Journal of FOOD SCIENCE

SEPTEMBER–OCTOBER, 1966 Volume 31, Number 5

CONTENTS

(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.

R. VINCENT,[a] W. D. POWRIE, and O. FENNEMA
University of Wisconsin, Madison, Wisconsin 53706

Surface Activity of Yolk, Plasma and Dispersions of Yolk Fractions

SUMMARY

Undiluted yolk, plasma, and a 4.8% livetin solution had surface tensions of about 44 dynes/cm and interfacial tensions of around 5 dynes/cm. Extensive dilution of the yolk and plasma with either water or 10% NaCl did not bring about marked decreases in the activity of the surfaces. Rehydrated dried yolk had surface energy values similar to those for yolk. Granule suspensions, diluted with water and 10% NaCl, had high surface and interfacial tensions. A yolk phospholipid fraction reduced the surface energy of sols to very low values. The contributions of proteins in a livetin fraction and of phospholipids to the surface properties of yolk are discussed.

INTRODUCTION

Egg yolk is regarded as an efficient emulsifier for the preparation of stable food emulsions. Actually, yolk contains many emulsifying agents such as phospholipids, cholesterol, and proteins. The contribution of these agents in yolk to the formation and stability of food emulsions has not been explored extensively. Sell *et al.* (1935) have suggested that a lipid-protein complex rather than lecithin is the emulsifying agent of yolk.

The reduction of interfacial tension is presumably the initial step in the formation of an emulsion. Harkins and Zollman (1926) indicated that an interfacial tension of less than 10 dynes/cm will tend to enhance emulsification, while an interfacial tension of less than 1 dyne/cm may bring about spontaneous emulsification. These concepts are in agreement with those of King (1941) and Clayton and Morse (1939). Thus surface-active agents in yolk are probably essential for effective emulsification. It should be noted, however, that surface-active agents may not stabilize an emulsion. Yeadon *et al.* (1958) showed that, with highly purified lecithin, stable emulsions could not be prepared.

[a] Research assistant. Present address: M and M Candies, Hackettstown, N. J.

Up to the present, little information has been available on the surface activity of yolk and its fractions. Amar (1924) indicated that the surface tension of yolk was about ⅔ of that for albumen, or approximately 35 dynes/cm. Powrie (1955) found that a 2% yolk lecithin sol had a surface tension of 23.3 dynes/cm.

This study was done to examine quantitatively the surface properties of yolk, plasma, and yolk fraction dispersions. Data from such an investigation should be useful to understand more clearly the emulsifying power of egg yolk.

Preliminary experiments indicated that moderate dilution of yolk and plasma did not appreciably influence the surface tension. Thus considerable dilution of all samples in this study was desirable so that changes in the surface activity-dilution curves would be apparent, and the emulsifying potential of diluted samples could be assessed.

MATERIALS AND METHODS

Materials. All eggs were less than 24 hr old at experimentation and were laid by hens on a diet consisting of 14–15% protein and about 3.4% fat.

Liquid yolk (about 53% solids) was prepared by washing the yolk in cold water, rolling the yolk on absorbent paper, and puncturing the vitelline membrane to allow the fluid yolk to flow into a container. Bromelain-treated yolk (0.5% bromelain) was prepared by mixing bromelain powder (bromelain concentrate F-26-1, Miles Chemical Company) with liquid yolk at about 27°C and allowing the mixture to stand for 4 hr.

Liquid yolk was dried by spray drying in a pilot-plant-size Niro dryer with an atomizer rotating at about 40,000 rpm. The air inlet temperature was maintained at 145–155°C and the outlet air temperature at 75–85°C. The solids content of the spray-dried sample was 96.9%. A sample of commercially spray-dried yolk with a solids content of about 97% was used for comparison. For rehydration of each dried yolk sample, an equal weight of water was added with stirring to the yolk sample, and the fluid was held overnight at about 6°C for optimum hydration. The solids contents of the original reconstituted yolk samples were

48.0% for the commercially spray-dried, 48.4% for the laboratory spray-dried and 48.4% for the bromelain-treated, laboratory spray-dried, all on a weight basis.

Granules and plasma (about 52% solids) were separated by centrifuging yolk at a high speed (78,000 × G) according to the method of Saari *et al.* (1964). The granules were washed three times with water to remove residual plasma. The solids content of the washed granules was 40.5% on a weight basis. The moist granules were mixed with either water or 10% NaCl to form a uniform suspension containing 50% moist granules.

The crude acetone-insoluble phospholipid fraction was prepared by a method similar to that of Pangborn (1951). To prepare a 10% (w/w) phospholipid sol, crude phospholipid dissolved in ether was added slowly to rapidly agitated water. The ether was removed by bubbling nitrogen gas through the sol.

The crude livetin fraction was prepared according to the method of Martin *et al.* (1957) and lyophilized. A solution containing 4.8% livetin was prepared by dissolving the powdered livetin in distilled water.

Methods. Yolk, plasma and dispersions containing yolk constituents were diluted with either water or 10% sodium chloride solution. The degree of dilution is termed "% of the original sample," which is defined as:

$$\frac{\text{weight of original undiluted sample}}{\text{weight of diluted sample}} \times 100.$$

All surface and interfacial tension measurements were made with a Cenco du Nouy direct-reading tensiometer. Each liquid sample was maintained at 25°C in a water-jacketed glass container with a diameter of 3 inches. Pure cottonseed oil was used as the liquid hydrophobic phase for interfacial tension measurements. Preliminary experiments indicated that the surface tension of diluted yolk samples did not change over a period of 1 hr. Apparently the surface-active agents in yolk migrate rapidly to the surface to form an equilibrium film.

The moisture content of samples was determined by drying an aliquot in a vacuum oven at about 70°C and a vacuum of 29 inches Hg.

RESULTS

The surface-tension values for undiluted and water-diluted yolk, plasma, and dispersions containing yolk components are shown in Figs. 1 and 2. Undiluted samples of yolk, plasma, and 4.8% livetin solution had surface tensions of around 44 dynes/cm. The surface tension of the original 10% phospholipid sol was about 27 dynes/cm. According to Powrie (1955) a 2% purified lecithin sol had a surface tension of 23.3 dynes/cm at 25°C. The original yolk granule suspensions (40.5% solids) and reconstituted yolk samples were so viscous that accurate surface-tension values could not be obtained without dilution. Used for dilution studies was distilled water with a surface tension of 71 dynes/cm (25°C). As shown in Fig. 1, the surface

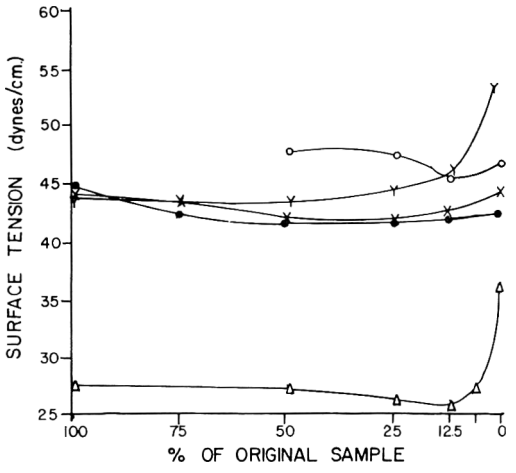


Fig. 1. Influence of dilution with water on the surface tension of yolk, plasma, and dispersions of yolk components.
- ● yolk
- X plasma
- Y livetin solution
- O granule suspension
- Δ phospholipid sol

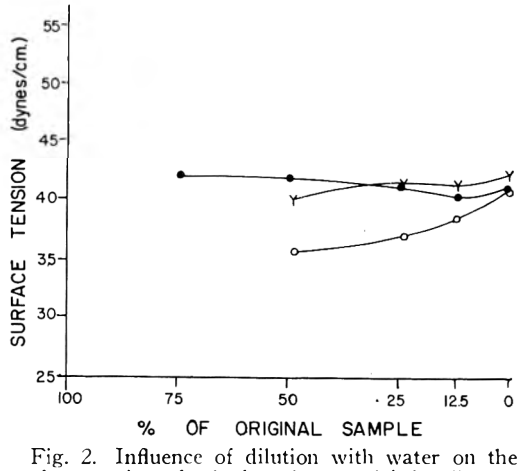


Fig. 2. Influence of dilution with water on the surface tension of rehydrated spray-dried yolk.
- ● commercially spray-dried yolk
- Y laboratory spray-dried yolk
- O laboratory spray-dried yolk with bromelain treatment

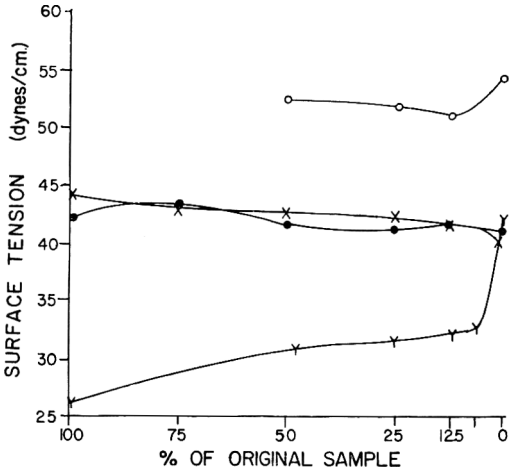


Fig. 3. Influence of dilution with 10% NaCl on the surface tension of yolk, plasma, and dispersions of yolk components.
- ● yolk
- X plasma
- Y phospholipid sol
- O granule suspension

tension of yolk and plasma did not change appreciably upon extensive dilution with water to 1% of the original sample. Apparently diluted yolk and plasma samples contained surface-active molecules in concentrations much higher than were needed for maximum surface adsorption. The surface tension of the livetin solution increased after the original sample was diluted to less than 25% (1.2% livetin solution). With the dilution value of 1%, the surface of the diluted livetin solution apparently was not completely saturated with surface-active agents, and as a consequence the surface tension was fairly high, 55 dynes/cm.

Both of the surface tension-dilution curves for crude phospholipid and granule suspensions have minima at the 12.5% dilution value. Such minima are typical of solutions with more than one surface-active agent (Miles and Shedlovsky, 1944).

The surface tension-dilution curves in Fig. 2 for rehydrated spray-dried yolk (both commercially- and laboratory-prepared) are similar to the curve for native yolk. Apparently drying does not modify the availability of surface-active molecules for adsorption at the surface of reconstituted yolk. When proteins in yolk were hydrolyzed by bromelain prior to drying, the surface tensions of the diluted, rehydrated yolk were lower than the untreated, rehydrated yolk with corresponding dilutions. Presumably surface-active compounds such as phospholipids or peptides were released more readily from lipoprotein complexes when their protein moieties were degraded by enzymic proteolysis.

A 10% NaCl solution was selected as a diluent because it was expected that the salt would bring about dissolution of protein aggregates or weaken electrostatic bonds between surface-active lipids and proteins in the yolk lipoprotein complexes. As a consequence, the surface activity could be enhanced. The surface tension of the 10% NaCl solution used for dilution was about 74 dynes/cm at 25°C. As shown in Fig. 3, sodium chloride did not have any marked effect on the surface tension of diluted samples of yolk or plasma. On the other hand, the surface-tension values of the granule suspension diluted with sodium chloride were at least 5 dynes/cm higher than those of the corresponding granule suspensions diluted with water. The granules in suspension were dispersed in the sodium chloride solution to form a clear, yellow solution. Perhaps the dissolved protein molecules of the granules competed with other more efficient surface-active molecules at the surface and thus caused a rise in the surface tension. The phospholipid sol diluted with 10% NaCl solution had higher surface tension values than corresponding water-diluted sols. In Fig. 4, the surface tension curve for spray-dried bromelain-treated yolk diluted with 10% NaCl has a pattern similar to that for the same sample with water dilution (Fig. 2). Comparison of curves for commercially spray-dried yolk (Figs. 2, 4) reveals the slight divergence of surface-tension values at the 1% dilution level.

As shown in Fig. 5, yolk and plasma had very low interfacial tensions, around 5 dynes/cm, even when diluted with water to 1% of the original weight. From these data, it is now apparent why an emulsion can be formed easily when egg yolk is an ingredient. According to Harkins and Zollman (1926), an interfacial tension below 10 dynes/cm is indicative of excellent emulsifiability.

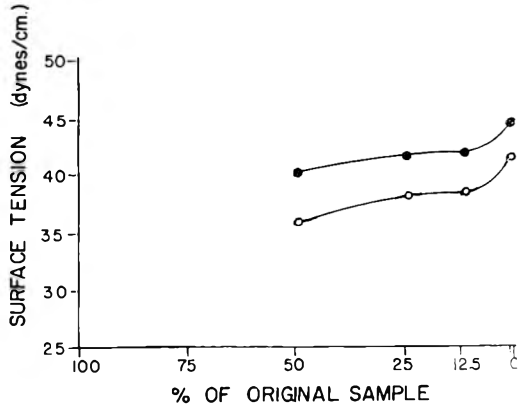


Fig. 4. Influence of dilution with 10% NaCl on the surface tension of rehydrated spray-dried yolk.
- ● commercially spray-dried yolk
- O laboratory spray-dried yolk with bromelain treatment

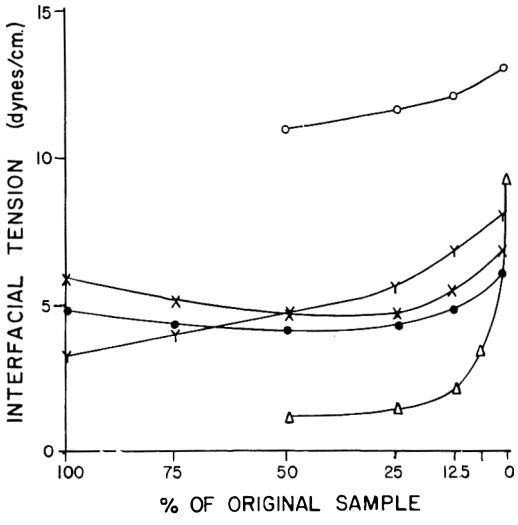


Fig. 5. Influence of dilution with water on the interfacial tension of yolk, plasma, and dispersions of yolk components.

- ● yolk
- X plasma
- Y livetin solution
- O granule suspension
- Δ phospholipid sol

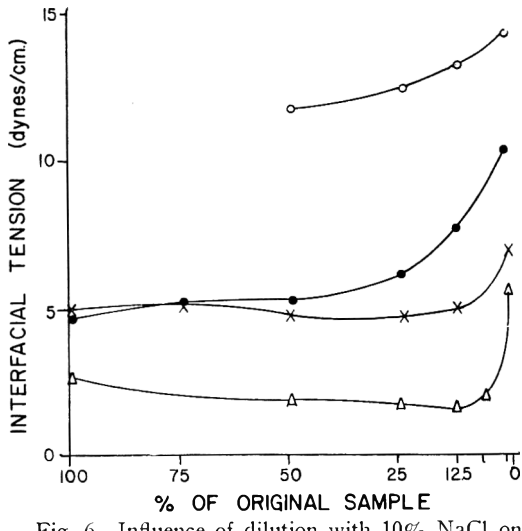


Fig. 6. Influence of dilution with 10% NaCl on the interfacial tension of yolk, plasma, and dispersions of yolk components.

- ● yolk
- X plasma
- O granule suspension
- Δ lecithin sol

It is apparent in Fig. 5 that crude livetin markedly reduced the interfacial tension. The gradual rise of the interfacial tension-dilution curve for the livetin solution was probably caused by a decrease in the concentration of specific surface-active compounds at the interface as dilution progressed. Probably crude livetin has a small amount of a specific protein with a very high surface activity. Crude phospholipid, even at low concentrations, was exceptionally effective in reducing surface energy. On the other hand, all interfacial tensions between water-diluted granule suspensions and oil were above 10 dynes/cm.

The dilution of plasma and granule suspension with 10% NaCl solution rather than water did not have any appreciable influence on their interfacial tensions (Fig. 6). When yolk was diluted below 50% of the original sample, the interfacial tension increased markedly. The interfacial tensions between reconstituted yolk (diluted with 10% NaCl) and oil ranged between 8.5 and 9 (Fig. 7). Significantly lower interfacial tensions were apparent with bromelain-treated reconstituted yolk.

DISCUSSION

It is apparent that the surface activity of yolk, undiluted and diluted with either water or 10% NaCl solution, is exceptionally high for a biological fluid. The surface tensions of native yolk samples ranged between 42 and 45 dynes/cm. These values are somewhat higher than the surface tension for yolk of about 35 dynes/cm reported by Amar (1924).

The contribution of granules to the surface activity of yolk can be considered minor, since yolk without granules, namely plasma, has surface properties similar to those of yolk. Although granules do possess surface-active agents, they are not as effective as the plasma surface-active agents for the reduction of surface energy.

Some proteins in the livetin fraction probably contribute in part to the low surface energy of yolk. The livetin content in yolk solids has been estimated to be about 7.5% (Sugano and Watanabe, 1961). Even when the original livetin solution was diluted to 12.5% of the original sample, the surface-energy values (Figs. 1 and 5) were near those for yolk. It is of interest to note that protein fractions in milk have the ability to lower surface and interfacial tensions. Aschaffenburg (1946) reported that σ-proteose, constituting only about 3% of the total milk proteins, was found to be the surface-active component of milk. The surface tension of σ-proteose solutions reached a minimum of around 54 dynes/cm. According to Jackson

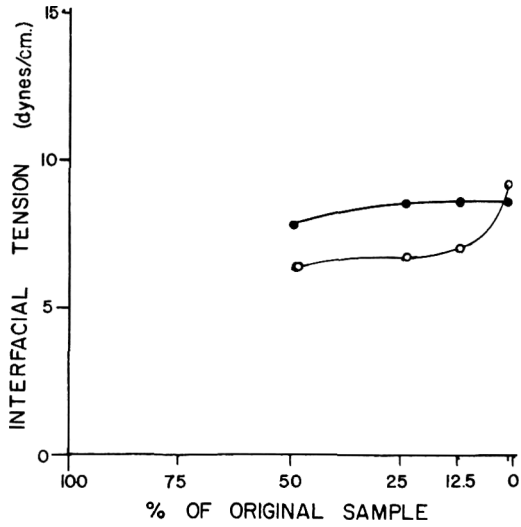


Fig. 7. Influence of dilution with 10% NaCl on the interfacial tension of rehydrated spray-dried yolk.

● commercially spray-dried yolk
O laboratory spray-dried yolk with bromelain treatment

and Pallansch (1961), a protein from the fat-globule membrane reduced the interfacial tension in a butter oil-water system to about 9 dynes/cm.

Phospholipids may also contribute to the low surface energy of yolk and plasma. As shown in this study, yolk phospholipids have the ability to lower surface and interfacial tensions markedly. Even though large amounts of phospholipids are present in yolk, only a small amount may be available for surface adsorption since the tensions of yolk and plasma are higher than that of the phospholipid sol. In pure lecithin sols, oriented phospholipid molecules in micelles are in equilibrium with molecularly-dispersed phospholipids in solution and at the surface or interface (Bangham, 1963). The low surface energy of a phospholipid sol is due to the adsorption of phospholipid molecules at the interface, with micelles as reservoirs for surface-active molecules. Robinson (1960) indicated that lecithin is in the micellar form even at concentrations as low as 5×10^{-5} g/ml. So far, no phospholipid micelles have been found in yolk. In fact, no mention has been made in the literature on molecularly-dispersed phospholipids. Phospholipids in yolk are considered to be bound in lipoproteins such as lipovitellins and low-density lipoproteins (Cook and Martin, 1962). Perhaps the lipoprotein complexes in yolk act as reserves for phospholipids to be released for interfacial adsorption. The migration of phospholipids from some lipoprotein complexes has been demonstrated. Kunkel and Bearn (1954) have shown the transfer of radioactive phospholipids from serum β-lipoproteins to α_1-lipoproteins. According to Greenbank and Pallansch (1961), any form of milk agitation can cause the migration of phospholipid from the fat-globule membrane to the skim milk.

Preliminary experiments have been conducted on the surface activity of solutions containing low-density lipoprotein, isolated from yolk plasma by a flotation procedure similar to that of Saari *et al.* (1964). This yolk fraction lowered the surface tension of water to about 42 dynes/cm when the concentration was between 15 and 0.25%. Apparently the solutions contained surface-active agents. In all probability, these agents were phospholipids released from the low-density lipoprotein.

REFERENCES

Amar, J. 1924. Physiologie générale—coagulation et structure de l'oeuf. *Compt. rend. Acad. Sci.* **178**, 803.

Aschaffenburg, R. 1946. Surface activity and proteins in milk. *J. Dairy Research* **14**, 316.

Bangham, A. D. 1963. Physical structure and behavior of lipids and lipid enzymes. R. Paoletti and D. Kritchevsky, eds. *Advances in Lipid Research* **1**, 65.

Clayton, W., and J. T. Morse. 1939. Direct observation of emulsification. *Chem. & Ind.* **17**, 304.

Cook, W. H., and W. G. Martin. 1962. Composition and properties of soluble lipoproteins in relation to structure. *Can. J. Biochem. Physiol.* **40**, 1273.

Greenbank, G. R., and M. J. Pallansch. 1961. Migration of phosphatides in processing dairy products. *J. Dairy Sci.* **44**, 1597.

Harkins, W. D., and N. Zollman. 1926. Interfacial tension and emulsification. I. The effects of bases, salts, and acids upon the interfacial tension between aqueous sodium oleate solutions and benzene. *J. Am. Chem. Soc.* **48**, 69.

King, A. 1941. Some factors governing the stability of oil-in-water emulsions. *Trans. Faraday Soc.* **37**, 168.

Kunkel, H. G., and A. G. Bearn. 1954. Phospholipid studies of different serum lipoproteins employing P_{32}. *Proc. Soc. Exptl. Biol. Med.* **86**, 887.

Jackson, R. H., and M. J. Pallansch. 1961. Influence of milk proteins on interfacial tension between butter oil and various aqueous phases. *J. Agr. Food. Chem.* **9**, 424.

Martin, W. G., J. E. Vandegaar, and W. H. Cook. 1957. Fractionation of livetin and the molecular weights of the alpha and beta components. *Can. J. Biochem. Physiol.* **35**, 241.

Miles, G. D., and L. Shedlovsky. 1944. Minima in surface tension-concentration curves of solutions of sodium alcohol sulfates. *J. Phys. Chem.* **48**, 57.

Pangborn, M. C. 1951. A simplified purification of lecithin. *J. Biol. Chem.* **188**, 471.

Powrie, W. D. 1955. A study of the gelation of hen's egg yolk. Ph.D. thesis. University of Massachusetts, Amherst, Mass.

Robinson, W. 1960. A light scattering study of lecithin. *Trans. Faraday Soc.* **56**, 1260.

Saari, A., W. D. Powrie, and O. Fennema. 1964. Isolation and characterization of low-density lipoproteins in native egg yolk plasma. *J. Food Sci.* **29**, 307.

Sell, H. M., A. G. Olson, and R. E. Kremmers. 1935. Lecitho-protein, the emulsifying ingredient in egg yolk. *Ind. Eng. Chem.* **27**, 1222.

Sugano, H., and I Watanabe. 1961. Isolations and some properties of native lipoproteins in egg yolk. *J. Biochem.* **50**, 473.

Yeadon, D. A., L. A. Goldblatt, and A. M. Altschul. 1958. Lecithin in oil-in-water emulsions. *J. Am. Oil Chemists' Soc.* **35**, 435.

Ms. rec'd 11/26/65.

This investigation was supported in part by Research Grant EF-505-01 from the U. S. Public Health Service and by a grant-in-aid from Poultry and Egg National Board.

FREDERICK J. KING
U. S. Bureau of Commercial Fisheries, Gloucester, Massachusetts 01930

Ultracentrifugal Analysis of Changes in the Composition of Myofibrillar Protein Extracts Obtained from Fresh and Frozen Cod Muscle

SUMMARY

Extracts prepared from stored frozen cod muscle showed that the extractability of components detected by ultracentrifugal analysis changed at different rates from the extractability of total myofibrillar protein. Analogous results were obtained by storing extracts as "model systems." The results consistently supported the postulated reaction equilibria: F-actomyosin ⇋ G-actomyosin ⇋ G-actin plus myosin ⇋ inextractable protein.

INTRODUCTION

Previous investigators have found a correlation between the textural quality of stored frozen fish muscle and the extractability of its myofibrillar protein. Their results have prompted several other investigations of components in the myofibrillar protein system. These subjects have already been thoroughly discussed in several reviews (Connell, 1962a, 1964; Dyer and Dingle, 1961; Love, 1960, 1962a; Hamoir, 1955).

However, there have been relatively few frozen-storage studies to characterize the specific myofibrillar component whose sensitivity to freezing-induced denaturation was responsible for the observed effects of storage on the extractability of total myofibrillar protein from fish muscle. In a series of frozen-storage studies of cod muscle, Connell employed selective extraction techniques for actin and myosin as well as extraction of "actomyosin" (measured as total myofibrillar protein). Using these techniques, he found no significant change in the amount of "native" actin that could be extracted from frozen cod muscle during a storage period in which the amount of total extractable myofibrillar protein fell from its initial value to a minimum (Connell, 1960). This result suggested that myosin was more sensitive to freezing-induced denaturation than actin. In a subsequent study, Connell (1962b) did find a decrease in the amount of myosin selectively extracted from frozen cod during the storage period. However, the amount of selectively extractable myosin was greater than the amount of total extractable myofibrillar protein. For example, almost 20% of the amount of myosin that was extractable at the beginning of the storage period was extracted from frozen cod muscle after a storage period had elapsed in which the amount of total extractable myofibrillar protein had decreased virtually to zero. Since neither myosin nor actin alone could have accounted for the observed effects of storage, Connell postulated an actin-myosin interaction in a hypothesis concerning the sensitivity of total myofibrillar protein to freezing-induced denaturation (Connell, 1962b). However, there was a possibility that one or both of the selective extraction techniques for actin or myosin might have reversed at least partially the unknown process of freezing-induced denaturation since this process was identified only on the basis of a different extraction technique to measure total extractable protein.

A more direct approach for characterizing the protein component which is responsible for the observed effects of storage on the textural quality of frozen fish muscle is to study the protein composition of the same extracts that have been used to measure the amount of total extractable myofibrillar protein from stored frozen fish muscle. Other workers have presumably considered this approach since they have used analytical ultracentrifugation for characterizing the components of myofibrillar protein extracts obtained from pre-rigor (Dingle *et al.*, 1963) or post-rigor (Ellis and Winchester, 1959) fresh cod muscle. Their schlieren patterns of post-rigor extracts contained two large peaks which were tentatively identified as actomyosin (fast moving peak) and myosin (slow moving peak). Their laboratory has also reported that dissociation of actomyosin was found in extracts from frozen stored cod muscle (Anon., 1962) as well as under conditions similar to frozen storage (Anon.,

1961; Dyer and Dingle, 1961), but these brief reports did not include experimental results. However, the results of very recent studies of stored frozen fish (Suzuki *et al.*, 1964) and stored unfrozen fish (Suzuki, 1965), although based on different species, do support the Canadian view that actomyosin dissociated before it became inextractable. The complex of actin and myosin that these Canadian and Japanese workers referred to as actomyosin is identical with F-actomyosin as defined in this paper. It should be distinguished from Connell's (1962b) postulated "actin-myosin" complex. Connell (1962b) has presented several reasons why the molecular characteristics of the actin and myosin components may be different in these two types of complexes.

The studies, based on myofibrillar protein extracts as well as selective extraction techniques, pointed to F-actomyosin or another type of "actin-myosin" complex instead of either myosin or actin alone as the component responsible for the observed rate of decrease in extractability of total myofibrillar protein during frozen storage of fish muscle. Both "actin-myosin" and F-actomyosin represented interactions of actin and myosin. However, it was difficult to reconcile Connell's interpretation that an actin-myosin interaction stabilized these proteins with the rapid dissociation of F-actomyosin as observed in storage studies by Canadian and Japanese workers.

Therefore, the present investigation was undertaken to: 1) characterize the components of myofibrillar protein extracts by quantitative as well as qualitative analytical ultracentrifugation techniques; 2) study the effect of storing extracts containing cod myofibrillar proteins by ultracentrifugal analysis; and 3) study the effect of storage on the amount of total myofibrillar protein in extracts of frozen cod muscle and to characterize the components of these extracts by ultracentrifugal analysis.

PART I. ULTRACENTRIFUGAL ANALYSIS OF COD MYOFIBRILLAR PROTEINS

EXPERIMENTAL METHODS

Material. Gutted cod (*Gadus morhua*) was obtained commercially. By the time it was used in an experiment its muscle was post-rigor, and it had been stored in ice for 24–48 hr after capture.

