confirmed that autolysis occurs, but concluded from histological evidence that it mainly affects the sarcoplasmic proteins of the meat.

Locker (1960), using another approach (terminal group analysis), found no significant increase in N-terminal groups indicative of protein breakdown in either the sarcoplasmic or myofibrillar proteins of beef during aging. In contrast, Solovjev *et al.* (1962) showed that a considerable increase occurs in N-terminal groups of myosin after 6 days' aging at 10°C. In this respect, Bodwell and Pearson (1964) pointed out that if reactions of transpeptidation and peptide elongation, known to be catalyzed by proteolytic enzymes occur during aging, then free end-group analysis would not neccessarily be an indication of the amount of protein breakdown in meat.

Landmann (1963) has reviewed briefly previous investigations of the muscle cathepsins and from his own studies he concludes that within the pH range of meat in rigor mortis (pH 5.4–6.8) catheptic activity is to be expected.

If proteolytic changes can be shown to occur and can be measured accurately, it should be possible to demonstrate the extent of the relationship between proteolysis and tenderizing occurring during aging. This paper shows that the rates of tenderizing and proteolysis are different in meat held at a storage temperature in the range normally used (2–37°). Future work to elucidate the mechanism of the tenderizing process will be directed to changes occurring in the fibrous proteins of meat.

EXPERIMENTAL

Meat samplings. Meat was obtained from Aberdeen Angus steers (1–3 years old) and Jersey cows (3–7 years old) slaughtered at the local meat

works. The sections of longissimus dorsi (LD) muscles adjacent to the 10th–14th ribs were removed after the halved carcasses had been held for 24 hr at chiller temperature (2–4°C). To ensure that the ultimate pH of the meat had been reached, the excised samples were stored a further 24 hr at 2°C in an atmosphere of nitrogen. Experience showed that only low rates of tenderizing occurred in meat held at this temperature so that zero time of aging was taken to be 48 hr post-mortem. Muscles having ultimate pH values of 5.5–5.9 were used in the following studies. Proteolysis and corresponding tenderness changes were measured on the right and left LD muscles from the beef carcasses.

Tenderness determinations. Transverse sections approximately 4 cm thick were cut from LD muscles vacuum-sealed in plastic bags and aged at the required temperature. Cooking to an internal temperature of 80°C and objective measurement of meat tenderness were carried out according to the procedure of Marsh *et al.* (1966), with the tenderometer used as described by Macfarlane and Marer (1966). Eight to ten evaluations of tenderness were made at each time during aging by obtaining shear-force values at right angles to the meat fibers.

Total and nonprotein nitrogen determinations. The increase in nonprotein nitrogen is used in these studies as a guide to the extent of proteolysis, although it is recognized that protein sub-units formed may not be determined. Since the muscle cathepsins have a variety of specificities (Landmann 1963), the possible production of such subunits should in fact be related to the increase in nonprotein nitrogen, itself made up mainly of end-products of proteolytic attack. For measuring proteolysis during aging (Fig. 1), 4-g samples of LD muscles used for nitrogen determinations were removed 1 cm from exposed surfaces and homogenized (cutting-blade homogenizer) for 2 min with KCl (40 ml, 0.1*M*). In other experiments (Figs. 3, 4) the proteolysis determinations were made on meat stored in the form of a mince. At intervals during the aging period, portions of the mince (40 g) were homogenized at high speed for 2 min with KCl (1 *l*, 0.1*M*) in a Waring blender with an antifoaming attachment. The major portion of connective tissue was removed by pouring the homogenized samples through nylon mesh (mesh

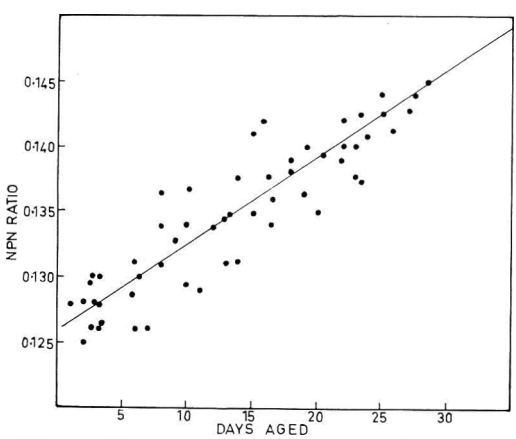


Fig. 1. The increase in the NPN-ratio during aging in nitrogen at 2°C, in the LD muscles of 10 Jersey cows (3–7 years old).

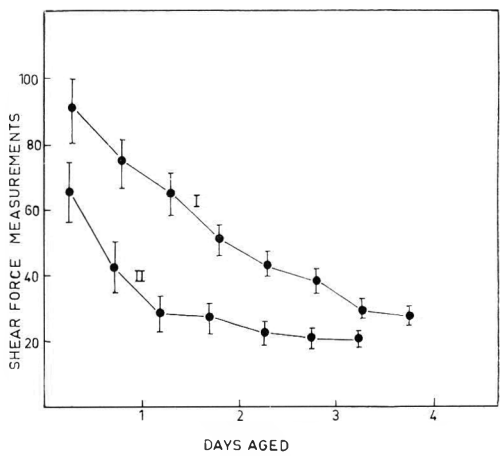


Fig. 2. The time-course of tenderizing in samples of LD muscles aged for 4 days in nitrogen at 15°C. Closed circles, mean of 9 determinations; Vertical lines, standard deviations.
Curve I Jersey cow (5 years old)
Curve II Aberdeen Angus steer (1½ years old)

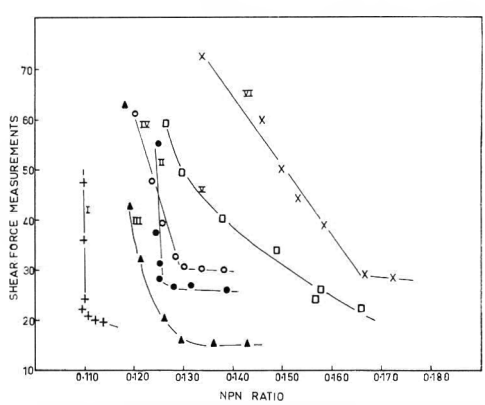


Fig. 3. The relationship between the tenderness and the NPN-ratios in LD muscles from 6 beef animals during aging for 3 days in nitrogen at 15°C. Estimations made at intervals of ½ day.
Curve I Aberdeen Angus steer (1 year old)
Curve II Aberdeen Angus steer (1½ years old)
Curve III Jersey cow (approximately 3 years old)
Curve IV Jersey cow (4 years old)
Curve V Jersey cow (approximately 6 years old)
Curve VI Jersey cow (approximately 7 years old)

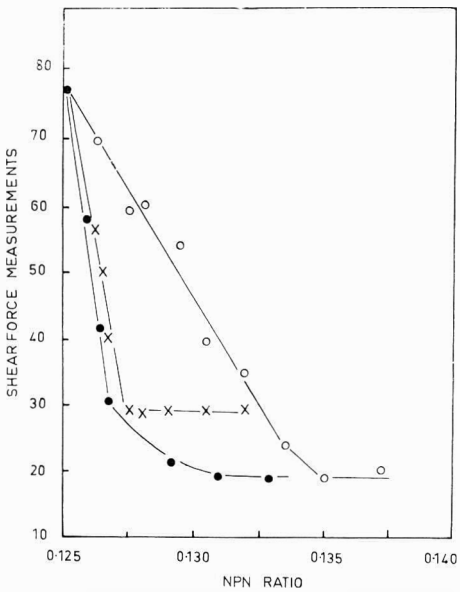


Fig. 4. The relationship between the tenderness and the NPN-ratios in samples of LD muscle from an Aberdeen Angus steer (approximately 2 years old) during aging in nitrogen.
O ——— O 21 days aging, 2°C
● ——— ● 3½ days aging, 15°C
X ——— X 3 days aging, 37°C

diameter 1.2 mm). In all experiments, 5 ml of the gently stirred homogenates were taken for nitrogen determinations with a graduated pipette from which the end had been removed. Nonprotein nitrogen values were determined in quadruplicate after precipitating the proteins of the muscle homogenates (5 ml) with 5 ml, 15% trichloroacetic acid (TCA).

A micro-Kjeldahl method was used for determining nitrogen concentration (AOAC, 1955).

The ratio of nonprotein nitrogen to total nitrogen in the homogenates (NPN-ratio) was used as a measure of proteolytic change.

Preparation of extracts for estimating hydrolysis products of nucleic acids. Samples of the muscle homogenates (3 ml) were shaken with ice-cold perchloric acid (10 ml, 0.5*M*) and the denatured protein removed immediately by centrifugation. Absorbency values at 260 mμ were determined on the clear extracts obtained.

Preparation of Supernatants. Samples of meat (200 g) were minced once and then homogenized at high speed for 2 min in a Waring blender with an equal volume of KCl (0.16*M*). The supernatants obtained by centrifugation (15 min, 1000 × G) were adjusted to pH 5.8 with sodium phosphate buffer (pH 7.0, 1.0*M*) and NPN-ratios determined as described above.

Bacterial control. In order to limit bacterial spoilage, the meat samples were processed and stored under aseptic conditions. Great care was taken to ensure the sterility of the glassware mincers, homogenizing cups, scalpels, etc. Whole meat was stored in a nitrogen atmosphere and sprayed with aureomycin (200 ppm) at intervals during the storage period. The samples of minced meat were collected in sterile plastic bags tied over the mincer plate. The bags of mince were vacuum-sealed and stored at 15°C until required. The extent of bacterial growth was determined on 1-g samples of the mince, and on whole meat samples (1 g) 1 cm from exposed surfaces. The samples were shaken with sterile peptone water (10 ml, 0.1%), and 1-ml portions of the rinsing solutions were plated on yeast extract nutrient agar. After incubating the plates for 5 days at 25°C the bacterial counts were determined. By adhering strictly to these procedures, bacterial numbers were maintained at the low levels of 10–20 organisms /g of whole meat aged 3 days at 15°C or 30 days at 2°C, and 10–100 organisms/g of minced meat aged 3 days at 15°C.

RESULTS

The increase in the NPN-ratio, presumed to indicate proteolysis occurring in the LD muscles of beef carcasses, is shown in Fig. 1. The NPN-ratio of the whole meat increases from 0.126 to 0.145 in 28½ days, based on the linear regression line of which aging time is the independent variable. The considerable scatter of values is partly accounted for by the different rates of increase in the NPN-ratio among the muscles of the animals examined.

The observed increase in nonprotein nitrogen may have arisen from hydrolysis of nucleic acids and not from proteolytic changes within the meat. The nucleic acids are normally insoluble in TCA whereas their hydrolysis products are soluble. There is approximately 3.2 mg nucleic acid/g meat (Young and Dinning, 1951). The nucleoside derivatives from a breakdown of this nucleic acid should double approximately the absorbancy/g meat at 260 mμ. In fact, decreases of 5–10% were observed in the LD muscles from the 6 beef carcasses studied. In a typical case the decrease was from 37.1 to 34.3 absorbancy units over 30 days of aging at 2°.

The demonstration of a relationship between rates of tenderizing and proteolysis in meat during aging largely depends upon the accuracy with which these two processes can be determined. Measurements of tenderness during the time-course of aging are subject to wide variations. Fig. 2 shows the tenderness changes in LD muscles, in this instance, from a 5-year-old Jersey cow and a 1½-year-old Aberdeen Angus steer. Of special

importance are the standard deviations, which decline in value with increasing tenderness of the meat. From more extensive analyses covering 400 separate determinations of tenderness of the LD muscles from 20 beef animals the standard deviations of the shear-force measurements are not very different from the values given in Fig. 2. For this reason, only the mean values are given in further diagrams. In determining the NPN-ratios, the scatter is largely reduced by sampling a mince of the LD muscles at intervals during aging. In these circumstances the coefficient of variation was found to be less than 1% for 15 determinations of the NPN-ratio. Since the activity of proteolysis is fully retained in sarcoplasmic extracts prepared from whole meat (Fig. 5), it is justified to conclude that mincing alone does not affect such activity.

If the increase in meat tenderness is a consequence of the observed proteolysis, it should be possible to show relationship between these processes that is independent of differences in tenderizing rates (see Fig. 2). Fig. 3 illustrates the relationship between tenderness values and NPN-ratios during aging at 15°C in the LD muscles from beef carcasses. The initial NPN-ratios of the unaged muscles from the older animals tend to be greater than those of the younger animals—in the extreme cases studied, 0.134 compared with 0.110. There also tend to be larger increases in the NPN-ratios over the period of 3 days' aging, indicative of a more rapid rate of proteolysis in the LD muscles of the older animals. Conversely, the muscles of the younger animals show a much more rapid tenderizing, more than 80% of the observed change having occurred in the first day of storage (animals I and II). The same percentage increase in tenderness is shown to take place in approximately 2½ days in the muscles of the older animals (animals V and VI). Fig. 3 shows that widely different curves relating tenderness to proteolysis are obtained from the LD muscles of different beef carcasses. The age of the animal appears to determine largely the position of the curve.

A study was made of the effect of temperature on the tenderness values and the NPN-ratios during the aging of the LD muscles of a 3-year-old steer (Fig. 4). Separate curves are obtained for the 3 temperatures studied, there being a greater difference between the 2 and 15°C curves than between the 15 and 37°C curves. This result was consistent with the results obtained on the LD muscles from 6 other beef animals studied.

The sample of muscle aged at the higher temperature (37°C) was finally less tender (shear-force 29) than the sample aged at either 15°C (shear-force 19) or at 2°C (shear-force 20).

This is not a consistent pattern and seems to depend upon the ultimate pH of the meat. It is possible that at 37°C denaturation effects at low ultimate pH (5.4) limit further tenderizing during aging, whereas at higher ultimate pH values (> 6.0) denaturation is less active thus allowing a more complete tenderizing of the undenatured meat.

A study has been made of the increase that occurs in the NPN-ratio during storage at 2°C of the supernatants obtained after low-speed centrifugation (1000 × G) of homogenates from the LD muscles of 7 beef animals. Fig. 5 shows a typical result, obtained on the LD muscles of a 5-year-old Jersey cow under conditions where complete sterility was not achieved.

Also shown in Fig. 5 is the line that would be expected if the increase in the NPN-ratio during storage is due entirely to proteolysis in the sarcoplasmic fraction of meat. It is derived from the regression line in Fig. 1 by assuming that the proteolytic enzymes remain saturated with substrate in the diluted supernatant and that the sarcoplasm is contained in 80% of the whole muscle volume. The bacterial count of the supernatant remained relatively low for the first 13 days of storage (6×10^4 organisms/ml), after which time bacterial population increased rapidly, rising to 2×10^7 organisms/ml in 28 days. The curve relating the NPN-ratio to storage period was, in this experiment, identical with the line expected

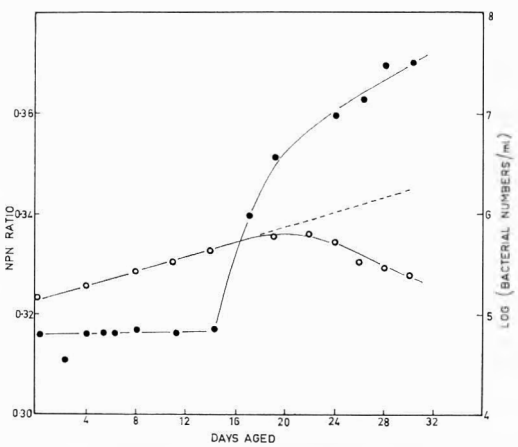


Fig. 5. The change on aging at 2°C in the NPN-ratio and the bacterial load of the sarcoplasmic fraction obtained from the LD muscle of a Jersey cow (5 years old).

- - - NPN-ratio based on the regression line (Fig. 1)

O —— O NPN-ratio of the supernatant

● —— ● log (bacterial numbers/ml supernatant)

from Fig. 1 for the first 20 days, after which time it began to fall.

In these studies the increase of nitrogen-containing compounds in the TCA-soluble fraction of meat has been considered to be a measure of proteolysis. Some degradation of proteins may have occurred into TCA-insoluble units before changes in the nonprotein nitrogen values could be detected, possibly negating the NPN-ratio as a meaningful parameter. However, Fujimaki and Deatherage (1964) have demonstrated a decrease during aging in eluted peaks obtained on chromatographing the sarcoplasmic proteins from the muscles of beef animals. This suggests that no significant sub-unit formation by endopeptidase attack of sarcoplasmic proteins can in fact be occurring during aging.

DISCUSSION

It has been established that, within the range of temperature normally used in aging meat, limited proteolytic changes independent of bacterial action occur in the LD muscles of beef carcasses. This is in broad agreement with the work of Zender *et al.* (1958), Radouco-Thomas *et al.* (1959), and Sharp (1963), but is contrary to the conclusions of Husaini *et al.* (1950) and Wierbicki *et al.* (1954). Of especial significance is the finding that the observed proteolytic changes are not related to the tenderizing of meat during aging.

The present results show a mean increase of 0.020 in the NPN-ratio in the LD muscles from 10 animals in 30 days at 2°C. The total nitrogen content of these muscles from 24 animals was shown to vary little, and to be 2.23 (S.D. $\pm$ 0.02) mM/g meat. Thus there is a mean increase in nonprotein nitrogen of 0.045mM/g meat in 30 days' storage at 2°C. This nitrogen could arise from either hydrolysis of nucleic acids or from proteolytic changes within the meat. The nucleic acids are normally insoluble in TCA, whereas their hydrolysis products are soluble. There is approximately 3.2 mg nucleic acid/g meat (Young and Dinning, 1951). With an average nitrogen content of 18.5%, 0.043mM/g of nitrogen could be released into the nonprotein nitrogen fraction from this source. This would account for the increase in the NPN-ratio during aging for 30 days at 2°C. However, the spectrographic evidence obtained shows that no such hydrolysis of nucleic acids is occurring. It must therefore be concluded that the increase of 0.02 in the NPN-ratio in meat during aging is derived wholly from proteolysis and represents 2.3% of meat protein degraded into TCA-soluble material.

It is the myofibrils which are called upon to withstand tensions in the living muscle of at least 5 Kg/cm^2 (Weber and Portzehl, 1952). For this reason alone, it can be assumed that the myofibrils will offer much greater resistance to transverse shearing-forces than will the sarcoplasmic components of muscle. Thus the finding that the proteolytic changes are located entirely within the sarcoplasm does not favor the view that such proteolytic changes are responsible for the tenderizing during aging.

Proteolysis and tenderizing are progressive changes occurring in meat during aging. A relationship between them will be meaningful only if it is independent of variations in characteristics such as breed, sex, and age of the animals under study. In this respect the assessment of the relationship will depend upon whether comparisons can be made of the results from separate animals. It has proved possible to make such comparisons in this study. Major differences in the rates of tenderizing during aging are observed in the LD muscles from different animals. However, as Fig. 3 has shown, no parallel differences in rates of proteolysis can be observed. Indeed, at least 80% of the tenderizing changes can be completed before proteolytic changes can be detected in some cases (Animals I and II, Fig. 3). This tells heavily against any view which suggests a relationship between proteolysis and tenderizing during aging. This conclusion is substantiated further by the studies of the effect of temperature on proteolysis and tenderizing during aging. It has been shown in Fig. 4 that separate curves relating tenderness and NPN-ratios are obtained for different temperatures of aging. In this respect the change in shear-force value per unit increase in NPN-ratio at 15°C was four times as great as at 2°C. Thus, no direct relationship between proteolysis and tenderizing during aging can be expected.

The finding that there are very few micro-organisms deep within whole aged meat,

indicates that neither the observed tenderizing nor proteolysis finds its origin in bacterial action.

The main conclusion from this study has been that the detectable proteolytic changes are not related to the tenderizing of meat during aging. Future work will therefore be directed to more specific possible causes of the tenderizing mechanism.

REFERENCES

AOAC. 1955. Official Methods of Analysis. 8th ed. Assoc. Office Agr. Chemists, Washington, D.C.

Balls, A. K. 1938. Enzyme action in food products at low temperature. *Ice and Cold Storage* **41**, 85.

Bodwell, C. E., and A. M. Pearson. 1964. The activity of partially purified bovine catheptic enzymes on various natural and synthetic substrates. *J. Food Sci.* **29**, 602.

Fujimaki, M., and F. E. Deatherage. 1964. Chromatographic fractionation of sarcoplasmic proteins of beef skeletal muscle on ion-exchange cellulose. *J. Food Sci.* **29**, 316.

Hoagland, R., C. M. McBryde, and W. C. Powick. 1917. Changes in fresh beef during cold storage above freezing. U. S. Dept. Agr. Bull. No. 433, 100.

Hodgkiss, W., and N. R. Jones. 1955. The free amino acids of fish. Post-mortem changes in sterile skeletal muscle of North Sea Codling Gadus callarius). *Biochem. J.* **61**, IV.

Kastelic, J. 1953. Cathepsins in animal tissue. Seminar at Iowa State College, Ames, Iowa.

Landmann, W. A. 1963. Enzymes and their influence on meat tenderness. Campbell Meat Tenderness Symposium.

Leinati, A. L. 1957. Investigations on the aging of meat from slaughtered animals by means of electrophoretic and chromatographic methods. *Arch, exptl. Veterinarmed* **11**, 13.

Locker, R. H. 1960. Proteolysis in the storage of beef. *J. Sci. Food Agr.* **9**, 520.

Marsh, B. B., P. R. Woodhams, and N. G. Leet. 1966. Studies in meat tenderness. I. Sensory and objective assessments of tenderness. *J. Food Sci.* **31**, 262.

Macfarlane, P. G., and J. M. Marer. 1966. An apparatus for determining the tenderness of meat. *Food Technol.* (submitted for publication).

Niewiarowicz, A. 1956. Changes in the amino-acid content and peptides during aging of beef and pork. *Przemys Spozywczy* **10**, 280. *Chem. Abstr.* **52**, 9470 (1958).

Radouco-Thomas, C., C. Lataste-Dorolle, R. Zender, R. Busset, H. M. Meyer, and R. F. Mouton. 1959. The anti-autolytic effect of epinephrine in skeletal muscle: non-additive process for preservation of meat. *Food Research* **24**, 453.

Sharp, J. G. 1963. Aspetic autolysis in rabbit and bovine muscle during storage at 37°. *J. Sci. Food Agr.* **7**, 468.

Shewan, J. M., and N. R. Jones. 1957. Chemical changes occurring in cod muscle during chill storage and their possible use as objective indices of quality. *J. Sci. Food Agr.* **8**, 491.

Solovjev, V. I., V. A. Adutskevitch, G. N. Kunzsetsova, A. G. Volkova, O. P. Shegoleva, Z. A. Agapova, and A. V. Agleetskoya. 1962. The chemistry of the changes occurring in meat both during aging and after treatment with proteolytic enzymes. *Rept. of All Union Inst. Research in Meat Ind. (Moscow)* 1962, No. **14**.

Weber, H. H., and H. Portzehl. 1952. Muscle contraction and fibrous muscle proteins. *Advances in Protein Chem.* **7**, 161.

Wierbicki, E., L. E. Kunkle, V. R. Cahill, and F. E. Deatherage. 1954. The relation of tenderness to protein alterations during post mortem aging. *Food Technol.* **8**, 506.

Young, J. M., and J. S. Dinning. 1951. A relationship of vitamin E to nucleic acid metabolism. *J. Biol. Chem.* **193**, 743.

Zender, R., C. Lataste-Dorolle, R. A. Collet, P. Rowinski, and R. F. Mouton. 1958. Aseptic autolysis of muscle: biochemical and microscopic modifications occurring in rabbit and lamb muscle during aseptic and anaerobic storage. *Food Research* **23**, 305.

Ms. rec'd 7/2/65.

The authors are grateful to Miss S. H. Smith and Mr. A. G. Lees for technical assistance, and to the Auckland Farmers' Freezing Co-operative Limited for provision of the meat samples.

S. H. SCHANDERL and DENISE Y. C. LYNN
Department of Food Science, Michigan State University
East Lansing, Michigan 48823

Changes in Chlorophylls and Spectrally Related Pigments During Ripening of Capsicum frutescens

SUMMARY

Hungarian yellow wax peppers *(Capsicum frutescens)* were selected at progressive stages of ripening. During these turning stages, compounds appeared which were separated by two-dimensional thin-layer chromatography and their spectra determined. Chlorophylls *a* and *b* and five or more pink fluorescent compounds were detected at different ripening stages of the immature fruit. Two compounds were the last to disappear and are thought to be somewhat more stable degradation products of chlorophylls *a* and *b*. No pink fluorescent compounds were found in the fully mature pepper.

INTRODUCTION

The degradation of chlorophylls in ripening fruits or autumn leaves has long been of interest, but investigations have in most cases been limited to quantitative measurement of the disappearance of chlorophyll or the appearance of the first degradation products of an oxidative or hydrolytic nature. A complete understanding of the sequence of reactions not only should provide additional information on the events that occur in plant tissues, but may also permit a better understanding of the degradation of heme and bile pigments which are pyrrolic in nature.

The types of degradation reactions were studied by Strain (1954) and found to be isomerization, hydrolysis, and oxidation, but no products of such reactions were found in living plants subjected to various treatments. It has been concluded by Sud'ina and Romanenko (1961) that chlorophyllase is not necessary as a step in the scheme of degradation of chlorophylls. Schanderl *et al.* (1962) have shown that a direct proportionality exists between length of side chain and activation energy for the acid hydrolysis of chlorophyll and chlorophyllides, a factor which may be of importance in the biodegradation of chlorophylls during ripening.

Earlier work in this laboratory has been conducted with bananas because of their availability throughout the year and because the change from deep green to full yellow occurs within 4–8 days at room temperature. Brief investigations were attempted with tomatoes and strawberries, but the work reported here was conducted on green peppers, for two main reasons: 1) the significant color change which some varieties of green peppers undergo leads ultimately to a fruit free of chlorophyll or derivatives; and 2) the concentration of easily extractable pigments in the carpel tissue is high, and there are no undesired interfering substances, such as the polyphenols in banana peel.

The timing of the color changes was observed to predict the stages of degradation. This particular variety of *Capsicum frutescens* undergoes a change from light green to dark green. The dark-green color remains unchanged for quite a period, during which it is assumed that the enzymes are formed which carry on the complete destruction of chlorophyll. During a period which can be as short as 24 hr, a change in color occurs from dark green through olive green and bright orange, to the dark-red color of the fully ripe fruit.

After this introductory study it was attempted to isolate the pigments and study typical patterns that might exist in the derivatives present at these stages. The literature on methods of separating these compounds was reviewed by Lynn and Schanderl (1965), and a new method was described permitting the separation of more than 18 chlorophylls and related compounds.

In this study it was attempted to identify as many compounds as possible that occur at the different stages of ripeness described above and to observe whether some of them are typical of certain stages.

METHODS AND MATERIALS

Five-gram samples of carpel tissue at 4 selected stages of ripeness were extracted repeatedly with acetone. The tissue was ground in a mortar at 0°C under minimum illumination. Pure glass sand was added to aid grinding, and a pinch of magnesium carbonate was used to neutralize the acids

liberated from the tissue. Five to 6 extractions of 5 ml acetone each were carried out speedily and the extracts combined. Upon the addition of saturated NaCl solution a binary system formed with all fluorescent pigments in the upper layer. The lower layer was discarded. After 3 washings with saturated NaCl solution, 5–10 ml of ether were added to the upper solvent layer. This mixture was washed repeatedly with H_2O to remove acetone and dried over Na_2SO_4. The amounts spotted were based on the final volume of extract. Spotting on the silica gel plates was done in the dark in less than 10 min.

The method described by Lynn and Schanderl (in press) was applied to separate the pigments using Bauer solvents I and II for two-dimensional development on 8-inch plates coated with silica gel G. The spots detected under UV light (366 mμ maximum output) were circled.

RESULTS AND DISCUSSION

Four stages of ripeness were selected for analysis and the results are shown in Fig. 1. The ripening stage 1 is whitish yellow; 2, dark green; 3, olive orange; and 4, bright orange. The subsequent stage of dark red did not yield any fluorescent chlorophyll derivatives. The spots were named *A*, *B*, *C*, *D*, *E*, *F*, *G*, and *H*, according to their appearance in the first dimension of the chromatogram in stage 3, when the maximum number of compounds was present. Some

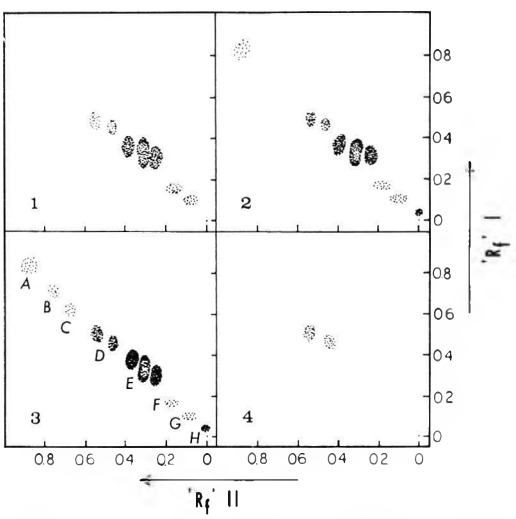


Fig. 1. A schematic drawing of two-dimensional chromatograms showing the changes in pigments during 4 progressive stages of ripening of *Capsicum frutescens*. Stages 1 and 2 represent those of increasing greenness, stage 3 the turning (breaking) point and 4 the one just prior to complete ripeness.

Table 1. Relative R_f values obtained from two-dimensional separation of chlorophylls and related compounds [a] extracted from ripening *Capsicum frutescens*.

Spots	R_f I	R_f II
A = pheophytin *a*	.83	.88
B	.71	.75
C	.63	.68
D_b	.50	.54
D_a	.45	.46
E_1 = chlorophyll a_1	.37	.37
E_2 = chlorophyll a_2 (+ b_1)	.35	.30
E_3 = chlorophyll b_1 (+ a_2)	.31	.30
E_4 = chlorophyll b_2	.31	.25
F	.18	.17
G	.10	.08
H	.05	0

[a] Compounds are labeled according to the first-dimensional separation at stage 3 of ripeness. Compound *D* was further separated into 2 compounds, while compound *E* was further separated into 4 chlorophylls and their isomers.

separate further in the second dimension. The first stage shows chlorophyll *a* and chlorophyll *b* and, between them, the two isomers, which are difficult to separate from each other and have the same spectra as chlorophylls *a* and *b*. Ahead of chlorophyll *a* are two spots. These belong to a compound here called *D*, which was later separated further. Behind the chlorophyll group appear spots *F* and *G*. Notably absent at this stage are spot *A* (pheophytin) and spot *H*. The fact that a chromatogram can be made without any formation of pheophytin indicates that the procedure of extraction and chromatography minimizes artifacts.

In stage 2, the same pigments are found as in stage 1 but in higher concentration, and the first indication is given of pheophytin and spot *H*. The appearance of spot *H* coincides with the beginning of the turning stage, as seen in Fig. 1, while stages 1 and 2 are those of a buildup of chlorophyll though some biodegradation may already occur. The next stage, 3, shows the appearance of two substances, *B* and *C*, of higher R_f values than either the chlorophylls or compound *D*. They are present in very small amounts, and at this stage do not permit good spectral identification. Stage 4 depicts the only two spots remaining after all derivatives have disappeared. They are D_b and D_a.

The spots on the silica gel plate were

scraped off and eluted with ether, and their spectra are shown in Figs. 2 and 3. The elution of the spot called *H* is difficult since it is very tightly adsorbed on the silica gel. The spectrum of this compound is therefore obtained by spotting the original extract on a sugar plate prepared according to Colman and Vishniac (1964) and developed with the solvent of Grob *et al.* (1961), neglecting all compounds but the most polar one. With this method, *H* was easily eluted for spectroscopy, and its identity was checked by rechromatography on silica gel.

Fig. 2 shows the spectrum of pheophytin *a* (Zscheile and Comar, 1941), found in the spot called *A*. No spectrum is available for *B*, since it was never present in large enough amounts to be eluted. A rather weak spectrum of compound *C* is shown, matched by the best spectrum obtained of *C* from spinach extracts, shown as a dotted line. A clear spectrum of *F* can be obtained, but *H* appears to contain more than one compound.

The upper left of Fig. 3 shows a spectrum of the fastest moving component of spot *D*. Since it showed characteristics of chlorophyll *b*, it was called D_b. The other component, shown below in the solid line, was called D_a. Both spectra are shown, matched by the spectra of the corresponding spots (dotted line) obtained in larger amounts from spinach extracts, to aid identification.

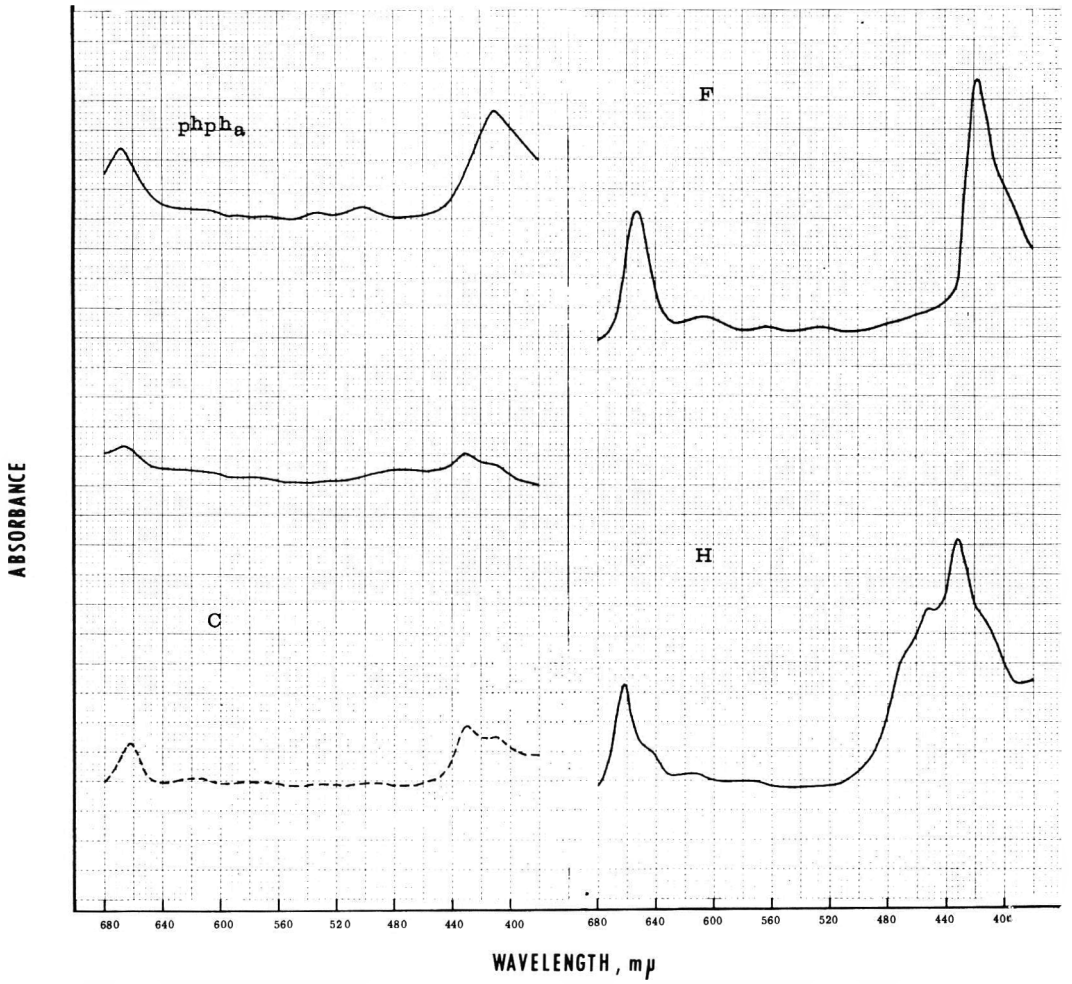


Fig. 2. Absorption spectra of pheophytin *a* (spot *A*) and 3 other typical compounds obtained from *Capsicum* extracts (solid lines) and from spinach extract (broken line). Spot *C* is a compound less polar than the chlorophylls and *F* and *H* are more polar derivatives. The spectrum of *H* shows some characteristics of chlorophyll *a* with trace amounts of *b*.

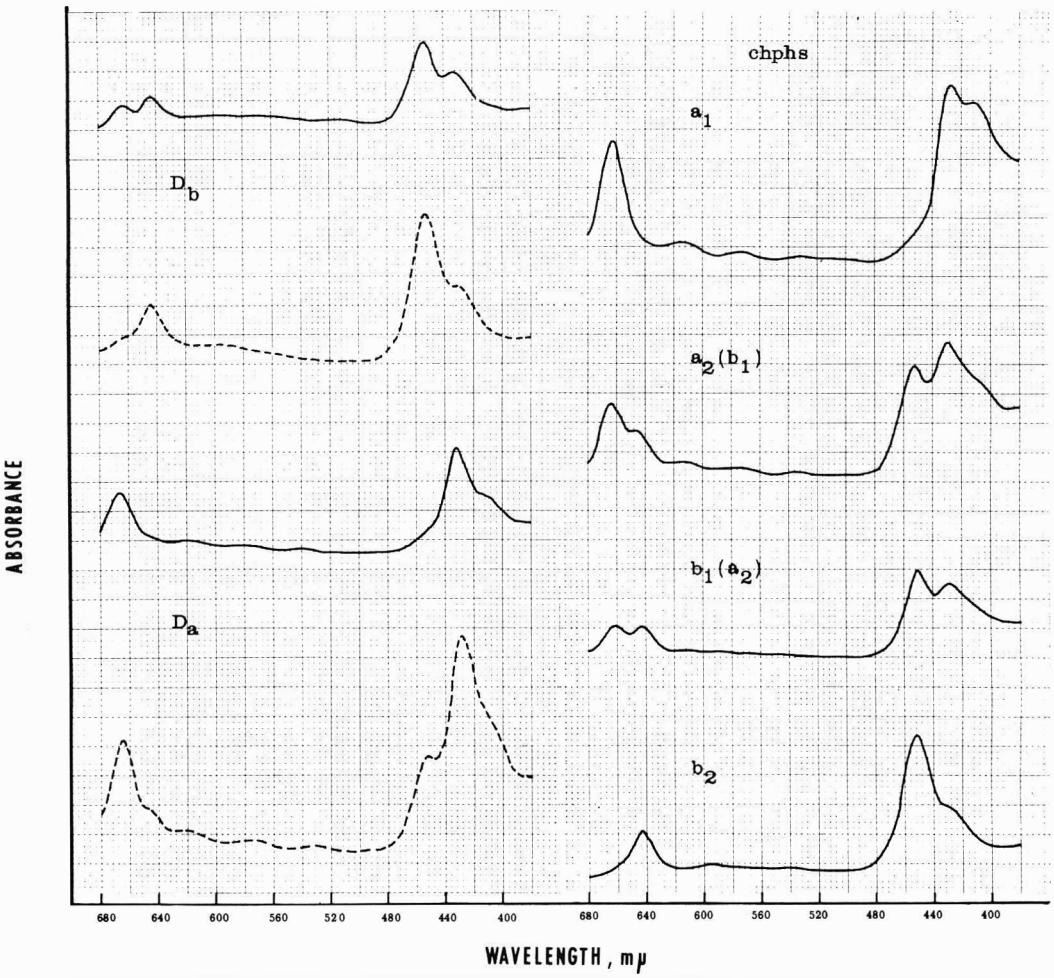


Fig. 3. Absorption spectra of 4 chlorophylls (right column) and 2 related compounds (left column) obtained from *Capsicum* extracts (solid lines) and from spinach extracts (broken lines). The spectra of the chlorophylls named here a_1 and b_2 are quite pure, with the middle spots showing overlap and doublepeaks both in the red and blue regions of the spectrum.

On the right side of Fig. 3 are the chlorophyll spectra. The first one is pure chlorophyll *a*. The second one is thought to be the isomer of *a* with traces of *b*. The third one is thought to be the isomer of *b* with traces of *a*, and the last one is pure chlorophyll *b*.

The *D* compounds are of particular interest during ripening since they disappear last. They are present in small amounts throughout the other stages, but appear to increase during stage 3. Their degradation appears to be slower and they are therefore retained longer than any other fluorescent compound. As with the others, it is not clear, at present, whether these compounds are degradation products of chlorophylls. They may be precursors or degradation products of chlorophyll precursors remaining from the biosynthesis.

REFERENCES

Colman, B., and W. Vishniac. 1964. Separation of chloroplast pigments on thin layers of sucrose. *Biochim. et Biophys. Acta* **82**, 416.

Grob, E. C., W. Eichenberger, and R. P. Pflugshaupt. 1961. Zur Bildung der Carotinoide in Blättern, Blüten and Früchten. *Chim. Aarau* **15**, 565.

Lynn, D. Y. C., and S. H. Schanderl. 1966. Separation of chlorophylls and related plant pigments by two-dimensional thin-layer chromatography. (in preparation)

Schanderl, S. H., C. O. Chichester, and G. L. Marsh. 1962. Degradation of chlorophyll and

several derivatives in acid solution. *J. Org. Chem.* **27**, 3865.

Strain, H. H. 1954. Oxidation and isomerization reactions of the chlorophylls in killed leaves. *J. Agr. Food Chem.* **2**(24), 1222.

Sud'ina, O. G., and E. Romanenko. 1961. Changes in pigment content and chlorophyllase activity in fruits. *Ukrain. Botan. Zhur.* **18**(4), 3.

Zscheile, F. P., and C. L. Comar. 1941. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. *Botan. Gaz.* **162**, 436.

Ms. rec'd 5/19/65.

Supported in part by NIH Grant No. EF-00531, Division of Environmental Engineering and Food Protection.

The technical assistance of Mrs. Dianne Carlton and Roger McFeeters is gratefully acknowledged.

M. HAMOSH, R. ATIA, and B. SHAPIRO
Department of Biochemistry,
Hebrew University Hadassah Medical School,
Jerusalem, Israel

Fatty Acid Uptake and Esterification by Fish Muscle

SUMMARY

Fresh carp muscle takes up fatty acid from a solution of bovine serum albumin–fatty acid complex, and converts a considerable part of the fatty acid taken up into glycerides. In this regard, brown muscle is much more active than white muscle. On storage of the muscle at −18°C, its ability to take up fatty acid is not impaired but its esterifying capacity is rapidly reduced to a low level. Upon storage at 0°C the esterifying capacity stayed intact for at least 24 hr.

Of various fatty acids tested, uptake is highest with the long-chain acids (C_{16}—C_{18}), with no marked difference due to unsaturation. Unsaturated acids (oleic and linoleic) and a short-chain acid (caprylic) are partly adsorbed to fish muscle proteins so strongly that they cannot be extracted with acid isopropanol-heptane.

INTRODUCTION

In recent years, changes in the concentrations of unesterified fatty acids have assumed an increased importance for certain aspects of fish technology. In particular, a relationship between the hydrolysis of lipids and the denaturation of muscle proteins during the cold storage of fish has been suggested (Dyer and Fraser, 1959; Dyer and Dingle, 1961). This relationship may be due either to a stabilizing effect of intact lipids on actomyosin, abolished by lipid hydrolysis, or to the effect of fatty acids formed during lipid hydrolysis causing actomyosin inextractability. King *et al.* (1962) were able to show that small concentrations of linoleic and linolenic acids reduced the solubility of purified cod actomyosin. This denaturation is important because it is supposed to be the main change underlying textural deterioration in fish.

The concentration of unesterified (so-called "free") fatty acid increases both during the cold storage of fish muscle (Dyer and Frazer, 1959; Olley and Lovern, 1960) and during storage in the unfrozen state of 0° (Lovern, Olley and Watson, 1959). In the present investigation, the uptake and esterification of various free fatty acids added to frozen and stored fish muscle were studied in order to throw further light on the mechanism by which the concentration of the acids changes. The study also adds to the few reports on the enzymatic activities of fresh fish muscle and their changes upon storage (e.g. Siebert, 1962).

EXPERIMENTAL

Fresh carp and several species of sea fish were used. The sea fish were kept on crushed ice for 14–24 hr after capture. They were then transported to the laboratory while frozen (usually 8–24 hr). Immediately upon arrival the fish were skinned, carefully wrapped in aluminum foil, and stored at −18°C. The carp were stunned, killed by decapitation, and skinned. Tissue slices were cut with a Stadie and Riggs (1944) tissue slicer. The tissue slices of brown muscle were prepared from about 1-cm^2 samples taken along the lateral line. The white muscle slices were prepared from the dorsal section of the muscle. Slices from frozen fish were prepared free-hand (1–2 mm thick).

Uptake and esterification of free fatty acids was determined by incubating 200–350 mg fish muscle slices in 2 ml of a medium containing 2–3 μc labeled fatty acid with 0.8–1.0 μmole carrier fatty acid dissolved in a 1% solution of bovine serum albumin (free fatty acid poor, Pentex Corporation, Kankakee, Ill.) in a calcium-free Krebs Ringer phosphate buffer at pH 7.4 (Umbreit *et al.*, 1957).

Incubation was carried out with shaking at 30 or 0°C, as specified. At the end of the indicated incubation period the fatty acids in the medium and tissue and the tissue glycerides were extracted and analyzed as described by Kerpel *et al.* (1961). All the samples were counted for radioactivity in the Packard Tri-Carb liquid scintillation counter.

The concentration of fatty acid strongly bound to the muscle protein was determined by re-extracting the tissue twice more with the extraction mixture of Dole (1956), namely, isopropanol, heptane, 1*N* H_2SO_4 (40:10:1 by volume). After these extractions the radioactivity of the tissue residue was determined and expressed as a percentage of that originally present in the whole incubation mixture. Uptake is calculated as percent of the added radioactivity found in the tissue and corrected to 100 mg of wet tissue. Esterification is expressed as the percent of radioactivity in the tissue extracts, present as esterified fatty acids. No esterified fatty acids were found in the medium. All the results given are the means of 2–4 experiments.

RESULTS

Carp was the only species available alive, so comparison of the metabolism of frozen tissue to that of fresh one was limited to this species. In preliminary experiments it was found that brown carp muscle released larger amounts of free fatty acids than white muscle, when stored at 0°C or at −18°C or when incubated fresh or after storage. It was therefore interesting to compare the free fatty acid uptake and esterification of these two types of muscle. With 1-C^{14} palmitic acid it was found that free fatty acid uptake levels off after 2 hr of incubation. In each experiment, 2–3 incubation times were included, but for the sake of clarity only the results obtained at the 2-hr incubation interval are given. As can be seen from Table 1, fresh brown muscle has a higher uptake and esterifying capacity than white muscle. Lowering the temperature to 0°C markedly reduced palmitate uptake and esterification in both types of tissue. Frozen storage reduced esterification to very low levels, whereas the uptake of unchanged free acid stayed unchanged or even increased.

In the results given in Table 1, the frozen tissue was kept at −18°C for one month. In the following the esterifying capacity was determined at much shorter storage intervals. From Table 2 it can be seen that about 70% of the initial esterifying capacity of the muscle is lost after 4 hr of storage at −18°C. Uptake of unchanged free acid was not decreased by frozen storage.

The effect of the temperature of storage on the

Table 1. 1-C^{14} palmitate uptake and esterification by frozen and fresh carp muscle.

	Uptake		Esterification	
	% per 100 mg wet tissue		Glycerides as % of total radioactive tissue lipids	
	30°	0°	30°	0°
Fresh tissue				
Brown muscle	5.60	1.70	61.0	16.7
White muscle	1.93	1.02	11.9	3.20
Frozen tissue				
1 month, −18°C				
Brown muscle	5.42	3.15	2.80	3.20
White muscle	4.37	3.32	2.90	2.45

For details, see Experimental.

Table 2. Effect of storage on 1-C^{14} palmitate uptake and esterification by brown carp muscle.

	Brown carp muscle incubated at 30°C				
Storage time at −18°C	0 hr	4 hr	7 hr	25 hr	47 hr
Uptake					
% per 100 mg wet tissue	5.25	4.38	4.68	3.57	4.75
Esterification					
Glycerides as % of total tissue lipids	35.0	11.5	8.30	4.65	4.20

Table 3. Effect of storage temperature on 1-C^{14} palmitate uptake and esterification.

	Incubation at 30°C		
	No storage	Storage for 24 hr	
		0°C	−18°C
Brown muscle			
Uptake % per 100 mg wet tissue	7.30	7.00	8.00
Esterification			
Glycerides as % of total tissue lipids	20.0	15.7	2.40
— ditto— + 10 μmoles glucose	20.5	11.0	2.20
White muscle			
Uptake % per 100 mg wet tissue	5.00	5.13	5.12
Esterification			
Glycerides as % of total tissue lipids	4.00	3.84	1.95
— ditto + 10 μmoles glucose	7.55	4.15	1.99

uptake and esterifying capacity of both white and brown muscle was studied next. From Table 3 it is evident that after 24 hr of storage at 0°C there is little change in the amount of palmitic acid esterified, while the same storage interval at −18°C results in a 90% reduction of the esterification by brown muscle and a 50% reduction of that by white muscle. Glucose added to the incubation medium (10 and 20 μmoles per 2 ml medium)—which enhances uptake and esterification of free fatty acid in mammalian tissues—affected esterification in fresh white fish muscle but not in brown one.

In the next experiments the uptake and esterification of 1-C^{14} palmitate were compared in several species of frozen sea fish and carp. All the specimens were previously stored for at least 1 month at −18°C. As can be seen from Table 4, in all the sea species tested, palmitate uptake was highest in cod muscle. Only a small fraction of the palmitic acid incorporated could be found in the glyceride fraction, while more than 90% was retained in the tissue as free fatty acid. There was no difference in results between 0°C and 30°C. Addition of glucose did not affect the results.

A number of free fatty acids of different chain length (C_8—C_{18}) and degree of unsaturation ($C_{18:1}$,

Table 4. 1-C^{14} palmitate uptake and esterification by several frozen fish species stored various periods.

	Carp (white muscle) 1 month	Cod [a]	Saurida 3 months	Diphadus Sargus 2 months	Blunt Jaw Barracuda 2 months	Pagellus Acarra 2 months
Uptake[b]						
Incubation at 30°C	3.92	16.5	4.35	7.00	6.60	7.20
Incubation at 0°C	3.80	14.2	3.30	.....	.....	.....
Esterification[c]						
Incubation at 30°C	2.45	1.50	2.77	7.30	6.25	5.35
Incubation at 0°C	2.45	1.69	3.06	.....	.....	

[a] The cod used in these experiments was commercially available frozen cod; the storage time is unknown.
[b] Percent uptake per 100 mg wet tissue.
[c] Glycerides as % of total labeled tissue lipids.

Table 5. Uptake and esterification of various labeled fatty acids and effect of storage.

	Carp-brown muscle incubated at 30°C					
	Uptake (% per 100 mg wet tissue)			Esterification (glycerides as % of total tissue lipids)		
	No storage	Storage at −18°C		No storage	Storage at −18°C	
Fatty acid		24 hr	10 days		24 hr	10 days
Caprylic C_8	5.0	3.5	.....	24.0	0.75	.....
Lauric C_{12}	7.6	5.6	.....	41.0	0.87	.....
Palmitic C_{16}	10.5	7.9	11.1	37.0	1.87	1.78
Oleic (C_{18}:1)	6.4	6.5	10.6	33.4	1.30	1.21
Linoleic (C_{18}:2)	7.7	7.9	4.8	28.6	1.35	1.60

Table 6. Irreversible adsorption of fatty acids by muscle proteins.

	Incubation at 30°C			
		Carp-brown muscle		
	Cod [a]	No storage	Storage at −18°C	
Fatty acid	white muscle		24 hr	10 days
Caprylic C_8	3.80	.....	.....	.....
Lauric C_{12}	0.043	.....	.....	.....
Palmitic C_{16}	0.076	0.073	0.050	0.045
Stearic C_{18}	0.070	.....	.....	.....
Oleic C_{18}:1	1.00	.....	.....	.....
Linoleic C_{18}:2		1.04	.....	3.50

The results are given as percent of total counts found in the tissue after repeated extraction with isopropanol-heptane (see Experimental).
[a] Commercially available cod, storage time unknown.

$C_{18:2}$) were next tested under identical conditions to those described for palmitic acid. The results in Table 5 on uptake and esterification by carp muscle show that fresh muscle esterifies all the fatty acids tested, and that storage for 24 hr at −18°C causes nearly complete loss of esterifying capacity.

Anderson *et al.* (1963) have shown that addition of unsaturated long-chain fatty acids to the extraction medium considerably reduces the extractability of actomyosin from cod muscle, indicating a strong interaction between acid and protein. Under the conditions of low ionic strength used in the present investigation, it has been found (Table 6) that certain fatty acids are adsorbed by fish muscle so strongly that they cannot be removed by a typical lipid solvent. In particular, a short-chain acid (caprylic) and unsaturated long-chain acids (oleic and linoleic) are adsorbed very strongly. With carp stored at −18°C, this adsorption of linoleic acid increases threefold in 10 days, but no increase occurs with palmitic acid in the same period.

DISCUSSION

Despite the presence of lipases and phospholipases (Olley *et al.*, 1962) the concentration of free fatty acids is low in fresh fish muscle but increases on storage of the unfrozen muscle at 0°C (Lovern *et al.*, 1959). The results presented in this paper indicate that the reasons for this may be as follows: Firstly, efficient re-esterification of free fatty acids occurs in the fresh muscle and thus keeps the concentration low. Secondly, storage at 0°C reduces the ability of the muscle to re-esterify, behavior which would be expected to lead to a progressive increase in the concentration of free fatty acid.

The esterifying system is inactivated much more rapidly with storage at −18°C, and since lipolytic enzymes are still active at about this temperature (Olley and Lovern, 1960), a net increase in free fatty acids will result. This effect occurs independently of any activation of the lipolytic enzymes by freezing.

As measured by the uptake values, the amount of fatty acid loosely bound to the tissue varies with the species of fish, the type of fatty acid, and the duration of storage of the tissue. With carp brown muscle, storage at −18°C results in an increased loose binding of palmitic and oleic acids, and a decreased loose binding of linoleic acid.

In addition to this loose binding, short-chain saturated and long-chain unsaturated fatty acids were firmly bound by the fish protiens, in a form which cannot be extracted by acid isopropanol-heptane. Storage at −18°C increased this binding of linoleic acid severalfold. Since a high percentage of fish fatty acids are highly unsaturated (Lovern, 1962), it is probable that the binding of these acids by muscle protein is responsible, and for the loss of solubility of actomyosin. The above-mentioned experiments of King *et al.* (1962) with purified actomyosin support this hypothesis.

The high activity of brown muscle should be pointed out. There is ample evidence that this muscle contains much higher lipid levels than white muscle (Lovern, 1962) and releases higher amounts of free fatty acids upon storage (authors' unpublished observation). It is thus not surprising to find also a higher fatty acid esterification rate in this part of muscle. In general, brown fish muscle has a higher metabolic activity than white muscle; thus, Bilinski (1963) showed that trout muscle contains an enzyme system that will oxidize free fatty acids and that the brown muscle is considerably more active in this respect than the white one.

REFERENCES

Anderson, M. L., F. J. King, and M. A. Steinberg, 1962. *J. Food Sci.* **27**, 363.

Bilinski, E. 1963. Utilization of lipids by fish. *Can. J. Biochem. Physiol.* **41**, 107.

Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35**, 150.

Dyer, W. J., and J. R. Dingle. 1961. Fish proteins with special reference to freezing. *In:* Fish as Food, vol. 1, pp. 275–328. G. Borgstrom, ed. Academic Press.

Dyer, W. J., and D. I. Fraser. 1959. Proteins in fish muscle 13. Lipid hydrolysis. *J. Fisheries Board Can.* **16**, 43.

Kerpel, S., E. Shafrir, and B. Shapiro. 1961. Mechanism of fatty acid assimilation in adipose tissue. *Biochim. et Biophys. Acta* **46**, 495.

King, F. J., M. L. Anderson, and M. A. Steinberg. 1962. Reaction of cod actomyosin with linoleic and linolenic acids. *J. Food Sci.* **27**, 363.

Lovern, J. A. 1962. The lipids of fish and changes occurring in them during processing and storage. *In:* Fish in Nutrition, pg. 86. H. Heen

and R. Kreuzer, eds. Fishing News Ltd., London.

Lovern, J. A., J. Olley, and H. A. Watson. 1959. Changes in the lipids of cod during storage in ice. *J. Sci. Food Agr.* **10**, 327.

Olley, J., and J. A. Lovern. 1960. Phospholipid hydrolysis in cod flesh stored at various temperatures. *J. Sci. Food Agr.* **11**, 644.

Olley, J., R. Pirie, and H. Watson. 1962. Lipase and phospholipase activity in fish skeletal muscle and its relationship to protein denaturation. *J. Sci. Food Agr.* **13**, 501.

Siebert, G. 1962. Enzymes in marine fish and their role in fish spoilage. *In:* Fish in Nutrition, pg. 80. H. Heen and R. Kreuzer, eds. Fishing News Ltd., London.

Stadie, W. C., and B. C. Riggs. 1944. Microtome for the preparation of tissue slices for metabolic studies of surviving tissues in vitro. *J. Biol. Chem.* **154**, 687.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques, p. 149, Burgess. Minn.

Ms. rec'd 8/26/65.

Supported by the Bureau of Commercial Fisheries, Department of Interior, U.S.A., under Foreign Research Agreement P.L. No. 480.

E. D. ABERLE and R. A. MERKEL

Departments of Food Science and Animal Husbandry
Michigan State University, East Lansing, Michigan 48823

Solubility and Electrophoretic Behavior of Some Proteins of Post-Mortem Aged Bovine Muscle

SUMMARY

The solubility characteristics and starch gel electrophoretic properties of intracellular bovine muscle proteins from the longissimus dorsi and semitendinosus muscles were investigated during a 336-hr post-mortem aging period. The relationship of solubility and electrophoretic behavior to post-mortem tenderization was also investigated. In the semitendinosus muscle, the solubility of sarcoplasmic protein was highest at slaughter and decreased as post-mortem aging proceeded, but no change was observed in the longissimus dorsi muscle. Fibrillar protein was least extractable at 24 hr post-mortem, but its solubility was significantly higher at 168 and 336 hr than at 24 hr post-mortem in both muscles studied. The amount of extractable fibrillar nitrogen was negatively related to Warner-Bratzler shear values as post-mortem aging proceeded. The increase in amount of soluble fibrillar nitrogen during the post-mortem aging periods of 24-168, 24-336, and 168-336 hr was positively related to the decrease in shear value for the respective periods. Fifteen zones were definable in the starch gel electrophoretic patterns of the sarcoplasmic proteins. At the pH used in this experiment, most of the zones were anionic. These anionic zones exhibited more discrete boundaries in the latter stages of aging, and certain new zones appeared or increased in intensity as aging progressed.

INTRODUCTION

The changes which occur in muscle proteins following death of the animal and the effect of such changes upon the properties of muscle have long been of interest to researchers. Deuticke (1932) and Weber and Meyer (1933) observed decreased protein extractability in muscle which had passed into rigor. The changes which occur during the development of rigor mortis are known to be associated with variation in meat tenderness. Ramsbottom and Strandine (1949) reported that beef was more tender 2 hr post-mortem than at 48 hr. It is well established that post-mortem aging improves the tenderness of meat. Wierbicki *et al.* (1954) were among the first to relate protein extractability to tenderness changes during post-mortem aging. They postulated that post-mortem tenderization may be related to the dissociation of actomyosin, which increases protein extractability. In a later report (Wierbicki *et al.*, 1956), however, they presented evidence that dissociation of actomyosin into actin and myosin was not responsible for post-mortem tenderization.

Electrophoretic properties of the muscle proteins have been studied (Kronman *et al.*, 1960; Hartshorne and Perry, 1962), but investigation of the gel electrophoretic properties during post-mortem aging of bovine muscle has not been reported. Fischer (1963) observed 7 bands upon separation of chicken muscle sarcoplasm by acrylamide gel disc electrophoresis, and found one band to disappear during 24-hr post-mortem aging. Neelin and Rose (1964) reported that the starch gel electrophoretic patterns of chicken muscle changed during post-mortem aging.

This study was undertaken to investigate the effects of post-mortem aging upon the solubility of the major protein fractions of bovine muscle from cattle varying in age and sex. Also studied were the effects of post-mortem aging upon starch gel electrophoretic properties of bovine sarcoplasmic proteins, as well as the relationship between solubility and electrophoretic properties of these proteins.

EXPERIMENTAL

Materials. Two cows (6–8 yr old), 2 heifers, and 2 steers (18–24 months old) were included. Samples were obtained from the longissimus dorsi and the semitendinosus muscles within 30 min of slaughter (0 hr) and at 24, 168, and 336 hr post-mortem. Samples of the left longissimus dorsi muscle were excised from the 1st to 2nd lumbar vertebrae region at zero hr. The right longissimus dorsi muscle was used for samples at 24, 168, and 336 hr, which were excised from the 1st to 2nd,

3rd to 4th, and 5th to 6th lumbar vertebrae regions, respectively. Samples of the left semitendinosus were excised from the anterior portion of the muscle at zero hr. The right semitendinosus muscle was used for samples at 24, 168, and 336 hr, starting at the anterior end and progressing posteriorly within each aging period. Paul and Bratzler (1955) reported that cutting bovine muscles prior to the development of rigor mortis interfered with the tenderizing process. Thus, sampling both sides of the carcass avoided use of samples from muscle which had been cut before passing into rigor.

The muscles were aged in the carcasses at 4°C. Steaks approximately 1¼ in. thick were removed from the longissimus dorsi muscle after aging for 24, 168, and 336 hr, for subsequent tenderness determinations. The samples of both muscles were ground once through a pre-chilled (4°C) sample grinder with a 2-mm plate. To minimize microbial contamination, the exposed muscle surfaces were covered with cellophane during aging. In addition, the outer surface of the muscle was removed before grinding. The muscle samples were fractionated into myofibrillar and sarcoplasmic proteins and nonprotein nitrogen, and also used for preparation of the sarcoplasmic extract for starch gel electrophoresis.

Fractionation of muscle proteins. The protein fractionation procedure was described by Lawrie (1961). The ratio of muscle tissue to extractant solution was 1:10 (w/v). Sarcoplasmic proteins were extracted with cold (4°C) 0.03*M* potassium phosphate buffer, pH 7.4. Myofibrillar proteins were extracted by cold 0.1*M* potassium phosphate buffer, pH 7.4, containing 1.1*M* potassium iodide. Nonprotein nitrogen was determined after precipitation of the proteins with 10% trichloroacetic acid. Fibrillar and sarcoplasmic protein nitrogen and nonprotein nitrogen in the extracted solutions were determined by the micro-Kjeldahl procedure (AOAC, 1960) and expressed as percentage of total nitrogen. Total nitrogen content of the tissue was determined by the macro-Kjeldahl procedure (AOAC, 1960). Stroma nitrogen was obtained by difference.

Preparation of sarcoplasmic extract for electrophoresis. Ground muscle tissue was homogenized with 2.5 volumes of deionized water in a Waring blender. After centrifugation for 30 min at 1750 × G, the extract was filtered through fine gauze to remove any fat particles and then through Whatman No. 1 filter paper. A fairly clear solution resulted. The extract was adjusted to pH 8.6 with 1.0*M* tris (hydroxymethylaminomethane) and then dialyzed against 0.01*M* tris-0.001*M* citric acid buffer containing 0.5*M* sucrose. Dialysis concentrated the extract from approximately 2½ to nearly 4% protein.

Starch gel electrophoresis. The electrophoresis apparatus described by Wake and Baldwin (1961), modified to include a water-cooled jacket, was employed in this experiment. The gels were suspended vertically and maintained at 4°C by pumping cold water through the apparatus. The discontinuous buffer system of Scopes (1964) was modified to include citric acid instead of diethylenetriamine-penta-acetic acid. The starch gel was prepared with 0.012*M* tris-0.0018*M* citric acid buffer, pH 8.6 at 4°C, using 12 g of starch (Connaught Medical Research Laboratories, Toronto) for each 100 ml of buffer. The electrode vessels contained 0.1*M* boric acid–0.06*M* tris buffer, also pH 8.6 at 4°C. A potential of 300 v and a maximum current of 12 ma were applied across the gel for 9 hr. Gels were sliced and stained by the method of Smithies (1955) with Amido-Black 10B used as the protein-staining dye. They were washed free of background stain with methanol-water-acetic acid mixture (5:5:1 v/v) and photographed immediately after destaining.

Tenderness determinations. Warner-Bratzler shear values were determined on steaks removed from the right longissimus dorsi muscle at the post-mortem periods described above. These steaks were cooked to an internal temperature of 63°C and allowed to cool 12 hr. Shear values were determined on ½-in. cores. All data were subjected to statistical analysis according to methods presented by Steel and Torrie (1960).

RESULTS AND DISCUSSION

Protein fractionation. As shown in Table 1, the longissimus dorsi muscle contained a higher percentage of sarcoplasmic and nonprotein nitrogen and a lower per-

Table 1. Distribution of nitrogen in various protein fractions of the longissimus dorsi and semitendinosus muscles (% of total N).

	Nitrogen fraction			
	Sarcoplasmic	Fibrillar	NPN	Stroma
Longissimus dorsi				
Steers	21.6	49.0	12.8	16.6
Heifers	23.6	53.7	14.2	7.3
Cows	24.3	53.5	13.5	8.7
Mean	23.2	52.0	13.5	10.9
Semitendinosus				
Steers	21.7	50.8	12.0	15.5
Heifers	21.2	52.6	12.9	13.2
Cows	23.1	52.8	13.1	10.9
Mean	22.0	52.1	12.7	13.2

centage of stroma nitrogen (obtained by difference) than the semitendinosus. Between-class (sex and/or age) variation in sarcoplasmic, fibrillar, nonprotein, and stroma nitrogen was not tested for significance, because there were so few cattle within each class. However, the amount of stroma nitrogen for both muscles was higher for steers than for heifers or cows. Sarcoplasmic, fibrillar, and nonprotein nitrogen in the longissimus dorsi muscles was lower for steers than for heifers or cows. Except for sarcoplasmic nitrogen, the observations were the same for the semitendinosus muscles.

Table 2 gives means and standard error of the means for percentages of nonprotein nitrogen and sarcoplasmic and fibrillar protein nitrogen extracted at 4 post-mortem periods. Soluble NPN increased significantly during post-mortem aging in both muscles, with the semitendinosus muscle exhibiting the greater increase. Most of the increase in nonprotein nitrogen occurred during the first 168 hr post-mortem, with only a slight increase observed at 336 hr. In general, this agrees with results of Sharp (1963), who, in studying aseptic autolysis of bovine muscle, found a slow but continuous breakdown of protein, with a concomitant formation of increased nonprotein nitrogen during extended storage.

With the semitendinosus muscle, the amount of extractable sarcoplasmic nitrogen decreased steadily during post-mortem aging. Only the amount extractable at 336 hr was significantly lower than for the other periods studied. However, soluble sarcoplasmic nitrogen of the longissimus dorsi muscle did not change significantly during post-mortem aging. No reason for this behavior is evident. In contrast, Kronman and Winterbottom (1960) and Goll *et al.* (1964) respectively studied the longissimus dorsi and semitendinosus muscle, and both groups found that sarcoplasmic protein was most extractable immediately after death and that its solubility decreased during post-mortem aging. Sharp (1963) concluded that the main autolytic effect of the muscle cathepsins during aseptic storage was upon the sarcoplasmic proteins, since the basic structure of the muscle fibers and fibrils, as evidenced by the presence of cross striations, remained intact during storage whereas nonprotein nitrogen increased continuously.

The extractability of fibrillar nitrogen in both the longissimus dorsi and semitendinosus muscles decreased during the period from 0 to 24 hr post-mortem (Table 2). The initial decrease in fibrillar protein solubility observed may be associated with the onset of rigor mortis during this period (Weber and Meyer, 1933; Bailey, 1954). However, in both muscles, the amount of extractable fibrillar nitrogen increased significantly from 24 to 168 hr. Although slightly higher values were found at 336 hr post-mortem, they were not significantly different from 168 hr. These data generally agree with data of Hegarty (1963), who found slightly increased fibrillar protein solubility during post-mortem aging for 168 hr. On the other hand, Goll *et al.* (1964) found

Table 2. Means and standard error of the means for percentage NPN, sarcoplasmic and fibrillar nitrogen.[A]

Muscle	Time-post-mortem				Standard error
	0 hr	24 hr	168 hr	336 hr	
NPN (% of total N)					
Longissimus dorsi	13.5[a]	14.1[b]	14.8[b]	14.8[b]	0.30
Semitendinosus	12.7[a]	13.6[ab]	14.1[b]	14.4[b]	0.40
Sarcoplasmic N (% of total N)					
Longissimus dorsi	23.2[a]	23.6[a]	23.3[a]	23.4[a]	0.92
Semitendinosus	22.0[b]	21.8[b]	20.8[b]	18.6[a]	0.66
Fibrillar N (% of total N)					
Longissimus dorsi	52.0[a]	50.2[a]	55.9[b]	57.2[b]	1.29
Semitendinosus	52.1[ab]	48.7[a]	55.6[bc]	57.9[c]	1.44

[A] Within-muscle means with the same superscript are not significantly different, $P < .05$.

no change in extractability during a 312-hr aging period, but they did not sample each carcass at each post-mortem period.

Simple correlation coefficients were calculated between Warner-Bratzler shear values and the quantity of extractable fibrillar protein. In addition, simple correlations were calculated between the increase in fibrillar protein extractability from one aging period to another and the decrease in shear value during these same periods. These correlations are presented in Table 3. Shear values

Table 3. Simple correlation coefficients for various factors related to Warner-Bratzler shear values.

Factor	Shear value (lb)	Decrease in shear value
Fibrillar nitrogen (% of total N)		
24 hr post-mortem	−.38	.....
168 hr post-mortem	−.77*	.....
336 hr post-mortem	−.44	.....
Fibrillar nitrogen, increase from:		
24 to 168 hr	.....	0.55
24 to 336 hr	.....	0.62
168 to 336 hr	.....	0.82*

* $P < .05$.

were negatively correlated with the amount of extractable fibrillar nitrogen at 24, 168, and 336 hr post-mortem. However, only the value ($r = -.77$) at 168 hr was significant ($P < .05$). The increase in amount of extractable fibrillar nitrogen during various post-mortem periods (24–168 hr, 24–336 hr, and 168–336 hr) was positively correlated with the decrease in shear value during the same periods. The correlation ($r = .82$) for the period 168–336 hr was significant ($P < .05$). These data indicate that only 30% of the variability in shear value was accounted for by the increase in fibrillar protein extractability during the 24–168-hr period, but 67% was accounted for by the changes during the 168–336-hr period.

Starch gel electrophoresis of sarcoplasmic proteins. Fig. 1 shows the electrophoretograms obtained from the sarcoplasmic proteins at 0, 24, 168, and 336 hr post-mortem. Zones are designated by numbers at the right margin. The majority of components migrated toward the anode. Separation of the anionic components was superior to that of the cationic components with this buffer system. The electrophoretograms of the longissimus dorsi and semitendinosus muscles were very similiar at all post-mortem periods, even though the muscles were quite divergent in tenderness. No major differences in the electrophoretograms of the sarcoplasmic proteins were found among animals differing widely in degree of tenderness. These data do not agree with the postulations made by Husaini *et al.* (1950), who suggested that muscle sarcoplasm may be important in meat tenderness. No variation was observed in the electrophoretograms of the two muscles which could be related to the different solubility behaviors of the sarcoplasmic proteins during post-mortem aging. Electrophoretic patterns did not appear to be related to sex and/or age of the cattle studied.

As shown in Fig. 1, some changes were evident in the electrophoretic patterns as post-mortem aging proceeded. Individual zones became more discrete and their boundaries more distinct at 168 and 336 hr post-mortem than at either 0 or 24 hr. In addition, a new protein zone became clearly visible at position 14 in the 336-hr electrophoretogram, and zone 15 was usually faintly visible. Zone 8 was more intense at 336 hr than at earlier periods. The changes which occurred in the electrophoretic pattern as aging proceeded may indicate that some qualitative changes have occurred in the sarcoplasmic proteins. Changes in sarcoplasmic protein solubility as discussed above may be related to the loss of electrophoretic components and may reflect denaturation or degenerative losses during aging. However, Neelin and Rose (1964) found that additional components were present in the starch gel electrophoretic pattern of post-mortem aged chicken breast muscle when compared to patterns of in-rigor and pre-rigor muscle. Their sarcoplasmic extracts were prepared by gentle homogenization in 0.44*M* sucrose. Rampton *et al.* (1965) detected alterations in bovine muscle sarcoplasmic proteins during post-mortem aging by DEAE-cellulose column chromatography. Such alterations included the loss of one fraction and appearance of new components, while other fractions di-

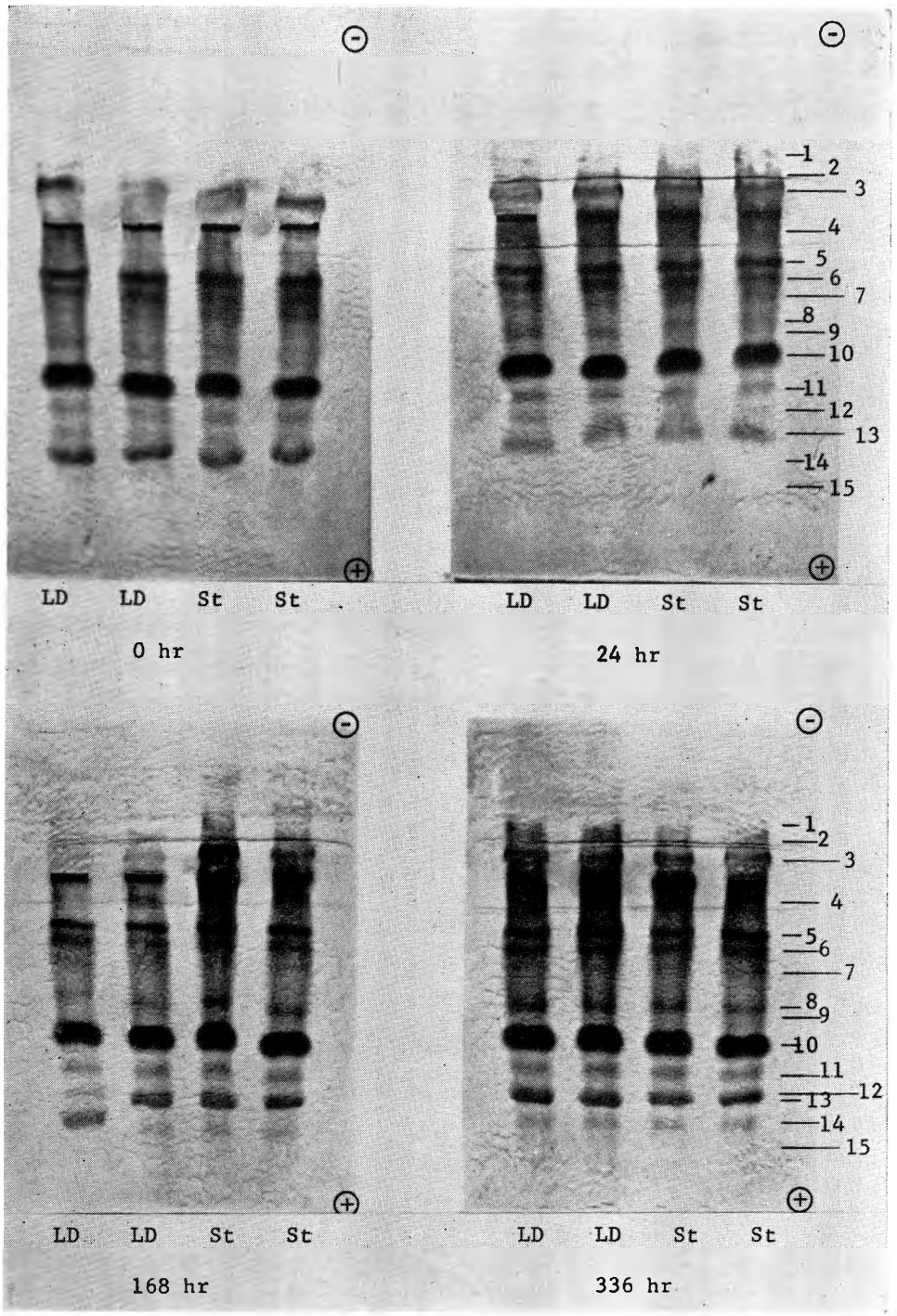


Fig. 1. Starch gel electrophoretic pattern of sarcoplasmic proteins at 4 post-mortem periods. LD = longissimus dorsi muscle; St = semitendinosus muscle. Polarity is designated by − or +. During electrophoresis, the cathode was oriented at the top of the gel, the anode at the bottom. Protein zones are designated by numbers at the right margin.

minished. Fujimaki and Deatherage (1964), also using ion-exchange cellulose chromatography, observed fewer fractions and a decreased level of certain fractions in sarcoplasm from aged bovine muscle.

The results of this electrophoretic study indicate the need for additional, more detailed investigation of the changes in muscle proteins during post-mortem aging.

REFERENCES

AOAC. 1960. "Official Methods of Analysis." 9th ed. Assoc. of Offic. Agr. Chemists, Washington, D. C.

Bailey, K. 1954. Structure of proteins. II. Muscle. *In:* "The Proteins." (H. Neurath and K. Bailey, eds.) Vol. 2, part B, p. 854. Academic Press, New York.

Deuticke, H. J. 1932. Kolloidzustandsanderungen der muskelproteine bei der muskeltätigkeit. *Z. Physiol. Chem.* **210**, 97.

Fischer, R. L. 1963. Change in the chemical and physical properties of protein during aging of meat. Proc. Meat Tenderness Symposium. Campbell Soup Co., p. 71.

Fujimaki, M., and F. E. Deatherage. 1964. Chromatographic fractionation of sarcoplasmic proteins of beef skeletal muscle on ion-exchange cellulose. *J. Food Sci.* **29**, 316.

Goll, D. E., D. W. Henderson, and E. A. Kline. 1964. Post-mortem changes in physical and chemical properties of bovine muscle. *J. Food Sci.* **29**, 590.

Hartshorne, D. J., and S. V. Perry. 1962. A chromatographic and electrophoretic study of sarcoplasm from adult and foetal rabbit muscle. *Biochem. J.* **85**, 171.

Hegarty, G. R. 1963. Solubility and emulsifying characteristics of intracellular beef muscle proteins. Ph.D. thesis. Michigan State University, East Lansing.

Husaini, S. A., F. E. Deatherage, and L. E. Kunkle. 1950. Studies on meat. II. Observations on the relation of biochemical factors to changes in tenderness. *Food Technol.* **4**, 366.

Kronman, M. J., and R. J. Winterbottom. 1960. Post-mortem changes in the water soluble proteins of bovine skeletal muscle during aging and freezing. *J. Agr. Food Chem.* **8**, 67.

Kronman, M. J., L. E. Weinberger, and R. J. Winterbottom. 1960. The water-soluble proteins of bovine skeletal muscle. *Arch. Biochem. Biophys.* **86**, 238.

Lawrie, R. A. 1961. Studies on the muscles of meat animals. I. Differences in composition of beef longissimus dorsi muscles determined by age and anatomical location. *J. Agr. Sci.* **56**, 249.

Neelin, J. M., and D. Rose. 1964. Progressive changes in starch gel electrophoretic patterns of chicken muscle proteins during aging post-mortem. *J. Food Sci.* **29**, 544.

Paul, Pauline C., and L. J. Bratzler. 1955. Studies on tenderness of beef. II. Varying storage times and conditions. *Food Research* **20**, 626.

Rampton, J. H., A. F. Anglemier, and M. W. Montgomery. 1965. Fractionation of bovine sarcoplasmic proteins by DEAE-cellulose chromatography. *J. Food Sci.* **30**, 636.

Ramsbottom, J. M., and E. J. Strandine. 1949. Initial physical and chemical changes in beef as related to tenderness. *J. Animal Sci.* **8**, 398.

Scopes, R. K. 1964. The influence of post-mortem conditions on the solubilities of muscle proteins. *Biochem. J.* **91**, 201.

Sharp, J. G. 1963. Aseptic autolysis in rabbit and bovine muscle at 37°C. *J. Sci. Food Agr.* **14**, 468.

Smithies, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629.

Steel, R. G. D., and J. H. Torrie. 1960. "Principles and Procedures of Statistics." McGraw-Hill Book Co., New York.

Wake, B. C., and R. L. Baldwin. 1961. Analysis of casein fractions by zone electrophoresis in concentrated urea. *Biochim et Biophys. Acta.* **47**, 225.

Weber, H. H., and K. Meyer. 1933. Dos kolloidale Verhalten der Muskeleiweisskorper. *Biochem. Z.* **266**, 137.

Wierbicki, E., L. E. Kunkle, V. R. Cahill, and F. E. Deatherage. 1954. The relation of tenderness to protein alterations during post-mortem aging. *Food Technol.* **8**, 506.

Wierbicki, E., L. E. Kunkle, V. R. Cahill, and F. E. Deatherage. 1956. Post-mortem changes in meat and their possible relation to tenderness together with some comparisons of meat from heifers, bulls, steers and diethylstilbestrol treated bulls and steers. *Food Technol.* **10**, 80.

Ms. rec'd 10/28/65.

Journal article No. 3740, published with the approval of the Director of the Michigan Agricultural Experiment Station, East Lansing, Michigan.

This study was supported in part by funds provided by graduate training grant No. T1-ES-16, Division of Environmental Health Sciences, U. S. Public Health Service. This manuscript reports a portion of the M.S. thesis by the senior author. Presented at the 25th Annual Meeting of the IFT, Kansas City, Mo., May 18, 1965.

NELL I. MONDY, SALLY B. GEDDE-DAHL, and EMILY O. MOBLEY
Graduate School of Nutrition, New York State College of Home Economics, Cornell University, Ithaca, New York 14850

Relationship of Specific Gravity to the Enzymatic Activity and Phenolic Content of Potatoes

SUMMARY

The relationship of specific gravity to the phenolic content and enzymatic activity of Ontario potatoes grown in each of two consecutive years was studied. Phenolic content was found to be highest in tubers of highest specific gravity. Cytochrome oxidase activity tended to increase with increasing specific gravity up to the highest level, but, in each of the harvests studied, cytochrome oxidase activity dropped at the highest specific gravity level. Polyphenol oxidase, in relation to specific gravity, showed a trend opposite to that of cytochrome oxidase. Polyphenol oxidase activity decreased significantly with increasing specific gravity.

INTRODUCTION

Specific gravity of potatoes has been used as a measure of their suitability for specific methods of food preparation. Potatoes of high specific gravity are preferred for chips and French fries. De Bruyn (1929) was among the first to observe that, within a given group of potatoes, tubers of high specific gravity showed the most discoloration. Storage in wet sand reduced the specific gravity of the tuber, and also the tendency to discolor. Scudder (1951) found that potatoes of high specific gravity were more susceptible to precooking discoloration, or black spot, than potatoes of low specific gravity. Sawyer and Collins (1960) related black spot susceptibility to specific gravity within a given variety. They observed that factors which cause turgor loss or increase in specific gravity influence black spot susceptibility. By controlling air circulation and humidity during storage, the addition or loss of moisture was shown to have a reversible effect on black spot susceptibility. Jacob (1959) found that the high variation among tubers prevented establishment of a critical specific gravity at which tubers would blacken. He suggested that the effectiveness of potassium in reducing black spot was partly related to its effect on the reduction of specific gravity, and thought it to be related to the influence on dry matter content.

Several workers (De Bruyn, 1929; Jacob, 1959; Mulder, 1949; Scudder, 1951; Sharma *et al.*, 1958) have reported that specific gravity varies in different parts of the tuber. De Bruyn (1929) observed that the stem end, the section which discolored most readily, had a higher specific gravity than the other portions of the tuber, and Mulder (1949) found that the stem end contained less water than other portions of the tuber. Jacob (1959) and Scudder (1951) reported that the specific gravity of the apical section of Green Mountain potatoes had a lower specific gravity than either the basal or center sections. Sharma *et al.* (1958) found that the cortex area of the tuber was higher in specific gravity than the pith.

Although the tendency of potatoes to black spot has been related to specific gravity, relatively little is known as to what constituents of the dry matter are involved. It is possible that factors such as the concentration of oxidative enzymes and their substrates, the phenols, may be responsible for the increased discoloration.

There are relatively few studies concerning the relationship between total phenolic content and specific gravity. Van Middelen (1941) found that tyrosine content was greater in the basal portion of the tubers than in either the middle or apical portions, and was highly correlated with the tendency to black spot, but the tyrosine content could not be related to the specific gravity of the whole tuber.

Black spot is believed to be due to the reaction of oxidative enzymes, such as polyphenol oxidase, with phenolic substances which serve as substrates. The role of polyphenol oxidase in the metabolism of the potato has not been clearly defined. Early workers associated this enzyme with terminal oxidation in the tuber (Baker and Nelson, 1943), but more recent studies have indicated

that cytochrome oxidase activity is high enough to account for the total respiration of the tuber (Goddard and Holden, 1950).

This study was made to relate specific gravity to the phenolic content and the enzymatic activity of potatoes.

EXPERIMENTAL

The effect of specific gravity on phenolic content and enzymatic activity of potatoes was studied in each of two consecutive years, 1959 and 1960. Ontario potatoes, a variety known to be susceptible to precooking discoloration, were used. The potatoes were grown at the Cornell Research Farm at Riverhead, Long Island. In 1959, potassium fertilizer was applied as 60% KCl at the rate of 140 lb per acre, and the potatoes were harvested 17 weeks (Harvest 1) and 22 weeks (Harvest 2) after planting. In 1960, potassium was applied at 400 lb per acre, and the potatoes were harvested 21 weeks after planting. The specific gravity in the individual tubers was determined by salt-water displacement after six months of storage at 40°F. For the phenol determination, four specific gravity groups were selected for each study, ranging from 1.06 to 1.09 in 1959, and from 1.05 to 1.08 in 1960. Used for cytochrome and polyphenol oxidase activity determinations, in addition to Ontario potatoes grown at the Cornell Research Farm, Riverhead, Long Island, were Ontario potatoes grown for 19 weeks at Ithaca, New York. Statistical treatment of the experimental data was made using analysis of variance.

Determination of phenols. Six tubers of each specific gravity group were selected at random for each extract. Two extracts were made for each specific gravity group. A 50-g sample of the cortex tissue was blended for 5 min with 150 ml of 95% ethanol. The total extract was measured and filtered, and 35 ml of the filtrate was used for phenol determination. Total phenolic constituents were determined by the method of Rosenblatt and Peluso (1941) with tannic acid the standard. Since this method is not specific for phenols but also includes ascorbic acid, corrections were made for ascorbic acid. Potato tissue was extracted with metaphosphoric acid and analyzed for ascorbic acid by the indophenol dye method, simultaneously with the analyses for total phenols.

Determination of enzyme activity. Three tubers of each variety were selected at random for each extract. Two extracts were made for each specific gravity group. A 50-g sample of cortex tissue taken longitudinally from the bud to stem end of potatoes was blended with 50 ml of 0.05M sodium barbituate for 1 min, the mixture was filtered through cheesecloth for 3 min and the supernatant liquid was used for enzyme activity determinations. All reagents and containers were chilled before and during preparation of the extract. Cytochrome oxidase and polyphenol oxidase activities were determined manometrically by a modification of the method described by Goddard and Holden (1950). For the determinations of cytochrome oxidase, the main compartment of the Warburg flask contained 1 ml of 0.5M phosphate buffer at pH 7.1 plus 0.6 ml of $2.25 \times 10^{-4}M$ cytochrome c; the center well 0.2 ml of 2N sodium hydroxide; one side arm 0.3 ml of the potato extract; the second side arm 0.8 ml of $6.82 \times 10^{-2}M$ hydroquinone. Water was added to the main compartment to bring the total volume of the liquid in the flask to 3.2 ml. The side-arm contents were added after a 5-min equilibration period at 30°C stopcocks were closed, and readings were taken every 10 min over a 1-hr period. Polyphenol oxidase activity was measured similarly but using 0.2 ml of extract in the side arm and 0.2 ml of $4.55 \times 10^{-3}M$ catechol in place of cytochrome c in the main part of the flask. Cytochrome oxidase and polyphenol oxidase activities were determined simultaneously on the same potato extract.

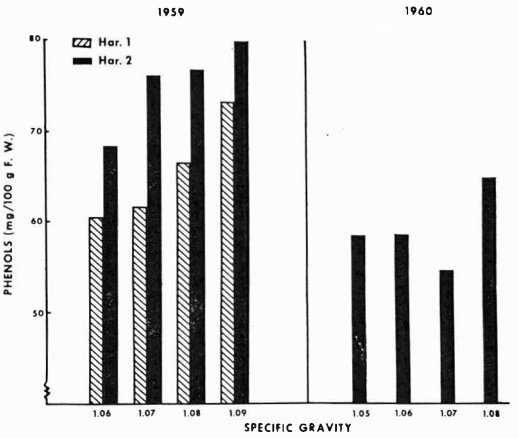


Fig. 1. Relationship of specific gravity to the phenolic content of Ontario potatoes (Har., harvest). Differences were significant at the 1% level.

RESULTS AND DISCUSSION

The phenolic content of tubers differing in specific gravity is given in Fig. 1. Phenolic content was generally found to be highest in tubers of highest specific gravity.

In 1959, the increase in the phenolic content with increasing specific gravity was significant at the 1% level. This trend was the same for both harvests. In 1960 a highly significant difference in phenolic content was

found between the highest and lowest specific gravity groups, but no definite trend was observed in the intervening groups. Generally, specific gravities were lower for potatoes grown in 1960 than for potatoes grown in 1959. The high levels of potassium fertilizer used in 1960 may account for the lowered specific gravity, for the application of potassium has been shown to lower specific gravity (Jacob, 1959; Massey, 1952; Scudder, 1951).

The cytochrome oxidase activity of potatoes of different specific gravities (Fig. 2) tended to increase with increasing specific gravity, but in each of the groups of potatoes there was a drop in cytochrome oxidase activity at the highest specific gravity level.

The decrease in polyphenol oxidase activity with increasing specific gravity (Fig. 3) was significant at the 5% level. This trend was opposite to that observed for cytochrome oxidase activity.

Although discoloration was not followed in this study, because of a limited number of potatoes in each of the specific gravity groups, there are reports that high specific gravity levels are positively correlated with black spot indices among potatoes of the same variety (Cotter, 1956; Massey, 1952; Scudder, 1951).

Cotter (1956) proposed that tubers of high specific gravity also tend to have high polyphenol oxidase activity. He studied Ontario and Pontiac varieties as examples respectively having high and low specific gravities, but did not study different specific gravity levels within a single variety of potato. Previous work in this laboratory (Mondy *et al.*, 1960) confirmed his findings that polyphenol oxidase activity is higher in Ontario than in Pontiac potatoes, but in the present study, using a single variety of potato, the Ontario, polyphenol oxidase activity decreased significantly with increasing specific gravity.

It is possible that the greater discoloration found in potatoes of high specific gravity may be more closely related to their higher phenolic content and their greater cytochrome oxidase activity than to their polyphenol oxidase activity.

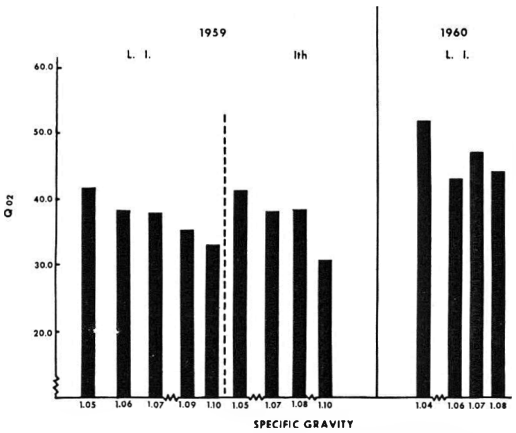


Fig. 3. Relation of specific gravity to polyphenol oxidase activity of Ontario potatoes. (Qo_2: microliters oxygen uptake/hr/mg dry weight of tissue; all values were corrected for endogenous respiration). (L. I. potatoes grown at Long Island; Ith., Ithaca). Differences were significant at the 5% level.

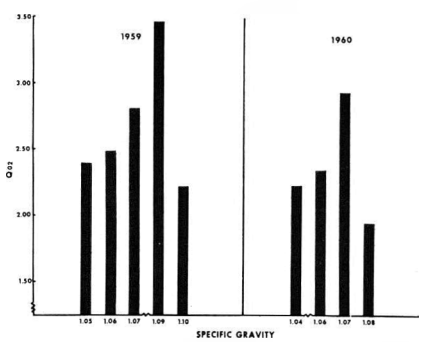


Fig. 2. Relation of specific gravity to cytochrome oxidase activity of Ontario potatoes. (Qo_2: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.)

REFERENCES

Baker, D., and J. M. Nelson. 1943. Tyrosinase and plant respiration. *J. Gen. Physiol.* **26**, 269.

Cotter, D. J. 1956. Some observations on black spot of potatoes. Ph.D. thesis. Cornell University.

De Bruyn, H. L. G. 1929. Het blaun worden van aardappelen (The blue discoloration of potatoes). *Tijdschr. Plantenziekten*, **35**, 185. (English summary).

Goddard, D. R., and C. Holden. 1950. Cytochrome oxidase in the potato tuber. *Arch Biochem.* **27**, 41.

Jacob, W. C. 1959. Studies on internal black spot of potatoes. *Cornell Univ. Agr. Expt. Sta. Memoir* No. **386**.

Massey, P. H. 1952. Field and storage experiment on internal black spot of potatoes. Ph.D. thesis. Cornell University.

Mondy, N. I., B. P. Klein, and L. I. Smith. 1960. The effect of maturity and storage on phenolic content, enzymatic activity and discoloration of potatoes. *Food Research* **27**, 693.

Mulder, E. G. 1949. Mineral nutrition in relation to the biochemistry and physiology of potatoes. Effect of sodium, potassium, magnesium, phosphorus, magnesium and copper nutrition on the tyrosine content and tyrosinase activity with particular reference to the blackening of the tubers. *Plant and Soil* **2**, 59.

Rosenblatt, M., and J. V. Peluso. 1941. Determination of tannins by photocolorimeter. *Assoc. Offic. Agr. Chemists* **24**, 170.

Sawyer, R. L., and G. H. Collins. 1960. Black spot of potatoes. *Am. Potato J.* **37**, 115.

Scudder, W. T. 1951. Black spot of potatoes. Ph.D. thesis. Cornell University.

Sharma, M. K., D. R. Isleib, and S. T. Dexter. 1958. Specific gravity of different zones within potato tubers. *Am. Potato J.* **35**, 784.

Van Middelen, C. H. 1941. Studies of the relations of some of the nitrogenous fractions of the potato tuber to the internal black spot. Ph.D. thesis. Cornell University.

Ms. rec'd 11/9/65.

The authors acknowledge the assistance of Drs. Richard Sawyer and Ora Smith, Department of Vegetable Crops; Dr. Robert Plaisted, Department of Plant Breeding; and Dr. L. C. Peterson, Department of Plant Pathology, for the arrangements made in growing, harvesting, transporting, and storing the potatoes. Grateful acknowledgment is made to Dr. John Skory for guidance and assistance in the statistical analyses.

F. HILL[a]

The Agricultural Institute, Dunsinea, Castleknock, County Dublin, Ireland

The Solubility of Intramuscular Collagen in Meat Animals of Various Ages

SUMMARY

The percentage of total intramuscular collagen solubilized by heating in ¼-strength Ringer's solution, muscles from 98 cattle, 15 sheep, and 9 pigs at 77°C for 1 hour decreased as chronological age increased. Total intramuscular collagen was high in very young (8–9 weeks) bovines and in two of the three very old (7–15 years) bovines studied. In the age range of 16 weeks to 4½ years, variation in total bovine intramuscular collagen was wide. There was no indication of a systematic increase as age increased. The results provide strong presumptive evidence of an increase with age in the number or strength of the cross links of intramuscular collagen and furnish an acceptable explanation as to why total amounts of intramuscular collagen are inadequate to explain toughness associated with increasing chronological age.

INTRODUCTION

Wilson *et al.* (1954) reported that the collagen content of the longissimus dorsi muscle of veal was greater than that of steers or cows and suggested that total amounts of collagen are not always adequate to explain different degrees of toughness. Loyd and Hiner (1959), however, found no significant differences between veal and beef in hydroxyproline content. Verzar (1960, 1963) found that the corium of cows' skin, and also human skin, when heated for 10 min at 65°C, liberated hydroxyproline complexes, and that the amount liberated decreased as chronological age increased. He explained these observations by suggesting that, as an animal ages, cross linkages increase within the collagen macromolecule. Verzar (1963) postulated that, since collagen does not have a metabolic turnover, molecular movements eventually bring the polypeptide chains nearer to each other, thus assisting the formation of cross linkages.

Jackson and Bentley (1960), in work on guinea pig skins, found that the collagen fraction which was most extractable also incorporated carbon-14 glycine most rapidly. They proposed that a continuous spectrum of collagen aggregates of varying degrees of cross linking exists at any given time in developing connective tissue, and that the longer the time that has elapsed since the synthesis of a collagen molecule, the more firmly it will be bound into a collagen aggregate. They explain the increase in cross linking with increasing age as follows: "The deeper in the fibre the collagen molecule is, the more firmly will it be cross linked, as with time the molecules move into more favourable steric apposition under the influence of thermal agitation." Schaub (1963) studied the aging of collagen in the connective tissue of the rat's skeletal musculature by determining the percentage of the total collagen solubilized in Ringer's solution in 10 min at 65°C. The values dropped from 30–40% in 5-week-old animals to 10% in 10-month-old ones, and to 3–4% in old age. Goll *et al.* (1963) suggested that a structural change may take place in collagen as an animal matures. Goll *et al.* (1964b) studied age-associated structural changes in collagenous residues isolated from the biceps femoris muscles of 11 bovines representing 4 different age groups. They reported that the susceptibility of this material to collagenase digestion decreased as chronological age increased. Scheraga (1961) pointed out that increased cross linking of protein molecules should result in slower proteolytic hydrolysis. In an extension of their studies on the residues, Goll *et al.* (1964a,c) found that, as chronological age increased, less hydroxyproline was released by heating in phosphate buffers at 60, 65, 70, and 100°C. The work reported in this paper deals with changes in the solubility of intramuscular collagen of meat animals as chronological age increases.

[a] Present address: Department of Biotechnology, Institute for Industrial Research and Standards, Dublin, 9, Ireland.

EXPERIMENTAL METHODS

The sternomandibularis was the muscle mainly used in this study. It is a uniformly straight-fibered neck muscle which is exposed at decapitation. It can be excised very soon after slaughter without mutilating the carcass. It contains a large amount of collagen. Also investigated in a few cases were other muscles, such as the psoas major, semitendinosus, and longissimus dorsi.

The muscles were dried overnight, over silica gel in an evacuated desiccator at 2°C. The desiccated muscle was then broken up in a micro hammer mill so that it passed through a 2-mm screen. A weight of dried muscle equivalent to 5 g of fresh tissue was put into a 50-ml centrifuge tube (containing a rubber stopper with thermometer and a vent), and 12 ml of ¼-strength Ringer's solution was added. It appears from Verzar's work (1963) that Ringer's solution is more effective than distilled water in weakening the intermolecular forces of collagen. The tube was put in a water bath for 63 min at 77°C and the contents stirred at intervals. Three minutes was the average time taken for the contents of the tube to reach 77°C. This temperature is considered desirable for the interior of a well done beef joint when removed from the oven (National Live Stock and Meat Board).

After centrifugation and removal of the supernatant, the residue in the tube was washed with 8 ml of Ringer's solution (at room temperature) and centrifugation was performed again. The supernatants were bulked and hydrolyzed for 7 hr. The residue of muscle was also hydrolyzed for 7 hr. Hydroxyproline was determined in the hydrolysates by the Mohler and Antonacopoulos (1957) modification of the Neumann and Logan (1950) method.

Table 1. Percentage total collagen and percentage of the total collagen solubilized (by heating 1 hr at 77°C) in the sternomandibularis muscles of 42 Friesian bovines.

Sex	No. of animals	Age	% total collagen in the muscles	% of total collagen solubilized by heating 1 hr at 77°
Male				
calves	2	8–9 weeks	2.21±.31	21.91± .57
	8	16 weeks	1.21±.06	24.59±1.06
	5	18 weeks	1.31±.11	20.66±1.16
Steers	5	4–6 months	1.75±.11	21.13±1.16
	14	10 months	1.30±.26	11.89± .61
	3	22 months	1.74±.16	8.47± .95
Cows	1	2½ years	1.51	3.62
	1	3 years	1.67	3.71
	2	3½ years	1.20±.13	4.51± .41
	1	4½ years	1.25	3.72

Table 2. Percentage of total collagen and percentage of the total collagen solubilized (by heating 1 hr at 77°C) in the sternomandibularis muscles of 24 Hereford and Hereford cross bovines.

Sex	Cross	No. of animals	Age	% total collagen in the muscles	% of total collagen solubilized by heating 1 hr at 77°C
Steers	H × H	4	4–6 months	1.27±.07	21.80±1.39
	H × S	8	17 months	1.08±.06	10.70± .33
	Hereford cross	7	2 years	1.48±.16	6.43± .27
Heifers	Hereford identical twins	2	3 5/12 years	1.14±.16	5.07± .78
Bull	H × H	1	12 years	1.45	1.98
Steer	AA × H	2	4–6 months	1.03±.03	16.24± .40

H, Hereford; S, Shorthorn; AA, Aberdeen Angus.

RESULTS

Table 1 shows the total collagen percent of wet weight, and the total collagen percent of wet weight solubilized (by heating for 1 hr at 77°C) in the sternomandibularis muscles of 42 Friesian bovines (15 calves, 22 steers, and 5 cows). The total collagen was high (2.21%) at 8–9 weeks and varied from 1.20% at 3½ years to 1.75% at 4–6 months. The high total collagen at 8–9 weeks is probably a reflection of poor muscle development at this age. Collagen solubility decreased with increasing age. This is illustrated in Fig. 1 (I). Table 2 shows similar data for 24 Hereford bovines. Solubility also decreased here with increasing chronological age. Fig. 1 (II) graphs the decrease in solubility with advancing age of the 22 Herefords and Hereford crosses listed in the top portion of Table 2. Table 3 shows a general decrease in solubility, illustrated in Fig. 1 (III), in the sternomandibularis muscles of 11 Shorthorn cows as chronological age increases. The oldest cow (7 years) had a very high total collagen content (2.79%), but there was no indication of a general increase in total collagen content with age. Table 4 shows pooled data on seven 13-week-old male Jersey calves and 12 old factory cows. Details of breed and age were not available for the latter, but it is probable that each cow was older than 3 years of age. Table 5 shows that there is little variation in the solubility of collagen in muscles from different anatomical locations of the same carcass. Results are shown for a 15-year-old bull and three young calves 8–9 weeks old. Table 6 shows data for 9 six-month-old Galway lambs, 6 miscellaneous ewes (each more than 5 years old), 2 Large White hogs, each 5 months old, 1 old boar, and 6 old sows. It appears that the collagen in the sternomandibularis muscles becomes insoluble at a faster rate in Galway lambs than in Friesians or Hereford crosses. While there is no overall increase in collagen with age, solubility decreases as in the bovine species. The six old factory sows and the boar had a collagen solubility of about 4%, compared to about 23% for the 5-month-old hogs.

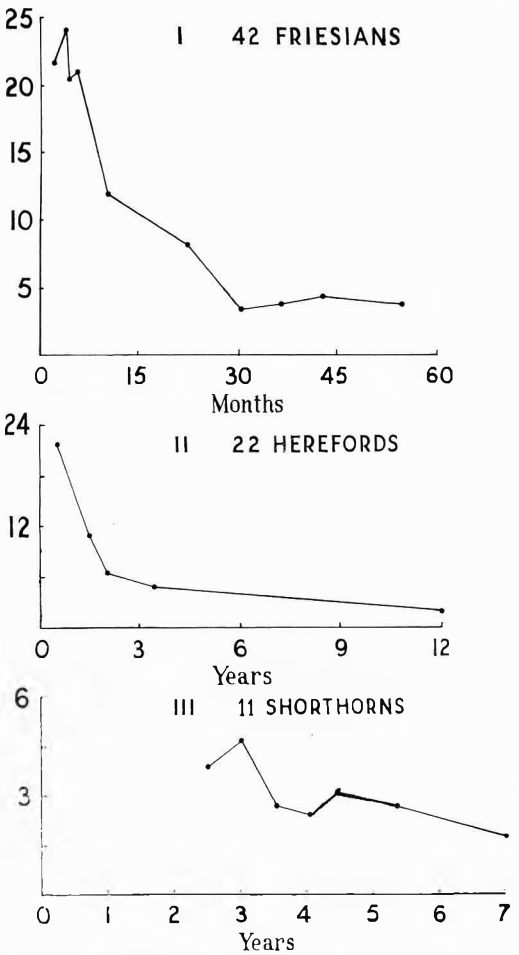


Fig. 1. Decreases in collagen solubility in the sternomandibularis muscle with age of the animal (indicated by percentage of total collagen solubilized in 1 hr at 77°C).

Table 3. Percentage total collagen and percentage of the total collagen solubilized (by heating 1 hr at 77°C) in the sternomandibularis muscles of 11 Shorthorn cows.

No. of cows	Age (years)	% total collagen in the muscle	% of total collagen solubilized by heating 1 hr at 77°
1	2½	1.19	3.82
1	3	1.58	4.61
2	3½	1.40±.002	2.74±.05
1	4	2.13	2.48
3	4 1/3	1.53±.26	3.21±.21
2	5 1/3	1.71±.05	2.65±.50
1	7	2.79	1.79

DISCUSSION

The results indicate that the intramuscular collagen in the sternomandibularis muscles of cattle, sheep, and pigs undergoes

Table 4. Percentages of total collagen and percentages of the total collagen solubilized (by heating 1 hr at 77°C) in the sternomandibularis muscles of seven 13-week-old male Jersey calves and 12 old factory cows.

	% total collagen	% of total collagen solubilized
7 Jersey calves	1.52±.14	19.24±1.06
12 old cows	1.17±.06	2.15± .23

Table 5. Percentage of total collagen and percentage of the total collagen solubilized (by heating 1 hr at 77°C) in muscles from different anatomical locations in 4 carcasses.

		% total collagen	% of total collagen solubilized
15-year-old Aberdeen Angus bull	Sternomandibularis	1.78	0.79
	Longissimus dorsi (10th–13th ribs)	.92	Nil
	Psoas major	.62	Nil
	Semitendinosus	2.19	Nil
8-week-old Friesian calf	Sternomandibularis	2.53	21.34
	Psoas major	.39	18.37
	Semitendinosus	.87	19.72
8-week-old Friesian calf	Psoas major	.27	18.13
	Semitendinosus	.60	18.84
9-week-old Friesian calf	Sternomandibularis	1.50	22.48
	Psoas major	.40	17.47
	Semitendinosus	.92	16.65

Table 6. Percentage total collagen and percentage of the total collagen solubilized (by heating 1 hr at 77°C) in the sternomandibularis muscles of 15 sheep and 9 pigs.

	No. of animals	Age	% total collagen in the muscle	% of total collagen solubilized by heating 1 hr at 77°
Sheep				
Male lambs	9	6 months	1.01±.05	8.16± .57
Old ewes	6	>5 years	1.09±.06	2.81± .23
Pigs				
Large White Hogs	2	5 months	1.93±.04	22.82±5.17
Old boar	1	.....	1.39	4.33
Old sows	6	.....	1.65±.32	3.94± .29

subtle chemical changes as the animals grow older. The results are consistent with those of Verzar (1960, 1963), Jackson and Bentley (1960), Schaub (1963), and Goll *et al.* (1964a,b). The aging phenomenon associated with collagen is not confined to the sternomandibularis muscle. This is indicated by the results for 3 other muscles (taken from different anatomical locations) of the 15-year-old Aberdeen Angus bull (Table 5). The work reported here can be considered as strong presumptive evidence of an increase in the number or strength of the cross linkages of intramuscular collagen in meat animals as they age.

Reed *et al.* (1963) pointed out that mature collagen is almost completely insoluble in acidic buffers even in the presence of hydrogen-bond-breaking reagents. This fact suggests the existence of strong types of linkages in mature collagen. Harding (1965), in his review, concluded that it would appear fairly certain that ester linkages occur in collagen and they take part in the intramolecular cross linking of the polypeptide chains. Hormann (1960), cited by Harding (1965), reported that procollagen gave rise to 0.64 moles of hydroxamic acid per 100 moles of amino acid and that mature collagen gave 1.07 moles. The hexose contents of both types of collagen were approximately equal. Hormann (1962a), cited by Verzar (1964), suggested that there are intramolecular hexose ester links in both procollagen and old collagen, and that old

collagen also contains intermolecular hexose ester linkages.

Reed *et al.* (1963), citing Hormann (1962b), stated that although solubilization of mature collagen can be achieved when ester and hexose type linkages are broken in the presence of hydrogen-bond breaking and other reagents, the rate of solution does not correspond with the rate of destruction of these specific linkages. They consider that although there seems no doubt that hydroxylamine-and-periodate-sensitive cross linkages are present in mature collagen, and so help to determine its degree of insolubility, it is likely that additional factors which depend on the age of the tissue are involved. They point out that as collagen fibrils mature, coatings of mucopolysaccharides and glycoproteins appear to make them more insoluble.

The results reported here indicate that the degree of solubility of the collagen, as well as the total amount, should be considered when biochemical explanations of toughness in meat are considered. During cooking, less collagen is solubilized in meat from older animals than in meat from younger animals. This situation results in an increased sensation of toughness when the meat of older animals is consumed.

An extension of the work on collagen solubility reported here could provide a chemical method of evaluating carcasses of different breeds and crosses for toughness. The hydroxyproline determination could be made more sensitive and accurate by using the modification of Woessner (1961). The method used in the work presented here is subject to interference by tyrosine and to partial inhibition of color development by the presence of other amino acids (Sharp, 1963). By raising the temperature of solubilization to a value above 77°C (say 95°C) sensitivity could also be increased.

Results from an investigation of the type suggested should indicate whether the collagen in different breeds and crosses becomes insoluble at different rates. If this is so, breeding of meat animals of improved tenderness should be possible by selection.

REFERENCES

Goll, D. E., R. W. Bray, and W. G. Hoekstra. 1963. Age associated changes in muscle composition. The isolation and properties of a collagenous residue from bovine muscle. *J. Food Sci.* **28**, 503.

Goll, D. E., R. W. Bray, and W. G. Hoekstra. 1964a. Age associated changes in bovine muscle connective tissue. III. Rate of solubilization at 100°C. *J. Food Sci.* **29**, 622.

Goll, D. E., W. G. Hoekstra, and R. W. Bray. 1964b. Age associated changes in bovine muscle connective tissue. I. Rate of hydrolysis by collagenase. *J. Food Sci.* **29**, 608.

Goll, D. E., W. G. Hoekstra, and R. W. Bray. 1964c. Age associated changes in bovine muscle connective tissue. II. Exposure to increasing temperature. *J. Food Sci.* **29**, 615.

Harding, J. J. 1965. The unusual links and cross links of collagen. *Advances in Protein Chemistry*. **20**, 109.

Hormann, H. 1960. *Beitr. Silikose-Forsch.* **4**, 205. Cited by Harding (1965).

Hormann, H. 1962a. *Leder* **13**, 79. Cited by Verzar (1964).

Hormann, H. 1962b. For full references, see Leder. 1962. **4**, 79. Cited by Reed *et al.* (1963).

Jackson, D. S., and J. P. Bentley. 1960. On the significance of the extractable collagens. *J. Biophys. Biochem. Cytol.* **7**, 37.

Loyd, E. J., and R. L. Hiner. 1959. Relation between hydroxyproline of alkali-insoluble protein and tenderness of bovine muscle. *J. Agr. Food Chem.* **7**, 860.

Mohler, K., and N. Antonacopoulos. 1957. Chemische Bestimmung von Bindegewebe in Fleisch und Seinen Zubereitungen. *Lebensmittel-Untersuch. u. Forsch.* **6**, 425.

National Livestock and Meat Board, Chicago. Ten Lessons on Meat. p. 75. 7th ed.

Neuman, R. E., and M. A. Logan. 1950. The determination of hydroxyproline. *J. Biol Chem.* **184**, 299.

Reed, R., G. Stainsby, and A. G. Ward. 1963. The development of fibrils from solutions of eucollagen. *J. Soc. Leather Trades' Chemist* **47**, 312.

Schaub, M. C. 1963. The aging of collagen in striated muscle. *Gerontologia* **8**, 16.

Scheraga, H. A. 1961. "Protein Structure." p. 67. Academic Press, New York.

Sharp, J. G. 1963. Aseptic autolysis in rabbit and bovine muscle during storage at 37°. *J. Sci. Food Agr.* **7**, 408.

Verzar, F. 1960. Nachweis der Zunahme der Bindung von Hydroxyprolin in Collagen der Haut mit dem Alter. *Gerontologia* **4**, 104.

Verzar, F. 1963. "Lectures on Experimental Gerontology" p. 36–59. Charles C Thomas, Springfield, Illinois.

Verzar, F. 1964. International Review of Connective Tissue Research. 2. p. 290. Academic Press, New York.

Wilson, G. D., R. W. Bray, and P. H. Phillips. 1954. The effects of age and grade on the collagen and elastin content of beef and veal. *J. Animal Sci.* **13**. 826.

Woessner, J. F. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* **93**. 440.

Ms. rec'd 7/13/65.

The technical assistance of Mr. D. Twomey in the laboratory and of Mr. T. Kendrick in drawing the graphs is acknowledged.

G. L. K. HUNTER[a] and M. G. MOSHONAS

U. S. Fruit and Vegetable Products Laboratory, Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Winter Haven, Florida 33882

Analysis of Alcohols in Essential Oils of Grapefruit, Lemon, Lime, and Tangerine

SUMMARY

Alcohols in the cold-pressed essential oils of grapefruit, lemon, lime, and tangerine were extracted from the whole oil with glycerol, isolated by column chromatography, separated by gas chromatography, and identified by infrared and mass spectroscopy. Nineteen alcohols were identified in grapefruit oil, 9 in lemon, 8 in lime, and 16 in tangerine. In addition, the fungicide, o-phenyl phenol, was found in the oils of grapefruit and tangerine.

INTRODUCTION

Little attention has been given to the alcoholic composition in citrus cold-pressed oils other than the orange. Kirchner and Miller (1953), investigating the volatile constituents of grapefruit juice, identified 6 alcohols. Kovats (1963), working on the composition of Brandis distilled lime oil, reported 11 alcohols, and Ikeda and Spitler (1964), reporting on a method for estimating esters and alcohols in California lemon oil, identified 3 alcohols. The alcoholic composition in tangerine oil had not been investigated. The present work was undertaken as part of a study into the composition of cold-pressed citrus oils, to isolate and identify the alcohols present in grapefruit, lemon, lime, and tangerine oils.

EXPERIMENTAL

The alcohols in the cold-pressed citrus oils were enriched by a procedure described by Hunter and Moshonas (1965), with some modifications. The primary change was the substitution of glycerol for propylene glycol during extraction of the alcohols from the citrus oils. Accordingly, 1 L of the citrus oil and 500 ml of glycerol were vigorously shaken for 20 min in a 2-L separatory funnel. The lower, or glycerol, layer was removed and extracted with four 125-ml portions of ethyl ether. The combined ether extracts were washed with 100 ml of water and dried with Na_2SO_4, and the ether was removed under reduced pressure, giving the following crude yields: 30 g from grapefruit, 21.5 g from lemon, 18.5 g from lime, and 17.3 g from tangerine oils.

The residue, consisting of alcohols, other oxygen-containing constituents, and hydrocarbons, was further enriched by column chromatography. Neutral alumina was used since basic alumina was found to saponify some of the esters in lemon and lime oils; however, basic alumina did not noticeably change the alcohol concentrations in orange, grapefruit, and tangerine oils. Accordingly, a 4-g sample of each oil residue was placed on a 5/8 × 18-in. column containing Fisher neutral alumina and eluted with 100 ml of n-hexane to remove the entrained oil hydrocarbons and 75 ml of a 50/50 solution of n-hexane and ethyl ether to remove the nonalcoholic oxygen-containing materials. Finally, the oil alcohols were stripped from the column with 100 ml of absolute ethanol, yielding the following residues upon removal of the solvent under reduced pressure: 0.3 g from grapefruit, 0.4 g from lemon, 0.3 g from lime, and 0.2 g from tangerine oils.

The alcohols were separated on an F & M gas chromatograph, model 810, using a thermal-conductivity cell and containing a 1/4-in. OD by 3/16-in. ID by 26-ft aluminum tube packed with 5% Carbowax-30M on 60–80-mesh acid-washed Chromosorb-G. The oven temperature was programmed from 150 to 240°C at 1.0°C per min with a helium flow rate of 60 ml per min. Fractions were collected in short capillary tubes for infrared and mass spectral analyses.

Mass spectra were obtained with a Bendix Model 12–100 whose source was operated at 70 e.v. in the continuous mode.

Infrared spectra were obtained on a Perkin-Elmer Infracord spectrometer, model 137.

RESULTS AND DISCUSSIONS

The use of glycerol as one of the partitioning agents for the extraction of alcohols from citrus oils provided a number of advantages over propylene glycol, which was formerly used by us (Hunter and Moshonas, 1965). Most important, CCl_4 was no longer needed as the other partitioning agent. Since glycerol is insoluble in the citrus oil hydrocarbons, the hydrocarbons served as the other phase. In addition, the glycerol layer

[a] Present address: Minute Maid Company, 1200 West Colonial Drive, Orlando, Florida 32802.

containing the oil alcohols and the hydrocarbon layer containing the remaining oxygen-containing components quickly separated into two distinct phases, eliminating the need for centrifugation. Also important, the citrus oil alcohols can be extracted directly from the glycerol layer with ethyl ether, obviating the need for water dilution as with propylene glycol. Finally, it was noted that cyclic acetals were formed, particularly during the analysis of grapefruit oil, by the condensation of propylene glycol with the alkane aldehydes present in the oil. These acetals did not form during the extraction with glycerol. To be certain that no acetals were formed or remained in the glycerol during extraction of the alcohols with ethyl ether, a blank was run in which the acetals were added to glycerol and subsequently extracted successfully with ethyl ether.

Fig. 1 is a chromatogram obtained from a 4-μl sample of the alcohols obtained from cold-pressed Duncan grapefruit oil. The names of 20 alcohols and nootkatone are indicated in the figure. The identity of each alcohol was determined by infrared and mass spectroscopy. Since all but four of these alcohols were isolated and identified in orange oil (Hunter and Moshonas, 1965), further verification of their identity is not repeated here. Linalool, one of the carveols, α-terpineol, geraniol, and one of the linalool monoxides have been reported in grapefruit juice (Kirchner and Miller, 1953). The remaining alcohols, the other linalool oxide, octanol, trans-2-8-p-menthadiene-1-ol, nonanol, cis-2,8-p-menthadiene-1-ol, decanol, citronellol, nerol, the other carveol, dodecanol, 1,8-p-menthadiene-9-ol, nerolidol, elemol, 8-p-menthene-1,2-diol, and o-phenol phenol are now shown to be constituents in grapefruit oil. Since *cis* and *trans*-linalool oxide, nerolidol, and o-phenyl phenol were not found in cold-pressed orange oil, they will be considered further. The identity of both cis and trans-

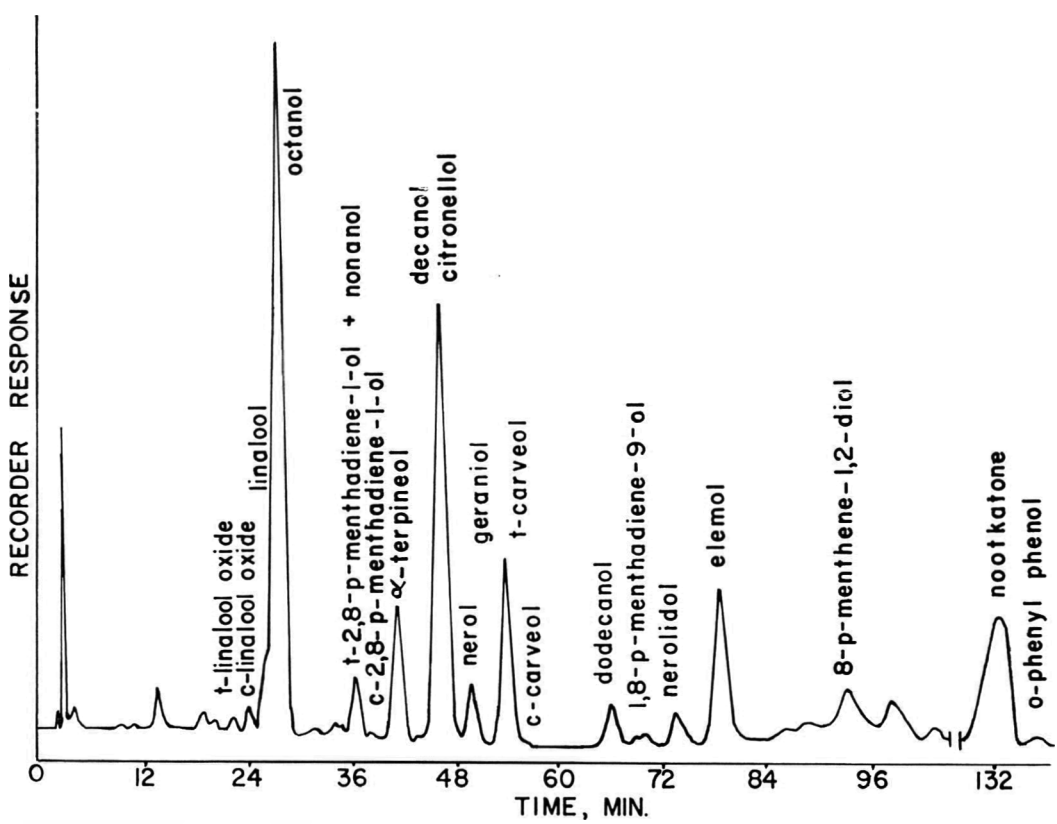


Fig. 1. Gas chromatographic curve of 4 μl of cold-pressed Duncan grapefruit oil.

linalool oxide was confirmed by comparing their infrared and mass spectra with that obtained on authentic samples from Klein *et al.* (1963). In the same way, the identity of nerolidol was confirmed by comparison with a sample of nerolidol from Hoffman-La Roche, Nutley, New Jersey. Finally, the identity of nootkatone was established by mass spectroscopy (Hunter and Brogden, 1965) and of o-phenyl phenol by comparing its infrared spectrum with that provided by Wyandotte ASTM spectral absorption data (1958). o-Phenyl phenol is used by the citrus industry as a fungicide.

The gas chromatogram shown in Fig. 2 was obtained from a 4-μl sample of the alcohols in Florida-produced lemon oil. Octanol, linalool, terpinene-4-ol, nonanol, α-terpineol, decanol, nerol, geraniol and 1,8-p-menthadiene-9-ol were readily identified by infrared and mass spectroscopy. The identity of terpinene-4-ol was confirmed by a spectral comparison with authentic material obtained from L. Light and Co., Colnbrook, England. Linalool, α-terpineol, and terpinene-4-ol have been reported in California lemon oil (Ikeda and Spitler, 1964). The early peaks in this chromatograph were not identified. They are not alcohols, and do not alter the alcohol analysis.

Fig. 3 shows a gas chromatogram of the Florida Avon lime oil alcohols. The alcohols in lime oil are very similar to those in Florida lemon oil. Octanol, linalool, terpinene-4-ol, nonanol, α-terpineol, and decanol have been reported in distilled Brandis lime oil (Kovats, 1963); however, nerol, and geraniol were not mentioned. Octanol is present in Florida lime oil in only trace amounts.

Fig. 4 shows a gas chromatogram obtained on a 4-μl sample of the alcohols from Florida cold-pressed tangerine oil. The composition of the alcohols in this oil is very similar to that of orange oil (Hunter and Moshonas, 1965) and grapefruit oil. The names of 17 alcohols are indicated in the figure. The identity of each alcohol was confirmed by infrared and mass spectroscopy. Thymol, the

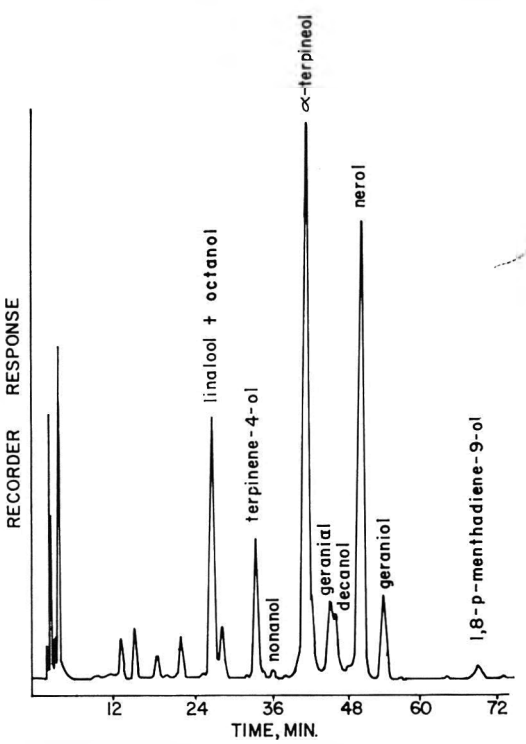


Fig. 2. Gas chromatographic curve of 4 μl of cold-pressed Florida lemon oil alcohols.

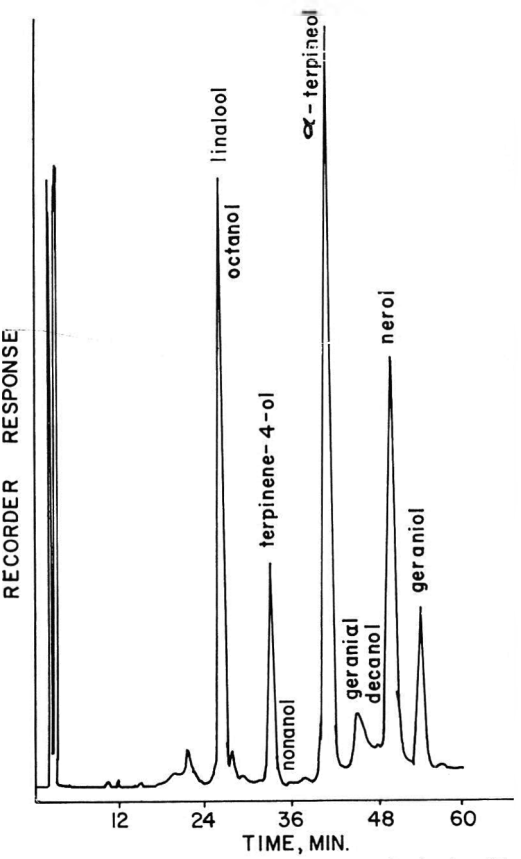


Fig. 3. Gas chromatographic curve 4 μl of cold-pressed Florida lime oil alcohols.

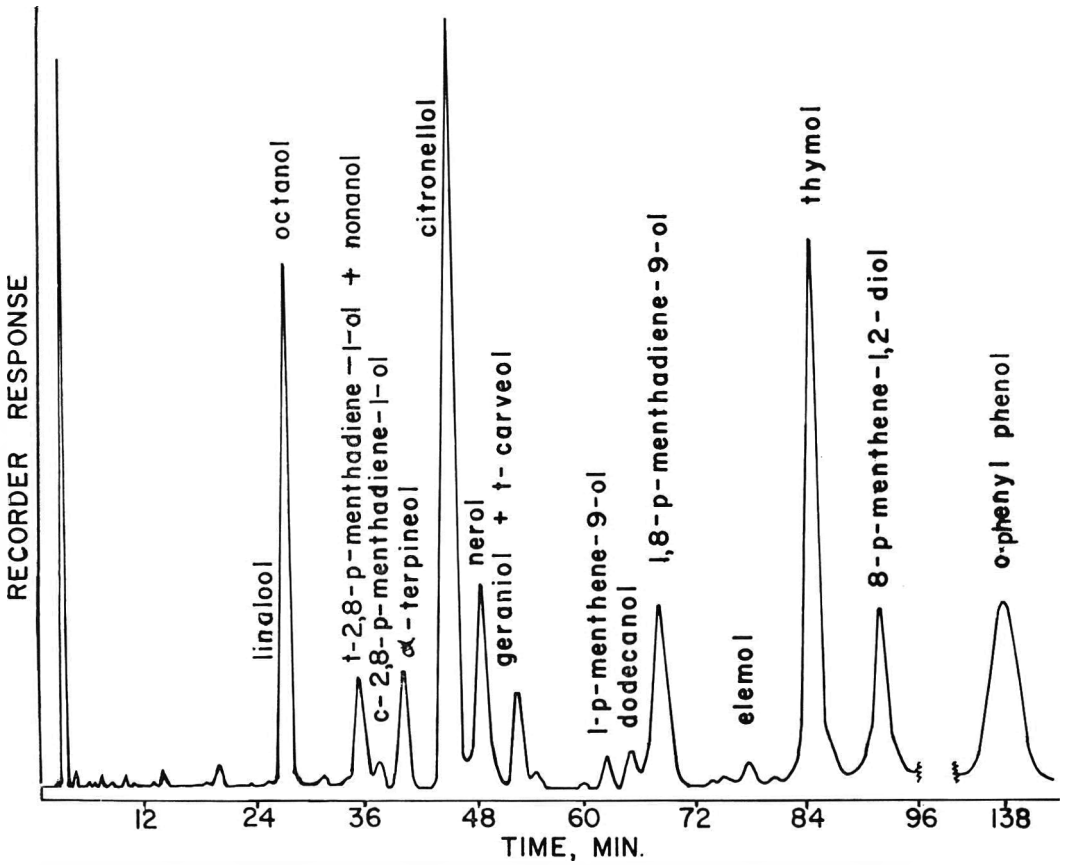


Fig. 4. Gas chromatographic curve of 4 μl of cold-pressed Florida tangerine oil alcohols.

one alcohol which had not been encountered in previous analyses, was identified by comparing its infrared and mass spectra with authentic material obtained from Fisher Chemical Company. The alcohols linalool, octanol, *cis-* and *trans*-2-8-p-menthadiene-1-ol, nonanol, α-terpineol, citronellol, nerol, geraniol, trans-carveol, 1-p-menthene-9-ol, dodecanol, 1,8-p-menthadiene-9-ol, elemol, thymol, and 8-p-menthene-1,2-diol are now reported to be in tangerine oil. o-Phenyl phenol probably enters the oil through its use as a fungicide by the industry.

Since the alcohols in orange, grapefruit, and tangerine are very similar, the distinctive differences between these oils would not be expected to be in this fraction. The alcohols in lemon and lime oils are all but identical; the large quantities of terpinene-4-ol, α-terpineol, and nerol distinguish these oils from the others. o-Phenyl phenol, a fungicide, was found in grapefruit and tangerine oils but not in the lemon and lime oils.

REFERENCES

Hunter, G. L. K., and W. B. Brogden, Jr. 1965. Conversion of valencene to nootkatone. *J. Food Sci.* In press.

Hunter, G. L. K., and M. G. Moshonas. 1965. Isolation and identification of alcohols in cold-pressed Valencia orange oil by liquid-liquid extraction and gas chromatography. *Anal. Chem.* **37**, 378.

Ikeda, R. M., and E. M. Spitler. 1964. Isolation, identification, and gas chromatographic estimation of some esters and alcohols of lemon oil. *Agr. Food Chem.* **12**, 1140.

Kirchner, J. G., and J. M. Miller. 1953. Volatile oil constituents of grapefruit juice. *Agr. Food Chem.* **1**, 512.

Klein, E. von, H. Farnow, and W. Rojahn. 1963. Zur Konstitution des linalooloxyds. *Tetrahedron Letters*. No. **17**, 1109.

Kovats, von E. sz. 1963. Zur Kenntnis des sog.

"distillierten" limetten-öls (Citrus medica, L., var. acida, Brandis; Citrus aurantifolia, Swingle). *Helv. Chem. Acta.* XLVI, 2705.

Wyadotte-ASTM Punched Cards. 1958. Spectral Absorption Data, American Society for Testing Materials. Philadelphia, Pa. No. 1884.

Ms. rec'd 9/13/65.

Mention of brand names does not imply endorsement.

The authors thank Birds Eye Division of General Foods, Florence Villa, Florida, for generous quantities of Commercial grapefruit, lemon, and lime oil and the Florida Citrus Canners Cooperative, Lake Wales, Florida, for the tangerine oil. In particular, we gratefully acknowledge the assistance of W. B. Brogden, Jr., for much of the mass spectral work.

L. W. AURAND, J. A. SINGLETON, T. A. BELL, and J. L. ETCHELLS
Department of Food Science and U. S. Food Fermentation Laboratory
North Carolina Agricultural Experiment Station
Raleigh, North Carolina 27607

Volatile Components in the Vapors of Natural and Distilled Vinegars

SUMMARY

Volatile components in the vapors of natural and distilled vinegars were identified by gas chromatography by comparing their retention volumes with compounds of known composition and by functional group analysis. Twenty-five volatile components were identified from three natural vinegars (cider, 19 components; wine, 17 components; tarragon, 20 components), and 11 volatile components were identified from five samples of distilled vinegar. Four components were present in all vinegars tested: acetaldehyde, acetone, ethyl acetate, and ethyl alcohol. The natural vinegars had the largest number of volatile components. This was attributed to the nature of the fermentable carbohydrate material used for production of the alcohol subsequently used for vinegar manufacture (e.g. apples and grapes). The importance of the carbonyls, alcohols, and esters to vinegar flavor is discussed.

INTRODUCTION

Vinegar (meaning sour wine) has been used as a food ingredient for over a thousand years, and it is only in the last one hundred years that the chemical and microbiological nature has been realized (Prescott and Dunn, 1959). By definition, vinegars are condiments made from alcoholic fermentation of carbohydrates and then subsequent bacterial fermentation to acetic acid. Although most of the commercial vinegar is made by this two-step process, vinegar is also made by the acetic acid bacteria directly from ethyl alcohol.

Vinegar methods of manufacture fall into two general classes: 1) "distilled," "grain," or "spirit," which denotes that the ethyl alcohol was concentrated by distillation; and 2) "natural," which designates the carbohydrate sources for the alcoholic fermentation and is so described on the labels as "apple," "cider," "wine," "grape," and "malt." Vinegars are measured in strength by their acetic acid concentration (1 grain equals 0.1% acid), and the commercial products range from 50 to 120 grains. Aqueous solutions of glacial acetic acid have a sharp unpleasant taste, so the characteristic flavors of vinegar are dependent on substances other than acetic acid, i.e., the flavor of a vinegar depends on the constituents formed during the fermentation of the raw materials as well as the subsequent acetous fermentation. Little is known about the volatile constituents that give a particular vinegar its characteristic aroma. Earlier studies (Jacobs, 1951) on cider vinegar gave the range of volatile esters as 0.30 to 0.91 g/100 ml, measured as ethyl acetate, and of alcohol as 0.03 to 2.00 g/100 ml. In vinegar generator operations, residual alcohol concentration of 0.3–0.5% is considered satisfactory. Conner *et al.* (1959) demonstrated that as the acetic acid in the final product is increased, the cycle time lengthens, the rate at which oxygen is utilized decreases, and the residual alcohol increases (e.g., an increase from 108 to 120 grain increases cycle time from 1 to 2 weeks and residual alcohol from 0.26 to 0.56%).

Suomalainen and Kangasperko (1963), using gas chromatography, identified the volatile components of a wine vinegar as acetoin, isoamyl alcohol, optically active amyl alcohol, isobutyl alcohol, ethyl acetate, isobutyl acetate, sec. butyl alcohol, isoamyl acetate, and diacetyl. They also reported that a distilled vinegar sample contained only ethyl acetate as the volatile component.

The standards of the Food and Drug Administration define the natural vinegars as a condiment made by the alcoholic and subsequent acetous fermentation of the juices of apples, and to contain not less than 4 g acetic acid per 100 ml (20°C). The same standard applies for other vinegars except that source of carbohydrate is replaced with natural products as grape, corn, barley, rye, and malt. With distilled vinegar the standard does not indicate the carbohydrate source or method of ethyl alcohol manufacture but requires the product to be made from the ace-

tous fermentation of dilute distilled ethyl alcohol and to contain not less than 4 g acetic acid per 100 ml (20°C) (Prescott and Dunn, 1959).

MATERIALS AND METHODS

Source and preparation of vinegar samples. Five commercial vinegars, purchased from the local markets, and three vinegar samples supplied from pickle companies which operate their own vinegar generators, were analyzed for volatile flavor constituents. The description of the vinegar samples, together with their percent acetic acid, is as follows: a) apple cider (5%); b) wine (6%) manufactured from California grape wine; c) tarragon flavored (5%), a mixture of distilled and malt vinegars with tarragon spices; d) distilled vinegar, sample No. 1, labeled "Crystal Distilled" (5%); e) distilled, sample No. 2 marked "Distilled White" (5%) and manufactured from corn, barley malt, and rye; f) distilled sample No. 3 (9%) manufactured by a pickle company located in Minnesota and using the "Frings Process"; g) distilled sample No. 4 (11.6%) from a pickle company in Michigan, made by "acetator process"; h) distilled sample No. 5 (11.6%) from a pickle company in Ohio, made by "Frings process." The above five commercial vinegars were diluted by the manufacturer to the acidities indicated for uniform pickling and table strength. Ten ml of each vinegar was added to a vial containing anhydrous sodium sulfate (1.2 g/ml). The vial was sealed with a rubber septum, shaken for 5 min, and then placed in a constant-temperature water bath (70°C) for a 3-min period to equilibrate the volatile vapors. A 5-ml vapor sample was withdrawn and subjected to gas chromatographic (GLC) analysis.

Separation and identification. A Barber-Colman gas chromatograph unit, model 10, equipped with a flame ionization detector, was used for the qualitative determination of the volatile compounds. The samples were chromatographed on two different stationary phases. The U-shaped columns were heavy-walled glass tubing, 5 mm

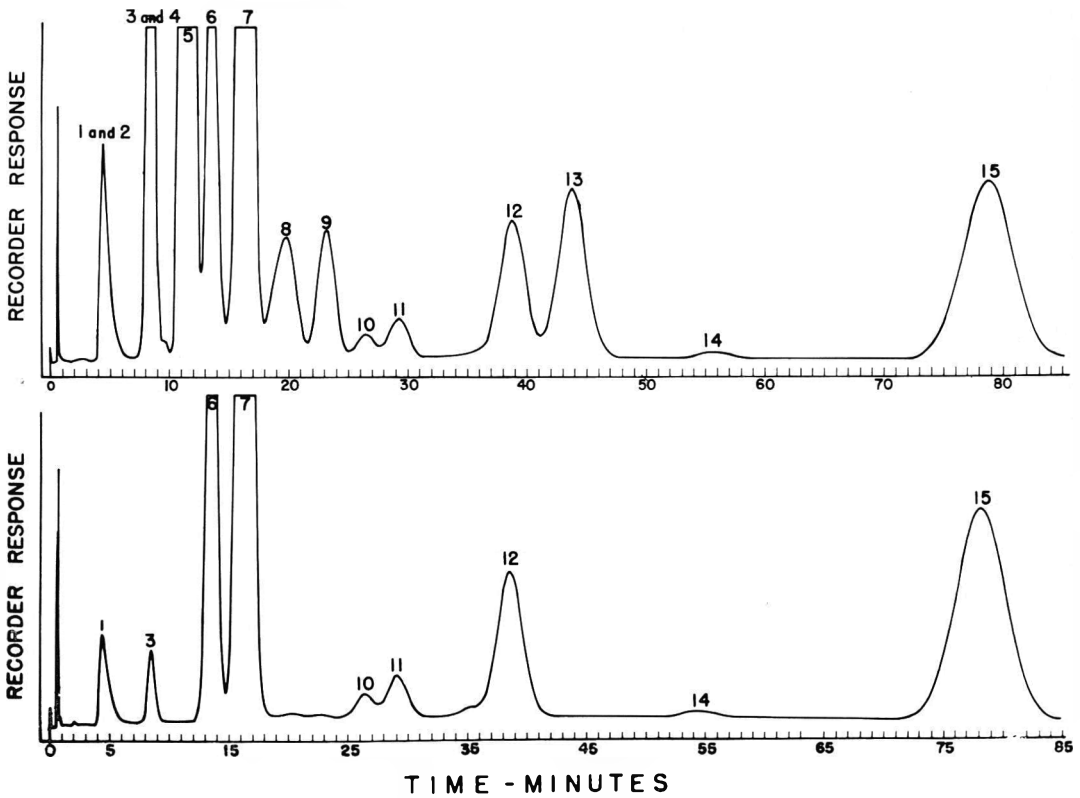


Fig. 1. Gas chromatogram of volatiles from a cider vinegar. Before (upper) and after (lower) treatment with 50% NaOH. The peaks represent 1) acetaldehyde, 2) methyl formate, 3) acetone, 4) ethyl formate, 5) ethyl acetate, 6) diacetyl, 7) ethyl alcohol, 8) sec-butyl acetate, 9) isobutyl acetate, 10) sec-butyl alcohol, 11) propyl alcohol, 12) isobutyl alcohol, 13) isoamyl acetate, 14) n-butyl alcohol, 15) pri-active amyl alcohol.