Buffer solution (0.05 μ) was made from 0.00338M KH_2PO_4 plus 0.0155M Na_2HPO_4. Its final pH was 7.5.

Buffer solution (0.5 μ) was made by adding KCl (final concentration 0.45M) to the 0.05-μ buffer solution. Its final pH was 7.2.

Method. All preparative operations were done in a 34°F (1°C) cold room using pre-chilled equipment and solutions. Muscle samples were blended with buffer solution in a Waring blender using a blender jar fitted with a rigid loose-fitting Teflon baffle (Anderson *et al.*, 1965). Before each extraction, the jar was checked for air bubbles by "blending" the buffer solution alone with the baffle in place. If air bubbles formed, the blending assembly was replaced with a new unit. If the jar passed this test, slices of muscle tissue were added and homogenized at reduced speed (7000 rpm) for 90 sec in nine 10-sec periods separated by 5-sec intervals to allow settling of undissolved material.

Preparative centrifugations were done for 30 min at 1000 × G in an International PR-2 Centrifuge refrigerated at 1°C using a type-284 rotor operated at 2400 rpm.

All extractions were based on a 1:19 flesh-solvent ratio to improve the yield of actomyosin. When a more dilute extract was desired, it was diluted with 0.5 μ buffer solution to the desired final concentration of total nitrogen (Anderson *et al.*, 1965).

Total protein extract. A 20.0-g sample of cod muscle myotomes was blended with 380 ml of 0.5-μ buffer solution and left overnight (18 hr). It was then centrifuged at 1000 × G to remove insoluble material. The upper portion of 150 ml was carefully removed and used as a total protein extract.

Myofibrillar protein extract. The principle of this method was to remove sarcoplasmic proteins by washing the sample of muscle tissue before dissolving its myofibrillar protein instead of precipitating myofibrillar protein from a total protein extract. A 20.0-g sample of cod muscle myotomes was blended with 380 ml of 0.05-μ buffer. The residue obtained by centrifugation at 1000 × G was resuspended by blending in 370 ml of 0.05-μ buffer for 30 sec in three 10-sec periods separated by 5-sec intervals, and then recentrifuged. This operation was repeated one more time. Then the residue was blended for three 10-sec periods in 360 ml of 0.5-μ buffer and left overnight (18 hr). It was then centrifuged at 1000 × G to remove insoluble material. The upper portion of 150 ml was carefully removed and used as a myofibrillar protein extract.

Total protein nitrogen of an extract was determined without further centrifugation by a biuret

procedure (Anderson *et al.*, 1965), assuming that the protein contained 16% nitrogen.

Ultracentrifugal analysis. A Spinco analytical ultracentrifuge, model E, was operated at 50740 rpm using the schlieren optical system with the rotor temperature control system set at 2.0°C (35.5°F). The operations of filling the 12-mm or 30-mm cells with aliquots of protein extracts and placing the cells into the An-E rotor were performed in a cold room at 34°F (1°C) using precooled equipment.

Sedimentation coefficients were calculated by the method of Svedberg and Pedersen (1940) using the relationship $s = d \ln x/\omega^2 dt$. Values of x were obtained from measurements of distances on photographs of the schlieren patterns with a Nikon optical comparator, model 6. The outer hole of the rotor was used as a reference point from which all distances were measured.

Peak areas were measured by enlarging the schlieren pattern in the optical comparator, tracing the area on paper, and weighing a cut-out of the area. Lower portions of these areas were drawn from the schlieren pattern obtained by centrifuging the buffer solution alone under identical operating conditions of the centrifuge and comparator. The area of peak III (slow moving peak) was measured from a photograph taken after 40 min of centrifugation at 50740 rpm to provide a constant position of the peak relative to the meniscus. The area of the fast-moving, hypersharp peak II was measured from any photograph in which the shape was clearly defined. The weight of a known area measured in the comparator from a photograph of the 306386 calibration cell taken in the centrifuge was used to convert grams of a peak area into square centimeters.

Calculation of protein concentration from peak area measurements was based on the method reviewed by Svedberg and Pedersen (1940) and Schachman (1959). All concentrations were corrected for radial dilution. However, no Johnston-Ogston corrections (reviewed by Schachman, 1959) were made, because of insufficient data for applying the theoretical assumptions underlying the corrections to mixtures of cod myosin and actomyosin and the pragmatic observation that, in the case of rabbit myosin and actomyosin at least, there was no significant difference in results whether Johnston-Ogston corrections were applied (Chiancone, 1962) or were not (Johnson and Rowe, 1964).

Some investigators have developed a factor to relate total protein concentration (biuret or Kjeldahl) to total area in the schlieren pattern of a protein extract. This type of factor has been used for cod myosin to average out variations between extracts arising from the contribution of contaminants such as lipids and nucleotides to the refractive index increment (Connell, 1963). Since his myosin extracts were ultracentrifugally homogeneous, Connell developed an accurate value for this factor. However, in the present study, different types of extracts were used which were obviously heterogeneous in the ultracentrifuge. To relate biuret values of these extracts to variations in area of each of the peaks seen in their schlieren patterns, one should separate the proteins in these extracts so that each schlieren peak can be studied individually. Attempts to perform this type of fractionation have not yet been completely successful. For this reason, a more empirical ratio was developed. Thirty total and myofibrillar protein extracts were used to calculate a ratio of total protein concentration as determined by biuret. An average value of 47.2% was obtained. No attempt was made to increase this value by subtracting the contribution to the biuret values of the gel fraction or sarcoplasmic protein in case of total protein extracts. To convert concentration units of g/ml into units of g protein nitrogen/ml it was assumed that the proteins contained 16% nitrogen.

RESULTS

Since the extracts used in this investigation usually contained a mixture of proteins, analytical ultracentrifugation was employed to identify the protein components. Since the schlieren patterns of these extracts usually contained more than one peak, the following sections describe the evidence by which the proteins responsible for the size and position of each peak are identified. In this description, a numerical system (Ellis and Winchester, 1959) is used to relate the results to the published literature. This numerical system is illustrated in Fig. 3.

The sedimentation data by which the schlieren peaks are classified came from several experiments. Data from myofibrillar protein extracts and total protein extracts were combined since there was no significant difference between these extracts in the size, shape, or position of the myofibrillar protein peaks on the schlieren pattern. A similar observation was made by Ellis and Winchester (1959). Since there is also no significant difference in sedimentation data based on extracts prepared from fresh or frozen post-rigor cod muscle, the data from these extracts have also been combined.

Peak II. This component has a characteristically high sedimentation rate which is very dependent on its concentration. Therefore the data (Fig. 1) were plotted from the equation $s = s_0/(1 + Kc)$ instead of the simpler equation $s = s_0 (1 - Kc)$ (Schachman, 1959). The s_0 at 2°C of 55 is almost the same as Ellis and Winchester's (1959) value

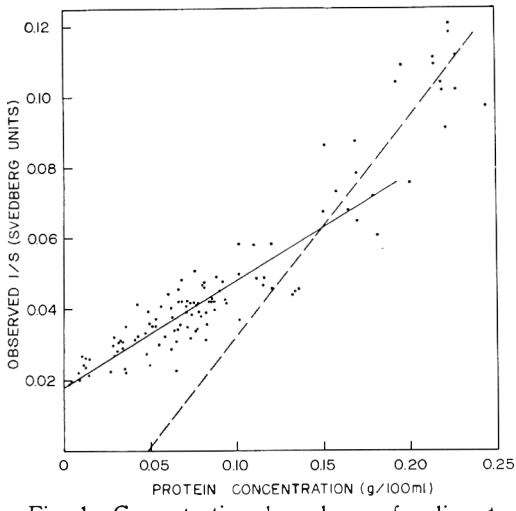


Fig. 1. Concentration dependence of sedimentation coefficient for peak II ("F-actomyosin") component of extracts containing cod myofibrillar proteins. Protein concentrations determined from area of the F-actomyosin peak in ultracentrifugal schlieren patterns.

of 60. When the s_0 is converted to standard conditions (method described by Svedberg and Pedersen, 1940), an $S^\circ_{20,w}$ of 92 is obtained. This value is comparable to reported values for F-actomyosin from other species (Table 1).

The concentration dependence of sedimentation rate is expressed as the slope of the s versus concentration line at low concentrations (Schachman, 1959). The slope of the line in Fig. 1 corresponds to a value of 0.18 at 20°C. Comparison of the results presented in Table 1 suggests that the slope is almost doubled if concentration is expressed as total protein (biuret determination) instead of the more specific determination of concentration from the ultracentrifugal data.

The sedimentation rate of concentrated F-actomyosin is only slightly dependent on its concentration as indicated by the dashed line in Fig. 1. Similar results were obtained from cod F-actomyosin (Ellis and Winchester, 1959) and carp F-actomyosin (Hamoir, 1955). In these special cases, the sedimentation rates of peaks II and III are nearly similar, so boundary effects (Schachman, 1959) may decrease the calculated concentration of F-actomyosin significantly. However, the sedimentation rate of F-actomyosin is also dependent on its tendency to form a gel-like physical state in concentrated solutions (Johnson and Rowe, 1964).

Table 1. Sedimentation data for F-actomyosin.

Species	$S^\circ_{20,w}$ in Svedberg units	Slope of line relating $1/S^\circ_{20,w}$ vs. protein concentration	Method used to obtain protein concentration	Reference
Cod	92	0.18	Schlieren peak of F-actomyosin	Present investigation
Cod	92	0.30	Total soluble protein of heterogeneous extract	Present investigation
Cod	101	0.25	Total soluble protein of heterogeneous extract	Calculated from Ellis and Winchester (1959)
Carp	90	0.16	Total soluble protein of homogeneous extract	Calculated from Hamoir (1955)
Rabbit	95	.....	.....	Hamoir (1955)
Mixed rabbit and pigeon extract	99.0	0.196	Schlieren peak of F-actomyosin	Fig. 1 of Chiancone (1962)
Homogeneous rabbit or pigeon extract	73.0	0.181	Schlieren peak of F-actomyosin	Fig. 1 of Chiancone (1962)
Rabbit	90	0.18	Schlieren peak of F-actomyosin	Calculated from Johnson and Rowe (1964)

Therefore, until more data are available, the concentration dependence of sedimentation rate for concentrated F-actomyosin is represented tentatively by the dashed line in Fig. 1.

Some of the concentrated F-actomyosin solutions contained two instead of one fast-moving peaks which were almost superimposed on each other. Ellis and Winchester (1959) presented similar data and classified the slower peak as II and the faster peak as I. However, evidence from rabbit F-actomyosin studies suggests strongly that the only difference between II and I is in the degree of polymerization of the F-actin component of F-actomyosin (Johnson and Rowe, 1964). Therefore, whenever such peaks were encountered, the average sedimentation rate and the total area were used to measure the sedimentation rate and concentration of F-actomyosin.

Peak IIIa. Some of the schlieren patterns of the extracts had a small peak sedimenting between peak II and peak III. The size of this peak depended principally on the extraction technique instead of the source of muscle used to extract myofibrillar protein. Specifically, the size of this peak depended on a rise in temperature during extraction or if air was incorporated when the muscle is homogenized. Therefore, no attempt was made to identify this peak. However, its sedimentation rate and the temperature effect of the extraction suggest a similarity with Martonosi's (1962) γ-component.

Peak III. This component had a characteristically moderate rate of sedimentation in contrast to peak II. Another distinguishing feature of this peak is that its rate of sedimentation is almost independent of concentration (Fig. 2). The sedimentation rate for similar concentrations of peak III in all of the extracts studied also remains essentially constant over a wide range of F-actomyosin concentrations. Similar results were obtained by Ellis and Winchester (1959). When the data in Fig. 2 are extrapolated to zero protein concentration an s_0 of 3.8 at 2°C is obtained, which corresponds to an $S^\circ_{20,w}$ of 6.4. Ellis and Winchester (1959) obtained a slightly lower value of 6.0 for peak III in similar extracts, but the difference is probably not significant considering the scatter of experimental data.

Peak IV. In preliminary ultracentrifugal analyses of total protein extracts, a small peak appeared when the boundary of peak III had moved almost halfway across the cell. Since this peak was very broad (an indication of heterogeneity) and since its area was always small in comparison with peaks III and II, it was not studied in detail. Nevertheless, the analyses of peak III were based on measurements in which peak III is near the meniscus, to minimize any possible contribution of the proteins represented by peak IV to the shape of peak III.

Gel fraction. Some of the properties of the gel fraction have been reported (Dingle *et al.*, 1963; Ellis and Winchester, 1959; Johnson and Rowe, 1964; Mommaerts, 1952), but its composition was not investigated in the present study.

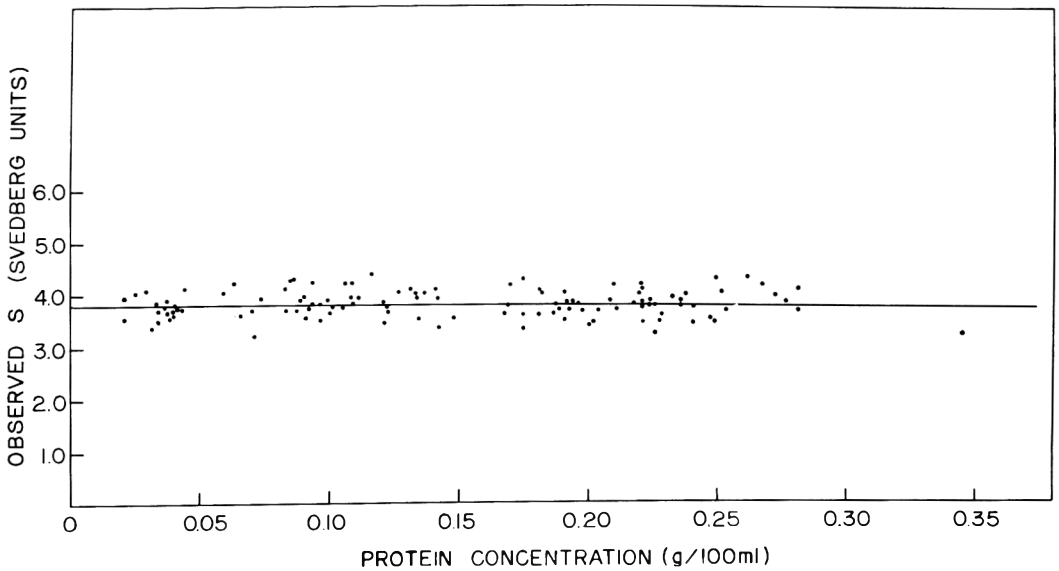


Fig. 2. Concentration dependence of sedimentation coefficient for peak III ("myosin plus G-actomyosin") component of extracts containing cod myofibrillar proteins. Protein concentrations determined from area of the myosin plus G-actomyosin peak in ultracentrifugal schlieren patterns.

DISCUSSION

The identification of peak II as F-actomyosin is reasonably certain. Its sedimentation rate and the pronounced dependence of this rate on its concentration are in excellent agreement with published data for mammalian as well as fish F-actomyosin. However, one should not infer that this component represents a single molecular species, since Johnson and Rowe (1964) have suggested that it may contain at least two different forms of F-actin.

Identification of the protein represented by peak III is complicated by the fact that the evidence suggests that it includes at least two myofibrillar protein components. The ultracentrifugal characteristics of peak III differ from those of homogeneous myosin selectively extracted from cod muscle (Connell, 1963). This difference could be due either to mistaken identification of peak III as myosin or that peak III really represents a mixture of myosin and another myofibrillar protein. Obviously, a decision between these possibilities has to be made.

One portion of the evidence is related to the possibility that myosin sediments as a component of peak III. The $S^{c}_{20,w}$ value of 6.4 is close to that for authentic myosin monomer (Connell, 1963) so that any myosin present in the extracts contributes to this peak. The results of our ultracentrifugal experiments in which adenosine triphosphate was added to myofibrillar protein extracts are similar to the results of Dingle *et al.* (1963) and Johnson and Rowe (1964), who suggested that a myosin-like protein was represented in their myofibrillar protein extracts.

Other lines of evidence suggest that the appearance of peak III in the schlieren patterns is not due to myosin alone. Peak III has a broader shape than homogeneous myosin monomer (Connell, 1963), and its concentration dependence of sedimentation is different. It is unlikely that this behavior is caused by significant interference from sarcoplasmic proteins ("albumins"), because the area of peak III was the same in total protein extracts and in myofibrillar protein extracts prepared from similar samples of either fresh or frozen cod muscle. The low concentration of tropomyosin in cod myofibrillar protein extracts (reviewed by Connell, 1964) and the low sedimentation rate of cod tropomyosin (Dingle and Odense, 1959) rule out the possibility of this protein also significantly affecting the area of peak III.

Johnson and Rowe's (1964) ultracentrifugal study of rabbit myofibrillar protein extracts provides strong supporting evidence for their hypothesis that they contained myosin bound to a relatively unpolymerized actin. Since this component was not isolated, they described it as "G-actomyosin" on the basis of its ultracentrifugally similarity to myosin and to distinguish it from F-actomyosin. Evidence for the existence of G-actomyosin was also derived from an experiment similar to that of Johnson and Rowe (1964) using cod instead of rabbit muscle (Anderson *et al.*, 1965). This ultracentrifugal study demonstrated that when the ionic strength of the extractant was increased from 0.5 μ to 0.8 μ the size of peak III (called "myosin" in that paper) increased at the expense of peak II (then called "actomyosin") since the total quantity of extractable protein remained essentially constant. This result is in excellent agreement with Johnson and Rowe's (1964) hypothesis that an increase in ionic strength displaces the equilibrium F-actomyosin $\rightleftharpoons$ G-actomyosin toward an increase in the concentration of G-actomyosin which is observed as an increase in the size of peak III.

In an ultracentrifugal study of myosin extracts obtained from frozen cod muscle, Connell (1962b) found other components that were derived from the fraction known as "extra-protein" and which were seen as small peaks sedimenting at almost the same rate as the myosin peak. The "extra-protein" fraction was defined in Connell's (1962b) paper as protein that is extracted together with the myofibrillar protein from muscle that has been previously washed with dilute buffer solutions, and after extraction it remains soluble when myosin and actomyosin are precipitated by dilution. The extracts examined in the present work will also contain the "extra protein" fraction, and there is a definite possibility that one major component is G-actomyosin. Perry and Zydowo's (1959) studies of the composition of "extra-protein" revealed that it is a heterogeneous mixture, but the component present in largest concentration is a "globulin" which they did not completely characterize. They found that this "globulin" gave a weak response when assayed for myosin adenosine triphosphatase activity, and essentially negative results when tested for the presence of actin or

Plate A—Extracts stored 0 days, 65.0°, 32 min, 30 mm cells.
Plate B—Extracts stored 6 days, 65.0°, 32 min, 30 mm cells.
Plate C—Extracts stored 21 days, 65.0°, 24 min, 30 mm cells.
Plate D—Cod stored 6 days (upper) or 1 day (lower), 65.0°, 32 min, 12 mm cells. →
Plate E—Cod stored 78 days (upper) or 73 days (lower), 55.0°, 24 min, 30 mm cells.
Plate F—As Plate E after 40 min.
Plate G—Cod stored 224 days (upper) or 219 days (lower), 55.0°, 4 min, 30 mm cells.
Plate H—As Plate G after 40 min.

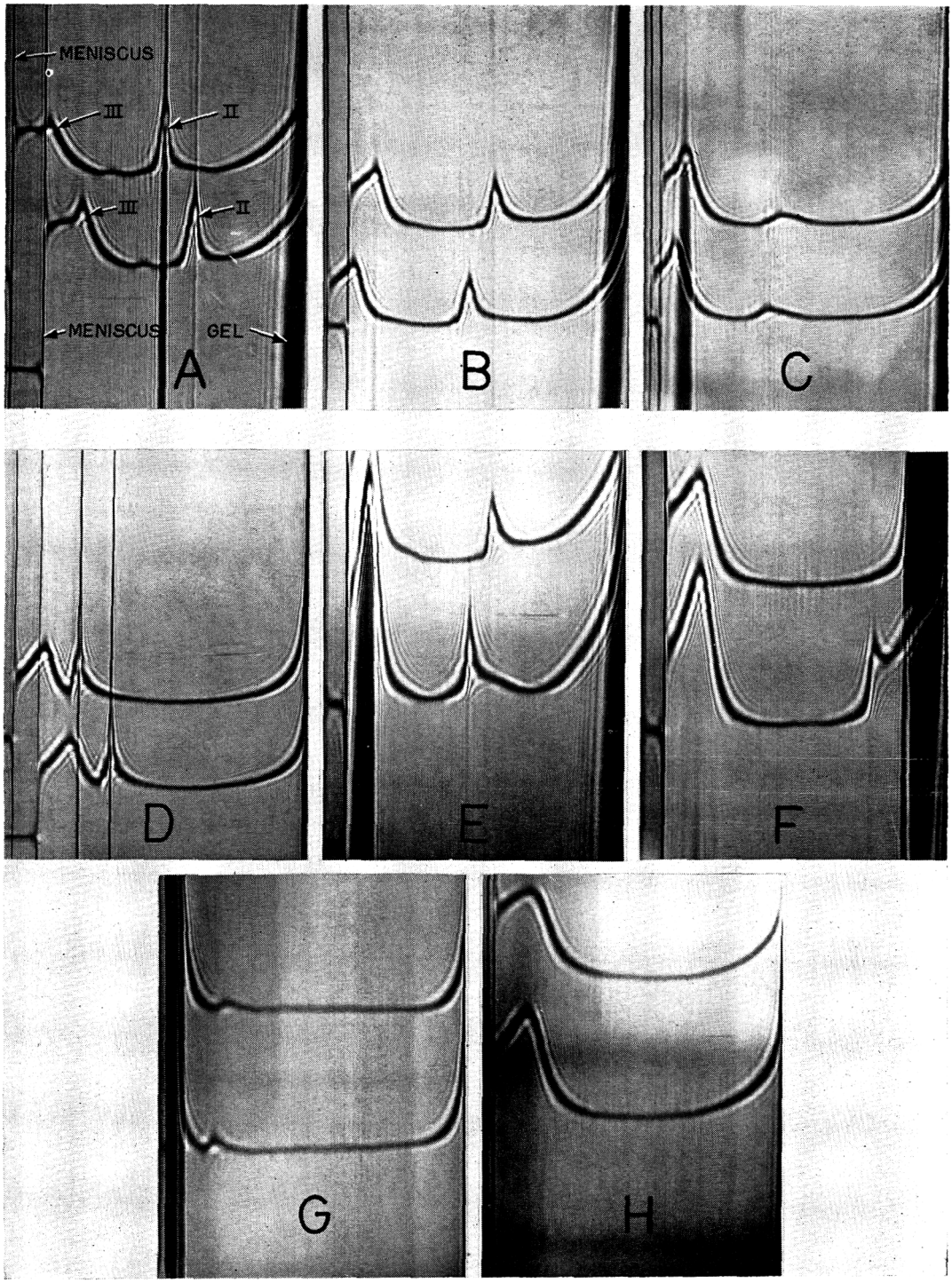


Fig. 3. Ultracentrifugal sedimentation of cod myofibrillar protein. Speed 50,740 rpm. Temperature 2.0°C. Direction of sedimentation from left to right. Lower patterns from cell with plain window. Upper patterns from cell with 1° positive wedge window. Times measured after rotor reached operating speed. Angles represent schlieren analyzer settings. Upper and lower patterns in Plates A, B, C represent two cod myofibrillar protein extracts stored at 34°F (1°C). Upper and lower patterns in Plates D through H represent two total protein extracts obtained from cod muscle stored at +3°F (−16°C).

myosin by applying either solubility criteria or viscometry to measure ability to combine with authentic myosin or actin. These results show that neither free actin nor free myosin was in this "globulin" fraction, but G-actomyosin as defined by Johnson and Rowe (1964) should react in this way. Connell's (1962b) solubility criteria for "extra-protein" give the impression that G-actomyosin cannot be a component of "extra-protein," because Johnson and Rowe's (1964) definition of G-actomyosin is based on their experimental procedure of precipitating myofibrillar protein at 0.2 μ or less and then dissolving the precipitate at a higher ionic strength. Johnson and Rowe (1964) have already suggested that an F-actomyosin precipitate at 0.2 μ can dissociate into G-actomyosin when it is solubilized by increasing the ionic strength. Thus, it is reasonable to propose that G-actomyosin is soluble at low ionic strengths even though it can also be present in F-actomyosin solutions that have been previously purified by precipitation at low ionic strengths.

In view of all the evidence from the other investigations as well as the evidence presented in the following sections, it is postulated that the protein observed as peak III during ultracentrifugation of the extracts used in this investigation, includes myosin and G-actomyosin. No attempt was made to determine specific concentrations of myosin or G-actomyosin by ultracentrifugal methods, since it was anticipated that these proteins had similar sedimentation rates (Johnson and Rowe, 1964). However, the results of the storage studies presented below provide a rough estimate of changes in their relative concentrations.

PART II. STORAGE STUDIES OF PROTEIN EXTRACTS AT 34°F (1°C)

EXPERIMENTAL METHODS

Six total protein extracts were prepared from fresh cod myotomes and diluted with 0.5-μ buffer solution to a final total protein concentration of 0.4–0.5 mg protein nitrogen per ml. These extracts were stored for up to 25 days at 34°F (1°C) in a cold room. An aliquot was removed from each extract at each of five regular intervals during the storage period for ultracentrifugal analysis and determination of total soluble protein nitrogen on its supernatant after centrifugation for 30 min at 1000 × G. The storage period was not carried beyond 25 days, since, during the period 20–25 days, the extracts developed a slight odor of putrefaction. The analytical methods were the same as those presented in Part I of this paper.

In a subsequent study, four myofibrillar protein extracts were stored and analyzed in the manner as described above.

RESULTS

The results were essentially the same whether actomyosin extracts or total protein extracts were stored at 34°F (1°C). The ultracentrifugal schlieren patterns demonstrated that the composition of these extracts changed during the storage period (Fig. 3). The concentration of the F-actomyosin component decreased linearly by an average of 0.44 ± 0.08 mg protein nitrogen per 100 ml per day, and the concentration of the myosin plus G-actomyosin component increased linearly at the same rate (Table 2). However, the total measured area of these schlieren peaks as well as the total soluble protein nitrogen content of each extract (0.4–0.5 mg per ml) remained essentially constant during the storage period.

DISCUSSION

The results of these studies clearly indicate dissociation of F-actomyosin. They do not support the hypothesis that F-actomyosin dissociates into F-actin and myosin at an ionic strength of 0.5. There is no ultracentrifugal evidence for a peak corresponding to F-actin. There is no evidence that F-actin is incorporated into the gel fraction or that it is precipitated, since the total area of the schlieren patterns as well as biuret determinations of total protein nitrogen remained constant throughout the storage period.

The results do provide additional evidence for the dissociation reaction F-actomyosin $\leftrightharpoons$ G-actomyosin as postulated by Johnson and Rowe (1964). Supporting evidence consists of the equal rates of change in the areas of schlieren patterns. The total area of the two peaks and the total protein concentration remained constant during the storage period. Since peak II was identified as F-actomyosin and peak III as a mixture of myosin and G-actomyosin (Part I of this paper), the results suggest that F-actomyosin was slowly dissociated to G-actomyosin during storage.

To fit the postulated reaction F-actomyosin $\leftrightharpoons$ G-actomyosin to the results, it is also necessary to assume that the ultracentrifugal component identified as myosin plus G-actomyosin contained more G-actomyosin and less myosin at the end of the storage period. Under similar storage conditions, a homogeneous extract of monomeric myosin aggregated spontaneously at a rapid rate (Connell, 1963). However, no evidence for the occurrence of this aggregation reaction was obtained in the present study, since a separate schlieren peak corresponding to an aggregated myosin was never observed. This result is in agreement with Connell's (1962b) observation that the presence of actin stabilized myosin against aggregating spontaneously.

Table 2. Ultracentrifugal analysis of the effect of storage at 34°F (1°C) on the composition of cod muscle protein extracts.

Sample	Rate of change in ultracentrifugal concentration: F-actomyosin (Mg protein nitrogen per 100 ml/day)	Rate of change in ultracentrifugal concentration: Myosin plus G-actomyosin (Mg protein nitrogen per 100 ml/day)	Storage time (days)	Area in ultracentrifugal schlieren patterns (arbitrary area units)
Total protein extracts				
A	−0.47	+0.50	3	.252
			6	.243
			10	.255
			13	.232
			18	.236
B	−0.45	+0.45	4	.250
			6	.248
			10	.254
			13	.249
			18	.258
C	−0.39	+0.47	4	.236
			6	.234
			10	.278
			13	.282
			18	.228
D	−0.44	+0.42	4	.280
			6	.235
			10	.249
			13	.229
			18	.216
E	−0.49	+0.51	4	.227
			6	.242
			10	.250
			14	.250
			18	.234
F	−0.50	+0.53	4	.217
			6	.211
			10	.239
			14	.230
			18	.222
Myofibrillar protein extracts				
1	−0.56	+0.57	3	.293
			5	.295
			11	.295
			14	.295
			21	.294
2	−0.41	+0.41	4	.292
			11	.292
			14	.292
			21	.293
3	−0.40	+0.37	5	.285
			11	.285
			14	.285
			21	.286
4	−0.29	+0.27	5	.192
			11	.193
Average rate of change	±0.44	±0.08	14	.193
			21	.193

The extent to which F-actomyosin was dissociated at 0.5μ is also analogous to a previous study in which higher ionic strengths dissociated more of the F-actomyosin immediately (Fig. 2 of Anderson *et al.*, 1965). Since the only difference between these experiments was the extent to which F-actomyosin dissociated, it is evident that soluble F-actomyosin in a low-ionic-strength medium can in time dissociate to the same extent as it can immediately in a higher-ionic-strength medium. In all of these cases, F-actomyosin is presumed to dissociate into G-actomyosin. This concept may also be applicable to the results of storage studies based on frozen cod muscle since freezing results in an increase in the ionic strength of those tissue fluids which remain unfrozen.

PART III. EXTRACTS PREPARED FROM STORED FROZEN COD MUSCLE

The following procedures were adopted in two separate frozen-storage studies. For each study, a very large cod (25–30 lb) was filleted. The paired fillets were wrapped securely in polyethylene bags, frozen in a plate freezer, and then stored at +3°F (−16°C). At intervals during the storage period, duplicate 20.0-g samples of frozen flesh were sliced from the anterior midsection of each fillet and used immediately to prepare total protein extracts. Total soluble protein nitrogen determinations and ultracentrifugal analyses were carried out on these extracts. The analytical methods were the same as those in Part I of this paper.

Two other frozen storage studies were also carried out. Their principal value was to demonstrate that the fillets should be wrapped securely to inhibit desiccation and that more samples should be obtained especially during the first 100–150 days of storage.

RESULTS

During the period of frozen storage, total extractable protein decreased at a steady rate (Fig. 4). Similar results have been widely used to indicate the effect of storage on specific extractability of myofibrillar protein from frozen cod (reviewed by Dyer and Dingle, 1961; Love, 1962b). Such results have also demonstrated that extractability of the water-soluble sarcoplasmic protein fraction from frozen cod muscle does not change during frozen storage (reviewed by Dyer and Dingle, 1961). Since the total extractable protein from fresh cod muscle contains about 22% water-soluble protein (reviewed by Dyer and Dingle, 1961), it is assumed that all the extracts on which Fig. 4 is based contained an average of 0.3 mg water-soluble protein nitrogen per ml. The total protein content of the extracts had dropped to almost this level by the end of the storage period. It is assumed, there-

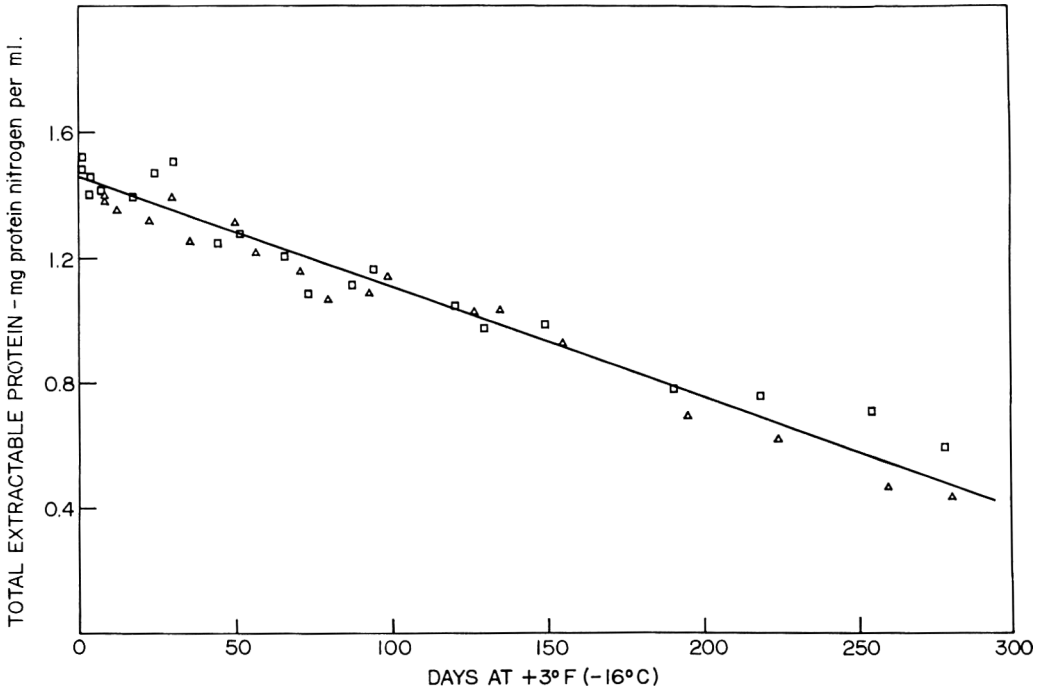


Fig. 4. Change in amount of total extractable protein from cod flesh stored at +3°F (−16°C). △ third storage study; □ fourth storage study.

fore, that the data given in Fig. 4 satisfactorily represent a storage period during which the extractability of myofibrillar protein from frozen cod decreases almost to zero.

The extracts obtained during this study were also examined in the ultracentrifuge. Their schlieren patterns reveal that the areas of the fast peak (F-actomyosin) and the slow peak (myosin plus G-actomyosin) are related to storage time of the frozen cod from which the extracts were prepared (Fig. 3). These peak areas were converted into units of protein concentration which are shown in Figs. 5 and 6. Fig. 5 shows a rapid initial decrease in extractability of the F-actomyosin component until about the 75th day of storage. Fig. 6 shows that the extractability of the myosin plus G-actomyosin component increased until a maximum was reached after about 75 days. Extractability of this component then decreased steadily until the end of the storage period. A peak that would have corresponded to F-actin was never observed.

The protein concentrations shown in Figs. 5 and 6 were not converted from units of mg protein nitrogen per ml of extract into mg protein per 100 g of muscle, because there was too much uncertainty in relating effects of lipid and nucleotide contaminants on the size of the schlieren peaks from which these protein concentrations were calculated (Part I of this paper). However, a rough estimation of the absolute extractability of these components may be useful. Extracts prepared from fresh cod muscle (zero time in Fig. 4) contained about 1.5 mg protein nitrogen per ml, equivalent to 3.0 g protein nitrogen per 100 g muscle. About 47% of this protein nitrogen was detected ultracentrifugally as F-actomyosin (0.72 g per 100 g, calculated from Fig. 5) and myosin plus G-actomyosin (0.64 g per 100 g, calculated from Fig. 6). The remainder (1.6 g per 100 g) includes about 22% sarcoplasmic protein (Dyer and Dingle, 1961), or 0.6 g per 100 g and about 20% gel fraction (Ellis and Winchester, 1959), or 0.6 g per 100 g. These calculations leave about 0.4 g per 100 g, or 13% of the total extractable protein, still unaccounted for. Included in this 13% are the small amounts of other myofibrillar proteins such as tropomyosin and errors in the experimental methods (Part I of this paper).

DISCUSSION

The observed rate of decrease of total extractable protein from frozen cod muscle can be partly explained on the basis that it is due to direct formation of aggregated and inextractable F-actomyosin. This concept has already been discussed in a previous report (Anderson *et al*, 1965). Direct formation of aggregated F-actomyosin can account for part of

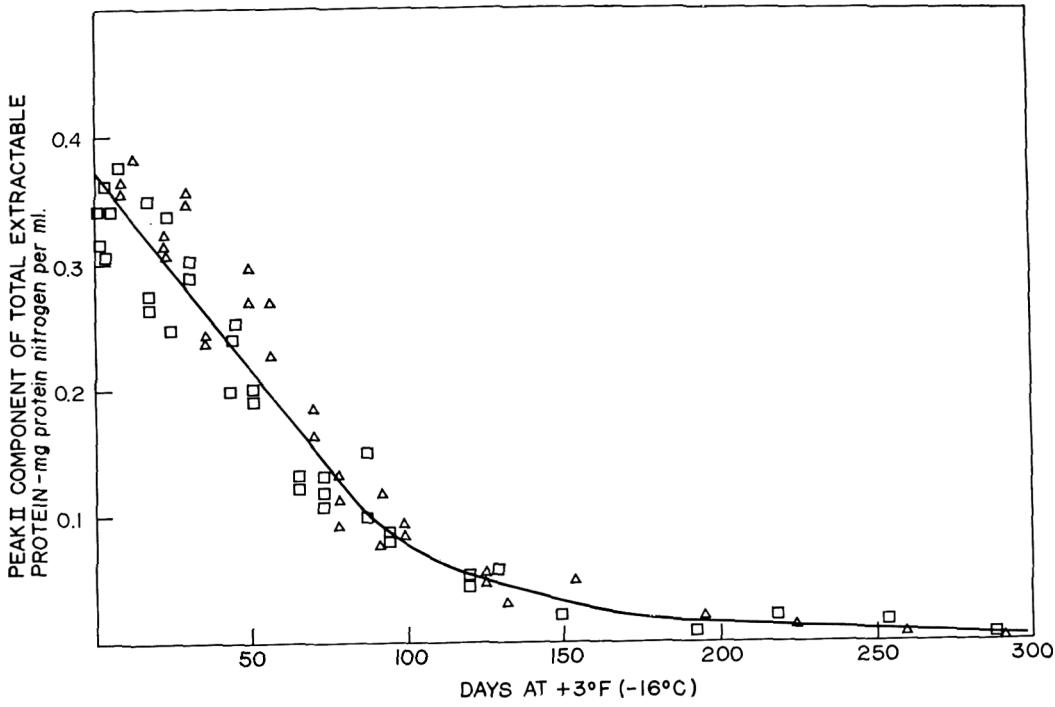


Fig. 5. Change in amount of the peak II ("F-actomyosin") component in total protein extracts obtained from cod flesh stored at +3°F (−16°C). F-actomyosin concentrations determined from area of its peak in ultracentrifugal schlieren patterns. △ third storage study; □ fourth storage study.

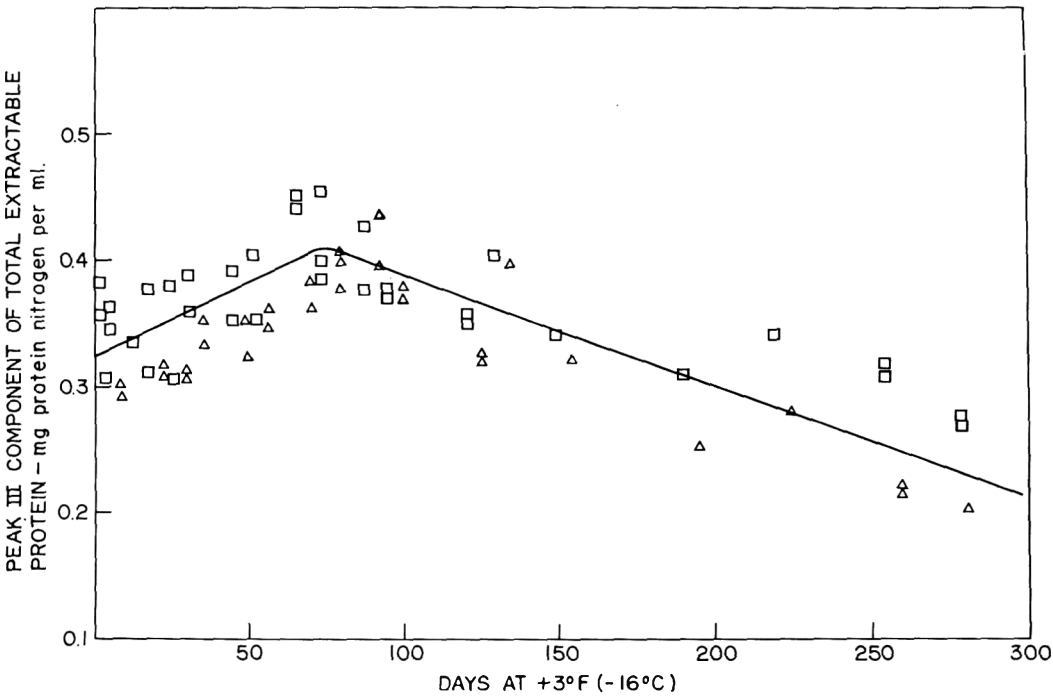


Fig. 6. Change in amount of the peak III ("myosin plus G-actomyosin") component in total protein extracts obtained from cod flesh stored at +3°F (−16°C). Myosin plus G-actomyosin concentrations determined from area of its peak in ultracentrifugal schlieren patterns. △ third storage study; □ fourth storage study.

the rapid loss in extractability of the ultracentrifugal F-actomyosin component (Fig. 5). However, the possibility of F-actomyosin aggregating directly into inextractable protein fails to provide a satisfactory cause-and-effect explanation for the different rates of change in extractability of F-actomyosin (Fig. 5), myosin plus G-actomyosin (Fig. 6), and total extractable protein (Fig. 4).

A more inclusive reaction scheme (Fig. 7) is proposed to integrate all of the results. In addition to a direct aggregation pathway it provides a dissociation pathway for the conversion of F-actomyosin into other forms of myofibrillar protein. This dissociation pathway was originally postulated in Johnson and Rowe's (1964) study of myofibrillar protein extracts obtained from fresh (unfrozen) rabbit muscle and has been adapted to the present investigation. According to this reaction scheme, the rapid decrease in amount of F-actomyosin (Fig. 5) with the slower increase in the amount of myosin plus G-actomyosin (Fig. 6) during the initial storage period (0 to 75 days) suggests a relatively rapid dissociation of F-actomyosin into G-actomyosin followed by a slower rate of dissociation of G-actomyosin into components which then aggregate to form inextractable protein. The assumption that G-actomyosin is more stable than F-actomyosin, even though it eventually dissociates into inextractable protein, also provides an explanation for the observed rate of decrease in Fig. 6 during the storage interval of 100–300 days in which only a minimum amount of F-actomyosin was extracted (Fig. 5). This reaction scheme is also consistent with the failure to observe an ultracentrifugal schlieren peak which would have corresponded to F-actin.

The postulated participation of G-actomyosin in this stepwise series of myofibrillar protein reactions is also suggested by the results of the extract storage study (Part II of this paper). Although that study is based on protein extracts prepared from fresh muscle, the ionic strength of the system is similar to those likely to occur in the cellular fluid of frozen cod (Anderson *et al*, 1965). In addition, the study on extracts is similar to the frozen muscle study in that it provides similar evidence for the greater stability of G-actomyosin over F-actomyosin under the storage conditions used.

Comparison of the results of this investigation with Connell's (1962b) work presents additional evidence for the involvement of G-actomyosin. The basis for comparing these studies is the similarity of results in measurement of total extractable myofibrillar protein (Fig. 4, compared with Fig. 2 of Connell, 1962b). By using a selective extraction method, Connell obtained a significant amount of

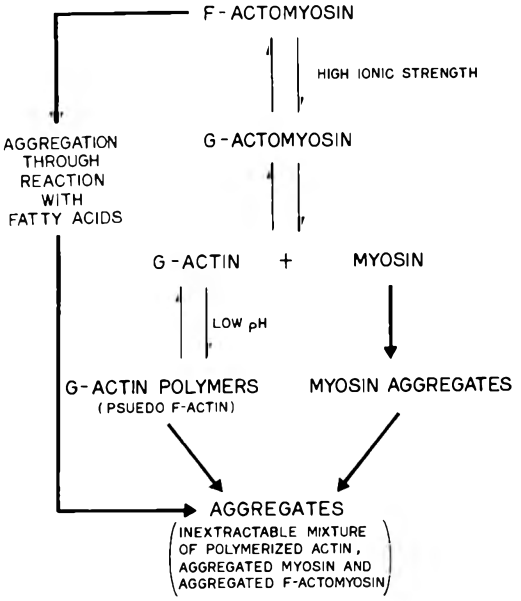


Fig. 7. Postulated mechanism for the conversion of myofibrillar protein into inextractable protein (adapted from Fig. 27.13 of Johnson and Rowe, 1964).

extractable monomeric myosin from frozen-stored cod that contained very little total extractable myofibrillar protein. However, since selective extraction of myosin is based on the capacity of adenosine triphosphate or pyrophosphate to split the actin-myosin complex, this extraction method is presumably incapable of distinguishing between myosin derived from F-actomyosin, G-actomyosin, or myosin not previously bound to actin. Connell (1962b) proposed that the myosin observed in his extract prepared from frozen-stored cod was partially derived from myosin bound to actin. The results of the present investigation suggest that the components of this complex are G-actin and myosin rather than F-actomyosin, especially in extracts prepared from aged frozen-stored cod.

Although the evidence suggests a gradual dissociation of F-actomyosin into G-actomyosin during frozen storage, it is still speculative whether this dissociation occurs entirely *in situ*. Procedures used to measure extractability of actomyosin from frozen muscle are relatively non-selective even though they are commonly used in investigations of deterioration of texture in stored muscle. During such extraction processes, it is possible that some actin and myosin molecules which were not combined *in situ* may form actin-myosin complexes *in vitro* (Connell, 1962b.) During extraction, there is also an opportunity for denaturation of actomyosin by reaction with fatty acids that are extracted together with the protein (Anderson *et al*, 1965). Considerably more work is needed to determine the effect of frozen storage on alterations of myofibrillar protein *in situ*.

CONCLUSIONS

The results of this investigation and those of a previous study (Anderson *et al*, 1965) consistently support the postulated series of stepwise dissociation and aggregation reactions illustrated in Fig. 7. Storage conditions which decreased the amount of soluble F-actomyosin displaced these reactions in favor of dissociation and aggregation into insoluble protein. The present evidence complements Johnson and Rowe's (1964) findings in providing strong supporting evidence for G-actomyosin which they originally distinguished from F-actomyosin on the basis of the molecular form of its actin component. The present results also suggest that G-actomyosin is a more stable form of actomoysin than F-actomyosin. The principal weakness in this suggestion is, of course, that, to the best of our knowledge, no one has yet developed a specific test for G-actomyosin in the presence of myosin, F-actomyosin, or other myofibrillar protein. We also need more information about the nature of the complexes that actin and myosin can form both *in situ* and *in vitro*, and the relation of the environments to their formation and stability. Nevertheless, by considering myofibrillar protein as an equilibrium system which includes G-actomyosin instead of as a simple mixture of actin, myosin, and F-actomyosin, a reaction scheme such as that of Fig. 7 may provide a useful working hypothesis to explain consistently the effect of several commercial storage or processing conditions of fish muscle on myofibrillar protein denaturation in terms of how these conditions displace equilibria between the actin, myosin, and actomyosin components.

REFERENCES

Anderson, M. L., M. A. Steinberg, and F. J. King. 1965. Some physical effects of freezing fish muscle and their relation to protein-fatty acid interaction. "The Technology of Fish Utilization." p. 105. Fishing News (Books) Ltd., London, England.

Anon. 1961. Annual Report of the Fisheries Research Board of Canada for the Fiscal Year ended March 31, 1961, p. 134.

Anon. 1962. Annual Report of the Fisheries Research Board of Canada for the Fiscal Year ended March 31, 1962, p. 149.

Chiancone, E. 1962. Observations on the concentration dependence of sedimentation coefficients in synthetic actomyosins. *Italian J. Biochem. (English ed.)* **11**(6), 375.

Connell, J. J. 1960. Changes in the actin of cod flesh during storage at −14°. *J. Sci. Food Agr.* **11**(9), 515.

Connell, J. J. 1962a. Fish Muscle Proteins. "Recent Advances in Food Science." Vol. 1, p. 136. (J. Hawthorn and J. M. Leitch, eds). Butterworth's, Ltd., London, England.

Connell, J. J. 1962b. Changes in amount of myosin extractable from cod flesh during storage at −14°. *J. Sci. Food Agr.* **13**(11), 607.

Connell, J. J. 1963. Sedimentation and aggregation of cod myosin: a reappraisal. *Biochim. et Biophys. Acta* **74**, 374.

Connell, J. J. 1964. Fish muscle proteins and some effects on them of processing. "Symposium on Foods: Proteins and Their Reactions." Chapt. 12, p. 255. (H. W. Schultz and A. F. Anglemier, eds.). Avi Publishing Co., Westport, Connecticut.

Dingle, J. R., and P. H. Odense. 1959. Proteins in fish muscle. 14. Cod tropomyosin. *J. Fisheries Research Board Can.* **16**(1), 53.

Dingle, J. R., D. G. Ellis, J. A. Hines, and J. T. Lauder. 1963. Proteins in fish muscle. 18. Sedimentation patterns of myosin-B extracts of prerigor cod muscle. *Can. J. Biochem. Physiol.* **41**, 1915.

Dyer, W. J., and J. R. Dingle. 1961. Fish proteins with special reference to freezing. "Fish as Food." Vol. I, p. 275. (Georg Borgstrom, ed.). Academic Press, New York.

Ellis, D. G., and P. M. Winchester. 1959. Proteins in fish muscle. 12. Ultracentrifuge studies on post-rigor extracts of structural protein. *J. Fisheries Research Board Can.* **16**(1), 33.

Hamoir, G. 1955. Fish proteins. *Advances in Protein Chem.* **10**, 227.

Johnson, P., and A. J. Rowe. 1964. An ultracentrifuge study of the actin-myosin interaction. "Biochemistry of Muscle Contraction." (John Gergely, ed.). Chapt. 27, p. 279. Little, Brown and Co., Boston, Massachusetts.

Love, R. M. 1960. Texture changes in fish and its measurement. "Texture in Foods." *Soc. Chem. Ind., Monograph* No. **7**, 109, London, England.

Love, R. M. 1962a. The effect of freezing on fish muscle. "Recent Advances in Food Science." **1**, 147. (J. Hawthorn and J. M. Leitch, eds.). Butterworths, Ltd., London, England.

Love, R. M. 1962b. Protein denaturation in frozen fish. VI. Cold-storage studies on cod using the cell fragility method. *J. Sci. Food Agr.* **13**(5), 269.

Martonosi, A. 1962. Studies on actin. VII. Ultracentrifugal analysis of partially polymerized actin solutions. *J. Biol. Chem.* **237**(9), 2795.

Mommaerts, W. F. H. M. 1952. The molecular transformations of actin. II. The polymerization process. *J. Biol. Chem.* **198**, 459.

Perry, S. V., and M. Zydowo. 1959. The nature of the extra protein fraction from myofibrils of striated muscle. *Biochem. J.* **71**, 220.

Schachman, H. K. 1959. "Ultracentrifugation in Biochemistry." Academic Press, New York.

Suzuki, T. 1965. Unpublished data. Tokai Regional Fisheries Research Laboratory, Tokyo, Japan.

Suzuki, T., K. Kanna, and T. Tanaka. 1964. Studies on protein denaturation of frozen fish. Comparison between super rapid freezing by liquid nitrogen (−196°C) and air (−20°C) freezing. *Bull. Japan. Soc. Sci. Fisheries* **30**(12), 1022.

Svedberg, T., and K. O. Pedersen. 1940. "The Ultracentrifuge." Clarendon Press, Oxford, England, reprinted by Johnson Reprint Corp., New York.

Ms. rec'd 5/19/65.

Presented at the 25th Meeting of the Institute of Food Technologists, Kansas City, Mo., May 16–20, 1965.

DEFINITIONS

Ionic strength of a solution is indicated by the symbol μ.

Total protein extract is defined by the experimental procedure as an extract containing all of the muscle proteins that are soluble in neutral 0.5-μ salt solution.

Myofibrillar protein extract is defined by the experimental procedure as an extract which contains myofibrillar proteins but not 0.05-μ neutral salt-soluble sarcoplasmic protein.

Actomyosin suggests a particular protein, but its usage has created confusion. Part of the ambiguity stems from the tendency of some investigators to describe actomyosin on a molecular basis even though their extracts were a more or less heterogeneous mixture of actomyosin and other proteins. However, even if the term "actomyosin extract" were used to identify actomyosin, there would still be an ambiguity in this paper, since it is essential to distinguish the more general evidence for actomyosin from the more specific evidence for F-actomyosin or other forms of actin-myosin interactions. Therefore, the more general term "myofibrillar protein extract" is used instead of "actomyosin extract" to refer to an extract con-

taining actomyosin. The term "F-actomyosin" is used only when the intention is to describe an actomyosin in which myosin has interacted with the highly polymerized forms of actin that other investigators recognize as F-actin. Since a definition of actomyosin specific for actin not as highly polymerized as F-actin prejudges the result of this investigation, the term "actomyosin" is used only when there is no reason to be specific about the size of the actin component.

Gel fraction is protein that is soluble in 0.5-μ neutral salt solution after preparative centrifugation (1000 $\times$ G for 30 min) but is removed from solution when the analytical ultracentrifuge is accelerated to operating speed (zero to 50,740 rpm or 187,000 $\times$ G).

KI SOON RHEE and BETTY M. WATTS
Department of Food and Nutrition
Florida State University, Tallahassee, Florida 32306

Evaluation of Lipid Oxidation in Plant Tissues

SUMMARY

Essential to application of the TBA test to plant tissues as a measure of prior lipid oxidation is inactivation of lipoxidase in the blending procedure by a strong acid. The production of TBA-reactive material during blending without acid may give a useful measure of the "lipid oxidation potential" of vegetable materials, as affected by their content of lipoxidase, substrate and antioxidants. Comparative data are presented on fatty acids and lipoxidase in dried Blackeye and Green (Burpee's blue bantam) peas, and the data are related to the TBA values in blending of these peas. Wide distribution of lipoxidase in vegetables and fruits has been demonstrated. Among the materials analyzed, the extracts of roots, tubers, botanical fruits with edible seed portion, and seeds usually showed a high lipid-oxidizing activity. Comparing families, the extracts of many vegetables belonging to Leguminosae, Solanaceae, and Cruciferae had relatively higher activity.

INTRODUCTION

Many plant tissues are known to contain the enzyme lipoxidase as well as the polyunsaturated fatty acids which serve as substrates for this enzyme. Lipid oxidation, resulting in rancid odors, might therefore be expected under conditions where raw or inadequately blanched vegetables are macerated, frozen, or otherwise treated in such a way that enzyme, substrate, and oxygen are brought together. Unfortunately, objective data implicating lipid oxidation are sparse in the literature.

The lack of established methods for evaluating lipid oxidation in plant materials has hampered progress in assessing the extent and importance of this reaction. Peroxide numbers have been used almost exclusively for this purpose. Peroxide determinations, which involve a preliminary extraction of the small amounts of lipid present, are cumbersome and unsatisfactory in several respects. They are usually limited to the easily extractable triglycerides. Furthermore, peroxides are only intermediates in the production of rancid end products, and in the presence of peroxide decomposers they may not accumulate even when lipid oxidation is far advanced.

Measurement of an end product of lipid oxidation would seem to be preferable to peroxide determination. In studies of stored peas it has been suggested that gas chromatographic evaluation of hexanal might be used as a measure of rancidity (Bengtsson and Bosund, 1964), but the reliability and applicability of such a test have not been examined.

Malonaldehyde, measured either by its reaction with 2-thiobarbituric acid (TBA) or by its absorption in the ultraviolet (Kwon and Watts, 1963), has proved highly useful in objective evaluation of lipid oxidation in animal tissues. Malonaldehyde is derived directly under test conditions from the hydroperoxides of fatty acids having three or more double bonds (Dahle *et al.*, 1962) and may arise from secondary oxidations of other lipid oxidation products (Lillard and Day, 1964). Tests for malonaldehyde have apparently not been applied to vegetable materials. In making such an application it may be anticipated that inactivation of lipoxidase in the blending step would be essential.

Rapid, precise methods for determining the rate of lipid oxidation by vegetable extracts or by purified lipoxidase preparations are also of interest in studies of enzyme stability, antioxidant effectiveness, etc. Surrey (1964) recently improved the methodology of lipoxidase assays by solubilizing fatty acid substrates. Although he used diene conjugation as a measure of the extent of lipid oxidation in his model mixtures, polarographic measurements of oxygen uptake are simpler and give a continuous record of reaction rates.

This paper describes: 1) malonaldehyde as a measure of lipid oxidation in plant tissues; 2) lipid-oxidizing activities of various vegetable and fruit extracts; and 3) variations in lipoxidase and substrate between two varieties of peas as related to the amount of malonaldehyde produced during blending.