1D and 6 ft long, packed with either polyethylene glycol 600 (15%) on 60–80-mesh GC-22 firebrick or with diisodecylphthalate (15%) on 60–80-mesh celite. The operating conditions were: column temperature, 105°C; injection port, 215°C; heater bath, 145°C; carrier gas (He), 18 psig; and sensitivity, 10^{-8} amps.

The resolved components were identified by comparing their retention volumes with compounds of known composition.

Functional group analysis. The syringe technique of Hoff and Feit (1964) was used for gas chromatographic analysis of functional groups of compounds in the vapor mixtures from various vinegar samples. Under the conditions used, saturated potassium permanganate in water removed aldehydes, leaving ketones; hydroxylamine hydrochloride (4 g/50 ml) in water removed carbonyl compounds; and sodium hydroxide removed esters. However, for the latter step it was necessary to modify the above technique in order to hydrolyze the esters effectively. Two ml of 50% sodium hydroxide was added to a vial containing 10 ml of the vinegar sample and allowed to stand for 1 hr prior to withdrawing the vapor sample for GLC analysis. The alcohols were determined by difference.

RESULTS AND DISCUSSION

The fact that members of different homologous series of organic compounds may have similar retention volumes has limited the use of gas chromatography for qualitative analysis of complex mixtures. Consequently, retention volume data without a knowledge of compound type lacks sufficient specificity to permit positive identification. This problem can be readily resolved by means of functional group classification reagents (e.g. Hoff and Feit, 1964) and may be illustrated by the chromatograms in Fig. 1. The chromatogram of the cider vinegar (Fig. 1, upper) appears to be that of a mixture having 13 components. Functional group tests showed that the first peak was due to an ester and an aldehyde, and the second peak to an ester and a ketone. Thus, 15 components were present rather than the 13 indicated by the chromatogram. Furthermore, if one compares the two chromatograms (upper and lower) it will be noted that peaks 5, 8, 9, and 13 were absent (Fig. 1, lower) following treatment with the sodium hydroxide reagent, thereby indicating that the peaks represented esters. Sodium hydroxide effectively hydrolyzed the esters and produced peaks of the corresponding alcohols. However, the yield of these products was poor and it will be noted methanol was not detected (probably because of high solubility in the reagent). Following assignment of the functional group to each peak in a chromatogram, comparison with relative retention volumes of pure reference compounds permitted identification of all compounds.

Representative chromatograms of the volatiles present in several of the vinegar samples are shown in Fig. 2. Examination of the chromatograms shows qualitative differences between the vinegars made by the alcoholic and subsequent acetous fermentations (Figs. 2-A,B) as compared to the vinegars made by the acetous fermentation of dilute distilled alcohol (Figs. 2-C,D). A qualitative difference existed between the two dis-

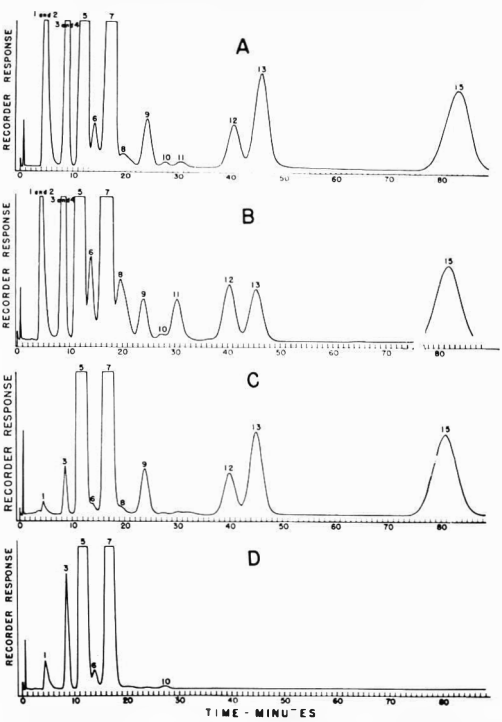


Fig. 2. Gas chromatograms of volatiles from several kinds of vinegar. Part *A*, wine vinegar; Part *B*, tarragon vinegar; Part *C*, distilled vinegar, sample No. 2; Part *D*, distilled vinegar, sample No. 1. These peaks represent: 1) acetaldehyde, 2) methyl formate, 3) acetone, 4) ethyl formate, 5) ethyl acetate, 6) diacetyl, 7) ethyl alcohol, 8) sec-butyl acetate, 9) isobutyl acetate, 10) sec-butyl alcohol, 11) propyl alcohol, 12) isobutyl alcohol, 13) isoamyl acetate, 14) n-butyl alcohol, 15) pri-active amyl alcohol.

tilled vinegars (Figs. 2-C,D). This difference resulted from the acetous fermentation of an alcohol (Fig. 2-D) prepared from fermentation of a mixture of corn, barley malt, and rye (distilled vinegar No. 2), whereas the vinegar (sample 1 in Fig. 2-C) was from the acetous fermentation of an alcohol whose source was not identified on the label. Similarly, close examination of the gas chromatograms reveals that there are quantitative differences among the various samples by comparing the peak heights of some of the components.

The importance to vinegar flavor of the 25 constituents in vinegar, as listed in Table 1, will require additional quantitative data. However, studies in other fermentation areas such as dairy products, wine, and beer, indicate that the flavor characteristics of these organic compounds are very important to the acceptability of the product.

Carbonyl compounds. Five of the eight vinegar samples contained acetaldehyde, acetone, and diacetyl. Amounts of diacetyl in the distilled vinegar samples were minute as compared with the natural vinegars (cider and wine). Diacetyl is undoubtedly one of the important flavor components of vinegar. In reviews on the flavor of beer (Stevens, 1960; Lawrence, 1964), diacetyl was re-

Table 1. Volatile compounds identified in the vapor mixtures from different vinegar samples.

	Natural[a] vinegars		Distilled[b] vinegars sample no.					Special[c] "mixed" vinegar
	Cider	wine	1	2	3	4	5	tarragon
Carbonyls								
acetaldehyde	+	+	+	+	+	+	−	+
acetone	+	+	+	+	+	+	+	+
diacetyl	+	+	+	+				+
acetoin						+		+
isobutyraldehyde	+	+	+		+	+	−	+
isovaleraldehyde					+	+	+	
a-methyl valeraldehyde						+		
methyl isobutyl ketone					+	+	+	
Esters								
methyl formate	+	+						+
ethyl formate	+	+						+
ethyl acetate	+	+	+	+	+	+	+	+
ethyl propionate	+							+
isobutyl acetate	+	+		+				+
n-butyl acetate	+							+
ethyl butyrate				+				
sec-butyl acetate	+	+		+				+
isoamyl acetate	+	+		+				+
Alcohols								
ethyl alcohol	+	+	+	+	+	+	+	+
propyl alcohol	+	+						+
n-butyl alcohol	+					+		
sec-butyl alcohol	+	+	+					+
pri-active isobutyl alcohol	+	+		+				+
amyl alcohol	+	+		+				+
isoamyl alcohol	+	+						+
sec-active amyl alcohol		+						+

[a] Natural vinegars: cider (5% acetic acid); wine (6%) from California grape wine.
[b] Distilled vinegars: Sample No. 1, crystal distilled 5% acetic; No. 2, distilled white (5% acetic) and made from corn, barley and rye; No. 3, 4 and 5 distilled vinegar samples from pickle companies which manufacture their own vinegar for pickle products. Samples No. 3 and 5 made by "Frings Process" (passed through shavings) and No. 4 by "The Acetator" (submerged fermentation).
[c] Special mixed vinegar: Tarragon flavored and a mixture of distilled and malt vinegar.

ported as contributing to an objectionable off-flavor at levels of 0.5 ppm and above. In dairy products, diacetyl has been considered a desirable flavor component, with threshold levels ranging from 0.01 to 0.20 ppm (Bennett *et al.*, 1965). Diacetyl has been reported in red wine in quantities of 2–4 ppm (Prescott and Dunn, 1959). However, in the present study the possible flavor contribution of the other carbonyl compounds such as acetaldehyde, acetone, isobutyraldehyde, and acetoin has not been established. It has been suggested that acetoin may be further oxidized to diacetyl (Lawrence, 1964).

Alcohols. Seven higher alcohols were identified in addition to ethyl alcohol (Table 1). The natural fruit vinegars (cider and wine) contained six of these alcohols, and the five distilled vinegar samples contained two of these alcohols. The flavor and aroma of fruit vinegars, when compared to distilled vinegars, might be explained by the differences in their alcohol contents. Conceivably, these alcohols could form esters with the organic acids, particularly acetic acid, and thereby contribute to the aroma of the vinegars. The higher alcohols are considered the most important group of volatiles in beer flavor (Stevens, 1960; Lawrence, 1964), and they are the principal components of the fraction known as "fusel oil." The composition of fusel oil is influenced by two main factors: a) the nature of the organisms affecting fermentation; and b) the nature of the substrate. A standard fusel oil, 1 part isobutyl to 4 parts isoamyl alcohol (containing 20% of the optically active isomer), has been used for flavor studies (Hudson and Stevens, 1960). In beer, fusel oil content ranged from 39 to 323 ppm, and a sample of cider contained 98 ppm of fusel oil. In a lager beer, a panel of tasters was able to differentiate between samples containing 45 and 50 ppm of isoamyl alcohol (Hudson and Stevens, 1960). Isoamyl alcohol was identified in the natural vinegars (cider, wine and tarragon) and absent in the five distilled vinegar samples. Thus the composition and concentration of fusel oil may be partially responsible for the characteristic odor and flavor of the vinegar samples.

Esters. In the two-step fermentation (alcohol and acetic acid) of vinegars it is not surprising that esters make up the largest group of volatile flavor compounds, representing nine of the 25 identified (Table 1). Esters are known for their aromatic odors. It is plausible to assume that ethyl acetate is the predominating ester in vinegar since vinegar contains considerable amounts of acetic acid and ethyl alcohol. Also, ethyl acetate is used for denaturing industrial ethyl alcohol at the rate of 4.25 gal per 100 gal of alcohol. Suomalainen and Kangasperko (1963) showed that the ester content of a distilled vinegar (10% acid), measured as ethyl acetate, increased from 200 mg/L in a 60-day storage period to a content of 1500 mg/L and that most of the esters were formed in the first 30 days. Eight additional esters (Table 1) were identified in the volatiles from cider, wine, and tarragon vinegar samples. In four of the five distilled vinegar samples, ethyl acetate is the only ester present and may account for the contrast in odor between natural and distilled vinegars. The esters which have been identified are those of the eight alcohols and short-chain fatty acids (Table 1). They may be direct fermentation products or they may be formed during conditioning of the vinegar. The chief difference in chromatogram profiles between distilled and natural vinegars appears to be due to the higher alcohols and ester contents of the natural vinegars.

Rentschler (1942) and Jacobs (1951) reported that the presence of acetylmethylcarbinol (acetoin) can be used to distinguish a product of acetous fermentation from artificial vinegar. In the present studies, acetoin was found only in the tarragon vinegar and in distilled vinegar sample No. 4.

Toth (1941) reported that diacetyl was not detected in distilled (spirit) vinegar. In contrast, diacetyl was present in two of the five distilled vinegars used in this study.

In conclusion, it would appear that the determination of fermentation products by gas chromatography would provide an index of the genuineness of a vinegar. Similarly, it appears that the standards of identity of a particular vinegar could be determined by the relative concentration of the volatiles, especially the alcohols and esters.

REFERENCES

Bennett, Grace, B. J. Liska, and W. L. Hempenires, 1965. Effect of other flavor components on the perception of diacetyl in fermented dairy products. *J. Food Sci.* **30**, 35.

Conner, H. A., G. B. Nickol, R. T. Wisthoff, and F. M. Hilderbrandt, 1959. Effect of grain strength on generator operation. Vinegar News Letter No. 27, U. S. Industrial Chemicals Co., 99 Park Avenue, New York 16, New York.

Hoff, J. E., and E. D. Feit, 1964. New technique for functional group analysis in gas chromatography syringe reactions. *Anal. Chem.* **36**, 1002.

Hudson, J. R., and J. Stevens, 1960. Beer flavor. II. Fusel oil content of some British beers. *J. Inst. Brewing* **66**, 471.

Jacobs, M. B. 1951. "Food and Food Products." pg. 1729, Vol II., Interscience Publishers, New York, N.Y.

Lawrence, W. C. 1964. Volatile compounds affecting beer flavor. *Wallerstein Labs. Communs.* **27**, 123.

Prescott, S. C., and C. G. Dunn, 1959. "Industrial Microbiology." 3d ed. McGraw-Hill Book Co., New York, N. Y.

Rentschler, E. 1942. The acetylmethylcarbinol content of vinegars. *Deut. Essigind.* **46**, 94.

Stevens, R. 1960. Beer flavor. I. Volatile products of fermentation. A review. *J. Inst. Brewing* **66**, 453.

Suomalainen, H., and J. Kangasperko, 1963. Aromatic components of natural vinegar. *Z. Lebensm.-Forsch.* **120**, 353.

Toth, E. 1941. Distinguishing between wine vinegar and spirit vinegar. *Z. Lebensm.-Forsch.* **82**, 439.

Ms. rec'd 9/15/65.

Published with approval of the director, North Carolina Agricultural Experiment Station, as Paper No. 2022 of the Journal Series.

J. A. Singleton is a Research Fellow, supported by Pickle Packers International, Inc., St. Charles, Illinois.

The U. S. Food Fermentation Laboratory is one of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

The mention of products or companies does not imply endorsement.

The authors gratefully acknowledge the contribution of the following persons and their companies for providing vinegar samples and for technical information relating to the commercial production of vinegar: Mr. Franklin C. Clements, Dailey Pickle Company, Saginaw, Michigan; Mr. Ivan D. Kittel, M. A. Gedney Company, Chaska, Minnesota; and Mr. Laurence J. Turney, H. W. Madison Company, Medina, Ohio.

This work was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, Illinois.

B. S. LUH and K. D. DASTUR[a]
Department of Food Science and Technology
University of California, Davis, California 95616

Texture and Pectin Changes in Canned Apricots

SUMMARY

The influence of ripeness level, heat processing, and post-canning storage on texture and pectin changes in canned apricots was investigated. Important factors influencing canned texture were ripeness level of the fresh fruit and processing time. The canned product gradually softened during storage, while water-soluble pectin in the syrup increased and protopectin in the fruit decreased. Riper apricots contained less protopectin, and the syrup derived from them contained more water-soluble pectin. Syrup viscosity gradually increased during storage of canned apricots. The characteristics of pectin were related to the texture of canned apricots.

INTRODUCTION

The grade of canned apricots is based on color, character, uniformity, and absence of defects (USDA, 1945). The character score is largely related to the texture of the canned product. Luh *et al.* (1959) have shown that fill weight and ripeness level are important factors influencing the drained weight of canned apricots. Riper fruits were lower in equilibrium drained weight after canning. Firmness and skin color are two criteria that have been used to determine the harvest maturity of apricots for canning (Allen, 1932; Hoos *et al.*, 1956; Strachan, 1951). Not much is known, however, about the factors that influence the texture of canned apricots.

This paper reports the influence of ripeness level, heat processing, and post-canning storage on texture and pectin changes in canned apricots.

EXPERIMENTAL MATERIALS AND METHODS

Apricots. Used in this study were Blenheim apricots grown on the University farm at Winters, California. One-hundred-lb lots were harvested on the same day at three ripeness levels based on skin color and firmness (Magness and Taylor, 1925): firm, medium, and soft ripe. Apricots of medium ripeness were used for studying the effect of heat processing and post-canning storage on texture and pectin changes.

Canning. The apricots were washed, sorted, and then pitted and halved in a Filice and Perrelli pitting machine. Each No. 2½ can (401 × 411) received 19½ oz of fruit and 10.5 oz of 40° Brix sucrose syrup, and was sealed in a double sealer under a vacuum of 16 in. Hg. Unless stated otherwise, the samples were processed for 19 min at 212°F in an Anderson-Barngrover rotary cooker and then cooled in a rotary water cooler. The effect of processing time on texture was determined by processing at 212°F for 11, 15, and 19 min. The processed cans were stored at 70°F for texture and pectin studies.

Texture determination. The texture of the canned apricots was determined with a L.E.E.-Kramer recording shear press. One hundred fifty grams of fruit were placed in a regular test cell. A 3,000-lb ring was used for testing the fresh fruit, and a 500-lb ring for the canned product. Dial settings for the fresh fruit ranged from 300 to 1,000 lb, depending on ripeness. Used for the canned apricots was a 200-lb setting. In all cases the readings were converted to a 100-lb basis, and are reported as such. The time interval from the beginning of the downward motion of the ring until it passed through the product in the test cell was 1 minute ± 5 seconds. The area under the curve, in square inches, represents the firmness of the product. The average of 12 readings on the contents of 4 cans for each sample is reported.

Water-soluble pectin. The syrup from 4 cans of apricots was combined. One hundred fifty ml of the syrup was mixed with 5 g of Super-Cel. The syrup was filtered under suction through Whatman no. 1 filter paper in a Buchner funnel. To 10 ml of the filtrate was added 5 drops of 0.5*N* HCl and 30 ml of 95% ethanol. The precipitated pectin was collected by centrifugation, washed twice with a 25-ml portion of 70% ethanol, suspended for 1 hr in 25 ml of 0.05*N* NaOH, and then diluted to 1 L with distilled water. Two ml of the test solution was added slowly, with shaking, into a 25 × 200-mm Pyrex tube containing 12 ml of conc. H_2SO_4 in an ice-water bath. The tube was heated 10 min in a boiling-water bath and then cooled in ice water to 2°C. One ml of 0.15% carbazole reagent was added and the mixture well shaken. After 25±5 min the absorbance of the resulting solution was read in a Klett-Summerson photo-

[a] Present address: Department of Dairy and Food Industries, University of Wisconsin, Madison, Wisconsin.

electric colorimeter with a No. 54 green filter. A standard curve was obtained by using galacturonic acid solutions of 0–80 μg (McCready and McComb, 1952; Postlmayr *et al.*, 1956).

Total pectin. The fruit from 4 cans of apricots was sampled, drained for 2 min on an 8-mesh screen, and then macerated in a Waring blender. Twenty-five g of the macerate was mixed with 250 ml of 95% ethanol. The mixture was allowed to stand for 1 hr with occasional stirring. The alcohol-insoluble solids (AIS) were collected on a Whatman no. 1 filter paper and washed once with 95% ethanol, twice with 70% ethanol, and finally with 20 ml of purified 95% ethanol (McCready and McComb, 1952). The AIS were treated with 300 ml of 0.5% Versene (tetrasodium salt of ethylenediaminetetraacetate), and the pH adjusted to 11.5 with 1*N* sodium hydroxide. The solution was permitted to stand for 30 min at room temperature, and then acidified to pH 5.0 with 1*N* acetic acid. To this mixture was added 4 ml of a freshly prepared and filtered 10% pectic enzyme solution (Pectinol R-10, Rohm and Haas) and a few drops of toluene. The mixture was allowed to stand overnight, diluted to 500 ml with distilled water, and then filtered. Four ml of the solution was diluted to 100 ml with distilled water. Two ml of the solution was used for determination of total pectin by the carbazole colorimetric method as described above.

Protopectin. Protopectin in the drained fruit was determined after removal of the water-soluble fraction (Postlmayr *et al.* 1956).

Characterization of pectic substances. The method described by Gee *et al.* (1958) was modified to determine the free acidity and methyl ester content of the pectins isolated from the fruit and the syrup.

Preparation of the marc. One hundred g of the macerate was mixed with 300 ml of 95% ethanol and allowed to stand for 1 hr. The AIS was collected on Whatman no. 1 filter paper, and then blended with 200 ml of 0.6*N* HCl in 70% ethanol for 2 min in a Waring blender. The mixture was allowed to stand 1 hr at room temperature. The supernatant was removed by filtration. The residue was washed three times successively with 350-ml portions of 70% ethanol, and then blended twice with 200 ml of acetone to remove pigments. The marc was dried at room temperature, ground in a mortar, and stored in covered bottles at room temperature.

Free acidity. One-half g of the dry marc was weighed into a beaker and mixed with 5 ml of 95% ethanol. One g sodium chloride and 100 ml distilled water were added to the mixture with vigorous stirring. The free acidity in the marc was determined by direct titration with 0.1*N* NaOH to pH 8.0 in a Beckman automatic titrator, model K. A blank was run with the same quantities of reagents.

For the pectin in the syrup, 50 ml of the clear serum was used to precipitate the pectin with acidified alcohol as described above. The precipitate was washed free of acids with 95% ethanol and dissolved in 100 ml of distilled water. The free acidity of the pectin in the syrup was determined by titration as described above. Free acidity is expressed as meq per g of pectin.

Degree of esterification. Ten ml of 0.5*N* NaOH was added to the neutralized solution. The mixture was stoppered and allowed to stand for 30 min at room temperature to saponify the methyl ester groups in the pectin. Then, 10 ml of 0.5*N* HCl was added and the mixture was titrated to pH 8.0 against 0.1*N* sodium hydroxide in a Beckman automatic titrator, model K. The titer was corrected for the reagent blank.

Acetyl groups. Acetyl groups in the pectin were determined by the method of McComb and McCready (1957). Four hundred mg of the marc was added slowly to 200 ml of distilled water in a Waring blender and then diluted to 250 ml with distilled water. Five ml of the solution was pipetted into a 25-ml volumetric flask. Two ml of a solution made from equal volumes of NaOH (9.4 g/100 ml water) and hydroxylamine hydrochloride (3.75 mg/100 ml water) was added. After standing for 4 min, 5 ml of acidified methanol (35.2 ml of 70% perchloric acid diluted to 500 ml with chilled absolute methanol) was added with constant mixing. The mixture was made to volume by adding small increments of ferric perchlorate solution. After holding for 5 min the precipitated pectin-hydroxamine acid-ferric complex was removed by filtering through a Whatman no. 12 filter paper. The absorbance of the filtrate was measured in a Klett-Summerson photoelectric colorimeter with a No. 54 green filter.

The pectin precipitated from 10 ml of clear syrup was washed with 95% ethanol and then dissolved in 5 ml water. This solution was used for determination of acetyl content in the same way as described above.

Syrup viscosity. The syrup from four cans was drained through an 8-mesh screen for 2 min. The syrup was centrifuged for 30 min at 600 ×G. The flow time of 10 ml of the centrifugate was determined at 30°C in a no. 300 Ostwald-Finske capillary viscometer. A pyknometer was used to measure the density of the syrup. An electrical timer with an accuracy of 0.1 sec was used to measure the flow time, in seconds. The viscosities are re-

Table 1. Chemical and physical properties of Blenheim apricots.

Sample	Ripeness level	Dimensions (mm)			Magness-Taylor pressure test (7/16") plunger (lb)	L.E.E.-Kramer shear press readings (work area in sq. inches)	pH	Soluble solids (° Brix at 20°C)	Total acidity as citric acid (%)
		Length	Check	Suture					
A	Firm	44.9	40.2	45.6	3.4	9.10	3.85	16.0	1.23
B	Medium	43.5	41.4	45.7	1.8	6.32	3.88	16.2	1.16
C	Soft ripe	43.9	41.5	46.1	1.0	4.42	3.90	17.1	1.05

ported in centipoises, with water at 30°C as a reference.

RESULTS

Chemical and physical properties. Table 1 shows the chemical and physical properties of Blenheim apricots at 3 ripeness levels. Respective average pressures on the Magness-Taylor pressure gauge with a 7/16-inch plunger were 3.4, 1.8, and 1.0 lb. Readings on a L.E.E.-Kramer shear press showed a similar trend. Firmer fruits were higher in pressure test and shear-press readings. As apricots ripen, soluble solids content increases and titratable acidity decreases. Among the more objective methods for evaluating ripeness levels are the pressure-test and shear-press readings. The former is simpler to perform, and the latter is more useful for correlating the ripeness level of the fresh apricot to that of the canned product.

Texture changes. Fig. 1 shows the effect of storage at 70°F up to 12 months on the texture of Blenheim apricots canned at different ripeness levels. The L.E.E.-Kramer shear press is useful for correlating the texture of the fresh fruit with that of the canned product. The canned product stored at 70°F gradually softened. The trend of softening in storage was similar regardless of initial ripeness level.

Figs. 2 and 3 show changes in the texture of canned apricots varying in processing time from 11 to 19 min. The longer processing time resulted in a canned product of softer texture. This may be

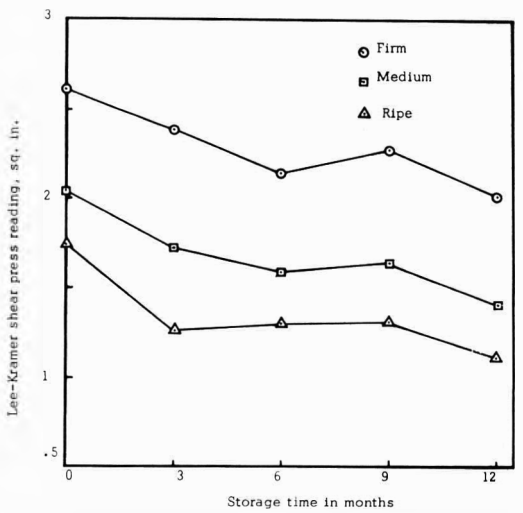


Fig. 1. Effect of storage at 70°F on texture of Blenheim apricots canned at different ripeness levels.

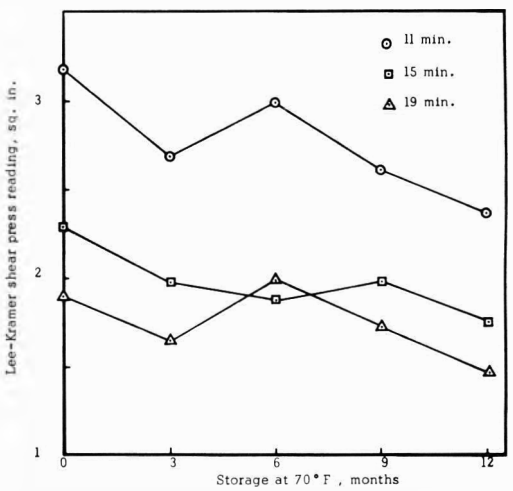


Fig. 2. Effect of storage at 70°F on texture of medium-ripe Blenheim apricots processed at 212°F for 11, 15, and 19 min.

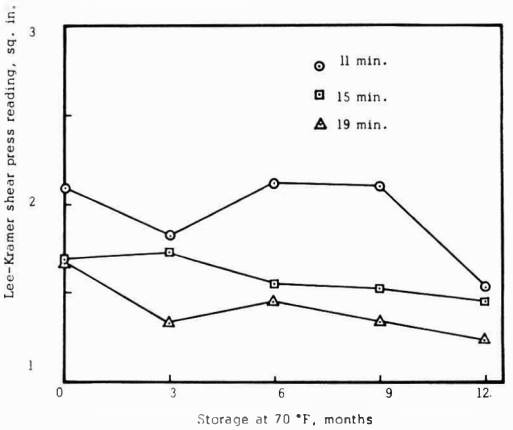


Fig. 3. Effect of storage at 70°F on texture of soft-ripe Blenheim apricots processed at 212°F for 11, 15, and 19 min.

explained by solubilization of the protopectin, which acts as binding materials in the middle lamella of the cell walls. The three samples used in this investigation were acceptable for choice grades even after storage for 12 months at 70°F. In some batches of unusually soft apricots, the L.E.E.-Kramer shear-press reading of the canned product may go lower than one square inch. Such samples were considered poor in texture because of lack of firmness. In several cases the fruit tissue disintegrated completely. The cause of the unusual softening is not known.

Water-soluble pectin. Protopectin acts as a binding material between cell walls and is related to fruit texture. Softening of fresh apricots during ripening may be related to gradual conversion of protopectin to water-soluble pectin. This action would reduce the adhesive force between cells and bring about softening. The gradual softening of canned apricots was related to the increase in water-soluble pectin in the syrup. Fig. 4 shows

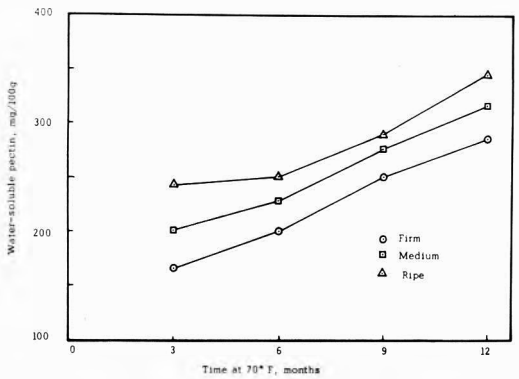


Fig. 4. Effect of storage on water-soluble pectin in the syrup of Blenheim apricots processed at different ripeness levels.

that the ripeness level of the fresh fruit was related to the water-soluble pectin in the syrup. Riper fruits yielded syrup of higher water-soluble pectin than the medium and firmer fruits. When the canned products were stored at 70°F, the water-soluble pectin in the syrup increased gradually. It appears that the cementing materials between cell walls were gradually moving into the syrup during storage, which explains, at least in part, the gradual softening in texture.

Firmer apricots may be made softer in texture by increasing the heat-processing time. This would indicate that heat energy accelerated acid hydrolysis of protopectin between the cell walls, causing an increase in water-soluble pectin and weakening the binding force between the cells.

Protopectin. One of the changes that takes place during ripening is conversion of protopectin into water-soluble pectin. This process decreases the fruit's textural strength. Fig. 5 shows the effect of ripeness level on protopectin in canned fruit during storage at 70°F. Firmer fruit contained higher amounts of protopectin than the medium and soft fruit. During storage, a good portion of the protopectin was converted to water-soluble pectin. Along with the progressive decrease in protopectin, the canned product softened.

Fig. 6 shows the effect of processing time on protopectin retention. Fruit processed for 19 min was lower in protopectin content than fruit processed 15 and 11 min. It appears that conversion of protopectin in the cell walls to water-soluble pectin was catalyzed by the heat energy received during processing. In order to retain the texture in the product, it would be advisable to adjust the processing time to get the more desired texture. Fruits that were higher in protopectin content were firmer.

Characterization of pectin in canned apricots. Table 2 lists the uronide, acetyl, and free and

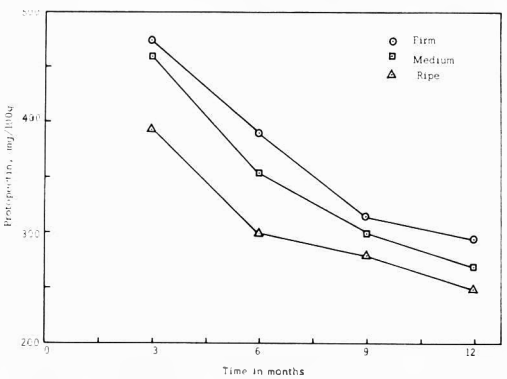


Fig. 5. Changes in protopectin content of canned apricots stored at 70°F. Fruits of different ripeness levels were processed for 19 min at 212°F.

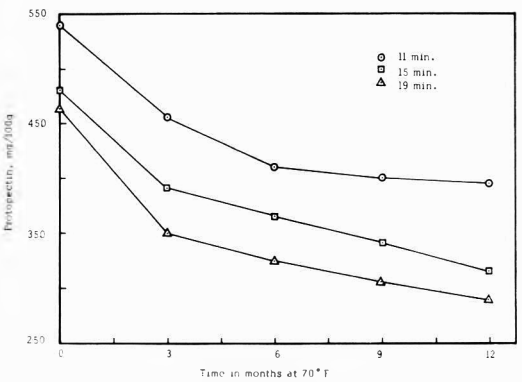


Fig. 6. Changes in protopectin content of canned Blenheim apricots stored at 70°F. Fruits of medium maturity were processed at 212°F for 11, 15, and 19 min.

Table 2. Effect of harvest maturity on certain characteristics of pectin isolated from fresh and canned Blenheim apricots.

Maturity level	Carboxyl groups (meq/g marc)	Esterified groups (meq/g marc)	Acetyl groups (meq/g marc)	Esterified carboxyls in uronic acid (meq/g marc)	Total uronic acid carboxyls (meq/g marc)	Anhydro-uronic acid (%)	Esterified carboxyls (%)
Fresh fruit							
Firm	0.394	1.42	0.343	1.08	1.47	25.9	73.2
Medium	0.404	1.35	0.331	1.02	1.42	25.0	71.6
Ripe	0.435	1.33	0.312	1.02	1.45	25.5	70.0
Canned apricot, fruit only, stored 6 months at 70°F							
Firm	0.414	1.24	0.305	0.938	1.35	23.8	69.4
Medium	0.425	1.20	0.299	0.903	1.33	23.4	68.0
Ripe	0.435	1.18	0.293	0.888	1.32	23.3	67.1

Table 3. Degree of esterification of pectin in syrup of canned Blenheim apricots stored 6 months at 70°F.

Maturity level of fresh fruit	Carboxyl groups (meq/g pectin)	Esterified groups (meq/g pectin)	Acetyl groups (meq/g pectin)	Esterified carboxyls in uronic acid (meq/g pectin)	Total uronic acid (meq/g pectin)	Anhydro-uronic acid (%)	% esteri-fication
Firm	1.533	3.74	0.656	3.08	4.61	81.2	66.8
Medium	0.990	3.63	0.749	2.88	3.87	68.1	74.4
Ripe	0.753	4.00	0.870	3.13	4.00	70.5	78.3

esterified carboxyl constituents of pectin isolated from canned apricots. Fresh and canned apricots differed in esterified carboxyls. The degree of esterification in the pectin decreased slightly with maturation.

Table 3 shows that canned apricots and their syrup differed markedly in degree of esterification of the pectin. Pectin isolated from the syrup was 66.8% esterified for firm fruit, 74.4% for medium-maturity fruit, and 78.3% for ripe fruit. The highly esterified pectin in the riper fruit probably diffused into the syrup more readily.

Table 4 shows the acetyl and methoxyl contents of pectin isolated from fresh and canned apricots. Acetyl content did not change appreciably during processing, but in the syrup pectin increased with increased fruit maturity. This indicates either that the water-soluble pectin fraction of the fruit increases in acetyl content with maturation or that it diffused into the syrup more readily from riper fruit than from firmer fruit.

Table 4. Acetyl and methoxyl content of pectin isolated from Blenheim apricots.

Ripeness level	Acetyl content (%)	Methoxyl content (%)
Fresh fruit		
Firm	5.58	11.7
Medium	5.60	11.4
Ripe	5.27	11.2
Canned fruit stored 6 months at 70°F		
Firm	5.52	11.1
Medium	5.50	10.9
Ripe	5.40	10.7
Syrup from canned fruit stored 6 months at 70°F		
Firm	3.47	10.7
Medium	4.73	11.9
Ripe	5.31	12.5

DISCUSSION

It appears from this investigation that the texture of canned apricots varied with the ripeness level of the fresh fruit, duration of heat processing, and post-canning storage. The softening of canned apricots can be explained, at least in part, by the conversion of protopectin to water-soluble pectin. This may occur either enzymically during the ripening process or chemically through hydronium ion catalysis during heat processing and post-canning storage. The increase in water-soluble pectin in the syrup and decrease in protopectin in the fruit during heat processing and storage substantiate the above hypothesis. There was a gradual decrease in methoxyl content of the pectin in the fruit as ripening proceeded. The difference be-

tween fruits of different ripeness levels in methoxyl content of the pectins was comparatively small compared with the conversion of protopectin to water-soluble pectin. It is believed that the binding force between cells which influences the texture of the canned product was largely dependent on the pectic material in the middle lamella of the cell wall. Movement of the pectic materials from the cell wall into the syrup during storage caused gradual softening of the texture.

Occasionally, canned apricot halves show an unusually soft texture. In severe cases, the fruit texture may break down completely. This phenomenon was observed occasionally in fruits from certain growing areas. One of the factors found related to the texture breakdown was higher acidity and low pH in the fresh fruit. The higher concentration of hydronium ions present in the high-acid fruits may accelerate the breakdown of the binding materials between the cell walls during heat processing. The possibility also exists that abnormalities in the cellular structure of apricots or in certain other chemical constituents may also be contributing factors.

Normal apricots for canning have pH values ranging from 3.6 to 4.0 and acidity of 0.8–1.3%. In certain growing areas, apricots had pH values as low as 3.4, and in others values as high as 4.6 . The unusual pH value may be related to the difference in varietal characteristics, soil property, horticultural practices, climatic conditions, and some unknown factors which contribute to the soft texture in the canned product. Perhaps the effects of air pollution, nutrient levels, soil types, irrigation practices, and chemical sprays are among the more important factors that need investigation for better understanding of the problem.

REFERENCES

Allen, F. W. 1932. Physical and chemical changes in ripening of deciduous fruits. *Hilgardia* **6**(13), 381.

Gee, M., E. A. McComb, and R. M. McCready. 1958. A method for the characterization of pectic substances in some fruit and sugarbeet marcs. *Food Research* **23**, 72.

Hoos, J. W., S. J. Leonard, and B. S. Luh. 1956. Effect of 2,4,5-trichlorophenoxyacetic acid spray on organic acids, pectin and quality of canned apricots. *Food Research* **21**, 571.

Luh, B. S., S. J. Leonard, and E. M. Mrak. 1959. Drained weight of canned apricots. *Food Technol.* **13**(5), 253.

Magness, J. R., and G. E. Taylor. 1925. An improved type of pressure tester for the determination of fruit maturity. *U. S. Dept. Agr. Circ.* No. **350**.

McComb, E. A., and R. M. McCready. 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. *Anal. Chem.* **29**, 819.

McCready, R. M., and E. A. McComb. 1952. Extraction and determination of total pectic material in fruits. *Anal. Chem.* **24**, 1986.

Postlmayr, H. L., B. S. Luh, and S. J. Leonard. 1956. Characterization of pectin changes in freestone and clingstone peaches during ripening and processing. *Food Technol.* **10**, 618.

Strachan, C. C., A. W. Moyls, F. R. Atkinson, and J. E. Britton. 1951. Chemical composition and nutritive value of British Columbia tree fruits. *Can. Dept. Agr. Exptl. Farms Service Publ.* No. **862**, 5–85.

1945. U. S. Standards for Grades of Canned Apricots. USDA.

Ms. rec'd 9/20/65.

The authors thank the Canners League of California for financial support.

A. M. PEARSON, B. G. TARLADGIS,[a] MILDRED E. SPOONER, and J. R. QUINN[b]

Department of Food Science, Michigan State University East Lansing, Michigan 48823

The Browning Produced on Heating Fresh Pork II. The Nature of the Reaction

SUMMARY

Studies are reported on the mechanism of browning occurring in model and meat systems. When model systems were heated, brownness appeared to be due largely to the amino-sugar reaction, although some brown color development apparently occurred from caramelization of the sugars. This was shown by blocking the amino group with acylation or the carbonyl group of sugars by addition of bisulfite or hydroxylamine. The same procedures were used for studying the development of brownness in meat systems, except that in some instances the free sugars naturally present in meat were removed by yeast fermentation or by the addition of glucose oxidase. Although the data with the meat systems were not clear-cut, evidence suggested that most of the browning occurred as a result of the amino-sugar reaction. However, a small but significant amount of browning seemed to be due to pyrolysis of the natural meat sugars.

INTRODUCTION

Recent work in this laboratory (Pearson *et al.*, 1962) demonstrated that the degree of brown color development during the heating of fresh pork is closely related to the level of reducing sugars in the tissues. Thus, results suggested that the browning of sugars is responsible for the color development that occurs on cooking fresh pork. Jenness and Patton (1959) stated that there are three types of browning in foods: 1) caramelization or nonamino browning; 2) the amino-sugar reaction or Maillard browning; and 3) oxidative or enzymatic browning, which is dependent upon the presence of oxygen and occurs in fruits, vegetables and some other foods. Several excellent reviews have been written on browning of foods, with major emphasis being given to the amino-sugar reaction (Danehy and Pigman, 1951; Hodge, 1953; Ellis, 1959; Reynolds, 1963). Hodge (1953) differentiated caramelization from the amino-sugar reaction by stating that caramel formation is due to the pyrolysis of sugars, whereas amino-sugar browning is due to the reaction of an aldehyde group with an amino group. Both reactions ultimately result in the development of brown to black color and the splitting off of water.

Although the major interest in the browning reaction in foods has been focused on its undesirable effects, both Hodge (1953) and Reynolds (1963) pointed out that the browning of foods during the cooking process produces desirable flavors and colors. The brown color and flavor development that occurs on cooking meat is one of the desirable aspects of browning, but seems to be closely related to the undesirable changes occurring in dehydrated meats that have been described by other workers (Henrickson *et al.*, 1955; Regier and Tappel, 1956; Sharp and Rolfe, 1958). Therefore, the current study was designed to ascertain whether the browning that occurs on heating fresh pork is due to caramelization or the amino-sugar reaction. Both model systems and meat systems were utilized to resolve this question.

PROCEDURE

Attempts to ascertain the nature of the reaction were made by comparing the free amino nitrogen and sugar levels, both before and after heating. In addition, the meat was fermented or treated with glucose oxidase and heated. Chemical inhibitors were also added, and their effects upon browning were observed and measured spectrophotometrically.

Free amino nitrogen and sugar levels. Free amino nitrogen was determined by the Van Slyke method as outlined in the AOAC (1960). The levels of reducing sugars were determined by the method of Folin and Wu (1920) and also checked by paper chromatography with a Spinco densitometer according to a modification of the method of Tarr (1953). Originally attempts were made to measure fructose, ribose, and glucose differentially with the densitometer. Since fructose could not be detected and ribose was present at a level of only 5 mg%, measurement of the individual reducing

[a] Present address: Unilever Research Laboratorium, Vlaardingen, Holland.

[b] Present address: Department of Agriculture, Food Research Institute, C.E.F., Ottawa, Canada.

sugars was abandoned. Furthermore, glucose was the only sugar added to the model systems.

Meat-fermentation and glucose oxidase treatments. The meat used in this study was ground fresh pork ham or longissimus dorsi muscle from the loin. The meat was ground thoroughly to give a uniform sample and was stored at −29°C until removed and thawed for various studies. In any one series of studies, the same original sample was used. In the model systems, reducing sugars and free amino nitrogen, or glycine, were added to give the same levels as found in the meat.

The meat was prepared for fermentation by adding 5 g of dried bakers yeast and 2.5 ml of distilled water to 10 g of meat. Then 100 ml of distilled water was added to the meat and the mixture was blended together in a Waring blender. The mixture was placed in a 250-ml Erlenmeyer flask, the flask stoppered, and the mixture incubated for 24 hr at 38°C. Glucose oxidase (Nutritional Biochemical Corp., Cleveland, Ohio) was added in the amount of 0.1 g and handled in the same manner as the yeast treatment. Following incubation, the samples were subjected to browning, measurement of brownness, and analysis for glucose and free amino nitrogen. Corrections were made for blanks by running glucose oxidase and yeast alone.

Preparation of meat fibers and water extract. The meat was divided into the fiber (residue) and the water extract. This was accomplished by extracting 25 g of ground meat with 225 ml of distilled water used in 3 equal portions. The final volume for both the meat and fiber was brought to 250 ml, which gave meat fibers equivalent to 25 g of meat in one flask and the water extract equivalent in another. In a third flask the fibers and water extract were recombined to give the recombined meat fibers and water extract in the same volume of solution. A control sample of meat was also made up to 250 ml. The samples were then subjected to browning and the determinations described herein. The design made it possible to compare the control meat sample, the reconstituted meat sample (water extract + fibers), the water extract, and the meat fibers from the standpoint of their contribution to brownness and the changes in free amino nitrogen and glucose.

Inhibitors. Sodium bisulfite and hydroxylamine were added to both the model system and to the meat at 0.25%, which was found to be the most effective level for inhibiting browning in preliminary studies. Since both of these inhibitors block the carbonyl group, a decrease in browning could be due to either the amino sugar reaction or to caramelization. Acylation of the glycine used in the model systems was accomplished by a modification of the methods described by Cheronis and Entrikin (1957). The meat samples were acylated by the method of Fraenkel-Conrat (1954), using dialysis to isolate the reacted product. Since acylation blocks the free amino groups, browning due to the amino-sugar reaction is stopped but browning due to caramelization can proceed. Thus, the mechanism of browning can be approximated by the amount of brownness developing on using the different inhibitors.

Since pH has a marked influence upon the amount of amino-sugar browning (Ellis, 1959; Reynolds, 1963), the model systems were buffered to the approximate pH of meat (5.6). Since meat has its own buffer system, no attempt was made to buffer the meat slurry. Blank determinations were also made using the inhibitors, which would remove any side effects from peroxide formation or pH effects due to gluconic acid formation. It was pointed out earlier by Ellis (1959) that even though the basic amino groups react more readily than the acidic groups and tend to depress pH, some browning occurs even in strongly acidic solutions.

Model systems. The model systems used in this study consisted of glycine and glucose or Casamino acids (acid-hydrolyzed casein supplied by Difco Laboratories, Detroit, Mich.) and glucose. The concentration of reducing sugars and free amino nitrogen was adjusted to the original level in the raw meat system. Inhibitors were added to the model systems as previously described. The model systems were buffered to the pH of meat (5.6).

Development of brownness. Brownness was developed in the meat-water slurry (20 g of raw meat in 200 ml of distilled water) and the model systems by heating them to dryness in an electric oven at approximately 100°C for 24–27 hr. In some cases, a crust formed over the top of the residue and trapped some of the water. Thus, during the latter phases of drying, the material in the flasks was frequently agitated to break up the crust and facilitate drying. As soon as the residue appeared to be dry, the flasks were removed from the drying oven.

Measurement of brownness. The dried samples were redispersed in the original amount of distilled water. After filtering, browning was measured in a Bausch and Lomb Spectronic 20 colorimeter at wave lengths varying from 350 to 450 mμ for different parts of the study. Original readings were made on a diluted sample at 350 mμ, but the method was modified for reading the undiluted sample at 450 mμ (Pearson *et al.*, 1962). Reading at different wave lengths was possible since the curve appeared to be solely dependent upon concentration and showed no distinct absorption peaks. Attempts

were also made to measure fluorescence with a Beckman DU spectrophotometer equipped with a fluorescence attachment at a wave length of 285 mμ. However, fluorescence readings were not in close agreement with the colorimetric readings and are not reported herein. Visual observations on color intensity were also recorded and are given when in obvious disagreement with spectral values.

RESULTS AND DISCUSSION

Influence of various meat fractions upon browning. Table 1 summarizes the changes in glucose (reducing sugars) and free amino nitrogen during development of the brown color as shown by optical density at 450 mμ. Both the water extract of meat and the fiber residue showed considerably less browning than either the control meat sample or the reconstituted meat (meat fibers + water extract at original concentration). Even though the reconstituted meat had a slightly lower optical density after browning than the control sample, some loss of glucose and free amino nitrogen would be expected and appears to be responsible for the slight decrease in brownness. The lower values for both free amino nitrogen and glucose also indicated some loss of these constituents during fractionation. However, the agreement between values is remarkably good and suggests that both the fibers and water extract of meat contain constituents that are essential to maximum color development.

For some unexplained reason, the amount of glucose was higher after browning for both the water extract of the meat and for the extracted fibers. This is not without precedent, since Ellis (1959) pointed out that changes in optical density due to brown color development is not a good index of the extent of the amino-sugar reaction. He further indicated that the decrease in free amino groups and reducing sugars was difficult to correlate with the amount of brownness.

Effect of glucose oxidase and yeast fermentation on browning. Table 2 demonstrates the influence of yeast fermentation and glucose oxidase on the levels of glucose and free amino nitrogen before and after browning and also gives the optical density reading following brown color development. Results show that fermentation resulted in a marked decline in color development as well as in a decline in glucose levels before browning. This is in agreement with the results obtained by Sharp and Rolfe (1958) from a similar study, in which they removed the reducing sugars by fermentation and largely prevented amino-sugar browning of

Table 1. Browning of various meat fractions.

Sample	Glucose (mg%)[a]		Free amino nitrogen (mg/g)[b]		Optical density at 450 mμ
	Before browning	After browning	Before browning	After browning	
Meat slurry	591	278	1.66	1.67	0.390
Water extract	435	479	0.52	0.33	0.175
Meat fibers	47	108	0.79	1.25	0.060
Water extract + fibers	539	396	1.28	1.92	0.330

[a] All values are expressed in terms of mg of glucose per 100 g of the original raw meat (mg %).
[b] Free amino nitrogen is expressed in terms of mg/g of the original raw meat.

Table 2. Effect of glucose oxidase and fermentation on browning.[a]

Treatment	Glucose [b] (mg%)		Free amino nitrogen [c] (mg/g)		Optical density at 450 mμ
	Before browning	After browning	Before browning	After browning	
Meat slurry	807	147	1.92	0.50	0.572
Meat + yeast [d]	119	191	3.18	2.02	0.120
Meat + glucose oxidase [d]	241	142	2.48	0.88	0.230

[a] Each value is an average of 6 replications.
[b] All values for glucose are expressed as mg per 100 g of the original raw meat.
[c] Free amino nitrogen is expressed in mg per g of the original raw meat sample.
[d] After an initial period of 24 hr at 38°C.

dehydrated cooked pork. Results also support the recommendations of Henrickson *et al.* (1955) that yeast fermentation can be utilized in prevention of browning of dehydrated pork tissues. Sharp and Rolfe (1958) have suggested that extra high levels of reducing sugars occur in pig muscle and are responsible for the greater problems from browning during dehydration and storage of pork.

Although the control meat sample and the meat with added glucose oxidase (Table 2) declined markedly in glucose and free amino nitrogen content, the meat plus yeast resulted in higher glucose values after browning—presumably from the reasons mentioned earlier. Removal of the sugar by either glucose oxidase or fermentation did, however, decrease development of brown color. It should be pointed out that the greater amount of brownness during cooking may contribute to the flavor, since Webb *et al.* (1961) demonstrated that flavor scores were higher for pork roasted to an internal temperature of 85°C than when cooked to lower internal temperatures.

Effect of browning upon glucose and free amino nitrogen. The results of heating both model systems and the meat system are reported in relation to the development of brownness and the effects upon glucose and free-amino nitrogen (Table 3). Heating the glucose alone in buffered solution resulted in development of some brownness. The change in sugar content indicated that approximately two-thirds of the free sugar had undergone pyrolysis. When glycine or Casamino acids were added to the glucose, about two-thirds of the glucose was again reacted, but the much greater optical density (0.450 compared 0.132) indicated the nature of the browning reaction to be different. Since the amount of color development was much more intense, a considerable portion of the colored material appeared to be due to the amino-sugar reaction as has been explained by Zerban (1947). Optical density readings after either glycine or casamino acids were heated alone were near zero, indicating that the amino groups are unreactive alone, but in the presence of free sugars react to produce colored compounds.

Further verification of the amino-sugar reaction is shown by the data on free amino groups (Table 3). Although the concentration of free amino nitrogen decreased only slightly when the pure amino acids were heated alone, it decreased considerably when the amino acids were heated with glucose. After browning, more free amino nitrogen remained in the solution than was true for free glucose, even though approximately one-third of the glucose was unreacted. This suggests that the conditions used for browning were not sufficiently favorable to react all of the free amino or sugar groups. The greater proportion of unreacted amino groups following browning—approximately 80%, compared with 33% for glucose—suggests that glucose is more likely to be the limiting factor in production of brownness than are the free amino groups. This is not sur-

Table 3. Effect of browning on the glucose and free amino nitrogen content and optical density of meat and model systems.

Sample solution	Glucose [a] (mg%)		Free amino nitrogen [b] (mg/g)		Optical density at 350 mμ
	Before browning	After browning	Before browning	After browning	
Glucose alone	331	124	–0–	–0–	0.132
Glycine alone	–0–	–0–	2.40	2.22	0.000
Casamino acid alone	–0–	–0–	2.38	2.10	0.010
Glucose + glycine	345	130	2.38	1.89	0.450
Glucose + casamino acid	342	112	2.44	1.91	0.465
Meat slurry	277	191	1.48	1.82	0.277

[a] Values for glucose are expressed as mg per 100 g of the raw meat sample. The amount of glucose added in the model systems was calculated to be the same as in the raw meat system, although actual analysis showed some discrepancies in the values.

[b] Values for free amino nitrogen are expressed as mg per g of the raw meat sample. Values in the model systems were calculated to give the same level as in the raw meat, but often did not agree in the actual analysis.

prising, since the model systems employed were made up to contain the same concentration of reducing sugars and free amino-nitrogen as the meat samples, which contained relatively high concentrations of free amino-nitrogen but quite low concentrations of reducing sugars.

Although the levels of glucose and free amino-nitrogen in the model systems were calculated to be the same as for the meat samples, Table 3 shows that both glucose and free amino nitrogen occurred at levels higher than those found in the meat. The fact that color development was less in the meat after heating than in the amino acid-glucose model system can probably be attributed to the lower concentration of reducing sugars in the meat. Although the level of reducing sugars in the meat had been determined by an earlier analysis, the analysis at the time of running the browning experiment was lower. This may have been a result of microbial destruction of some of the reducing sugars as the meat had been frozen and thawed again prior to analysis.

The data on free amino nitrogen changes during browning of the meat slurry are somewhat conflicting, since Table 1 shows no change, Table 2 shows a decline and Table 3 shows an increase. Although the data are difficult to reconcile, Reynolds (1959) has pointed out some of the problems in following browning by changes in the chemical constituents. One of the possible causes for variability observed in the meat slurries could be the difference in the levels of glucose in the various samples, since they varied from a low value of 277 (Table 3) to a high of 807 mg% (Table 2). Ellis (1959) has concluded that the proportion of amino acids to sugar should be 1:2, although more sugar reacts per mole of amino acids at higher temperatures. Results of the present study indicate that even though the model systems were buffered to the pH of meat (5.6), the meat appeared to be less reactive to browning since some 65% of all glucose was still unreacted after heating. This would be expected, since the Van Slyke method (AOAC, 1960) measures the total free amino groups of the protein and does not differentiate between accessible and unaccessible groups.

Effects of inhibitors on browning systems. In order to determine the effectiveness of blocking the browning reaction by various inhibitors, model systems were used in which the free amino groups from the proteins were blocked by acylation and the aldehyde groups of the sugars were blocked by the addition of sodium bisulfite or hydroxylamine. The amount of browning after heating is shown in terms of optical density in Table 4. The data show that glucose alone had undergone some pyrolysis, whereas considerably more browning occurred on adding glycine to glucose.

The acylation of glycine with the concomitant blocking of the amino groups resulted in nearly as much browning on heating as for glucose alone. This indicated that the browning in this case was due to caramel formation from glucose since the reactive amino groups were blocked by acylation. The addition of either sodium bisulfite or hydroxylamine resulted in a marked decrease in color formation during heating. Since these two compounds block the reaction with glucose, it is obvious that the inhibition of reactions involving glucose was not complete or no color formation would have occurred. Results with the model system show that color formation was partially due to caramel formation although the amino-sugar reaction was responsible for the largest amount of colored material.

Effect of fermentation and inhibitors upon color development during heating of meat. Table 5 summarizes the effects of fermentation and the use of inhibitors upon development of brownness of meat solutions.

Table 4. Effects of inhibitors upon the browning of model systems.[a]

Sample solution	Optical density at 370 mμ
Glucose alone	0.098
Glucose + glycine	0.257
Glucose + acylated glycine	0.107
Glucose + glycine + 0.25% sodium bisulfite	0.145[b]
Glucose + glycine + 0.25% hydroxylamine	0.062

[a] All samples contained glucose at a level of 436 mg% and glycine at a level 128 mg.
[b] Yellow in color; did not appear visually to be as dark as the reading indicates.

Table 5. Effect of fermentation and inhibitors upon the browning of meat solutions.

Sample solution	Optical density at 450 mμ
Meat slurry	0.222
Meat + hydroxylamine	0.070
Meat + bisulfite	0.120[a]
Meat fermented	0.170[a]
Meat fermented + glucose[b] + bisulfite	0.147[a]
Meat fermented + glucose[b] + hydroxylamine	0.187
Meat fermented and acylated	0.080
Meat fermented and acylated + glucose[a]	0.126

[a] Colloidal solution.
[b] Glucose added back to give the amount originally present in the meat, i.e., 300 mg/100 g of meat.

The control sample of meat contained 300 mg of reducing sugars per 100 g. Fermentation lowered the concentration to 39 mg. Visual observations on color were not always in agreement with spectral readings, since colloidal suspensions occurred in a few treatments. Centrifugation or filtration techniques with paper or various filter aids were not effective in removing the colloidal material. The problem was largely confined to fermented samples both with and without added bisulfite.

It is obvious that all of the inhibitors were effective in reducing development of the brown color (Table 5). Hydroxylamine and bisulfite both blocked much of the browning. Similarly, there was very little browning in the fermented acylated meat. On adding glucose back to the fermented meat, the amount of browning again increased for all treatments. However, the relative brownness was less in the acylated meat. The residual browning was apparently due to caramelization of the sugars, since the amino groups were blocked by acylation. Thus, results indicate that the major part of browning on heating meat occurs as a result of the amino-sugar reaction, yet a small but significant amount of browning appears to be associated with pyrolysis of the reducing sugars present in meat.

The results obtained in preventing browning by adding bisulfite are in agreement with the effects of adding SO_2 to dehydrated meat (Sharp and Rolfe, 1958) and other foods (Ellis, 1959). However, Reynolds (1959) indicated that the probable mode of action in prevention of browning differed for SO_2 and bisulfite. The variation of the results observed in the meat systems of the present study is not surprising, since water content (Wolfram and Rooney, 1953), the specific sugars and amino acids involved (Ellis, 1959), the temperature (Ellis, 1959), the sugar phosphates (Jones, 1959; Sharp, 1957) and other treatments are known to have variable effects on brown color development.

REFERENCES

AOAC. 1960. "Official Methods of Analysis." 9th ed. Association of Official Agricultural Chemists, Washington, D. C. p. 300.

Cheronis, N. D., and J. B. Entrikin. 1957. "Semimicro Qualitative Organic Analysis. The Systematic Identification of Organic Compounds." 2nd ed. Interscience Publishers, N. Y. p. 346.

Danehy, J. P., and W. W. Pigman. 1951. Reactions between sugars and nitrogenous compounds and their relationship to certain food problems. *Advances in Food Research* **3**, 241.

Ellis, G. P. 1959. The Maillard reaction. *Advances in Carbohydrate Chem.* **14**, 63.

Folin, A., and H. Wu. 1920. A simplified and improved method for determination of sugar. *J. Biol. Chem.* **41**, 367.

Fraenkel-Conrat, H. 1954. Methods for investigating the essential groups for enzyme activity. *Methods in Enzymol.* **4**, 247.

Henrickson, R. L., D. E. Brady, C. W. Gehrke, and R. F. Brooks. 1955. Dehydrated pork studies. Removal of glucose by yeast fermentation. *Food Technol.* **9**, 290.

Hodge, J. E. 1953. Dehydrated foods. Chemistry of browning reaction in model systems. *J. Agr. Food Chem.* **1**, 928.

Jenness, R., and S. Patton. 1959. "Principles of Dairy Chemistry." John Wiley & Sons, N. Y., p. 347.

Jones, N. R. 1959. Browning reactions and the loss of free amino acid and sugar from lyophilized muscle extractives of fresh and chill-stored codling (Gadus callarias). *Food Research* **24**, 704.

Pearson, A. M., G. Harrington, R. G. West, and Mildred E. Spooner. 1962. The browning produced on heating fresh pork. I. The relation of intensity of browning to chemical constituents and pH. *J. Food Sci.* **27**, 177.

Reynolds, T. M. 1963. Chemistry of nonenzymic browning. I. The reaction between aldoses and amines. *Advances in Food Research* **12**, 1.

Reynolds, T. M. 1959. Chemistry of non-enzymic browning. III. Effect of bisulphite, phosphate and malate on the reaction of glycine and glucose. *Austral. J. Chem.* **12**, 265.

Sharp, J. G., and E. J. Rolfe. 1958. Deterioration of dehydrated meat during storage. *In*: "Fundamental Aspects of The Dehydration of Foodstuffs" p. 197. Soc. Chem. Ind., London.

Sharp, J. G. 1957. Deterioration of dehydrated meat during storage. II. Effect of pH and temperature on browning changes in dehydrated aqueous extracts. *J. Sci. Food Agr.* **8**, 21.

Tarr, H. L. A. 1953. Ribose and the Maillard reaction in fish muscle. *Nature* **177**, 344.

Webb, N. L., N. B. Webb, D. Cedarquist and L. J. Bratzler. 1961. The effect of internal temperature and time of cooking on the palatability of pork loin roasts. *Food Technol.* **15**, 371.

Zerban, F. W. 1947. The color problem in sucrose manufacture. Sugar Research Foundation Report 2, Sugar Research Found., New York, p. 1.

Ms. rec'd 2/18/65.

Journal Article No. 3573, Michigan Agricultural Experiment Station, East Lansing.

REX ELLIS, A. M. GADDIS, and G. T. CURRIE

Meat Laboratory, Eastern Utilization Research and Development Division
Agricultural Research Service, United States Department of Agriculture
Beltsville, Maryland 20705

Carbonyls in Oxidizing Fat. IX. Aldehydes Isolated from Autoxidized Methyl Arachidonate

SUMMARY

The monocarbonyl compounds developed by oxidation of methyl arachidonate under mild conditions were measured by three methods of isolation. Aldehydes from scission of hydroperoxides of the pentadiene system furthest removed from the carboxyl group accounted for 80% of the total, and the same ones are characteristic of oxidized linoleate. The C_6 alkanal was the major aldehyde and C_7 alkanal, a compound not readily accountable for by conventional mechanisms of autoxidation, was second in quantity. The mild quantitatively equal Girard T and Schwartz methods broke down some precursors, but in a qualitatively different manner. The Pool and Klose alumina chromatographic method decomposed precursors, but not to the extent that has been observed in autoxidized lard. Two unsaturated aldehydes isolated by this method may be alk-2,4,6-trienals.

INTRODUCTION

Arachidonic acid is present in animal depot fats and tissue phospholipids. Hornstein *et al.* (1961) found 0.1% in the triglycerides of beef and pork and 19.2% and 16.3%, respectively, in their phospholipids. The oxidation of this highly susceptible fatty acid component of lipids apparently has not been extensively studied. According to Badings (1960) the methyl ester oxidizes at a rate approaching twice that of methyl linolenate.

Little appears to have been done on determination of the carbonyl compounds formed in the autoxidation of arachidonic acid and its esters. Sulzbacher *et al.* (1963) reported examination of the monocarbonyls obtained by steam distillation at 100°C of an oxidized sample of methyl arachidonate and found that the aldehydes were qualitatively rather similar to those observed from methyl linoleate by Ellis *et al.* (1961). Notable exceptions were the C_7 alkanal, C_{11} enal, and C_{11}, C_{12} dienals. This work was undertaken to determine the quantitative relationships between the monocarbonyl compounds formed by methyl arachidonate. Since recent investigations concerned with the isolation of free aldehydes have shown a sensitivity of precursors to method of isolation, with resultant quantitative and sometimes qualitative effects (Gaddis *et al.*, 1965), several different methods of isolation were used: the Girard T method of Gaddis *et al.* (1964), the Schwartz procedure (Schwartz *et al.*, 1963), and the Pool and Klose method (Keith and Day, 1963). An isolation of volatile aldehydes would also have been desirable, but it was feared that results would be complicated by the short path of the Lea method (Lea and Swoboda, 1962), which might result in some collection of primary oxidation products. The triglyceride of the fatty acid, which would have been suitable, was not available.

EXPERIMENTAL

Solvents, reagents, materials, and methods. Solvents were purified as described in previous reports (Gaddis *et al.*, 1964, 1965). The methyl arachidonate (lot 5M) used, obtained from the Hormel Foundation, Austin, Minnesota, had an estimated purity of higher than 90%. One gram of methyl arachidonate was oxidized for 24 hr at 23°C under artificial light, by which time it had a peroxide value of 550 meq/1000 g. Samples varying from 0.12 to 0.19 g were weighed out exactly, and duplicate isolations of carbonyl compounds were made simultaneously by the Girard T method, the Schwartz method, and the Pool and Klose method. Monocarbonyl derivatives, isolated by the alumina reaction method of Pool and Klose, were separated from the fat by the Seasorb method of Schwartz *et al.* (1963). Carbonyl compounds isolated by the Girard T and Schwartz methods were fractionated to simple monocarbonyl compounds by chromatography on alumina. Before paper chromatography, all monocarbonyl fractions were further purified by the ion-exchange method of Schwartz *et al.* (1962). Isolated monocarbonyl derivatives were measured by paper chromatographic separations into classes and individual compounds by previously reported methods and applications (Ellis, Gaddis, and Currie, 1958; Gaddis and Ellis, 1959; Ellis and Gaddis, 1959; Gaddis *et al.*,

1961). The $\lambda_{max.}$ of the classes and values used for conversion to μm are described by Gaddis *et al.* (1964). Corrections were applied to the classes isolated by the Pool and Klose method. All results were calculated on the basis of 10.00 g of methyl arachidonate. Absorbance values represent the amount of 2,4-dinitrophenylhydrazones in 100 ml of carbon tetrachloride. Suitable corrections were made for blanks.

RESULTS AND DISCUSSION

Differences between mild methods of isolation. The Girard T and Schwartz methods give quantitative reaction with aldehydes (Gaddis *et al.*, 1964; Schwartz *et al.*, 1963; Gaddis *et al.*, 1965). Table 1 shows that the Girard T and Schwartz monocarbonyl compounds had the same total absorbance values. However, the class compositions were considerably different. In total μm, the Girard T hydrazones were 93.5% of the Schwartz hydrazones. The Schwartz aldehydes had higher amounts of alkanal and enal classes, and only a trace of the dienal class. On the other hand, the Girard T hydrazones had nearly as much alk-2,4-dienal as alk-2-enal class. In lard oxidized at 23°C, higher Schwartz alkanal and enal class isolation than the Girard T values has been found characteristic (Gaddis *et al.*, 1965). This has been assumed to indicate some precursor breakdown by the Schwartz reaction conditions. Dienal values for the two methods were similar for autoxidized lard. In the case of the oxidized arachidonate, it would appear that the Girard T method contributed to a dienal precursor breakdown. The availability of an appreciable amount of dienal precursors may be an abnormal condition (Gaddis *et al.*, in ms.). Comparison of these results with a vacuum distillation technique would have been helpful in interpretation, but this could not be used, for reasons already stated.

The proportions of saturated aldehydes were similar, but the enal aldehydes differed because of higher proportions of the C_8 and

Table 1. Comparison of monocarbonyl isolation methods.

		Girard T			Schwartz			Pool and Klose		
		Amounts	% each class	% total	Amounts	% each class	% total	Amounts	% each class	% total
Total absorbance values		19.59			19.60			53.84		
Class absorbance values										
Alkanal		14.13		72.1	16.67		85.0	34.73		64.5
Enal		2.51		12.8	2.93		15.0	5.55		10.3
Dienal		2.95		15.1	trace			7.86		14.6
Unknown		....		....	....		....	5.70		10.6
μm/10 g										
Alkanal		68.94		80.2	81.32		88.5	169.42		80.5
Enal		9.09		10.6	10.62		11.5	20.11		9.5
Dienal		7.91		9.2	trace			21.06		10.0
Unknown		....		....	....		....	?		
Total		85.94			91.94			210.58		
Individual aldehydes										
Alkanal	C_6	2.62	3.8	3.0	2.93	3.6	3.2	4.47	2.6	2.1
	C_8	57.45	83.3	66.9	68.49	84.2	74.5	131.30	77.5	62.4
	C_7	8.87	12.9	10.3	9.90	12.2	10.8	33.65	19.9	16.0
Enal	C_7	1.70	18.8	2.0	1.88	17.7	2.0	3.97	19.8	1.9
	C_8	3.87	42.5	4.5	5.17	48.7	5.6	5.96	29.6	2.8
	C_9	1.93	21.2	2.3	3.57	33.6	3.9	7.08	35.2	3.3
	C_{11}	1.59	17.5	1.8	trace			3.10	15.4	1.5
Dienal	C_9	1.18	14.9	1.4				trace		
	C_{10}	5.27	66.7	6.1				13.87	65.9	6.6
	C_{11}	0.91	11.5	1.1				4.09	19.4	1.9
	C_{12}	0.55	6.9	0.6				3.10	14.7	1.5

C_9 enals and negligible amounts of C_{11} enal in the Schwartz method. The major alkanals and enals isolated by both methods were the C_6 and C_7 alkanals and C_8 enal. The C_{10} dienal in the Girard T method exceeded the C_8 enal.

Aldehydes isolated by the Pool and Klose method. Aldehydes extracted by the hydrated alumina method (Keith and Day, 1963) exceeded the mild methods considerably, although not nearly to the extent observed in oxidized lard (Gaddis *et al.*, in ms.). This was probably due to a low proportion of monomeric hydroperoxides in the oxidized arachidonate. Early work on method comparison by Gaddis *et al.* (1960) showed that the monocarbonyl compounds isolated by the Pool and Klose method (Pool and Klose, 1951) were higher than those isolated by the Lea mild vacuum distillation method (Lea and Swoboda, 1958). Lea and Jackson (1964) have also demonstrated that Keith and Day (1963) modification is invalid for free aldehydes due to a partial conversion of hydroperoxides to monocarbonyl compounds.

There was an unknown class present of about the same magnitude as the enal class. This had properties similar to those of unknown classes isolated by the method from oxidized lard (Gaddis *et al.*, in ms.). The maximum of the unknown class in carbon tetrachloride was 390–95 mμ, and in alkali there was no secondary maximum or fading in the course of 30 min. Such properties suggest an unsaturated aldehyde with a conjugated triene system, an alk-2,4,6-trienal. The class composition of the known aldehydes was similar to that of the Girard T aldehydes, except that dienals exceeded the enals.

The proportions of individual aldehydes differed from the two "mild" methods. In the alkanal class the C_7 compound was higher and C_6 aldehyde lower, and in the enals the C_9 aldehyde was the major compound. There was little difference between the Schwartz and Pool and Klose amounts of the C_8 enal.

The unknown unsaturated class was separated by paper chromatography into two compounds which retained the maximum in carbon tetrachloride at 390–95 mμ. These compounds were related quantitatively in the ratio of 1:3. The major compound had an R_F value close to that of a C_{11} dienal, and the minor compound that of a C_{12} dienal. The compounds are probably of longer carbon chain than these dienals, since mobility increases with the number of conjugated double bonds (Gaddis and Ellis, 1959).

Parent primary oxidation products. The polyunsaturated unconjugated fatty acids encountered in meat animal lipids are composed of pentadiene units, each with a methylene group between two double bonds. Farmer's theory of autoxidation (Frankel, 1962) favors initial attack at the methylene group and formation of a resonance hybrid of three valence bond structures. Since the products would be resonance-stabilized by a conjugated diene system, preferential reaction with oxygen at the end positions of the pentadiene system might be expected. Such a mechanism is indicated by reports that 90% of linoleate hydroperoxides are conjugated (Privett *et al.*, 1953). Frankel *et al.* (1961) found only 4 isomeric linolenate hydroperoxides of a possible 6 isomers. These were products of end-group attacks in the two pentadiene systems. The structure of methyl arachidonate is shown in Table 2 with the sensitive methylene groups labeled *a*, *b*, and *c*. The monohydroperoxides theoretically possible, a total of nine, are shown with their aldehyde decomposition products. If the oxygen attack is mainly at the end carbons of the pentadiene systems, the major hydroperoxides present would be the 15, 11, 12, 8, 9, and 5. Referring back to Table 1, it will be noted that hydroperoxide products of the *a* methylene group pentadiene system were the sources of the major known aldehydes measured. These scission products were 77.5% in the Girard T isolates, 80.1% in the Schwartz, and 71.8% in the Pool and Klose aldehydes of the total aldehydes. It should also be observed that the hydroperoxide the greatest distance from the carboxyl group furnished the major amount of aldehyde. Similar results have been observed in the case of autoxidized linoleate and linolenate (Gaddis *et al.*, 1961). It is not certain whether this is due to the formation of

Table 2. Hydroperoxides and aldehydes possible in the autoxidation of methyl arachidonate.

Methyl arachidonate	Methylene group	Isomeric hydroperoxides	Main aldehydes expected
$CH_3-(CH_2)_4-\underset{15}{CH}=CH-\underset{13}{CH_2}-\underset{12}{CH}=$	a	15-hydroperoxy $\Delta^{5,8,11,13}$	C_6-al
		13-hydroperoxy $\Delta^{5,8,11,14}$	C_8-Δ^2-enal
		11-hydroperoxy $\Delta^{5,8,12,14}$	C_{10}-$\Delta^{2,4}$-dienal
$\underset{11}{CH}-\underset{10}{CH_2}-\underset{9}{CH}=\underset{8}{CH}-\underset{7}{CH_2}-CH=$		12-hydroperoxy $\Delta^{5,8,10,14}$	C_9-Δ^3-enal
$CH-(CH_2)_3-COOCH_3$	b	10-hydroperoxy $\Delta^{5,8,11,14}$	C_{11}-$\Delta^{2,5}$-dienal
		8-hydroperoxy $\Delta^{5,9,11,14}$	C_{13}-$\Delta^{2,4,7}$-trienal
		9-hydroperoxy $\Delta^{5,7,11,14}$	C_{12}-$\Delta^{3,6}$-dienal
	c	7-hydroperoxy $\Delta^{5,8,11,14}$	C_{14}-$\Delta^{2,5,8}$-trienal
		5-hydroperoxy $\Delta^{6,8,11,14}$	C_{16}-$\Delta^{2,4,7,10}$-tetraenal

larger amounts of such hydroperoxides, or to differences in stability. The observation of Frankel *et al.* (1961) that the 15-hydroperoxy $\Delta^{9,12,14}$ linolenate was 48% of the total hydroperoxides isolated may be extremely significant. Evidence concerned with autoxidized lard (Gaddis *et al.*, in ms.) suggested that the 13-hydroperoxy $\Delta^{9,11}$ linoleate is the major primary oxidation product of linoleate, but that conditions, such as heat, produced its isomer, the 9-hydroperoxy $\Delta^{10,12}$ compound. The aldehydes possible from *b* and *c* methylene systems are difficult to relate to the remaining aldehydes because they may contain unconjugated double bonds which, as indicated by Badings (1960), cannot be detected by the methods used in this work. However, some of these might have undergone shifts to conjugated positions. The predicted C_9 3-enal could have shifted to form the C_9 2-enal found; the C_{11} 2,4-dienal might have been measured as a C_{11} enal or shifted to give the C_{11} 2,4-dienal found. The predicted C_{12} 3,6-dienal may have shifted to give the C_{12} 2,4-dienal determined. The two unknown compounds with properties similar to trienals separated by the Pool and Klose method could have come from the two trienals, or the tetraenal predicted by theory. This leaves unexplained the C_2, C_7 alkanal, C_7 enal, and C_9 2,4-dienal compounds measured. These, except for the C_7 alkanal, were present in very minor quantities and could be explained by the numerous side reactions possible. The C_7 alkanal was the second largest fraction and, in comparison with aldehydes developed by autoxidized oleate, linoleate, and linolenate (Gaddis *et al.*, 1961), is apparently quite characteristic of arachidonate. It increased more proportionally than any other compound in the hydroperoxide-destructive Pool and Klose method (Gaddis *et al.*, in ms.). This would appear to suggest that it comes from a primary oxidation product, but there is no assurance that its source is arachidonate since a highly unsaturated impurity might be present.

REFERENCES

Badings, H. T. 1960. Principles of autoxidation processes in lipids with special regard to the development of autoxidation off-flavors. *Ned. Melk-en Zuiveltijdschrift* **14**, 215.

Ellis, R., and A. M. Gaddis. 1959. Paper chromatography of 2,4-dinitrophenylhydrazones. Estimation of 2-alkanone, n-alkanal, alk-2-enal, and alk-2,4-dienal derivatives. *Anal Chem.* **31**. 1997.

Ellis, R., A. M. Gaddis, and G. T. Currie. 1958. Paper chromatography of 2,4-dinitrophenylhydrazones of saturated aliphatic aldehydes. *Anal. Chem.* **30**, 475.

Ellis, R., A. M. Gaddis, and G. T. Currie. 1961. Carbonyls in oxidizing fat. IV. The role of various fatty acid components in carbonyl generation. *J. Food Sci.* **26**. 131.

Frankel, E. N. 1962. Symposium on foods: "Lipids and their Oxidation." H. W. Schultz, E. A. Day, and R. O. Sinnhuber, eds. Avi Publ. Co., Westport, Conn., 51.

Frankel, E. N., C. D. Evans, D. G. McConnell, E. Selke, and H. J. Dutton. 1961. Autoxidation of methyl linolenate. Isolation and characterization of hydroperoxides. *J. Org. Chem.* **26**. 4663.

Gaddis, A. M., and R. Ellis. 1959. Paper chromatography of 2,4-dinitrophenylhydrazones. Resolution of 2-alkanone, n-alkanal, alk-2-enal, and alk-2,4-dienal derivatives. *Anal. Chem.* **31**, 870.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1960. Carbonyls in oxidizing fat. III. The distribution of volatile and non-volatile carbonyls. *Food Research* **25**, 495.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1961. Carbonyls in oxidizing fat. V. The composition of neutral volatile monocarbonyl compounds from autoxidized oleate, linoleate, and linolenate esters, and fats. *J. Am. Oil Chemists' Soc.* **38**, 371.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1964. Carbonyls in oxidizing fat. VI. The Girard T reagent in the isolation and determination of micro amounts of n-aliphatic aldehydes and 2-alkanones. *J. Food Sci.* **29**, 6.

Gaddis, A. M., R. Ellis, J. Shamey, and G. T. Currie. 1965. Carbonyls in oxidizing fat. VII. A comparison of methods of isolating monocarbonyl compounds. *J. Am. Oil Chemists' Soc.* **42**, 620.

Gaddis, A. M., R. Ellis, and G. T. Currie. Carbonyls in oxidizing fat. VIII. The effect of the Pool and Klose method on monocarbonyl precursors in autoxidized lard. In manuscript.

Hornstein, I., P. F. Crowe, and M. J. Heimberg. 1961. Fatty acid composition of meat tissue lipids. *J. Food Sci.* **26**, 581.

Keith, R. W., and E. A. Day. 1963. Determination of the classes of free monocarbonyl compounds in oxidizing fats and oils. *J. Am. Oil Chemists' Soc.* **40**, 212.

Lea, C. H., and H. A. F. Jackson. 1964. Determination of the volatile or "free" carbonyl content of fats. *Chem & Ind. (London)* **52**, 1429.

Lea, C. H., and P. A. T. Swoboda. 1958. A new procedure for the determination of volatile carbonylic substances in autoxidizing edible fats. *Chem. & Ind. (London)* **46**, 974.

Lea, C. H., and P. A. T. Swoboda. 1962. Simple vacuum distillation procedure for determination of the volatile carbonyl content of autoxidising edible fats. *J. Sci. Food Agr.* **13**, 148.

Pool, M. F., and A. A. Klose. 1951. Estimation of monocarbonyl compounds in rancid foods. *J. Am. Oil Chemists' Soc.* **28**, 215.

Privett, O. S., W. O. Lundberg, N. A. Khan, W. E. Tolberg, and D. H. Wheeler. 1953. Structure of hydroperoxides obtained from autoxidized methyl linoleate. *J. Am. Oil Chemists' Soc.* **30**, 61.

Schwartz, D. P., A. R. Johnson, and O. W. Parks. 1962. Use of ion-exchange resins in the micro analysis of 2,4-dinitrophenylhydrazones. *Microchem. J.* **6**, 37.

Schwartz, D. P., H. S. Haller, and M. Keeney. 1963. Direct quantitative isolation of monocarbonyl compounds from fats and oils. *Anal. Chem.* **35**, 2191.

Sulzbacher, W. L., A. M. Gaddis, and R. Ellis. 1963. Oxidative rancidity in meat and meat products. Proc. 15th Am. Meat Inst. Found. Res. Conf., Chicago, Ill., 111.

Ms. rec'd 8/13/65.

DAVID F. DYER[a]
School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia
DEWEY K. CARPENTER
School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332
and
J. EDWARD SUNDERLAND
School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia

Equilibrium Vapor Pressure of Frozen Bovine Muscle

SUMMARY

An experimental study of the equilibrium vapor pressure of frozen bovine muscle is presented for a temperature range of −23°C to −1°C. Measurements are presented for round, sirloin, and T-bone steak. The results show that the equilibrium vapor pressure of beef is approximately 20% lower than the vapor pressure of pure ice at the same temperature. An interpretation of the experiments attributes the vapor-pressure depression to dissolved solute species in the frozen liquid phase of the beef muscle. To determine the role of the solid meat matrix in the vapor-pressure depression, experimental measurements are also presented for the vapor pressure of the frozen liquid phase, which can be mechanically removed from the meat by squeezing at room temperature. The vapor pressure of this phase at a given temperature lies between that of beef muscle and pure ice. This result is interpreted by the use of a simple model which idealizes the beef muscle as a cross-linked ionic network which contains not only soluble and mobile ionic species but also ions and other hydrophilic groups which are permanently attached to the network. Because of the presence of these permanently attached groups, more solute particles are present in the frozen liquid phase when it is incorporated in the meat matrix than when it is removed. Since the magnitude of the vapor-pressure depression increases with the concentration of dissolved solute species, the depression is greater when the meat matrix is present. These ideas are consistent with the observed temperature coefficient of the vapor pressure, which is larger for frozen beef muscle than for the separate frozen liquid phase.

INTRODUCTION

To predict freeze-drying rates accurately, it is important to know the equilibrium vapor pressure of the product being dried. During freeze-drying, a dried layer develops which is distinctly separate from the frozen wet layer. In analytical calculations of freeze-drying rates, a boundary condition generally used is that the temperature of the interface separating the dried and undried regions is equal to the equilibrium temperature of pure ice at a pressure equal to that which exists at the interface. Recent calculations by us for freeze-drying of beef show that errors of ±2°C in the assumed interface temperature can cause an error of 100% in the calculated heat flux through the undried layer. Consequently, it is desirable to know as accurately as possible the equilibrium vapor pressure-temperature relationship for frozen meat.

Bovine muscle consists essentially of a liquid phase dispersed throughout a solid matrix. The liquid phase is an aqueous solution which contains dissolved salts and proteins. When the meat is frozen, it might well be expected that the thermodynamic properties of the frozen liquid phase would be different from those of ice. In particular, the equilibrium vapor pressure of the frozen liquid in the meat might be expected to deviate from that of pure ice. The present study of the equilibrium vapor pressure of beef steak has been prompted by these considerations.

EXPERIMENTAL

The apparatus used for the vapor pressure measurements is shown in Fig. 1. A 2000-ml vacuum flask was submerged in acetone; the temperature of the system was controlled with a Freon refrigeration unit. The temperatures of the acetone bath and of two positions in the sample were measured with 28 gage copper-constantan thermocouples. The pressure in the flask was measured with a type-160 absolute-pressure gage (manufactured by Wallace

[a] Current address: Department of Mechanical Engineering, Auburn University, Auburn, Alabama.

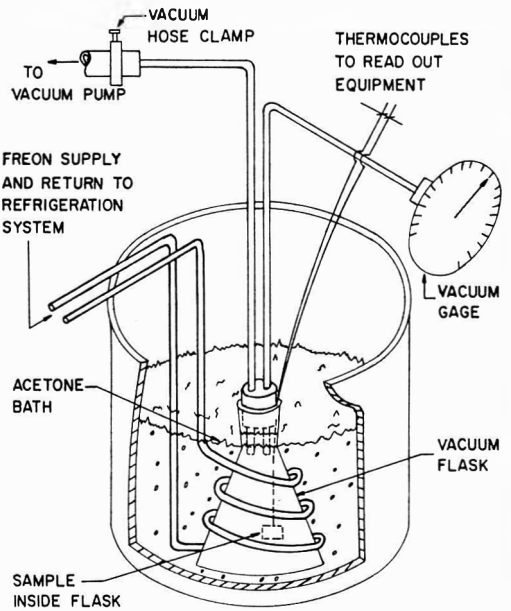


Fig. 1. Experimental apparatus.

and Tiernan, 25 Main Street, Belleville 9, New Jersey). The thermocouples were calibrated with secondary standard mercury-in-glass thermometers and the pressure gage was calibrated with the micromanometer arrangement described by Mayne (1965).

The following test procedure was used. For solid meat samples, two thermocouples were inserted into a piece of approximately $1 \times 2 \times 3$ cm. The sample was subsequently frozen. For measurements made on the frozen meat juice, the juice was first squeezed out of the meat at room temperature and then placed in a small open cylinder. Thermocouples were placed in the desired positions in the liquid and then the liquid was frozen. The frozen sample, either solid meat or frozen meat juice, was suspended by the thermocouple wires in the vacuum flask and the rubber stopper was placed in the flask as shown in Fig. 1. The flask was subsequently evacuated to a pressure of about 0.3 Torr with a mechanical vacuum pump. The clamp between the flask and the pump was closed. The acetone bath temperature was regulated by means of the refrigeration system until it reached the temperature at which the vapor pressure measurement was desired. The sample slowly reached thermal equilibrium with the bath. It was assumed that thermal equilibrium existed when the temperature of the sample was within ± 0.5°C of the bath temperature. No changes of pressure were observed for periods up to 10 min following the establishment of thermal equilibrium. To determine the residual air present in the flask, the system was cooled to a low temperature by placing dry ice in the acetone bath (see Dyer, 1964). Most of the vapor froze during this process and could be seen on the inside surface of the flask. The final temperature and pressure were measured; the residual air pressure was calculated assuming that the secondary gas present at this temperature was water-vapor at its saturation pressure. From this information, the equilibrium vapor pressure was calculated using the ideal-gas law and Dalton's law of partial pressures. The vapor pressures calculated in this manner are precise to within $\pm 3\%$. The accuracy of the apparatus was checked by measuring the vapor pressure of pure ice at -17, -9, and -3°C. The results shown in Fig. 2 agreed within 2% with data reported by Keenan and Keyes (1936).

Results for round, sirloin, and T-bone steak are summarized graphically in Fig. 2. The points shown represent runs for both ascending and descending temperatures. Since there is no evidence of hysteresis, we conclude that the pressures measured are equlibrium values. The legend in this figure indicates the various grades of beef used. Some results are also presented for a sample which was freeze-dried for several hours before the clamp on the vacuum line to the pump was closed. This sample was completely surrounded by a thin layer of dried meat.

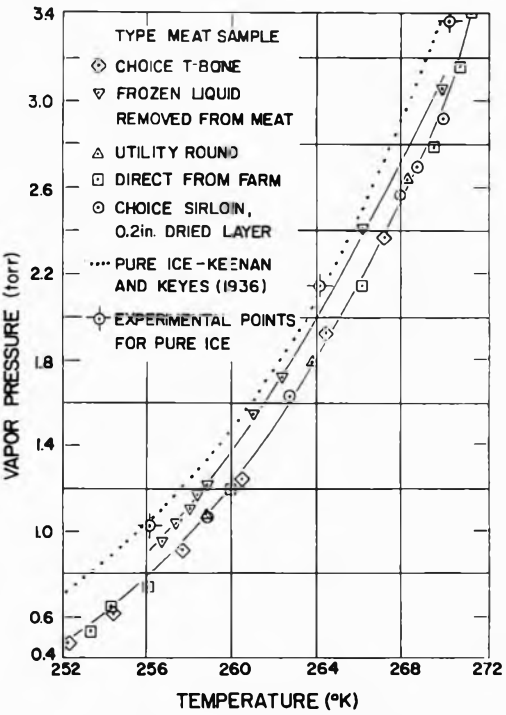


Fig. 2. Equilibrium vapor pressure as a function of temperature.

DISCUSSION

The experimental results consist essentially of two parts: vapor-pressure data for frozen bovine muscle itself, and data for the frozen liquid squeezed from the muscle. From Fig. 2, it is clear that the vapor pressure is depressed below that of pure ice at the same temperature, and that the depression is larger for the frozen meat than for the meat juice.

In discussing these results, we shall consider a piece of meat to consist of a frozen liquid phase which is dispersed throughout a solid matrix. This matrix may contain cavities and capillaries. The liquid-phase, frozen meat juice, is a dilute aqueous solution containing dissolved proteins and salts. The equilibrium vapor pressure of meat is a function of the nature of the frozen liquid phase, of the solid matrix, and of the interactions between these two. In interpreting the difference between the equilibrium vapor pressure of frozen meat and pure ice at the same temperature, three possible contributing factors will be considered.

1) The influence of the concentration of solute species in the frozen liquid.

2) The influence of concave interfaces between the vapor phase and the frozen liquid phase.

3) The restriction of the maximum possible size of crystals of the frozen substance by the solid matrix.

Consider first the effect of dissolved solute species in the frozen phase. If the latter is an ideal solid solution, then Raoult's Law as given by Lewis and Randall (1961) will apply:

$$P/P^\circ = 1 - X \qquad [1]$$

where P is the partial pressure of water above the solid solution, P° is the vapor pressure of pure ice at the same temperature, and X is the total mole fraction of all solute species in the solid solution. Since the solute consists of a number of (unknown) components,

$$X = \sum_i X_i \qquad [2]$$

where X_i is the mole fraction of solute species i. Thus,

$$P/P^\circ = 1 - \sum_i X_i \qquad [3]$$

Clearly, the greater the total concentration of solute species present in the frozen liquid phase, the lower will be the vapor pressure of water over a frozen solution compared to that of pure ice at the same temperature. Of course Eq. 3 is an oversimplification, since Raoult's Law is valid only for ideal solutions, and in view of the electrolytic nature of the frozen liquid phase in meat, deviations are certainly to be expected. Nevertheless, Eq. 3 should serve to account for the influence of dissolved solute species to a degree of approximation sufficient for our purpose. A more accurate treatment of the solid-solution behavior would require knowledge of the chemical and physical constitution of meat that we do not possess.

The second effect has its origin in the influence of the curvature of a surface on the vapor pressure. Consider two surfaces, one planar and the other with a concave indentation of radius r. The molecules at the surface in the latter case are surrounded by more nearest neighbors than are the molecules at the planar surface. This is the molecular basis for the fact that the energy required to generate a unit area of fresh surface is less for a concave than for a planar surface. The surface free energy and the vapor pressure are accordingly lower for a concave surface. This result can be expressed thermodynamically by the Gibbs-Kelvin equation, which is presented in many references such as Swalin (1962):

$$RT [\ln(P_r/P_\infty)\ ] = -2\sigma\bar{V}/r \qquad [4]$$

where P_r and P_∞ refer to the vapor pressures above the concave and planar surfaces, respectively; σ is the surface free energy of the solid in equilibrium with its vapor; $\bar{V}$ is the partial molar volume of the volatile substance; R is the universal gas constant; and T is the absolute temperature. Thus, if the surface of a piece of beef muscle contains many concave indentations, the equilibrium vapor pressure will be lowered to an extent given by Eq. 4. Such concave indentations could exist at the openings of capillary-like channels in the meat.

The third effect mentioned above is suggested by the possibility of the existence in the meat matrix of cavities which might by

their size limit the extent to which ice crystals could grow. If the maximum size of the crystals is restricted to a small value in this manner, then the ratio of the surface area to the volume of these crystals would be large. This would in turn give rise to a surface free energy which would be *higher* than the surface free energy at a planar surface. This would in turn increase the vapor pressure of the water over such confined ice crystals because, at equilibrium, the chemical potential must be uniform throughout the system.

To summarize, effects 1 and 2 would tend to cause a decrease in the equilibrium vapor pressure, while effect 3 would tend to increase it. We now proceed to estimate the magnitudes of these effects.

Consider first the effect of concave surfaces, effect 2. A capillary radius of 0.075 mm is suggested by the data of Luyet (1959). Weast (1964) gives a value for the surface free energy of 77 ergs/cm^2. Using these values in Eq. 4, the calculated vapor pressure depression turns out to be less than 0.01%.

Regarding effect 3, we note that if the capillary radius of 0.075 mm is also characteristic of the internal cavity size (as seems to be the case), then the increase in the vapor pressure due to the restriction of the size of crystals of ice will also be of the order of 0.01%.

We thus see that the magnitudes of effects 2 and 3 are both negligible compared with the observed vapor-pressure depressions.

We accordingly conclude that the lowering of the vapor pressure is due to the formation of a solid solution in the frozen liquid phase by dissolved solute species and ice. This conclusion immediately raises the question of why the vapor pressure of the frozen beef muscle is depressed more than that of the frozen meat juice. To answer this question we shall use Fig. 3, in which we depict an idealized model of meat which we shall use to interpret the vapor-pressure data. The model depicted is essentially that of a cross-linked ionic network which is pervaded by an aqueous solution. The latter contains dissolved ionic species, and perhaps electrically neutral protein molecules. These solute species are mobile at temperatures above the

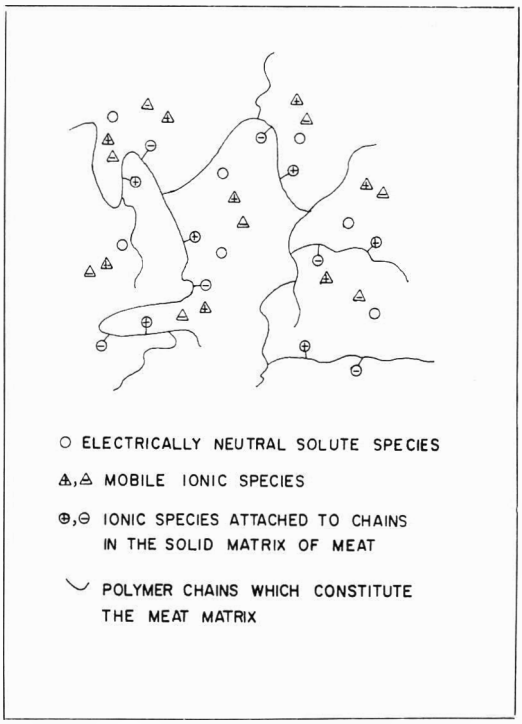


Fig. 3. Idealized model of meat.

freezing point. In addition to these solute species, however, there are also ions which are chemically bound to the polymer chains of the network, and there are other hydrophilic groups on these chains. As long as the aqueous phase remains inside the network, both these immobile ions and hydrophilic groups are able to act as solute species. However, when the aqueous phase is squeezed out of the network, only the mobile solute species are removed; those solute species that are permanently attached to the network chains necessarily remain behind. Therefore, the total concentration of solute species in the meat juice which is removed is less than the concentration present in the meat itself. It is for this reason that the vapor pressure of the frozen meat juice is higher than that of frozen meat. These ideas can be put on a quantitative basis through use of Eq. 3. The results of such calculations are to be found in Table 1, where we tabulate the values of X, the total mole fraction of solute species in the aqueous phase when the latter is inside the meat, and after it has been squeezed out. The variation of these calculated mole fractions with temperature is due

Table 1. Mole fraction of solute species in the solid solution.

T (°K)	$X_s = \Sigma X_{i,s}$	$X_L = \Sigma X_{i,L}$	$X_s - X_L$
252.8	0.32	0.13	0.19
255.5	0.27	0.11	0.16
258.3	0.19	0.11	0.08
261.1	0.18	0.09	0.09
263.9	0.15	0.09	0.06
266.7	0.14	0.06	0.08
269.4	0.15	0.05	0.10

X_s = total mole fraction of solute species in the aqueous phase when the latter is in the meat.
X_L = total mole fraction of solute species in the aqueous phase after the latter has been squeezed out.

primarily to the oversimplification that is introduced by the assumption of ideal solution behavior. The fourth column of Table 1 shows the increment in solute concentration between the frozen meat juice and the frozen meat itself.

Additional confirmation of our model of frozen meat can be obtained by examining the temperature-dependence of the vapor-pressure data. These data are plotted in the customary manner in Fig. 4, as ln p vs. $1/T$. To interpret the slopes of the lines we use a variant of the Clausius-Clapeyron equation,

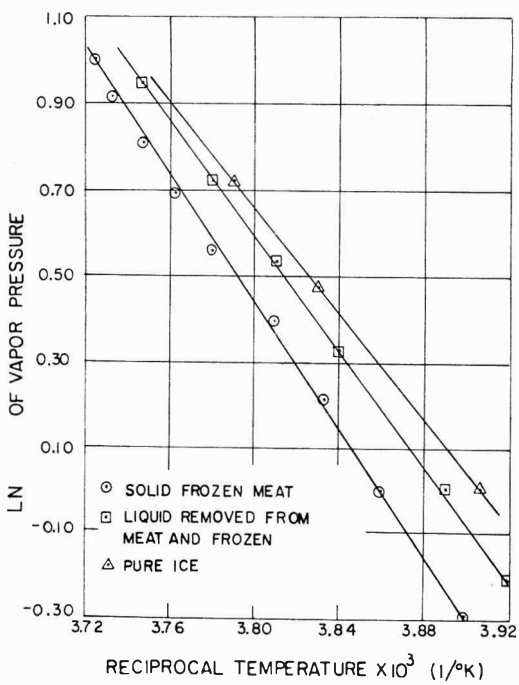


Fig. 4. Logarithm of vapor pressure vs. reciprocal of temperature for meat and meat juices.

$$\frac{d \ln P}{d\, 1/T} = -\Delta H/R \qquad [5]$$

where

$$\Delta H = \Delta H_{SUB} - \Delta H_M \qquad [6]$$

ΔH_{SUB} = molar heat of sublimation of ice
ΔH_M = change in enthalpy occurring when one mole of ice is transferred to a solid solution over which the partial pressure is P.

For convenience, we shall call ΔH_M the "heat of mixing." From the slopes of the lines in Fig. 4, and using a value of ΔH_{SUB} of 12,200 cal/mole given by Keenan and Keyes (1936), we find that

$\Delta H_M = -2680$ cal/mole for frozen bovine muscle
and $\Delta H_M = -1030$ cal/mole for frozen meat juice

The negative signs signify that the formation of solid solution from ice and solute species is an exothermic process. The magnitudes indicate that considerably more heat is liberated in the formation of the solid solution in meat itself than in meat juice. In fact, the enthalpy change for the process:

1 mole H_2O in meat juice $\rightarrow$ 1 mole H_2O in meat is $\Delta H' = (-2680$ cal/mole$) - (-1030$ cal/mole$) = -1650$ cal/mole. These values are quite compatible with our ionic network model for meat. The excess enthalpy, $\Delta H' = -1650$ cal/mole, represents, according to this model, the additional heat of hydration of ions and hydrophilic chain groups that occurs per mole of water when the water is inside the meat matrix.

The foregoing analysis of the vapor-pressure data assumes that the pressures measured were true equilibrium values. Effects due to the rate of transfer of water through membrane materials in the meat have not been deemed significant. Partial justification for this simplification is to be found in the lack of hysteresis in the observed pressure-temperature relationships.

REFERENCES

Dyer, D. F., and J. E. Sunderland. 1964. A standard for vapor pressure measurement. *Vacuum* **14**, 396.

Keenan, J. H., and F. G. Keyes. 1936. Thermodynamic properties of steam. John Wiley, 76.

Lewis, G. N., and M. Randall. 1961. Thermodynamics. McGraw-Hill, 233.

Luyet, B. J. 1959. Rehydration of freeze dried beef. Am. Foundation for Biological Research, Rept. No. 1, Quartermaster Food and Container Inst., Chicago.

Mayne, R. W. 1965. Humidity measurement under low vacuum conditions. M. S. Thesis. Georgia Institute of Technology, Atlanta, Ga.

Swain, R. A. 1962. Thermodynamics of solids. John Wiley, 147.

Weast, R. C., Ed. 1964. Handbook of chemistry and physics. The Chemical Rubber Co., Sect. F, p. 18.

Ms. rec'd 6/28/65.

The authors gratefully acknowledge partial support for this work by the Public Health Service, Grant EF 00102-02, from the Division of Environmental Food Protection.

T. A. ROHAN and T. STEWART
British Food Manufacturing Industries Research Association
Leatherhead, England

The Precursors of Chocolate Aroma: Changes in the Free Amino Acids During the Roasting of Cocoa Beans

SUMMARY

The destruction of free amino acids during a factory roast of Accra cocoa beans was studied. Different amino acids were destroyed at different rates, but none were destroyed completely. Of the total free amino acids present in the unroasted beans, some 50% were found in the roasted product.

INTRODUCTION

This investigation, a continuation and extension of work carried out in recent years in these laboratories on the nature of the precursors of chocolate aroma, involves a study of the chemical changes which take place in the flavor and aroma constituents of cocoa beans and the precursors of these compounds, during factory processing.

The free amino acids of cocoa beans have already been shown to constitute one of the major components of the precursors of chocolate aroma (Rohan, 1963, 1964), and there is also some evidence (Rohan and Stewart, 1965) that the amino acids are responsible, in part, for the auxiliary aroma of chocolate. Changes which occur in these constituents of cocoa beans, during roasting, are consequently of some importance. It has been demonstrated that amino acids are oxidatively deaminated either by reducing sugars (Schonberg and Moubasher, 1952) or by quinones (Isherwood and Niavis, 1956); and Purr *et al.* (1963) have postulated the formation of amino acid-quinone complexes, the quinone moiety arising as an intermediate product in the action of cocoa polyphenol oxidase on polyphenols, and that the addition product may catalyze the oxidative deamination of amino acids without intervention of polyphenol oxidase. Bailey *et al.* (1962) identified a number of aldehydes among the volatile constituents of chocolate, and ascribed their existence to a Strecker degradation of the amino acids of cocoa during roasting. They did not, however, suggest the nature of degrading reagent.

In the present investigation, the rate at which the amino acids were destroyed during factory roasting of Accra cocoa beans was estimated, and the results are an extension of those obtained by Diemair *et al.* (1959) who roasted beans on laboratory scale and sampled only at the beginning and the end of the process.

EXPERIMENTAL

Factory roasting conditions. Accra cocoa beans (500 lb) were roasted in a Barth automatic gas roaster at an air temperature of 182–3°C for 28 min. Samples were withdrawn after 5, 10, 15, 20, 25, and 28 min. A replicate roast was carried out and sampled likewise. The roasts and samples were those described in a previous publication (Rohan and Stewart, 1965).

Extraction of free amino acids. Finely ground shell-free cocoa beans (20 g) were blended with water (200 ml) in a Paladin blender for 5 min, and the resultant suspension was filtered. An aliquot (100 ml) of the filtrate was passed through a column (20 × 1 cm) of Amberlite 1R.120(H) resin to remove the amino acids. The resin was washed with 50 ml of a 20% solution of iso-propanol in water followed by 50 ml of an 80% solution of the same alcohol in water. This treatment freed the resin from the bulk of the adsorbed flavonoids, and the amino acids were then eluted with 4*N* ammonium hydroxide solution. The effluent, which was collected until the pH was 10, was evaporated to dryness on a rotary evaporator and the powdery residue used for subsequent paper chromatography.

Group separation and semiquantitative estimation of free amino acids This was effected by paper chromatographic separation of the amino acids into groups and spectrophotometric estimation of the ninhydrin complexes, as previously described (Rohan and Stewart, 1965).

Nitrogen estimations. *Total nitrogen.* Finely ground shell-free cocoa beans (0.1 g) were accurately weighed and transferred to a Kjeldahl flask with distilled water (2–3 ml). Sulfuric acid (5 ml SG, 1.84 contained 0.5 g selenium) was added, and the mixture heated, slowly at first and then more strongly, for a total 5 hr. The resultant solution was cooled, diluted with water (25 ml), and transferred to the steam distillation unit. Sodium hydroxide (20 ml of a 40% w/v solution)

was added, and the liberated ammonia was distilled into a conical flask containing a saturated aqueous solution of boric acid. The ammonia was titrated against 0.01*N* sulfuric acid using bromophenol blue indicator.

Soluble nitrogen. Finely ground shell-free cocoa beans (approximately 2 g, weighed accurately) were heated to boiling with water (100 ml) and then kept in a boiling-water bath for 10 min. Potash alum (2 ml of a 10% w/v solution) was added to the hot suspension, followed by copper sulfate (25 ml of a solution containing 60 g hydrate/liter) and sodium hydroxide (25 ml of a 1.25% w/v solution) with stirring. The suspension was allowed to stand overnight and then filtered. The precipitate was washed with warm water until the combined filtrate and washings totaled 250 ml. An aliquot (25 ml) of this solution was transferred to the Kjeldahl flask and digested in the manner described above.

RESULTS AND DISCUSSION

Changes in amino acids. For convenience, the amino acids were arbitrarily separated into eight groups based on their chromatographic properties. Semiquantitative assessment of the amino acid content of each group was made by measurement of the optical density of aqueous acetone (50% v/v) solutions of the ninhydrin complexes (*cf.* Rohan and Stewart, 1965). The results in Table 1 show the overall destruction of amino acids in the various groups. The rates of destruction and final concentrations are seen more readily in Table 2, and Fig. 1 shows the rate of destruction of the total free amino acids during roasting. The destruction of the amino acids was by no means complete, and different groups of amino acids reacted at different rates and to different extents under the conditions observed. The reason for the incomplete destruction of the amino acids is discussed in the following contribution in this series, which describes destruction of the reducing sugars during roasting. Anticipating the discussion in that paper, it is pertinent that the reducing sugars were almost completely destroyed during the roasting process. Recent work in these laboratories (Rohan, unpublished results) suggests that deamination of the amino acids occurs in the absence of the flavonoid moiety of the aroma precursor concentrate (Rohan, 1963, 1964). The role of the reducing sugars is consequently magnified in importance, and the high residual amino acid content of roasted cocoa beans, also observed by Diemair *et al.* (1959), might be attributed to complete destruction of the deaminating reagent.

Table 2. Destruction of amino acids during roasting.

Fraction	% of initial concentration of amino acids in unroasted beans at intervals shown (min).						
	0	5	10	15	20	25	28
a	100	92	88	81	75	65	60
b	100	100	85	84	76	65	47
c	100	94	89	81	80	71	65
d	100	93	85	76	74	61	58
e	100	91	83	76	73	64	55
f	100	99	89	80	64	51	38
g	100	93	77	74	70	65	53
h	100	100	90	86	80	67	47
Mean	100	95	86	80	74	64	53

An interesting feature of Fig. 1 is that the loss of amino acids begins early in the roasting process and their destruction is approximately linear with the time of heating. Table 2 shows that the rates of destruction of the various amino acids in cocoa

Table 1. Optical densities of ninhydrin complexes of amino acids during roasting.

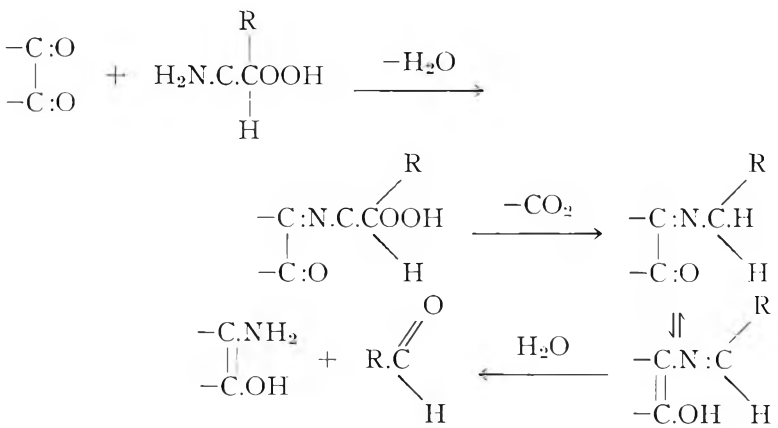
Fraction	Optical densities (λ,570 mμ) at intervals shown (min)							Composition of fraction
	0	5	10	15	20	25	28	
a	0.56	0.51	0.49	0.45	0.42	0.36	0.33	Leu; iso-Leu
b	0.28	0.28	0.24	0.23	0.21	0.18	0.13	Ph.al
c	0.33	0.31	0.29	0.27	0.27	0.23	0.21	Val; Tryp.
d	0.44	0.41	0.38	0.34	0.33	0.27	0.27	β-Al; Tyr.
e	0.55	0.51	0.46	0.42	0.40	0.35	0.30	Al.
f	0.39	0.38	0.34	0.31	0.25	0.20	0.15	Threo; Glu.
g	0.36	0.33	0.27	0.26	0.25	0.23	0.19	Asp; Ser.
h	0.28	0.28	0.26	0.25	0.23	0.19	0.13	Cyst; $Asp.NH_2$ Hist; Arg.

beans are not uniform, and that only in certain groups does the deamination appear to commence with heating. In others, notably groups *c* and *h*, there is a pronounced time lag. The rates of reaction of the various groups of amino acids also vary during the process.

Nitrogen values. An attempt was made to follow destruction of the α-amino acids during roasting by estimating the water-soluble nitrogen contents of the different samples. The soda alum solution used in the clarification precipitated phosphates, and the Stutzer reagent (alkaline copper sulfate) precipitated protein and, by adsorption, the purines. The filtrate comprised predominantly amino acids and peptides accompanied by small amounts of nitrate and ammonia nitrogen. The soluble nitrogen determination on each of the seven samples showed that there was no change during the course of roasting; the values at the beginning and at the end of the process were 0.75% of the shell-free bean, and this is the average value observed for fermented Accra cocoa beans. A very small reduction in total nitrogen was observed. The evidence in support of the destruction of the amino acids of cocoa during roasting is irrefutable and it can only be assumed, since the soluble and total nitrogen contents of the beans remained unchanged during processing, that the nitrogenous degradation products were retained in the system and that they were soluble in water.

The fat of the amino group in the oxidative deamination of amino acids (Strecker degradation) has been discussed by Schonberg *et al.* (1948), who postulated that this is dependent upon the nature of the carbonyl compound which effects the degradation. The amino group may be eliminated as ammonia, or it may become linked, in a transamination reaction, to the carbonyl compound which effects the degradation, converting it into an amino compound of similar structure; or it may enter into combination with the carbonyl compound which effects the degradation, to produce a nitrogenous compound of complicated character not related to the original compound.

The degradation cannot be effected by mono-carbonyl compounds and requires the presence of molecules with the group $-CO.(C:C)_nCO-$ (where n may be zero or contained in aromatic systems). The mechanism suggested by Schonberg *et al.* would account for the lack of change in soluble nitrogen content of the cocoa beans during roasting (*cf.* Baddar, 1949):



In conclusion, the experimental results presented above demonstrate the selective but incomplete destruction of amino acids during cocoa bean roasting. The rates of destruction varied among the different groups into which the amino acids had been arbitrarily divided. There was no loss of nitrogen to the system and this was probably due to the formation of soluble nitrogenous complexes of the reducing sugars which almost certainly play an important part in the Strecker degradation by which the amino acids are destroyed.

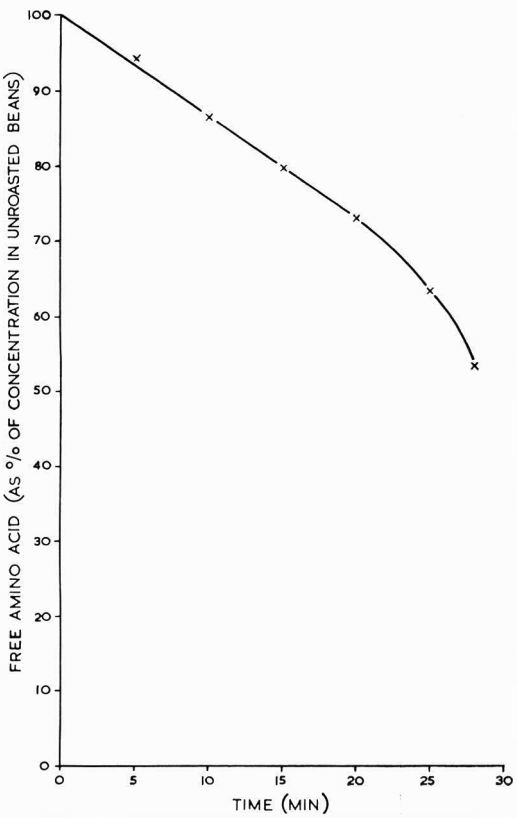


Fig. 1. Destruction of reducing sugars during roasting.

REFERENCES

Baddar, F. G. 1949. The electronic intrepretation of the Strecker degradation. *J. Chem. Soc.* **1949**, 163–7.

Bailey, S. D., D. G. Mitchell, M. L. Bazinet, and C. Weurman. 1962. Studies on volatile components of different varieties of cocoa beans. *J. Food Sci.* **27**. 165.

Diemair, W., L. Acker, and H. Lange. 1959. Modification of cocoa beans by roasting. 3. Proteins, amino acids, furfurol. *Susswaren* **3**, (10), 542.

Isherwood, F. A., and C. A. Niavis. 1956. Estimation of α-keto acids in plant tissues: A critical study of various methods of extraction as applied to strawberry leaves, potato slices and peas. *Biochem. J.* **64**. 549.

Purr, A., A. Helfenberger, and J. Nadai. 1963. Use of vacuum infiltration for fermentation of cocoa beans with special reference to enzymatic changes and the formation of flavour and flavour precursors. *Intern. Choc. Rev.* **18**, 194.

Rohan, T. A. 1963. Precursors of chocolate aroma. *J. Sci. Food Agr.* **14**, 799.

Rohan, T. A. 1964. Precursors of chocolate aroma: A comparative study of fermented and unfermented cocoa beans. *J. Food Sci.* **29**, 456.

Rohan, T. A., and T. Stewart. 1965. The precursors of chocolate aroma: The distribution of free amino acids in different commercial varieties of cocoa beans. *J. Food Sci.* **30**, 416.

Schonberg, A., and R. Moubasher. 1952. The Strecker degradation of α-amino acids. *Chem. Revs.* **50**. 267 .

Schonberg, A., R. Moubasher, and A. Moustafa. 1948. Degradation of α-amino acids to aldehydes and ketones by interaction with carbonyl compounds. *J. Chem. Soc.* **1948**, 163.

Ms. rec'd 10/23/65.

The authors are indebted to the Caxton Chocolate Co. for providing factory facilities and assistance in sampling during processing.

T. A. ROHAN and T. STEWART
British Food Manufacturing Industries Research Association, Leatherhead, England

The Precursors of Chocolate Aroma: Changes in the Sugars During the Roasting of Cocoa Beans

SUMMARY

The destruction of reducing sugars during the roasting of cocoa beans was investigated and found to be almost complete. The significance of this observation is discussed in relation to the possible role played by the reducing sugars in the deamination of the free amino acids of the cocoa bean, and to flavor development.

INTRODUCTION

In a study of the destruction of free amino acids in cocoa beans during factory roasting (Rohan and Stewart, 1966), the point was made that, since the precursors of the primary aroma of chocolate had already been shown to comprise reducing sugars (Rohan, 1963, 1964) these components may, at least in part, be responsible for amino acid degradation and flavor development. Since there is some evidence that reducing sugars are capable of degrading amino acids (Akabori, 1933), a study of these constituents of cocoa beans was made in relation to the roasting process. The changes which occur in the reducing sugar content of Accra cocoa beans was investigated in some detail during processing, and a number of different geographical types of cocoa beans were also examined before and after roasting.

EXPERIMENTAL

Factory roasting. Samples of beans were withdrawn at 5-min intervals from a factory roast of Accra cocoa beans (1½ cwt) in a Barth automatic gas roaster operated at an air temperature of 182–3°. The complete roast occupied 28 min.

Laboratory roasting. A sample of the factory roasted beans was subjected to an additional roasting process in a simple laboratory roaster in the form of an electrically heated cylindrical vessel. The beans were heated at 140° in this apparatus for 1 hr. Nine different geographical types of cocoa beans were likewise roasted in this equipment.

Estimation of reducing sugars. *Reagents.* The Somogyi micro copper reagent for reducing sugar analyses, as modified by Patterson and Savage (1957), was prepared in the following manner. Sodium potassium tartrate (30 g) and anhydrous sodium carbonate (30 g) were dissolved in hot water (200 ml) and an *N*/2 sodium hydroxide solution (80 ml) was added followed by a solution of copper sulfate (80 ml of a 10% w/v solution of the hydrate). The solution was then boiled. In a separate vessel, sodium sulfate (290 g of the hydrate) was dissolved in water (300 ml) and the solution boiled. The two solutions were mixed, and potassium iodide (8 g) and *N*-potassium iodate (10 ml) added. The resultant mixture was diluted to 1 L with boiled distilled water.

Extraction of sugars. Shell-free cocoa beans (50 g) were finely ground in a Moulinex coffee mill, and the resultant powder (5 g) was added to distilled water (50 ml contained in a 250-ml flask). The suspension was heated at 50° with occasional stirring, for 30 min, then cooled in ice-water to solidify the fat, which was removed, together with the insoluble residue, by vacuum filtration. The insoluble residue was washed with 2 × 10 ml of cold distilled water, and the combined filtrate and washings were well shaken with a saturated aqueous solution of basic lead acetate (5 ml). The resultant precipitate was removed by filtration and washed with 2 × 10 ml distilled water. The pale yellow filtrate and washings were made alkaline with 0.3*N* NaOH (*ca.* 5 ml) and the suspension passed through a column (12 × 1 in.) containing layers, each 2 inches deep, of Celite 545; Celite 545–zinc metal powder (50:50 w/w); and Celite 545.

Sugar analyses. An aliquot of the eluant solution from the zinc column estimated to give an approximate titer of 5 ml 0.005*N* sodium thiosulfate, was analyzed by the Patterson and Savage modification of the Somogyi copper-iodometric method.

To the sugar solution, in a 6 × 1-in. boiling tube, was added Somogyi reagent (5 ml). The tube was fitted with a loose-fitting glass stopper, heated 10 min in a boiling-water bath, and then cooled for 2½ min in cold water. The stopper was rinsed into the tube with water, and 1 ml of 6*N* H_2SO_4 was added quickly, with gentle shaking, to dissolve any precipitate formed. The liberated iodine was then titrated against 0.005*N* sodium thiosulfate with freshly prepared starch indicator (1% soln). A blank was run on 5 ml distilled water.

Estimation of total reducing and non-reducing sugars. The sugar solution (5 ml) prepared as described above, was hydrolyzed by heating for 30 min with 6*N* H_2SO_4 (1 ml). The cooled hydrolysate was neutralized with a 5% aqueous solution of

sodium carbonate, and Somogyi reagent (5 ml) added. The reducing sugar content of the hydrolyzed solution was then estimated as described above.

Paper chromatography of sugars in roasted beans. Shell-free powdered and roasted beans (5 g) were extracted with methanol-water (80:20 v/v, 2 × 10 ml) at 50°. After removal of the insoluble residue, the filtrate was concentrated in a rotary evaporator at 50° and a vacuum of 10 mm Hg. The aqueous concentrate was filtered and the insoluble residue washed with water. The combined filtrate and washings were made alkaline (pH 10) with ammonia (SG 0.88) and a saturated solution of lead acetate added until there was a small excess of lead ions in solution. The precipitated polyphenols were removed by vacuum filtration and washed with water. The combined filtrate and washings were passed through a column of cation-exchange resin (25 × 1 cm Amberlite IR.120.H) followed by 100 ml distilled water. The effluent and washings were passed through an anion-exchange resin (25 × 1 cm of Amberlite IR.4B.OH) which was washed with distilled water. The combined eluant solution and washings were concentrated to 10 ml, and an aliquot (5 μl) was chromatographed on Whatman No 1 paper by the ascending method. The paper was irrigated with ethyl acetate–acetic acid–water (140:30:30 v/v) and the reducing sugars revealed on the dried papers with aniline phosphate (Smith, 1960). Similar chromatograms were run with solutions of glucose and fructose whose concentrations approximated to the apparent reducing sugar content of roasted cocoa beans as found by the copper-iodometric titration.

RESULTS AND DISCUSSION

Changes in the reducing sugars. Modification of the reductometric method of analysis for reducing sugars was necessitated because of the presence of large amounts of oxidizable polyphenols in the cocoa bean extract. These polyphenols competed with the reducing sugars for the reagent, and their removal was therefore imperative. Zinc ferricyanide, used by Diemair *et al.* (1959) in a similar study, did not remove all the polyphenols, and experiments in which this precipitant was used gave abnormally high values for reducing sugars. The use of saturated basic lead acetate, followed by removal of excess lead on a column of zinc powder, removed polyphenols more effectively, and analysis of the polyphenol-free solution gave a more precise notion of the rate and extent of destruction of reducing sugars during the roasting of cocoa beans. The precipitation procedure was carried out on bean extracts containing added glucose and fructose, in accurately measured amounts, and recovery of the added reducing sugars was approximately 95%.

The destruction of reducing sugars in cocoa beans during roasting can be observed from results in Table 1 and Fig. 1. The effect of added heating for 1 hr in a laboratory roaster at 140° on the residual reducing sugars was slight, suggesting that the reaction had reached completion. The previous paper (Rohan and Stewart, 1966) showed that the amino acids, with which the reducing sugars are assumed to react in a Strecker degradation, were only partly degraded. The amino acid changes during roasting are superimposed on the sugar results in Fig. 1 in order to emphasize this point. The tentative suggestion is therefore made that the partial destruction of amino acids was due to the system becoming depleted of the degrading reagent, viz. the reducing sugars.

An apparent residual reducing sugar content of about 8% was observed in the roasted beans (see Fig. 1). To determine whether this was in fact reducing sugar, the extract

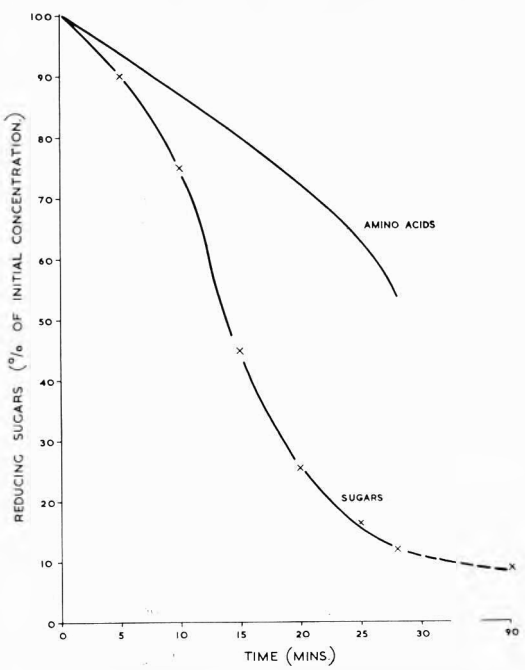


Fig. 1. Destruction of total free amino acids during roasting.

Table 1. Changes in soluble sugars in Accra cocoa beans during roasting.

Roasting time (min)	Reducing sugar content (% by wt of shell-free bean)	Non-reducing sugar content (% by wt of shell-free bean)
0	1.48	0.61
5	1.33	
10	1.11	
15	0.66	
20	0.38	
25	0.24	
28	0.18	
28 + 1 hr at 140°	0.12	0.14

of the beans, which had been subjected to extra heating in the laboratory, was chromatographed on paper. A second chromatogram was run using a solution of glucose and fructose of similar concentration to that which would have resulted from the roasted beans if the observed reducing sugar content were true. Development of the sugars on these chromatograms showed much more intense spots than were observed in the cocoa bean extract, confirming that the reducing sugar content of the roasted beans was very much less than the observed 8% of the initial concentration. Destruction of the reducing sugars was therefore complete for all practical purposes.

Changes in non-reducing sugars. The total soluble sugars were estimated as reducing sugars after hydrolysis of the aqueous extracts of roasted and unroasted cocoa beans with acid. The results in Table 1 suggest that the non-reducing sugar (sucrose) was largely destroyed during roasting. This may be explained by the hydrolysis of the sucrose in the hot acid medium of the beans during the heating process and the reaction of the resultant reducing sugars with amino acids.

A number of different geographical types of cocoa beans were roasted for 1 hr in the laboratory at an air temperature of 140°, and the reducing sugar contents determined before and after treatment. The results (Table 2), again, show considerable reduction in reducing sugar content in each sample, even under conditions much more gentle than are normally observed in factory processing.

CONCLUSIONS

During the roasting of cocoa beans, which is a fundamentally important stage in the development of chocolate flavor and aroma, there is almost complete destruction of the reducing sugars in the beans. Considerable destruction of the non-reducing sugar content has also been observed. It is tentatively concluded that the non-reducing sugar fraction, which is predominantly sucrose, is hydrolyzed to reducing sugars during the heat-

Table 2. Changes in reducing sugars of different geographical types of cocoa beans during laboratory roasting.

Sample	Reducing sugar content of unroasted beans (nib) (% by wt) (A)	Reducing sugar content of roasted beans (nib) (% by wt) (B)	$B/A \times 100$
Accra	1.48	0.12	8.1
Arriba	0.34	0.08	23.6
Bahia	1.27	0.27	21.3
Carupano	0.84	0.19	22.6
Grenada	0.68	0.12	17.7
New Guinea	0.45	0.09	20.0
Puerto Cabello	1.38	0.15	10.9
Samoa	0.92	0.09	9.8
Sanchez	0.68	0.13	19.1
Trinidad	1.27	0.18	14.2

ing process, and that these, together with the reducing sugars normally present in the fermented bean, react with the free amino acids to produce characteristic volatile products. That the amino acid fraction is only partly degraded (*cf.* previous paper) is now attributed to a deficiency of reducing sugars, and potential reducing sugars, in the system.

REFERENCES

Akabori, S. 1931. Amino acids and their derivatives. 1. Oxidative decomposition of α-amino acids with sugars. *J. Chem. Soc. Japan.* **52**, 606.

Diemair, W., L. Acker, and H. Lange. 1959. Modification of cocoa bean constituents during roasting. 2. Soluble carbohydrates. *Susswaren* **3**(9), 488.

Patterson, S. J., and R. I. Savage. 1957. Use of selective desorption from carbon for the determination of dextrose and maltose in starch conversion products. *Analyst.* **82**, 812.

Rohan, T. A. 1963. Precursors of chocolate aroma. *J. Sci. Food Agr.* **14**, 799.

Rohan, T. A. 1964. Precursors of chocolate aroma. A comparative study of fermented and unfermented cocoa beans. *J. Food Sci.* **29**, 456.

Rohan, T. A., and T. Stewart. 1966. Precursors of chocolate aroma: Changes in the free amino acids during roasting. *J. Food Sci.* **31**, 202.

Smith, I. 1960. Chromatographic and electrophoretic techniques. Vol. 1, Chromatography. p. 251. Wm. Heinemann, London.

Ms. rec'd 10/23/65.

The authors are indebted to the Caxton Chocolate Co. for providing factory facilities and assistance in sampling during processing.

MARVEL-DARE NUTTING and ROBERT BECKER

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

Chlorophylls a and b in Green Vegetables: A Comparison of Procedures

SUMMARY

A critical comparison was made of a resin separation and two sets of equations for quantitative determination of chlorophyll *a* and chlorophyll *b* and pheophytin *a* and pheophytin *b* in green vegetables. Each of the three procedures proved equally good, depending on the product analyzed, the time available, and whether the results are desired as total chlorophyll or chlorophyll *a*/*b* ratios.

INTRODUCTION

Comar and Zscheile (1942) suggested an equation for calculating chlorophylls *a* and *b* based on the different spectral absorptions of the two chlorophylls in an 80%-acetone extract of plant material. Vernon (1960) later developed a number of formulae for determining the chlorophylls and pheophytins in acetone extracts of raw and frozen vegetables. These formulae involved wavelengths slightly different from those of Comar and Zscheile. At most wavelengths the absorbance must be read on the steep slope of a curve, where the maxima for chlorophyll *b* and pheophytin *b* are masked by higher proportions of chlorophyll *a* and pheophytin *a*. Therefore, these formulae lose accuracy as the fraction of converted chlorophyll rises.

To simplify spectral measurements and determine chlorophylls in solutions that had undergone considerable conversion, Wilson and Nutting (1963) used cation-exchange resin columns to remove the magnesium from the chlorophyll molecule and thereby physically separate pheophytins *a* and *b*. The pheophytins were measured with a Cary 15 spectrophotometer, and the results presented as mg of chlorophyll *a* and *b* per 100 g of frozen vegetables. The method is independent of the original percent conversion. The water content of each solvent system is known, and shifts of the apparent absorption maxima of the pheophytins due to instrument variation do not influence the results.

In previous work (Wilson *et al.*, 1962), Dowex 50W-X4 separated the chlorophylls of spinach, but conditions for successful operation of the resin columns appear to vary with the source of the pigment. With slight but critical modifications for each vegetable studied, the determinations are routine and good reproducibility can be obtained.

EXPERIMENTAL

Acetone extracts of spinach were easily evaporated to dryness. However, evaporation of the acetone extracts of peas, beans, and Brussels sprouts to dryness is more difficult. In order to minimize pigment destruction (as shown by thin-layer chromatography; Nutting *et al.* 1965) we modified the procedure slightly for these vegetables. The pigment was transferred from the 80%-acetone extract to petroleum ether (bp 65–68°C), and the latter solvent was evaporated rapidly at 20°C under a pressure of 15 mm Hg. All extractions and evaporations were carried out in a constant temperature room (22°C), in dim light. The evaporations were further protected by covering with black paper.

We subsequently found that the presence of a small amount of pyridine greatly lowered the sensitivity of the resin to traces of water which might have remained after vacuum evaporations. Therefore the dried pigment was taken up in a mixture of acetone and anhydrous pyridine. The amount of anhydrous pyridine required for complete separation of pheophytin *a* from *b* was different for each vegetable: spinach 0, green beans 1.5%, Brussels sprouts 3%, and peas 4%.

In the present study, a number of trials showed that Dowex 50W-X2 readily separated the pheophytin *b* from the pheophytin *a* of peas, beans, Brussels sprouts, and spinach. Aliquots of the pyridine-acetone extracts were applied to the resin column and the pigments separated. The little pyridine that washed off the resin with the pheophytins did not affect the spectrophotometric measurements.

The pigments in acetone extracts of green beans, peas, and Brussels sprouts were separated by the resin method of Wilson and Nutting (1963), and absorbances read on the Cary 15 spectrophotometer. Spectra were also run on identical samples containing chlorophyll *a* and *b* in the same solution before separation, and Vernon's formulae (1960) applied.

RESULTS AND DISCUSSION

Table 1 shows the results obtained by: resin separation (procedure 1), the use of Vernon's equations for the calculations of chlorophyll *a* and chlorophyll *b* (procedure 2), and the use of Vernon's equations for the calculations of pheophytin *a* and pheophytin *b* (procedure 3). All values were converted to mg of chlorophyll per 100 g of total solids for each vegetable. The figures given for procedure 1 are averages of 4 to 10 resin column separations. The experimental error in replicate aliquots of extract from the same sample was reflected by a miximum difference in spectrophotometric reading of ±0.005 absorbancy units. At low pigment concentrations, this gave results with mean deviations of ±0.09 mg/100 g of chlorophyll *a* and chlorophyll *b*. As the pigment concentrations increase, the mean deviations decrease to ±0.06 mg/100 g. The values under procedure 2 were obtained from Vernon's (1960) equations 7 and 8. The values under procedure 3 are averages of his equations 10 and 10a for pheophytin *a*, and 11 and 11a for pheophytin *b*. Averaging the results of the two pairs of equations for procedure 3 minimized the error in absorbance reading or wavelength shift. Although the results of the three procedures in Table 1 do not vary uniformly, the difference in total chlorophyll is less than 10% of the mean of the chlorophyll content of each of the six products.

The figures for percent conversion (Table 1) were calculated from Cary 15 spectra of the 80%-acetone extract of chlorophylls according to the method of Dietrich (1958). The absorbance of an identical sample of pigment extract was read on the Beckman DU spectrophotometer. The concentrations of pigment needed to give peak heights on the Cary 15 spectrophotometer for appropriate Vernon's calculations resulted in absorption readings near 0.1 at the wavelengths used by Dietrich. Nevertheless the conversion figures agreed within 6 percentage units with the results from the same samples read on the Beckman DU spectrophotometer with concentrations and cuvette lengths adjusted to give absorbance readings near 0.5, where its precision is best.

Table 1. Three procedures for determination of chlorophylls in green vegetables (mg/100 g).

	1	2	3
Pole beans I			
Chlorophyll *a*	8.25	7.74	7.97
Chlorophyll *b*	3.21	2.97	3.42
Total chlorophyll	11.46	10.71	11.39
a/b ratio	2.57	2.60	2.34
% conversion	18.3		
Pole beans II			
Chlorophyll *a*	8.34	7.86	8.41
Chlorophyll *b*	3.27	3.10	3.40
Total chlorophyll	11.61	10.96	11.80
a/b ratio	2.55	2.54	2.48
% conversion	13.1		
Bush beans I			
Chlorophyll *a*	7.58	7.02	7.50
Chlorophyll *b*	2.76	2.49	3.27
Total chlorophyll	10.34	9.51	10.77
a/b ratio	2.75	2.82	2.29
% conversion	14.2		
Bush beans II			
Chlorophyll *a*	7.87	7.21	7.65
Chlorophyll *b*	2.84	2.57	3.18
Total chlorophyll	10.71	9.78	10.83
a/b ratio	2.78	2.81	2.40
% conversion	17.8		
Peas I			
Chlorophyll *a*	8.50	8.64	8.33
Chlorophyll *b*	3.42	3.29	3.09
Total chlorophyll	11.92	11.93	11.42
a/b ratio	2.48	2.62	2.69
% conversion	3.66		
Peas II			
Chlorophyll *a*	8.18	8.67	8.44
Chlorophyll *b*	3.11	3.45	3.57
Total chlorophyll	11.29	12.12	12.01
a/b ratio	2.63	2.51	2.36
% conversion	2.13		

Procedure 2 gave lower chlorophyll *a* and chlorophyll *b* values than did procedure 1 in the two varieties of beans, but it gave higher values in peas. The *a/b* ratios, however, are similar. This close agreement of the ratios suggests that either procedure gives satisfactory results. Procedure 3 gave the highest values for chlorophyll *b*, and therefore *a/b* ratios lower than by either procedure 1 or 2. The lower total chlorophyll concentrations determined by 2 may be explained by the conversion of chloro-

phyll to pheophytin, since pheophytin is not included in the equation. Vernon (1960) estimates the error in procedure 2 as one-half the percentage of chlorophyll converted to pheophytin.

Table 2 gives the data for chlorophyll in several lots of Brussels sprouts as determined by the 3 procedures. The total chlorophyll figures are lower than those from the other three products, because of the low chlorophyll content of the sprout interiors.

Table 2. Three procedures for determination of chlorophylls in Brussels sprouts (mg/100 g).

	1	2	3
Lot I			
Chlorophyll *a*	4.92	4.08	4.69
Chlorophyll *b*	0.97	1.18	1.60
Total chlorophyll	5.89	5.26	6.29
a/b ratio	5.10	3.44	2.93
% conversion	32.5		
Lot II			
Chlorophyll *a*	4.12	3.53	3.75
Chlorophyll *b*	0.70	1.03	1.81
Total chlorophyll	4.82	4.56	5.56
a/b ratio	5.88	3.43	2.07
% conversion	41.6		
Lot III			
Chlorophyll *a*	4.71	4.42	4.46
Chlorophyll *b*	1.44	1.81	3.54
Total chlorophyll	6.15	6.23	8.00
a/b ratio	3.27	2.44	1.26
% conversion	25.4		

Procedure 1 is best suited for determinations of the *a/b* ratios in green beans because the components are quantitatively separated and determined in the absence of interfering carotenoids. If a variety of products are to be analyzed and relative figures are acceptable, procedure 2 is faster and the *a/b* ratios are consistent. If only total chlorophyll is desired, the three procedures provide comparable results.

REFERENCES

Comar, C. L., and F. P. Zscheile. 1942. Analysis of plant extracts for chlorophylls *a* and *b* by a photoelectric spectrophotometric method. *Plant Physiol.* **17**, 198.

Dietrich, W. C. 1958. Determination of the conversion of chlorophyll to pheophytin. *Food Technol.* **12**, 428.

Nutting, M-D., R. Becker, and M. Voet. 1965. Powdered sugar adsorbent for detection of chlorophyll components in thin layer chromatography. *Anal. Chem.* **37**, 445.

Vernon, L. P. 1960. Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. *Anal. Chem.* **32**, 1144.

Wilson, J. R., and M-D. Nutting. 1963. Use of ion exchange resin for conversion, separation, and determination of chlorophylls as pheophytins. *Anal. Chem.* **35**, 144.

Wilson, J. R., M-D. Nutting, and G. F. Bailey. 1962. Use of tetracyanoethylene for removal of visual carotenoid spectra from solutions of pheophytins. *Anal. Chem.* **34**, 1331.

Ms. rec'd 11/3/65.

Reference to a company or product name does not imply endorsement.

R. C. MORGAN

Food Preservation Research Laboratory,
Queensland Department of Primary Industries, Brisbane, Queensland, Australia

Chemical Studies on Concentrated Pineapple Juice
1. Carotenoid Composition of Fresh Pineapples

SUMMARY

The carotenoid composition of fresh pineapples was examined by counter-current distribution and column chromatography. The number of different compounds found, not including isomers, was 21. The major constituents of the carotenoid mixture were violaxanthins (50%), luteoxanthins (13%), beta-carotene (9%), and neoxanthins (8%). Less abundant carotenoids included zeta-carotene, hydroxy alpha-carotene, cryptoxanthins, lutein, auroxanthins, and neochromes.

INTRODUCTION

In recent years the Food Preservation Research Laboratory of the Queensland Department of Primary Industries has been investigating the behavior of pineapple juice during concentration and subsequent storage (Leverington, 1962; Leverington and Morgan, 1964), and it became evident that the color of the juice was undergoing changes. Very little was known about the carotenoids of the pineapple, except that they contained a high proportion of epoxide groups and that these were easily transformed into furanoid forms by the fruit acids (Singleton *et al.*, 1961). These last authors stated that the conversion of carotenoids containing epoxide groups into furanoid rearrangement products was responsible for the change in absorption spectra associated with processed pineapple products.

Curl and Bailey (1954) treated violaxanthin—a 5,6,5′6′-diepoxide diol—with citric acid, and obtained as the major reaction product luteoxanthin—a 5,6,5′8′-diepoxide diol. Luteoxanthin has an absorption spectrum similar to violaxanthin except that the maxima have shifted some 17 mμ toward shorter wave lengths. Minor products such as auroxanthins, which have maxima at even shorter wave lengths, were also obtained. One would expect that if similar types of reactions occurred in the pineapple carotenoids there would be a higher percentage of 5,8-epoxides in the processed product. A preliminary investigation has now been made of the carotenoids of fresh pineapples, the major components being violaxanthins and luteoxanthins.

EXPERIMENTAL

Whole pineapples varying in external color from half-green to fully yellow were obtained from a nearby cannery during May 1965. Each fruit was carefully inspected before selection, and only those showing no external injury or other defects were used.

The skins and any internal blemishes were removed, and the flesh cut into small pieces. These were then blended with 1% w/w calcium carbonate and 2% w/w filter aid. To minimize isomerization of the pigments the blending was carried out within 1 min of dicing. The blended pulp was allowed to stand 45 min and the juice filtered off in a Buchner funnel through a filter paper precoated with filter aid. After extraction of the filter cake with acetone, the solvent was filtered off and the residue washed until the washings were colorless. Combined acetone extracts were evaporated *in vacuo*, the aqueous residue was transferred to a separatory funnel with water, 2% w/v sodium chloride, and 10% v/v methanol were added, and the carotenoids were extracted with diethyl ether (peroxide-free). The ethereal extract was washed several times with water, dried over sodium sulfate, and finally concentrated to a small volume *in vacuo*. An equal volume of a 20% solution of potassium hydroxide in methanol was added, and the solution was set aside in the dark for 24 hr. After standing, the saponified pigment extract was diluted with water and the carotenoids re-extracted with diethyl ether until no color remained in the aqueous layer. The combined ethereal extracts were washed repeatedly with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*, and the residue was taken up in the upper phase of solvent system I (see below).