MATERIALS AND METHODS

Lipoxidase and fatty acids. Purified soybean lipoxidase was obtained from Nutritional Biochemicals Corporation, and linoleic and linolenic acids were from the Hormel Institute.

Procedure for TBA test on vegetables. A distillation method developed by Tarladgis *et al.* (1960) was slightly modified by the addition of acid during blending, in order to inactivate lipoxidase. The method finally adopted for TBA test in the presence of lipoxidase follows. A given weight of vegetable was blended for 2 min with 5 ml of HCl (1:2) plus distilled water to make 60 ml in a Virtis "45" homogenizer. The mixture was quantitatively transferred into a Kjeldahl flask by washing with 40 ml distilled water. The remainder of the procedure was carried out as described by Tarladgis *et al.* TBA numbers are expressed as mg malonaldehyde per 1,000 g vegetable tissue.

A polarographic method for lipoxidase assay. Polarographic measurements of oxygen tension were adapted for determination of lipoxidase activity as follows. The rates of oxygen consumption of fatty acid solutions or emulsions, pH 6.2, in the presence of purified lipoxidase or aqueous crude vegetable extracts were measured with the Beckman oxygen analyzer, model 777, and recorder. The decrease of oxygen tension, in mm Hg per minute, at the maximum slope is taken as a comparative measure of the rate of oxygen consumption.

The detailed procedure was as follows. A 50-ml Erlenmeyer flask with Teflon magnet was placed on an asbestos-covered foam pad on a magnetic stirrer. Then, 10 ml crude enzyme extract was quickly mixed with 50 ml emulsion in a 250-ml Erlenmeyer flask and immediately transferred to the 50-ml flask. The sensor was promptly inserted into the neck of the flask, and measurement was begun.

Changes in oxygen tension could not be precisely translated into moles of oxygen consumed, since the solubility of oxygen in the reaction mixture at any particular oxygen tension is not known. However, assuming that the low concentrations of linoleic acid, Tween 20, phosphate buffers, and pea extract would not significantly affect the amount of dissolved oxygen, the figure of oxygen solubility of 8.4 ppm O_2 in pure water in equilibrium with water-saturated air at 25°C and 760 mm Hg (160 mm PO_2) may be used as a rough approximation. A fall of 1 mm in the oxygen tension measured is roughly equivalent to a loss of 0.053 ppm oxygen or 1.7×10^{-3} μmoles of oxygen per milliliter of reaction mixture.

Gas-liquid chromatographic (GLC) analysis of fatty acids. Lipids were extracted from peas by the method described by Folch *et al.* (1957) using 2:1 (v/v) chloroform-methanol mixture. Dried peas were soaked in a small volume of distilled water before lipid extraction. The extracted lipids were saponified with a 1:1 (v/v) mixture of 50% aqueous sodium hydroxide and absolute ethanol. The saponification procedure was similar to the method of Ast (1963). After saponification and acidification the free acids were extracted with n-hexane. The solvent was removed and the fatty acids converted to their methyl esters with boron trifluoride-methanol reagent following the procedure given by Metcalfe and Schmitz (1961).

GLC analysis of the methyl esters was carried out using a flame ionization gas chromatograph F & M Model 609, with 6-ft × ¼-in. stainless-steel column packed with 15% diethylene glycol succinate on 80–100-mesh Chromosorb W. The runs were isothermal, at 190°C, and the column carrier helium flow was 62.5 ml per minute. The area of a peak was measured by a planimeter.

The gas chromatograph was standardized, using the model mixture of known pure fatty acid methyl esters, the esters of palmitic, stearic, oleic, linoleic, and linolenic acids, from Hormel Institute. Quantitative results on the standards agreed well with the known composition.

Determination of lipid-oxidizing activity in fruit and vegetable extracts. Extracts were prepared from fresh vegetables and fruit purchased from the local market. Variety and harvest time were not determined. Prior to chopping, vegetables and fruit were washed, rinsed with distilled water, blot-dried, and weighed. Roots, stem ends, cores of fruit, and other parts not commonly consumed, were discarded unless otherwise indicated.

Ten to fifty grams of minced tissue were blended with 100 ml distilled water for 2 min, using a Virtis "45" homogenizer equipped with an ice-water bath. The slurry was filtered through a fat-free filter paper, and the filtrate was used as crude enzyme extract in Surrey's method (1964) for lipoxidase activity.

RESULTS AND DISCUSSION

Malonaldehyde production in lipoxidase-catalyzed oxidation of linolenic acid. To determine whether malonaldehyde is produced when linolenate is oxidized by lipoxidase, model systems were prepared containing lipoxidase (.025%), phosphate buffer (pH 6.2), and increasing amounts of linolenic acid emulsified with Tween 20. Rate of oxygen consumption was measured on each mixture with the oxygen analyzer. Aliquots (20 ml) of the same mixtures were

allowed to react at room temperature for 10 min, then the enzyme was inactivated with 2.5 ml HCl (1:2), and the mixture was made up to 100 ml and distilled for TBA analysis.

Table 1 demonstrates that malonaldehyde is produced in increasing amounts with increased linolenate oxidation under these conditions. Saslaw and Waravdekar (1965), on the other hand, found that linolenate hydroperoxide prepared by the action of lipoxidase, was inactive in the TBA test. These apparently conflicting results cannot be reconciled in the absence of more detailed information on experimental procedure.

Inactivation of lipoxidase for TBA test on vegetables. The most important step in applying the TBA test to plant tissues is inactivation of lipoxidase, which catalyzes lipid oxidation in the blending step. Since lipoxidase, like most enzymes, can be easily inactivated at low pH, the experiment was designed to determine the extent of lipoxidase inhibition by acid in the TBA test. The material used was fresh White Acre peas. Different amounts of HCl (1:2) were added to 10-g samples in blending. The results are shown in Table 2.

The extensive oxidation of the lipid matter of blended raw peas shown in Table 2 was also found by Wagenknecht and Lee (1956). The addition of 2.5 ml HCl (1:2) to a 10-g portion of peas was sufficient to inhibit enzyme action. The TBA numbers obtained from enzyme-inactivated samples may be attributed mainly to autoxidation of pea lipids during blending and distillation.

Malonaldehyde produced during blending without acid as related to fatty acid content and lipoxidase activity. Since extensive lipid oxidation occurs during blending of raw vegetables in the absence of lipoxidase inactivator, the amount of malonaldehyde formed during blending has some usefulness as an index of the tendency of the vegetable to undergo lipid oxidation under conditions of tissue breakdown. Polyunsaturated fatty acid content, lipoxidase activity, and antioxidants present would all be expected to influence such oxidation.

Table 1. Relation of TBA reaction to oxygen consumption in linolenate-lipoxidase model systems.

O_2 consumption (mm Hg/min)	TBA value (O.D. at 532 mμ)
93	1.17
138	1.70
166	2.58
186	3.25
192	3.35

Table 2. Lipoxidase inhibition by acidity.

ml of HCl (1:2) to 10 g peas	pH of blended pea slurry	TBA no.
0	6.20	9.31
1.25	1.21	1.00
2.50	0.92	0.82
5.00	0.53	0.90

Table 3 demonstrates that of two varieties of peas differing markedly in malonaldehyde produced during blending, that with the higher TBA number was also higher in lipoxidase activity and in polyunsaturated fatty acids, especially linolenic. Antioxidants in these peas were not determined. Earlier studies on cooked vegetable extracts by Cofer (1964) showed relatively little antioxidant activity in legumes.

Lipid-oxidizing activity in fruit and vegetable extracts. Lipid-oxidizing activity is expressed as the catalytic activity of an aqueous crude extract on oxidation of externally supplied substrate, linoleate, using Surrey's spectrophotometric procedure. The concentration of vegetable tissue present in the reaction mixtures ranged from 1 to 5%, depending on their lipid-oxidizing activities. Table 4 shows the data in decreasing order of lipid-oxidizing activity per 10 g fresh tissue. The activity per gram protein is also presented. The protein values were obtained from food composition tables (Watt and Merrill, 1963).

The following vegetables and fruits did not have lipid-oxidizing activity: spinach, mustard green, collard, lettuce, white squash, purple hull pea, banana pepper, grape, peach, and cantaloupe. The crude extracts of roots, tubers, botanical fruits with edible seed portion, and seeds usually showed a high catalytic effect on linoleate oxidation. Most of the leafy vegetables and many fruits had little or no activity. Comparing families, many vegetables belonging to Leguminosae, Solanaceae, or Cruciferae had higher activ-

Table 3. Comparison of TBA numbers with lipoxidase activity and fatty acid composition in two varieties of peas.

Pea	TBA no. after 5 min blending [a]	Lipoxidase activity of crude extracts [b]	Polyunsat. ratio [c]	Linolenic % of total fatty acids
Blackeye	5.5	1.16	1.5	22.6
Green	1.1	0.55	.78	6.5

[a] Blended for 5 min in absence of acid.

[b] Lipoxidase units/0.025 g pea as defined by Surrey (1964). Polarographic measurements gave an almost identical ratio between the activity of the two peas.

[c] (Linoleic + linolenic)/(oleic + stearic + palmitic) as determined from GLC.

ity. Süllmann (1945) showed high activity of leaf extracts of Solanaceae; it is evident that fruits of this family also have the catalytic effect.

The lipid-oxidizing activities listed in Table 4 do not represent a true measure of lipoxidase content, because of the fact that the crude extracts were analyzed without

Table 4. Lipid-oxidizing activity of vegetable and fruit extracts.

	Latin name [a]		Units per 10 g fresh tissues	Units per g protein
	Family	Genus and species		
Garden pea	Leguminosae	*Pisum sativum*	8.53	13.55
Blackeye pea	Leguminosae	*Vigna sinensis*	5.72	6.36
Pole bean	Leguminosae	*Phaseolus vulgaris*	5.48	28.84
Potato	Solanaceae	*Solanum tuberosum*	2.98	14.19
Lima bean, immatured	Leguminosae	*Phaseolus limensis*	1.20	1.43
Egg-plant	Solanaceae	*Solanum melongena*	1.11	9.25
Tender bean	Leguminosae	*Phaseolus vulgaris*	1.07	5.63
Sweet potato	Convolvulaceae	*Ipomoea batatas*	0.96	5.65
Asparagus	Liliaceae	*Asparagus officinalis*	0.85	3.49
Hot pepper, whole	Solanaceae	*Capsicum annuum*	0.79	6.08
Squash, yellow	Cucurbitaceae	*Cucurbita pepo* var. melopepo	0.66	5.50
Green pepper pod	Solanaceae	*Capsicum annuum* var. grossum	0.62	5.17
Radish	Cruciferae	*Raphanus sativus*	0.61	6.10
Rutabaga	Cruciferae	*Brassica napobrassica*	0.55	5.00
Corn, immatured	Gramineae	*Zea mays*	0.52	1.49
Cabbage	Cruciferae	*Brassica oleracea* var. capitata	0.51	3.92
Carrot	Umbelliferae	*Daucus carota* var. sativa	0.47	4.27
Green bean	Leguminosae	*Phaseolus vulgaris*	0.44	2.32
Onion, yellow skin	Liliaceae	*Allium cepa*	0.44	2.93
Cauliflower	Cruciferae	*Brassica oleracea* var. botrytis	0.43	1.59
Apple, golden delicious	Rosaceae	*Malus sylvestris*	0.37	18.50
Apple, red delicious	Rosaceae	*Malus sylvestris*	0.36	18.00
Cucumber	Cucurbitaceae	*Cucumis sativus*	0.36	4.00
Pear	Rosaceae	*Pyrus communis*	0.34	4.89
Banana	Musaceae	*Musa paradisaca*	0.29	2.64
Tomato	Solanaceae	*Lycopersicon esculentum*	0.29	2.64
Celery	Umbelliferae	*Apium graveolens*	0.25	2.78
Rhubarb	Polygonaceae	*Rheum rhaponticum*	0.13	2.17
Squash, acorn	Cucurbitaceae	*Cucurbita maxima*	0.12	0.23
Turnip root	Cruciferae	*Brassica rapa*	0.10	1.00
Zucchini	Cucurbitaceae	*Cucurbita pepo* var. medullosa	0.05	0.10
Orange	Rutaceae	*Citrus sinensis*	0.02	0.29
Turnip green	Cruciferae	*Brassica rapa*	0.02	0.23
Green Crowder pea	Leguminosae	*Vigna species*	0.01	0.01

[a] Makino (1956); Watt and Merrill (1963).

further purification. Antioxidants could be present in the crude extracts. Pratt and Watts (1964) and Cofer (1964) found that hot-water extracts of many vegetables have antioxidant activity.

A spot check of the antioxidant activities of several vegetables in Table 4 showed wide differences. Cold-water extracts of turnip green and banana pepper, when added in concentrations of approximately 4% fresh tissue to model systems containing purified soybean lipoxidase and linoleic acid, showed approximately 90% inhibition of the reaction as estimated by oxygen consumption, whereas with cucumber and egg-plant the inhibition was in the range 0–20%. Evidently, as might be expected, the lipid-oxidizing activity determined in a crude extract of vegetable is the result of the true lipoxidase content and concentration of endogenous antioxidants.

REFERENCES

Ast, H. J. 1963. Inadvertent isomerization of polyunsaturated acids during ester preparation. *Anal. Chem.* **35**, 1539.

Bengtsson, B., and I. Bosund. 1964. Gas chromatographic evaluation of the formation of volatile substances in stored peas. *Food Technol.* **18**, 773.

Cofer, A. C. 1964. Antioxidant activity of vegetable extracts. Ph.D. thesis. Florida State University, Tallahassee.

Dahle, L. K., E. G. Hill, and R. T. Holman. 1962. The thiobarbituric acid reaction and autoxidation of polyunsaturated fatty acid methyl esters. *Arch. Biochem. Biophys.* **98**, 253.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497.

Kwon, T. W., and B. M. Watts. 1963. Determination of malonaldehyde by ultraviolet spectrophotometry. *J. Food Sci.* **28**, 627.

Lillard, D. A., and E. A. Day. 1964. Degradation of monocarbonyls from autoxidizing lipids. *J. Am. Oil Chemists' Soc.* **41**, 549.

Makino, T. 1956. "Makino's Illustrated Flora of Japan." 36th ed. Kokuryukan Co., Tokyo.

Metcalfe, L. D., and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. *Anal. Chem.* **33**, 363.

Pratt, D. E., and B. M. Watts. 1964. The antioxidant activity of vegetable extracts. I. Flavone aglycones. *J. Food Sci.* **29**, 27.

Saslaw, L. D., and V. S. Waravdekar. 1965. Behavior of unsaturated fatty acids in the thiobarbituric acid test after radiolysis. *Radiation Research* **24**, 375.

Süllmann, H. 1945. Über die Oxidation ungesättigter Fettsäuren durch Pflanzensäfte. *Experientia* **1**, 322.

Surrey, K. 1964. Spectrophotometric method for determination of lipoxidase activity. *Plant Physiol.* **39**, 65.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chemists' Soc.* **37**, 44.

Wagenknecht, A. C., and F. A. Lee. 1956. The action of lipoxidase in frozen raw peas. *Food Research* **21**, 605.

Watt, B. K., and A. L. Merrill. 1963. "Composition of Foods." Agriculture Handbook No. 8. United States Department of Agriculture, Washington, D. C.

Ms. rec'd 3/29/66.

Work supported in part by a grant, EF-00179 from the National Institutes of Health, U. S. Public Health Service.

KI SOON RHEE and BETTY M. WATTS
Florida State University, Tallahassee, Florida 32306

Effect of Antioxidants on Lipoxidase Activity in Model Systems and Pea (Pisum sativum) Slurries

SUMMARY

The antioxidants BHA, propyl gallate, quercetin, gallic acid, turnip green extract, and sodium tripolyphosphate were tested for their inhibitory effect on linoleate oxidation catalyzed by purified soybean lipoxidase or crude pea lipoxidase, and on lipid oxidation occurring in blending of peas. In artificial lipoxidase-linoleate systems, most phenolic inhibitors, especially BHA and propyl gallate, were very effective, but sodium tripolyphosphate was ineffective. The effectiveness of antioxidants decreased with increase in lipoxidase concentration. The relative effectiveness of the various antioxidants was quite different in pea slurries from that in artificial systems. Higher concentrations of propyl gallate, turnip green extract, and sodium tripolyphosphate retarded the pea lipid oxidation, but no significant inhibitory effect was found with other antioxidants tested. Factors which might contribute to variations in the behavior of antioxidants in pure model systems versus raw pea slurries are discussed. Hydrogen peroxide at concentrations of 0.005% or higher rapidly destroyed lipoxidase activity.

INTRODUCTION

Lipoxidase, present in plant tissues, catalyzes the peroxidation of polyunsaturated fatty acids to produce rancid end products. All the published work on the inhibition of lipoxidase is, however, confined to pure systems, and no attempt has been made to inhibit the enzyme in complex food systems.

In the pure lipoxidase systems, certain phenolic antioxidants have been shown to inhibit enzymatic action of lipoxidase. Nordihydroguaiaretic acid, propyl gallate, and α-tocopherol strongly inhibited the oxidation of linoleate by this enzyme (Tappel *et al.*, 1953; Siddiqi and Tappel, 1956). Since nordihydroguaiaretic acid and tocopherols are insoluble or sparingly soluble in water, their use is limited in foodstuffs such as vegetables, in which the naturally occurring lipids are surrounded by an aqueous phase.

Sodium tripolyphosphate, water-soluble, retarded rancidity of refrigerated cooked beef when used as a cover or dip solution (Ramsey and Watts, 1963). The mechanism of its antioxidant action is not known, but presumably differs from that of the phenolic antioxidants, which donate hydrogen to break chain reactions. Recently, the antioxidant activity of aqueous vegetable extracts has been investigated in heme-catalyzed and autoxidizing systems (Ramsey and Watts, 1963; Cofer, 1964; Pratt and Watts, 1964). In most of these vegetable extracts, the flavonoid quercetin contributed significantly to antioxidant activity although in turnip greens the amine fraction was most effective, with phenolic antioxidants of secondary importance.

The phenolic antioxidant butylated hydroxy anisole (BHA) has apparently not been tried in lipoxidase systems, although this is now the most widely used food antioxidant. While not water-soluble, this compound is commercially available in the form of a stable aqueous emulsion marketed under the trade name of Sustane E (Universal Oil Products Chemical Company). The emulsion has been very successful in protecting meats from rancidity. In view of these facts the following antioxidants were tested: sodium tripolyphosphate, gallic acid, propyl gallate, quercetin, turnip green extract, and Sustane E.

Peas were selected for antioxidant studies because they are relatively high in lipoxidase content and frozen peas are of great economic importance. It is difficult, however, to bring an antioxidant into contact with the enzyme in intact peas. For this reason, antioxidants were added directly to the peas as they were blended. This paper reports studies of the effect of antioxidants on the following systems: 1) purified soybean lipoxidase with linoleate; 2) crude pea lipoxidase with linoleate; 3) lipoxidase-catalyzed oxidation of pea lipids during blending of peas.

MATERIALS AND METHODS

Peas. Garden peas were purchased from the local market when needed, without determining the harvest time or variety.

Composition of Sustane E. Sustane E (Universal Oil Products Chemical Company) has the following composition: 10% BHA, 40% vegetable oil, 2.5% sorbitan monostearate, 0.1% lecithin, 47.4% water. In using Sustane E as an antioxidant the concentration is expressed as that of BHA.

Preparation of turnip green extract. Hot-water extract was prepared by the procedure employed by Cofer (1964) and Pratt and Watts (1964). "Percentage extract" denotes the grams of turnip greens to prepare 100 ml of extract solution.

Crude enzyme preparation from peas. A given weight of peas was blended with 100 ml distilled water for 2 min with a Virtis "45" homogenizer equipped with an ice-water bath. The slurry was filtered, and the filtrate was used as crude enzyme extract.

Effect of antioxidant on lipoxidase activity. For routine assay, the activity of lipoxidase was measured by the polarographic method described by Rhee and Watts (1966a). Antioxidants and purified soybean lipoxidase (Nutritional Biochemicals Corporation) dissolved in phosphate buffer (0.01*M*) of pH 6.2, or crude pea lipoxidase solution, was incorporated as indicated in each experiment.

The spectrophotometric method of Surrey (1964), slightly modified, was also used in some antioxidant experiments. The concentration of linoleic acid was 1%, and the reaction mixture consisted of 9 ml substrate, 10 ml antioxidant solution, and 1 ml enzyme solution.

For both methods the reaction was carried out at room temperature, 26–28°C.

Determination of phenolase activity in peas. The colorimetric method used by Boscan *et al.* (1962) was adapted. Phenolase activity of 1 ml aqueous pea extract (50 g/100 ml) was measured at room temperature with DOPA used as substrate. Distilled water was used as a blank, and the optical density of the 0.05% DOPA solution and that of 100 ml distilled water plus 1 ml pea extract were subtracted from sample readings.

RESULTS AND DISCUSSION

Effect of antioxidants on lipoxidase reaction in the model systems. Table 1 shows the effects of the various antioxidants on oxygen consumption of soybean lipoxidase– and crude pea lipoxidase–linoleic acid emulsion systems. Variable inhibitions were obtained with the phenolic antioxidants and turnip green extracts, whereas sodium tripolyphosphate was ineffective. Except for the polyphosphate, all antioxidants showed an increase in effectiveness as the concentration increased. The antioxidants themselves, in the presence of an even higher concentration of pea extract (0.625% as fresh pea) but without linoleic acid, showed no measurable oxygen uptake.

Table 1. Effect of antioxidants on oxygen consumption of lipoxidase–linoleic acid emulsion system.

Antioxidant	Concentration (*M*)	% inhibition: Soybean lipoxidase [a]	% inhibition: Pea lipoxidase [b]
BHA	2.6×10^{-5}	99	69
	7.8×10^{-5}	99	82
Propyl gallate	8.3×10^{-5}	87	.....
	2.5×10^{-4}	93	.....
Quercetin	8.3×10^{-5}	66	31
	2.5×10^{-4}	86	48
Gallic acid	8.3×10^{-5}	13	15
	2.5×10^{-4}	23	13
Sodium tripolyphosphate	5.7×10^{-4}	6	13
	1.7×10^{-3}	− 3	2
Turnip green extract	0.83(%)	31	28
	2.6(%)	81	45

[a] 0.0067% lipoxidase, 0.417% (v/v) linoleic acid in the reaction mixture; rate of oxygen tension decrease for control is 50.4 mm Hg/min.

[b] 0.125%, as fresh pea, of crude pea lipoxidase, 0.417% (v/v) linoleic acid in the reaction mixture; rate of oxygen tension decrease for control is 100.8 mm Hg/min.

Table 2 shows the inhibitory effect of the antioxidants on production of conjugated dienes in linoleate oxidations catalyzed by purified soybean lipoxidase. Diene conjugation as measured at 234 mμ was inhibited to approximately the same extent as oxygen consumption. Hence, the simpler polarographic method, measuring oxygen consump-

Table 2. Effect of antioxidants on production of conjugated dienes in soybean lipoxidase-catalyzed oxidation of linoleate.[a]

Antioxidant	Concentration (*M*)	% inhibition
BHA	7.8×10^{-5}	100
Quercetin	2.5×10^{-4}	87
Gallic acid	2.5×10^{-4}	−8
Sodium tripolyphosphate	1.7×10^{-3}	8
Turnip green extract	2.5(%)	89

[a] 0.006% purified soybean lipoxidase and 0.225% (v/v) linoleic acid in the reaction mixture.

tion, was used throughout the later inhibition studies.

Effect of lipoxidase concentration on the effectiveness of antioxidants. It was demonstrated with purified soybean lipoxidase that a given concentration of antioxidant is less effective for higher concentrations of lipoxidase (Table 3). Therefore, the higher inhibitory effect of antioxidants on soybean than on crude pea lipoxidase systems, observed in the preceding experiment (see Table 1), can be ascribed to the lower concentration of soybean lipoxidase in the reaction.

Whereas Siddiqi and Tappel (1956) stated that the inhibition of pea lipoxidase is greater than that of soybean lipoxidase with nordihydroguaiaretic acid, propyl gallate, and α-tocopherol, they did not give the activity or concentration of these two lipoxidases in the reaction system. Unless the enzyme concentrations were similar, their hypothesis that the antioxidants tested might be more effective hydrogen donors for pea lipoxidase is not justified.

Effect of antioxidants on lipid oxidation in pea slurries. The oxidation of pea lipids was caused by a 5-min blending of peas with distilled water and antioxidant. After blending, the extent of lipid oxidation was measured by the 2-thiobarbituric acid (TBA) test (Tarladgis *et al.*, 1960). The results are given in Table 4. Among the antioxidants tested, only turnip green extract and sodium tripolyphosphate inhibited lipid oxidation in pea slurries. Since the TBA chromogen of the turnip green samples had an orange color, indicative of interfering absorption in the visible region, malonaldehyde in distillates of those samples was measured by the ultraviolet spectrophotometric method of Kwon and Watts (1963). BHA in Sustane E, quercetin, and gallic acid decreased in effectiveness or even accelerated the oxidation of pea lipids as the concentration increased. This finding is in contrast with the observed effect of these antioxidants in model lipoxidase systems.

To determine the effect of increasing and much higher concentrations of turnip green extract and sodium tripolyphosphate, and to test the effectiveness of propyl gallate, which became available later, additional experiments were performed. Since the stock solution of sodium tripolyphosphate was high in pH, the effect of pH-adjusted sodium tripolyphosphate was also determined. The pH was adjusted to 6.50, close to that of control pea slurries (6.35 to 6.62) by adding

Table 3. Effect of lipoxidase concentration on the inhibitory effect of antioxidants.

Antioxidant	Concentration (M)	% inhibition	
		0.003% lipoxidase[a]	0.012% lipoxidase[b]
BHA	2.6×10^{-5}	99	98
Quercetin	8.3×10^{-5}	74	37
Gallic acid	8.3×10^{-5}	17	10
Sodium tripolyphosphate	5.7×10^{-4}	0	6
Turnip green extract	0.83(%)	50	15

[a] Rate of oxygen tension decrease for control is 28.8 mm Hg/min.
[b] Rate of oxygen tension degree for control is 96.0 mm Hg/min.
Reaction mixtures contained 0.417% (v/v) linoleic acid.
Soybean lipoxidase (Nutritional Biochemical Corp.) used.

Table 4. Effect of antioxidants on lipid oxidation in pea slurries.

Antioxidant	Concentration (M)	% inhibition
BHA	3.9×10^{-5a}	9
	1.6×10^{-4b}	2
	3.1×10^{-4c}	−2
Quercetin	1.25×10^{-4a}	7
	5×10^{-4b}	−8
	1×10^{-3c}	−9
Gallic acid	1.25×10^{-4a}	−3
	5×10^{-4b}	−3
	1×10^{-3c}	−16
Sodium tripolyphosphate	8.5×10^{-4a}	13
	3.4×10^{-3b}	20
	6.8×10^{-3c}	27
Turnip green extract	1.25 (%)[a]	9
	5 (%)[b]	43
	10 (%)[c]	61

[a] TBA number of control is 3.0.
[b] TBA number of control is 2.0.
[c] TBA number of control is 1.9.

droplets of concentrated HCl. The data are presented in Table 5.

The inhibitory effect of very high concentrations of unadjusted sodium tripolyphosphate was, in large part, attributed to the alkalinity of the solution. The effect of the pH-adjusted polyphosphate was still considerable. Turnip green extract and propyl gallate were also shown to be good antioxidants at higher concentrations for protecting the pea lipids from oxidation.

Effect of peroxidase and polyphenol oxidase on the inhibition of lipoxidase reaction by antioxidants. Garden peas contain enzymes other than lipoxidase. On blending peas with phenolic antioxidants, enzymatic destruction of these antioxidants was assumed to be possible. Phenol oxidase, and peroxidase plus H_2O_2, catalyze the oxidation of phenolic compounds. To test the possible oxidation, polyphenol oxidase, or peroxidase along with hydrogen peroxide, was added to systems consisting of linoleic acid emulsion, an antioxidant, and purified soybean lipoxidase. Horseradish peroxidase (200 units/mg) and polyphenol oxidase obtained from Nutritional Biochemicals Corporation were dissolved in 0.1*M* phosphate buffer of pH 6.2.

The added polyphenol oxidase did not affect the inhibitory effect of any of the antioxidants, and the presence of the oxidase did not significantly influence the oxygen uptake of the lipoxidase reaction mixture. Phenolase activity of raw garden peas was negligible. The increment of optical density at 470 mμ was only about 0.0004 per minute with the reaction mixture described under Materials and Methods.

A high level of peroxidase in raw garden peas has been demonstrated (Rhee and Watts, 1966b). In attempting to assess the possible effects of peroxidase activity on phenolic antioxidants added to pea slurries, it was discovered that H_2O_2, either alone or in the presence of peroxidase, rapidly destroyed lipoxidase activity as measured by oxygen consumption (Fig. 1). Similar levels of inhibition (over 90% with 0.03% or more H_2O_2) of conjugated diene production were obtained.

In view of this destructive effect of H_2O_2 on lipoxidase, experiments involving the addition of peroxidase plus H_2O_2 to oxidizing lipid systems become difficult to control. These experiments are not presented in detail, since their interpretation is open to question. However, in several such experiments at various levels of lipoxidase activity, of the phenolic inhibitors, only quercetin showed any marked loss of antioxidant activity in the presence of peroxidase and H_2O_2. The addition of peroxidase plus H_2O_2 consistently increased the antioxidant action of gallic acid and turnip green extract.

In addition to possible destruction of antioxidants by peroxidase and H_2O_2, it was thought possible that some antioxidants might inhibit peroxidase action, thus protecting pea phenolic antioxidants which

Table 5. Effect of high concentration of sodium tripolyphosphate, turnip green extract and propyl gallate on lipid oxidation in pea slurries.

Antioxidant	TBA number of control	Concentration (*M*)	pH of slurry	% inhibition
Sodium tripolyphosphate	3.8	2.7×10^{-3}	6.40	27
		2.7×10^{-2}	7.50	56
		5.4×10^{-2}	8.21	90
pH-adjusted sodium tripolyphosphate	4.4	2.7×10^{-3}	6.40	27
		2.7×10^{-2}	6.48	46
		5.4×10^{-2}	6.50	57
Propyl gallate	4.9	2.5×10^{-4}	6.50	32
		5.0×10^{-4}	6.50	37
		1.5×10^{-3}	6.53	61
Turnip green extract	5.0	2.5 (%)	6.43	36
		5.0 (%)	6.40	49
		15.0 (%)	6.33	78

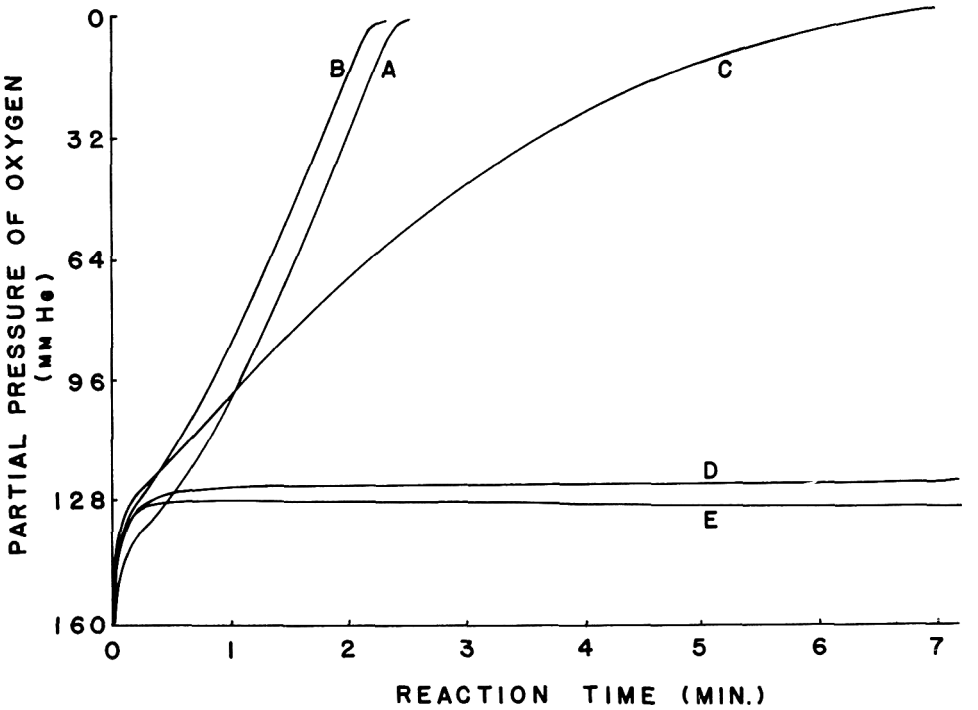


Fig. 1. Inhibition of lipoxidase by H_2O_2; control or 0% H_2O_2 (A); 0.00013% H_2O_2 (B); 0.005% H_2O_2 (C); 0.01% H_2O_2 (D); 0.025% H_2O_2 (E). Lipoxidase concentration is 0.0067% and linoleic acid concentration is 0.417% (v/v). Soybean lipoxidase (Nutritional Biochemical Corp.) used.

might otherwise have been oxidized by peroxidase. The complete inhibition of turnip peroxidase by $2.7 \times 10^{-4}M$ propyl gallate was reported by Tappel and Marr (1954), but even much higher concentrations of propyl gallate did not inhibit pea peroxidase, aqueous extract, as measured by the method of Masure and Campbell (1944) using 0.5% guaiacol (in 50% ethyl alcohol) as substrate. Sodium tripolyphosphate, in concentrations up to $0.054M$ (pH adjusted to that of distilled water by adding concentrated HCl) did not show any inhibition of pea peroxidase. The observed antioxidant effect of sodium tripolyphosphate cannot be explained at present, although it is known to inhibit heme-catalyzed lipid oxidation of meats very effectively.

Other factors could contribute to variations in the behavior of antioxidants in pure model systems versus raw pea slurries. Whereas the substrate is uniformly distributed in soluble form in the model systems, the polyunsaturated fatty acids of peas would be expected to remain largely bound to formed elements in the pea tissues. Under these conditions, there may be very limited access of some antioxidants to the site of the oxidation. This might explain the fact that BHA, which was extremely effective in stopping lipid oxidation in model systems, was useless in pea slurries.

Increased protection in the raw pea slurry versus the model system, shown for example by turnip green extract and sodium tripolyphosphate, may be an example of synergisms between the added substances and antioxidants present in the pea.

REFERENCES

Boscan, L., W. D. Powrie, and O. Fennema. 1962. Darkening of red beets, Beta vulgaris. *J. Food Sci.* **27**, 574.

Cofer, A. C. 1964. Antioxidant activity of vegetable extracts. Ph.D. thesis. Florida State University, Tallahassee.

Kwon, T. W., and B. M. Watts. 1963. Determination of malonaldehyde by ultraviolet spectrophotometry. *J. Food Sci.* **28**, 627.

Masure, M. P., and H. Campbell. 1944. Rapid

estimation of peroxidase in vegetable extracts—an index of blanching adequacy for frozen vegetables. *Fruit Prods. J.* **23**, 369.

Pratt, D. E., and B. M. Watts. 1964. The antioxidant activity of vegetable extracts. I. Flavone aglycones. *J. Food Sci.* **29**, 27.

Ramsey, M. B., and B. M. Watts. 1963. The antioxidant effects of sodium tripolyphosphate and vegetable extracts on cooked meat and fish. *Food Technol.* **17**, 1056.

Rhee, K. S., and B. M. Watts. 1966a. Evaluation of lipid oxidation in plant tissues. *J. Food Sci.* **31**, 664.

Rhee, K. S., and B. M. Watts. 1966b. Lipid oxidation in frozen vegetables in relation to flavor change. *J. Food Sci.* (in press).

Siddiqi, A. M., and A. L. Tappel. 1956. Catalysis of linoleate oxidation by pea lipoxidase. *Arch. Biochem. Biophys.* **60**, 91.

Surrey, K. 1964. Spectrophotometric method for determination of lipoxidase activity. *Plant Physiol.* **39**, 65.

Tappel, A. L., and A. G. Marr. 1954. Effect of α-tocopherol, propyl gallate, and nordihydroguaiaretic acid on enzymatic reaction. *J. Agr. Food Chem.* **2**, 554.

Tappel, A. L., W. O. Lundberg, and P. D. Boyer. 1953. Effect of temperature and antioxidants upon the lipoxidase-catalyzed oxidation of sodium linoleate. *Arch. Biochem. Biophys.* **42**, 293.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chemists' Soc.* **37**, 44.

Ms. rec'd 3/29/66.

Work supported in part by a grant, EF-00179, from the National Institutes of Health, U. S. Public Health Service.

KI SOON RHEE and BETTY M. WATTS
Department of Food and Nutrition, Florida State University, Tallahassee, Florida 32306

Lipid Oxidation in Frozen Vegetables in Relation to Flavor Change

SUMMARY

The TBA test, adapted for vegetable material, was useful in following lipid oxidation in frozen peas. Gas-liquid chromatographic analysis for the loss of unsaturated fatty acids was not feasible for determining lipid oxidation in unblanched vegetables. This work has established that rancidity is not a main cause of flavor deterioration in frozen Blackeye peas (*Vigna sinensis*) and possibly in other frozen vegetables. The amount of lipid oxidation occurring in frozen raw peas was too small to produce rancid odors. Lipoxidase was rapidly inactivated by a short blanching time, and no regeneration of the enzyme occurred during frozen storage of garden peas (*Pisum sativum*).

INTRODUCTION

The development of off-flavor in unblanched vegetables during frozen storage has been ascribed to the rancidification of lipid matter, catalyzed by lipoxidase, although very little direct evidence is available to support this. Wagenknecht and Lee (1956) demonstrated the presence of lipoxidase in fresh and frozen peas and increases in peroxide number of lipids extracted from the raw vegetable after extended periods of frozen storage. A similar finding was made in underblanched frozen corn-on-the-cob (Wagenknecht, 1959). Lee and Wagenknecht (1958) used model systems in which the enzymes lipoxidase, lipase, catalase, and peroxidase were added to enzymatically inert macerated blanched peas. After frozen storage, examination by a test panel showed the production of mild off-flavor with catalase and peroxidase, a moderate off-flavor with lipoxidase, and an intensely disagreeable off-odor with lipase. While the results of these studies demonstrate that lipoxidase is present and can function at freezer temperatures, it is not clear to what extent the products of lipoxidase activity contribute to the deterioration of flavor of the natural vegetables in which various enzymes are present.

Further studies are needed for correlating objective tests for lipid oxidation with organoleptic evaluations of frozen vegetables. The usefulness of the 2-thiobarbituric acid (TBA) test for following lipid oxidation in plant tissues has been described by Rhee and Watts (1966). Gas chromatographic techniques have simplified the quantitative estimations of polyunsaturated fatty acids, but this technique has been used successfully to follow oxidative loss of these fatty acids in stored vegetables only in a single study with dehydrated potato granules (Buttery *et al.*, 1961).

Although the enzymes causing flavor deterioration in unblanched vegetables have not been clearly identified, it is common practice to use tests for catalase and peroxidase as indices of adequacy of blanching in vegetables to be frozen. Lipoxidase could become a factor in flavor deterioration if it is not completely inactivated by blanching procedures which destroy other enzymes.

This paper determines the role of lipid oxidation in the flavor deterioration of frozen vegetables as measured by an adaptation of the TBA test, gas-liquid chromatographic (GLC) analysis of fatty acids, and organoleptic evaluation of frozen peas. Also described are the effects of blanching and freezing storage on the activity of lipoxidase and other enzymes in peas.

METHODS

Blanching procedure. Blanching of the peas was accomplished by lowering 400 g peas in a perforated basket into about 6 L of boiling water. The water temperature dropped to 89 or 90°C and then gradually increased, even to boiling with longer blanching time. The heating conditions were controlled for any one experiment but differed somewhat between experiments. Time was determined from the moment peas were dropped into boiling water. Blanched peas were blot-dried without crushing and immediately frozen at —10°C.

Preparation of extracts for enzyme inactivation test. Each 30-g portion of peas was blended for 2 min with 100 ml distilled water and ½ teaspoon of $CaCO_3$ in a Virtis "45" homogenizer,

using an ice-water bath. The slurry was again filtered after centrifugation. The filtrate was used as crude enzyme preparation.

Peroxidase test. The semiquantitative method of Masure and Campbell (1944) for peroxidase activity was used. To each of two test tubes, 22 ml distilled water and 2 ml filtrate were added. To one of the two, 1 ml of 0.08% H_2O_2 and 1 ml of 0.5% guaiacol (in 50% ethanol) were added. The time required for appearance of color was measured. There should be no detectable color change in 210 seconds in a negative test. This test has a margin of safety. Extent of color is roughly proportional to the enzyme concentration.

Catalase test. A semiquantitative assay was made by the fermentation tube method, frequently used in the freezing industry. To 4 ml filtrate, 8 ml of 3% H_2O_2 was added. The contents were quickly mixed and immediately placed in a fermentation tube. Three minutes were allowed for reaction. Less than 0.1 ml gas is considered negative. Amount of gas evolved is roughly proportional to the enzyme concentration.

RESULTS AND DISCUSSION

Lipid oxidation in stored frozen Blackeye peas measured by the TBA test. Shelled fresh Blackeye peas *(Vigna sinensis)* were purchased at a local market, and bruised peas were screened out. As far as possible, peas of similar size were selected. For frozen storage, three treatments were carried out: 1) untreated raw peas; 2) 90-sec blanching in boiling water; 3) 10-min cooking in boiling water. The TBA test, adapted for vegetable material (Rhee and Watts, 1966) by adding HCl in blending, was used to measure the extent of lipid oxidation during storage. TBA numbers of frozen raw, blanched, and cooked peas are presented in Table 1. The increasing TBA numbers of frozen raw peas indicate that lipoxidase is still active at the low storage temperature, −10°C. However, the highest TBA numbers were low as compared to values obtained in animal tissues or to those obtained when lipoxidase was not inactivated during blending. There are no significant differences in TBA numbers of frozen blanched and frozen cooked peas.

Table 1. TBA numbers of raw, blanched, and cooked Blackeye peas in frozen storage.

Pea sample	TBA number			
	0 day	206 days	311 days	406 days
Raw pea	0.28	0.44	0.55	0.64
Blanched pea	0.12	0.17	0.18	0.14
Cooked pea	0.14	0.26	0.17	0.13

Fatty acid changes. GLC analysis of fatty acids in peas has been carried out as described by Rhee and Watts (1966). The fatty acids having longer retention times than linolenic acid were identified with the stabilized diethylene glycol succinate (DEGS) column (Analytical Engineering Laboratories) at 210°C with other operating conditions the same. Since there were not enough standard unsaturated fatty acid samples available to plot accurate curves for the logarithm of retention times against the number of carbon atoms, the identification of C 12:2 and C 14:1 acids is tentative. Table 2 gives the fatty acids found in fresh Blackeye peas.

The fatty acid composition of fresh Blackeye peas has also been reported by Korytnyk and Metzier (1963). They did not, however, identify any fatty acids having shorter retention times than myristic acid and longer ones than behenic acid.

Peas stored at −10°C were removed at intervals to carry out GLC analysis of the fatty acids. The ratio of linoleic plus linolenic acid to palmitic plus stearic acid was used to express the degree of oxidative loss.

In the nine analyses made on blanched

Table 2. Fatty acid composition of fresh Blackeye peas by GLC analysis.

Fatty acid	mg/20 g peas	Relative %
Lauric	Trace	Trace
Tridecanoic	Trace	Trace
Dodecadienoic [a]	0.7	0.5
Myristic	0.9	0.7
Tetradecenoic [a]	Trace	Trace
Pentadecanoic	Trace	Trace
Palmitic	34.5	26.6
Heptadecanoic	0.8	0.6
Stearic	4.7	3.6
Oleic	5.8	4.5
Linoleic	41.6	32.1
Linolenic	37.4	28.8
Eicosadienoic	Trace	Trace
Behenic	Trace	Trace
Lignoceric	1.5	1.2
Cerotic	Trace	Trace
Octacosanoic	1.8	1.4

[a] Identity tentative.

and cooked peas, ranging from 0 to 406 days of storage, this ratio did not deviate beyond the range 2.0–2.2, demonstrating no measurable loss of polyunsaturated fatty acids. With the raw frozen peas the range was from 1.7 to 2.0, with no consistent trend. These fluctuations in the raw peas, though small, are believed to be real rather than the result of experimental or sampling errors, and may be ascribed to metabolic changes of pea lipids even at this low temperature of storage. Lee and Mattick (1961) also observed changes in the fatty acids of frozen unblanched peas (*Pisum sativum*) stored at −17.8°C, which could not be interpreted as the oxidative loss of unsaturated fatty acids. Similar changes were also found in frozen untreated spinach stored at the same temperature for prolonged times (Mattick and Lee, 1961).

In any case, the TBA numbers of less than 1 obtained in the raw stored peas (Table 1) correspond to a molar ratio of polyunsaturated fatty acids to malonaldehyde of more than 1,000 to 1. It is unlikely that the oxidative loss of polyunsaturated fatty acids is large enough to be measurable.

Organoleptic evaluation of frozen Blackeye peas. After 206 and 311 days of storage the samples were presented to a panel of trained judges for rating the intensity of off-odor. Numerical values ranging from 6 (no off-odor) to 1 (very strong off-odor) were assigned to judgments, and the average sensory score was calculated for each sample. The judges were encouraged to comment on odors. Various comments were made of frozen raw pea odor, such as "sour," "beany fermented," or "spoiled or fermented." The frozen cooked peas were judged to have a normal cooked pea odor. Table 3 shows the sensory scores and statistical evaluation by Wilcoxon matched-pairs signed-ranks test (Siegel, 1956). There was a sharp differentiation between raw and blanched samples or raw and cooked samples, although TBA numbers on all frozen samples were well below the approximate threshold level of 1.0 for rancid odor established for animal products. The judges definitely preferred cooked peas to blanched peas even though the TBA numbers of the two samples did not show significant differences. Statistical evaluation of the sensory scores by analysis of variance with randomized block design (Cochran and Cox, 1957) gave similar significance of differences among the three samples.

The low magnitude of TBA numbers and the judges' descriptions of frozen raw pea odor indicate that rancidity was not the main cause of off-odor in these peas. In contrast, the odor of peas blended in water, with the high TBA number of 7.8, was definitely rancid, quite different from the odor of frozen raw peas.

Comparative enzyme activities after blanching. Prior to the storage experiments for the effect of blanching and storage on the enzyme activity, garden peas (*Pisum sativum*) from a different lot were blanched and tested for enzyme activity and lipid oxidation in blending. Determinations for enzyme activity were made immediately after blanching. The lipoxidase test was made by the method of Surrey (1964). The results are presented in Table 4.

Peroxidase was obviously the most heat-resistant of the three enzymes tested. Heat treatment required for inactivation of lipoxidase was intermediate between that for catalase and peroxidase. Walker (1964a) found the heat sensitivities of lipoxidase and catalase of French beans (*Phaseolus vulgaris*) to be similar. No catalysis of lipid oxidation occurred in blending of the peas heated sufficiently to inactivate lipoxidase. The slight lipid oxidation taking place with more than 60 sec of blanch may be ascribed to autoxidation of pea lipids during blending and distillation.

Table 3. Statistical analysis of the odor rating scores for frozen Blackeye peas.

Pea sample	Mean sensory score[a] 206 days			311 days		
Raw pea	1.4		b	1.3		b
Blanched pea	4.5	b	c	4.5	b	c
Cooked pea	5.5			5.5		

[a] Based on a scale of 1–6 (very strong to no off-odor).
[b] Highly significant differences.
[c] Significant difference.

Table 4. Heat inactivation of enzymes in garden peas and lipid oxidation in blending.

Time of blanch (sec)	Peroxidase [a]	Catalase [b]	Lipoxidase [c]	Lipid oxidation [d]
0	++++	+++	100	100
10	++++	++	83	93
20	+++	+	73	75
30	++	....	36	30
60	+	....	0	4
90	....	....	0	4
120	....	....	0	4

[a] ++++: color in 5 sec; +++: color in 15 sec; ++: color in 60 sec; +: color in 210 sec.
[b] +++: more than 5 ml gas; ++: more than 2 ml gas; +: more than 0.1 ml gas.
[c] Percent raw pea lipoxidase activity.
[d] Percent raw pea TBA number.

Effect of blanching and freezing storage on the activity of enzymes in garden peas. Table 5 shows activities of peroxidase, catalase, and lipoxidase during freezing storage. Reactivation of peroxidase occurred during freezing storage of peas blanched 60 sec or less. No reactivation has been shown in frozen peas blanched 90 sec or more, the time required to pass the commercial test. Other investigators also observed peroxidase regeneration in peas that had been blanched just enough to reduce the original peroxidase activity (Dietrich *et al.*, 1955; Pinsent, 1962). It should be pointed out that the conditions of blanching were slightly different from the preceding experiment on Blackeye peas, in which some flavor deterioration occurred after a 90-sec blanch.

No regeneration of catalase or lipoxidase activity was found with any of the blanching times employed. Instead, lipoxidase activity of raw peas or 15-sec-blanched peas decreased during freezing storage, possibly because of gradual freezing denaturation of the enzyme protein at −10°C.

Significance of lipid oxidation in frozen vegetables for flavor deterioration. This work, contrary to the concept under which it was undertaken, has established that rancidity is not a main cause of flavor deterioration in the frozen peas examined here, and possibly in other frozen vegetables. The highest TBA number obtained was much below the threshold level (approximately 1.0) for rancid odor established for animal products. In addition, the frozen raw pea

Table 5. Effect of blanching and freezing storage on activity of enzymes in garden peas.

Enzyme tested	Days of storage	Time of blanch (sec) 0	15	30	60	90	120	600
Peroxidase [a]	0 [d]	50	17	8.3	0.91	0.31	0.22	<0.02
	30	50	25	14	1.0	0.26	....	<0.02
	70	50	33	20	1.3	0.35	0.24	<0.02
	100	50	33	25	1.3	0.29	0.22	<0.02
Catalase [b]	0 [d]	>5	2.9	<0.1	<0.1	....	....	....
	30	>5	2.6	<0.1	<0.1	....	....	....
	70	>5	2.7	<0.1	<0.1	....	....	....
	100	>5	2.0	<0.1	<0.1	....	....	....
Lipoxidase [c]	0 [d]	1.05	0.88	0.05	0	....	....	....
	30	0.82	0.79	0.17	0	....	....	....
	70	0.81	0.74	0.05	0	....	....	....
	100	0.72	0.68	0.02	0	....	....	....

[a] Activity expressed as reciprocal sec × 100 for appearance of the color; 210 sec (.48 expressed as 100/sec) set for a negative test.
[b] Activity expressed as ml gas in fermentation tube; less than 0.1 ml gas set for a negative test.
[c] Activity expressed as optical density at 234 mμ.
[d] Determinations made immediately after blanching.

odor is quite different from the rancid odor which accompanies high TBA numbers of aerobically blended raw pea slurries. Rapid inactivation of lipoxidase by short blanching time and absence of reactivation of the enzyme during frozen storage of peas make it unlikely that this enzyme plays an important role in off-odors of slightly underblanched frozen vegetables.

Other enzymatic pathways of flavor deterioration in frozen vegetables, involving anaerobiosis and fermentative changes accompanied by increases in acetaldehyde and ethyl alcohol, have been elucidated by several workers (Joslyn and David, 1952; David and Joslyn, 1953; Buck and Joslyn, 1956). Our judges' descriptions of the off-odors produced in frozen peas suggest this type of deterioration rather than rancidity. It is quite possible, of course, that the relative contribution of lipid oxidation versus fermentative changes to flavor deterioration in frozen vegetables may depend upon the initial composition of the vegetable as well as processing factors.

Based on his observations of chlorophyll losses, Walker (1964b) postulated that lipids of vegetables heated beyond the optimum blanching period would show greater autoxidation during freezer storage. This did not occur in the present study with peas, even after a 10-min cook.

REFERENCES

Buck, P. A., and M. A. Joslyn. 1956. Formation of alcohol, acetaldehyde, and acetoin in frozen broccoli tissue. *J. Agr. Food Chem.* **4**, 548.

Buttery, R. G., C. E. Hendel, and M. M. Boggs. 1961. Autoxidation of potato granules. *J. Agr. Food Chem.* **9**, 245.

Cochran, W. G., and G. M. Cox. 1957. "Experimental Designs." 2nd ed. Wiley, New York.

David, J. J., and M. A. Joslyn. 1953. Acetaldehyde and related compounds in frozen green peas. *Food Research* **18**, 390.

Dietrich, W. C., F. E. Lindquist, G. S. Bohart, H. J. Morris, and M-D. Nutting. 1955. Effect of degree of enzyme inactivation and storage temperature on quality retention in frozen peas. *Food Research* **20**, 480.

Joslyn, M. A., and J. J. David. 1952 (Nov.). Acetaldehyde and alcohol in raw or underblanched peas. *Quick Frozen Foods* **15**, 51.

Korytnyk, B., and E. A. Metzier. 1963. Composition of lipids of lima beans and certain other beans. *J. Sci. Food Agr.* **14**, 841.

Lee, F. A., and L. R. Mattick. 1961. Fatty acids of the lipids of vegetables. I. Peas (Pisum sativum). *J. Food Sci.* **26**, 273.

Lee, F. A., and A. C. Wagenknecht. 1958. Enzyme action and off-flavor in frozen peas. II. The use of enzymes prepared from garden peas. *Food Research* **23**, 584.

Masure, M. P., and H. Campbell. 1944. Rapid estimation of peroxidase in vegetable extracts—an index of blanching adequacy for frozen vegetables. *Fruit Prods. J.* **23**, 369.

Mattick, L. R., and F. A. Lee. 1961. The fatty acids of vegetables. II. Spinach. *J. Food Sci.* **26**, 356.

Pinsent, B. R. W. 1962. Peroxidase regeneration and its effect on quality in frozen peas and thawed peas. *J. Food Sci.* **27**, 120.

Rhee, K. S., and B. M. Watts. 1966. Evaluation of lipid oxidation in plant tissues. *J. Food Sci.* **31**, 664.

Siegel, S. 1956. "Nonparametric Statistics for the Behavioral Sciences." McGraw-Hill, New York.

Surrey, K. 1964. Spectrophotometric method for determination of lipoxidase activity. *Plant Physiol.* **39**, 65.

Wagenknecht, A. C. 1959. Lipoxidase activity and off-flavor in underblanched frozen corn-on-the-cob. *Food Research* **24**, 539.

Wagenknecht, A. C., and F. A. Lee. 1956. The action of lipoxidase in frozen raw peas. *Food Research* **21**, 605.

Walker, G. C. 1963a. Color deterioration in frozen French beans (Phaseolus vulgaris). *J. Food Sci.* **29**, 383.

Walker, G. C. 1964b. Color deterioration in frozen French beans (Phaseolus vulgaris). 2. The effect of blanching. *J. Food Sci.* **29**, 389.

Ms. rec'd 3/29/66.

Work supported in part by a grant, EF-00179, from the National Institutes of Health, U.S. Public Health Service.

L. G. SCHARPF, JR.,[a] W. W. MARION, and R. H. FORSYTHE
Iowa State University of Science and Technology, Ames, Iowa 50010

Post-Rigor Changes in Selected Physicochemical Properties of Myosin B Fraction of Turkey Muscle

SUMMARY

Changes in selected physicochemical properties of the myosin B fraction of turkey muscle were followed during a 44-hr post-rigor aging period. Sedimentation patterns of myosin B fractions prepared 48 hr after death indicated a decrease in a high-molecular-weight component thought to be myosin B gel. It is concluded that myosin B fractions lose gel-forming ability with resolution of rigor. Density-gradient centrifugal patterns showed small increases in slowly sedimenting components of myosin B fractions with aging. These components were high in ATP-ase activity in the presence of calcium ions and showed no reduction of viscosity with addition of ATP. Some evidence favors the concept of dissociation of myosin B into myosin A and actin with resolution of rigor. This dissociation, however, may also represent disruption of myosin B gel formed during and after extraction from muscle.

INTRODUCTION

Toughness in meats is related directly to the biochemical state of muscle proteins. Fundamental research has thus been undertaken to determine the nature of post-mortem physicochemical changes that occur as the meat becomes uniformly tender. The turkey and chicken are ideal experimental animals to be used in such study since they are easy to handle and process, and undergo onset and resolution of rigor quite rapidly.

The most obvious site of post-mortem changes is the myofibrillar proteins, since they are involved directly in the processes of contraction and relaxation. Furthermore, the individual myofibrillar proteins *in vivo* are associated at least to some extent as a myosin B complex. This complex, therefore, serves as a focal point for such study.

The extent and nature of involvement of myosin B in the development and resolution of rigor in skeletal muscle has not been clearly elucidated. Rigor development has been described as a combination of actin and myosin filaments resulting from the post-mortem disappearance of ATP (Szent-Gyorgyi, 1964; Bendall, 1964). The decrease in post-mortem solubilities of myofibrillar proteins (Sayre and Briskey, 1963; Khan and Van den Berg, 1964) supports this hypothesis.