The pigments were separated into six fractions by counter-current distribution in a semi-micro all-glass Craig apparatus (3 ml upper and lower phases) using two solvent systems. With system I, using 1.8:1 (v/v) hexane and 99% methanol (Curl, 1953), the pigments were readily separated into three fractions—hydrocarbons, monols, and diols plus polyols—in 100 transfers (Fig. 1). The diol-polyol fraction was subjected to a further 100 transfers with system II, which used 10:7.35:2.65 (v/v)

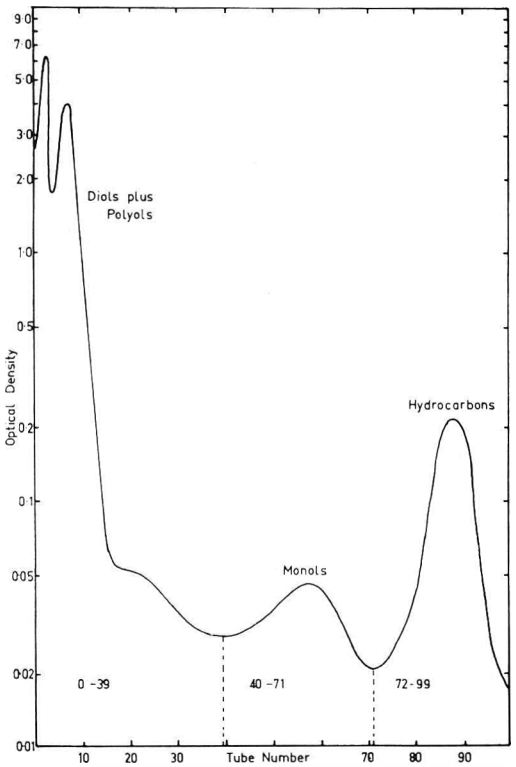


Fig. 1. Counter-current distribution of the carotenoids from pineapple pulp with solvent system I.

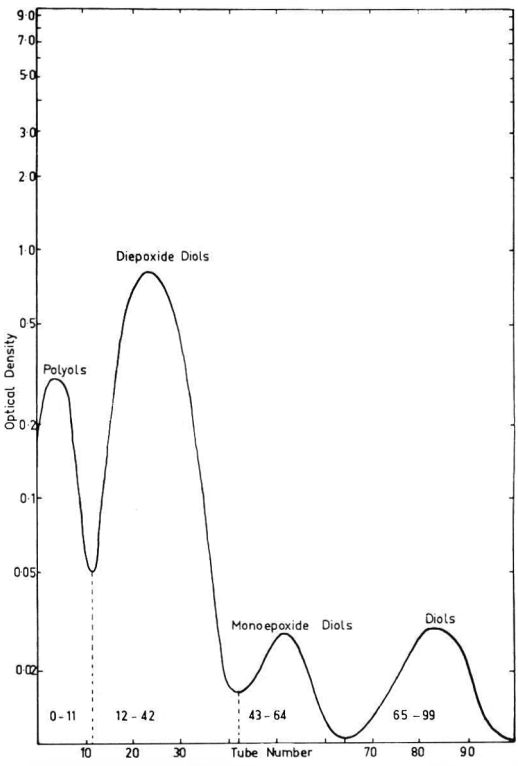


Fig. 2. Counter-current distribution of the carotenoids from pineapple pulp with solvent system II.

hexane, methanol, and water (Curl, 1960). This system separated the diol-polyol fraction into four sub-fractions with tube maxima at 3, 22, 54, and 84 (Fig. 2). These values were very close to those obtained by Curl (1960) with the xanthophylls of orange juice, indicating that these sub-fractions respectively consisted of polyols, diepoxide diols, monoepoxide diols, and diols. The position of the tube maxima for each solvent system was obtained by diluting the contents of each tube with acetone to 25 ml and measuring the absorption at 440 mμ in an Optica CF4 spectrophotometer.

The six fractions obtained by counter-current distribution were evaporated to dryness *in vacuo*, the residues taken up in either hexane (hydrocarbons and monols only) or benzene, and each fraction chromatographed on columns of 1:1 (v/v) magnesium oxide and filter aid. Eluants used were those recommended by Curl (1959): 14% benzene in hexane for hydrocarbons, and 0.3, 3.5, 3.5, 3.5, and 7% absolute ethanol in hexane for monols, diols, monoepoxide diols, diepoxide diols, and polyols, respectively. Subsequent eluants for each fraction were 1½ times as strong as the preceding one.

Spectral absorption curves were obtained on a Cary recording spectrophotometer, model 14.

RESULTS AND DISCUSSION

The total carotenoid content (before saponification) of the fruit was 0.98 mg/kg (as beta-carotene and measured at 438 mμ).

Counter-current distribution. The fractional composition of the carotenoid mixture is given in Table 1. The diol-polyol fraction predominates, in this respect resembling many of the fruits examined by Curl (1964). On further separation, however, this fraction

Table 1. Partition of saponified carotenoids by counter-current distribution.

Fraction	% of total [a]	Tube no. of maximum
System I [b]		
Hydrocarbons I	9.5	89
Monols II	2.5	59
Diols and Polyols	88.0	7 and 3
System II [b]		
Diols III A	3.5	84
Monoepoxide diols III B	2.9	54
Diepoxide diols III C	68.1	22
Polyols IV	13.5	3

[a] Based on the total absorbance of 440 mμ.
[b] For composition of solvent systems, see text.

was found to contain over 75% diepoxide diols, much more than any other fruit so far examined by Curl (1964).

Column chromatography. Thirty-two components were obtained, but only 28 of these were present in sufficient quantity to allow identification (Table 2). Considerable quantities of substances showing strong uv absorption were detected in each fraction and may have been terpenes. In many cases the presence of these compounds masked any minor peaks in the spectra appearing at wavelengths below 360 mμ.

Hydrocarbons. Five constituents were found in this fraction. The first two eluted were identified as alpha-carotene and beta-carotene by their spectral absorption curves and position on the column on elution. The absorption spectra of alpha-carotene contained two peaks at 367 and 347 mμ and are probably those of phytofluene although no fluorescence in ultraviolet light was observed on the column. Fraction I-3 was eluted just after beta-carotene and gave a spectral absorption curve which closely resembled that of zeta-carotene (Nash, *et al.*, 1948). Fraction I-4 was strongly absorbed on the column, and from its spectral absorption was tentatively identified as neurosporene, a hy-

Table 2. Carotenoids obtained from pineapples.

Fraction	Probable identity [d]	Solvent [c]	Absorption maxima (mμ)	Approx. % [a]	
I — 1	Phytofluene	H	367;347	0.5	
I — 2	α-Carotene	H	474;445;421	0.5	
I — 3	β-Carotene	H	476;449;(427) [b]	6.7	
I — 4	Zeta-carotene	H	426;400;377	0.9	
I — 5	Neurosporene	H	468;439;416	0.7	
II — 1	Hydroxy-α-carotene	H	472;445;420	0.9	
II — 2	Cryptoxanthin	H	475;449;(426)	0.9	
II — 3	Cryptoxanthin-like	H	470;445;(427)	0.4	0.7
II — 4	Cryptoxanthin-like	H	470;445;(424)	0.3	
IIIA — 1	Lutein	B	483;453;426	2.5	
IIIA — 2	Cis-lutein	B	480;445;(426)	1.0	
IIIB — 1	Lutein 5,6-epoxide	B	481;450;426	0.3	
IIIB — 2	Antheraxanthin	B	482;454;(432)	1.0	1.7
IIIB — 3	Cis-antheraxanthin	B	480;452;(430)	0.7	
IIIB — 4	Flavoxanthin	B	456;432;408	0.3	
IIIB — 5	Mutatoxanthin	B	462;434;410	0.6	
IIIC — 1	Cis-violaxanthin	B	477;447;422	25.0	52.0
IIIC — 2	Di-cis-violaxanthin	B	471;441;416	27.0	
IIIC — 3	Luteoxanthins	B	460;432;407	6.2	13.4
IIIC — 4	Cis-luteoxanthins	B	458;430;406	7.2	
IIIC — 5	Auroxanthins	B	436;410;386	3.4	
IV — 1	Neoxanthin-like	B	480;449;426	0.7	1.5
IV — 2	Neoxanthin-like	B	480;450;426	0.8	
IV — 3	Neoxanthin	B	480;450;425	8.1	
IV — 4	Trollixanthin-like	B	480;451;427	1.1	2.1
IV — 5	Trollixanthin-like	B	480;450;428	1.0	
IV — 6	Neochromes	B	457;431;408	0.7	1.5
IV — 7	Neochromes	B	458;430;406	0.8	
IV — 8	Substance 407 [e]	B	432;407;385	0.8	

[a] Based on absorbance at principal wave lengths.
[b] Values in parentheses represents shoulders on spectral absorption curves.
[c] H = hexana; B = benzene.
[d] Tentative identification indicated by absorption spectra etc.
[e] Substance 407 refers to that pigment with absorption maximum at 407 mμ.

drocarbon found in tomatoes (Trombly and Porter, 1953).

Monols. The major constituents of this fraction were found to be hydroxy-alpha-carotene and cryptoxanthin. Four other constituents were eluted after cryptoxanthin, but only two of these were present in sufficient quantities to permit further analysis. The spectral absorption of these compounds was very similar to that of cis-cryptoxanthin. Treatment with hydrochloric acid and ether (Curl and Bailey, 1961) yielded inconclusive results, and hence it could not be established whether these constituents were isomeric forms of cryptoxanthin or cryptoxanthin epoxides.

Diols. Lutein and a compound having an absorption curve resembling cis-lutein (Curl and Bailey, 1957) were found. Accompanying the cis-lutein band was a reddish band which may have been zeaxanthin but which could not be isolated.

Monoepoxide diols. On chromatography this fraction yielded a total of 5 constituents. These were identified on the basis of their spectral absorption curves and on the hydrochloric acid-ether test as lutein 5,6-epoxide, antheraxanthin, cis-antheraxanthin, flavoxanthin, and mutatoxanthin.

Diepoxide diols. The main constituents of this fraction were apparently cis-violaxanthin and a dicis-isomer. These two carotenoids were present in approximately equal amounts and were predominant carotenoids in the pineapple. They were accompanied by the isomeric 5,8-epoxides luteoxanthins and auroxanthins.

Polyols. Column chromatography showed this fraction to be quite complex. Eight bands were obtained, of which the first five exhibited almost identical absorption spectra. The two lowest bands were pale yellow on the column, and both gave only light-blue colors with the hydrochloric acid-ether test. Eluted after these by 10% ethanol in hexane was the major constituents of this fraction, which gave a light-blue color on treatment with ether and hydrochloric acid, and it was probably a neoxanthin. The next two bands were eluted with 15% ethanol in hexane and were incompletely separated on the column. Both gave pale-blue colors with hydrochloric acid and ether and could be either trollixanthins or trolliflors. Two of the remaining three bands had identical absorption spectra: both gave blue colors with hydrochloric acid and ether, and could be neochromes or trollichromes. The last component eluted from the column had spectral absorption maxima similar to but somewhat lower than auroxanthin.

Further studies on the polyol fraction are in progress, and it is proposed to report more fully on the identity of the carotenoids of this fraction in a subsequent paper.

In the carotenoids extracted from fresh pineapple, greater amounts of 5,6-epoxides were found than the corresponding 5,8-epoxides, indicating that acid-catalyzed isomerization of 5,6-epoxides to 5,8-epoxides has not occurred to any extent. Changes occurring in the carotenoid composition of pineapples during processing are now under investigation and will be the subject of a separate communication.

REFERENCES

Curl, A. L. 1953. Application of counter-current distribution to Valencia orange juice carotenoids. *J. Agr. Food Chem.* **1**, 456.

Curl, A. L. 1959. The carotenoids of cling peaches. *Food Research* **24**, 413.

Curl, A. L. 1960. Two new solvent systems for the countercurrent distribution of carotenoids. *J. Agr. Food Chem.* **8**, 356.

Curl, A. L. 1964. The carotenoids of several low-carotenoid fruits. *J. Food Sci.* **29**, 241.

Curl, A. L., and G. F. Bailey. 1954. Polyoxygen carotenoids of Valencia orange juice. *J. Agr. Food Chem.* **2**, 683.

Curl, A. L., and G. F. Bailey. 1957. Comparison of xanthophylls from leaves and orange juice. *J. Food Research* **22**, 323.

Curl, A. L., and G. F. Bailey. 1961. An improved test for carotenoid epoxides. *J. Agr. Food Chem.* **9**, 403.

Leverington, R. E. 1962. Problems associated with the processing of pineapple juice in Queensland. *Food Technol. Australia* **14**, 594.

Leverington, R. E., and R. C. Morgan. 1964. An experimental pineapple juice concentrate plant incorporating flavour recovery. *Queensland Agr. J.* **28**, 33.

Nash, H. A., F. W. Quackenbush, and J. W. Porter. 1948. Studies on the structure of zeta-carotene. *J. Am. Chem. Soc.* **70**, 3613.

Singleton, V. L., Willis A. Gortner, and H. T. Young. 1961. Carotenoid pigments of pine-

apple fruit. 1. Acid-catalyzed isomerization of the pigments. *J. Food Sci.* **26**, 49.

Trombly, H. J., and J. W. Porter. 1953. Additional carotenes and a colourless polyene of Lycopersicon species and strains. *Arch. Biochem. Biophys.* **43**, 443.

Ms. rec'd 11/3/65.

The author thanks Mr. H. Schmitzerling and the CSIRO Veterinary Parasitology Laboratory, Brisbane, for the use of the laboratory's countercurrent distribution apparatus, and Mr. J. P. Callaghan, of the Department of Pathology, Brisbane Hospital, who ran the spectral absorption curves.

SUZANNE LOVE and L. J. BRATZLER
Food Science Department, Michigan State University, East Lansing, Michigan 48823

Tentative Identification of Carbonyl Compounds In Wood Smoke by Gas Chromatography

SUMMARY

Isolation, separation, and partial identification of 21 carbonyl compounds found in wood smoke are described. Isolation was by precipitation of carbonyls by 2,4-dinitrophenylhydrazine. Separation was by gas chromatography using Carbowax 20M and diisodecylphthlate as the liquid phase. Tentative identification of the eluted components was based upon retention times of the unknowns compared with those of authentic compounds on the two columns used. Sixteen of the twenty-one components were tentatively identified: formaldehyde, acetaldehyde, propanal, acetone, acrolein, isobutyraldehyde, butanal, 2-butanone, diacetyl, isovaleraldehyde, 3-methyl-2-butanone, crotonaldehyde, pinacolone, 4-methyl-3-pentanone, α-methylvaleraldehyde, and tiglic aldehyde. Trace amounts of 3-hexanone and 2-hexanone were found. Also present were 1–3 five-carbon compounds, but no separation could be achieved to identify them.

INTRODUCTION

More attention has been given to the chemical composition of wood smoke in the last ten years than ever before. There are still many unanswered questions why wood smoke decreases spoilage and improves the taste and appearance of food. To improve the process of wood smoking in terms of increased efficiency and enhancing product quality, knowledge must be obtained of the chemical nature of the compounds affecting the product (Hoff and Kapsalopoulou, 1964).

Most of the flavoring materials contained in smoke have been attributed to the steam-distillable fraction (Husaini and Cooper, 1957; Porter *et al.*, 1964). There is some question, however, whether smoke flavor is due directly to smoke compounds or to reaction products formed when food is smoked. Therefore, considerable research is needed on the chemical composition of smoke and the interaction of smoke constituents with food components to determine what causes smoke flavor.

The carbonyls are one group of compounds found in the steam-distillable fraction of smoke. It is of interest to isolate and identify these compounds directly from smoke. Carbonyls previously identified in wood smoke are: formaldehyde, acetaldehyde, methyl glyoxal, propanal, acetone, isobutyraldehyde, pentanal, 2-pentanone, methacryaldehyde, methyl vinyl ketone, furfural, and 5-methyl furfural (Callow, 1927; Jahnsen, 1961; Hoff and Kapsalopoulou, 1964; Hollenbeck and Marinelli, 1963; Ockerman *et al.*, 1964; Pettet and Lane, 1940; Porter *et al.*, 1965). Three of these compounds (formaldehyde, acetaldehyde, and acetone) were identified most frequently; others were identified in a few instances. The major causes of the differences among these identifications are presumably due to variations in the generation, collection, and identification procedures, and in the types of wood used.

This study was made to isolate, identify, and compare qualitatively the carbonyl compounds present in whole wood smoke produced by a laboratory smoke generator and a commercial generator and the vapor phase of wood smoke produced by the laboratory smoke generator equipped with an electrostatic precipitator.

EXPERIMENTAL PROCEDURE

Sample treatment. Wood smoke was collected from the smoke-generating unit described by Porter *et al.* (1965). Both whole smoke and vapor phase were collected with the hot-plate temperature ranging between 490 and 500°C. The smoke temperature before the electrostatic air cleaner was between 105 and 125°C, and its temperature before entering into the traps was between 90 and 110°C. Whole smoke was also collected from a commercial air-conditioned smoke house equipped with a Mepaco smoke generator. The smokehouse temperature was 48.0–49.5°C, and the relative humidity 11%.

Hardwood sawdust, predominantly hard maple, which could be sifted through a ¼-inch but not ⅛-inch mesh screen was used. The moisture content of the sawdust was 7.1% for the generator unit and 56.0% for the smoke house.

Three suction flasks containing a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* HCl were placed in series. The last flask was connected to a

vacuum line (¼-inch ID), and the vacuum was so adjusted that there was an air flow from the generator or smoke house of 7–9 lpm. Collection time for one run was about 7 hr. A bright orange-red precipitate was formed in each flask.

Preparation of sample for gas chromatography. The solutions were stored overnight at room temperature and filtered the following day with a Buchner funnel and No. 2 Whatman filter paper. The collected precipitate was washed thoroughly with a 2*N* HCl solution, rinsed with distilled water, and air-dried.

The hydrazones were regenerated to free carbonyls by the method of Keeney (1957). Reagent-grade levulinic acid and 2*N* HCl in 10:1 volume ratio were added to a 10-ml distilling flask containing a hydrazone sample weighing less than 0.2 g. The mixture was heated slowly in an oil bath up to 160°C and held for 5 min. This allowed the freed carbonyls to distill over with water into a small tube immersed in an ice bath. The carbonyls were extracted with a small amount (20–30 μl) of methyl phenyl ether (Anisole) and injected in 10-μl quantities into a gas chromatograph. Several trials were also made with only the levulinic mixture present in the flask.

Vapor analysis. The regenerated carbonyls were separated with a Barber-Colman gas chromatograph, model 20, equipped with a radium ionization detector. Two 6-ft columns were made of ¼-inch-OD copper tubing. The primary analytical column, *A*, was packed with 10% Carbowax 20M, a polyethylene glycol, on 60–80-mesh Chromasorb W.A.W. (F & M Scientific Corp.). Column *B* was packed with 10% diisodecylphthlate coated on 60–80-mesh Chromasorb W.A.W. Both were packed with an electric vibrator and then coiled to a diameter of 5 inches. Columns *A* and *B* were preconditioned for at least 24 hr at 150°C with respective argon flow rates of 40.0 and 39.2 ml/min

For all trials the following conditions were maintained: split flow rate (used only with standards) 571.4 ml/min, scavenger flow rate 71.43 ml/min, detector cell temperature 150°C, flash-heater temperature 150°C, cell voltage 1250, and sensitivity 1×10^{-7} amps (for unknowns only).

For column *A*, operating conditions were: column temperature 75°C, inlet pressure 12 psig, and argon flow rate 40.0 ml/min. For column *B*, these conditions were: column temperature 75°C, inlet pressure 12 psig, and argon flow rate 66.7 ml/min.

The solvent, methyl phenyl ether, was retained on the column until after all unknown compounds were eluted off. It was found, however, that this solvent would change the retention times of the compounds on column *B* during the time of its elution. Therefore, to make retention times comparable, the standards were preceded with an injection of methyl phenyl ether on this column.

Table. 1. Retention times (min) for column A.

	Laboratory generator			Commercial generator whole smoke
	Standards	Whole smoke	Vapor phase	
Formaldehyde	1.7	1.7	1.7	1.7
Acetaldehyde	2.7	2.7	2.7	2.7
Propanal	4.2	4.3	4.3	4.3
Acetone	4.7	5.0	5.0	4.9
Isobutyraldehyde	4.8	5.0	5.0	4.9
Acrolein (2 propanal)	5.8	5.9	6.0	5.9
Butanal	6.9	7.2	7.3	7.2
2-Butanone	8.0	8.2	8.2	8.1
Isovaleraldehyde	9.1	9.1	9.2	9.1
3-methyl-2-butanone	9.8	9.8	9.6	9.6
Pinacolone (3, 3-dimethyl-2-butanone)	11.1	10.8	10.9	10.7
Diacetyl (2,3-butanedione)	12.5	12.0	12.4	12.1
3-pentanone	13.1	13.3	13.1	13.0
2-pentanone	13.2	.....	.....	.....
Pentanal	13.2	.....	.....	.....
4-methyl-3-pentanone	14.6	14.3	14.5	14.4
α-methyl valeraldehyde	15.6	15.7	15.9	15.7
Crotonaldehyde (2-butenal)	19.6	19.5	19.5	19.6
3-hexanone	20.7	.....	.....	.....
2-hexanone	25.0	.....	.....	.....
Tiglic aldehyde (2-methyl-2-butenal)	27.7	27.7	27.7	27.6
Unknown	.....	33.6	34.3	33.7

Table 2. Retention times (min) for column B.

	Laboratory generator			Commercial generator whole smoke
	Standards	Whole smoke	Vapor phase	
Formaldehyde	1.0	1.1	1.1	1.1
Acetaldehyde	1.4	1.4	1.4	1.4
Propanal	2.4	2.6	2.5	2.5
Acetone	2.5	2.6	2.6	2.5
Acrolein (2-propanal)	2.5	2.6	2.6	2.5
Isobutyraldehyde	3.5	3.7	3.6	3.5
Unknown	.....	4.3	4.2	4.1
Butanal	4.7	4.9	4.8	4.7
2-butanone	5.0	5.3	5.2	5.1
Diacetyl (2,3-butanedione)	6.1	6.5	6.3	6.3
Isovaleraldehyde	7.5	7.7	7.7	7.6
3-methyl-2-butanone	9.1	9.1	9.1	9.0
Crotonaldehyde (2-butenal)	9.4	9.6	9.5	9.3
2-pentanone	10.1	10.8	10.8	10.3
3-pentanone	10.6	.....	.....	.....
Pentanal	11.0	.....	.....	.....
Pinacolone (3,3-dimethyl-2-butanone)	11.2	.....	.....	.....
4-methyl-3-pentanone	14.5	14.6	14.4	14.0
Tiglic aldehyde (2-methyl-2-butenal)	17.2	17.4	17.4	17.2
α-methyl valeraldehyde	17.4	17.4	17.4	17.2
3-hexanone	21.1	21.0	20.7	20.1
2-hexanone	23.2	23.7	23.8	23.1

RESULTS AND DISCUSSION

Analysis of carbonyls in wood smoke using gas chromatography revealed at least 21 components. Sixteen were tentatively identified (Tables 1, 2). The data in each of these tables (except standards) represent an average of the six regenerations made from three separate collections on each type of smoke. No difference was found between the three types of smoke on either column. Therefore, a typical chromatogram is included from each column (Figs. 1, 2) rather than one for each type of smoke.

Formaldehyde was present in small but distinct amounts in all samples. Its quantity was probably reduced by its rapid evaporation between the times of collection, extraction and injection. Separation of propanal, acetone, and acrolein was excellent on column *A*, whereas on column *B* these three compounds eluted as one peak with only an occasional separation of propanal from acetone and acrolein. Isobutyraldehyde could not be separated from acetone on column *A*, but the size of the peak on this chromatograph indicates that acetone is present, since isobutyraldehyde appeared only in small amounts from column *B*.

An unidentified peak was eluted between isobutyraldehyde and butanal on column *B*. The lack of this peak on column *A* may be due to its elution time being identical with that of another standard compound. Butanal is one suggestion in this case, because of the difference in peak size in relation to 2-butanone as compared to the results with column *B*.

Diacetyl was detected on column *A* as merging with the five-carbon compounds. The small amount present was completely separated and verified by column *B*. The five-carbon compounds referred to in the chromatograms are 2-pentanone, 3-pentanone, and pentanal. The retention times of these compounds are so nearly the same that the peaks eluted on the chromatograms could be any one or combination of these three. All efforts failed to separate these three compounds on the two columns. On column *B* the retention time of pinacolone caused its peak to overlap with the five-carbon compounds, but its presence is verified by column *A*.

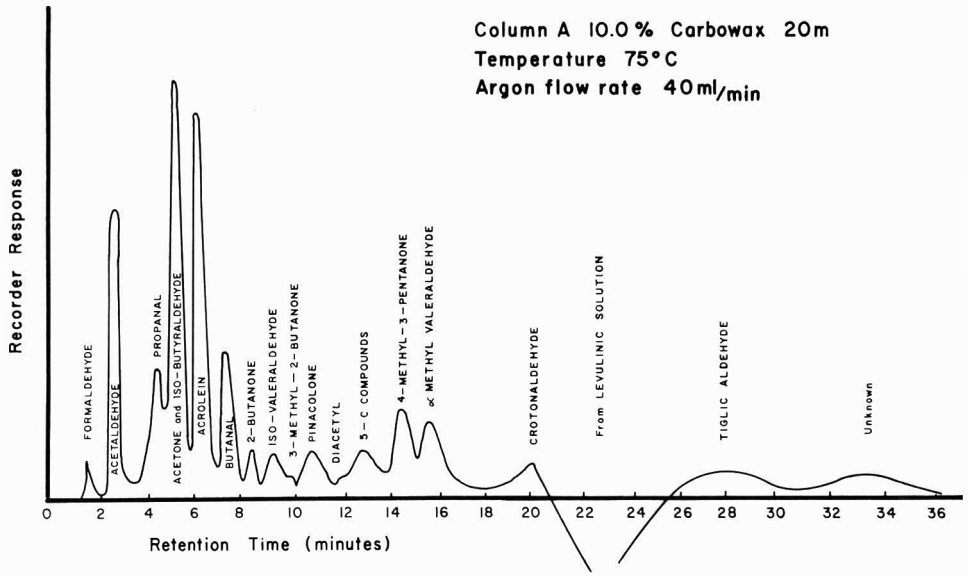


Fig. 1. Chromatogram of carbonyls isolated from wood smoke with Column A.

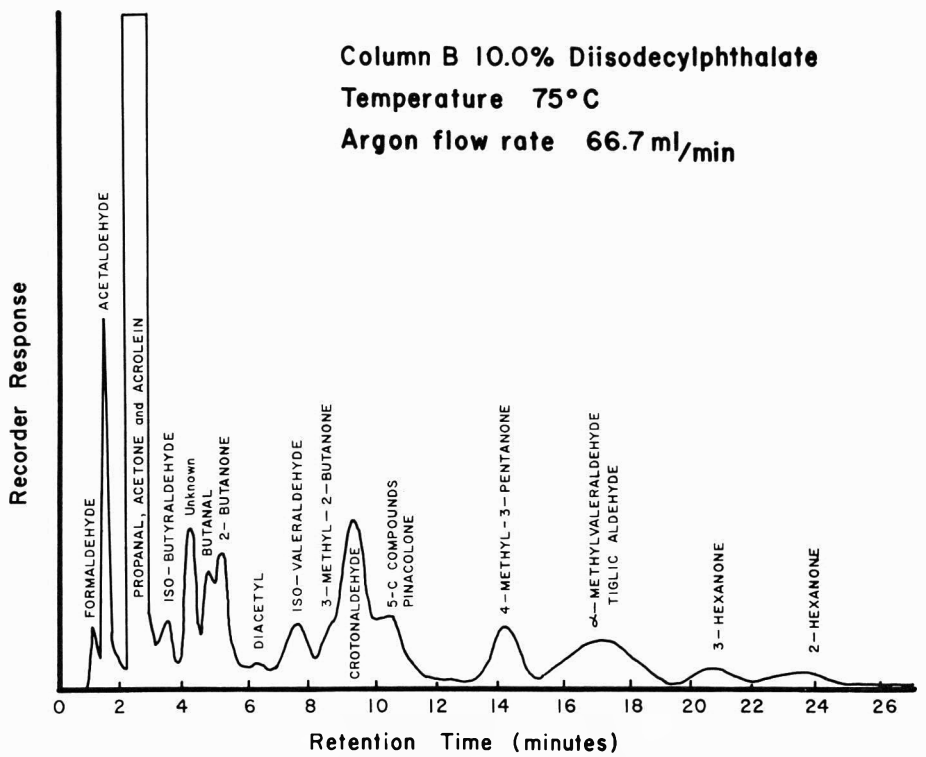


Fig. 2. Chromatogram of carbonyls isolated from wood smoke with Column B.

With column *A*, a negative peak follows immediately after crotonaldehyde. This peak appeared in the trials made with the levulinic acid mixture containing no hydrazones, and thus was attributed to it. Because of this negative peak, 2- and 3-hexanones could not be verified with column *A*, since it occurred in the region where the standards of these compounds were eluted.

With column B, α-methyl valeraldehyde and tiglic aldehyde were eluted close together. This usually gave one broad flat peak, but occasionally they separated enough to identify two peaks. Column *A* separated and verified that both were present. A long-chain compound was found with column *A* but not with column *B*. It was not identified.

Any small variation in retention times was probably due to normal experimental error such as the speed of injection, injection-port temperature, and measuring to the center of peak height.

REFERENCES

Callow, E. 1927. The presence of formaldehyde in wood smoke and in smoked foodstuffs. *Analyst* **52**, 391.

Hoff, J. E., and A. J. Kapsalopoulou. 1964. Low boiling components of wood smoke—identification by means of syringe reactions. *J. Gas Chromatog.* **2**, 296.

Hollenbeck, C. M. and L. J. Marinelli. 1963. Some chemical constituents of smoke flavoring. Proc. 15th Research Conf. p. 67. Univ. of Chicago, Chicago, Illinois.

Husaini, S. A., and G. E. Cooper. 1957. Fractionation of wood smoke and the comparison of chemical composition of sawdust and friction smokes. *Food Technol.* **11**, 499.

Jahnsen, V. J. 1961. The chemical composition of hardwood smoke. *Dessert. Abstr. 22* No. 1, July.

Keeney, M. 1957. Regeneration of carbonyls from 2,4-dinitrophenylhydrazones with levulinic acid. *Anal. Chem.* **29**, 1489.

Ockerman, H. W., T. N. Blumer, and H. B. Craig. 1964. Volatile chemical compounds in dry-cured hams. *J. Food Science* **29**, 123.

Pettet, A. E., and F. G. Lane. 1940. The chemical composition of wood smoke. *J. Soc. Chem. Ind.* **59**, 114.

Porter, R. W., L. J. Bratzler, and A. M. Pearson. 1965. Fractionation and study of compounds in wood smoke. *J. Food Science* **30**, 615.

Ms. rec'd 11/13/65.

Journal article 3750, Michigan Agricultural Experiment Station.

This study was supported in part by research grant No. EF-00435, provided by Division of Environmental Health and Food Protection, U.S. Public Health Service.

T. W. KWON and MARIDA J. NORGAARD
Department of Nutritional Sciences, Institute of Marine Resources, University of California, Berkeley, California 94720

Observations on the Reactivity of 2-Thiobarbituric Acid-Reactive Substances in Dried Whole Egg Powder

SUMMARY

The production of 2-thiobarbiturie acid-reactive substances (TBRS) during storage of dried whole egg powders was temperature- and time-dependent. Some of the TBRS occurred as a bound form with egg proteins, the lipoprotein fraction contained considerably more bound TBRS than did the other water-soluble proteins. Malonaldehyde (MA), as an example of TBRS, was more reactive than glucose with egg white proteins.

INTRODUCTION

The oxidation of egg lipids and the interaction of their oxidation products with other egg constituents cause chemical and physical changes associated with the deterioration of dried egg (Lightbody and Fevold, 1948). Kline *et al.* (1964) recently measured 2-thiobarbituric acid-reactive substances (TBRS) in dried whole egg samples and found good correlation between the amounts of distillable TBRS and loss of organoleptic quality for powders stored at low temperatures. It is likely that malonaldehyde (MA) is the most important of the TBRS (Kwon and Olcott, 1965). Reactions of MA with proteins and other food constituents have been reported (Kwon and Brown, 1965; Kwon *et al.*, 1965).

The present communication describes the increase of TBRS in egg powder during storage and evidence for their presence in bound form with proteins. Some data on the reaction of MA with ovalbumin and conalbumin are also presented.

EXPERIMENTAL METHODS

Dried whole egg powders with and without added sucrose (Kline *et al.*, 1964) were held at 6, 25, and 35° in air. The moisture contents of the samples were approximately 3% as received (Kline *et al.*, 1964), and did not change significantly during storage.

Analyses of distillable TBRS (Tarladgis *et al.*, 1960) were carried out at one-week intervals. Four g of egg powder, 97.5 ml of distilled water, and 2.5 ml of 4*N* HCl were combined in a Kjeldahl flask and distilled until 50 ml of distillate was collected. Two ml of the distillate were mixed with an equal volume of 0.02*M* 2-thiobarbituric acid (TBA) in 90% glacial acetic acid and heated for 35 min at 100°. Relative amounts of TBRS were expressed as absorbance at 532 mμ, corrected for interference by absorbance at 450 mμ, according to the empirical method of Yu and Sinnhuber (1962).

A water-soluble protein fraction was prepared from egg powder which had been stored for 20 days at 25° by mixing 2 g with 8 ml distilled water. After separation of the solids by centrifugation, the solution was dialyzed extensively against cold glass-distilled water, to remove free TBRS. The dialyzed protein solution was then fractionated on a Sephadex G-100 column (2 × 95 cm) with 0.01*M* acetate buffer containing 0.1*M* NaCl, pH 6.0, according to a method previously described (Kwon *et al.*, 1965). The solution (12 mg protein/1.3 ml) was introduced on the column, and the proteins were eluted by the same buffer (0.66 ml/min) at room temperature. The absorbance of each fraction was measured at 280 mμ and, after reaction with TBA, at 532 mμ.

Ovalbumin and conalbumin (Sigma) were reacted with MA as follows: 50 mg protein and 0.2 mg MA were mixed in 10 ml of 0.08*M* sodium chloride–0.01*M* sodium bicarbonate buffer, pH 9.0, and incubated at 25 or 30°. Glucose-protein reaction mixtures were also prepared in the same manner (cf. Feeney *et al.*, 1964). At intervals, aliquots were dialyzed extensively against cold glass-distilled water. The amount of MA bound to the protein was determined by analyses for MA and protein in the dialyzed reaction mixtures, by TBA and biuret reactions, respectively. The dialyzed reaction mixtures were compared with the original proteins and with the dialyzed glucose-protein reaction mixtures, by starch gel electrophoresis with 0.01*M* succinate buffer, pH 5.4.

RESULTS AND DISCUSSION

Fig. 1 shows the increase in distillable TBRS with time of storage of dried whole egg powders. More TBRS was produced at the higher temperatures. The results for the whole egg powders with sucrose were almost parallel to those for the whole egg powder, except that slightly higher amounts

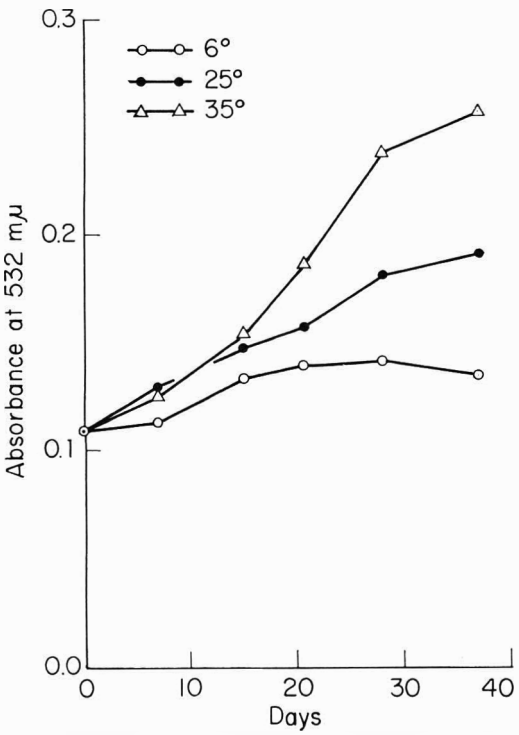


Fig. 1. Relationship between distillable TBRS (absorbance at 532 mμ) production and storage of dried whole egg powders at different temperature.

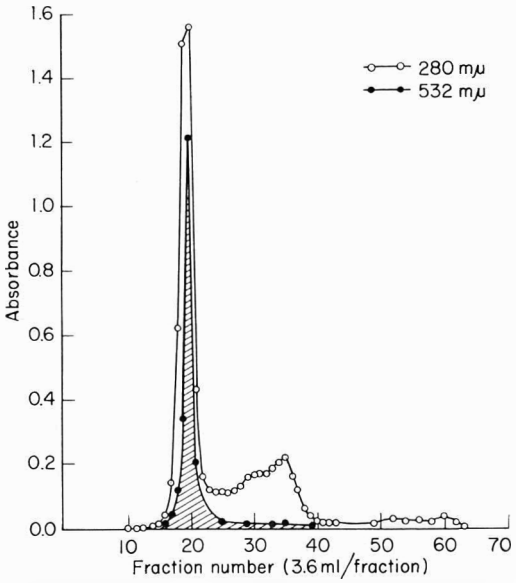


Fig. 2. Elution of TBRS-protein reaction products extracted from dried whole egg powder. Sephadex G-100 column, 2 × 95 cm; buffer, 0.01*M* acetate containing 0.1*M* NaCl, pH 6.0.

of TBRS were produced (not shown). When the dialyzed solution containing the water-soluble proteins (somewhat turbid) was chromatographed on a Sephadex G-100 column, the results shown in Fig. 2 were obtained. The absorbance at 280 mμ coincided with the amounts of TBRS (absorbance at 532 mμ) for the main peak (turbid). The other protein peaks contained very little TBRS or none. A preparation was submitted to fractionation in a preparative ultracentrifuge (Beckman L-2), and the floating lipoprotein layer was carefully removed. The lipoprotein fraction was turbid and had an ultraviolet absorption spectrum (max. at 277 mμ) similar to that of the main peak in the chromatogram. The residual material from the ultracentrifuge was examined by column chromatography. In this case the main peak was drastically reduced but the other protein peaks were approximately the same. The main peak (lipoprotein fraction) contained considerably more bound TBRS than the other peaks.

After 4 days of interaction with MA at 30°, and dialysis, ovalbumin contained 0.63 μg of bound MA per mg protein. The absorbance of the MA-treated protein at 280 mμ was about 25% higher than that of the untreated protein. The difference spectrum of the former against the latter had an absorption maximum at 287 mμ. These phenomena are similar to those observed with bovine serum albumin and MA (Kwon and Brown, 1965). On the other hand, the dialyzed ovalbumin-glucose reaction mixture showed no difference in ultraviolet absorption spectra compared with that of the untreated protein. The conalbumin-MA product had 0.11 μg of bound MA per mg protein after 3 days of incubation at 25°, and 0.18 and 0.35 μg after 6 and 10 days, respectively. Starch gel electrophoresis of the MA-protein reaction products gave different patterns from those of untreated proteins; the bands were more diffuse and thinner. The protein that had been incubated with glucose could not be differentiated from the untreated control.

Feeney *et al.* (1964) found changes in egg proteins which had been incubated with

glucose and other reducing sugars at levels of 0.4 and 4%. In the present case the levels were 0.002%. Thus the observations show that MA will react with egg proteins at a much lower concentration than is required for the reaction with glucose.

The production of TBRS and the reactivity of TBRS and pure MA with isolated egg white proteins were temperature- and time-dependent. The reaction products were usually yellow or brown in color and less soluble in water, as has been observed also with serum albumin-MA reaction products. Hence, at least one reaction of MA or other TBRS with egg proteins is probably a type of amine-carbonyl browning (Kwon and Brown, 1965).

The quantitative significance of the effects of TBRS, probably mostly MA, on changes in the functional properties of egg powders during storage requires further study.

REFERENCES

Feeney, R. E., J. J. Clary, and J. R. Clark. 1964. A reaction between glucose and egg white proteins in incubated eggs. *Nature* **201**. 192.

Kline, L., R. F. Sugihara, and J. J. Meehan. 1964. Properties of yolk-containing solids with added carbohydrates. *J. Food Sci.* **29**. 693.

Kwon, T. W., and W. D. Brown. 1965. Complex formation between malonaldehyde and bovine serum albumin. *Federation Proc.* **24**. 592.

Kwon, T. W., and H. S. Olcott. 1966. Malonaldehyde from the autoxidation of methyl linolenate. *Nature*. In press.

Kwon, T. W., D. B. Menzel, and H. S. Olcott. 1965. Reactivity of malonaldehyde with food constituents. *J. Food Sci.* **30**. 808.

Lightbody, H. D., and H. L. Fevold. 1948. Biochemical factors influencing the shelf life of dried whole eggs and means for their control. *Advances in Food Research* **1**, 149.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan, Jr. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chemists' Soc.* **37**. 44.

Yu, R. C., and R. O. Sinnhuber. 1962. Removal of interfering pigments in determining malonaldehyde by the 2-thiobarbituric acid reaction. *Food Technol.* **16**. 115.

Ms. rec'd 11/6/65.

The dried whole egg powders were kindly provided by Dr. L. Kline, Western Regional Research Laboratory, USDA, Albany, California.

ELIAS D. DEKAZOS

Horticultural Crops Research Branch, Market Quality Research Division
Agricultural Research Service, U. S. Department of Agriculture
Beltsville, Maryland 20705

Anthocyanin in Red Tart Cherries as Related to Anaerobiosis and Scald

SUMMARY

A study was made of some causes and anthocyanin changes associated with scald in red tart cherries. Scald is defined as the localized translocation of the red pigment from the skin to the flesh of the cherry. Cherries, bruised and held in 100% nitrogen, showed that migration of pigment from the skin to the flesh left light-pink scalded areas. When held in 100% oxygen, bruised cherries did not scald, but the skin became dark red, and the flesh, when peeled, revealed oxidative browning. The total amount of anthocyanin pigments in the bruised cherries, scalded and unscalded, was about the same as in fresh unbruised fruit, demonstrating that anthocyanase does not participate in scald development. Analysis of volatiles emanating from scalded and unscalded cherries showed no differences, except from severely scalded fruit, which produced ethyl alcohol. Distribution of pigment was shown histologically in fresh unbruised cherries and in bruised cherries, scalded and unscalded.

INTRODUCTION

The anthocyanins, a group of glycosidic plant pigments that are usually dissolved in the cell sap, give red tart cherries their bright color. Basic information about cherry scald, which has troubled processors from time to time, has been sought in an effort to inhibit or delay this color loss.

LaBelle (1956), Whittenberger and Hills (1956), and LaBelle *et al.* (1958) observed that the initiation of scald is always preceded by bruising of the fruit. Scald, however, should be differentiated from subsequent browning. Scald may be defined as a localized translocation of red pigment from the skin to the flesh of the cherry. These scald areas are light pink and show a rather sharp line of demarcation between the affected and the healthy tissue. Scalded areas and the underlying tissue later become brown as a result of an enzymatic oxidation, which normally follows disruption of cellular structure (Joslyn and Ponting, 1951).

Wagenknecht *et al.* (1960) reported that anthocyanase participates in the early stages of scald development in red tart cherries through destruction of anthocyanin pigments. They used enzyme preparations from tart cherries to demonstrate the oxidative nature of the enzyme. And the exclusion of air completely inhibited the decolorization reaction.

Yeatman *et al.* (1961) reported that bruised cherries held in contact with each other at 40°F in water, at 80°F in air, or in sunlight did not show significant changes in the total amount of anthocyanin when the cherries scalded.

In the present investigation, a new technique was developed for artificial induction of scald in order to demonstrate specific phases of its development. Anthocyanin contents of scalded and unscalded cherries were determined; volatiles emanating from cherries were analyzed; and changes in pigmentation were demonstrated histologically.

MATERIALS AND METHODS

Red tart cherries (*Prunus cerasus* L., var. Montmorency) were harvested from an orchard of the C. H. Musselman Company, Biglerville, Pennsylvania. They were cut carefully, with their stems attached, from a number of similar trees and were selected for uniform size. All cherries, harvested at the same time, were separated by color into three stages of maturity: bright red, mature cherries, having maximum color development; intermediate red, immature; and a light red color, very immature. These cherries were placed in a container with ice water and transported to the laboratory.

Induction of scald. Fruits uniform in size and color were stemmed without bleeding, washed in distilled water, dried, and dropped three times from a height of 18 in. onto a hard, flat surface. The cherries were bruised to become softened, but their skins were not broken. The data cover 4 treatments, 2 dates, and 20 replications of 12 cherries per sample.

Bruised cherries were placed in air-tight 1000-ml flasks: half of the flasks were flushed with prepurified nitrogen, and the others were flushed with oxygen. The same was done for unbruised

fresh cherries. Bruised areas of the cherries were in contact neither with each other nor with the glass container. The air-tight flasks were placed in an incubator at 90°F to simulate the daytime temperature in the orchard. Samples of bruised and unbruised cherries placed in air at 90°F were also treated for scald development.

Determination of anthocyanins. Mature (overripe) scalded and unscalded cherries were sampled for chemical analyses. The scalded cherries were peeled with a scalpel, and separate samples were taken of the scalded skin and of the tissue beneath. The sampling areas of bruised unscalded cherries under oxygen were determined by the dark red spots on the skin and the brown flesh directly beneath them. Adhering flesh was removed from the skin. The clean skins and the flesh were frozen immediately in liquid nitrogen and stored in polyethylene bags at −15°F until analyzed. In a typical sample of the scalded cherries, the total amount of scalded skin was 1.76 g and the flesh directly beneath the scald area was 22.27 g, and these weights were used for every chemical analysis.

Pigment was extracted from the skin and flesh by macerating each sample separately for 2 min in a chilled Waring blender with 50–100 ml of cold 1% methanolic HCl. This mixture was stored for 2 days at −4°F and filtered through Whatman no. 1 filter paper in a Buchner funnel under mild vacuum. The residue was re-extracted at 35°F with cold 1% methanolic HCl. Soaking and extracting the residue 4–5 times removed all the anthocyanins. The combined extracts were centrifuged, filtered through celite, concentrated to a smaller volume under reduced pressure in a nitrogen atmosphere at 104°F, and then recentrifuged. All centrifugations were at 10,000 × G for 5 min in a refrigerated Servall centrifuge at 32°F.

The above solutions were diluted with 1% methanolic HCl to give the same total volume for the absorption determinations. The absorption spectra of these pigment extracts were measured in the range of 240–700 mμ with a Cary recording spectrophotometer, model 14, using 1-cm cuvettes, against a blank solution of 1% methanolic HCl. All of the optical density values are expressed on the same basis: the density of the 400-ml solution diluted 4 times. The maximum absorption peak of these extracts in the visible region of the spectrum was 530 mμ ($\lambda_{max} = 530$ mμ). The absorbance at 530 mμ was also measured with a Beckman quartz spectrophotometer, model DU, using 1-cm quartz cuvettes. Calculations of anthocyanin content were based on the molar-extinction coefficient for Idaein ($C_{21}H_{21}O_{11} \cdot 2.5\ H_2O$) at 530 mμ ($E = 3.43 \times 10^4$), the peak absorption of Idaein in methanolic 1% HCl (Siegelman and Hendricks, 1958).

Volatiles emanating from scalded and unscalded cherries. Volatile emanations from cherries were measured in the following treatments: a) cherries bruised and held in 100% nitrogen; and b) cherries bruised and held in 100% oxygen. The cherries in the air-tight flasks were incubated at 90°F until the bruised cherries under nitrogen became scalded (18 hr).

For each analysis of the headspace gas over scalded and unscalded cherries, an appropriate volume of gas was withdrawn by gas-tight syringe and introduced into the chromatographic column. Hydrogen flame ionization detectors were operated isothermally throughout the investigation. Columns and conditions were as follows: a capillary glass column of alumina, 1½ ft × 2 mm, 86°F; spiral packing column of 15% DC 710 silicone oil, 7 ft × ¼ in., 88°F; and a spiral column of 30% DC 550 silicone oil, 18 ft × ¼ in., 95°F. The silicone substrates, 550 and 710, were respectively coated on firebrick, 60–80-mesh, and on untreated Chromosorb W, 60–80-mesh. The carrier gas was hydrogen-nitrogen (50:50 mixture) at a rate of 45 ml/min. The flow rate of the compressed air was 500 ml/min.

Histological investigation. Preparation of sections for the histological examination of pigmentation was difficult because of the soft flesh of the cherries and the solubility of the anthocyanins. Using Griswold's method (1944), saturated neutral lead acetate was used to precipitate the anthocyanins within the cells and change their color to blue. In this work, rectangular pieces of cherry of about 4 × 7 mm were placed in the above solution, and suction was applied until the pieces were infiltrated (2½ hr).

RESULTS AND DISCUSSION

Changes associated with cherry scald. When bruised cherries were held in 100% nitrogen, migration of pigment from the skin to the flesh left light-pink scalded areas (Fig. 1). The first indication of incipient scald were slight color loss and mottling of the skin.

Progessive changes in the flesh were observed in cherries that were peeled at various times after the first scald symptoms were noted. The flesh immediately under the scald area of the skin first showed a red coloration (Fig. 2). In later sampling, all the flesh of this area became impregnated with the migrated pigment and was pink to

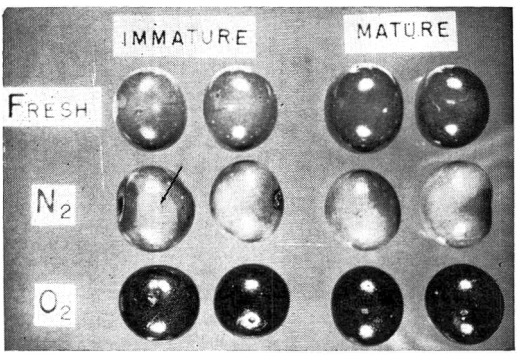


Fig. 1. Comparative appearance of scalded and unscalded red tart cherries:

Fresh: unbruised
N_2: scalded (see arrow), bruised cherries held 18 hr in 100% N_2 atmosphere at 90°F
O_2: unscalded bruised cherries held 18 hr in 100% O_2 atmosphere at 90°F

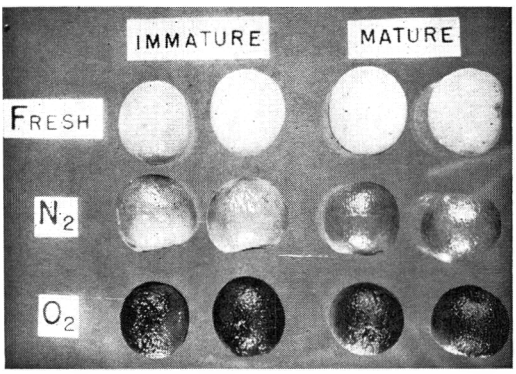


Fig. 2. Comparative appearance of scalded and unscalded peeled red tart cherries:

Fresh: unbruised, normal yellowish flesh
N_2: scalded bruised cherries held 18 hr in 100% N_2 atmosphere at 90°F; flesh appeared red
O_2: unscalded bruised cherries held 18 hr in 100% O_2 atmosphere at 90°F; flesh turned brown

light red. No skin or flesh browning could be observed on these cherries.

The skin of bruised cherries held in 100% oxygen was dark red, and the tissue beneath became brown (Figs. 1, 2). Although discolored, these cherries were not scalded. The dark-red color may have been caused by partial masking of the red pigment in the skin by miscellaneous brown products that developed in the flesh because of oxidative changes.

Thus, the anaerobic process caused the characteristic scalded areas in the skin, whereas the aerobic process resulted in an apparent enzymatic oxidation in the tissue beneath the skin as well as in the skin itself. The scald and the oxidative browning which appeared are two processes which stem from the same cause (bruising) but are not interdependent.

Bruised and unbruised cherries in air at 90°F showed no scald damage. Since the bruised cherries were not in contact with each other, there was an oxygen supply sufficient to prevent scald. The bruised areas were the same as those of bruised cherries held in 100% oxygen.

Very immature cherries in which scald was artificially induced, developed distinct scald areas somewhat faster than did mature fruit. This difference might be due to the fact that softer fruit gives more on impact, whereas firm fruit is more easily punctured or bruised by hitting a hard, flat surface. Damage to prunes from dropping on ground prepared only for hand pickup, has been shown to be related directly to flesh firmness (Miller *et al.*, 1963). Whittenberger and Marshall (1950) found that immature cherries were firmer than mature fruits as measured by percent compression. Scald development in red tart cherries has also been correlated with the extent of bruising (Pollack *et al.*, 1958).

The fact that unbruised cherries placed in a nitrogen atmosphere did not scald (Fig. 3) indicates that bruising is the primary factor in scald development. However, bruised cherries in a nitrogen atmosphere showed

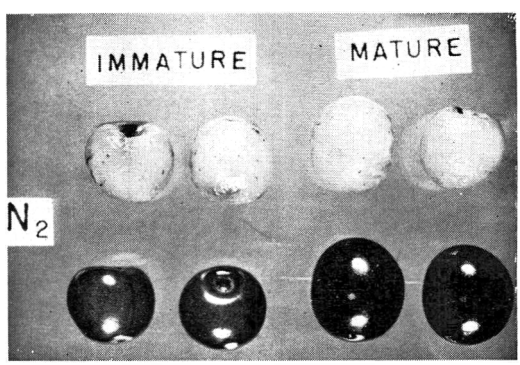


Fig. 3. Unbruised red tart cherries held 18 hr in 100% N_2 atmosphere at 90°F were unscalded. The bright red color of the skin was retained, and the normal yellowish flesh was visible for the peeled cherries of the upper row.

that lack of oxygen was a contributing factor in the development of scald.

The findings permitted extension of the work to soak tanks, where, in normal commercial operation, cherries are soaked in water after harvesting and tank scald develops. A second paper presents the relationship between oxygen and scald in red tart cherries in soak tanks.

Quantitative estimation of anthocyanin pigments. The skin of fresh unbruised cherries contained 423 mg of anthocyanin pigments per 100 g of the fresh skin. The whole fruit (flesh, skin, and pit) contained slightly more pigment than the skin. The flesh contained very little pigment.

Li and Wagenknecht (1956) found only two pigments — cyanidin-3-rhamnoglucoside and cyanidin-3-diglucoside—of nearly equal concentration in the skin of red tart cherries. In the present investigation, chromatograms of anthocyanin pigments from the skin of tart cherries showed seven pigments. Two of these are the major pigments. One is present in twice the concentration of the other. Work is in progress to isolate and identify these pigments. The differences reported in the number of pigments present could be due to real differences in the composition of the fruit or to the experimental techniques.

To establish a chemical profile of the pigments in the skin and flesh of red tart cherries, the total pigment of these tissues was estimated, by measurement of optical density at 530 mμ, for fresh bruised (scalded and unscalded) fruit.

Figs. 4 and 5 show three maxima, 530, 320, and 282 mμ, in the absorption spectra of the anthocyanin pigment extracts. Fig. 6 shows a small fourth absorption peak at 370 mμ.

The visible region of the absorption spectra of anthocyanin pigments of fresh unbruised red tart cherries shows that the pigments are concentrated in the skin (Fig. 4). In the bruised unscalded cherries the absorption spectra (Fig. 6) were similar to those of fresh cherries except that a small portion of the pigments migrated into the flesh; the total amount of pigment is almost the same as for fresh unbruised cherries. The anthocyanin pigments of bruised scalded cherries (Fig. 5) show migration from the skin into the flesh, but the total amount of pigment remained the same as in fresh unbruised cherries.

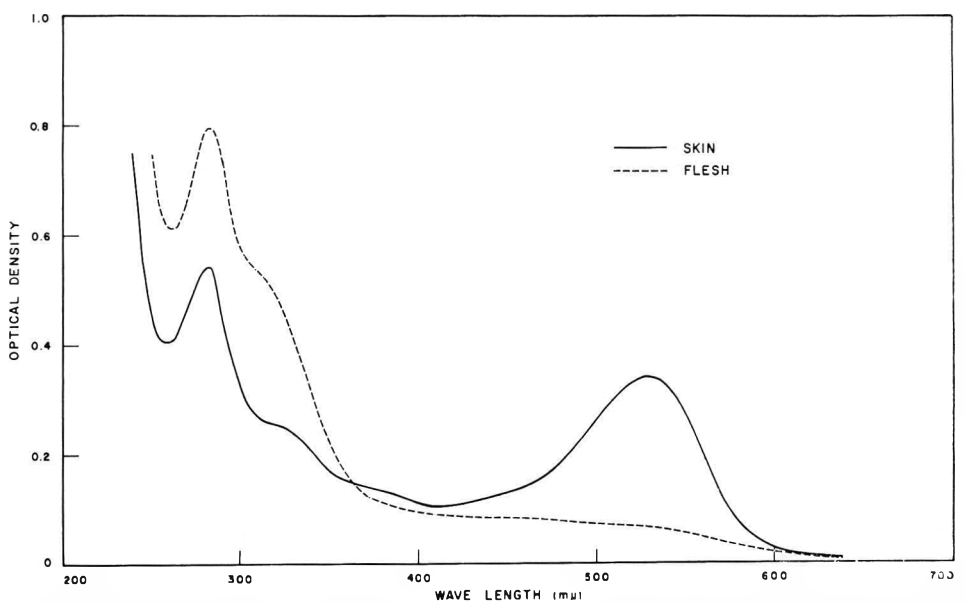


Fig. 4. Absorption spectra of anthocyanin pigments from fresh red tart cherries (unbruised) in MeOH (1% HCl).

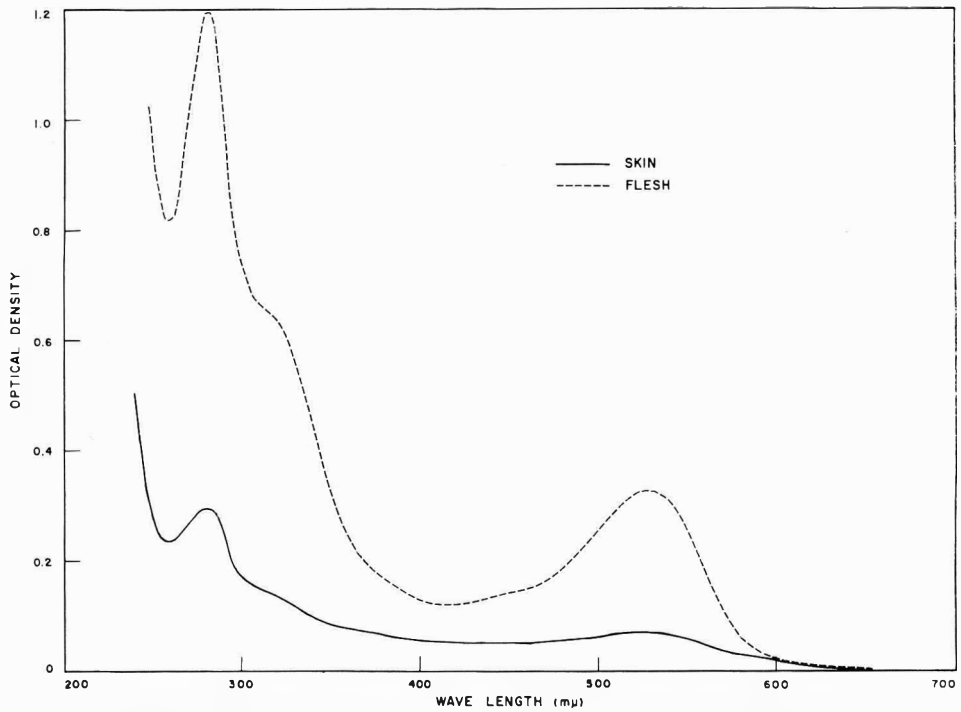


Fig. 5. Absorption spectra of anthocyanin pigments from scalded red tart cherries (bruised and held in 100% N_2) in MeOH (1% HCl).

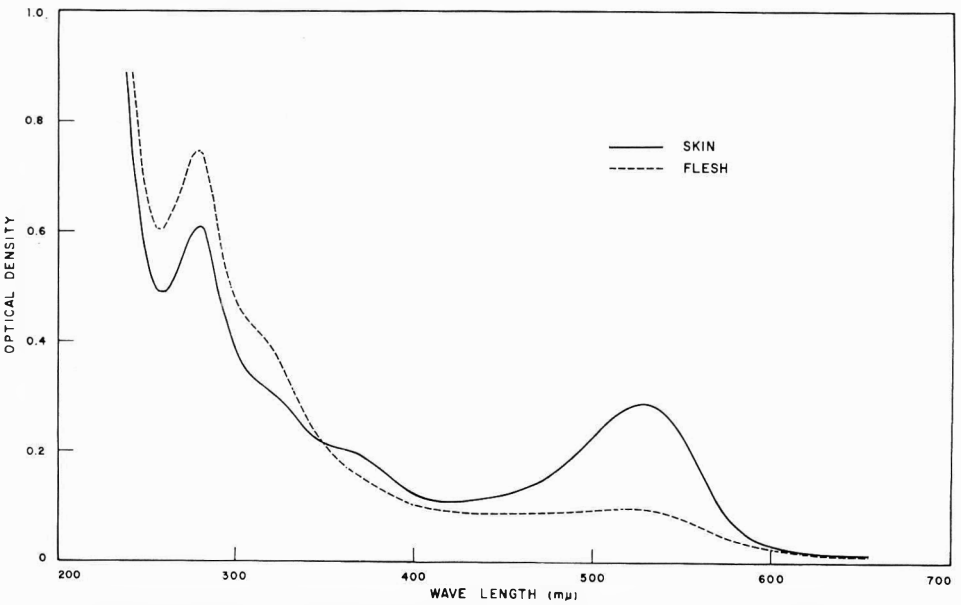


Fig. 6. Absorption spectra of anthocyanin pigments from unscalded red tart cherries (bruised and held in 100% O_2) in MeOH (1% HCl).

Anthocyanin contents, calculated as Idaein, were as follows [mg per sample of tissue (skin 1.76 g, flesh 22.27 g)]:

	Fresh	Bruised unscalded	Bruised scalded
Skin	7.44	6.24	1.19
Flesh	1.22	2.34	7.42
	(mg per 100 g of skin)		
	423.0	344.5	67.6

These results demonstrate that the pigments migrate but are not destroyed during scald development.

The chemical data and induction of scald under anaerobic conditions are contrary to findings of Wagenknecht *et al.* (1960). It is their belief that anthocyanase, which requires oxygen for activity, participates in the scald development in red tart cherries, through the destruction of anthocyanin pigments. In the present study the anthocyanin pigment content of the skin is not less in bruised unscalded cherries held in 100% oxygen than in bruised scalded cherries held in 100% nitrogen. The findings are different because the histological studies of this work showed no cellular breakdown, whereas Wagenknecht *et al.* (1960) worked *in vitro* and assumed disruption of cellular structure in bruised fruit.

Volatiles of scalded and unscalded cherries. Comparison of the chromatograms of the organic volatiles in the headspace of scalded and unscalded cherries showed no difference. When the samples were allowed to stand for over 24 hr, however, a large fraction appeared in addition to the components found in the headspace gas of unscalded cherries. Comparison of the retention time of this fraction, 97 min, with the time of known standards, when passed through the silicone 550 column, indicated that this additional fraction was ethyl alcohol, arising from anaerobic fermentation of scalded cherries.

Histological changes associated with scald. Histological studies were made of the location of the pigment in the fresh unbruised cherries and bruised unscalded and scalded cherries. All tissues were fixed in the manner previously described.

Microscopic examinations showed that the fresh cherry is surrounded by a continuous waxy cuticle. The epidermis and subepidermis, each 1 cell layer thick, appeared to be colored (Fig. 7). Whether the subepidermal cells of the fresh cherry are colored, or whether pigment diffused from the epidermal cells of the cut fruit before the lead acetate penetrated, could not be determined.

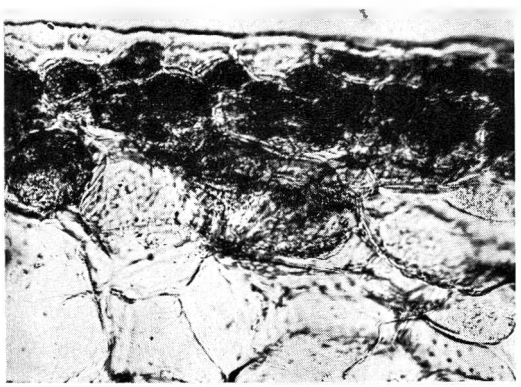


Fig. 7. Photomicrograph of a section of fresh unbruised red tart cherry showing concentration of pigment in epidermal and subepidermal layers. 150×.

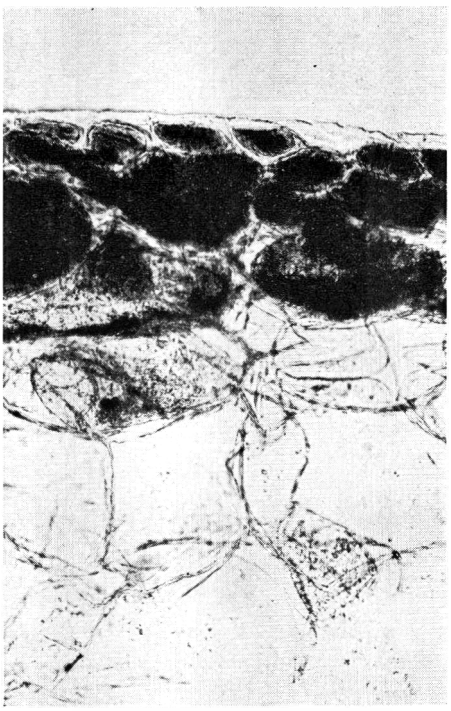


Fig. 8. Photomicrograph of a section of unscalded bruised red tart cherry held 18 hr in 100% O_2 atmosphere at 90°F. Pigment primarily in epidermal and subepidermal layers, with slight internal migration into adjacent cells. 150×.

In the bruised unscalded cherries held in 100% oxygen (Fig. 8), the anthocyanin pigments are restricted to the above type of cells, but some transformation appeared in the shape of the bruised cells. There are cases where aggregates of cells beneath the hypodermal cells which appear to be colored with pigment, probably diffused from broken epidermal or subepidermal cells.

In the bruised scalded cherries held in 100% nitrogen, the anthocyanins are no longer restricted to epidermis and subepidermis cells but are distributed throughout the tissues. Diffusion of the pigment throughout the fleshy tissue is closely associated with cell walls thickness and perhaps with the size of intercellular spaces (Fig. 9).

The following physiological hypothesis can be advanced for the development of cherry scald as induced in the present studies. Under conditions of stress such as bruising and 90°F temperature, oxygen consumption would be expected to rise. This increased demand for oxygen was not satisfied for the bruised fruits held under 100% nitrogen, and anaerobic respiration was induced. There are at least two probable reasons for the injurious effects of substitution of anaerobic for aerobic respiration. One of these is that anaerobic fermentation releases a much lower energy output. This curtailed rate of energy release might be expected to affect permeability since the structure of cytoplasmic membranes is maintained by the expenditure of metabolic energy (Kramer, 1955). Another probable cause is that the occurrence of anaerobic fermentation resulted in the accumulation of ethyl alcohol which affects permeability (Stiles *et al.*, 1917). Changes in the semipermeability of the cell membranes would result in the diffusion of pigments into the intercellular spaces, leaving behind scald areas.

Furthermore, bruised cherries held under 100% oxygen can satisfy their needs for oxygen, and as a result no shift would be expected in the aerobic phase of respiration. Thus, the cell walls and membranes maintain their semipermeability and prohibit diffusion of the pigment from cell to cell. The small amount of pigment which migrated into the flesh probably came from disrupted cells of the anthocyanin-containing skin, leaving behind no visible external spot.

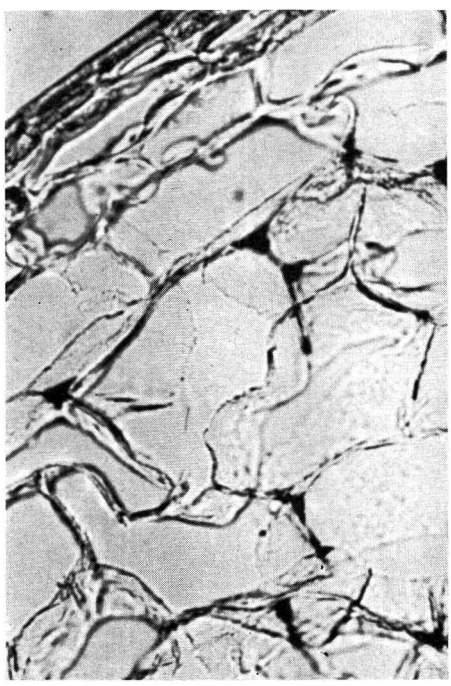


Fig. 9. Photomicrograph of a section of scalded bruised red tart cherry held 18 hr in 100% N_2 atmosphere at 90°F. Illustrates lack of pigment concentration as a result of diffusion throughout internal fleshy tissue. 150×.

REFERENCES

Griswold, R. M. 1944. Factors influencing the quality of home-canned Montmorency cherries. *Mich. Agr. Expt. Sta. Tech. Bull.* No. **194**.

Joslyn, M. A., and J. D. Ponting. 1951. Enzyme-catalyzed oxidative browning of fruit products. *Advances in Food Research* **3**, 1.

Kramer, P. J. 1955. Water relations of plant cells and tissues. *Ann. Rev. Plant Physiol.* **6**, 252–72.

LaBelle, R. L. 1956. Cherry scald—a harvesting and handling problem. *Farm Research* **22**(2), 15.

LaBelle, R. L., J. C. Moyer, W. B. Robinson, and D. B. Hand. 1958. Causes of scald in red tart cherries. *Food Technol.* **12**(2), 94.

Li, Kuang C., and A. C. Wagenknecht. 1956. The anthocyanin pigments of sour cherries. *J. Am. Chem. Soc.* **78**, 979.

Miller, M. W., R. B. Fridley, and A. A. McKillop. 1963. The effects of mechanical harvesting on the quality of prunes. *Food Technol.* **17**(11), 113.

Pollack, R. L., R. T. Whittenberger, and C. H. Hills. 1958. Studies on cherry scald. II. Relationship between bruising and respiration in air. *Food Technol.* **12**, 106.

Siegelman, H. W., and S. B. Hendricks. 1958. Photocontrol of anthocyanin synthesis in apple skin. *Plant Physiol.* **33**, 185.

Stiles, W., and I. Jorgensen. 1917. Studies in permeability. *Ann. Botany* **31**, 47–76.

Wagenknecht, A. C., D. M. Scheiner, and J. P. Van Buren. 1960. Anthocyanase activity and its possible relation to scald in sour cherries. *Food Technol.* **14**, 47.