Resolution of rigor has been proposed as a disorganization or a disruption of the myosin B complex (Erdos, 1946; Szent-Gyorgyi, 1951). More recent work by Partmann (1963) suggests that a partial dissociation of myosin B can occur, in that ATP added to post-rigor muscle resulted in contraction. Deatherage (1963), however, indicated that, during resolution, essentially no increase in solubilization of proteins or dissociation of myosin B into actin and myosin A occurs. Fujimaki *et al.* (1965) recently showed that, in rabbit muscle, the interaction between myosin A and actin decreases with aging.

In light of the conflicting views, the present investigation on turkey muscle has been carried out with the following objectives: 1) to follow changes in selected physicochemical properties of the myosin B fraction associated with resolution of rigor; 2) to investigate the fundamental nature of the observed changes. It is likely that the extraction and preparation procedures induce some changes in the proteins differing from their native state. Although it is difficult to assess the degree of alteration, the use of mild extraction techniques perhaps minimizes the amount of damage to protein components.

Since myofibrillar proteins have frequently been a subject of inconsistent use of terminology, the following nomenclature will be used: Myosin A denotes myosin free of actin. Myosin B denotes the natural complex between actin and myosin A obtained by direct extraction from muscle.

EXPERIMENTAL

Chemicals. All chemicals were analytical grade where obtainable. The disodium salt of ATP was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and was stored at 0–5°C.

[a] Present address: Inorganic Research Department, Monsanto Co., St. Louis, Mo. 63166.

Stock and age of turkeys. Muscle samples were taken from Keithley White females approximately 10–12 months of age, raised at the Iowa State University Poultry Science Center.

Method of exsanguination. Slaughter was carried out as previously described (Scharpf and Marion, 1964).

Storage conditions. Turkeys were placed in plastic bags and were held for 48 hr in a walk-in cooler maintained at 3–5°C. Skin, depot fat, and membranes were carefully replaced over muscles after sampling to minimize changes in muscle proteins.

Preparation of muscle proteins. Muscle samples weighing 15 g were taken from the pectoralis major muscle and were trimmed free of visible fat, tendons, and connective tissue. Samples were cut into small pieces, placed in a Servall Omni-mixer jar containing 250 ml of distilled water, and were homogenized at high speeds for 1 min and at low speeds for 9 min. Low speeds (25–30 rpm) were adjusted so as to achieve a gentle stirring action. Homogenates were centrifuged 10 min at 7000 × G in an International Model HR-I centrifuge with a No. 856 head, and residues were washed with distilled water to remove traces of water-soluble proteins. Myosin B was extracted from these residues with 1.0*M* KCl/0.01*M* $NaHCO_3$, pH 7.2, by homogenization at low speeds in the Omni-mixer for 30 min. Following centrifugation of the homogenate at 20,000 × G for 30 min, the supernatant, containing myosin B, was filtered through two layers of cheesecloth to remove lipids. All steps in the preparation, including centrifugation, were carried out at 0–5°C.

Protein estimation. Protein determinations were carried out by the biruet method (Layne, 1957).

Enzymic assays. ATPase determinations were carried out at 25°C for 5 min. Incubation mixtures contained 2 ml of protein solution, 5m*M* ATP, and 10mM of either $CaCl_2$ or $MgCl_2$, and total volumes were 3.0 ml. Reactions were stopped by the addition of 1.0 ml of 15% (w/v) trichloroacetic acid, and P_i liberated was determined by the method of Taussky and Shorr (1953). All determinations were made in duplicate.

Ultracentrifugal analyses. Sedimentation behavior of myosin B fractions was observed in a Spinco model E analytical ultracentrifuge equipped with schlieren optics. The temperature was regulated at 20°C by a rotor temperature indicator and control unit. Sedimenting boundaries were photographed at 8-min intervals at 44,770 rpm, and sedimentation coefficients were calculated according to Schachman (1957). No corrections were made for the Johnston-Ogston effect or for radial dilution of the protein. An An-D rotor with a 12-mm standard cell was used with all protein solutions.

Density-gradient centrifugation. Linear sucrose gradients were prepared at room temperature in 1.0*M* KCl/0.01*M* $NaHCO_3$. The device for preparing gradients consisted of two 50-ml beakers connected at bottom outlets by a short piece of Tygon tubing containing a screw clamp. An outflow tube extended from the mixing beaker, which was stirred with a small magnetic stirrer. Stirring speeds were adjusted to give good mixing without disturbance of the meniscus. The less dense sucrose solution was added to one chamber and, by adjusting the screw clamp, was allowed to flow through the tubing to the outlet of the mixing beaker. The more dense solution was added to the mixing beaker, and the stirrer was adjusted. The tip of the outflow tube was placed at the top of centrifuge tubes, and the flow was started by simultaneously adjusting the clamps on the outflow and connection tubes. Fluid levels in the two beakers were kept equal during emptying to assure linearity of the gradients. Tubes containing the gradient, as well as the rotor, were equilibrated at 5°C for several hours. Samples containing 10% or less of total protein were layered over the gradient. Centrifugation was carried out in a Spinco model L-2 preparative ultracentrifuge equipped with a swinging bucket rotor fitted with cellulose nitrate centrifuge tubes. Other experimental conditions concerning the centrifugation and sampling of fractions are given in Table 1.

Viscosity measurements. Relative viscosities

Table 1. Experimental conditions for sucrose gradient centrifugation and sampling of fractions.

Spinco model L rotor	Range of sucrose concentration in gradient (w/w)	Maximum speed (rpm)	Vacuum chamber: Temperature (°C)	Vacuum chamber: Pressure (μ Hg)
SW 25	4.92–20.99%	23,000	10	<50

Time of centrifugation at maximum speed (hr)	Gauge of needle	No. of drops per fraction	Volume of each fraction (ml)
23	20	25	2.5

were determined at 3°C on 5-ml samples in an Ostwald viscometer with an outflow time for water of 70 sec. Viscosities in the absence and presence of ATP were determined from the average of three consecutive readings made before and immediately after the addition of 0.1 ml of 60m*M* ATP.

Ultraviolet extinction. Ultraviolet extinction ratios were obtained at room temperature with a Beckman DK-2A recording spectrophotometer.

RESULTS

Ultracentrifugal analyses. Myosin B preparations showed considerable turbidity at temperatures of 0–20°C. Much of the turbidity was removed during acceleration in the ultracentrifuge, and a distinct shoulder in the pictures indicated considerable sedimented material in the bottom of the cell. Some turbidity, however, remained after attaining the set speed, but this was essentially removed halfway through each run. An interface between the turbid and clear solution appeared to move down the cell as shown clearly in Fig. 1A. From the photographs, it is obvious that considerably more of this high-molecular-weight material was present in the 4-hr myosin B preparations.

Only small qualitative differences in sedimentation patterns of individual components were observed between fractions prepared 4 and 48 hr after death. Essentially three peaks were seen during sedimentation for 80 min. In both preparations a small, rapidly sedimenting component aggregated or interacted in some way with the already sedimented material which resulted in the formation of an ultra-sharp boundary at the bottom of the cell. A smaller, slower-moving component followed with a sedimentation coefficient (S_{20}) of 15S. The major component sedimented at a rate of 7.9S. Significant amounts of slowly sedimenting components remained at the end of runs, as indicated by shoulders in the left portion of frames.

Density gradient centrifugation. Typical results of sucrose gradient centrifugation of myosin B preparations are shown in Fig. 2. One major component was observed in all gradient patterns. This component, about one-third the distance between the meniscus and the bottom, made up a variable proportion of the total protein. In addition to the major fraction, gradients frequently contained significant amounts of 280-mμ-absorbing material at the top of tubes. A minor, rapidly sedimenting fraction was present near the bottom, and a small pellet was always found in the bottom of tubes. Only small qualitative differences were observed between the 4- and 48-hr myosin B fractions. The major component of the 48-hr preparation was slightly displaced from that of the 4-hr preparation, and component I showed slight increases in the amount of slowly sedimenting material.

Analyses of the individual fractions are shown in Table 2. ATPase activity of material near the meniscus (I) was quite high, particularly the calcium-activated ATPase. The major component showed comparatively low ATPase activity. In this fraction, activation by magnesium ions was

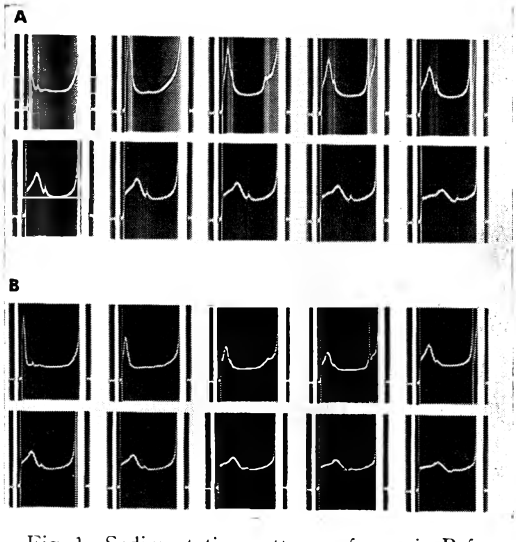


Fig. 1. Sedimentation patterns of myosin B fractions in 1.0*M* KCl/0.01*M* $NaHCO_3$. Speed: 44,770 rpm; temperature, 20°; bar angle, 40° in first frame, 35° in subsequent frames. Sedimentation proceeds to the right. All pictures taken at 8-min intervals. A) Myosin B fraction prepared 4 hr after death; protein concentration, 9.2 mg/ml. B) Myosin B fraction prepared 48 hr after death; protein concentration, 7.1 mg/ml.

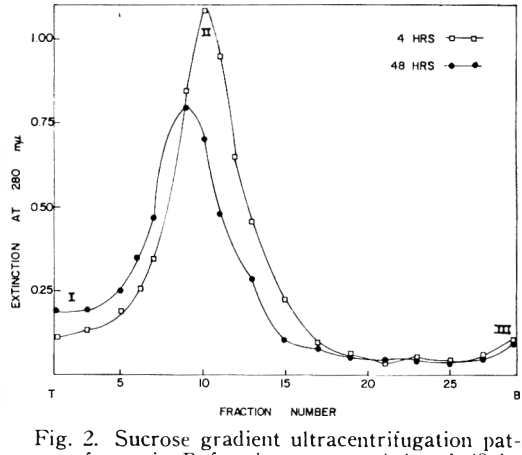


Fig. 2. Sucrose gradient ultracentrifugation patterns of myosin B fractions prepared 4 and 48 hr after death. Sucrose gradient, 4.92 to 20.99%, containing 1.0*M* KCl; rotor, SW 25; time of centrifugation, 23 hr at 23,000 rpm. Total protein in 4-hr fraction, 18.4 mg; 48-hr fraction, 16.0 mg. T and B on the abscissa respectively signify top and bottom fractions.

Table 2. Analyses of fractions from sucrose gradient ultracentrifugation of myosin B fractions.

Fraction	Adenosine triphosphatase activity [a] (μM P_i/mg protein/5 min)		Viscosity fall with addition of ATP [b] percent	Ultraviolet extinction ratio (A_{280}/A_{260})
	Ca^{++}-activated	Mg^{++}-activated		
I	3.72	2.28	0	0.57
II	0.09	0.16	15	1.56
III	1.78	1.10	2	0.71

[a] Incubation mixtures were 5mM in adenosine triphosphate and 10mM in either $MgCl_2$ or $CaCl_2$.
[b] Final adenosine triphosphate concentration, 5mM.

greater than activation by calcium ions. Material near the bottom of tubes (III) exhibited moderate enzymic activity, in which the calcium-activated enzyme gave highest values. The fall in viscosity with the addition of ATP was highest in the major fraction, while values for I and III fractions were negligible. Ultraviolet extinction ratios were lowest in fractions I and III.

DISCUSSION

Ultracentrifugal analyses. Concentrated myosin B fractions exhibited considerable turbidity before runs, which was partially removed during the early stages of acceleration. The high rate of sedimentation suggests that these components are probably cellular fragments and/or particulate cell particles not completely solubilized during extraction. Large protein aggregates or gel forms were perhaps responsible for the turbidity remaining after attainment of set speeds.

The first, small, rapidly sedimenting component obviously interacted in some way with material already sedimented as shown by the formation of the ultrasharp boundary particularly evident in frame 4 of Figs. 1A and 1B. The formation of this and another smaller component near the bottom of the cell possibly represents the formation of large aggregates of myosin B, perhaps corresponding to 90S to 280S sedimenting components observed by Portzehl (1950). According to Johnson and Rowe (1964), these higher values probably refer to the typical gel component of myosin B solutions first observed by Snellman and Erdos (1948). The material sedimented during acceleration may have consisted in part of this gel component, which, on interaction with the 15S myosin B species, gave rise to yet another sedimenting component.

Immediately to the right of the ultrasharp boundary near the bottom of the cell in frame 4 of Fig. 1B, a small but distinct component was observed which was absent in the corresponding frame of Fig. 1A. This peak may represent an additional gel component not present in the 4-hr myosin B fraction. The slowly sedimenting material observed in the myosin B fractions is similar to small amounts of polydisperse material observed by Johnson and Rowe (1964) in natural actomyosin systems. Those workers are of the opinion that the material must be derived from the formation of an equilibrium system between actin and myosin, since the sedimentation rate of its broad peak changes with the weight fraction of myosin A.

It would be difficult to make any further assumptions as to the nature of the sedimenting species without assessing relative concentration dependencies of each component and the extent of Johnston-Ogston effects common to such multicomponent systems.

Density-gradient centrifugation. Ultracentrifugation of myosin B fractions in a continuous sucrose gradient provided a degree of resolution similar to that obtained with the analytical ultracentrifuge. Although density-gradient patterns lacked the detail of schlieren patterns, the major features of sedimentation were comparable. The small fraction near the cell bottom, the major ultraviolet-absorbing fraction, and the material remaining near the meniscus of gradient tubes, correspond favorably with schlieren patterns of the same preparation.

The high-calcium-activated ATPase activity of slowly sedimenting material (I of Fig. 2) suggests the presence of myosin A. The presence of myosin B in the major fraction

II is indicated by the high-magnesium- and low-calcium-activated ATPase and the 15% fall in viscosity with addition of ATP. The relatively high-calcium- and magnesium-activated ATPase in material near the bottom of the tube (III) is perhaps due to the presence of both myosin A and myosin B. This material may correspond in part to the myosin B gel observed in moving-boundary ultracentrifugation. Some myosin B is present, as indicated by the small drop in viscosity of fraction III with the addition of ATP.

Calcium ions exert a strong activating effect on myosin A ATPase, while magnesium ions (Mommaerts and Green, 1954) inhibit the enzymic activity. Myosin B possesses ATPase activity which is highly accelerated by magnesium ions and to some extent by calcium ions (Weber and Herz, 1963). In solutions of high ionic strength, ATP dissociates myosin B into myosin A and actin. The result is an immediate fall in viscosity back to the sum of the viscosities of the individual components. Accompanying this dissociation is the appearance of the characteristic myosin A ATPase activity (Hasselbach and Schneider, 1951). Endo (1964), however, showed that at concentrations as high as 1m*M* in ATP, only a partial dissociation of myosin B occurs. Apparently some myosin B remained in the undissociated form in fractions showing high ATPase activity in the presence of magnesium ions. Since high ionic strengths were used, however, a part of the high-calcium-activated ATPase (myosin A) may have been a direct result of the dissociating action of ATP in incubation mixtures.

As in schlieren patterns, only small differences were apparent between density-gradient ultracentrifugal patterns of 4- and 48-hr myosin B fractions. The increase in the highly active ATPase (I) suggests either a change in degree of aggregation or an increase in myosin-A-containing material.

Aging for 44 hr causes a decrease in the amount of rapidly sedimenting high-molecular-weight material and increases in lower-molecular-weight components possessing high specific ATPase activity. This evidence perhaps favors the concept of dissociation of myosin B into myosin A and actin with resolution of rigor. On the other hand, the high-molecular-weight material as observed in the analytical ultracentrifuge, may be myosin B gel, and the decrease in this component may be due to depolymerization as well as dissociation into myosin A and actin.

REFERENCES

Bendall, J. R. 1964. Meat proteins. *In:* "Proteins and Their Reactions." Schultz and Anglemier, eds. p. 225. Avi Publishing Co., Westport, Conn.

Deatherage, F. E. 1963. The effect of water and inorganic salts on tenderness. Proc. Meat Tenderness Symposium, Campbell Soup Co., pp. 45–68.

Endo, M. 1964. The superprecipitation of actomyosin and its ATPase activity in low concentration of ATP. *J. Biochem.* **55**, 614.

Erdos, T. 1946. Rigor, contraction and ATP. *Stud. Inst. Med. Chem. Univ. Szeged.* **3**, 51.

Fujimaki, M., N. Arakawa, A. Okitani, and O. Takagi. 1965. The changes of "myosin B" ("actomyosin") during storage of rabbit muscle. II. The dissociation of "myosin B" into myosin A and actin, and its interaction with ATP. *J. Food Sci.* **30**, 937.

Hasselbach, W., and G. Schneider. 1951. L-Myosin and actin contents of rabbit muscle. *Biochem. Z.* **321**, 462.

Johnson, P., and A. Rowe. 1964. An ultracentrifuge study of the actin-myosin interaction. *In:* "Biochemistry of Muscle Contraction." Gergely, ed. pp. 279–299. Little, Brown Co., Boston.

Khan, A. W., and L. Van den Berg. 1964. Some protein changes during post-mortem tenderization in poultry meat. *J. Food Sci.* **29**, 597.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. *In:* "Methods in Enzymology." Vol. I., p. 450. Colowick and Kaplan, eds. Academic Press, New York.

Mommaerts, W. F., and I. Green. 1954. Adenosine triphosphatase systems of muscle. III. A survey of the adenosine triphosphatase activity of myosin. *J. Biol. Chem.* **208**, 833.

Partmann, W. 1963. Post-mortem changes in chilled and frozen muscle. *J. Food Sci.* **28**, 15.

Portzehl, H. 1950. Measurements and dimensions of L-myosin. *Z. Naturforsch.* **5b**, 75.

Sayre, R. V., and E. J. Briskey. 1963. Protein solubility as influenced by physiological conditions in the muscle. *J. Food Sci.* **28**, 675.

Schachman, H. K. 1957. Ultracentrifugation, diffusion and viscometry. *In:* "Methods in En-

zymology." Vol. IV., p. 32. Colowick and Kaplan, eds. Academic Press, New York.

Scharpf, L. G., and W. W. Marion. 1964. Extraction of fibrillar and sarcoplasmic proteins of turkey muscle. *J. Food Sci.* **29**, 303.

Snellman, O., and T. Edros. 1948. Ultracentrifugal analysis of crystallized myosin. *Biochim. et Biophys. Acta* **2**, 650.

Szent-Gyorgyi, A. 1951. Contractile muscle protein. *Discussions Faraday Soc.* **11**, 179.

Szent-Gyorgyi, A. G. 1964. Proteins of the myofibril. *Recip. Meat Conf. Proc.* **17**, 131.

Taussky, H. H., and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem* **209**, 675.

Weber, A., and R. Herz. 1963. The binding of calcium to actomyosin systems in relation to their biological activity. *J. Biol. Chem.* **238**, 599.

Ms. rec'd 1/20/66.

Journal Paper No. J-5248 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project 1400. Departments of Poultry Science and Biochemistry and Biophysics cooperating.

The authors thank Dr. John Ayres and Dr. Darrell Goll, of the Department of Dairy and Food Industry, for use of the preparative ultracentrifuge and other equipment.

Abbreviations used are as follows: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase.

C. T. MORROW and N. N. MOHSENIN
Department of Agricultural Engineering, The Pennsylvania State University
University Park, Pennsylvania 16802

Consideration of Selected Agricultural Products as Viscoelastic Materials

SUMMARY

A demand currently exists for information on the mechanical behavior of foods and agricultural products during handling, processing and quality evaluation. Many of the older subjective tests have now been replaced by various techniques from which empirical and partially objective measurements may be obtained; however, the majority of these measurements are not well-defined in terms of accepted physical constants, thereby making their interpretation difficult. To provide consistency between various investigations, all mechanical properties may be evaluated in terms of common engineering parameters as the first approximation. From the point of view of mechanics, many products such as fruits, vegetables, and cereal grains in their natural state may be considered as convex bodies for which classical solutions can be applied for evaluation of compression, shear and tension properties.

In this paper previous and current investigations in which the engineering approach to mechanical property evaluation has been used are reviewed. In all of these investigations, the common methods for product testing are hydrostatic and uniaxial compression. Instrumentation and techniques are described for obtaining and interpreting data from these tests in terms of physical constants.

The mechanical behavior of most agricultural products is time dependent. Therefore, characterization of mechanical behavior requires the application of viscoelasticity principles in which both viscous and elastic responses are combined. The fundamental principles of viscoelasticity are presented briefly and analogies are developed for relating the observed behavior to well-established mechanical systems.

To illustrate the application of the engineering approach to mechanical property evaluation, a study on McIntosh apple fruits is described. This study demonstrates that many of the principles of classical mechanics are applicable to selected agricultural materials.

INTRODUCTION

Many agricultural products are subjected to mechanical treatments in harvesting, handling and processing. To establish an efficient mechanical process and at the same time have a minimum degree of product damage and highest quality, the behavior of the various products when subjected to stress and strain must be fully understood. Knowledge of the general stress-strain-time behavior of the products is needed in addition to information regarding the critical levels of stresses and strains at which damage will result.

In these respects it appears that many of the techniques used in the engineering sciences to study the behavior of engineering materials might be applicable to agricultural products. The critical stress and strain levels may be considered from the same standpoint as yield strength, ultimate strength, and similar parameters are for common engineering materials.

Any mechanical properties which are evaluated for an agricultural product must be specified as being associated with a particular stage of the material's living process, such as maturity level. This means that properties must be evaluated for the same product at various stages in order to specify completely its mechanical behavior. A large number of tests will be needed to compensate for inter-replicatory variations for a given product.

Considering the above limitations, if it is possible to show that the stress-strain behavior of a selected product is similar in form to that of engineering materials, it will be possible to evaluate the mechanical behavior of that product by using established testing and analysis procedures.

LITERATURE REVIEW

Only a few investigators have studied the mechanical behavior of agricultural products by using engineering terminology and techniques. Many different instruments are available within the food industry for qualitative study of the texture of products such as fruits and vegetables. These instruments, although more useful than subjective panel evaluations, do not characterize the products or provide data usable in engineering analyses.

Shpolyanskaya (1952) has studied the structural-mechanical properties of the wheat grain. She found that the wheat grain behaved as an elastic-plastic-viscous body which exhibited creep, stress relaxation, and elastic after-effects. She also proposed a rather complicated mechanical model to represent the time-dependent behavior of a grain subjected to uniaxial compression. Of particular interest is the fact that she utilized the classical Hertz solution for contact stresses to evaluate the moduli of deformability for the grain.

Zoerb and Hall (1960) have also studied the mechanical and rheological properties of cereal grains, using a variable-speed testing apparatus. Compressive tests were conducted in two series: 1) the whole kernel was subjected to load; 2) core specimens, made by cutting off each end, were tested. The load-deformation curves obtained were converted to stress-strain curves by utilizing the measured cross-sectional area and initial length of the specimens. Information derived from these studies was used for evaluation of hysteresis losses, modulus of resilience, and modulus of elasticity. They also conducted stress relaxation studies on pea beans, using varying loading rates. A significant variation was found to exist between relaxation time and loading rate, thereby indicating the viscoelastic nature of the seeds. In reality, only force-deformation studies were conducted, because stress and strain distributions for the seeds have not yet been established. The relaxation data were fitted to a two-element Maxwell Model, giving a close approximation of real behavior.

Mohsenin and associates have been studying the viscoelastic behavior of fruits and vegetables for several years. Realization of the viscoelastic nature of such products has been shown in several of their early reports. Mohsenin *et al.* (1963) proposed a qualitative model to represent the viscoelastic nature of creep behavior for fruits subjected to uniaxial compression. At that time, no attempt was made to evaluate the model parameters quantitatively.

Finney *et al.* (1964) and Finney (1963) have considered the potato as a linear viscoelastic body. They derived a physical basis for this consideration by studying the constitutive components of the potato tuber. They also studied the stress relaxation properties of the tubers when axially loaded between parallel plates. The relaxation times were found to vary with initial rate of mechanical loading. The data obtained from the relaxation tests were fitted to mechanical models which simulated the viscoelastic behavior of the force relaxation process. While Finney proposed the Hertz and Boussinesq techniques for evaluation of the viscoelastic compression moduli, no attempt was made to interpret the relaxation process in terms of modulus changes.

Suggs and Splinter (1965) studied the behavior of tobacco stalks in bending. They found variations between compression and tension moduli. They also observed a viscoelastic effect as exhibited in the stress relaxation behavior of the stalks. This effect was observed to be predominant at low strain rates.

Halyk and Hurlburt (1964) applied materials-testing procedures to alfalfa stems in order to determine their ultimate tensile and ultimate shear strength. They did not conduct time-dependent studies, but they did utilize testing procedures which had previously been accepted for common engineering materials.

Timbers *et al.* (1965) and Timbers (1964) have studied some mechanical and rheological properties of the Netted Gem potato. They studied both creep and stress relaxation behavior. The significance of this work was in recognizing the need for an analysis of viscoelastic changes of moduli and compliances (compliance $=$ 1/modulus $=$ strain/stress).

Although Timbers recognized the need for studying actual modulus changes, his stress relaxation studies on whole tubers were analyzed from the viewpoint of force relaxation. He also proposed a suitable mechanical model to represent the tuber behavior, but did not attempt to actually evaluate viscoelastic parameters as are commonly specified for polymeric materials.

Recent work at Pennsylvania State University has indicated the feasibility of considering bulk corn silage as a viscoelastic material. Investigated in the work was the variation of relaxation time constants with the density and composition of corn silage. The information derived from these studies may prove useful in designing a maximum compaction system. Investigations in the same laboratory on corn grains, subjected to bulk compression, have led to the evaluation of certain viscoelastic bulk parameters for this product.

Morrow (1965) studied the viscoelastic nature of McIntosh apple fruits subjected to both uniaxial and bulk compression. Mechanical models were chosen to represent both creep and relaxation behavior. The viscoelastic parameters derived from these studies were based upon apparent moduli in all instances. It was possible to obtain good agreement between the model response and experimental creep and relaxation behavior.

EXPERIMENTAL METHODS

Techniques for determining viscoelastic parameters. Examination of procedures used by various

investigators of mechanical behavior of agricultural products indicates a wide variety of testing and analysis techniques. For results to be meaningful and comparative, it is imperative that some attempt at procedural standardization be made. While it is realized that testing procedure is often dictated by available facilities, a more uniform method of data analysis may be adopted. The techniques described herein are suggested as a possible means for achieving such standardization. Certainly, considerable modification of these methods is required when working with such complex bodies as agricultural products. As a first approximation, however, many familiar solutions may be applied verbatim to agricultural products

Choice and preparation of specimens. This discussion will be concerned mainly with products as found in their natural state. No preparation will be used other than to cut or slice a uniform specimen, if the natural state of the material is not disturbed. With complex products in the form of convex bodies, it is desirable to test the intact product. With fruits, for example, while useful information may be obtained using samples cut from the flesh, it is difficult to correlate mechanical properties derived for the flesh specimens with the predicted properties of the intact product. This problem of testing of the product in its natural state has been pointed out by Mohsenin (1965).

It is realized that an intact product such as a fruit violates all of the fundamental assumptions of homogeneity, isotropy, and continuity that are normally required in solving elementary materials science problems. Disregarding the violation, however, it is possible to consider a "black-box" approach to modulus evaluation. Such an approach is often utilized in electrical network analysis, where only input parameters and output quantities are considered, with intermediate behavior disregarded. By using such an approach, it is more valid to consider moduli for agricultural products as apparent rather than actual parameters. Timbers (1964) has suggested the use of such terminology. It may be noted that the term "apparent modulus" is often used for comparing the plastic behavior of common engineering materials.

Testing methods. In studying viscoelastic behavior, it is possible to apply either a constant load or a constant deformation to the material. If a constant load is applied, creep behavior is studied; with a constant deformation, stress relaxation results. When deriving viscoelastic parameters, it is often desirable to study both forms of fundamental behavior.

There are three fundamental methods of straining specimens for testing purposes: 1) bulk compression; 2) uniaxial compression; and 3) application of shearing forces. Of these methods, only the first two are easily adapted to agricultural products in their natural form. Very uniform specimens with special shapes are required for conducting shear studies. Several other techniques are available for general materials science testing. Tension testing seems to be difficult to utilize, because of specimen shape and size requirements, and gripping problems.