Whittenberger, R. T., and C. H. Hills. 1956. Bruising causes cherry discoloration. *Canner and Freezer* **123**(4), 14.

Whittenberger, R. T., and R. E. Marshall. 1950. Measuring firmness of red tart cherries. *Food Technol.* **4**, 311.

Yeatman, J. N., G. S. Birth, Jane V. Ernest, R. W. Bender, and A. P. Sidwell. 1961. Spectrophotometric evaluation of anthocyanin pigment development and scald damage in intact red tart cherries. *Food Technol.* **15**(12), 521.

Ms. rec'd 6/21/65.

The author is grateful for use of the facilities of the Plant Virology Laboratory and the Pioneering Research Laboratory for Post-Harvest Physiology of ARS.

Mention of specific instruments, trade names, or manufacturers does not imply endorsement.

D. F. SPLITTSTOESSER and ISABEL GADJO
New York State Agricultural Experiment Station
Cornell University, Geneva, New York 14456

The Groups of Microorganisms Composing the "Total"-Count Population in Frozen Vegetables

SUMMARY

The types of microorganisms contaminating frozen peas, snap beans, and corn were studied quantitatively. An average of 40-75% of the isolates were species of *Leuconostoc* and *Streptococcus*. The incidence of some of the less numerous groups is also given. The data suggested that a characteristic microflora, dependent upon vegetable product, developed on the surfaces of processing equipment. Thus, similar distributions of microorganisms were found on vegetables processed at different factories, as well as on vegetables in different stages of processing. The characteristic microbial distributions were observed, however, only in samples collected after the processing seasons had been in effect for a number of days.

INTRODUCTION

The effect of certain commercial processing conditions on the "total" plate counts of microorganisms contaminating vegetables was described in previous publications (Splittstoesser *et al.*, 1961 a,b). This research, a continuation of the earlier work, was done to isolate and identify the groups of microorganisms composing the total count population in order to determine whether the incidence of certain types was related to specific conditions on the processing line.

Little quantitative information is available on the predominant microflora of commercially-processed frozen vegetables. Smart (1939) reported *Sarcina*, *Flavobacterium*, and *Bacillus* to be the genera isolated most frequently from a variety of vegetables. Hucker *et al.* (1952) found *Micrococcus*, *Flavobacterium*, *Achromobacter*, and *Streptococcus* to make up the greatest percentage of isolates from frozen beans, peas, and corn. A more recent study showed *Leuconostoc* and *Streptococcus* to constitute more than 90% of the organisms isolated from frozen peas (White and White, 1962). None of these reports related microbial types to conditions on the processing line.

EXPERIMENTAL METHODS

The vegetables were obtained from the lines of area factories. Details of the methods used in collecting, transporting, and culturing the samples have been described (Splittstoesser *et al.*, 1961a). In general, appropriate dilutions of blended vegetables were plated on tryptone-glucose-yeast extract agar with standard pour-plate techniques (American Public Health Association, 1958). The isolates were obtained by picking all discernible colonies from plates that had incubated two days at 32°C. When possible, plates containing between 50 and 100 colonies were chosen for this work.

Following purification, the cultures were initially examined for gram reaction, morphology, and catalase production. The catalase-negative cocci were then tested for growth at 10 and 45°C, for changes in litmus milk, and for the ability to grow in broth containing 6.5% (w/v) sodium chloride (Sherman, 1937). Carbon dioxide production in broth plugged with vaspar and dextran formation in sucrose media separated the leuconostoc from the streptococci. The catalase-positive rods were examined for flagella (Casares-Gil stain), breakdown of gelatin and casein, oxidation, and/or fermentation of carbohydrates (Hugh and Leifson, 1953). Details of the different tests are given in the publications of Skerman (1959) and the Committee on Bacteriological Technique (1957).

RESULTS AND DISCUSSION

Examination of a large number of isolates was deemed necessary in order to establish whether a certain distribution of microbial types was characteristic of a particular vegetable or processing condition. Over a period of four processing seasons, 4,652 cultures were isolated from 80 samples of peas, snap beans, and corn processed in five different factories.

When grouped according to morphology, gram-stain and catalase-reaction organisms resembling streptococci were found to be the predominant group in vegetables collected from processing stages following the blanch (Table 1). Corn contained the greatest proportion of these organisms (75%), while

Table 1. Percent distribution of the microbial groups responsible for the "total" counts obtained in frozen vegetables.

Vegetable	Microbial group [a] % distribution					No. of samples	No. of isolates	Median "total" count
	1	2	3	4	5			
								($\times 10^{-3}$)
Green peas								
Pre-blanch	14	37	17	26	6	6	382	4,200
Post-blanch [b]	21	21	13	42	3	19	1,169	110
Snap beans								
Pre-blanch	38	29	7	17	9	8	433	130
Post-blanch [b]	14	19	17	41	9	25	1,290	140
Whole-kernel corn								
Post-blanch [b]	8	5	6	75	6	22	1,378	480

[a] Microbial groups: 1) rods, catalase-positive, Gram-negative; 2) rods, catalase-positive, gram-positive; 3) cocci, catalase-positive, Gram-positive; 4) cocci, catalase-negative, gram-positive; 5) others, including yeasts, antinomyces, lactobacilli.

[b] Post-blanch refers to samples collected from the different processing stages following the blanch.

peas and snap beans yielded figures quite similar to each other (about 40%). In contrast to the catalase-negative cocci, catalase-negative rods resembling lactobacilli were rarely isolated. With respect to the less numerous catalase-positive organisms, it can be seen that the three groups were present in about equal numbers on a given vegetable.

Unblanched peas and beans contained a lower proportion of catalase-negative cocci than did blanched samples. The difference in microflora between blanched and unheated beans is understandable when one considers that this vegetable is subject to soil-borne contamination in the growing field. Less difference in microbial distribution was obtained in the two types of pea samples. The major source of contamination of unblanched peas was the viner, which, as illustrated by the high median counts (Table 1), often permitted the build-up of large microbial populations. It appears that this equipment was contaminated with a microflora similar to that found within the processing factory.

The vegetables were collected from many different areas along the processing lines. Except for samples obtained immediately after the blanch, where sporeformers predominated, the percent distribution of microbial groups was similar at all stages. In one survey, for example, the incidence of catalase-negative cocci in corn collected from an inspection belt, a conveyor, and a filling machine was respectively 84, 95, and 90%.

Similar microbial distributions were found for a given vegetable even though processed in different factories. As illustrated in Fig. 1, the distribution patterns for snap beans processed in three factories were relatively similar to each other, as were the distribution patterns obtained with corn processed in two factories. These findings were not expected, since each factory used somewhat different equipment and procedures for processing the vegetables. Factory A, for example, used belts exclusively to convey corn to the different unit operations, while the Factory B corn line also included several long flumes through which the vegetable was transported. The finding of a similar microflora in the different factories suggested that the different microbial types had reached some sort of equilibrium on the surfaces of processing equipment and that an important factor influencing this equilibrium or characteristic distribution was the composition of the available nutrients, the vegetable solubles.

A comparison of the types of contaminants on vegetables collected from a given factory on different processing days provided further evidence that a characteristic microflora did, in fact, develop on vegetable processing lines. It was found (Table 2) that the catalase-negative cocci did not predominate at the start of the pea and corn processing seasons. However, as the processing period progressed, the percentage of these organisms

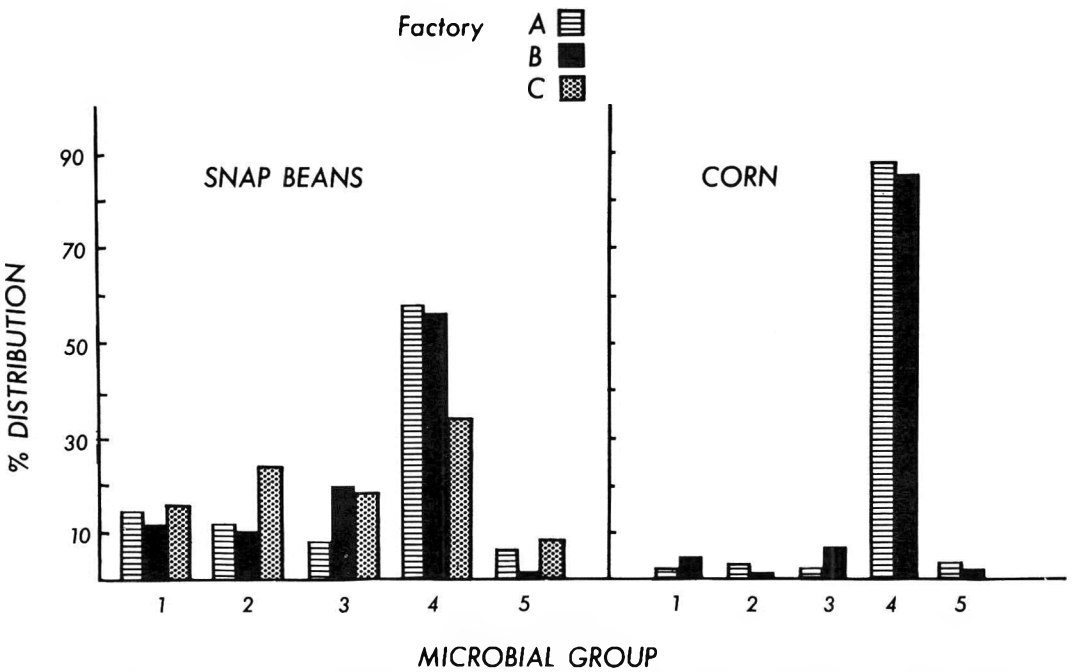


Fig. 1. Comparison of the distribution of microbial types in vegetables processed in different factories.

increased until, as shown with corn, an equilibrium was reached. This change in distribution of microbial types should not have occurred if conditions on the processing line had been the same throughout the season. A possible explanation is that a build-up of microorganisms took place in areas that were not being cleaned effectively, and it was at these sites that the microbial equilibrium was first established. With time, these organisms were disseminated throughout the processing line and became the predominant contaminants.

A number of cultures within the main groups of isolates were given additional tests in order to learn more about their identity. The cultures were selected randomly so that the relative frequency of the different types within a group could be estimated.

Further separation of the catalase-positive gram-negative rods, Group 1, was based on type of flagella, pigmentation, and whether acid was produced oxidatively or fermentatively from sugars (Table 3). The organisms possessing polar flagella, subgroup 1A, most closely resembled *Pseudomonas* species,

Table 2. Changes in the percent distribution of microbial types as the processing season progressed.

Vegetable	Processing day	Microbial group [a] % distribution					Av. "total" count $\times 10^{-3}$
		1	2	3	4	5	
Peas	1st	73	.....	17	4	6	140
	11th	52	28	11	9	.....	220
	16th	17	32	6	46	.....	240
	18th	18	3	14	60	4	120
Corn	1st	30	16	14	28	12	84
	8th	3	4	6	72	15	490
	19th	2	1	1	93	3	4,000
	23rd	4	2	4	88	2	990
	30th	4	1	6	87	2	1,300

[a] See footnote, Table 1.

Table 3. Types of catalase-positive rods isolated from frozen vegetables.

Group properties	% distribution
1) Gram-negative (71 isolates studied)	
A) Polar flagella; pigmentation variable (some water soluble); 15/22 [b] oxidative [a]; 7/22 negative; 15/22 proteolytic	31
B) Non-motile; unpigmented; 17/28 oxidative; 11/28 proteolytic	40
C) Non-motile; yellow colonies; 10/10 oxidative; 7/10 proteolytic	14
D) Peritrichous flagella; 11/11 fermentative; 8/11 produced copious gas; 2/11 proteolytic	15
	100
2) Gram-positive (101 isolates studied)	
A) Aerobic sporeformers	2
B) Unpigmented; 25/32 oxidative; 5/32 fermentative; 2/32 negative; 16/32 proteolytic	32
C) Yellow colonies; 43/67 oxidative; 24/67 fermentative; 30/67 proteolytic	66
	100

[a] Acid from glucose, xylose, or arabinose using the test of Hugh and Leifson (1953).
[b] Refers to fraction of cultures possessing the characteristics.

although within this group were three cultures whose yellow pigmentation was suggestive of *Xanthomonas*. The non-motile, non-pigmented isolates, subgroup 1B, resembled *Archromobacter* (Shewan *et al.*, 1960). The 11 cultures that failed to produce acid from sugars might also be placed in the genus *Alcaligenes*. The less numerous organisms within subgroups 1C and 1D appeared to be *Flavobacteria* and members of the family Enterobacteriaceae, respectively.

Except for the sporeforming cultures, most of the gram-positive rods isolated from frozen vegetables remain unclassified. As shown in Table 3, approximately two-thirds of the isolates formed yellow colonies on agar media. Both the pigmented and unpigmented subgroups were quite heterogeneous as to sugar metabolism and proteolysis of casein and gelatin. At present, a large number of characters are being recorded for these organisms in preparation for comparing them with known species of *Microbacteria*, *Arthrobacter*, and *Corynebacteria* by numerical taxonomic procedures. It is suspected that many of the unknown gram-positive organisms may fall within these groups.

Almost all of the catalase-positive cocci, Group 3, metabolized sugars oxidatively rather than fermentatively, and thus would be classified as *Micrococci* rather than *Staphylococci*. The incidence of the latter in frozen vegetables has been reported recently (Splittstoesser *et al.*, 1965). Undoubtedly, members of the genus *Sarcina* also made up a part of the catalase-positive cocci, since microscopic study showed that some of the cultures formed packets of cells.

Over 1,200 cultures of catalase-negative cocci, the predominant group, were given additional study. All were low-temperature organisms that grew at 10°C and lower. On the basis of the different tests, these organisms appeared to be heterofermentative leuconostoc, lactic streptococci, and enterococci (Table 4).

The lactic streptococci were the most numerous of the catalase-negative isolates if one includes in this subgroup all homofermentative cocci that grew at 10°C but not at 45°C. A majority of these organisms were atypical in that some produced little or no detectable change in litmus milk whereas others vigorously reduced but failed to coagulate this medium. It is possible that some of these isolates were *Pediococci*. They were all placed in the lactic group, however, because tetrad forms were not observed, and because strains of *Streptococcus lactis* that do not

Table 4. The types of catalase-negative cocci isolated from frozen vegetables.

Group properties	% distribution in [a]		
	Peas	Beans	Corn
Typical lactic streptococci—growth at 10°C but not at 45°C, reduce litmus milk prior to coagulation, no growth in 6.5% NaCl	10	11	16
Atypical lactic streptococci—as above but fail to coagulate milk	23	7	15
Atypical lactic streptococci—growth at 10°C but not at 45°C, only slight reduction and/or acid in litmus milk, CO_2 not detected	35	57	30
Enterococci—growth at 10°C and 45°C, litmus milk variable, growth in 6.5% NaCl	6	12	17
Leuconostoc—growth at 10°C but not 45°C, significant CO_2 from glucose, almost all produce dextran from sucrose	27	13	23

[a] Number of isolates studied: 415 from peas, 204 from beans, 656 from corn.

ferment lactose have long been known (Yawger and Sherman, 1937) and were recently isolated from frozen peas (White and White, 1962).

The numbers of enterococci isolated from the plates seem to agree fairly well with counts obtained with selective media (Splittstoesser and Wettergreen, 1964). No evidence was obtained indicating that enterococci in frozen vegetables have any special sanitary significance.

The finding that a characteristic microflora developed on vegetable processing lines was both of interest and reassuring. The fact that these acid-producing organisms were capable of growing at low temperatures strongly suggests that this group would continue to predominate in vegetables that had been exposed to a defrost temperature for an extended period.

It is not known whether the patterns of microflora on vegetables processed in other geographical locations would closely resemble those found in upstate New York. The fact that streptococci are not emphasized in some publications dealing with the microflora of frozen vegetables would seem to indicate different patterns (Lochhead and Jones, 1936; Smart, 1937, 1939). On the other hand, in some of those early studies the vegetables were processed in pilot plants, where a characteristic flora might not have had a chance to build up. The report from Great Britain that streptococci and leuconostoc organisms predominated in commercially frozen peas (White and White, 1962) lends support to the idea that vegetables processed in other areas may possess a microbial pattern for the predominant types similar to that found in this study.

REFERENCES

American Public Health Association. 1958. Recommended Methods for the Microbiological Examination of Foods. American Public Health Association, New York, p. 207.

Committee on Bacteriological Technique. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., New York, p. 315.

Hucker, G. J., R. F. Brooks, and A. J. Emery. 1952. The source of bacteria in processing and their significance in frozen vegetables. *Food Technol.* **6**, 147.

Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bacteriol.* **66**, 24.

Lochhead, A. G., and A. H. Jones. 1936. Studies of numbers and types of microorganisms in frozen vegetables and fruits. *Food Research* **1**, 29.

Sherman, J. M. 1937. The streptococci. *Bacteriol. Rev.* **1**, 3.

Shewan, J. M., G. Hobbs, and W. Hodgkiss. 1960. A determinative scheme for the identification of certain genera of Gram-negative bacteria, with special reference to the Pseudomonadaceae. *J. Appl. Bacteriol.* **23**, 379.

Skerman, V. B. D. 1959. A Guide to the Identification of the Genera of Bacteria. Williams and Wilkins Co., Baltimore, p. 217.

Smart, H. F. 1937. Types and survival of some microorganisms in frozen-pack peas, beans, and

sweet corn growth in the east. *Food Research* **2**, 515.

Smart, H. F. 1939. Microbiological studies on commercial packs of frozen fruits and vegetables. *Food Research* **4**, 293.

Splittstoesser, D. F., and W. P. Wettergreen. 1964. The significance of coliforms in frozen vegetables. *Food Technol.* **18**, 134.

Splittstoesser, D. F., W. P. Wettergreen, and C. S. Pederson. 1961a. Control of microorganisms during preparation of vegetables for freezing. I. Green beans. *Food Technol.* **15**, 329.

Splittstoesser, D. F., W. P. Wettergreen, and C. S. Pederson. 1961b. Control of microorganisms during preparation of vegetables for freezing. II. Peas and corn. *Food Technol.* **15**, 332.

Splittstoesser, D. F., G. E. R. Hervey, II, and W. P. Wettergreen. 1965. Contamination of frozen vegetables by coagulase-positive staphylococci. *J. Milk and Food Technol.* **28**, 149.

White, A., and H. R. White. 1962. Some aspects of the microbiology of frozen peas. *J. Appl. Bacteriol.* **25**, 62.

Yawger, E. S., Jr., and J. M. Sherman. 1937. Variants of Streptococcus lactis which do not ferment lactose. *J. Dairy Sci.* **20**, 83.

Ms. rec'd 9/17/65.

This investigation was supported in part by Public Health Service Grant EF-255 from the Division of Environmental Engineering and Food Protection.

Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1453.

J. H. SILLIKER, ALEX SCHMALL, and JOSEPH Y. CHIU
Department of Pathology, St. James Hospital, Chicago Heights, Illinois 60412

The Fluorescent Antibody Technique as a Means of Detecting Salmonellae in Foods

SUMMARY

The indirect immunofluorescence (FA) technique was used. Absolute correlation between culture and FA results was observed in the analysis of dried foods. The FA procedure was significantly more sensitive than cultural methods for quantitative and qualitative analysis of *Salmonella* in liquid eggs.

INTRODUCTION

The technique of immunofluorescence (FA) as developed by Coons *et al.* (1941) has proven a powerful tool in diagnostic microbiology. The technique has been particularly useful in identification of Group A streptococci in nasopharyngeal exudates, serogrouping of enteropathogenic *Escherichia coli* in fecal smears, and identification of gonococcus in exudates from the reproductive tract. The indirect FA test is used widely to detect the presence of syphilitic antibody in human sera (Cherry and Moody, 1965).

Application of the FA technique to other diagnostic problems has been less successful. One of the greatest problems has been the scarcity of reliable diagnostic sera. This difficulty is illustrated by the reported lack of specificity of the FA technique for rapid detection of *Salmonella* in feces (Thomason *et al.*, 1959). Using fluorescein-labeled conjugates derived from polyvalent *Salmonella* O sera, those workers observed that cross-reactions with enteric organisms other than salmonellae were common.

Recent work at this laboratory (Silliker *et al.*, 1964, 1965) has indicated that commercially available *Salmonella* O antisera lack specificity, whereas H antisera give reliable results. Recent work of Haglund *et al.* (1964) supports these conclusions. Employing the FA technique, they found extensive cross-reactions between *Salmonella* O antisera and a variety of bacterial isolates from egg products. After absorption of the O antisera with a mixture of *E. coli* strains, the cross-reactions with salmonellae were eliminated. However, it should be noted the absorbed O sera were not tested with paracolons, and these organisms are known to share a broad array of O antigens with *Salmonella* (Edwards and Ewing, 1962). Haglund *et al.* (1964) reported no cross-reactions with a polyvalent H antiserum. Using the indirect FA staining technique (Weller and Coons, 1954), *Salmonella* was detected in eggs or egg products within 24 hr with either H or absorbed O antisera. The technique combined selective culture methods with the FA procedure. Georgala and Boothroyd (1964) utilized the indirect FA technique for detecting *Salmonella* in carcass and boneless meats. They concluded that the procedure lacked precision but could be used effectively as a rapid "presumptive" *Salmonella* test. Their technique involved the use of a polyvalent O *Salmonella* antiserum.

This paper reports qualitative and quantitative results obtained with the indirect FA technique using a polyvalent H antiserum for the detection of salmonellae in eggs and a variety of other food products.

MATERIALS AND METHODS

Culture procedures. *Food samples.* Twenty-five g of food was pre-enriched in 250 ml of lactose broth. After 24 hr of incubation at 37°C, 10-ml portions of the lactose cultures were inoculated into 100 ml of tetrathionate and cystine selenite broths. After 24 hr of incubation, the latter cultures were streaked onto brilliant green (BG) and *Salmonella-Shigella* (SS) agars. Biochemical and serological identification of *Salmonella* colonies developing on these media was according to procedures described by Silliker *et al.* (1965).

Quantitative studies were carried out on liquid egg samples. Since we have found that pre-enrichment of liquid samples in lactose broth results in extensive coliform overgrowth, direct inoculation of selenite was used. Subcultures were made on BG only. As indicated, quantitative estimates of the *Salmonella* population were made by the most probable number technique. This involved inoculation of 3 tubes of selenite broth at each dilution tested. The presence or absence of

salmonellae in the individual tubes was accomplished as indicated below (fluorescent antibody techniques). On certain samples, the population was estimated by serial dilutions, with the highest dilution giving a positive result being the end-point.

Feces specimens. Approximately 1 g of feces was emulsified in 10 ml of cystine selenite broth. After 24 hr of incubation at 37°C, the cultures were streaked onto BG, SS, and desoxycholate agars. Identification of salmonellae was as indicated above.

The selenite broth cultures of feces were transferred into BHI broth after 24 hr of incubation. The BHI broth was incubated for 6–8 hr.

Fluorescent antibody techniques. Tetrathionate and selenite broth cultures from foods were transferred to brain-heart infusion broth (BHI) after 8 hr of incubation at 37°C. One-tenth ml of the enrichment culture was inoculated in 3 ml of BHI, and these cultures were incubated for 16 hr at 37°C. It was found that if the selenite and tetrathionate broth cultures were subcultured before 8 hr, false negative results tended to occur. However, the incubation time of the BHI cultures did not appear to be critical, satisfactory results being obtained in 6–8 hr. The 16-hr period was used for convenience, since this corresponded to overnight incubation.

After incubation, an equal volume of 0.6% formalinized saline was added to the BHI broth cultures. One loopful of the formalinized culture was then evenly distributed over the etched circle of a Trident Fluoro-slide (Aloe Scientific Co., St. Louis, Mo.). The slide was then allowed to air-dry at room temperature and was then fixed by immersion in acetone for 5 min. Early in the investigation the ethanol-chloroform-formalin fixative recommended by Haglund *et al.* (1964) was tried. With this procedure there was a tendency for the antigen to float off the slide, and, further, we observed brighter fluorescence if the formalin was added to the broth culture before the antigen was placed on the slide for fixation.

After fixation, a drop of polyvalent H antiserum was placed on the smear and the slide incubated in a moist chamber for 30 min at 37°C. The polyvalent antiserum was prepared by mixing the 7 individual sera in the Spicer-Edwards "kit" (Difco) and diluting them so that the final titer of the serum was 1:500 with respect to each of the 7 component sera. Although the supplier recommends that the Spicer-Edwards sera be used at a titer of 1:1000 for tube agglutination tests, when the indirect antibody technique was used it was necessary to use a titer of 1:500. We have tested multiple lots of antisera at this titer and observed no variation.

After incubation, the excess antiserum was removed from the slide by gentle tapping on a piece of blotting paper. The slides were then washed for 5 min in 2 successive baths containing phosphate-buffered saline, pH 7.2 (Haglund *et al.*, 1964). During these washing operations, the buffered saline was continuously agitated with a mechanical stirrer. The improved circulation that resulted greatly decreased the tendency toward non-specific fluorescence. Following the second rinse in buffered saline, the slides were dipped in distilled water in order to wash off excess saline. The slides were then air-dried.

Since the Spicer-Edwards antisera are derived from immunized rabbits, the indirect fluorescent staining technique demands the application of a fluorescein-tagged anti-rabbit globulin. For this purpose we used goat anti-rabbit (GAR) fluorescein-labeled serum. Although we found that both Difco and BBL sera gave satisfactory results, the BBL product appeared to be more stable after reconstitution. One drop of the GAR serum was added to the smear which had previously been treated with untagged polyvalent H antiserum. It is important that the drop be spread evenly over the entire etched circle containing the antigen. The treated smear was held for 15 min at 37°C in a moist chamber. Following the staining with GAR the excess fluorescent antiserum was removed from the slide by gentle tapping, and the smear was then washed as previously described through 2 baths of buffered saline. After the slide was washed, it was air-dried. The slides were then cover-slipped using FA mounting fluid (Difco), with care being taken to avoid air bubbles.

It is essential that each bottle of GAR antiserum be carefully titered before use. We observed that, at low dilution, this serum stained a variety of enteric organisms non-specifically, including *Proteus,* coliforms, and, especially, pseudomonads. In determining the dilution of the fluorescent antiserum to be used, not only is it important to test the serum against known *Salmonella* for positive fluorescence after staining with untagged polyvalent H antiserum, but it is equally important to check the serum against non-salmonellae, particularly pseudomonads. In our experience, results have been satisfactory at a dilution of 1:35.

The treated smears were examined with a Leitz Ortholux Fluorescence microscope (Universal Light Source, model 250). The microscope contained a high-pressure mercury lamp, OSRAM HBO 200. The light was used with the UG 12 blue light exciter filter, together with an orange barrier filter ("BLAU-ABS"). The fluorescent cells, under these conditions, are greenish-yellow. The background tends to be bluish-black. With the procedures described above, we observed very little fluorescence in the amorphous material, and this,

we feel, is attributable to mechanical agitation of the phosphate buffer used in washing the slides, together with careful titering of the GAR antiserum.

The presence of fluorescent rod-shaped bacteria was designated as a positive result. We have not encountered difficulty with non-specific fluorescence in other morphological forms, viz. yeasts and cocci. In most cases, a positive sample will show large numbers of fluorescent organisms in every field examined. Occasionally, however, a sample may show only small numbers of fluorescent rod-shaped bacteria. However, correlation of culture and FA results has led us to conclude that the presence of any rod-shaped bacteria showing fluorescence must be called a positive result. Without exception, the organisms reacting with the GAR show bright fluorescence and typical morphology. Thus, virtually no subjective interpretation of the slide has been necessary, since the cells are either clearly fluorescent or are negative.

Source of samples. The food samples examined consisted of a variety of samples submitted to this laboratory for routine *Salmonella* analysis in connection with the *Salmonella* product-control programs of the companies submitting the samples. However, in connection with the quantitative evaluation of the technique, a large egg processor cooperated by supplying a variety of unpasteurized products so that samples with varying levels of *Salmonella* contamination could be studied.

The feces specimens were those submitted to the clinical laboratories of this hospital for routine culture.

RESULTS

Four hundred and twenty different dried food samples were analyzed, comparison being made between cultural and FA detection of *Salmonella*. The results are summarized in Table 1. Salmonellae were isolated from 26 of these samples, and in each case a positive FA result was obtained. Three hundred and ninety-four samples were negative by both culture and immunofluorescence techniques. Thus, on these dried food samples the agreement between the two procedures was absolute.

Table 1. Dried food samples analyzed for *Salmonella* by immunofluorescence (FA) and culture techniques.

Product	No. samples analyzed	No. +[a]	No. −[b]
Whole egg	20	2	18
Yolk	180	9	171
Albumen	187	12	175
Meat scrap	19	3	16
Miscellaneous	14	0	14
Total	420	26	394

[a] All positive by culture and FA.
[b] All negative by culture and FA.

Table 2. Comparison of culture and FA techniques for the analysis of liquid egg samples.

Total samples, 45
No. culture positive, FA positive, 14
No. culture positive, FA negative, 2
No. culture negative, FA positive, 13
No. culture negative, FA negative, 16

Forty-five liquid egg samples were analyzed qualitatively. The data (Table 2) indicate a marked lack of correlation between FA and culture results in analysis of this type of sample. Salmonellae were detected by one or the other procedure in 29 of the 45 samples. However, only 14 samples were positive by both culture and FA procedures. Two samples were positive by culture but negative by FA, and 13 were positive by FA and negative by culture. Quantitative studies on the two samples that were positive only by culture gave MPN values of 0.03 per g.

Quantitative *Salmonella* determinations were conducted on 43 liquid egg samples. Included among these were samples of yolk and whole egg, as well as raw and fermented albumen. The results are summarized in Table 3. Fourteen of the samples were negative by both culture and FA techniques (<0.03 salmonellae per g). Sixteen samples were positive by both procedures. On 12 of the 16 samples the FA result was higher than the culture MPN; on 2, the culture result was higher; while on the remaining 2, the culture and MPN results were identical. Of greatest significance is the fact that with most samples the value obtained with the FA procedure was 10–100 times that determined by the culture method. On the other hand, in the 2 instances where the culture MPN was higher than the FA, the differences were within the range of experimental error (see samples 8 and 11). Eleven of the samples were negative by the MPN technique but positive by FA. Because of the dilutions selected, the sensitivity of the procedure varied from <0.03 to 30.0 salmonellae per g. It will be noted (samples 1A and 6A) that egg products with as high as 2,300 salmonellae per g on the basis of the FA procedure were negative by culture. As previously indicated, the 2 samples that were positive by culture and negative by FA contained only 0.03 salmonellae per g.

A limited number of feces specimens have been analyzed by the indirect FA technique. To date, the correlation between the FA and culture results has been excellent. However, this work is still in a preliminary stage and will be reported

Table 3. Quantitative *Salmonella* determinations on liquid egg products.

	Culture MPN/g	FA MPN/g
Samples positive by culture and FA		
1	2,800	430,000
2	2,100	> 23,000
3	0.20	11.0
4	3.6	110
5	36.0	15,000
6	15.0	930
7	910	2,300
8	440	430
9	0.91	2.3
10	430	2,300
11	93.0	75.0
12	0.91	0.91
13	36.0	440
14[a]	100	100
15[a]	100	1,000
16[a]	positive in 10 g	100
Samples negative by culture and positive by FA		
1A	<3.0	> 2,300
2A	<0.03	0.93
3A	<0.03	0.091
4A	<30.0	73.0
5A	<0.03	> 24.0
6A	<3.0	> 2,300
7A	<30.0	72.0
8A	<0.03	1.50
9A	<0.03	0.06
10A	<0.03	0.091
11A	<0.03	0.23
Samples positive by culture, negative by FA		
1C	0.03	> 0.03
2C	0.03	> 0.03
Samples negative by both culture and FA: 14		

[a] Determination by serial dilution, result denoting highest dilution where positive result obtained.

in a separate publication when the data are more definitive.

DISCUSSION

The results indicate an absolute correlation between FA and culture techniques in the analysis of dried food samples and a lack of correlation between the two procedures in the examination of liquid egg products. The FA procedure has proven far more sensitive, both qualitatively and quantitatively, for the detection of salmonellae in liquid eggs.

It might he argued that the positive FA results on liquid egg samples which were negative by culture might reflect nonspecific fluorescence. However, this seems unlikely, since not a single false positive FA result was obtained in the analysis of over 400 dried food samples, 387 of which were egg products. The same general microflora is encountered in both the liquid and dried products, and thus false positive fluorescence is equally likely in both types of sample.

The comparison between FA and culture techniques using the MPN procedure gave results suggesting strongly that the negative culture results on liquid samples simply reflect a failure to detect salmonellae present in the enrichment broths, using plating procedures. Liquid egg samples generally contain large numbers of coliform bacteria, and these organisms compete with the salmonellae in enrichment media. When, subsequently, the detection of *Salmonella* is attempted by streaking onto solid differential agars, unfavorable coliform/*Salmonella* relationships in the inoculum may result in failure to detect non-lactose-fermenting colonies, i.e. possible salmonellae. With the FA techniques, coliforms and other non-salmonellae are not seen under fluorescent light, and the salmonellae are readily seen even if the coliform-*Salmonella* ratio is greatly in favor of the former.

Our results with dried egg products confirm results reported by Haglund *et al.* (1964), except that we have encountered no difficulty with "nonspecific" fluorescence by yolk particles. We feel that the agitation of phosphate buffer baths effects much better washing of the slides after they have been treated with antiserum. The lack of precision of the FA technique for detection of salmonellae in meats (Georgala and Boothroyd, 1964) can probably be attributed to the use of polyvalent O *Salmonella* antiserum. The use of the more specific H antiserum would certainly be worthy of investigation for this purpose. The limited studies which we have carried out on the detection of *Salmonella* in feces have given no indication of the false positive reactions described by Thomason *et al.* (1959). The cross-reactions with non-salmonellae appear to be avoided if the proper dilutions of antiserum are selected. This application is being intensively investigated in our laboratory.

Aside from the greater sensitivity of the

FA technique under the conditions described, the procedure offers the obvious advantage of speed, as compared with conventional cultural procedures. While it is still necessary to enrich the food sample, the detection of salmonellae in the enrichment cultures can be accomplished within 6–8 hr after these cultures are inoculated into BHI broth. This avoids the necessity of streaking onto differential agars and the subsequent procedures involved in the identification of non-lactose-fermenting colonies developing on these media. The disadvantage of the method is that the culture itself is not available for complete serological and biochemical testing, unless conventional isolation procedures are used. However, for the detection of salmonellae in connection with food quality-control programs, the much faster FA procedure has considerable merit.

The methodology suggested by Haglund *et al.* (1964) involved direct enrichment of dried egg samples in selective media. With this approach it was possible to detect salmonellae in certain samples within 24 hr. However, numerous workers have shown the necessity for pre-enrichment of dried egg samples in a non-selective medium prior to inoculation of selenite or tetrathionate broths, if maximum recovery is to be achieved (Sugiyama, *et al.*, 1960; North, 1961; Taylor and Silliker, 1961; Taylor, 1961; Montford and Thatcher, 1961). Before this pre-enrichment step can be eliminated in connection with the use of the FA technique, critical studies are needed comparing recovery with and without pre-enrichment. The development of a sufficiently selective non-inhibitory enrichment medium should ultimately make possible the detection of salmonellae within 24 hr if the culture procedure is combined with the fluorescent antibody methodology.

REFERENCES

Cherry, W. B., and M. D. Moody. 1965. Fluorescent-antibody techniques in diagnostic bacteriology. *Bacteriol. Rev.* **29**, 222.

Coons, A. H., H. J. Creech, and R. N. Jones. 1941. Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exptl. Med.* **47**, 200.

Edwards, P. R., and W. H. Ewing. 1962. Identification of Enterobacteriaceae. Minneapolis; Burgess Pub. Co.

Georgala, D. L., and M. Boothroyd. 1964. A rapid immunofluorescence technique for detecting salmonellae in raw meat. *J. Hyg.* **62**, 319.

Haglund, J. R., J. C. Ayres, A. M. Paton, A. A. Kraft, and L. Y. Quinn. 1964. Rapid detection of Salmonella in dried eggs and egg products by the FA technique. *Appl. Microbiol.* **12**, 447.

Montford, J., and F. S. Thatcher. 1961. Comparison of four methods for isolating salmonellae from foods, an elaboration of a preferred procedure. *J. Food Sci.* **26**, 510.

North, W. R. 1961. Lactose pre-enrichment method for isolation of Salmonella from dried egg albumen. Its use in a survey of commercially produced albumen. *Appl. Microbiol.* **9**, 188.

Silliker, J. H., R. H. Deibel, and J. Y. Chiu. 1964. Occurrence of Gram-positive organisms possessing characteristics similar to Salmonella and the practical problem of rapid and definitive Salmonella identification. *Appl. Microbiol.* **12**, 395.

Silliker, J. H., P. T. Fagan, J. Y. Chiu, and A. Williams. 1965. Polyvalent H agglutination as a rapid means of screening non-lactose-fermenting colonies for Salmonella organisms. *Am. J. Clin. Pathol.* **43**, 548.

Sugiyama, H., G. M. Dack, and G. Lippitz. 1960. Agglutinating antiserum for the isolation of Salmonella with special reference to isolation of egg albumen. *Appl. Microbiol.* **8**, 205.

Taylor, W. I. 1961. Isolation of salmonellae from food samples. V. Determination of the method of choice for enumeration of Salmonella. *Appl. Microbiol.* **9**, 487.

Taylor, W. I., and J. H. Silliker. 1961. Isolation of salmonellae from food samples. IV. The comparison of methods of enrichment. *Appl. Microbiol.* **9**, 484.

Thomason, B. M., W. B. Cherry, and P. R. Edwards. 1959. Staining bacterial smears with fluorescent antibody. VI. Identification of salmonellae in fecal samples. *J. Bacteriol.* **77**, 478.

Weller, T. H., and A. H. Coons. 1954. Fluorescent antibody studies with agents of Varicella and Herpes zoster propagated in vitro. *Proc. Soc. Exptl. Biol. Med.* **86**, 789.

Ms. rec'd 9/27/65.

This investigation was supported by Public Health Service Research Grant AI-06149-01 of the National Institutes of Health.

IGNACIO S. PABLO, GERALD J. SILVERMAN and SAMUEL A. GOLDBLITH
Department of Nutrition and Food Science, Massachusetts Institute of Technology Cambridge, Massachusetts 02139

Microbial Growth Patterns of Rehydrated Freeze-Dried Foods. II. Chicken

SUMMARY

Microbial growth patterns in rehydrated freeze-dried chicken at 4, 20, 30, and 37°C are similar to those of rehydrated freeze-dried shrimp, and are greatly influenced by storage temperature, time, and initial number of microorganisms present. Lag time was shortest at 37 and 30°C, four times as long at 20°C as at 37°C, and 120 times as long at 4°C as at 37°C. At 37°C, the maximum population was 300,000 times the initial population, while at 30°C it was 115,000 times after 26 hr. The microbial population increased 100,000-fold at 55 hr at 20°C, and 500-fold at 15 days at 4°C.

A natural contaminant in the freeze-dried chicken examined, coagulase-positive *Staphylococcus aureus*, was found to be at a level of 30–40 organisms per gram, out of a total population of 10^9 organisms per gram. Fecal enterococci were also present in the freeze-dried material at a level of about 10^2 to 10^3 per gram.

Rehydration studies show that *Staphylococcus aureus* and "fecal enterococci" as natural contaminants in freeze-dried chicken can grow in competition with the natural flora at 20°C or above and that the competition may be due to the nature of the microbial distribution on the chicken surface. Refrigeration temperatures in the vicinity of 4°C completely suppressed the growth of *S. aureus* and greatly extended the shelf life of rehydrated chicken.

INTRODUCTION

Pablo *et al.* (1966) found that the microbial growth patterns of rehydrated freeze-dried shrimp are greatly influenced by temperature, time of storage, and initial number of microorganisms present. The present paper is a companion study to compare the microbial growth patterns in rehydrated freeze-dried chicken with those in shrimp. *Staphylococcus aureus*, fecal enterococci, and coliform were not present as natural contaminants of rehydrated shrimp in our previous study. This paper deals with the growth patterns of "fecal enterococci" and *Staphylococcus aureus*, as part of the natural flora found in rehydrated chicken stored at different temperatures. This may be of significance for rehydrated products in order to predict specific situations which may be of potential public-health hazard for rehydrated products.

A number of previous publications have dealt with competition between *S. aureus* and the natural microflora of food products at different temperatures. Peterson *et al.* (1962a,b) noted that *S. aureus* was not able to multiply in the presence of a saprophytic flora below 10°C. Slabyj *et al.* (1965) found that *S. aureus* was unable to multiply, and in fact decreased, in foods kept at 1 and 8°C, and was inhibited at higher temperatures (12 and 22°C) in nonsterile crab meat. They reviewed the importance of ratios of *S. aureus* to the general microflora and also the possibility of the production of inhibitory substances by the microflora or the removal of essential nutrients as the causative agents in the inhibition of *S. aureus* at moderate growth temperatures (12–20°C). In all of these studies the cultures of *S. aureus* being considered have been pure inocula and not natural contaminants of food material. Since competition depends not only on the temperature and substrate but also on the actual physical distribution of the contaminants, it was felt of interest to examine these aspects of the problem by the use of natural contaminants and enumeration by selective media.

MATERIALS AND METHODS

Cooked, diced, and freeze-dried chicken meat was obtained from a commercial source and stored at 20°C until analyzed. The chicken meat was in the form of ⅜-inch dice and packaged in 8-oz cans under nitrogen.

A 420-g portion of freeze-dried chicken meat was rehydrated for 15 min with 1000 ml of either sterile distilled water or trypticase diluent. The excess rehydration liquid was drained, and the chicken meat was placed on a wire screen inside a wide-mouth evaporation dish. Rehydration and storage were performed in incubators at 4, 20, 30, and 37°C. The rehydration liquid was tem-

pered at the corresponding storage temperature prior to use. Unless otherwise indicated, distilled water was normally used for rehydration.

For each sampling period, two 40-g portions of rehydrated chicken meat were blended for 3 min in a semi-micro jacketed monel container with cap and blending assembly. Added to the 40 g of rehydrated chicken meat prior to grinding was 114 ml of 0.1% trypticase diluent. This resulted in a 1:10 dilution (dry basis). The trypticase diluent consisted of 0.0003M of KH_2PO_4 containing 0.1% trypticase (BBL, pH 7.2). Crushed ice was placed in the jacket of the monel containers, and the diluent was always cooled to 5°C before use and each serial dilution was plated in triplicate. The pH of the chicken homogenate was determined with a Model H-2 Beckman pH meter.

Total aerobic pour plate counts were made in either trypticase soy agar (BBL) supplemented with 0.5% yeast extract (Difco) (TSY) or veal infusion agar (BBL). Serial dilutions were made in trypticase diluent (Sinskey *et al.*, 1964). Plates incubated at 37 and 30°C were counted after 3 days, plates incubated at 20°C after 5 days, and plates incubated at 4°C after 7 days.

"Fecal enterococci" were enumerated with the KF medium (Difco) of Kenner *et al.* (1961), which had been found (Saleh *et al.*, 1966) to be equivalent to that of the Tween-carbonate medium of Burkwall and Hartman (1964) for chicken. Typical colonies were repurified and grown in tryptone glucose extract (TGE) agar slants and tested for catalase (3% H_2O_2, Merck) and by the benzidine test for iron porphyrin compounds (Diebel and Evans, 1960).

Coagulase-positive staphylococci were detected on two selective and differential media, that of Baird-Parker (1962), ETGPA, and Crisley *et al.* (1964), TPEY, by surface plating. The plates were incubated at 37°C and examined at 24 hr and again at 48 hr. Clear zones appeared after 24 hr and the opaque reaction after 48 hr in ETGPA media.

Representative colonies showing positive egg-yolk reactions were isolated from both media and repurified on Staph 110 medium (Difco), with egg-yolk added (Carter, 1960). Isolates were then transferred into brain-heart infusion broth (Difco). Free coagulase was detected by the agglutination tube test with coagulase plasma (Difco).

For coliform organisms, aliquots were plated on violet red bile agar (Difco) and incubated 48 hr at 37°C.

For total nitrogen, 100 g of freeze-dried chicken was rehydrated with 500 ml of distilled water. After 15 min the excess of the rehydration medium was drained and the rehydrated chicken was placed on a 150-mm glass funnel with wire screen and the drip collected in a 500-ml flask. The total nitrogen content of the excess rehydrating media and drip collected during storage was determined by direct Nesslerization of Kjeldahl digest (Minari and Zilversmit, 1963).

RESULTS

Two selective and differential media were evaluated for the recovery of *Staphylococcus aureus* from rehydrated chicken stored at 20 and 37°C (Table 1). These were the tellurite-polymyxin-egg yolk

Table 1. Total aerobic plate counts and the recovery of *Staphylococcus aureus* rehydrated and stored at two temperatures.

Storage time (hr)	*S. aureus*/egg-yolk-positive strains		*S. aureus*/g dry material		Total plate counts (org/g dry material)		pH of homogenate
	ETGPA	TPEY	ETGPA	TPEY	TSY	Veal	
			20°C				
0	1/5	1/6	6	7	12×10^4	70×10^3	6.4
8	3/5	4/6	50	40	35×10^4	70×10^4	6.4
14	6/6	6/6	10×10^2	11×10^2	65×10^5	65×10^5	6.4
32	6/6	6/6	19×10^4	28×10^4	30×10^8	30×10^8	6.4
55	8/8	8/8	29×10^4	32×10^4	25×10^9	25×10^9	7.0
			37°C				
0	1/8	1/8	10	5	10×10^4	65×10^3	6.3
4	8/8	8/8	20×10^2	30×10^2	11×10^8	10×10^8	6.3
8	8/8	8/8	40×10^4	45×10^4	10×10^8	13×10^8	6.5
11	8/8	7/8	10×10^5	18×10^5	35×10^8	75×10^8	6.7
25	8/8	8/8	25×10^9	15×10^9	25×10^9	30×10^9	8.1

ETGPA: Egg yolk tellurite glycine pyruvate medium (Baird-Parker).
TPEY: Tellurite-polymixin egg yolk medium (Crisley).
TSY: Trypticase soy agar + 0.5% yeast extract (BBL).
Veal: veal infusion agar (BBL).

medium (TPEY) of Crisley *et al.* (1964) and egg yolk-tellurite-glycine-pyruvate medium (ETGPA, Baird-Parker, 1962). Neither of the two test media was highly selective for presumptively isolating coagulase-positive staphylococci initially from freeze-dried chicken. The presumptive test was based on the egg yolk reaction, and only about 20% of the egg-yolk-positive strains were coagulase-positive. Both media gave comparable recovery of coagulase-positive strains. The TPEY was found to be generally more inhibitory to saprophytic growth, and much less crowded than ETGPA.

The egg-yolk reaction from the ETGPA medium is shown in Fig. 1. Noted in this study were two distinct types of reactions on ETGPA media, all of which yielded, upon examination, coagulase-positive staphylococci: the typical opaque halo reaction noted by Silverman *et al.* (1961) and Carter (1960), which also gives a metallic sheen in the presence of tellurite in reflected light after 48 hr at 37°C; and an opaque zone which is surrounded with clearing after 48 hr of incubation. Colonies giving only a clear zone reaction after 48 hr of incubation were found to be coagulase-negative. The development of opacity and clearing in egg yolk was used by Crisley *et al.* (1965) as the basis for presumptive identification of coagulase-positive staphylococci. In this study, only the opaque halo reaction without clearing was found to be present on TPEY. A great majority of egg-yolk-reacting colonies from the ETGPA and TPEY were found to be coagulase-positive micrococci.

Table 1 also compares the efficiency of trypticase soy-yeast extract agar (TSY) and veal infusion agar for total aerobic plate counts. It had been found in shrimp (Sinskey *et al.*, 1964) that TSY and veal infusion agar had given higher total plate counts than Eugon, plate count, or tryptone glucose extract agar. In chicken stored at 37°C, veal infusion gave a slightly higher recovery than TSY. At 20°C, TSY gave slightly higher initial recovery than veal infusion, but at subsequent intervals of storage the recovery of both media was the same.

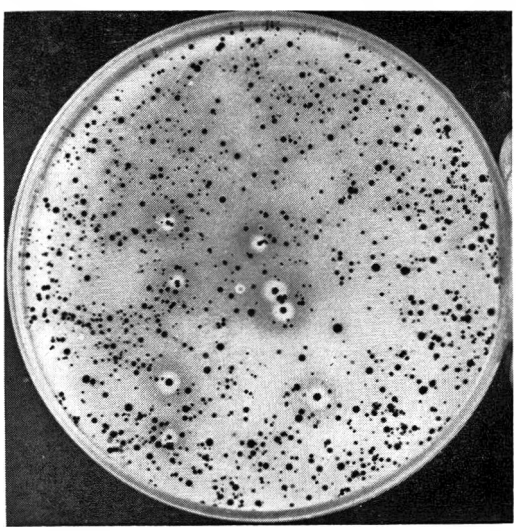


Fig. 1. Typical egg-yolk reaction of *Staphylococcus aureus* in ETGPA media.

In comparison with shrimp (Pablo *et al.*, 1966) for which the enumeration of the aerobic microbial flora was increased by rehydration with trypticase diluent, this effect was not noted in chicken. Distilled water as the rehydration medium gave slightly higher or equal recovery as compared to trypticase diluent at 20, 30, and 37°C (Fig. 2).

Higher temperature greatly enhanced the growth of microorganisms in rehydrated chicken, where growth rates were higher at 37°C than at 30 and 20°C (Fig. 3). The shortest lag time occurred at 37 and 30°C, approximately one-fourth that at 20°C. The longest lag time was at 4°C, approximately 120 times that at 37°C (Table 2). A population of 4×10^{10} organisms per gram of dry material was reached at 37 and 30°C in 24 hr, while 10^9 organisms was achieved at 20°C in 24 hr. At 37°C, the rehydrated chicken became putrid, with very strong ammoniacal odor, at 16 hr, while the same odor was noticed at 24 hr at 30°C, and at 54 hr at 20°C. At 4°C the shelf life of rehydrated chicken was greatly extended and no odor was detected even at 16 days.

The temperature of the rehydration medium did not seem to have any effect on the initial plate counts obtained from rehydrated chicken stored at 37, 30, 20, and 4°C, as shown by the similarity of the initial plate counts at zero time (Fig. 3).

If one is to consider the adoption of realistic standards that would reflect the degree of potential public-health hazards (Goldblith, 1963), it is important to include organisms of public-health significance in a study of this type. A population of

Table 2. Total aerobic plate counts and the incidence of *Staphylococcus aureus* of freeze-dried chicken rehydrated and incubated at 4°C.

Storage time (days)	Total aerobic plate counts (org/g dry material)	*Staphylococcus aureus*[a]	pH of homogenate
0	12×10^4	35	6.3
1	19×10^4	35	6.4
3	27×10^4	35	6.4
6	40×10^4	35	6.2
10	75×10^4	35	6.3
16	60×10^6	35	6.3

[a] Determined on the medium of Baird-Parker.

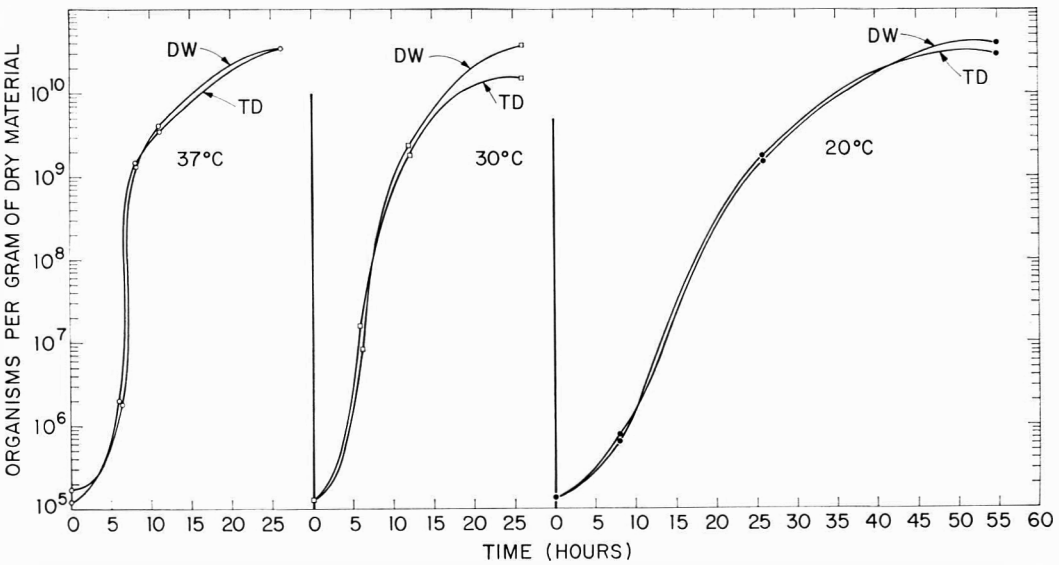


Fig. 2. Growth patterns at 20, 30, and 37°C of chicken meat rehydrated in distilled water (DW) or in trypticase diluent (TD).

30–40 *Staphylococcus aureus* organisms per gram of dry material was found to be a natural contaminant in a total population of 10^5 organisms (Fig. 3). It can be seen from Fig. 3 that *Staph. aureus* can grow in competition with the natural flora of rehydrated chicken at 20°C or above. Rate of growth was highest at 37 and 30°C, and appreciably lower at 20°C. The population of *Staph. aureus* remained constant at 4°C, while the total plate count increased from 10^5 to 6×10^7 in 16 days of storage (Table 2). There was a millionfold increase at 37°C, and a 100,000-fold increase at 30°C, in 24 hr of storage, and a 3,000-fold increase at 20°C in 55 hr. Thus, refrigeration is an excellent means, as well as a necessary one, in preventing the multiplication of *Staph. aureus* as well as retarding the growth of other naturally occurring species of organisms in rehydrated freeze-dried foods if these are to be stored after rehydration.

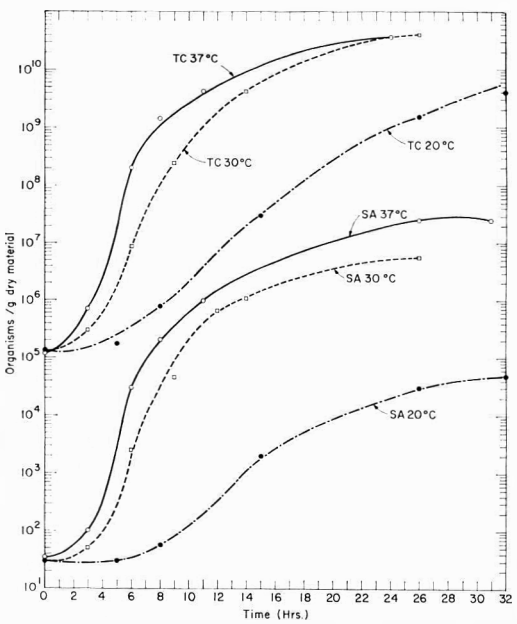


Fig. 3. A comparison at 20, 30, and 37°C of the growth patterns of total aerobic plate counts (TC) and *Staphylococcus aureus* (SA) in rehydrated freeze-dried chicken.

"Fecal enterococci" using KF medium (Kenner *et al.*, 1961) were found to be present in the natural flora of rehydrated chicken, and appear to grow well at storage temperatures of 20 and 37°C (Fig. 4). Coliforms were not detected in the microflora of the chicken sample studied. Rehydrated chicken stored at 20°C had an initial fecal enterococcal population of approximately 0.3% of the total plate counts and at the end of 55 hr it was also 0.3% of the total plate counts. At 37°C, fecal enterococci were initially 1.6% of the total plate counts, and at the end of 24 hr were 2.5%.

The pH of the homogenate at three storage temperatures varied from 6.2 to 8.2 (Tables 2, 3) and appeared to be a reflection of the degree of bacterial growth. A rise in pH to above 6.5 was noted only when the total aerobic plate count increased to over 10^9 cells per gram of dry weight. This occurred after 15 hr of storage at 37 and 30°C, and 31 hr at 20°C. The pH was unchanged during 16 days of storage at 4°C since the level of contamination did not exceed 10^7 cells per gram.

Table 3. Microbial analysis, nitrogen content of drip, and pH of homogenate of chicken rehydrated and stored at 37, 30, and 20°C.

Storage time (hr)	37°C				30°C			20°C			
	Total aerobic plate counts[a]	*S. aureus* counts[a]	mgN/100 ml	pH	Total aerobic plate counts	*S. aureus* counts	pH	Total aerobic plate counts	*S. aureus* counts	mgN/100 ml	pH
0	12×10^4	35	45	6.3	13×10^4	30	6.2	14×10^4	30	50	6.4
6	20×10^7	30×10^3	125	6.3	85×10^5	25×10^2	6.2	75×10^4	55	45	6.4
15	11×10^{10}	35×10^5	155	6.5	80×10^8	20×10^5	6.4	30×10^6	15×10^2	40	6.4
26	35×10^9	25×10^6	230	8.0	40×10^9	55×10^5	7.5	15×10^8	30×10^3	65	6.4
31	.....	25×10^{11}	....	8.2	.....	.....	....	40×10^9	50×10^3	60	6.5
55	.....	.....	....	....	.....	.....	.....	40×10^9	85×10^3	....	7.0

[a] Organisms per gram of dry material.

The microbial growth at 37°C had a significant influence on the total nitrogen content of the drip, while at 20°C the microbial population did not seem to cause any increase in total nitrogen.

The excess rehydrating medium was also analyzed for total plate counts. Calculations based on the total number of organisms showed that 6.5% of the microbial flora was recovered in the rehydration water at 37°C, and 0.5% at 20°C.

Fifty-eight randomly selected isolates from the KF medium were examined microscopically and tested for catalase and the presence of iron porphyrin compounds by the benzidine test (Diebel and Evans, 1960). Fifty-four of the isolates were found to be cocci and were benzidine- and catalase-negative. Three of the catalase- and benzidine-positive isolates were obtained at the initial sampling period.

DISCUSSION

Initially, the efficacy in enumeration of coagulase-positive strains on the two differential media (ETGPA and TPEY) is quite low but increases with storage time. This observation may possibly indicate: 1) that damage incurred by freeze-drying is most probably lost and new cells are now functioning normally; or 2) that as multiplication of *S. aureus* occurred in the chicken the effect of serial dilutions minimizes the concentration of food materials and increases selectivity. Saleh *et al.* (1965) indicated on a similar chicken sample that nonspecificity due to damage appears to be minor. Of 13 egg-yolk-positive coagulase-negative cultures, only one was DNase-positive and fermented mannitol. TPEY and ETGPA were of equal efficiency in the recovery of *S. aureus* as part of the natural flora of chicken (Table 1). This is not in agreement with Crisley's report that ETGPA did not recover staphylococci from food as well as four other selective media evaluated (Crisley *et al.*, 1965). This may be due to cultural differences among strains, which are of primary importance in influencing the efficacy of selective and differential media.

Figs. 3 and 4 show that *Staphylococcus*

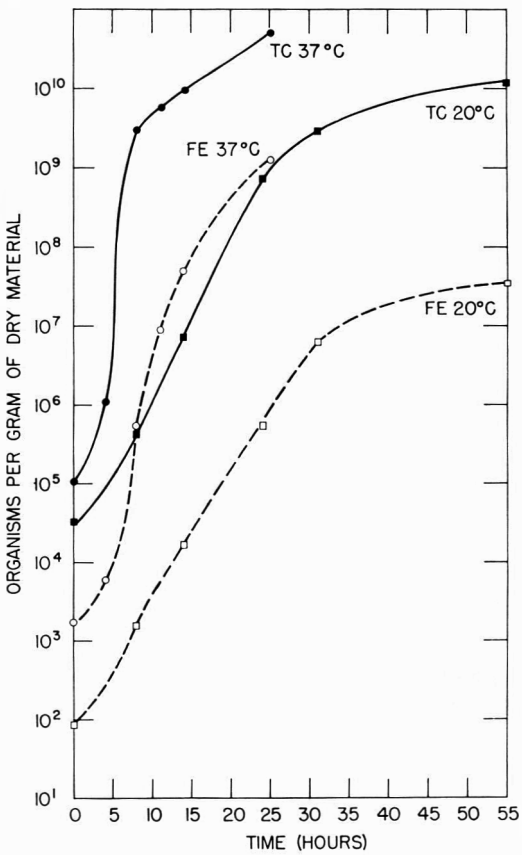


Fig. 4. A comparison at 20 and 37°C of the growth patterns of total aerobic plate counts (TC) and fecal enterococci (FE) in rehydrated freeze-dried chicken.

aureus and "fecal enterococci" can grow in competition with the natural flora of rehydrated chicken meat. To illustrate this point, the data from Figs. 3 and 4 are replotted to compare the levels of either *Staphylococcus aureus* or "fecal enterococci" against total aerobic plate counts (Fig. 5). The initial population of *S. aureus* of 30 per gram was able to grow at temperatures at 20°C or above against a saprophytic population which is 4,000 times as great. The initial counts of *S. aureus* are quite low, and it is seen that if the initial contamination of this organism is at a very high level, then appreciable growth could occur and even toxin formation might possibly result. At 4°C, neither multiplication nor decrease in the number of *S. aureus* takes place in rehydrated chicken meat. If the level of contamination were higher, there is a danger, especially if toxin were already produced, because 4°C cannot be depended on to eliminate *S. aureus* or destroy toxin.

This appears to be in only partial agreement with studies of Appleman *et al.* (1964) who also found the presence of normal fish muscle flora did not interfere with the recovery of staphylococci at 30 and 37°C; however, he found no appreciable increase at 20°C within 48 hr or at 0°C within 10 days. The failure of staphylococci to grow at 20°C in Appleman's study may be due to the use of pure culture inoculum staphylococci in a fish homogenate. Our study showed successful competition at 20°C within 32 hr which is probably due to the growth of organisms as discrete colonies in the rehydrated chicken meat. Competition from other organisms in the natural flora may he less severe on isolated clones of *Staph. aureus* on a surface than in a homogenate. Fig. 6 shows the growth of microorganisms on freeze-dried chicken which was rehydrated with 0.2% 2,3,4-triphenyl tetrazolium chloride and incubated at 20°C. The colonies appeared to grow as distinct clones with no interference from neighboring groups. The inhibition of the growth of *Staphylococcus aureus* at 4°C is due to the fact that staphylococci do not grow at 4°C (Elliot and Michener, 1960).

"Fecal enterococci" at an initial population of approximately 2×10^3 organisms were able to grow in competition with saprophytes which are 100 times as great at 37°C, and at an initial population of 100 "fecal enterococci" at 20°C against a saprophytic population which is 500 times as great. Ap-

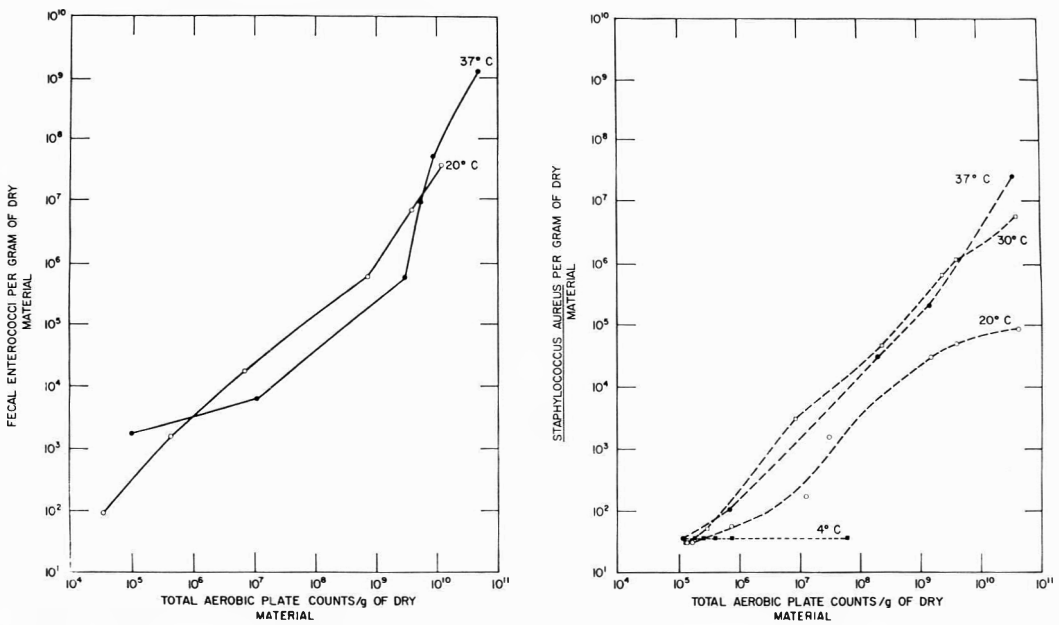


Fig. 5. The relationship between fecal enterococci or *S. aureus* levels and the total aerobic plate count.

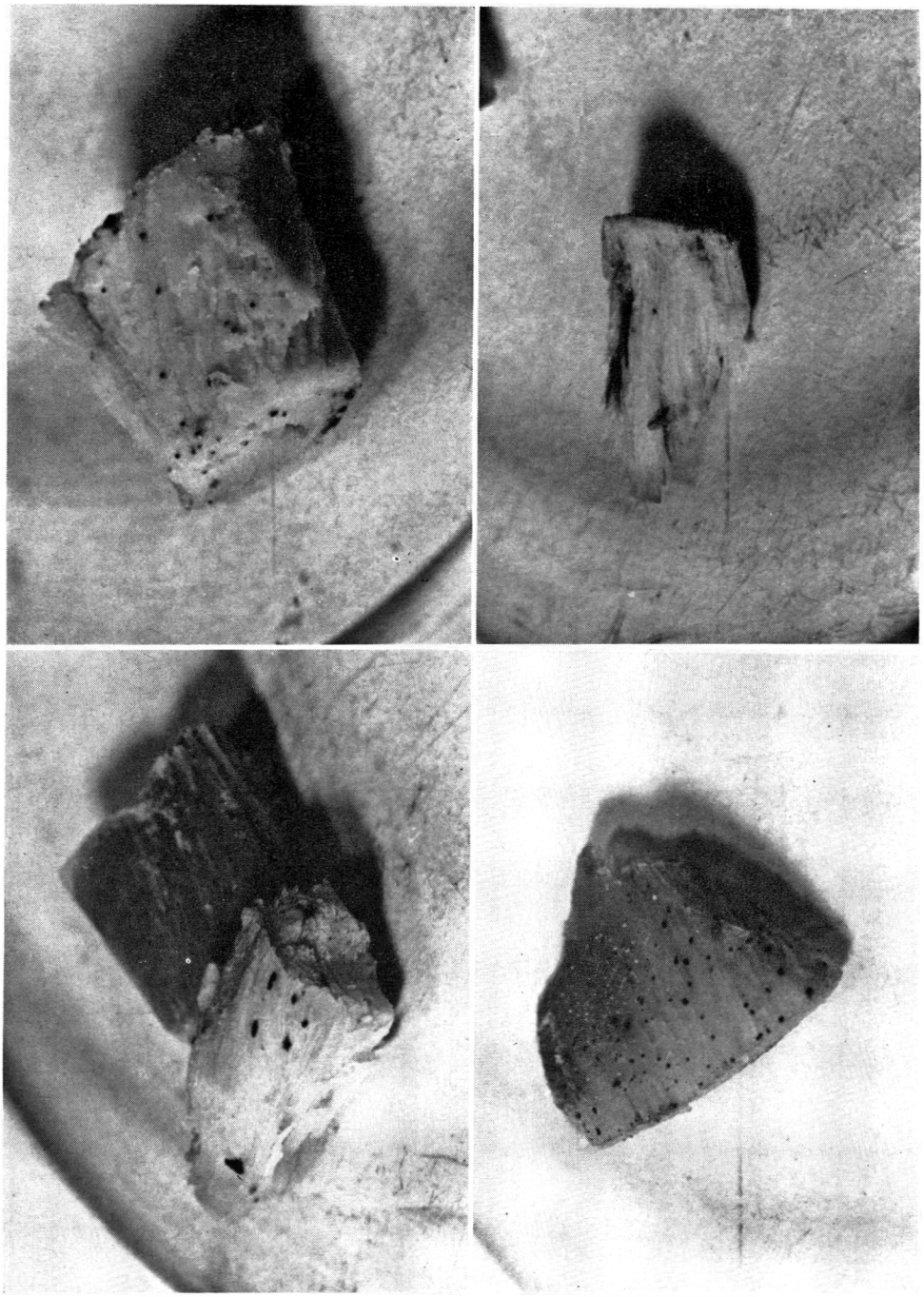


Fig. 6. Colony formation on freeze-dried chicken meat rehydrated with 0.2% of 2,3,4-triphenyl tetrazolium chloride.

parently, this indicates that "fecal enterococci" and other organisms which are part of the natural flora of rehydrated chicken can grow together at 20 and 37°C.

Shrimp rehydrated and stored at 4, 20, and 37°C was found to be organoleptically spoiled at a microbial population level of about 15 to 20 $\times$ 10^8 organisms per gram of

dry material (Pablo *et al.*, 1966). In this study, rehydrated chicken appeared to be organoleptically spoiled, as determined by odor, when a population level of 2.5 to 3.0 $\times$ 10^{10} organisms per g (dry wt) was reached at 20, 30, and 37°C. Elliot and Straka (1964) found that unfrozen chicken stored at 2°C was organoleptically spoiled when a bacterial population of approximately 5 $\times$ 10^9 organisms per gram (of wet material) was achieved. At 4°C storage, this microbial population in rehydrated chicken meat was not reached even in 16 days of storage, and no off-odor was detected.

The aerobic plate count was higher in the sample of chicken reported in this study than in other samples examined. These other samples did not contain *S. aureus* or "fecal enterococci" organisms, and the data presented in this paper do not intend to suggest the average quality of commercial freeze-dried chicken.

REFERENCES

Appleman, M. D., N. Bain, and J. M. Shewan. 1964. A study of some organisms of public health significance from fish and fishery products. *J. Appl. Bacteriol.* **27**, 69.

Baird-Parker, A. C. 1962. An improved diagnostic and selective medium for isolating coagulase-positive staphylococci. *J. Appl. Bacteriol.* **25**, 12.

Burkwall, M. K., and P. A. Hartman. 1964. Comparison of direct plating media for the isolation and enumeration of enterococci in certain frozen foods. *Appl. Microbiol.* **12**, 18.

Carter, C. H. 1960. Egg yolk agar for isolation of coagulase-positive staphylococci. *J. Bacteriol.* **79**, 753.

Crisley, F. D., R. Angelotti, and M. J. Foter. 1964. Multiplication of *Staphylococcus aureus* in synthetic cream fillings and pies. *Public Health Rept. U. S.* **79**, 369.

Crisley, F. D., J. T. Peeler, and R. Angelotti. 1965. Comparative evaluation of five selective and differential media for the detection and enumeration of coagulase-positive staphylococci in foods. *Appl. Microbiol.* **13**, 140.

Diebel, R. H., and J. B. Evans. 1960. Modified benzidine test for the detection of cytochrome-containing respiratory systems in microorganisms. *J. Bacteriol.* **79**, 356.

Elliot, R. P. and H. D. Michener. 1960. Review of the microbiology of frozen foods. In Conference on Frozen Food Quality. *U. S. Dept. Agr., ARS-***74-21**, 40.

Elliot, R. P., and R. P. Straka. 1964. Rate of microbial deterioration of chicken meat at 2°C after freezing and thawing. *Poultry Sci.* **43**, 81.

Goldblith, S. A. 1963. *In*: Exploration in Future Food Processing Techniques. (S. A. Goldblith, ed.) p. 70. MIT Press, Cambridge, Mass.

Kenner, B. A., H. F. Clark, and P. W. Kabler, 1961. Fecal streptococci. I. Cultivation and enumeration of streptococci in surface waters. *Appl. Microbiol.* **9**, 15.

Minari, O., and D. B. Zilversmit. 1963. Use of KCN for stabilization of color in direct Nesslerization of Kjeldahl digests. *Anal. Biochem.* **6**, 320.

Pablo, I. S., G. J. Silverman, and S. A. Goldblith. 1966. Growth patterns of rehydrated freeze-dried foods. I. Shrimp. *J. Food Sci.* In press.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962a. Staphylococci in competition. I. Growth of naturally occurring mixed populations in precooked frozen foods during defrost. *Appl. Microbiol.* **10**, 16.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962b. Staphylococci in competition. II. Effect of total numbers and proportion of staphylococci in mixed cultures on growth in artificial culture medium. *Appl. Microbiol.* **10**, 23.

Saleh, B., G. J. Silverman, and S. A. Goldblith. 1966. Microbial evaluation of commercial freeze-dried foods. *J. Food Sci.* (in press).

Silverman, G. J., N. S. Davis, J. T. R. Nickerson, D. W. Duncan, I. Tezcan, and M. Johnson. 1961. Microbial analysis of frozen raw and cooked shrimp. II. Certain characteristics of staphylococcus isolates. *Food Technol.* **15**, 458.

Sinskey, T. J., A. H. McIntosh, I. S. Pablo, G. J. Silverman, and S. A. Goldblith. 1964. Considerations in the recovery of microorganisms from freeze-dried foods. *Health Lab. Sci.* **1**, 297.

Slabyj, B. M., A. M. Dollar, and J. Liston. 1965. Post-irradiation survival of *Staphylococcus aureus* in sea foods. *J. Food Sci.* **30**, 344.

Ms. rec'd 5/19/65.

Contribution no. 704 from the Department of Nutrition and Food Science. The research reported was supported by the Division of Environmental Engineering and Food Protection, Public Health Service, research grant EF-00314.

M. F. NELSON, T. A. POULOS, L. E. GONGWER, and J. C. KIRSCHMAN
Chemical Research Department
Bio-Medical Research Department
Atlas Chemical Industries, Inc.
Wilmington, Delaware 19899

Preparations of Carbon-14-Labeled Polyoxyethylene (20) Sorbitan Monolaurate and their Metabolic Fate in Rats

SUMMARY

Preparation is given and metabolic fate is shown in rats for both fatty-acid-C^{14}-labeled and polyoxyethylene-C^{14}-labeled polyoxyethylene (20) sorbitan monolaurate. The emulsifier's metabolic pattern, following oral administration, appears to be as follows: The ester bond between the polyoxyethylated polyol and fatty acid moieties undergoes almost complete hydrolysis, with the fatty acid portion being metabolized in a manner similar to that for trilaurin. The ether linkage between the polyoxyethylene chain and the hexitan molecule is not disrupted appreciably, and this polyoxyethylene-polyol emulsifier moiety is only slightly absorbed. The data also show that none of the polyol moiety is stored in the body of the animal. The fate of each labeled form of the emulsifier following intravenous administration is also shown.

INTRODUCTION

The literature contains several references to studies conducted on the metabolic fate of sorbitan monostearate and polyoxyethylene (20) sorbitan monostearate (Wick and Joseph, 1953, 1956). The latter product was carbon-14 labeled only in the polyol moiety, and although studies with this material gave some indication as to its fate in rats, several questions were not answered concerning metabolism of this class of compound.

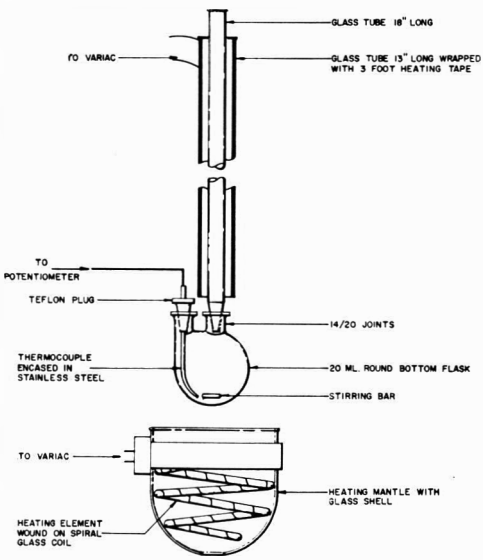
Experiments were planned to study more thoroughly the metabolic fate of a similar emulsifier-polyoxyethylene (20) sorbitan monolaurate (sold by Atlas Chemical Industries, Inc., as Tween® 20; also known as polysorbate 20) hereinafter referred to as PSML.

Two PSML emulsifiers were prepared: one was labeled with carbon-14 in the polyoxyethylene chain, and the second was labeled with carbon-14 in the fatty acid moiety. Both of these materials were studied in fasted as well as nonfasted rats, and both oral and intravenous administration were used.

EXPERIMENTAL METHODS

Syntheses of the labeled emulsifiers. *Fatty-acid-labeled PSML.* Pilot syntheses were conducted using nonradioactive materials to determine optimum conditions for preparation of the labeled compounds. With all pilot runs the acid, saponification, and hydroxyl numbers were determined to serve as an index of conformity of these preparations with production lots of PSML.

After optimum conditions were established, fatty-acid-labeled sorbitan monolaurate, the intermediate compound, was prepared by condensing 0.530 g of fatty acid (containing 1.500 mc of dodecanoic acid-1-C^{14} and commercial lauric acid used in production of PSML) and 0.511 g of crystalline sorbitol in the presence of disodium phosphate catalyst for 2 hr at 250°C. The equipment used is shown in Fig. 1.



-18-

Fig. 1. Apparatus used to prepare sorbitan monolaurate.

The resultant sorbitan monolaurate-C^{14} was then condensed with 2.9 g of nonradioactive ethylene oxide using 6 mg of fused sodium oleate catalyst. The equipment used for the condensation is shown in Fig. 2. The reaction was run at 155°C and

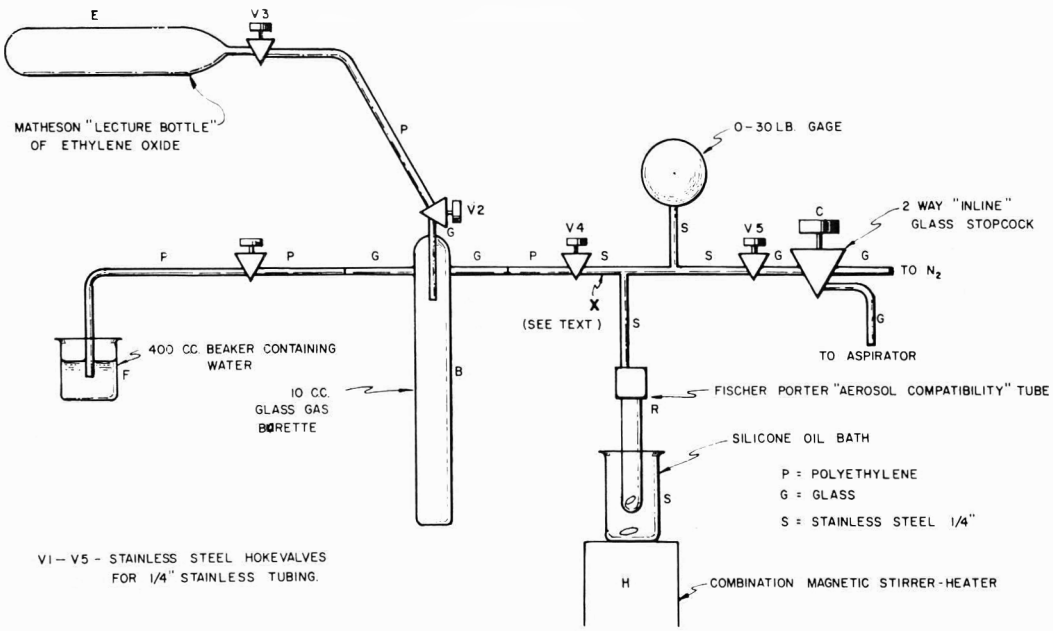


Fig. 2. Basic apparatus used to prepare PSML.

25 lb/sq in. pressure until all ethylene oxide had reacted (about 6 hr). The product, fatty-acid-labeled PSML-C^{14}, was found to have a specific activity of 0.355 mc/g.

Polyoxyethylene-labeled PSML. Development of techniques for synthesis of the polyoxyethylene-labeled emulsifier was also undertaken, by first using pilot syntheses with nonradioactive materials. A modified apparatus was used so that carbon-14-labeled ethylene oxide could be inserted into the condensation train. This involved incorporation, at point *X* Fig. 2, of a special series of valves as well as the vial containing the carbon-14-labeled ethylene oxide. This modification is shown in Fig. 3.

After the technique had been developed, polyoxyethylene-labeled PSML was synthesized by condensing 1.00 g of nonradioactive sorbitan monolaurate from regular production with 2.9 g of ethylene oxide containing 1.500 mc of ethylene oxide-1,2-C^{14}. The reaction was run at 155°C and 25 lb/sq in. pressure until all ethylene oxide had reacted (about 6 hr). The resultant labeled emulsifier was found to have a specific activity of 0.341 mc/g.

Design of the metabolic studies. "Blocks of four" 100-g immature rats were used for all studies. A "block of four" consisted of a male and female rat studied 12 hr, and a male and female rat studied 24 hr. Both fasted and nonfasted rats were used, with the former being fasted 16 hr prior to dose administration.

The labeled emulsifier was administered either orally or intravenously as a 40% aqueous solution. Specially devised pipettes, each composed of a microliter transfer pipette with a polyethylene delivery tube, were used. These pipettes, after calibration, possessed an accuracy and precision within 1%.

The oral dose level was 1 g/kg of body weight, and although the intravenous dose level was originally intended to be the same, difficulties were encountered which necessitated lowering the dose to 0.5 g/kg. This is covered more completely under "Results and Discussion."

In the studies, after the animals were dosed they were placed in modified Roth chambers (Roth *et al.*, 1948) for the course of the individual experiment, water permitted *ad libitum*, and urine, feces, and respired air samples were collected. At the desired time, 1 ml of 50 mg/ml sodium pentobarbital solution was given intraperitoneally. After the animals' death the tissues were removed and stored frozen until assayed. The carcasses were frozen, homogenized by grinding in an electric meat grinder, and then stored frozen until assayed. No tissues were stored more than 5 days before being combusted.

Radioassay techniques. The frozen tissues were quickly thawed, placed in porcelain combustion boats, and combusted at 850°C in the presence of oxygen in a specially devised combustion train. This train used a large combustion tube which permitted combustion of total tissues, except for

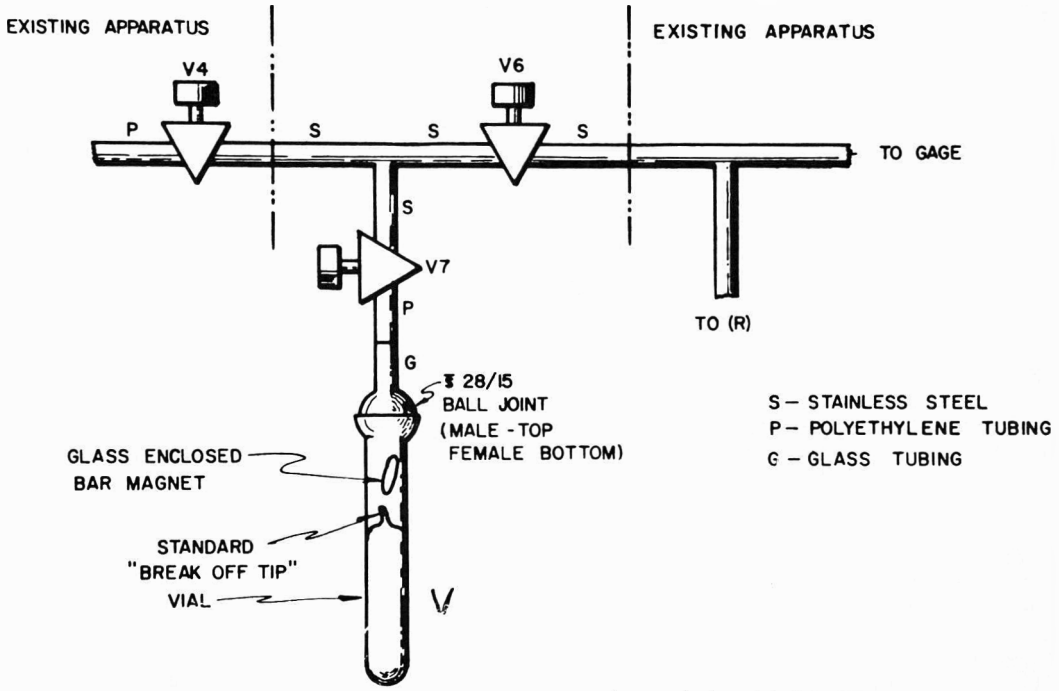


Fig. 3. Modified apparatus used to prepare polyoxyethylene-labeled PSML.

the carcass, of which 1/5 was assayed. This total-tissue combustion eliminated the need to try to homogenize samples and take aliquots. The carbon dioxide from the combustions was collected in sodium hydroxide as sodium carbonate.

Duplicate aliquots of the sodium carbonate solution were withdrawn from standard volumes and the carbonate was precipitated as barium carbonate using a mixture of barium and ammonium chlorides. After filtration the precipitates were dried to a constant weight. Portions of each weighed, dried precipitate were packed into planchets of the same geometry and were assayed in triplicate as "infinitely thick" samples with a Baird Atomic Model 750 automatic counting system. Since the cross-sectional area of the planchet, the total weight of precipitate ($BaCO_3$) in aliquot, and the infinite thickness value of barium carbonate were known, the relative amount of carbon-14 per sample was determined.

The time of counting was selected so that the standard counting error was less than 1% with samples that contained higher activity. The samples with lower activity were counted for periods that resulted in a 5% standard counting error.

RESULTS AND DISCUSSION

All results in the tables are expressed in percent of administered dose found in the tissues; standard deviations for the assays are also shown. Aliquots of the solutions used to dose the animals were combusted and asayed in a manner identical to the tissues, and these served as controls for determining the administered dose.

Oral administration of PSML. Table 1 gives results for rats administered oral doses of fatty-acid-labeled PSML; Table 2 gives results for orally administered polyoxyethylene-labeled PSML. Results show that when the fatty-acid-labeled emulsifier was used the carbon-14 moiety was almost completely absorbed. Only about 4% was found in the feces. In contrast the polyoxyethylene-labeled PSML gave results which show that at least 80% of the carbon-14 tracer appeared in the feces. These results indicate that the PSML ester bond is easily hydrolyzed in the rat intestine and the acid moiety is metabolized similar to trilaurin (Kirschner and Harris, 1961). The polyoxyethylene moiety, in contrast, is not absorbed and passed through the animal.

Additional conclusions can also be drawn by comparing the results with results of Wick and Joseph (1956). They labeled the polyol portion of polyoxyethylene (20) sorbitan monostearate by using sorbitol-C-^{14},

Table 1. Oral administration of fatty-acid-labeled PSML (1.0 g/kg dose level).

Tissue	Fasted				Unfasted			
	12 hr Male	12 hr Female	24 hr Male	24 hr Female	12 hr Male	12 hr Female	24 hr Male	24 hr Female
Liver	2.12±.05	1.64±.07	1.34±.09	1.45±.07	0.78±.04	1.89±.08	0.80±.06	1.00±.05
Urine and bladder content	2.25±.04	2.82±.09	2.71±.04	2.75±.07	2.34±.09	3.06±.06	2.71±.09	1.79±.12
Feces and GI tract	5.06±.06	4.52±.10	4.27±.08	3.51±.08	3.40±.06	5.24±.10	2.98±.06	4.01±.14
Carcass	15.8±.9	12.7±.7	11.7±.5	11.3±.5	12.1±.4	23.5±.4	11.8±.7	30.5±.9
Respired air	81.1±.6	79.7±.5	82.7±1.3	81.4±1.1	80.8±.7	64.5±.8	80.5±.8	55.8±1.1
Total recovery	106±2	101±2	103±2	100±2	99.5±1.3	98.2±1.4	98.9±1.7	93.1±2.3

Values under each column are obtained on a single animal.
Standard deviations shown are that of the assay procedure.

whereas we labeled the ethylene oxide portion of the PSML. The two studies show that with the two methods of labeling, the metabolic fates are similar. Most of the activity is eliminated in the feces, and this indicates little or no cleavage of the ether bond between the ethylene oxide chain and the sorbitan. If the ether bond did split, the carbon-14 distribution pattern would be similar to that for polyol-labeled sorbitan monostearate (Wick and Joseph, 1953).

One difference is noted between these data and those of Wick and Joseph. They reported that 2–7% of the carbon-14 appears in the respired air when hexitol-labeled polyoxyethylene (20) monostearate emulsifier is given. The present studies with ethylene-oxide-labeled PSML gave no detectable carbon-14 in the respired air samples under the conditions established. This difference may be due in part to the presence of unreacted labeled sorbitan monostearate in the Wick and Joseph material. Any of this material, if not reacted with ethylene oxide, will undergo intestinal hydrolysis and metabolize to give some carbon-14 in respired air. It should be pointed out that the two emulsifiers also differ in fatty acid component.

The studies also show that fasting does not have a pronounced effect on absorption or metabolism of PSML. This is in contrast to some carbohydrates, whose metabolism and absorption are altered by fasting (Kirk *et al.*, 1959).

Intravenous administration of PSML. Table 3 illustrates the distribution of carbon-

Table 2. Oral administration of polyoxyethylene-labeled PSML (1.0 g/kg dose level).

Tissue	Fasted				Unfasted			
	12 hr Male	12 hr Female	24 hr Male	24 hr Female	12 hr Male	12 hr Female	24 hr Male	24 hr Female
Liver	nil	nil	nil	nil	nil	nil	nil	nil
Urine and bladder content	8.70±.10	14.0±.2	8.91±.12	9.41±.04	7.23±.09	8.19±.12	8.51±.15	7.50±.09
Feces and GI tract	92.9±.7	70.7±.7	95.9±.7	88.2±1.1	74.8±.4	95.2±1.0	85.5±.8	95.9±1.5
Carcass	2.85±.35	1.69±.27	nil	nil	5.18±.41	0.81±.30	nil	nil
Respired air	nil	nil	nil	nil	nil	nil	nil	nil
Total recovery	104±1	86.4±1.2	105±1	97.6±1.1	87.2±.9	104±1	94.0±1.0	103±2

Values under each column are obtained on a single animal.
Standard deviations shown are that of the assay procedure.

Table 3. Intravenous administration of fatty-acid-labeled PSML (0.5 g/kg dose level).

Tissue	Fasted				Unfasted			
	12 hr Male	12 hr Female	24 hr Male [a]	24 hr Female [a]	12 hr Male	12 hr Female	24 hr Male [b]	24 hr Female [b]
Liver	1.98±.08	2.08±.05	—	—	1.47±.08	1.03±.05	0.56±.05	0.86±.14
Urine and bladder content	5.35±.18	5.18±.14	—	—	4.39±.26	4.12±.11	5.43±.13	4.84±.05
Feces and GI tract	2.26±.11	1.85±.07	—	—	2.28±.26	2.11±.14	2.85±.08	2.44±.05
Carcass	25.5±.7	17.2±.9	—	—	25.4±.9	18.2±.8	22.9±.6	22.6±.8
Respired air	63.4±1.4	61.2±.5	—	—	65.3±.90	62.7±.8	67.9±1.3	69.7±.6
Total recovery	98.5±2.5	87.5±1.7	—	—	98.8±1.50	88.2±1.1	99.3±2.4	101±1

[a] 24-hr study with fasted animals not completed. See text.
[b] 24-hr study with unfasted animals was at 1.8-g/kg dose level.
Values under each column are obtained on a single animal.
Standard deviations shown are that of the assay procedure.

14 following intravenous administration of the fatty-acid-labeled PSML; Table 4 similarly shows values obtained following intravenous administration of the polyoxyethylene-labeled emulsifier. In both cases standard deviations for the assays are included.

Two dose levels were used in the studies. Originally, the intended dose was 1.0 g/kg body weight of PSML, but this amount was not well tolerated intravenously in fasted immature rats. Therefore the dose was decreased to 0.5 g/kg. Because of lack of sufficient fatty-acid-labeled PSML, values were not obtained for fasted animals which were to be studied 24 hr.

Results show that with intravenous dosing, cleavage of the ester bond again occurs. This is shown in the fatty-acid-labeled PSML studies by the high degree of oxidation as illustrated by the large percentage of carbon-14 in the respired-air values.

In contrast, the animals given polyoxyethylene-labeled PSML show no carbon-14 in the respired air. More than 75% of the carbon-14 appears in the urine, probably as the polyol-polyoxyethylene moiety, although this was not proven experimentally with the intravenous dosing of polyol-labeled PSML. It is interesting to note the presence of activity in the feces (about 2% with fatty-acid-labeled PSML and 11% with polyoxyethylene-labeled emulsifier). Since intravenous administration precludes nonabsorption, the fecal carbon-14 may have been transported

Table 4. Intravenous administration of polyoxyethylene-labeled PSML (0.5 g/kg dose level).

Tissue	Fasted				Unfasted			
	12 hr Male	12 hr Female	24 hr Male	24 hr Female	12 hr Male	12 hr Female	24 hr Male	24 hr Female
Liver	0.26±.02	0.40±.06	0.17±.02	0.22±.04	0.26±.03	0.22±.03	0.18±.05	0.15±.06
Urine and bladder content	80.8±.5	72.3±.2	82.0±.5	80.3±.4	79.9±.7	81.7±.1	82.9±.8	87.2±1.0
Feces and GI tract	6.06±.11	15.0±.3	11.2±.2	12.2±.3	13.2±.2	11.9±.1	8.78±.20	11.4±.2
Carcass	4.37±.27	3.91±.10	2.44±.29	1.66±.24	3.98±.32	1.97±.44	1.98±.25	1.48±.20
Respired air	nil	nil	nil	nil	nil	nil	nil	nil
Total recovery	91.5±.9	91.6±.7	95.8±1.1	94.4±1.0	97.3±1.3	95.8±.7	93.8±1.3	100±1

Values under each column are obtained on a single animal.
Standard deviations shown are that of the assay procedure.

there by an enterohepatic cycle or a reverse "absorption" process through the intestinal wall.

Results again indicate that fasting does not appreciably affect metabolic fate of the emulsifier.

The data in Table 2 indicates that, with oral administration, radioactivity of the carbon-14-labeled polyol remains in the animal body after 12 hr but none remains after 24 hr.

REFERENCES

Kirk, M. R., S. Lepkovsky, and B. M. Tolbert. 1959. $C^{14}O_2$ respiration from labeled compounds in feeding trained rats. *Metabolism, Clin. and Exptl.*, **8**, 769.

Kirschner, S. L. and R. S. Harris. 1961. Effects of chain length on the metabolism of saturated fatty acids by the rat. *J. Nutrition* **73**, 397.

Roth, L. J., E. Leifer, J. R. Hogness, and W. H. Langham. 1948. Studies on the metabolism of radioactive nicotinic acid and nicotinamide in mice. *J. Biol. Chem.* **176**, 299.

Wick, A. N., and L. Joseph. 1953. The metabolism of sorbitan monostearate. *Food Research* **18**, 79.

Wick, A. N., and L. Joseph. 1956. The fate of injested polyoxyethylene (20) sorbitan monostearate in rats. *Food Research* **21**, 250.

Ms. rec'd 3/30/65.

KIN WA CHENG[a] and WENDELL W. KILGORE
Agricultural Toxicology and Residue Research Laboratory, University of California, Davis, California 95616

Determination of 2,6-dichloro-4-nitroaniline Residues in Fruits by Electron-Capture Gas Chromatography

SUMMARY

A procedure for the analysis of 2,6-dichloro-4-nitroaniline by electron-capture gas chromatography is described. In this technique, samples are extracted with benzene and analyzed in a gas chromatograph without involving any additional cleanup procedures. It is applicable to a variety of stone fruits, and as low as 0.01 ppm of residue can be detected.

INTRODUCTION

DCNA (Botran; Upjohn Company trade name), 2,6-dichloro-4-nitroaniline, has been approved for use on a variety of stone fruits for the control of postharvest fungus rots. For effective control of these diseases, fruits may be treated with the chemical either before or after harvesting. Preharvest spray treatment, however, appears to be more feasible than postharvest dipping.

The analytical procedure originally developed by Kilgore *et al.* (1962) for the extraction and determination of DCNA residues in canned fruits has been used extensively for both processed and unprocessed samples. This colorimetric procedure is based on the development of an intense yellow color characteristic of certain mononitro aromatic compounds in the presence of strong alkali and acetone. Beckman and Bevenue (1962), using the Kilgore *et al.* (1962) methods of extraction and cleanup, found that DCNA could be analyzed by two gas chromatographic procedures: one utilizing a thermal-conductivity detector, and the other a micro-coulometer detector.

In the present study, a simplified but sensitive procedure is described for the extraction and gas chromatographic determination of DCNA residues in a variety of unprocessed stone fruits. In this procedure, samples are extracted with benzene by a one-step process and are analyzed without further cleanup in a gas chromatograph equipped with an electron-capture detector.

[a] Present address: Department of Agricultural Engineering, University of California, Davis, California 95616

MATERIALS AND METHODS

Gas chromatograph and recorder. Used for all analyses was an Aerograph HY-FI gas chromatograph (Model 600B, Wilkins Instrument and Research, Inc.) equipped with an electron-capture detector. The detector, which is partially exposed to the outside of the instrument, was covered with a small wide-mouth Thermos bottle to avoid extreme temperature fluctuations. The detector signal was supplied to a 1-mv Brown recorder having a 1-second pen response. The recorder was operated at a speed of 1 in./min.

Column and operating conditions. The column used routinely was a ⅛-in. × 5-ft spiral Pyrex glass column prepacked with 5% Dow-11 (w/w) silicone oil on acid-washed 60/80-mesh Chromosorb W. This prepacked column was obtained from Wilkins Instrument and Research, Inc.

The column was maintained in the gas chromatograph at 185°C. The nitrogen carrier-gas, which was passed through a small molecular-sieve filter (Wilkins Instrument and Research, Inc.), was regulated to provide a flow rate of 25 cc/min. All solutions were injected into the column with a 10-μl Hamilton syringe (No. 701).

Planimeter. A compensating polar planimeter (Keuffel and Esser Co.) was used to measure the area within the boundaries of the recorder response curve.

Reagents. Redistilled reagent-grade benzene, DCNA standard (Upjohn Company, Kalamazoo, Michigan).

PROCEDURE

Preparation of standard curve. An aliquot of a stock solution of DCNA (100 μg/ml benzene) is diluted with benzene to contain 0.05 μg/ml (50 picograms/μl). Aliquots of this solution, ranging from 1 μl to 8 μl, are injected directly into the gas chromatograph.

An adequate interval, 7–8 min, is allowed for the recorder to respond to the detector signal and to recover to the previous baseline before a subsequent injection is made. The area under the curve is then determined, and the average area of three injections for each concentration is plotted against the concentration.

Extraction. Macerate a representative sample of the fruit in a suitable food chopper. Place 100 g of the macerated material into a 1-gallon tin can, equipped with a metal baffle for mixing, and add 200 ml of benzene. Seal the can tightly with a lid,

and roll it on a mechanical roller (35 rpm) for 30 min. For recovery studies, fortify the control samples with a known amount of DCNA after placing the macerated material in the tin can, but before the addition of the benzene.

The can should not be opened after the rolling procedure for at least 10 min. This will permit the dispersion of any emulsion which may form during the extraction step. After standing, decant the mixture into a beaker containing about 100 g of anhydrous sodium sulfate and mix thoroughly. Filter through a fluted filter paper and store in a tightly capped bottle until analyzed.

Gas chromatographic analyses. Undiluted 1-μl aliquots of the extracts from the control and the fortified samples are injected separately into the gas chromatograph. This is done to determine whether any interfering substances are present and to establish actual recovery values.

Depending on the amount of DCNA residues in the samples, the volume of extract which can be placed on the column will vary considerably. Preliminary response curves should be made for each treated sample before trying to make an accurate measurement. If the residues are high, the extracts from the treated samples may require diluting with benzene. After analysis, the amount of residue in the sample is determined by comparing the area under the curve (average of 3 determinations) with the figures on the calibration curve.

RESULTS AND DISCUSSION

The linearity of response of the electron-capture detection system to DCNA was measured over a range of 50–400 picograms. The area vs. concentration plot of these results is shown in Fig. 1. The peak height and column retention time for a 120-picogram sample of DCNA are shown in Fig. 2.

The amount of interfering substances present in untreated control samples of peaches, apricots, prunes, cherries, and nectarines was found to be almost negligible. The response curve for cherry extracts shown in Fig. 3 was typical of most fruits. The only extracts from untreated control samples that contained substances which responded in the electron-capture system were prepared from Halford peaches. In these samples a small peak was observed 3 min after the extracts were placed onto the column, but this did not interfere with the DCNA peak emitted 6 min after injection.

Recoveries of DCNA were excellent, ranging from 83 to 97.5%, when fortified check samples were analyzed. The overall average recovery for all fruits was 90%. A sensitivity of 0.01 ppm was readily obtained,

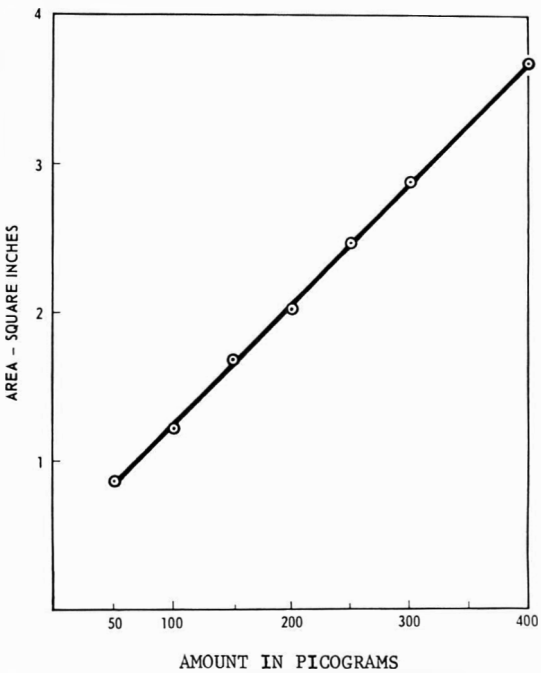


Fig. 1. Linearity curve of DCNA. Each point represents the area (average of 3 determinations) of the response of DCNA given by the electron-capture detector.

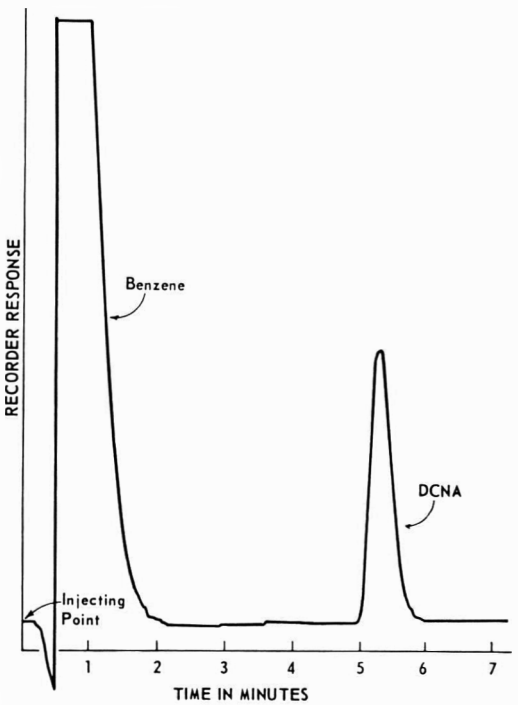


Fig. 2. Gas chromatogram of DCNA.

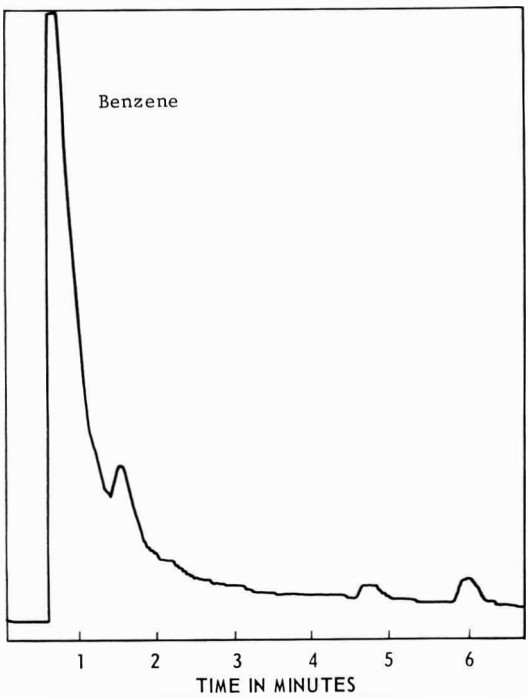


Fig. 3. Gas chromatogram of cherry extract from untreated control sample.

average recovery for all fruits was 90%. A sensitivity of 0.01 ppm was readily obtained, but this could have been pushed even lower had a greater sensitivity been required.

To show the applicability of this procedure, several field-treated samples of peaches and nectarines were analyzed (Table 1). The technique has also been used successfully to detect DCNA residues on apricots, prunes, and cherries. The degradation of DCNA residues on apricots appeared to be logarithmic, and less than 1.0 ppm of the chemical remained on the fruit four days after spraying (Fig. 4).

This gas chromatographic procedure for the detection of DCNA residues on fruits

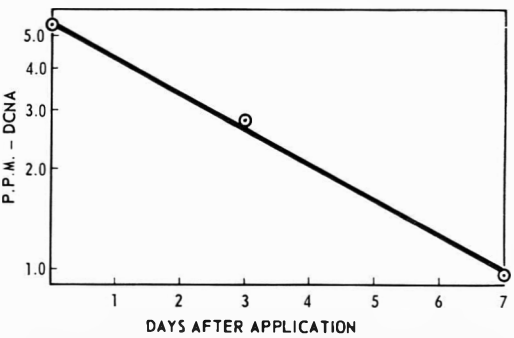


Fig. 4. Degradation curve of DCNA residues on apricots.

has several distinct advantages over the colorimetric technique (Kilgore *et al.*, 1962) now used in many laboratories. Of particular importance is the absence of bothersome interfering substances in the benzene extracts. This, however, is due to the specificity of the electron-capture detector in the gas chromatograph, rather than to the complete absence of other materials in the extracts. Also, a direct analysis of the extracts without additional cleanup is a decided advantage because the time required for each test is reduced and the chances of improved recovery values are increased. In addition, it is not necessary to condense large volumes of extracting solvent to obtain a desirable sensitivity.

REFERENCES

Beckman, H., and A. Bevenue. 1962. Pesticide residue analysis. Gas chromatographic analysis of 2,6-dichloro-4-nitroaniline. *J. Food Sci.* **27** (6), 602.

Kilgore, W. W., K. W. Cheng, and J. M. Ogawa. 1962. Extraction and determination of 2,6-dichloro-4-nitroaniline in processed fruits. *J. Agr. Food Chem.* **10** (5), 399.

Ms. rec'd 10/26/65.

The authors thank Dr. Joseph Ogawa, Department of Plant Pathology, for providing the field-treated samples.

Table 1. DCNA residues on nectarines and peaches after spraying.[a]

Nectarines		Peaches	
Spray application (No. of days before harvest)	DCNA residue[b] (PPM)	Spray application (No. of days before harvest)	DCNA residue[c] (PPM)
0	1.83	0	10.2
0[d]	1.58	4	6.9
4	1.18	11	1.5
7	0.63		

[a] Application rate: 6 gallons (1 lb DCNA in 100 gallons water)/tree.
[b] Figures uncorrected for a recovery value of 95%.
[c] Figures uncorrected for a recovery value of 93%.
[d] Fruit harvested same day as spray application, passed through a defuzzing machine, and stored at 68°F for 13 days before analysis.

B. B. MARSH, PAMELA R. WOODHAMS, and N. G. LEET
Meat Industry Research Institute of New Zealand, Hamilton, New Zealand

Studies in Meat Tenderness. I. Sensory and Objective Assessments of Tenderness

SUMMARY

As an introduction to a long-term investigation of meat tenderness, seven series of comparisons were made between sensory and objective evaluations of the tenderness of roasted lamb rib-loins. Using a tenderometer based on that of Volodkevich, high coefficients of correlation (0.68-0.94) were found despite the occasional occurrence of large variations in tenderness within single muscles and the detection of slight panel fluctuation in three of the more prolonged series. Sensory assessment of tenderness was shown to be more nearly linearly related to either the reciprocal or the square root of shear force than to shear force itself.

INTRODUCTION

An extended investigation of some of the factors likely to affect meat tenderness has been initiated in this laboratory. It was realized that some aspects of the proposed study would be amenable to sensory evaluation while others would require an objective assessment of tenderness. It was of considerable importance, therefore, to establish at the outset that the two methods were, in fact, measuring the same quality, and doing so with comparable sensitivities.

This paper, the first of a continuing series, is concerned with descriptions of the cooking methods for beef and lamb used in this and later studies, and with the methods employed in tenderness assessment. Evidence is presented of the correlation found between sensory and objective methods of evaluation, for on this will depend many of the results of later papers.

EXPERIMENTAL

Meat. Beef "neck muscles" (sternomandibularis muscles) were obtained within 20 min of slaughter from a variety of beef animals, including large bulls when an experiment called for many samples from one animal. The neck muscle is particularly valuable in meat investigations, being straight-fibered, available very soon after death without mutilating the carcass, and of little commercial value. Almost invariably it has an initial pH characteristic of a well-rested muscle (7.3–7.5) and a moderately low ultimate pH (5.4–5.7), these factors ensuring a prolonged prerigor phase (Bate-Smith and Bendall, 1949).

The lamb rib-loins studied were from animals 4–9 months old and of predominantly Southdown and Romney breeds. To the point of weighing of the dressed carcass, 20–30 min post-mortem, the lambs followed normal slaughter-house procedure. Beyond this point, different experimental cooling and freezing patterns were imposed. The carcasses, wrapped in single stockinette bags, were then stored 5–8 months at −12°C (except for the lambs of series II and VIII, which were stored for only 1–4 weeks). Rib-loins (pin-bone to ribs 5–6) were cut from the frozen carcasses, and the breast (flap) of each was trimmed to leave an exterior surface 7 in. wide. In any one series, rib-loins from only one side of the carcasses were used, and only 12 carcasses, enough to satisfy requirements for a week, were cut at any one time. The rib-loins were labeled, wrapped separately in polyethylene sheets, and stored at −10°C.

Cooking procedures. *Beef neck.* To simulate the time-temperature relationship of a roasting treatment despite the small size (80–200 g) of the samples, the following procedure was devised. The samples, thawed for 16 hr at 2°C if previously frozen, were placed in polyethylene bags. The bags were then vacuum-shrunk on the samples, sealed, and immersed in water (8 L) in an open cylindrical glass tank (diam. 24 cm) heated by a Tempunit heater (1070 watts; Techne Ltd., Cambridge, England). After an equilibration period of 1 hr at 30°C, the thermostat was raised to 80°C, and exactly 1 hr later the samples were removed and cooled in running tap water. The initial bath temperature of 30°C was selected because it could be achieved the year around without supplementary cooling equipment. The cooking period of 1 hr was dictated by the finding that in this time (± 5 min) the internal meat temperature reached 80°C. This simple and reproducible procedure can be used for any small-scale cooking, including that of excised lamb muscles, and is being employed in studies to be reported later.

Lamb cuts. Before each cooking period the required number of polyethylene-wrapped rib-loins were thawed for 24–28 hr at 5°C. The psoas muscle was removed from each to assist even cooking of the longissimus dorsi muscle (LD), and thermo-

couples were inserted in the LD ¾–1 in. from the vertebrae and one-third of the length of the cut from each end. The cuts were supported by stainless-steel wire racks in aluminum pans, and roasted in separate preheated household ovens fitted with thermostat-controlled radiant elements. Mean oven temperatures during cooking were 163±5°C. Oven and internal meat temperatures were read on a thermocouple galvanometer every 10 min until a temperature of 75°C was reached, and then every 2 min; each roast was taken from the oven when the lower of the two internal temperatures reached 80°C. The LD was removed 1 hr later, wrapped in aluminum foil, and stored overnight at 5°C.

Sensory assessment. Eight panel members were selected initially for ability to detect tenderness differences produced by simmering ½-in. cubes of lamb LD for 15, 30, and 50 min. The final six members were chosen from these for ability to reproduce scores for similar samples on different days.

Tasting sessions were held midmorning and, when necessary, midafternoon, the judges refraining from food, drink, and tobacco during the preceding 30 min. The samples were served on coded plates 1 hr after removal from overnight storage at 5°C. Tasters occupied individual booths, the lights of which were fitted with red filters to mask occasional slight differences in pigment content. Tap water at room temperature was available to remove lingering flavors of previous samples.

At each session, 4 LD muscles were tested in Series I, II, IV, V, and VII, 3 in Series VI, and 2 in Series III. Each taster received four adjacent ½-in. cubes from the same position in each LD (Fig. 1). To reduce the problem of tenderness variation along the muscle, the allocation of sample positions to panel members was changed for each session so that, after 6 tasting days, each taster had received samples from all 6 positions. In addition, the order of presentation of samples was randomized for each day. During each series, performance was checked by presenting adjacent samples of the same LD coded as if to represent different randomized samples. The judges were asked to assess tenderness on a scale ranging from 1 (very tough) to 9 (very tender).

Objective assessment. From the central area of the cooked, cooled LD of lamb (Fig. 1), or of the beef neck sample after cooking by the water-bath method, a section 15 mm wide and parallel to the fibers was cut with a double-bladed scalpel (Love, 1958). A slice was cut from this section about 5–8 mm thick and 3–6 cm long, the longer axis parallel to the fiber direction.

Tenderness was assessed (in Series I–IV) in the apparatus of Nottingham (1956); it is based on that described by Winkler (1939), who simplified the apparatus devised by Volodkevich (1938). A blunt brass wedge is forced by a linearly-increasing load (in our equipment applied by a stream of mercury) through the section of cooked meat, the leading edge of the wedge being at right angles to the fiber axis. The time required (sec) to cleave the sample provides a convenient relative measure of toughness, a clear end-point being obtained at final cleavage after an initial compressive phase. In contrast to Shrimpton and Miller (1960), we have found that sensory assessment correlates as well with this simple linear measure as with the work done in cleaving the sample, which requires the measurement of the area under the force-penetration curve.

The success of this instrument early in the investigation prompted the construction of a much-improved version (used in Series V, VI and VII). In this, the increasing load is applied by a weighted trolley propelled along a beam by an electrically-powered screw-drive (Macfarlane and Marer, 1966).

Proved very useful in initial calibration trials and periodic reproducibility checks was ethyl cellulose ("ethocel" hot-melt coating, experimental resin X-2203.10, Dow Chemical Co.). It is homogeneous, stable, thermoplastic, and easily cut: in contrast to a number of other materials tested, it has a compression-cleavage pattern very similar to that of cooked meat, with a sharp end-point. Ethocel has proved markedly superior to other materials suggested as calibration standards (Spencer *et al.*, 1962).

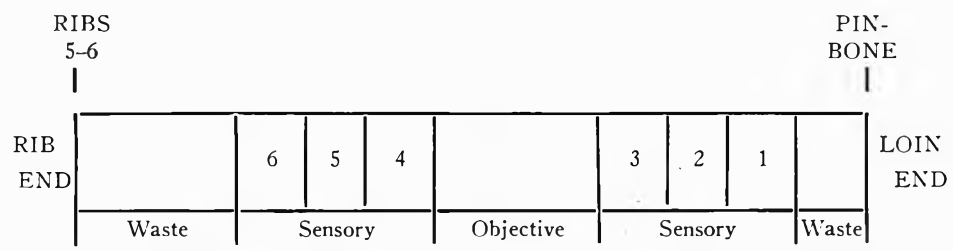


Fig. 1. Allocation of samples of lamb longissimus dorsi muscle for sensory and objective evaluation.

RESULTS

Reliability of the tenderometer. Several experiments were conducted to assess both the reproducibility of the objective test and the variability of tenderness within a single muscle. The results are given in Table 1.

The very low coefficient of variation observed when ethocel was sheared demonstrates both the excellence of this substance as a standard and the consistent results to be expected of this type of tenderometer. The result may be compared with that of Spencer *et al.* (1962), who found coefficients of variation of 6.6 and 13.5% for beeswax and plastic modeling clay, respectively, sheared in a conventional Warner-Bratzler apparatus, and of 6.6 and 10.6% in their modified apparatus.

Beef neck muscle is seen from the table to be a material almost as uniform in its resistance to shearing as ethocel, and consequently is ideally suited to the needs of our proposed studies on tenderness. Lamb LD, on the other hand, showed considerable variation in tenderness within a single muscle, a feature noticed particularly in those specially treated to produce tougher meat (see later papers). A range of shear values covering 6 times the minimum value has been occasionally observed within a single LD; such a case is illustrated in Fig. 2, together with one showing the more usual tenderness pattern produced by normal post-mortem processing.

In view of the high degree of reproducibility found in experiments with ethocel, it is concluded

Table 1. Reproducibility of objective results by multiple shearing of single samples.

Material	Tenderometer	No. of shears	Range	Mean ± SD	CV %
Ethocel	Original	20	27.5–30.5	29.0 ± 1.0	3.5
Beef neck A	Original	8	33.2–41.2	38.2 ± 2.9	7.6
Lamb loin	Original	14	17.2–36.2	25.5 ± 6.3	24.7
Beef neck B	Improved	26	37.8–50.0	44.1 ± 2.7	6.1
Beef neck C	Improved	18	29.0–33.0	31.3 ± 1.1	3.5

SD, standard deviation.
CV, coefficient of variation.
A, B, C are 3 different animals.

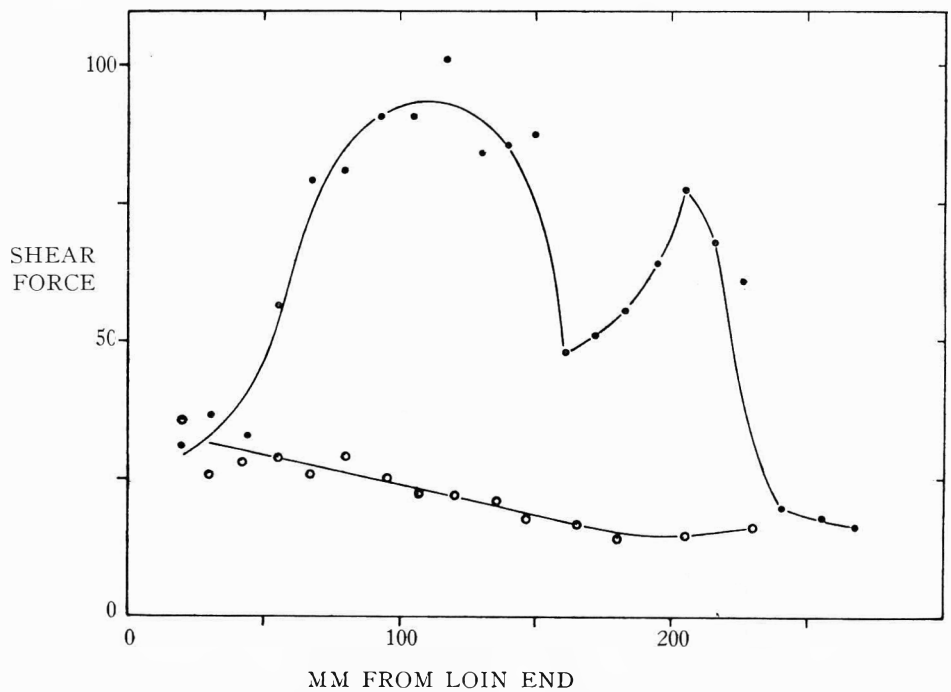


Fig. 2. The pattern of variation in resistance to shearing force within two lamb longissimus dorsi muscles.

that the variations observed within single muscles—small in beef neck and large in lamb LD—are genuine, and cannot be ascribed to erratic behavior of the tenderometer. Conversely, the detection of such variations within a lamb muscle indicates that the small coefficient of variation observed with ethocel or beef neck is not due to a low instrument sensitivity.

The effect of sample thickness on resistance to shearing was investigated, using the very nearly uniform muscle of beef neck C in the first table. The mean (±SD) of 12 shears on samples 3.9–4.5 mm thick was 31.3±1.2, while that of 6 shears on samples 9.0–9.5 mm thick was 31.3±0.8. However, 6 shears on samples only 2.7–3.2 mm thick averaged 25.2±1.6. Within limits, therefore, thickness does not affect resistance to shear, and the range of 5–8 mm selected for this work is well within the zone which is independent of thickness. This conclusion was found to apply also to lamb LD; no significant difference in shear value was detected between two groups of 7 samples each from the same muscle, the mean thicknesses of the groups being 3.5 and 8.0 mm.

Correlation between panel and tenderometer. During a period of three years, 372 direct comparisons were made between the tenderness of lamb rib-loin as judged subjectively and the force required to shear the same meat as assessed by tenderometer. In this time the composition of the panel altered, and for this reason the comparisons are presented as separate results based on seven distinct series of comparisons; for each of the seven series, panel composition was constant, but for the final six-membered panel only two of the original judges were still available.

In each of the seven series a direct plot of panel score against shearing force produced a scatter about a curve which could be fitted reasonably and almost equally well by equations of the form

$$S = a + \frac{b}{F}$$

or

$$S = c - d\sqrt{F},$$

where S = mean sensory score and F = force required to shear the sample. This is illustrated in Fig. 3, in which S is plotted against F, $\sqrt{F}$, and $1/F$ for the smallest of the seven series. It will be seen that the curvature of the relationship between S and F has been reduced by plotting S against $\sqrt{F}$ and eliminated by plotting S against $1/F$. Similar relationships were found in the other six series of comparisons, though with somewhat greater scatters; in all cases, plots of S against F were pronounced curves, whereas those of S against $\sqrt{F}$ and S against $1/F$ were much nearer to linearity. Neither of these two latter plots, however, was consistently better than the other, and for this reason correlation coefficients have been calculated for both relationships (Table 2).

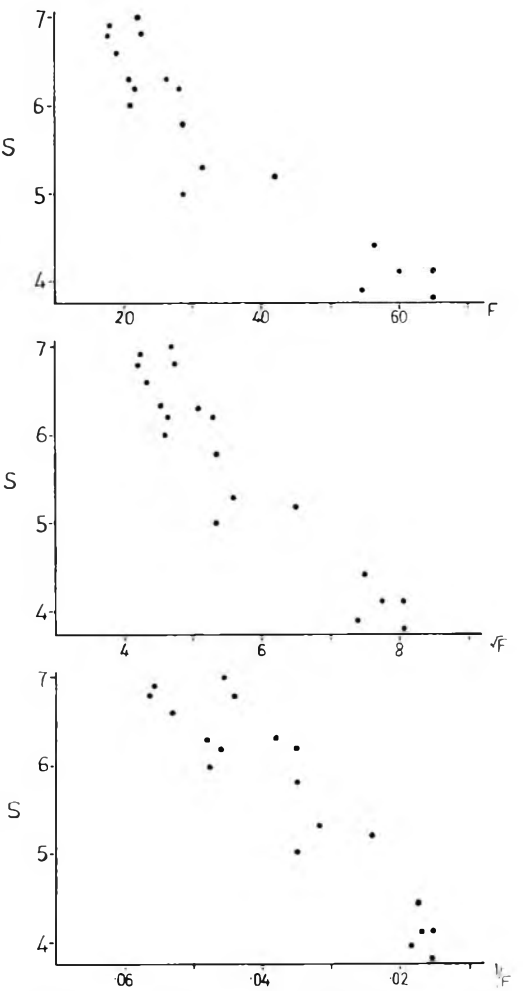


Fig. 3. The relation between sensory score (S) and functions of shearing force (F) in Series III.

In five of the series (II–V and VII) the tenderness spread was extended by special toughening treatments. That the high correlations were not a consequence of this artificial elongation of the range, however, is shown by the results of the two series in which no toughening treatment was applied. The highest shear value recorded in series I was only 31, whereas in series VI only two samples required forces exceeding 40, a value which, in our experience, is still reasonably tender. Despite these limited ranges of tenderness, however, very satisfactory correlations were observed.

Although all correlations were gratifyingly high, we believe that results with this tenderometer

Table 2. Correlations of sensory score and shearing force for roasted lamb rib-loins.

Series	No. of comparisons	Days from first to last panel	Range of scores (S)	Range of shear values (F)	Correlation, S with: $1/F$	Correlation, S with: $\sqrt{F}$
I	48	56	4.8–8.0	31–12	+.740	−.741
II	60	30	2.3–7.2	98–19.5	+.723	−.682
III	19	7	3.8–7.0	65–18	+.934	−.946
IV	74	38	3.2–8.3	83–11.5	+.760	−.729
V	60	28	1.8–7.7	112–13	+.817	−.893
VI	71	38	2.8–7.7	54–4	+.749	−.775
VII	40	23	2.4–6.5	87–22	+.755	−.735

would have been even more highly significant had the muscle selected for study been more uniform in tenderness than lamb LD. As earlier shown, this muscle may be highly variable, and consequently it is by no means an ideal material if the production of a high correlation is the sole aim.

A second reason for our inability to achieve more nearly perfect correlations is revealed by a more detailed study of the results. In the small series (III) of 19 comparisons, requiring only five tasting sessions spread over seven days, the correlation was considerably higher than in any of the larger series, the assessment of which occupied periods of 3–8 weeks. This suggests the possibility of minor fluctuation of panel scores in the more extended series, and in three of them there is evidence to support this view.

For each sample a score was calculated from the shearing force by reference to the regression equation of S on $1/F$. The difference between the calculated score and that actually found ($\Delta S = S_{observed} - S_{calculated}$) is shown in Table 3 in relation to the order of assessment. In series I, a highly significant increase in severity of judgment occurred about one-third of the way through the assessments. In series IV, an alternation of lenient and severe phases of tenderness assessment was apparent. In series VI, a prolonged phase of steady judgment was preceded by a period of leniency, but toward the end a considerable increase in the severity of assessment became apparent.

Table 3. Fluctuation of sensory tenderness judgment.

Series	Sample numbers (sequentially)	ΔS (mean ± S.E.)	Significance of difference
I	1–16	+ 0.46 ± 0.10	
			$p < .001$
	17–48	− 0.22 ± 0.10	
IV	1–16	+ 0.65 ± 0.14	
			$p < .001$
	17–40	− 0.42 ± 0.12	
			$p < .001$
	41–52	+ 0.47 ± 0.19	
			$p < .01$
	53–74	− 0.26 ± 0.15	
VI	1–15	+ 0.95 ± 0.19	
			$p < .001$
	16–57	− 0.09 ± 0.10	
			$p < .01$
	58–71	− 0.72 ± 0.13	

SE, standard error.

DISCUSSION

The mere establishment of a correlation, even though of very high significance, is not necessarily sufficient for the satisfactory prediction of taste-panel assessments from mechanical measurements (Shrimpton and Miller, 1960). It has been calculated for Series IV, for instance, that a correlation coefficient of only 0.38 would have been very highly significant ($P = .001$), yet such a scatter would have allowed only very poor estimation of panel reaction from tenderometer results. The coefficient of 0.76 actually found in that series, on the other hand, permits the prediction of sensory score with a relatively high degree of accurary: 78% of the samples were judged within one unit of the values calculated from shear force values, and in only 4% was the error greater than 1.5 units.

It is concluded that a highly satisfactory degree of correlation exists between sensory and objective methods which will be used separately in later studies, and that tenderometer results can be accepted with confidence as a reflection of taste-panel reaction.

REFERENCES

Bate-Smith, E. C., and J. R. Bendall. 1949. Factors determining the time course of rigor mortis. *J. Physiol.* **110**, 47.

Love, R. M. 1958. Measurement of toughness in cold-stored fish. *Nature* **182**, 108.

Macfarlane, P. G., and J. M. Marer. 1966. An apparatus for determining the tenderness of meat. *J. Food Sci.* (submitted.)

Nottingham, P. M. 1956. The connective-tissue content and toughness of sheep muscles. *J. Sci. Food Agr.* **7**, 51.

Shrimpton, D. H., and W. S. Miller. 1960. Some causes of toughness in broilers. II. Effects of breed, management and sex. *Brit. Poultry Sci.* **1**, 111.

Spencer, J. V., M. Jacobson, and J. T. Kimbrell. 1962. Recording strain-gage shear apparatus. *Food Technol.* **16**(2), 113.

Volodkevich, N. N. 1938. Apparatus for measurements of chewing resistance or tenderness of foodstuffs. *Food Research* **3**, 221.

Winkler, C. A. 1939. Tenderness of meat. I. A recording apparatus for its estimation, and relation between pH and tenderness. *Can. J. Research* **17**D, 8.

Ms. rec'd 7/2/65.

The authors are grateful to the Auckland Farmers' Freezing Co-operative Limited for providing meat and works' facilities, to Mr. K. E. Jury (Ruakura Agricultural Research Centre) for advice on statistics, to Mrs. D. R. K. Smith and Mrs. H. A. Swarbrick for technical assistance, to Messrs. P. G. Macfarlane and J. M. Marer for constructing the tenderometer, and to the staff members who served on the taste panels.

DAVID A. KENDALL and ANNE J. NEILSON
Food and Flavor Section, Life Sciences Division, Arthur D. Little, Inc.
Cambridge, Massachusetts 02140

Sensory and Chromatographic Analysis of Mixtures Formulated from Pure Odorants

SUMMARY

Continuing the leads from previously published studies of four chemicals having different odors but similar molecular weights and boiling points, this work centers on sensory examinations of the vapors over essentially water solutions of GLC-purified anethol, geranial, methyl salicylate, and safrol. Experienced profile-trained panelists examined the odors from two- and four-component mixtures of the odorants at multiple decimal fractions of odor-recognition threshold concentrations in terms of molarity. Findings for the two-component mixtures showed these possible phenomena: 1) intensification of one odorant with suppression of the other; 2) increase in total intensity of odor; 3) decrease in total intensity with development of a different odor character (i.e., blending); and 4) infrequently, no describable difference (i.e., no interaction). Interactions were also found for the four-component systems, which, because of greater complexity, more frequently showed tendencies toward blending. Sucrose, salt, and monosodium glutamate were added separately at their food-use levels (respectively 10, 1, and 0.1%) to a quaternary mixture and were found to have different effects. Sucrose emphasized the mixture's lemon-candy identity; salt clouded the solution and emphasized its root beer character; MSG disrupted the blend. In addition to these nonvolatiles, two odorous substances known to have odor-blending or fixative properties were examined for recognition threshold and effect on the quaternary blend when added at 1/10 their threshold concentrations. Nonalactone, at 1/10 10^{-8} g/ml, increased the soapy-oily character of the geranial. Exaltone®, at 1/10 10^{-9} g/ml, blended the odor still further. The minimum change in concentration to give a detectable difference in odor strength (just noticeable difference) was determined for geranial at the profile intensity range of moderate. The jnd was found to be of the order of $10^{-0.2}M$, representing about a 60% increase in concentration, which coincides with the 58% increase cited by other workers.

INTRODUCTION

This work is one increment of our basic odor research program, initiated at ADL in the 1920's by the late Ernest Crocker and continued as time has permitted in the succeeding years. It is a continuation of that reported at the Gordon Conferences in 1962 and at the N. Y. Academy of Sciences Odor Symposium in 1963 (Kendall and Neilson, 1964). The program objective is to obtain meaningful information on sensory perceptions. Threshold concentrations, intensity measurement in terms of just noticeable differences (jnd), odor quality and description, and the effects of impurities and known mixtures on the perception of odor quality and intensity are all important facets which must be defined before adequate theories on the mechanism of olfaction, for example, can be postulated. Modern instrumentation can be helpful in developing meaningful data, but at present it cannot supplant the abilities of humans in odor and flavor measurement. Previously, we reported data on: 1) the relative sensitivities and analytical qualities of human olfaction and gas liquid chromatography (GLC); 2) the effects of purification on odor recognition thresholds of four selected odorants; and 3) the odor effects of threshold, sub- and suprathreshold quantities of one odorant on a threshold quantity of another. Besides developing proper techniques for conducting further work, these studies provided an approach for probing into the quantification of sensory odor intensity measurements and the impetus for learning more about what happens to specific odors when odorants are mixed at various ratios of their recognition threshold over a range of concentration. By this approach we hoped to approximate formulations on the basis of odor strength. For this work we used the following four chemical compounds purified by GLC: anethol, geranial (i.e. citral A), methyl saliclyate, and safrol. These had been selected for the prior studies because they are useful flavoring materials, can be isolated from natural essential oils or produced synthetically, represent a range

of odor types, and have similar elemental compositions, molecular weights (148–162), and boiling points (224–233°C).

EXPERIMENTAL PROCEDURES

Purification of odorants. The odorants were purified chromatographically by collecting the major component in all-glass traps cooled with dry ice–acetone. Chromatography was carried out with a Barber Coleman instrument using a 10-ft glass column packed with 5% Carbowax 20M on Chromosorb W. Temperatures ranged from 125 to 150°C. The argon flow rate was approximately 130 ml/min. Conditions were varied for each odorant to get optimum separation from impurities. The major component of the impure material was collected from multiple 10-μl injections and eluted from the glass trap with absolute ethanol. The purity and concentration of the odorant in the collected fractions were determined by chromatographing aliquots of the sample and comparing peak areas with calibration curves.

Molar dilutions of odorants. The concentration of purified odorant in solution was adjusted to 0.1M with absolute ethanol, and these purified samples were stored at 0° until used. Subsequent solutions of single odorants were prepared at 100 times threshold (approximately 10^{-4} molar) by dilution in ethanol and water so that the final alcohol concentration was 0.1%. Serial dilutions were then made with 0.1% ethanol so that the ethanol content was kept constant.

The selection of a diluent for this type of work presents considerable problems, particularly from the standpoint of odor contribution. Ethanol at 0.1% in water was not itself apparent in the odor studies although it must be considered as a factor in the solution concentrations reported for odorant thresholds. The ethanol concentration was the minimum possible to maintain apparent solution of anethol (as well as the other three odorants) at its maximum concentration (i.e. $10^{-4}M$). The ethanol contributed no interference by odor or GLC and we observed no interactions with the odorants whose solutions were stored properly and used promptly. Of the several diluents previously surveyed, the water-ethanol system was the most suitable and was superior to repurified hexadecane, used in the original studies, with regard to odor and GLC behavior.

Binary mixtures were prepared by measuring a calculated amount of 0.1 molar solution of purified odorant to obtain the desired multiple of threshold concentration. In one series the odorants were each present at threshold concentrations, and serial decimal dilutions were made with the maximum concentration at 100 times threshold. In subsequent series the odor concentration of one odorant was 10 times or 100 times as strong as that of the other odorant.

The quaternary mixtures were prepared by adding a calculated amount of 0.1 molar solution of each odorant and adjusting the concentration with ethanol to obtain 0.1% ethanol solutions.

For the triangle tests a 10^{-4} molar stock solution of geranial was prepared in 0.1% ethanol, and the desired solutions made by dilution.

The solutions were presented to the odor panel in 250-ml Erlenmeyer flasks covered with watch glasses. Each flask contained 25 ml of solution, and the solutions were allowed to equilibrate at 70°F for 1 hr prior to panel examination. For profile analyses each panel member was given an individual set of samples.

Panel operations. The odor examinations were carried out in air-conditioned panel rooms maintained at 70°F. All the panel members are on the technical staff of our flavor laboratory, which means that they are schooled in analytical techniques for both odor and flavor measurement. Ten panelists represented as little as 3 and as much as 15 years of odor-measurement experience. It is understood that all glassware was odor-free and that other extraneous variables were minimized.

For triangle testing, 10 panel members were used. For odor profile analyses the panel consisted of four of us. Briefly, an odor profile is a tabular report of the total intensity of odor and a chronological listing of terms describing the separately distinguishable odor-integers and the intensity at which each was perceived. The descriptive terms have the same meaning to all panel members and the intensity values are usually recognition threshold, slight, moderate and strong. Intensity symbols are)(, 1, 2, and 3, respectively. Obviously, the only point on the intensity scale is recognition threshold. The others are ranges, which a part of the work reported below attempts to define.

GLC analyses. In addition to the odor panel analyses, liquid and vapor samples of the four pure odorants were analyzed chromatographically with a MicroTek 2500 R, with flame ionization detector and a 6-ft column packed with 5% Carbowax 20M on Chromeport. The helium flowrate was 100 ml/min @ 150°C. Ten microliters of liquid sample or 10 ml of vapor from a closed 250-ml Erlenmeyer flask were injected. Under these conditions, it was possible to measure anethol and safrol in the vapor samples over 10^{-4} and $10^{-3.75}$ with retention times of 10 and 10.5 minutes and possible to detect them in vapor samples of 10^{-5} and $10^{-4.75}$, respectively. Geranial was just detect-

able over $10^{-1.25}$, while the results with methyl salicylate were inconsistent with vapor samples over 10^{-1}. We have not explained the difficulty with methyl salicylate here, or with hexadecane solutions in our previous studies.

With liquid samples, the limit of detectability was 100× threshold, or approximately $10^{-4}M$ for all odorants. For geranial, 10 μl of $10^{-4}M$ gave a peak of 1 cm, representing approximately 2 nanograms.

The sensitivity of our instrumentation precluded the possibility of demonstrating any changes in vapor samples around threshold for these particular compounds, limiting its usefulness to measuring anethol and safrol at only the highest concentration studied.

RESULTS AND DISCUSSION

Determination of jnd for geranial. As previously reported, odor recognition threshold is obtained in the vapor above a geranial solution at $10^{-6.25}$ molar. Triangulation tests on geranial were carried out at three odorant concentrations—$10^{-5.8}$, $10^{-5.0}$, and $10^{-4.0}M$—to determine whether there is a variation in the concentration needed to detect a difference in odor at various odor strengths. The results of the triangulation tests showed the following:

Not significantly discriminable

$10^{-5.8}$ vs. $10^{-5.7}M$

$10^{-5.8}$ vs. $10^{-5.65}M$

Significantly discriminable

$10^{-5.8}$ vs. $10^{-5.6}M$

$10^{-5.0}$ vs. $10^{-4.8}M$

$10^{-4.0}$ vs. $10^{-4.2}M$

This means that: 1) over a range of about two powers of 10, and the jnd is reasonably constant; and 2) the jnd for geranial at a moderate intensity is $10^{0.2}$ (this represents approximately 60% increase in concentration).

Hainer *et al.* (1954) cited work of Currie and Sjöström which defined the jnd values for anethol and isovaleric acid preparations in mineral oil. Their jnd's, also determined statistically in the middle of the perceptual range, required a 58% increase in concentration.

Binary mixtures. Binary combinations were prepared for all possible pairs of the four odorants. Each pair was examined in five different ratios based on the threshold concentration of each odorant alone. The concentrations used for each odorant are listed in Table 1. Table 2 shows the concentration range over which each odorant pair in its various ratios was studied.

The resultant odor from a mixture of two odorants can be expected to be one of the following phenomena: a suppression of total odor; and intensification of one component's odor; or a blend or complex in which most, if not all, of the qualities of each odor are no longer identifiable as such.

Rather than describe the results of all of these studies we have picked two binary mixtures which represent two extremes of the kind of interaction observed. The first is the combination of geranial with anethol, in which we find a minimum of interaction in producing blends or new odors but rather an intensification or enhancement of the odor level. In combinations of 10:1, 1:1, and 1:10 the odors of both components are detectable and recognizable as such at all concentrations. When the concentrations were 1:100 and 100:1 the minimum component was not recognizable as such, but there was an increase in strength at the threshold of the major component. Where the two compounds were in equal odor strength concentration, geranial was recognizable at 1/100 of its threshold concentration in pure diluent. This is consistent with studies by Baker (1964), reported most recently at the NYAS

Table 1. Concentrations of odorant solutions based on threshold concentrations.

	Molar concentrations				
Purified odorants	T[a]	100T	10T	T/10	T/100
A = anethol	10^{-9}	10^{-7}	10^{-8}	10^{-10}	10^{-11}
G = geranial	$10^{-6.25}$	$10^{-4.25}$	$10^{-5.25}$	$10^{-7.25}$	$10^{-8.25}$
M = methyl salicylate	10^{-6}	10^{-4}	10^{-5}	10^{-7}	10^{-8}
S = safrol	$10^{-5.75}$	$10^{-3.75}$	$10^{-4.75}$	$10^{-6.75}$	$10^{-7.75}$

[a] Recognition threshold; from Kendall and Neilson (1964).

Table 2. Binary mixtures studied for the six possible pairs.

Ratios	Combinations				
1:1	100T[a] + 100T	10T + 10T	T + T	T/10 + T/10	T/100 + T/100
100:1	100T + T	10T + T/10	T + T/100		
10:1	100T + 10T	10T + T	T + T/10	T/10 + T/100	
1:10		10T + 100T	T + 10T	T/10 + T	T/100 + T/10
1:100			T + 100T	T/10 + 10T	T/100 + T

[a] Recognition threshold.