Of the many dynamic techniques which are available, wave propagation appears to be an excellent approach to the evaluation of viscoelastic parameters. Transducer techniques also appear to be very applicable. Very few studies have been conducted on agricultural products utilizing dy-

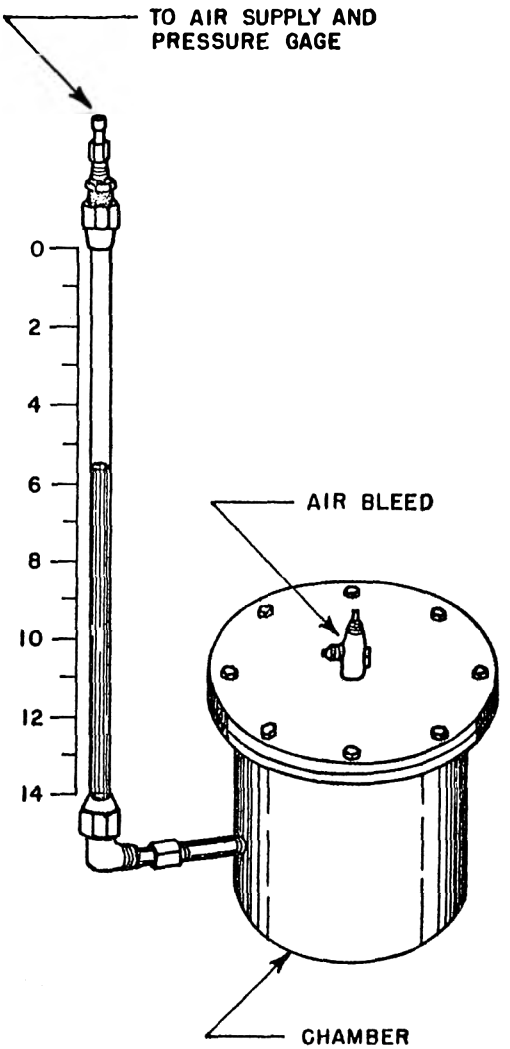


Fig. 1. Low-pressure bulk compression apparatus used for obtaining data reported in this paper.

namic measurement methods, but such an approach appears highly desirable for future investigations.

Bulk compression. When static bulk compression is utilized, either creep or relaxation studies may be performed. Two instruments have been built in the Pennsylvania State University Rheology Laboratory for studying bulk behavior. The device shown in Fig. 1 is used for applying low hydrostatic pressures to fruits and vegetables. Its design is such that it may be utilized only for bulk creep studies. A similar apparatus has been used by Finney (1963) for bulk compression studies of potatoes.

An apparatus designed for studying the compressibility of cereal grains is fully described by White (1966). This device is suitable for both creep and stress relaxation. By using this instrument, it is possible to simultaneously record bulk stress and strain for a product.

Constrained compression characteristics of unconsolidated materials such as forage, silage, or grouped grains may be studied by using a device such as described by Mohsenin (1963). The studies performed with this device could be conducted on any commercially available testing machine, but at a much higher cost.

Data obtained from bulk compression studies may be analyzed by considering either moduli or compliances at various time increments. In terms of bulk modulus:

$$K(t) = -\frac{p}{\Delta V/V_0} \qquad [1]$$

The bulk compliance or compressibility is equivalent to the inverse of the bulk modulus and may be defined as

$$B(t) = -\frac{\Delta V/V_0}{p} \qquad [2]$$

Uniaxial compression. Many different devices have been used for applying uniaxial compressive loads to agricultural products. Creep studies may be conveniently conducted with a device similar to that in Fig. 2. A dead load is applied to the specimen, and time-dependent deformations are recorded. Relaxation studies may be performed with any universal testing machine. The Instron testing machine and environmental control unit are found to be very suitable. The P.S.U. testing machine, as previously described by Mohhenin (1963), is also suitable for uniaxial relaxation studies and creep at higher pressures.

Regardless of the type of loading instrument used, a choice must be made as to the most desirable form of loading head to contact the fruit. Both flat plates and cylindrical plungers have been used in the past, but very few attempts have been made

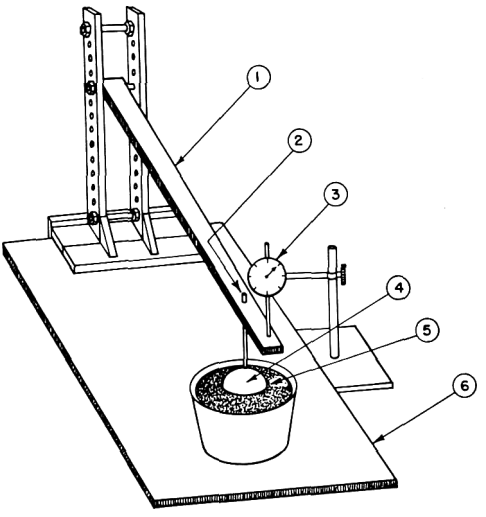


Fig. 2. Dead-weight testing apparatus used for obtaining data reported in this paper.

at analyzing the stress distribution within the product. This information is very important if meaningful parameters are to be derived. The approximate solutions presented in the following sections have also been proposed by Finney (1963) and Timbers (1964), but few attempts have been made at their proper application.

Plunger loading. Stress-strain relations for convex bodies loaded by cylindrical plungers require the use of classical theory of elasticity principles. Finney (1963) suggested using a solution derived by Boussinesq (1885) for analysis of potato tubers subjected to plunger loadings. The original solution, as proposed by Boussinesq, developed relations for semi-infinite bodies (half-spaces) subjected to concentrated compressive loads. This solution has been expanded by Timoshenko and Goodier (1951) to include three-dimensional considerations of a half-space loaded by a cylindrical plunger. Using the elastic solution proposed by Boussinesq and modified by Timoshenko and Goodier (1951), it is necessary to assume homogeneity and isotropy. It is also necessary to assume that a half-space exists. It is realized that because of the small radius of curvature of certain products, the latter assumption may not be completely valid. *Examination of* the constitutive equations of the Boussinesq solution, as discussed in the following, will provide an insight into the validity of assuming such products to be semi-infinite bodies.

Establishing a cylindrical coordinate system in which the z direction is parallel to the longitudinal axis of the cylindrical plunger, it is possible to evaluate normal stresses in the z direction at various points below the surface of the half-space. These normal stresses are shown by Timoshenko and Goodier to be of the form

$$\sigma_z = F\left[-1 + \frac{z^3}{(a^2 + z^2)^{3/2}}\right] \qquad [3]$$

where the origin of the z direction is at the surface of the half-space. Considering a cylindrical plunger of radius 0.125 inch and a depth of two inches below the surface of the product, the above expression shows that stress σ_z at this level is approximately zero. The reverse pressure effect caused by the product's support may be neglected if the product is supported over a very large contact area. Under these conditions, it is permissible to consider the product as a half-space from the viewpoint of σ_z normal stresses. Using the same type of procedure, it is possible to show that the assumption of a half space is also valid for σ_r and σ_θ stresses. Note that the product diameter was equal to at least ten times the plunger diameter in this illustration.

For an absolutely rigid plunger in the form of a circular cylinder pressed against the plane boundary of a semi-infinite elastic solid, the displacement is constant over the circular base of the cylinder. The distribution of pressures, p, at the surface of the semi-infinite body is not uniform, and its intensity is given by the equation:

$$p = \frac{F}{2\pi a\sqrt{a^2 - r^2}} \qquad [4]$$

The closed form of this pressure distribution is shown in Fig. 3 as obtained from Finney (1963). Plastic yielding will occur along this ($r = a$) boundary, but this yielding will not greatly affect the pressure distribution. At points beyond the outer boundary of the plunger, the pressure distribution will not be affected by the singularity at $r = a$.

The relationship between the deformation, d, of the material beneath the plunger and the applied force, F, has been shown by Timoshenko and Goodier to be of the form

$$E = \frac{F(1 - \mu^2)}{2ad} \qquad [5]$$

where E and μ are respectively Young's modulus and Poisson's ratio for the material.

Under the conditions stated above, the Boussinesq solution appears to be a good approximation to the stress distribution in agricultural products loaded by small cylindrical plungers. As shown by Finney (1963), it does not appear valid for cases in which the plunger diameter is of nearly the same magnitude as the loaded body. Recent studies by Timbers *et al.* (1965) neglected the restriction of the Boussinesq solution.

Flat-plate loading. Evaluation of stress-strain relations for convex bodies subjected to uniaxial compression by means of flat plates also requires the use of classical theory-of-elasticity techniques. Assuming deflections to be small when compared with the dimensions of the convex body, it is possible to use a technique proposed by Heinrich Hertz (1896) for evaluating contact stresses between two convex bodies. This solution may be generalized to flat-plate compression by taking orthogonal radii of curvature of a flat plate as being infinite. Such a generalization has been shown by Timoshenko and Goodier (1951) and Kazma and Cunningham (1962).

The form of the solution proposed by Kozma and Cunningham requires that eight fundamental assumptions be satisfied. In addition to normal requirements of homogeneity and isotropy, these specify that the contacting bodies are infinitely large and that the radii of curvature of the contracting solids are large when compared with the dimensions of the contacting area. These assumptions appear approximately justified for normal sizes and shapes of convex agricultural products. Morrow (1965) has discussed the fundamental assumptions in detail.

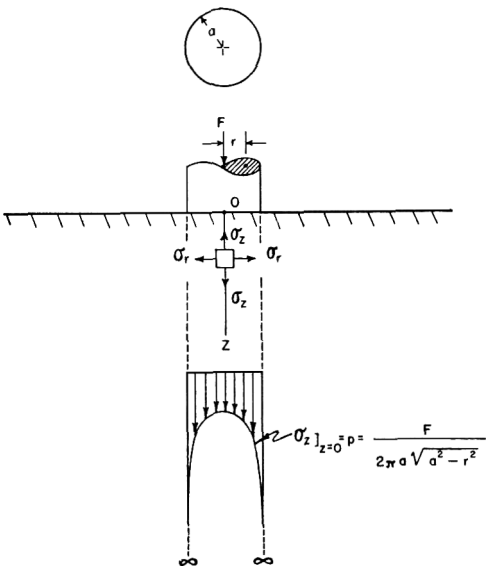


Fig. 3. Theoretical stress distribution under a rigid plunger acting against a semi-infinite elastic body (from Finney, 1963).

Kozma and Cunningham give the following relation for the deformation of two convex bodies subjected to uniaxial compression:

$$d = \frac{k}{2}\left[\frac{9}{16\pi^2} F^2 (Q_1 + Q_2)^2 \left(\frac{1}{R_1} + \frac{1}{R_1'} + \frac{1}{R_2} + \frac{1}{R_2'}\right)\right]^{1/3} \quad [6]$$

where: $Q = 4(1-\mu^2)/E$

1 denotes primary convex body
2 denotes secondary convex body

Considering a product compressed between two flat steel plates and assuming a spherical shape for the product

$$(R_1 = R_1' = \frac{D}{2},\ R_2' = \infty,\ Q_2 \simeq o),$$

Eq. 6 may be simplified into the following form:

$$E = \left[\frac{16\ (1-\mu^2)^2}{14.285} \frac{F^2}{Dd^3}\right]^{1/2} \quad [7]$$

which permits computation of Young's modulus for the products using experimental parameters and a known or calculated value of Poisson's ratio.

Evaluation of Poisson's ratio. Both the Bossinesq and Hertz solutions require knowledge of Poisson's ratio for the material being studied. Direct measurement of this ratio is very difficult for convex bodies such as most agricultural products. However, it is possible to calculate the ratio based upon consideration of elastic parameters. The relation between elastic bulk modulus and Young's modulus is of the form

$$K_0 = \frac{E_0}{3(1-2\mu)} \quad [8]$$

Eq. 8 can be solved simultaneously with either the Hertz or the Boussinesq solution, using the linear portion of a typical load-deformation diagram, and the elastic bulk modulus to obtain an approximate value for Poisson's ratio. Assuming that the Poisson's ratio is not of a viscoelastic form such as that shown by Theocaris (1964), it may be used as a "constant" in evaluating all compliances and moduli.

Model representation. After the moduli and compliances have been evaluated at incremental times, it is necessary to consider their composite behavior for characterization of the material. By utilizing classical elasticity and Newtonian theory, it is possible to formulate an approximate theory of viscoelastic behavior. One method of developing such theory is by the use of model representation of elastic and viscous elements combined in such a way as to resemble the viscoelastic behavior. An elastic element will exhibit a strain which is directly proportional to the applied stress. Such a behavior may be represented by a spring. If a load is applied to such a spring, a deflection will result. This condition is analogous to the relation between applied stress and resulting strain in an elastic material.

For a Newtonian material, stress is proportional to the rate of strain and is independent of the strain itself. Such a relationship is analogous to the behavior of a dashpot consisting of a loose-fitting piston moving through a viscous fluid. Physical understanding of viscoelastic behavior is made much easier by using mechanical models consisting of elastic springs and viscous dashpots. Mechanical models have been proposed by many researchers, including Ferry (1961), Sharma (1964), and Mohsenin *et al.* (1963).

In the past, many objections have been raised to the use of mechanical models to represent viscoelastic behavior of engineering materials. It should be noted that agricultural products with high water content are very similar to the actual mechanical models. The cells behave in what is believed to be a nearly elastic manner while the cellular fluids are actual liquids representing the viscous element in the model. Therefore, it seems to be logical to represent the mechanical behavior of selected agricultural products by using mechanical models.

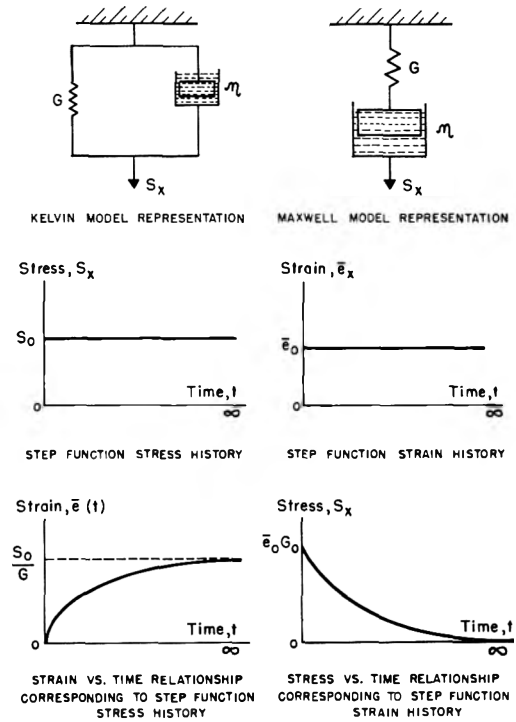


Fig. 4. Kelvin and Maxwell model characteristics.

Simple viscoelastic models. By combining an elastic and a viscous element in series, a Maxwell model is obtained as described by Ferry (1961) and others. By combining an elastic and a viscous element in parallel, a Kelvin or Voigt model is obtained, as described by those researchers. Both the Maxwell and the Kelvin models are graphically depicted in Fig. 4 as given by Sharma (1965).

The behavior of most real viscoelastic materials is too complex to be adequately represented by either a simple Maxwell or a simple Kelvin model. For complete characterization of many materials, an infinite combination of elastic and viscous elements is required. As a first approximation, however, finite combinations are suggested for use.

Degenerate Kelvin model. A degenerate Kelvin model as defined by Sharma (1964) is very useful for representing some types of viscoelastic behavior. This model as shown in Fig. 5 consists of a Kelvin element in series with a viscous and an elastic element. The same model is defined as a Burger's model by Mohsenin *et al.* (1963) and Reiner (1960).

If this model is assumed to represent shear behavior, elastic and viscous components may be assigned to each of the four elements as shown in Fig. 5. Examination of the model will show that the total strain ($\bar{e}_x$) will be the sum of the strains in each of three sections, *A*, *B*, and *C*. By Newton's third law, it is easily shown that the stress in each of these sections must be equal. Therefore the following relations are known to exist:

$$\begin{aligned} \bar{e}_x &= \bar{e}_A + \bar{e}_B + \bar{e}_C \\ S_A &= S_X = G_0 \bar{e}_A \\ S_B &= S_X = \eta \dot{\bar{e}}_B + G\bar{e}_B \\ S_C &= S_X = \eta_v \dot{\bar{e}}_C \end{aligned} \qquad [9]$$

These differential relations can now be solved for any stress and strain history to which the material is subjected. Considering a creep experiment in which the specimen is subjected to a constant-stress history, the total solution will be of the form

$$\bar{e}_X = \frac{1}{G_0} S_0 + \frac{1}{G} (1 - e^{-t/a}) S_0 + \frac{S_0 t}{\eta_v} \qquad [10]$$

Defining a compliance as the ratio of strain to stress, Eq. 10 can be written in the form

$$H(t) = H_0 + H(1 - e^{-t/a}) + t/\eta_v \qquad [11]$$

which defines the response of a degenerate Kelvin model to an application of constant stress. Behavior of this sort is shown in Fig. 5.

The above expressions are given for shear parameters, but are equally valid for tension, compression, or bulk functions except that η_v will be infinitely large for a specimen subjected to bulk compression. While Eq. 9 could have been solved for a constant-strain history, it is much easier to study relaxation behavior by considering a degenerate Maxwell model.

Degenerate Maxwell model. A degenerate Maxwell model is useful for studying viscoelastic behavior of materials subjected to a constant-strain history (stress relaxation). This model, as shown in Fig. 6, consists of an elastic element in parallel with a simple Maxwell model. The response of this model can be shown to be functionally equivalent to placing a limiting stop in the viscous dashpot of a simple Maxwell model.

As in the degenerate Kelvin model, the degenerate Maxwell model will be developed for shear relations having elastic and viscous constants assigned as shown in Fig. 6. The elastic constants G and G_e do not have the same magnitude as in the Kelvin model, as will become apparent after examination of the constitutive equation.

Fig. 6 indicates that the total strain rate of the model is equal to the sum of the strain rates of the elastic and viscous element in the simple Maxwell model, as shown by

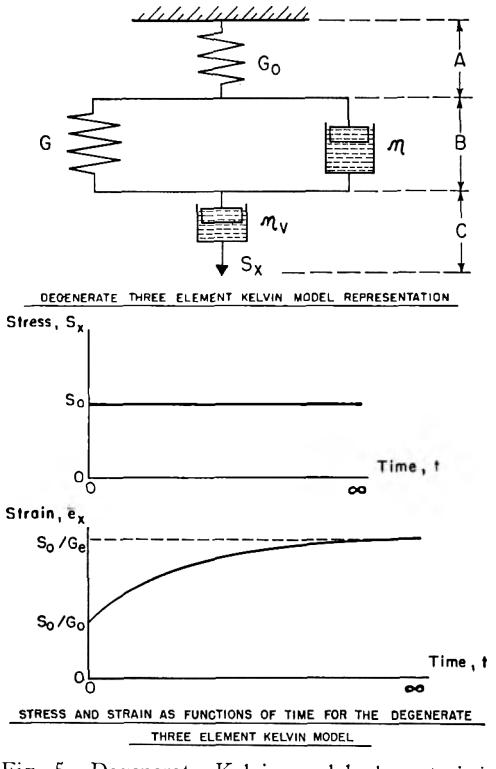


Fig. 5. Degenerate Kelvin model characteristics.

$$\dot{e}_x = \frac{\dot{S}_x}{G} + \frac{S_x}{\eta} \qquad [12]$$

If the model is subjected to a constant-strain history, Eq. 12 can be solved to yield the following relation

$$G(t) = Ge^{-t/\beta} + G_e \qquad [13]$$

This relation gives instantaneous values of the relaxation modulus for a material subjected to a constant strain history. Most real materials require the use of several Maxwell elements to completely describe the viscoelastic behavior. It must be emphasized that only an approximation to viscoelastic behavior is made by using the preceding three-element model. The response of such a model to a constant-strain history is shown in Fig. 6.

RESULTS AND DISCUSSION

The preceding techniques for evaluation of moduli and compliances and viscoelastic model fitting have been utilized in a study of the viscoelastic properties of McIntosh apple fruits (Morrow, 1965). This study did not contain sufficient replications to establish statistically valid viscoelastic parameters; however, several interesting observations were made concerning the viscoelastic nature of the material and the validity of the techniques being used.

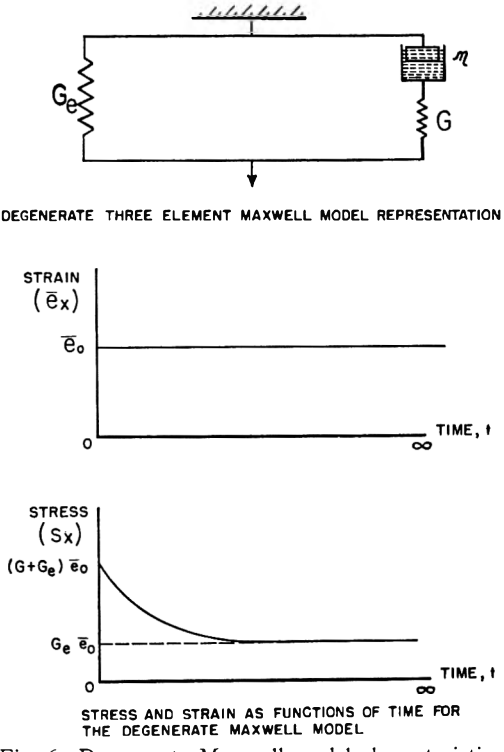


Fig. 6. Degenerate Maxwell model characteristics.

Poisson's ratio for the apple fruits was calculated by using the average bulk modulus and the average Young's modulus as found in plate and plunger creep tests using Hertz and Boussinesq solutions as previously given. For the plate loadings, $\mu = 0.22$; and for the plunger loadings, $\mu = 0.37$. The latter value was believed to be the more reliable and was used in the analysis of all moduli and compliances. Investigations are now being conducted to re-evaluate the Poisson's ratio. Actually, Poisson's ratio is entered as a second-power term and has no great effect on the magnitude of moduli and compliances determined by the Hertz or Boussinesq solutions. Since its value varies from 0.0 to 0.5, the extent of modulus variation will be from 100%, when $\mu = 0.0$, to 75%, when $\mu = 0.5$.

All compliances for hydrostatic compression tests were fitted to degenerate Kelvin models having the behavior shown by Eq. 11. The mean behavior of these models was found to be

$$B(t) = 0.00196 + 0.00031\ (1 - e^{-t/30.8}) \qquad [14]$$

This behavior is depicted in Fig. 7. The deviation between the experimental creep behavior and that predicted by a particular model is shown for one specimen in Table 1. The deviation could have been reduced by incorporating additional Kelvin elements into the model, thereby obtaining a better fitting curve to the experimental data.

Specimens were subjected to uniaxial compression using both plate and plunger loadings. The compliances were obtained using the inverse of Eqs. 7 and 5 for plate and plunger loading, respectively. All results were fitted to degenerate Kelvin models having the behavior shown by Eq. 11. The mean behavior of specimens subjected to plate loading was

$$J(t) = 0.00128 + 0.00077\ (1 - e^{-t/6.57}) + t/492{,}000 \qquad [15]$$

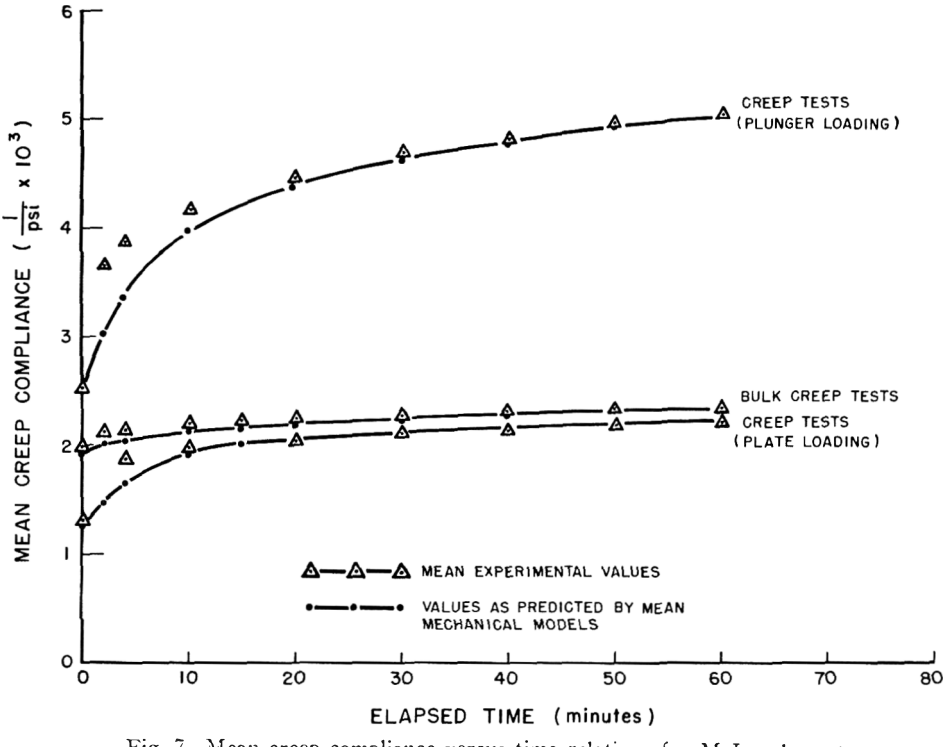


Fig. 7. Mean creep compliance versus time relations for McIntosh apples.

Table 1. Typical moduli and compliances versus time for McIntosh apple fruits.

Time (min)	Bulk creep compliance $(\frac{1}{psi} \times 10^3)$		Axial creep compliance $(\frac{1}{psi} \times 10^3)$				Axial relaxation modulus (psi)			
			Plate loading		Plunger loading		Plate loading		Plunger loading	
	E	T	E	T	E	T	E	T	E	T
0	1.87	1.87	1.26	1.26	2.53	2.53	476	392	329	252
1	2.05	1.89	1.82	1.37	3.55	2.80	400	389	268	251
2	2.12	1.91	1.90	1.47	3.65	3.02	388	386	253	249
3	2.14	1.92	1.96	1.56	3.72	3.21	380	382	247	248
4	2.15	1.94	2.00	1.65	3.77	3.38	373	379	242	246
5	.....	1.06	.....	1.72	.....	3.52	.....	376	.....	245
10	2.20	2.03	2.14	1.99	4.09	3.96	350	362	228	238
15	2.26	2.09	2.21	2.15	4.25	4.20	337	350	222	232
20	2.27	2.13	2.28	2.25	4.38	4.34	328	338	219	227
25	2.27	2.17	.....	2.31	.....	4.45	313	319	212	218
30	2.27	2.20	2.39	2.36	4.55	4.54	300	302	208	210
35	.....	2.23	.....	2.40	.....	4.63	291	289	204	205
40	2.29	2.25	2.46	2.43	4.76	4.72	284	278	202	200
45	.....	2.27	.....	2.46	.....	4.80	278	269	198	196
50	2.31	2.28	2.49	2.48	4.87	4.88	270	262	195	193
55	.....	2.30	.....	2.50	.....	4.97	264	256	193	191
60	2.32	2.31	2.53	2.53	5.05	5.05	258	251	190	189

E, experimental values.
T, values predicted by mechanical models.

The mean behavior of specimens subjected to plunger loading was

$$J(t) = 0.00254 + 0.00167 (1-e^{-t/6.61}) + t/98{,}000 \quad [16]$$

Comparison of Eqs. 15 and 16 indicates that the difference between the response was the result of variation between methods of calculating compliances. The retardation times of 6.61 min for plunger loadings and 6.57 min for plate loading were approximately equal. The form of the viscoelastic decay is therefore the same for both types of loading, as can be seen from Fig. 7. In general the solutions for plunger loadings are believed to be more nearly correct than those for plate loadings, using the Hertz method of analysis. Additional experimentation is needed in order to determine the nature of the stress distribution within the convex body of the fruit. Until the distribution is fully understood, it is not possible to combine the Hertz solution and the Boussinesq solution for the fruits into one representative creep behavior.

Moduli for specimens subjected to axial relaxation studies using both plate and plunger loadings were fitted to degenerate Maxwell models having the behavior shown by Eq. 13. The representative behavior of specimens subjected to plunger loading is

$$E(t) = 104e^{-t/56.6} + 134 \quad [17]$$

The mean behavior of specimens subjected to plate loadings is

$$E(t) = 213e^{-t/52.5} + 200 \quad [18]$$

Comparison of Eqs. 17 and 18 indicates that the differences between the responses were the result of variation between the calculated moduli. The relaxation times of 56.6 min for plunger loadings and 52.5 min for plate loadings indicate that the viscoelastic decay function was of the same form for both types of loading as can be seen from Fig. 8. As for the creep tests, the stress distribution needs to be more fully understood before the comparative merits of the Hertz and Boussinesq solutions may be fully evaluated.