Odor Symposium (1963). The second example of binary mixtures was the combination of methyl salicylate and anethol in the same range of combinations and concentrations. With 1:1 mixtures of these two components the odor produced was similar to that of safrol with minor characteristics of both methyl salicylate and anethol. The recognition threshold for anethol in this combination may have been slightly reduced from that of the pure compound, but the odor characteristic for this concentration was covered by fragrant, floral aromatics. In ratios of 1:10 and 10:1 (10T + 100T and 100T + 10T), both components were detectable, but at 10× threshold for the maximum component (10T + T and T + 10T), new blends appeared which were characterized as musty, fragrant, and fruity. The addition of methyl salicylate to anethol in the ratios 10:1 and 100:1 introduced pain sensation or pungency, particularly at the high concentrations, with little of the odor quality characteristic of methyl salicylate; the addition of anethol to methyl salicylate in the same ratios also intensified the pungency but altered the characteristic odor of the methyl salicylate by introducing a rooty, woody note. In these instances the detection threshold for the major component, particularly where it was methyl salicylate, was raised above that for pure methyl salicylate alone, i.e., methyl salicylate not recognizable at 10^{-6}, which corresponds to the effect observed by Jones and Woskow (1964). It was not possible to measure chromatographically a change in odorant concentration or composition in the vapor state where sensory measurement indicated an increase or decrease in odor strength at the threshold concentration. However, Shiftan and Feinsilver (1964) suggested that the source of the odor changes produced by so-called fixatives may be related to hydrogen bondings, which can be measured instrumentally.

To summarize—with simple binary combinations (ratios of 1:1) we most frequently observed an intensification or enhancement of total odor and a lowering of the threshold of one or both components by as much as two powers of 10. This trend was also evident in both the 10:1 mixtures and the 1:10 mixtures of the two components.

A different situation was also observed where the odor character of one component dominated that of the second component even when the combination was T/10 + T. Finally, we observed a third situation where different odor characteristics were produced: for example, in 10:1 and 1:10 combinations of safrol and methyl salicylate, from which the odor characteristics perceived were associated with other odorants not present in the system, and with 1:1 combinations of methyl salicylate and anethol, which resulted in a floral fragrance uncommon to either component. These we call blends. Where such blending occurs, there can be an intensification of odor as indicated by a lowering of the threshold for odor recognition, but usually there is suppression or decrease in odor intensity evidenced by an increase in concentration of odorant required to produce a recognizable odor.

Thus, on the basis of these preliminary data it appears that when little blending occurs there may be an enhancement of intensity of one or both components. Where blending does occur and new odor types develop, there may be a suppression. Whether one can go further and say that there are both positive and negative types of blending (that is, where one has a masking of odor as compared to where one has a synthesis of new odor) is still a question. Also, it appears that there are very few

instances where one can say that there is little or no interaction between the two components.

Quaternary mixtures. On the basis of the odor results of the binary combinations, several quaternary mixtures with varying ratios were prepared with the major component(s) at a concentration equivalent to 100× threshold. Two of these combinations are taken as representative of the extremes in interaction observed in this series. The first, a simple 1:1:1:1 ratio (T+T+T+T), resulted in an enhancement of the total odor intensity in which the odor characteristics of all four of the components could be readily recognized. This increase in odor intensity apparently results from an emphasis of the sharp, harsh characteristics of each of the four components: the pungency of methyl salicylate; the sourness of geranial; the woodiness of safrol, and the sweet chemical character of anethol. Related to flavor profile terminology, this is an increase in odor intensity with a low amplitude or blend. In the chromatogram of this 1:1:1:1 solution where each component was present at 100T the anethol and safrol appeared as separate peaks, approximately 10 chart units high at the maximum operable sensitivity. The anethol peak obscured the geranial and methyl salicylate, both of which when chromatographed alone had been just barely distinguishable from the baseline. Although the chromatogram was consistent with the behavior of the pure compounds we did find a noticeable change in the sensory impression.

In the second example of quaternary mixtures (later referred to as Blend *B*), anethol and safrol were present at 1/10 the odor strength of geranial and methyl salicylate (i.e., A = 10T, G = 100T, M = 100T, S = 10T). From the binary-mixture studies we would have predicted that the lemon character of geranial would dominate the odor of this mixture. However, a strong complex, described as candy sweet, was produced which exhibited some facets reminiscent of, but not clearly identifiable as, each of the four components. An oily character note associated with citral (the parent substance from which purified geranial had been isolated) was apparent, and also a spicy, woody characteristic related to either safrol or methyl salicylate. The total intensity of this aroma was somewhat lower than would be expected of a mixture containing two odorants each at a concentration which alone produces a moderate intensity. Of all the quaternary mixtures examined, this represented the closest to a complex or blend. In chromatographing the vapors above this solution it was not possible to differentiate any of the four odorants from background.

The effects of seasoners and known odor-blenders on quaternary mixtures. *Seasoners.* Because it has been recognized that nonvolatile materials affect the taste and flavor of food, 10% sucrose, 1% salt, and 0.1% monosodium glutamate were added to aliquots of the second quaternary mixture (Blend *B*). Note that each seasoner was studied at a level conventionally used in foods or beverages, and these levels are well above the respective taste thresholds. Odor profiles of these mixtures are presented in Table 3. Except with salt there was no apparent change in the solubility of the odorants in the mixture.

The addition of sucrose suppressed anethol and safrol odors and gave the complex a definite lemon-candy connotation with some sharp sourness and a slight increase in total intensity of aroma. In retrospect, this result should not be unexpected; in addition to its obvious sweetening power for food flavors, sucrose has traditionally demonstrated salutary effects on food aromas, particularly in fruits, candies, and soft drinks (Sjöström and Cairncross, 1955).

The addition of monosodium glutamate decreased the complexity of the odor mixture and emphasized the sour, minty, pun-

Table 3. Odor profiles of blend *B* alone and with added seasoners.

Blend *B* 10:1:1:10	+ 10% sucrose	+ 1% salt	+ 0.1% MSG
TIA 1	TIA 1+	TIA 1½	TIA 1
Sweet	Sweet	Anethol	Sour
Oily	Lemon drop	Safrol	Minty
Root beer	Sour	Oily	Sweet
			Pungent

TIA: total intensity of aroma; 1: intensity symbol meaning low.

gent character. Past experience with MSG indicates that, with some food aromatics, particularly those of cooked meats, vegetables, and their derivatives, it acts as a blender, but with fruits and confections its effects may be disruptive to whatever blend exists (Cairncross and Sjöström, 1948).

Salt, as indicated above, produced a cloud in the solution, indicating insolubilities (probably anethol and safrol) with an attendant increase in the root beer character of the vapor.

Odor-blenders. In order to observe the effect of odorants which are used primarily for their blending or fixative value, odor thresholds were first determined for nonalactone and Exaltone®: 10^{-8} and 10^{-9} g/ml, respectively. These two odorants were studied at 1/10th of their threshold value to aliquots of the second quaternary mixture.

The addition of nonalactone at this subthreshold level emphasized or enhanced the soapy-oily character associated with geranial, although soapy-oily is also a characteristic of nonalactone at high concentrations. The odor complex of the mixture was destroyed almost entirely.

On the other hand, the addition of 2.5 nanograms (2.5×10^{-9} g) of Exaltone® to 25 ml of the quaternary solution (i.e., at 1/10T for Exaltone®) changed the sensory impression to one of a floral, almost rose-like, complex, with a slightly reduced total intensity of aroma. This was definitely a blending effect, created by the addition of a subthreshold level.

INTERPRETATION

While these and our preceding experiments are of more than academic interest, there are certainly insufficient data to postulate an odor theory and not a sufficient range of odorants to attempt classifications on the basis of their enhancement or blending capabilities. However, there is strong odor-panel evidence that there are various types of interactions of these four pure odorants which are not defined by measurable changes in the chromatographic behavior of the components. Possibly the least frequent situation is where no sensorially apparent interaction occurred. Most frequently we observed that interactions resulted in an increase of the odor intensity of one or both components, with little change in the character of the odor. When another type of interaction (i.e., blending) occurred, we most often noted a decrease in the total odor intensity and concomitant alteration in the character of the odor. This type of action increases in frequency as the mixtures become more complex, as in the quaternary mixtures. This was evident in earlier work done at ADL and reported by Sjöström *et al.* (1957), and may in part explain the difficulty of achieving odor blends with synthetic materials in relatively noncomplex mixtures.

It is impressive that odorants such as these can produce a considerable effect on odor when added in concentrations below their respective recognition thresholds. This suggests that the odor activity of a compound might be evaluated by first comparing the odors resulting from addition of respective quantities to produce detection threshold vs. recognition threshold in a medium with low odor intensity, and then comparing these same concentrations in a more complex medium. Further, a comparison of the jnd's in each medium would provide interesting information.

Finally, the jnd information obtained for purified geranial in a 0.1% ethanol solution nicely matches the work cited by Hainer for anethol and isovaleric acid in mineral oil dilutions and which he used to substantiate his proposed theory of olfaction.

REFERENCES

Baker, R. A. 1964. Response parameters including synergism-antagonism in aqueous odor measurement. *Ann. N. Y. Acad. Sci.* **116**, 495.

Cairncross, S. E., and L. B. Sjöström. 1948. What glutamate does in food. *Food Ind.* **20**, 982.

Hainer, R. M., A. G. Emslie, and Ada Jacobson. 1954. An information theory of olfaction. *Ann. N. Y. Acad. Sci.* **58**, 170.

Jones, F. N., and M. H. Woskow. 1964. On intensity of odor mixtures. *Ann. N. Y. Acad. Sci.* **116**, 484.

Kendall, D. A., and A. J. Neilson. 1964. Correlation of subjective and objective odor responses. *Ann. N. Y. Acad. Sci.* **116**, 567.

Shiftan, E., and M. Feinsilver. 1964. Practical

research of the art of perfumery. *Ann. N. Y. Acad. Sci.* **116**, 692.

Sjöström, L. B., and S. E. Cairncross. 1955. Role of sweeteners in food flavor. *Advances in Chemistry Series*, **108**.

Sjöström, L. B., S. E. Cairncross, and J. F. Caul. 1957. Methodology of the flavor profile. *Food Technol.* **11**(9), 20.

Ms. rec'd 5/29/64.

The authors acknowledge particularly the help and cooperation of Dorothy L. Jones and Sara B. Irons, members of the Food and Flavor Section of Arthur D. Little, Inc., and of Theodore E. Dickelman, a member of the Analytical Section of Arthur D. Little, Inc.

LLOYD M. BEIDLER
Department of Biological Sciences, Florida State University
Tallahassee, Florida 32306

A Physiological Basis of Taste Sensation

SUMMARY

Taste stimuli are adsorbed to the receptors of taste buds. This leads to a change in the receptor membrane permeability and an excitation of the taste nerves. The quantitative relation between the magnitude of taste receptor response and the concentration of the stimulus can be expressed by a hyperbolic equation. Application of this equation to human taste responses is useful for further predictions and generalizations.

Tastes and smells of the environment greatly affect mammals in many ways. It has been shown that certain tastes and smells are important not only in the appreciation of food but also in the detection and obtainment of food, attraction of one sex by another culminating in reproduction, and, in the mouse, even the inhibition of pregnancy (Parkes and Bruce, 1961). Tastes and odors have also been used by some animals as a means of communication (Wilson, 1963). The primary use of tastes and smells by man is in the appreciation of food flavors.

Describing a pleasant flavor is a problem similar to describing a beautiful painting. The painting cannot be described in terms of intensity of light or the amount and colors of paint used by the artist. Similarly, the flavor of food cannot be adequately described in terms of the kinds and amounts of chemicals making up the flavor. It has been useful, however, to consider the ingredients of a flavor in terms of the various types of taste, odors, and other sensations that can be described by an individual. Thus, the profile method of flavor analysis originated by A. D. Little is based on a knowledge of the sensations associated with a flavor rather than a listing of the types of chemicals making up the taste or odor stimulus. The flavor profile method is useful to those interested in improving food products but of little value to the laboratory scientist who wishes to know how the taste or olfactory receptors function. Let us simplify the flavor problem and consider only how taste receptors may respond to chemical stimuli. After obtaining a better view of how these receptors work, the more complex problem of food flavor can be considered.

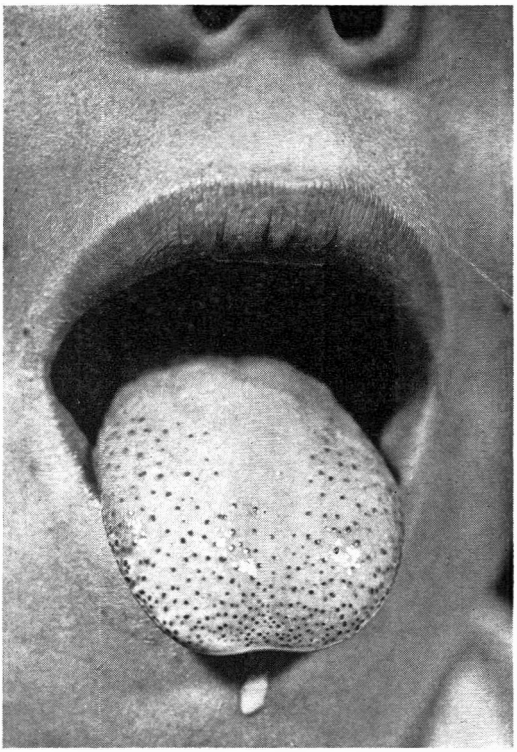


Fig. 1. Tongue of young child showing fungiform papillae as red spots each of which contains 3 to 5 taste buds.

Taste is that sensation produced when food is taken into the mouth and stimulates the receptors of the taste buds. Taste is often simply described by the four classical qualities—sourness, bitterness, saltiness and sweetness. The organs of taste concerned with the four qualities are clusters of receptor cells located in the taste bud, most of which are found on the surface of the tongue (see Fig. 1). Autoradiographic studies using tritiated thymidine, a component of DNA, suggest that these cells are continually being reproduced and destroyed so that an average individual taste receptor lives only about ten days (Beidler and

Smallman, 1965; Beidler, 1961). In this manner, damage to the taste buds during normal use can be repaired.

The individual taste cell within the bud is like a living battery with the source of electrical energy being an uneven distribution of potassium and sodium ions across the cell surface. The inside of the cell is electrically negative with respect to the outside (see Fig. 2). Each cell has finger-like projections, taste microvilli, that extend into the saliva coating the tongue where contact is made with various food chemicals. These chemicals are adsorbed to specific sites on the microvilli, slightly changing the spatial arrangement of the molecules making up the surface of the taste receptor.

In some unknown manner, the influence of the filled sites is projected further down the surface of the receptor cell where minute holes are created in the 75 Å lipid-protein cell membrane. The Na^+ and K^+ ions leak through these holes and exchange with similar ions in the thin layer of solution encircling the body of the receptor cell. This increase in ion permeability of the receptor cell membrane results in a decrease in the electrical voltage normally present across the receptor cell membrane. The magnitude of the voltage change is proportional to the number of microvilla receptor sites filled by the taste molecules, the maximum change being about 30 to 40 mv as measured by microelectrodes inserted into the cells (Kimura and Beidler, 1961; Tateda and Beidler, 1964). This voltage change is associated with a change in current which crosses and thereby stimulates the nerve endings that are closely encircled by the receptor cell membrane. (Electron microscopy indicates that the nerve membrane may be separated from the receptor cell mem-

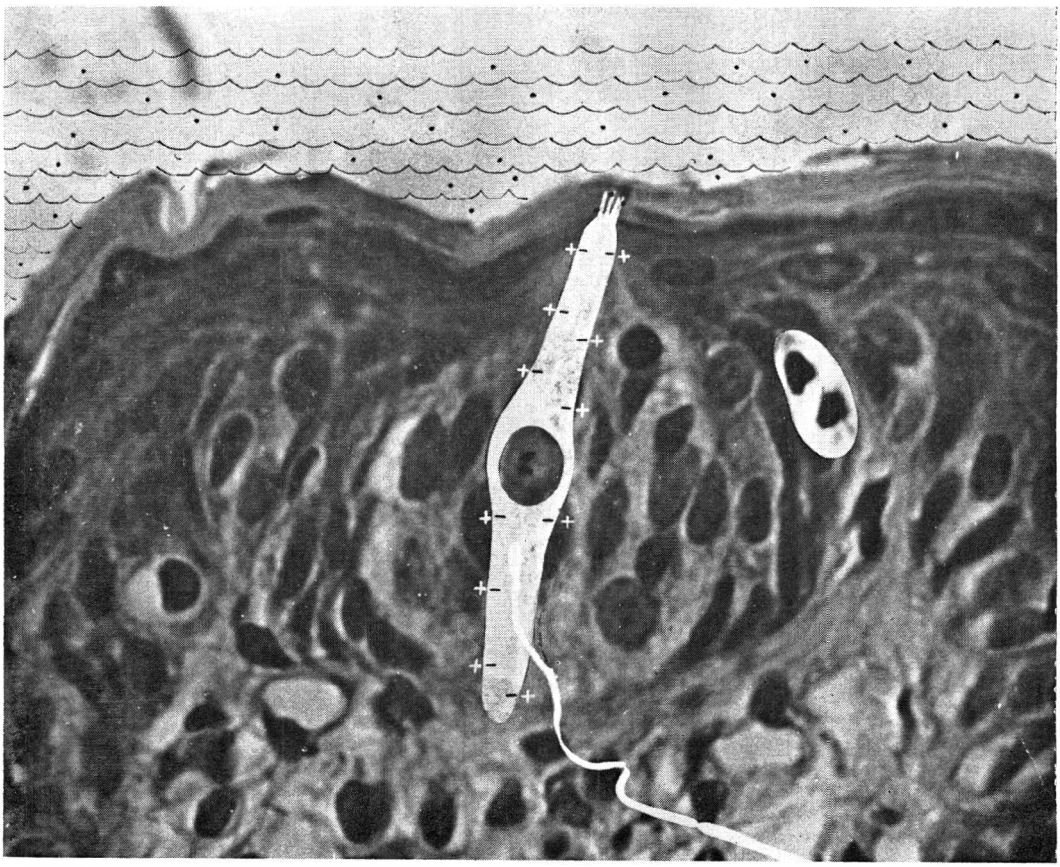


Fig. 2. Diagram showing a rat fungiform papilla taste bud with a cell bordering the taste bud undergoing mitotic division. A taste cell within the bud is innervated by a fine nerve fiber.

brane by a distance as little as 200 Å.) The nerve then develops a series of brief (1–3 msec) electrical impulses that are carried to the brain, which in turn acts as a computer to determine whether the food is salty, sour, bitter, or sweet.

There is no one taste receptor cell that responds only to acids, or another to salts, or another to sweets. Rather, most individual taste cells within a bud respond to all of the taste substances, with sensitivities differing from one taste cell to the next (Kimura and Beidler, 1961; Tateda and Beidler, 1964). Thus, it is only the simultaneous stimulation of a group of taste cells of varying sensitivities that can result in a person's knowing the taste quality of a given stimulus.

How can the experimenter measure the magnitude of the response of the taste cell to a given chemical substance? It is possible to insert very fine micropipette electrodes (0.5-μ tips) into a single taste cell and record the small change in electrical potential across the cell membrane in response to various taste stimuli. The taste nerve messages, coded in the form of short electrical impulses, can also be recorded as they travel from the taste cell to the brain (Pfaffmann, 1941; Fishman, 1957).

The quantitative relation between the stimulus and the magnitude of the response obtained from such recordings (Fig. 3) can be described mathematically. Such a description is consistent with the idea that the chemical stimulus is adsorbed to given receptor sites on the surface of the microvilli and that such adsorption initiates a series of electrical and chemical events that lead to nerve excitation. The basic taste equation is:

$$\frac{C}{R} = \frac{C}{R_s} + \frac{1}{KR_s}$$

where C is the concentration of the stimulus, R is the magnitude of response to that stimulus, and R_s is the maximum magnitude of response obtained with high concentrations of that stimulus (Beidler, 1954). The equilibrium constant of the reaction K can then be easily calculated. This equation can be applied to experimental data to determine how strong a given taste substance adsorbs to the microvilla surface of the taste cell and to obtain a relative measure of the number of taste molecules that are adsorbed at any given time. The rather weak forces involved in such adsorption are but a few kilocalories per mole (Beidler, 1954, 1961).

Fig. 3. Summated electrical activity recorded from rat chorda tympani taste nerve in response to: 0.1M NH_4Cl, 0.1M NaCl, 1.0mM Quinine, 0.5M sucrose, 0.01M HCl, and 0.1M NH_4Cl interspaced with water rinses. Time mark = 20 sec.

Such mathematical expressions are of great use when we try to understand the physiological basis of taste sensation. Measuring the electrical activity of the taste nerve as well as of the receptors themselves in a quantitative and objective manner allows us to study and compare the taste processes that exist in a large number of different species of animals. This is of particular importance when one realizes that each animal species may have taste characteristics that are quite different from man. For example, the taste sensitivity of the chicken may be quite different from that of the cat. Is it possible to perform similar electrical experiments on man and compare the results with those obtained on laboratory animals? Fortunately, the chorda tympani taste nerve passes through the middle ear on its path from the tongue to the brain and is available for experimentation during various middle ear surgeries. Dr. Zotterman (Diamant *et al.*, 1960) and his associates in Sweden have recorded sucessfully from a number of patients and have shown that the response characteristics of the human taste receptors are similar but not identical to those of other animals.

Can the fundamental taste equation which was determined from a study of the electrical responses of receptor cells in experimental animals be applied to human be-

havioral measures of taste? To apply the taste equation one must first have a measure of the magnitude of a human taste response. One such measure that has been found to be useful is the number of JND's (just noticeable differences). The first JND is that between water and a threshold concentration of a given substance. The second JND would then be obtained by using a slightly higher concentration that can just be discriminated from the threshold value, and so forth. If the number of JND's is plotted against the concentration of stimulus, the curve generated is markedly similar to that obtained with experimental animals when the magnitude of electrical neural activity is used as the measure of response. In fact, the taste equation can be applied equally as well to this type of human subjective data. Thus, we may obtain the important equilibrium constant of the reaction between the chemical stimulus and the human taste receptor. The magnitude of the equilibrium constant obtained in this way for man is similar to that obtained from experimental animals using other methods. Unfortunately, the measurement of JND's is time-consuming and only a few investigators in the past have made a series of precise and adequate measurements that can be used for such calculations. In our laboratory we are measuring JND's of young children between the ages of four and fourteen using a variety of taste stimuli. We chose to study children rather than adults since we want to use subjects that can be highly motivated. A taste-testing machine was designed that automatically dispenses taste substances into three different glasses. The subject then tastes the solution in each glass, determines which of the three is different from the other two (traditional triangle test), and pushes a button above the chosen glass (see Fig. 4). If the child makes the right

Fig. 4. Taste discrimination of young boy being determined with the aid of an automatic taste-testing machine. The recorder plots the successive concentrations of the taste solution that were tested.

choice, he is rewarded immediately with a nickel and the machine automatically changes the concentration for the next series of experiments. The taste-tester can select one out of a thousand different taste stimuli and the record of the child's choice is plotted automatically. Such measurements are dependent not only on how well the taste receptor can respond but also on the state of the human subject and how well he is motivated. The application of the theoretical taste model to human taste sensation is a great step forward because it offers a simple basis from which we can predict important characteristics of taste sensations. Most measurements obtained with experimental animals result in data that accurately describe the relative magnitudes of responses to various substances but, unfortunately, little data are obtained about the quality of taste sensation. Since the coding used by the nervous system to carry information concerning taste quality is much more complex than originally realized, a clear understanding of this matter may have to wait until more sophisticated techniques are developed to study the taste receptors and nerves. This should not be too shocking, since it is only in the past few years that scientists studying the visual system have obtained a direct proof of the way the eye codes information concerning color, even though theories relating to color vision have been prominent for many, many decades.

The adsorption of taste substances to the surface of the microvilli is dependent upon the type of receptor sites available. Some may simply be anionic sites that attract various cations such as Na^+ or K^+ which stimulate the receptor cell. Others may be quite complex and discriminate molecules on the basis of shape, such as that possessed by *d*- and *l*-amino acids. Some receptor sites may be normally filled or even hidden, available only after being exposed to specific molecules. For example, certain anionic receptor sites may complex heavy metals and are exposed only for further reactions after the heavy metals are removed when natural chelators are placed on the surface of the tongue. After this, the receptor sites would again be available for reaction with simple monovalent ions. Electrophysiological experiments with rats indicate that the response to 0.1*M* NaCl is greater after the tongue is exposed to citric acid, a natural chelator. Novel effects on foods might be expected with other natural chelators such as glutamic acid, cysteine, glycine, or certain 5′-nucleotides that form metal complexes (see Furia, 1964; Frieden and Alles, 1958; Sanui and Pace, 1965; Sato and Akaike, 1965).

The specificity of a site may be genetically controlled. Some people can taste the bitterness of all compounds containing a -C-N
||
S
group whereas other people find these compounds inoffensive. Such differences in molecular architecture of taste receptors may be related to the likes and dislikes of people to foods containing such substances.

The relation between molecular structure and taste sensation has intrigued many. Cohn (1914) listed the taste elicited by thousands of different molecules. Unfortunately, our knowledge in this area has not progressed very far since then. The reasons are simple. First, although we may know many details concerning the shape and functional properties of the stimulating molecule, similar properties of the receptor site with which it reacts are unknown. Second, there must exist many types of receptor sites, occasionally all on the same receptor cell, that elicit the same given taste sensation when stimulated. Thus, it is difficult to determine the properties of any one given receptor site. One powerful technique that may be used is that of genetic control, particularly with substances that are personalized by a single functional group, such as the -C-N formation.
||
S

Sometimes we are not so interested in asking whether food is sweet, bitter, sour, or salty, but rather how we can change the taste of food. This involves interactions at the surface of the cell between two or more chemical stimuli as well as facilitory and inhibitory events between taste receptors

and neural structures. One may systematically search for a taste inhibitor in two ways. First, the mathematical basis of taste can be applied to the case of two chemicals competing for the same receptor site on the surface of the taste cell. In this way one soon learns that the best inhibitors probably have little or no taste themselves and the experimenter must then study the effect various tasteless chemicals have when added to well-known taste solutions. Another way of searching for an inhibitor of a given taste quality is to locate those substances that change the normal taste of foods as found by various people in different lands. Such substances have recently been studied in several laboratories. One occurs in the leaf of a plant, *Gymnema sylvestre,* found in India (Warren and Pfaffmann, 1959). If such a leaf is chewed for a short time, the ability to taste sweetness disappears for an hour or so, and table sugar taken into the mouth retains only its sand-like property. After such sweetness is subtracted from various foods, one really enters a different world of taste. A drink such as Coca-Cola is no longer inviting, and chocolate milk suddenly appears to be more creamy with a flatter taste. The artificial flavors contained in most candies are now enhanced but sweetness is gone. It is apparent that isolation, characterization, and synthesis of the active ingredient is not necessary before gymnemic acid is used in food products. The pulverized dried leaf was sold as a tablet in Germany in the 1920's as a remedy for diabetes. Our own studies show that pulverized *Gymnema sylvestre* leaves imbedded in chewing gum are quite effective in reducing the sweetness of foods. The psychological effects associated with sweetness inhibition should be very interesting in diet control.

In West Africa, natives chew a small red berry called "miraculous fruit" before drinking their sour wine or eating their sour bread (Inglett *et al.,* 1965). After chewing this berry for a short time, we found that the sourness of a raw lemon changes to the sweet taste of an orange and a raw grapefruit becomes very pleasant. Our laboratory is presently trying to isolate and identify the chemical responsible for such inhibitions so that we may better understand its physiological properties. The radical change in flavor of natural products, such as citrus, by this inhibitor suggests that in this case a flavor "potentiator" may be obtained. Since food flavors are so complex, it is quite possible that food preferences can be increased by inhibiting some components to allow others to dominate and increase flavors that are natural to the particular food. It is apparent that such inhibition would result in exactly the type of effect postulated for monosodium glutamate and certain nucleotides.

It should be remembered that we have confined our attention to but four major taste qualities. This we do, not because we think there are but four primary taste qualities—indeed the whole concept of taste qualities might be questioned—but rather because we need to simplify the taste problem before we can attack it with present technology. Certainly, the flavor of soap or the taste of a metal cannot be described adequately in terms of sweetness, bitterness, sourness, or saltiness. Furthermore, the color of a food may also be very important in determining its flavor. Many of you have probably tried the simple experiment of giving a blindfolded child five or six differently flavored popsickles and asking him to describe whether a given popsickle is orange, lemon, lime, strawberry, and so forth. Without seeing the color, the child is no longer able to judge the flavor of the popsickle correctly. So, again, we see that the information that is necessary for us to completely describe a flavor is almost as complex as that used in describing a painting. However, the physiologist has tried to better understand the basis of such descriptions by systematically investigating one factor at a time. We do not expect in the near future to be able to understand completely all the forces that are operating when we place food in our mouths and consider its flavor. However, there have been notable advances that have led to a better theoretical basis of flavor. It is now possible for the food technologist to better plan his own flavor experiments, consider the theories that

exist, and discover the best ways of changing flavors.

The problems involved in the study of the interaction of odors and tastes with the receptor cells are very similar to those present in modern pharmacology. In fact, those of us interested in taste would be well advised to study the results of the pharmacologist who is interested in drug-receptor interactions as well as those of the entomologist who is worried about biological control of insects. It is only after the science of taste and smell has been put in the proper perspective that major breakthroughs can occur which will lead to a better understanding of flavor and its control.

REFERENCES

Beidler, L. M. 1954. A theory of taste stimulation. *J. Gen. Physiol.* **38**, 133.

Beidler, L. M. 1961. Taste receptor stimulation. *In:* "Progress in Biophysics and Biophysical Chemistry." p. 107 Pergamon Press, London.

Beidler, L. M., and R. Smallman. 1965. Renewal of cells within taste buds. *J. Cell Biol.* **27**, 263.

Cohn, G. 1914. "Die organischen Geschmacksstoffe," Franz Siemenroth, Berlin.

Diamant, H., T. Skoog, and Y. Zotterman. 1960. The taste of water. *Acta oto-laryng.* **51**, 308.

Fishman, I. Y. 1957. Single fiber gustatory impulses in rat and hamster. *J. Cellular Comp. Physiol.* **49**, 319.

Frieden, E. and J. Alles. 1958. Subtle interactions of cupric ion with nuclei acid components. *J. Biol. Chem.* **230**, 797.

Furia, T. A. 1964. EDTA in foods. *Food Technol.* **18**, 50.

Inglett, G. E., B. Dowling, J. J. Albrecht, and F. A. Hoglan. 1965. Taste-modifying properties of Miracle Fruit (Synsepalum dulcificum). *J. Agr. Food Chem.* **13**, 284.

Kimura, K., and L. M. Beidler. 1961. Microelectrode study of taste receptors of rat and hamster. *J. Cellular Comp. Physiol.* **58**, 131.

Parkes, A. S., and H. M. Bruce. 1961. Olfactory stimuli in mammalian reproduction. *Science* **134**, 1049.

Pfaffmann, C. 1941. Gustatory afferent impulses. *J. Cellular Comp. Physiol.* **17**, 243.

Sanui, H., and N. Pace. 1965. Mass law effects of adenosine triphosphate on Na, K, Mg and Ca binding by rat liver microsomes. *J. Cellular Comp. Physiol.* **65**, 27.

Sato, M., and N. Akaike. 1965. 5'-ribonucleotides as gustatory stimuli in rats. Electrophysiological studies. *Japan. J. Physiol.* **15**, 53.

Tateda, H., and L. M. Beidler. 1964. The receptor potential of the taste cell of the rat. *J. Gen Physiol.* **47**, 479.

Warren, R. M., and C. Pfaffman. 1959. Suppression of sweet sensitivity by potassium gymnemate. *J. Appl. Physiol.* **14**, 40.

Wilson, E. O. 1963. Pheronomes. *Sci. American* **208**, 100.

Ms. rec'd 7/7/65.

This paper is based upon some of the material from "The Physiological Basis of Taste Sensation," the Fred W. Tanner Lecture delivered to the Chicago Section of the Institute of Food Technologists, March 8, 1965.

This research has been supported in part by National Science Foundation Grants GB-1491 and G-14334.

M. C. BOURNE
New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456

Measure of Shear and Compression Components of Puncture Tests

SUMMARY

A study was designed to separate and measure the compression components (proportional to area) and the shear components (proportional to perimeter) of a simple puncture test. Two sets of punches were used (one with a constant area and a variable perimeter, and the other with a constant perimeter and a variable area) to measure the compression and shear components in puncture tests on foam plastic board and representative foods. The puncture force for both can be expressed by the equation $F = K_s P + K_c A + C$, where K_s, K_c, and C are constants, P is the perimeter of the punch, and A is the area of the punch. K_s represents the shear coefficient and K_c the compression coefficient of the food being tested. For a given punch, F depends upon K_s and K_c, which are properties of the material being tested. For a given material, F depends on the perimeter and area of the punch. These results can be used as a basis for design of punch shape and size in a practical puncture test. The shear component of a puncture test can be increased or decreased relative to the compression component by manipulating the perimeter/area ratio of the punch.

INTRODUCTION

Puncture testing is one of the simplest and most widely used methods for objective measurement of the firmness of many food products. The Magness-Taylor pressure tester (Magness and Taylor, 1925) is probably the best known puncture testing device, but other puncture testers are in general use. The best known are the Christel Texturemeter (Christel, 1938), which uses a nest of 25 punches that are approximately 3/16 inch in diameter; the Maturometer (Lynch and Mitchell, 1950 and Mitchell *et al.*, 1961), which uses a battery of 143 punches that are ⅛ inch in diameter; and the Chatillon Pressure Tester (John Chatillon & Sons, 85 Cliff Street, New York, New York).

There is a certain amount of confusion in the literature concerning use of the words "pressure test" and "puncture test." The following definitions are given to clarify the type of test that is described in this report:

Pressure test. Any type of test where pressure is applied to a food product by any means. It includes, but is not restricted to, puncture tests, deformation tests, and penetration tests.

Puncture test. Measures the force required to push some kind of a punch into a food product. The test is characterized by: a) using a force-measuring instrument; b) penetration of the punch into the food; and c) a distance usually held constant. Many examples of puncture testers are listed in the literature review, below. This report is concerned only with puncture testers and has nothing to do with other kinds of pressure testers.

Deformation test. Measures the distance that the product deforms under a force before penetration. The force may be applied by a punch, by extensive flat surfaces, or by other means. The test is characterized by: a) using a distance-measuring instrument; b) no penetration into the food; and c) a force usually held constant (though some deformation tests measure the force required to obtain a constant deformation). An example of a recently developed deformation tester is the Hampden instrument, used to measure the firmness of Brussels sprouts (Rowlands, 1964).

Penetration test. Measures the depth of penetration of a punch into a food under a force in a given time. This test is characterized by: a) using a distance-measuring instrument (though some penetrometers measure time to reach a standard depth); b) penetration into the food; and c) a force held constant. Examples of penetration testers are found in the various penetrometers used to test shortenings, mayonnaise, and similar products.

[It is unfortunate that the term "pressure tester" is applied indiscriminately to these different families of testers when different variables (force vs. distance, deformation vs.

penetration) are being measured. It would be more meaningful if a tester were described more specifically as a puncture tester, a deformation tester, etc. It should be pointed out that a puncture tester is also a pressure tester, but that a pressure tester may or may not be a puncture tester. The author would be pleased to receive correspondence about the definitions given above.]

The first food puncture tester was probably the one developed by Carpi (1884), who measured the weight required to force a 2-mm-diameter iron rod 1 cm deep into olive oil and cottonseed oil at 20°C. Brulle (1893) used a similar principle for measuring the hardness of butter, and Sohn (1893) spelled out the procedure necessary to obtain reproducible results with Brulle's instrument. Perkins (1914) used needles with cross-sectional areas varying from 5 up to 100 sq mm to measure the hardness of butter fat. Gallup (1936) designed a puncture tester for butter using a plunger 5 mm in diameter and measuring the grams of force required to push it through a 6-mm-thick slab of butter. Kruisheer (1939) developed for butter a puncture tester with a 4-cm-diameter plunger.

The first puncture tester for fruit was developed in 1917, by Prof. Morris of Washington (Morris, 1925), who partly embedded a ⅝-inch-diameter marble in paraffin wax and measured the force required to push the exposed portion of the marble into an apple. Lewis *et al.* (1919) modified Morris's tester by using a 7/16-inch-diameter steel ball embedded in a block of wood, and called it an "iron thumb." Murneek (1921) used a blunt plunger ½-inch in diameter and added electrical contacts which caused a bell to ring when the depth of penetration was reached. The Magness-Taylor instrument (Magness and Taylor, 1925) used a cylindrical plunger with a rounded end in place of the balls of the earlier testers. Haller (1941) has reviewed some of the early history of fruit pressure testers. Other pressure testers that are similar in principle to the Magness-Taylor instrument or are modified versions of it have been described by Clark (1928), who reported on the New Jersey Pressure Tester developed by Prof. Blake; Allen (1929) added a peeling blade and pistol-type grip; Blake (1929) tried plungers of various diameters and concluded that a 3/16-inch-diameter plunger was large enough for accuracy; Shoemaker and Greve (1930) used a ¼-inch-diameter plunger, a ⅜-inch penetration depth, and a metric force scale; MacGillivray (1933) used a ⅛-inch-diameter plunger for testing asparagus; Margruder and Knight (1933) used a ⅛-inch-diameter plunger on onions; Culpepper and Magoon (1924) used a size-16 brass wire with squarred-off ends in a pressure tester with a metric scale for work with sweet corn; Hartman and Bullis (1929) used a 2-mm-diameter glass rod and measured the force in grams to penetrate sweet cherries; Scott Blair and Coppen (1941) used a standard cheese skewer attached to a spring scale to measure the hardness of cheese; Lutz (1944) measured the force in grams required to push a wire of 0.035-inch diameter into tomatoes; Gangstad and Snell (1948) used a pressure tester with a 0.038-inch-diameter point in work with sweet corn; and Longree and Fenton (1950) used the blunt head of a small nail in studies with kale.

Other instruments that use the puncture principle have been described. Rudnick and Bakke (1920) used a glass needle of 0.5-mm diameter to puncture corn; Davis (1921) used a rounded-end plunger to pierce cookies resting on a flat surface; Bloom (1925) measured the weight of lead shot required to force a plunger 4 mm into jellies; Sayre *et al.* (1931) developed a puncture tester for testing single peas; Bonney *et al.* (1931) loaded a 5/32-inch rod with mercury until it penetrated the flesh of peaches; Sweetman and Lancaster (1931) fitted a penetrometer with a knitting needle of 0.081-inch diameter in studies with cooked vegetables; Tressler *et al.* (1932) used a 5/16-inch-diameter rod attached to a Schrader tire-pressure gauge to measure tenderness of meat; Saxl (1938) measured the force necessary to push a cylindrical plunger of 12.75-mm diameter 4 mm deep into gelatin gels; Johannessen (1949) used a platinum wire with an area of 0.20268 sq

mm to puncture tomatoes; Lampe (1959) used a 3-mm-diameter plunger to puncture potatoes embedded in a tray of sand; Garrett *et al.* (1960) described the McCollum Firmness Meter; Hartman *et al.* (1963) mounted various puncture tips in the shear press; and Landreth (1929) used a 1-mm-diameter needle in a modified Vicat apparatus to measure the doneness of cooked vegetables. Hawkins and Harvey (1919) measured the toughness of potato skins by the force necessary to puncture the skin with rounded glass needles 58 to 71 μ in diameter; the needle was suspended from a spring, and the force was applied by lowering the spring. This principle was used by Rosenbaum and Sando (1920) in a puncture tester for tomato skins, and also by Willaman *et al.* (1925) in a puncture tester to measure the toughness of plum skins. Tarr (1926) used compressed air to force a plunger into jellies; Ross (1949) and Witz (1954) also used compressed air as the driving force in their pressure testers. Schomer and Olsen (1962) described a Magness-Taylor tester modified to read at a very shallow penetration. Smith and Davis (1963) used a 1¼-inch-diameter punch mounted in the shear press to measure the texture of reconstituted potato flakes. Morris (1963) used metal plungers of 1/16-inch-diameter and weighing 90 g to measure the softening time of cooking dry beans. Simon *et al.* (1965) used an incisor-shaped punch to measure the texture of frankfurters.

A recent development in puncture testing has been mounting of the punch in a machine that will draw out a complete force-distance curve for the test (Pflug *et al.*, 1960; Mohsenin, 1963; Kulwich *et al.*, 1963; Bourne, 1965; Voisey and MacDonald, 1964). Voisey *et al.* (1964) attached a digital computer to their puncture instrument in order to increase speed and accuracy in handling the data.

Instruments of the wide range described above have been used principally to measure the *force* necessary to effect some degree of penetration of a punch into the food. The type of test, which is defined as a "puncture test," is the subject of the research reported here. This paper restricts the term "puncture tester" to force-measuring instruments in which some kind of plunger is caused to penetrate a product to a certain depth. Further, it considers only those punches that are of uniform cross section throughout their length and that have flat contact surfaces. It specifically excludes punches with dome-shaped contact surfaces or conical-shaped cross-sections.

THE POSTULATE

Consider Fig. 1. As a punch contacts and penetrates a food it must compress and shear that commodity. The compression force should be proportional to the area under the punch and to the *compressive* strength of the food. The shear force should be proportional to the perimeter of the punch and to the *shear* strength of the food. The following equation can therefore be postulated for the force required of a puncture test:

$$F = K_c A + K_s P + C \qquad [1]$$

where F = measured force (kg)

K_c = compression coefficient of the commodity being tested (kg cm^{-2})

K_s = shear coefficient of the commodity being tested (kg cm^{-1})

A = area of punch (sq cm)

P = perimeter of punch (cm)

C = constant (kg)

In the special case of a punch that is circular in cross-section the above equation can be written as:

$$F = K_c' D^2 + K_s' D + C \qquad [2]$$

where D = diameter of punch

$$K_s' = \pi K_s$$

$$K_c' = \frac{\pi K_c}{4}$$

Eq. 2 points out that the relation of puncture force to diameter of a cylindrical punch is not

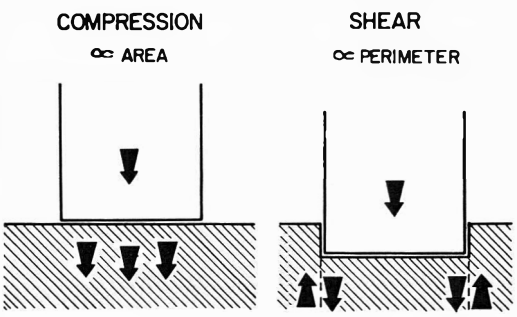


Fig. 1. Schematic representation of a punch test.

a simple one; the puncture force is the sum of a force that is directly proportional to punch diameter and of a force that is proportional to the square of the punch diameter.

EXPERIMENTAL

To establish whether the equation postulated above is correct, it is necessary to measure puncture forces on a commodity using punches of differing areas and perimeters. Considerable difficulty is encountered in separating the shear and compression components using circular punches because the area is proportional to (diameter)^2 and perimeter is directly proportional to diameter. Therefore, two sets of flat-faced steel punches approximately 2 cm long were made that were rectangular in cross-section but of varying shapes. One set of punches had a constant area of one square centimeter and perimeters ranging from 4 cm up to 8.5 cm. The second set of punches had a constant perimeter of 4 cm and areas ranging from 0.25 up to 1.00 sq. cm. Each set also contained that unique circular punch that had an area of 1 sq cm and a perimeter of 4 cm. The dimensions of the punches are given in Table 1.

These punches were mounted one at a time in a metal holder shown in Fig. 2 and used to puncture different commodities. The punch and holder were mounted in an Instron universal testing machine, which draws a force-distance curve for each puncture test on a strip-chart recorder (Fig. 3). Adaptation of the Instron universal testing machine to use on foodstuffs has been described elsewhere (Bourne *et al.*, 1965). The yield point on each curve was read from the chart and used in subsequent calculations. Punch speed was 20 cm/min for all tests.

Sheets of foam plastic board were punched first because they present a large area of fairly uniform character. The sheets were ruled off into 1-inch squares and each punch was allotted 50 squares at random for a puncture test. The mean yield point for the 50 puncture tests was taken as the puncture force for each punch. When the validity of Eq. 1 had been established with three

Table 1. Details of punch dimensions; each punch was made in mild steel and is ¼ inch long.

Punch no.	Area (cm^2)	Perimeter (cm)	Dimension (cm)
Constant-area set			
1	1.00	4.0	1.000 × 1.000
2	1.00	5.5	2.318 × 0.432
3	1.00	7.0	3.186 × 0.314
4	1.00	8.5	4.000 × 0.250
8	1.00	3.55	1.128 (diam)
Constant-perimeter set			
1	1.00	4.0	1.000 × 1.000
5	0.75	4.0	1.500 × 0.500
6	0.50	4.0	1.707 × 0.293
7	0.25	4.0	1.866 × 0.134
9	1.274	4.0	1.274 (diam)

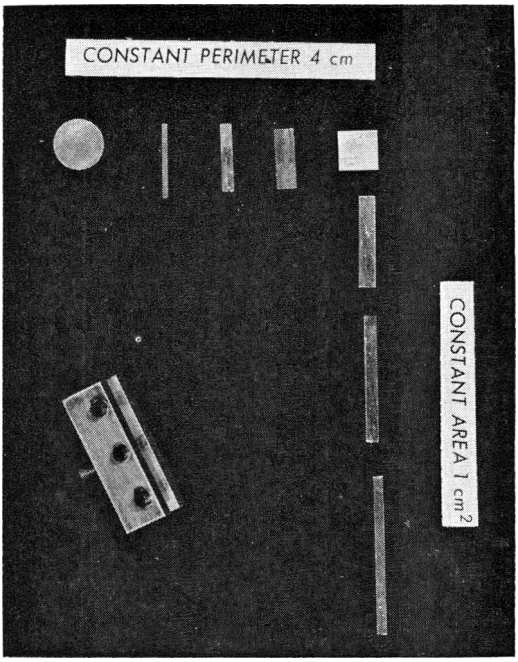


Fig. 2. Set of punches and the punch holder.

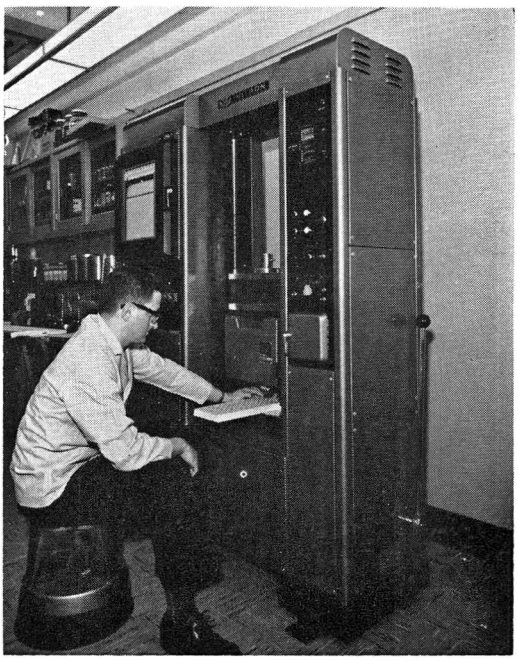


Fig. 3. Sheet of foam plastic board being punched in the Instron universal testing machine.

different kinds of foam plastic board, various food commodities were tested in a similar manner. Fruits and vegetables were sliced open to expose the maximum area, and all punches were made on the freshly cut surface. Twenty-five units of the fruits and vegetables were used, and each punch was used once on each unit. Each punch was used 25 times on the cream-filled wafers, but many wafers were required because of their small size and tendency to crumble and split after a single punch. The agar gels were poured hot into shallow trays about ½ inch deep and allowed to set overnight before punching. Each punch was used 25 times at random positions on each agar gel. The mean force from the 25 punches was taken as the puncture force for that punch on that commodity. The samples were supported on the bed of the machine during punching and were not restrained in any way. Only those foods that show the break in the force-distance curve known as the "yield point" were used for testing. The yield point represents the commencement of penetration of the punch into the tissue and is also a good reference point from which to take a force reading. The least-squares method was used to calculate the line of best fit and the slopes and intercepts on the graphs.

RESULTS

Figs. 4 and 5 give data for the foamed polystyrene board. The graph of puncture force vs. punch area (Fig. 4) is rectilinear, showing that the puncture force is related directly to area when the perimeter is constant. The slope of the line gives the compression coefficient K_c. The intercept with the ordinate (at zero area) gives the constant term $(K_s P' + C)$ where $P' = 4$ cm for this set of punches.

The graph of puncture force vs. punch perimeter (Fig. 5) is also rectilinear, showing that the puncture force is directly proportional to perimeter when the area is constant. The slope of this line gives the shear coefficient K_s. The intercept with the ordinate (at zero perimeter), gives the constant term $(K_c A + C)$ where $A = 1.00$ sq cm for this set of punches. The numerical value of C can be calculated for any commodity once K_s, K_c, A, and P' are known. This procedure, although lengthy, enables every coefficient in Eq. 1 to be evaluated for a given commodity.

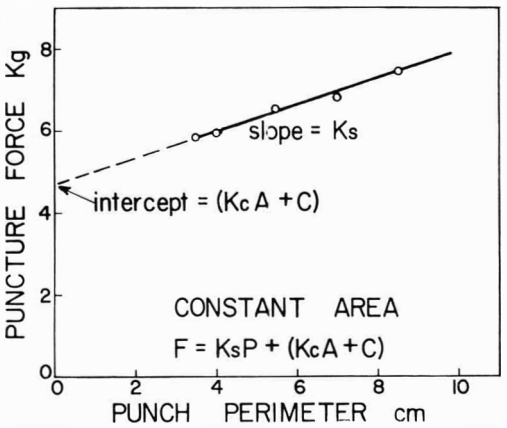


Fig. 5. Puncture force vs. punch perimeter with constant area on polystyrene board.

Figs. 6 and 7 respectively show the relationships between puncture force vs. punch area and puncture force vs. punch perimeter for representative food commodities. Table 2 gives the numerical values of the coefficients K_c, K_s, and C for a number of commodities. In every case the experimental data obtained fitted Eq. 1 very closely, with correlation coefficients of 0.97 and higher. The values in Table 2 are illustrative only. The numerical values of these coefficients can be expected to vary over

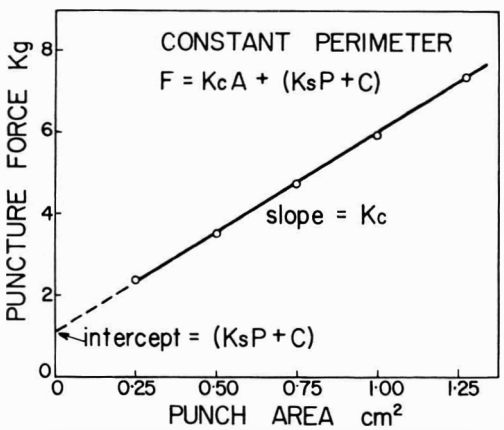


Fig. 4. Puncture force vs. punch area with constant perimeter on polystyrene board.

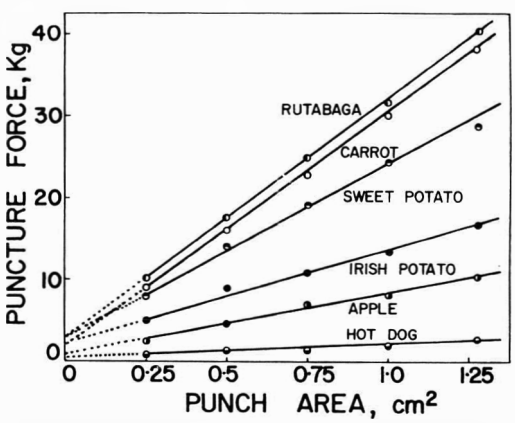


Fig. 6. Puncture force vs. punch area with constant perimeter on various foods.

Table 2. Numerical values of coefficients for various commodities.

Commodity	Compression coefficient K_c (Kg/cm^2)	Shear coefficient K_s (Kg/cm)	Constant C (Kg)
Expanded polystyrene	4.86	0.34	−0.23
High-density polystyrene	13.14	2.20	−2.75
Polyurethane	3.57	0.29	−0.47
Apples (raw, Limbertwig variety)	7.52	0.16	0.03
Apples (raw, Fr. von Berl variety)	6.43	0.07	0.40
Banana (ripe, yellow)	0.43	0.06	−0.06
Creme filled wafers	1.06	0.14	0.64
Carrot (uncooked core tissue)	28.0	−0.03	2.18
Wiener (cold)	1.69	0.004	0.15
Potato (Irish, uncooked)	10.79	0.52	0.60
Rutabaga (uncooked)	29.58	0.86	−0.15
Sweet potato (uncooked)	19.8	0.90	0.35
1% agar gel	0.15	0.005	−0.01
2% agar gel	0.63	0.029	−0.02
3% agar gel	1.21	0.16	−0.33

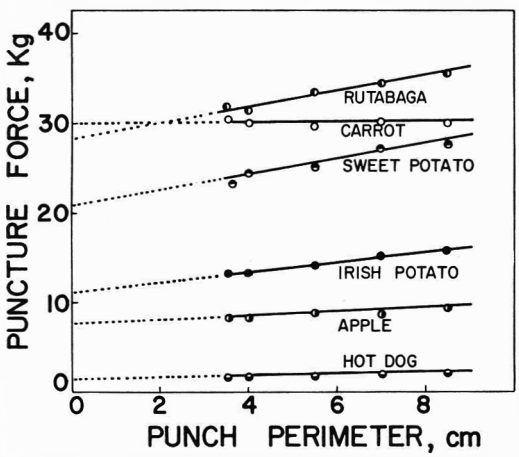


Fig. 7. Puncture force vs. punch perimeter with constant area on various foods.

a wide range depending upon the composition, maturity, processing conditions, and other variables that affect textural quality.

DISCUSSION

The excellent agreement of the experimental data with Eq. 1 establishes the validity of this equation in describing the forces involved in a puncture test. The values obtained for K_c, K_s, and C depend upon the nature of the commodity. It seems probable that K_c and K_s are a measure of the textural quality of foods. Further experimental work in this direction is planned to determine whether the coefficients K_s and K_c correlate with textural quality as measured by sensory tests. The values for A and P depend upon the dimensions of the punch. When K_c, K_s and C are known for a commodity, Eq. 1 can be used to calculate the puncture force that will be obtained with a punch of any area and perimeter. It can also be used to calculate the puncture force that will be obtained if the punch size is altered. It seems that there is no "corner effect" with the rectangular punches, because the force values obtained with the two circular punches fall right into line with those obtained with the rectangular punches.

The physical meaning of the constant C would be interpreted from Eq. 1 as being the force required to puncture a commodity with a punch of zero area and zero perimeter. C has a value close to zero for most of the commodities tested, and in such cases could be neglected without introducing any great inaccuracy. Some commodities, however, have a value for C that is numerically too high to be attributed to experimental error, and these C values are usually negative. It seems probable that in these cases there is a zone of influence round the punch such that the actual compression area on the commodity is larger than the area of the punch. On the other hand, Eq. 1 might lose its rectilinear nature for small punches, thus making inaccurate the extrapolation to zero area and zero perimeter.

The relationship between punch dimensions and puncture force may be represented

as a three-dimensional graph. Fig. 8, a diagram for polystyrene board, is drawn from the same data used in Figs. 4 and 5. Puncture force is plotted on the vertical axis; punch perimeter and punch area are plotted on the horizontal axes. In this kind of three-coordinate plot, the force-area-perimeter relationship appears as an inclined plane surface. The slope of the plane in each dimension is given by the coefficients K_c and K_s, and the intercept of the plane on the vertical axis (force) is given by the constant C. C has a value of zero in Fig. 8. The position and inclination of the plane are determined by the nature of the test material because K_c, K_s, and C are properties of the material. When the position of the plane is known, the puncture force can be read off for a punch with any perimeter or area. This is not a recommended procedure for routine testing, because a new plane surface must be constructed for every lot of each commodity.

It follows from Eq. 1 that the puncture force for a given punch on any food will depend upon the nature of the food, because the area, A, and perimeter, P, of the punch are constant and the coefficients K_c, K_s, and C, which are properties of the food, are the variables. It also follows from Eq. 1 that the puncture force on a given food depends on the dimensions of the punch, because the coefficients K_c, K_s and C will be constant, and the area, A, and perimeter, P, of the punch are the variables.

Eq. 1 explains why a simple doubling of the area of a punch usually fails to double the puncture force. When the area of a circular punch is increased by a factor of 2.0, the perimeter is increased by a factor of only 1.41. The puncture force will be doubled only if the shear coefficient is zero or if the shape of the punch is changed so that both perimeter and area are doubled. For example, Mulder (1953) has pointed out that the butter puncture tester that is used officially in the Netherlands gives a force reading that is only 1.7 times as large when the area of the punch is doubled. It can be calculated from Mulder's factor of 1.7 that the compression and shear components contribute approximately equally to the puncture force in the official butter test.

In designing punches for a test device it is possible to give added or less weight to the shear component simply by increasing or decreasing the ratio: perimeter/area of the punch. Fig. 9 shows two methods of manipulating the perimeter/area ratio of a punch.

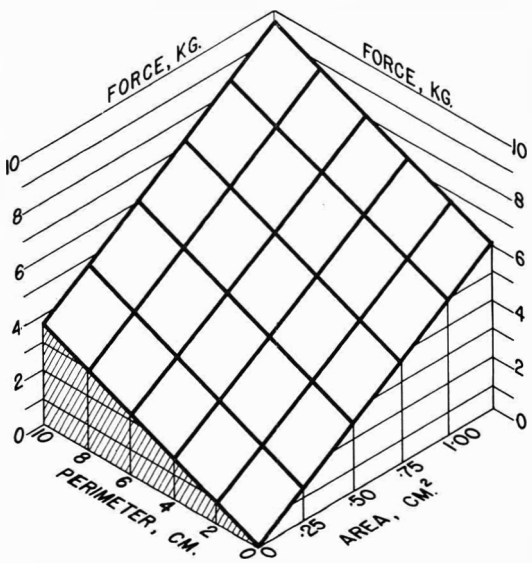


Fig. 8. Three-dimensional representation of force-area-perimeter puncture test for polystyrene board.

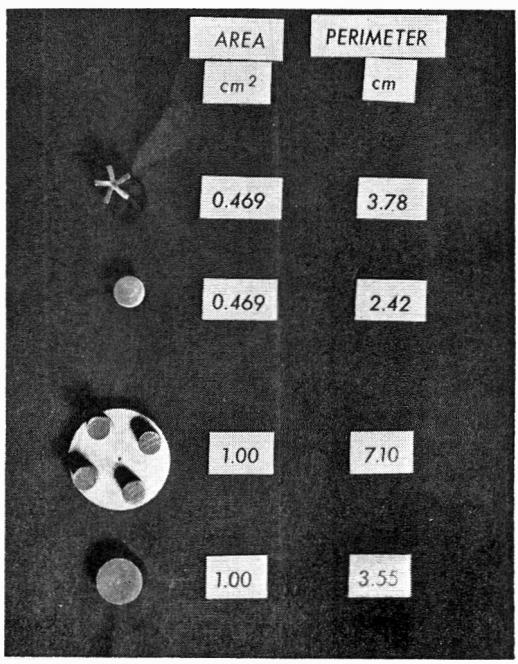


Fig. 9. Two sets of punches with equal area but unequal perimeter.

The first single punch has an area of 1.00 sq cm and a perimeter of 3.55 cm. The nest of four punches has a combined area of 1.00 cm and a combined perimeter of 7.10 cm. The nest of four punches gives a higher puncture force reading than the single punch because the amount of shearing in the test is double that of the single punch. The second circular punch has an area of 0.469 sq cm and a perimeter of 2.42 cm, and its star-shaped partner has the same area but a perimeter of 3.78 cm. A third method of manipulating the perimeter/area ratio is to use rectangular punches of varying shape as shown in Fig. 2. A fourth method of manipulation is suggested by the stepped punch described by Kulwich *et al.* (1963), where a punch of 0.125 inch diameter is stepped up to 0.372 inch diameter and both sections of the punch are used to test slices of pork.

This report has shown that the force required to puncture a food depends on the area and perimeter of the punch and on the compressive strength and shear strength of the food being tested. The puncture force for different punches on the same food will be a function of both the area and perimeter of the punch used. The puncture force with the same punch on different foods will be a function of both the compressive strength and shear strength of that food. The numerical values given for the coefficients K_c and K_s in Table 2 illustrate application of the method for separating and independently evaluating each of the coefficients. Further work is planned with this technique to evaluate the manner in which variety, maturity, composition, storage, and processing conditions affect the coefficients K_c and K_s for different foods and to find how changes in K_c and K_s relate to sensory quality.

REFERENCES

Allen, F. W. 1929. Maturity standards for harvesting pears for Eastern shipment. *Calif. Univ. Ag. Expt. Sta. Bull.* **470**.

Blake, M. A. 1929. A device for determining the texture of peach fruits for shipping and marketing. *New Jersey Agr. Expt. Sta. Circ.* **212**.

Bloom, O. J. 1925. Machine for testing jelly strength of glues, gelatines and the like. U.S. Patent No. 1,540,979.

Bonney, V. B., P. A. Clifford, and H. A. Lepper. 1931. An apparatus for determining the tenderness of certain canned fruits and vegetables. *US Dept. Agr. Circ.* No. **164**.

Bourne, M. C. 1965. Studies on punch testing of apples. *Food Technol.* **19**, 113.

Bourne, M. C., J. C. Moyer, and D. B. Hand. 1965. Measurement of food texture by a universal testing machine. In press.

Brulle, R. 1893. Méthode générale pour l'analyse des beurres. *Compte rend.* **116**, 1255.

Carpi, S. 1884. *Arch. Pharm. Acol.* **221**, 963 cited in *Z. Anal. Chem.* **23**, 566, by F. Hofmeister and W. Lenz.

Christel, W. F. 1938. Texturemeter, a new device for measuring the texture of peas. *Canning Trade* **60** (34), 10.

Clark, J. H. 1928. Forty-ninth annual report of the New Jersey State Agr. Expt. Sta. p 227.

Culpepper, C. W., and C. A. Magoon. 1924. Studies upon the relative merits of sweet corn varieties for canning purposes and the relation of maturity of corn to the quality of the canned product. *J. Agr. Research* **28**, 403.

Davis, C. E. 1921. Shortening: Its definition and measurement. *Ind. Eng. Chem.* **13**, 797.

Gallup, W. D. 1936. Studying the hardness of butter fat. *Ind. Eng. Chem. Anal.* ed. **8**, 123.

Gangstad, E. O., and R. S. Snell. 1948. A practical method for quantitative evaluation of texture in sweet corn inbreds and hybrids. *J. Am. Soc. Agron.* **40**, 885.

Garrett, A. W., N. W. Desrosier, G. D. Kuhn, and M. L. Fields. 1960. Evaluation of instruments to measure firmness of tomatoes. *Food Technol.* **14**, 562.

Haller, M. H. 1941. Fruit pressure testers and their practical application. *U.S. Dept. Agr. Circ.* No. **627**.

Hartman, H., and D. E. Bullis. 1929. Investigations relating to the handling of sweet cherries. *Oregon State Coll. Agr. Expt. Sta. Bull.* No. **247**.

Hartmann, J. D., F. M. Isenberg, and J. K. Ang. 1963. New applications of the shear press in measuring texture in vegetables and vegetable products. I. Modifications and attachments to increase the versatility and accuracy of the press. *Proc. Am. Soc. Hort. Sci.* **82**, 465.

Hawkins, L. A., and R. B. Harvey. 1919. Physiological study of the parasitism of Pythium debaryanum Hesse on the potato tuber. *J. Agr. Research* **18**, 275.

Johannessen, G. A. 1949. Skin puncture studies on red-ripe tomatoes. *Proc. Am. Soc. Hort. Sci.* **54**, 272.

Kruisheer, C. I. 1939. An analytical study on the

quality and especially the firmness of butter. *Chem. Ind.* **58**, 735.

Kulwich, L., R. W. Decker, and R. H. Alsmeyer. 1963. Use of a slice-tenderness evaluation device with pork. *Food Technol.* **17**, 201.

Lampe, K. 1959. Moglichkeiten zur Messung der Beschadigungsempfindlichkeit von Kartoffelknollen und anderen Fruchten. *Landtech Forsch., Munch.* **9**, (2), 50.

Landreth, C. 1929. A method of standardizing doneness in cooked vegetables. *J. Home Econ.* **21**, 826.

Lewis, C. I., A. E. Murneek, and C. C. Cate. 1919. Pear harvesting and storage investigations in Rogue River Valley. *Oregon State Coll. Agr. Expt. Sta. Bull.* No. **162**.

Longree, Karla, and Faith Fenton. 1950. Objective and subjective studies on factors affecting toughness and stringiness of kale (Brassica oleracea acephala). *Food Research* **15**, 471.

Lutz, J. M. 1944. Maturity and handling of greenwrap tomatoes in Mississippi. *U.S. Dept. Agr. Circ.* **695**.

Lynch, L. J., and R. S. Mitchell. 1950. The physical measurement of quality in canning of peas. *Australia Commonwealth Sci. Ind. Research Organization Bull.* No. **254**.

MacGillivray, H. H. 1933. Seasonal variation in the tenderness of asparagus. *Proc. Am. Soc. Hort. Sci.* **30**, 558.

Magness, J. R., and G. F. Taylor. 1925. An improved type of pressure tester for the determination of fruit maturity. *U.S. Dept. Agr. Circ.* No. **350**.

Margruder, R., and E. Q. Knight. 1933. The relative firmness of 15 onion varieties. *Proc. Am. Soc. Hort. Sci.* **30**, 563.

Mitchell, R. S., D. J. Casimir, and L. J. Lynch. 1961. The Maturometer-instrumental test and redesign. *Food Technol.* **15**, 415.

Mohsenin, N. M. 1963. A testing machine for determination of mechanical and rheological properties of agricultural products. *Penn. State Univ. Agr. Expt. Sta. Bull.* No. **701**.

Morris, O. M. 1925. Studies in apple storage. *Wash. State Coll. Agr. Expt. Sta. Bull.* No. **193**.

Morris, H. J. 1963. Cooking quality of dry beans. Proc. Sixth Ann. Dry Bean Conf. Los Angeles, Calif. 11–23.

Mulder, H. 1953. Consistency of butter. In: "Foodstuffs, their Fluidity and Consistency." G. W. Scott Blair, ed. Interscience Publ., New York. p. 107.

Murneek, A. E. 1921. A new test for maturity of the pear. *Oregon State Coll. Agr. Expt. Sta. Bull.* No. **186**.

Perkins, A. E. 1914. An apparatus for determining the hardness of butter fat. *Ind. Eng. Chem.* **6**, 136.

Pflug, I. J., F. M. Joffe, and R. C. Nicholas. 1960. A mechanical recording pressure tester. *Mich. State Univ. Agr. Expt. Sta. Quart. Bull.* **43**, 117.

Rosenbaum, J., and C. E. Sando. 1920. Correlation between size of the fruit and the resistance of the tomato skin to puncture and its relation to infection with Macrosporium tomato Cooke. *Am. J. Botany* **7**, 78.

Ross, E. 1949. A quantitative hardness tester for food products. *Science* **109**, 204.

Rowlands, D. G. 1964. Measurement of varietal differences in firmness of Brussels sprouts. *Hort. Research* **3**, 102.

Rudnick, R. A. and A. L. Bakke. 1920. The mechanical penetration of the sweet corn pericarp. *Proc. Iowa Acad. Sci.* **27**, 129.

Saxl, E. J. 1938. Load versus compression characteristics of gelatins, fibers, and other materials. *Ind. Eng. Chem. Anal. Ed.* **10**, 82.

Sayre, C. B., J. J. Willaman, and Z. I. Kertesz. 1931. Factors affecting the quality of commercial canning peas. *N.Y. State Expt. Sta. Tech. Bull.* No. **176**.

Schomer, H. A. and K. L. Olsen. 1962. A mechanical thumb for determining firmness of apples. *Proc. Am. Soc. Hort. Sci.* **81**, 61.

Scott Blair, G. W., and F. M. V. Coppen. 1941. The consistency of cheese curd at the pitching point and its bearing on the firmness and quality of the finished cheese. *J. Dairy Research* **12**, 44.

Shoemaker, J. S., and E. W. Greve. 1930. Relation of nitrogen fertilizers to the firmness and composition of strawberries. *Ohio Agr. Expt. Sta. Bull.* **466**.

Simon, S., J. C. Field, W. E. Kramlich, and F. W. Tauber. 1965. Factors affecting frankfurter texture and a method of measurement. *Food Technol.* **19**, 110.

Smith, O., and C. O. Davis. 1963. Potato quality. XV. Improving texture and measuring textural changes in potato flakes. *Am. Potato J.* **40**, 67.

Sohn, C. E. 1893. Simple appliances for testing the consistency of semi-solids with a note on a new method of examining butter. *Analyst* **18**, 218.

Sweetman, M. D., and M. C. Lancaster. 1931. Penetrometer measurements of doneness in cooked vegetables. *J. Home Econ.* **23**, 565.

Tarr, L. W. 1926. Fruit jellies. III. Jelly strength measurements. *Delaware Univ. Agr. Expt. Sta. Bull.* No. **142**.

Tressler, D. K., C. Birdseye, and W. T. Murray. 1932. Tenderness of meat. *Ind. Eng. Chem.* **24**, 242.

Voisey, P. W., and D. C. MacDonald. 1964. An instrument for measuring the puncture resistance of fruits and vegetables. *Proc. Am. Soc. Hort. Sci.* **84**, 557.

Voisey, P. W., M. Kloek, and D. C. MacDonald. 1964. A rapid method of determining the mechanical properties of fruits and vegetables. *Proc. Am. Soc. Hort. Sci.* **85**, 547.

Willaman, J. J., N. C. Pervier, and H. O. Triebold. 1925. Biochemistry of plant diseases. V. Relation between susceptibility to brown rot in plums and physical and chemical properties. *Botan. Gaz.* **80**, 121.

Witz, R. L. 1954. Measuring resistance of potatoes to bruising. *Agr. Eng.* **35**, 241.

Ms. rec'd 10/23/65.

Approved for publication by the Director of the New York State Agricultural Experiment Station as Journal Paper No. 1457, Oct. 12, 1965.

Presented at the 25th Annual Meeting of the Institute of Food Technologists, at Kansas City, Missouri, May 19, 1965.

The author is indebted to Dr. D. F. Farkas for valuable discussions during the experiment, and to Dr. R. S. Shallenberger for assistance in drawing Fig. 8.