Both the Kelvin and Maxwell models used to represent the viscoelastic behavior in this study were simplified sufficiently that only smooth curves resulted. The relations

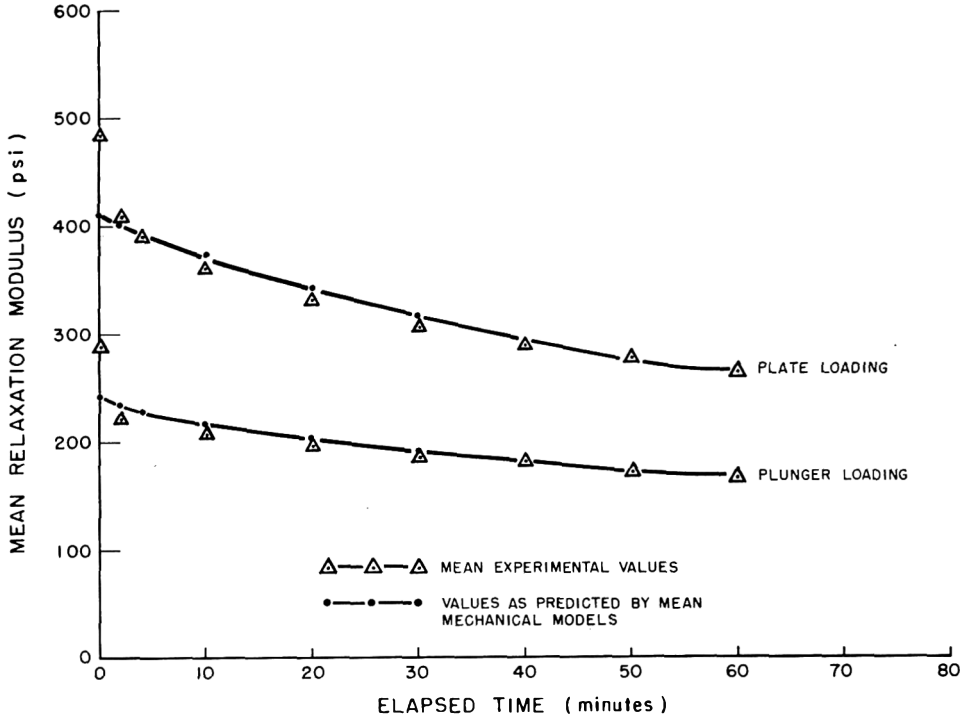


Fig. 8. Mean relaxation modulus versus time relations for McIntosh apples.

plotted in Figs. 7 and 8 represent only a mean behavior and are not indicative of any particular specimen. For any individual specimen, the three elements in the models yield only a trend curve and not necessarily the best-fitting one.

Interconversion of viscoelastic functions. Table 2 summarizes the methods for evaluating each of the viscoelastic parameters. In real viscoelastic materials it is possible to transfer from one viscoelastic function to another by using LaPlace Transforms, Heaviside Unit Functions, operator notation, or similar techniques if behavior has been represented by continuous models. Such conversions were not made in the investigation, for several reasons.

The quantity of data which was obtained for bulk creep, axial creep, and axial relaxation was insufficient to establish a complete spectrum of relaxation or retardation times for each of the tests. Many more replications would be needed at varying pressures, loads, and deformation levels. Within the regions studied, there was no apparent variation of viscoelastic parameters for different stress and strain levels, but the replications were insufficient to verify this observation completely. It must be remembered that all applied loads must be small enough not to exceed the bio-yield point as defined by Mohsenin *et al.* (1963).

Another obstacle to inter-conversion of creep and relaxation parameters results from the manner in which the relaxation parameters were obtained. The use of a testing machine required a finite time for initial straining of the specimens. During this loading period, it is probable that the specimens were actually undergoing viscoelastic changes. The elastic relaxation modulus, E_0, in such a situation will not correspond to the inverse of the elastic creep compliance which was obtained by instantaneous application of load.

From the results obtained in studying the McIntosh apple fruits, the following conclusions appear to be justified:

1) McIntosh apple fruits can be considered as viscoelastic bodies with an approximately linear viscoelastic behavior.

2) The viscoelastic behavior of the fruits can be adequately approximated by three-element degenerate Maxwell and Kelvin mechanical models.

3) Relaxation and retardation times are equal for fruits subjected to either plate or plunger uniaxial compression.

4) It is justifiable to consider an apple fruit as a semi-infinite body when it is loaded by means of a 0.025-inch-diameter rigid plunger.

5) Hertz and Boussinesq solutions do not yield completely compatible results for fruits subjected to uniaxial compression.

6) Relaxation studies in apple fruits must utilize a very fast rate of load application in order to obtain a true representation of the relaxation behavior.

7) Hydrostatic compression is a very convenient and useful method of analyzing

Table 2. Summary of methods for analysis of viscoelastic parameters of McIntosh apples.

	Hydrostatic compression	Uniaxial compression			
		Plunger loading		Plate loading	
	Creep	Creep	Relaxation	Creep	Relaxation
Viscoelastic parameter	$B\ (t)$	$J\ (t)$	$E\ (t)$	$J\ (t)$	$E\ (t)$
Model representation	Degenerate Kelvin	Degenerate Kelvin	Degenerate Maxwell	Degenerate Kelvin	Degenerate Maxwell
Method for experimental values	$\frac{-\Delta V}{p\,V_0}$	$\frac{2\,ad}{F\,(1-\mu^2)}$	$3.45\,\frac{F}{d}$	$\sqrt[2]{\frac{1.207\,D\cdot d}{F^2}}$	$\sqrt[2]{\frac{0.824\,F^2}{D\cdot d^3}}$
Method for theoretical values	$B_0+(1-e^{-t/\lambda b})\,B_1$	$J_0+(1-e^{-t/\lambda})\,J_1+t/\eta_v$	$E_1e^{-t/\tau}+E_e$	$J_0+(1-e^{-t/\lambda})\,J_1+t/\eta_v$	$E_1e^{-t/\tau}+E_e$

viscoelastic behavior of certain agricultural products.

LIST OF SYMBOLS

$K(t)$ = instantaneous bulk modulus in psi
p = pressure in psi
$\Delta V/V_0$ = bulk strain in in.^{3}/in.^{3}
$B(t)$ = instantaneous bulk compliance in psi^{-1}
F = compressive force in lbs
a = plunger radius in inches
r = distance from the center of the area over which the plunger is acting in inches
d = axial deformation in inches
μ = Poisson's ratio
E = uniaxial modulus in psi
J = uniaxial compliance in psi^{-1}
z = vertical coordinate below specimen surface in inches
σ_z = normal stress in psi
σ_r = radial normal stress in psi
σ_θ = transverse normal stress in psi
k = constant determined from elliptic integral tables
R = major radius of curvature in inches
R' = minor radius of curvature in inches
D = mean product diameter in inches
K_0 = elastic bulk modulus in psi
E_0 = elastic uniaxial modulus in psi
S = deviatoric stress in psi
$\bar{e}$ = deviatoric strain in in./in.
$H(t)$ = instantaneous shear compliance in psi^{-1}
H_0 = $1/G_0$ = initial shear compliance in psi^{-1}
H = $1/G$ = decay shear compliance in psi^{-1}
α = η/G = retardation time in shear in min
t = time after load application in min
η_v = Kelvin viscosity coefficient in psi-min
η = Newtonian viscosity coefficient in psi-min
e = 2.718 = Naperian logarithm base
G = decay shear modulus in psi
G_0 = elastic shear modulus in psi
$G(t)$ = instantaneous shear modulus in psi
G_e = equilibrium shear modulus in psi
β = η/G = relaxation time in shear in min
$E(t)$ = instantaneous uniaxial modulus in psi
$J(t)$ = instantaneous uniaxial compliance in psi

REFERENCES

Boussinesq, J. 1885. Applications des potentiels a l'étude de l'équilibre et du mouvement des solides élastiques. Paris, France.

Ferry, J. D. 1961. Viscoelastic Properties of Polymers. John Wiley and Sons, New York.

Finney, E. E. 1963. The Viscoelastic Behavior of the Potato, Solanum Tuberosum Under Quasi-Static Loading. Unpublished Ph.D. thesis, Department of Agricultural Engineering, Michigan State University.

Finney, E. E., C. W. Hall, and G. E. Mase. 1964 Theory of linear viscoelasticity applied to the potato. *J. Agri. Eng. Research* **9**(4), 307.

Halyk, R. M., and L. W. Hurlburt. 1964. Tensile and shear strength characteristics of alfalfa stems. ASAE Paper No. 64-816. American Society of Agricultural Engineers, St. Joseph, Michigan.

Hertz, Heinrich. 1896. Miscellaneous papers, Macmillan Co., New York.

Kozma, A., and H. Cunningham. 1962. Tables for calculating the compressive surface stresses and deflections in the contact of two solid elastic bodies whose principal planes of curvature do not coincide. *J. Ind. Math.* **12**(1), 31.

Mohsenin, N. N. 1963. A testing machine for determination of mechanical and rheological properties of agricultural products. *Penna. State Agr. Expt. Sta. Bull. Number* **701**.

Mohsenin, N. N. 1965. Physical properties of agricultural products. *Transactions of the ASAE* **8**(1), 25.

Mohsenin, N. N., H. E. Cooper, and L. D. Tukey. 1963. An engineering approach to evaluating textural factors in fruits and vegetables. *Transactions of the ASAE* **6**(2), 85.

Morrow, C. T. 1965. Viscoelasticity in a Selected Agricultural Product. Unpublished M.S. Thesis. Department of Agricultural Engineering, The Pennsylvania State University.

Reiner, M. 1960. Deformation, Strain, and Flow. Lewis and Company, London.

Sharma, M. G. 1965. Testing of Polymers. J. V. Schmitz, ed. Interscience Publishers, London.

Shopolyanskaya, A. L. 1952. Structural and mechanical properties of the wheat grain. *Colloid J. (USSR).* **14**(1), 137. English translation by Consultants Bureau, New York.

Suggs, C. W., and W. E. Splinter. 1965. Mechanical properties of tobacco stalks. *Transactions of the ASAE* **8**(3), 361.

Theocaris, P. S. 1964. Creep and relaxation contraction ratio of linear viscoelastic materials. *J. Mech. and Phys. Solids* **12**, 125.

Timbers, G. E. 1964. Some Mechanical and Rheological Properties of the Netted Gem Potato. M.S.A. thesis. Department of Agricultural Engineering, University of British Columbia.

Timbers, G. E., L. M. Staley, and E. L. Watson. 1965. Determining modulus of elasticity in agricultural products by loaded plungers. *Agr. Eng.* **46**(5), 274.

Timoshenko, S., and J. N. Goodier. 1951. Theory of Elasticity. McGraw-Hill, New York.

White, R. K. 1966. Swelling Stress in the Corn Kernel as Influenced by Moisture Sorption. M.S. thesis. Department of Agricultural Engineering, The Pennsylvania State University.

Zoerb, G. C., and C. W. Hall. 1960. Some mechanical and rheological properties of grains. *J. Agr. Eng. Research* **5**(1), 83.

Ms. rec'd 12/27/65.

Approved as Journal Paper No. 3099 of the Pennsylvania Agricultural Experiment Station, University Park. Authorized for publication February, 1966.

This work was supported by National Science Foundation Grant GK 15 to the Agricultural Engineering Department of The Pennsylvania State University.

F. SHAMA and P. SHERMAN
Unilever Research Laboratory, Welwyn, Hertfordshire, England

The Texture of Ice Cream
2. Rheological Properties of Frozen Ice Cream

SUMMARY

The creep behavior of frozen ice cream can be represented satisfactorily by a six-element model. The parameters involved are the instantaneous elasticity (E_0), two elastic moduli (E_1 and E_2), and two viscosity components (η_1 and η_2) associated with retarded elasticity, and a Newtonian viscosity (η_N). From the effect of fat, overrun, and temperature on the magnitude of these parameters it is suggested that E_0 is affected primarily by the ice crystals, E_1 and η_2 by the weak stabilizer-gel network, E_2 by protein-enveloped air cells, η_1 by the fat crystals, and η_N by both fat and ice crystals. When ice cream mix is whipped and frozen, the fat globules undergo some coagulation from rupture of the protective protein-emulsifier layer around the globules. Coagulation is hindered by the simultaneous conversion of water to ice.

INTRODUCTION

In Part 1 of this series the structure of frozen ice cream was discussed in terms of a solid foam in which a network of coagulated fat plays an important part (Sherman, 1965). Fat globules coagulate when ice cream mix is frozen at low temperature. Agitation and whipping distribute the solidified fat particles throughout the liquid phase between the air cells and ice crystals. The fat globules are held together by strong London–van der Waals' attraction forces.

Part 2 provides additional evidence in support of this theory. It describes structural changes occurring when ice cream mix is frozen, and also the rheological assessment of frozen ice cream texture. Rheological examinations of ice cream mix and thawed ice cream are described in Part 3.

EXPERIMENTAL

Ice cream mix recipes. The standard 10% vegetable-fat mix had the same composition as before (Sherman, 1965). Mixes containing 0–8% fat were prepared by reducing the fat content to the desired level and either increasing the water content accordingly, or increasing the total proportion of water phase (i.e. including milk, sugar, vegetable gum). The rheological properties of frozen ice cream prepared from mixes of a given fat content blended in either of these ways were not significantly different.

The influence of overrun levels of 2, 5 and 110% on the properties of frozen ice cream containing 10% fat was investigated.

Also investigated was the influence of small temperature variations within the range −11 to −15°C on ice cream containing 10% fat and 110% overrun.

All mixes were pasteurized 15 sec by the HTST process at 79.5°C, homogenized at 2,500 psi, and cooled to 4.5°C.

Overrun determination. Overrun content was checked quantitatively in the following way. A small sample of frozen ice cream was introduced into a flask containing weakly alkaline water, and the flask was then attached to an inverted micro-burette. The neck of the flask and the upper end of the micro-burette were fitted with ground-glass joints of opposing types so that one fitted into the other. Below the ground-glass joint the flask had a narrow side-arm which was connected by rubber tubing to a wider glass reservoir of alkaline water. The whole apparatus was filled with alkaline water and inserted in a water bath. When the temperature of the bath was slowly raised the sample melted, and the released air collected at the upper end of the micro-burette. The volume of air was corrected to NTP.

Structural changes during the freezing of ice cream mix and model O/W emulsions. Ice cream mixes, and liquid paraffin-in-water emulsions stabilized with 2.5–10.0% spray-dried milk powder, were frozen on a Reichert-Köfler micro-cold stage supported on a microscope stage. The temperature was reduced slowly from 4.5° C to about −7°C.

Rheology of frozen ice cream. Creep behavior under shearing stress was studied at various temperatures (−7 to −15°C) in an insulated cabinet using an improved version of the parallel-plate viscoelastometer originally designed for studying the viscoelastic behavior of polymers (Holde and Williams, 1953). The present design differs from the original modification (Shaw, 1963) in the following points. The teeth on all parts of equipment in contact with the test sample were reduced in size to 1/16 in. and increased in number; the original pulley carrying the cord to which the weight is attached was replaced by a system of ball-bearing pulleys so as to eliminate continued lubrication when the instrument is at a low temperature, and a device was incorporated to ensure that the sample was compressed 1/64 in. when the frame

was tightened. The ice cream samples were clamped between the heavy plates of the frame and left overnight (18 hr) at the appropriate temperature before testing. Low cabinet temperatures were achieved by blowing air cooled by CO_2 through it with a refrigerator unit.

RESULTS AND DISCUSSION

Changes in emulsion structure on freezing. When ice cream mix, or liquid paraffin-in-water emulsions containing milk powder, are frozen there is no marked change in the size, or size distribution, of the globules. If the emulsion is remelted, the globules move together and coalesce.

The protective protein-emulsifier layer around the globules ruptures during freezing, but coagulation in the absence of agitation is prevented by the simultaneous development of an ice crystal barrier. When the ice cream melts, coagulation is no longer hindered, and the globules increase in size but decrease in their number per unit volume. This latter process, which will be much more pronounced when freezing is accompanied by agitation, should influence subjective assessment of texture on the palate.

If the freezing process is followed microscopically through an oil-immersion lens the viscosity of the immersion oil deposited on the coverslip increases as the temperature falls. By refocusing now at a slightly lower level, the gentle pressure applied to the emulsion is sufficient to rupture the protein-emulsifier films around some of the globules. Adjacent globules link up through narrow "necks" of oil or fat, and sometimes several connections of this type may develop within a cluster of globules.

With vigorous conditions prevailing in a freezer unit, "neck" formation and rupture will be accelerated, leading to more pronounced coagulation or churning.

Rheology of frozen ice cream. All samples showed viscoelastic behavior, i.e. they behaved in part like solids and in part like fluids "in the sense that work of shearing deformation is not completely conserved, as in solids, nor is it completely dissipated as in fluids" (Fredrickson, 1964). Within the range of shearing stresses up to 4000 dynes/cm^2 the strain was linearly related to stress.

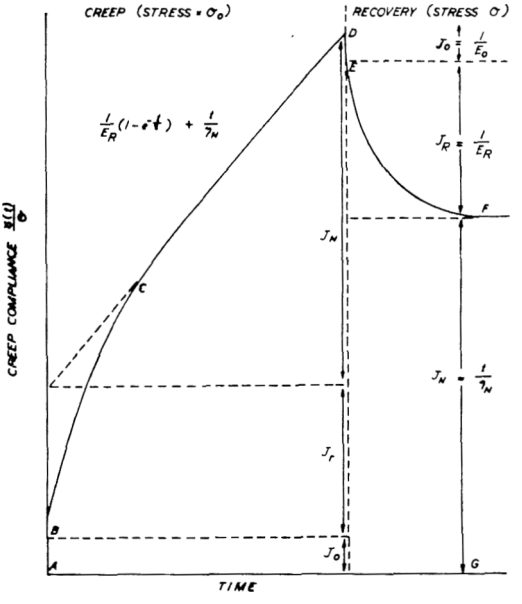


Fig. 1. Model creep curve.

The rheological parameters were calculated by the method described in Appendix 1, Part 3. Fig. 2 shows a typical creep-compliance time plot, and the way in which this is treated will now be described in detail.

thickness of each sample (h) = 2.50 cm.
total weight applied (m) = weight of pan + load = 250.80 g
so that $m \times g$ = 250.80 × 981 dynes
area (A) of ribbed plate placed between 2 samples = 31.82 cm^2

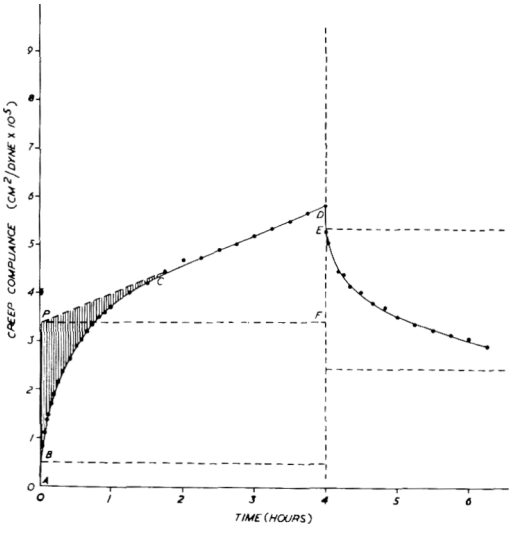


Fig. 2. Typical creep curve for 10% fat ice cream.

reciprocal magnification factor (f) of microscope = 0.57

so that, in accordance with eq. c of Appendix 1

$$J(t) = \frac{\beta \times 0.57 \times 2 \times 31.82}{2.50 \times 250.80 \times 981} = \beta \times 5.91 \times 10^{-5} \text{ cm}^2/\text{dyne}$$

where β is the displacement of the ribbed plate.

From Fig. 2

$$J_0(\text{AB}) = \frac{1}{E_0} = 0.5 \times 10^{-5} \text{cm}^2/\text{dyne}$$

so that $E_0 = 2.0 \times 10^5$ dynes/cm^2

Analysis of retarded elastic compliance is carried out by Inokuchi's procedure (eq. g, Appendix 1). Table 1 gives details of Q for different time intervals (t). When this data is plotted in the form ln Q against t a straight line is obtained which curves upwards at values of t below about 10 (Fig. 3). Extrapolating the linear part of the graph back to the ordinate axis gives J_1, since $Q = \sum_{i=1}^{n} J_i e^{-t/\tau_i}$ also, and in this case

$\ln Q = \ln J_1 - t/\tau_i \cdot J_1 = 2.19 \times 10^{-5}$ cm^2/dyne, and from the gradient of the straight line $\tau_1 = 31.1$ min, and $\eta_1 = 8.50 \times 10^7$ poise.

Since these data do not adequately reproduce the whole graph a second plot is necessary. In this case ln $[Q - J_1 e^{-t/\tau_1}]$ is plotted against t (Table 1). A straight line results (Fig. 4) so that no further plots are required. The intercept on the ordinate axis yields $J_2 = 8.91 \times 10^{-6}$ cm^2/dyne, and the gradient of the graph leads to $\tau_2 = 2.4$ min, so $\eta_2 = 1.60 \times 10^7$ poise.

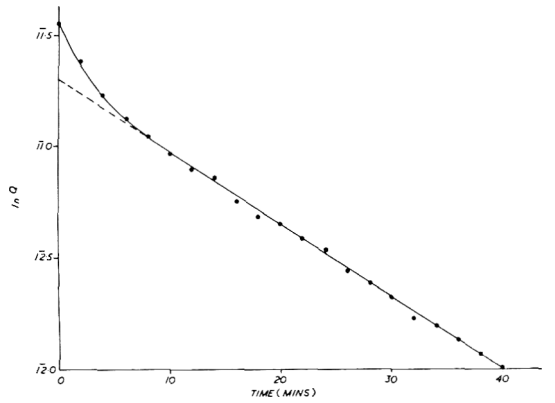


Fig. 3. Plot of ln Q v. time.

Table 1. Analysis of retarded elastic compliance by Inokuchi's method.

(a) ln Q — t		
t (min)	Q (cm^2/dyne × 10^5)	ln Q
0	2.90	11.5518
2	2.45	11.3832
4	2.12	11.2385
6	1.90	11.1290
8	1.75	11.0464
10	1.60	12.9570
12	1.50	12.8925
14	1.45	12.8586
16	1.30	12.7495
18	1.22	12.6859
20	1.18	12.6526
22	1.10	12.5824
24	1.05	12.5359
26	0.95	12.4358
28	0.90	12.3817
30	0.85	12.3246
32	0.80	12.2639
34	0.75	12.1994
36	0.70	12.1304
38	0.65	12.0563
40	0.62	12.0090

When a less detailed analysis of the retarded elastic compliance suffices eq. l, m, and n in Appendix 1 are used. Table 2 shows the relevant data derived from Fig. 2.

From Fig. 2

$$\frac{1}{\eta_N} = \frac{\text{DF}}{\text{PF}} = \frac{2.40 \times 10^{-5}}{4 \times (60)^2} = 1.67 \times 10^{-9}$$

so $\eta_N = 6.0 \times 10^8$ poise

Inserting the calculated values of J_0 and

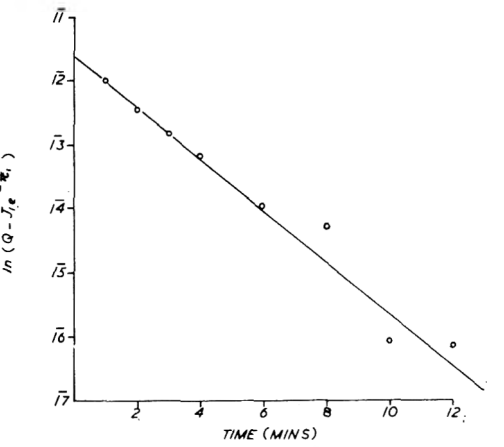


Fig. 4. Plot of ln $Q - J_1 e^{-t/\tau_1}$ v. time.

Table 2. Analysis of retarded elastic compliance by Inokuchi's method.
(5) $\ln (Q - J_1 e^{-t/\tau_1}) - t$

t (mins)	t/τ_1	$J e^{-t/\tau_1}$ (cm^2/dyne $\times 10^5$)	Q (cm^2/dyne $\times 10^5$)	$Q - J e^{-t/\tau_1}$ (cm^2/dyne $\times 10^5$)
1	0.0321	2.1252	2.7500	0.6248
2	0.0642	2.0623	2.4500	0.3877
3	0.0963	1.9815	2.2500	0.2685
4	0.1284	1.9230	2.1200	0.1970
6	0.1926	1.8109	1.9000	0.0881
8	0.2568	1.6885	1.7500	0.0615
10	0.3210	1.5901	1.6000	0.0099
12	0.3852	1.4903	1.5000	0.0097

η_N in eq. 1 leads to $E_a = \dfrac{1}{J_a} = 3.5 \times 10^4$ dynes /cm^2

$\tau = 25.8$ min, and $\eta_a = 5.34 \times 10^7$ poise

The parameter J_a is derived by calculation rather than by measuring PB (Fig. 2) because of the difficulty, at the time, of defining AB accurately. This difficulty has now been eliminated by using a transducer and transducer meter.

The curves for creep compliance $J(t)$ versus time t at $-11°C$ for ice creams containing 0–10% fat, and prepared with 110% overrun, are shown in Fig. 6. If $J(t)$ is plotted against log t, a single plateau is derived corresponding to a compliance of $\sim 10^{-6}$ cm^2/dyne (Fig. 7). The curve then rises steeply. When the elastic part of the creep compliance $J(t) - t/\eta_N$ is plotted against log t, two plateaus are observed (Fig. 8). The first, at low values of t, corresponds to a retarded elastic process with a compliance $\sim 10^{-6}$ cm^2/dyne, whereas the second elastic process has a compliance of $\sim 10^{-5}$ cm^2/dyne at high values of t. Both compliances increase slightly as fat content increases (Table 1). With the present experimental technique it was not possible to derive an accurate value of $J(t) - t/\eta_N$ for $t \to 0$, i.e. for the instantaneous elastic compliance J_0.

The retarded elasticity region of creep curves can be analyzed in greater detail by the graphical procedure of Inokuchi (1955)

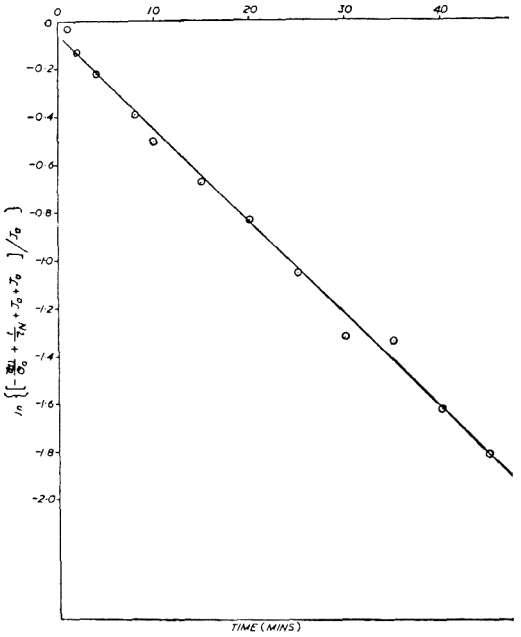


Fig. 5. Plot of $\ln \left[\dfrac{-J(t) + J_0 + J_a + t/\eta_N}{J_a}\right]$ v. time.

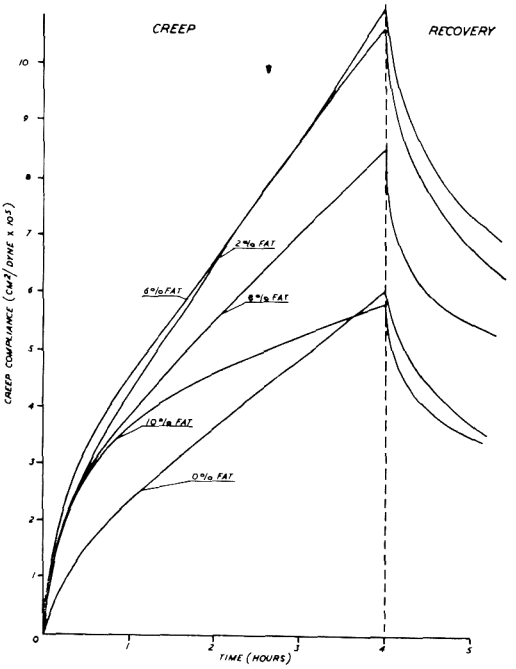


Fig. 6. Creep compliance curves for ice creams with different fat contents.

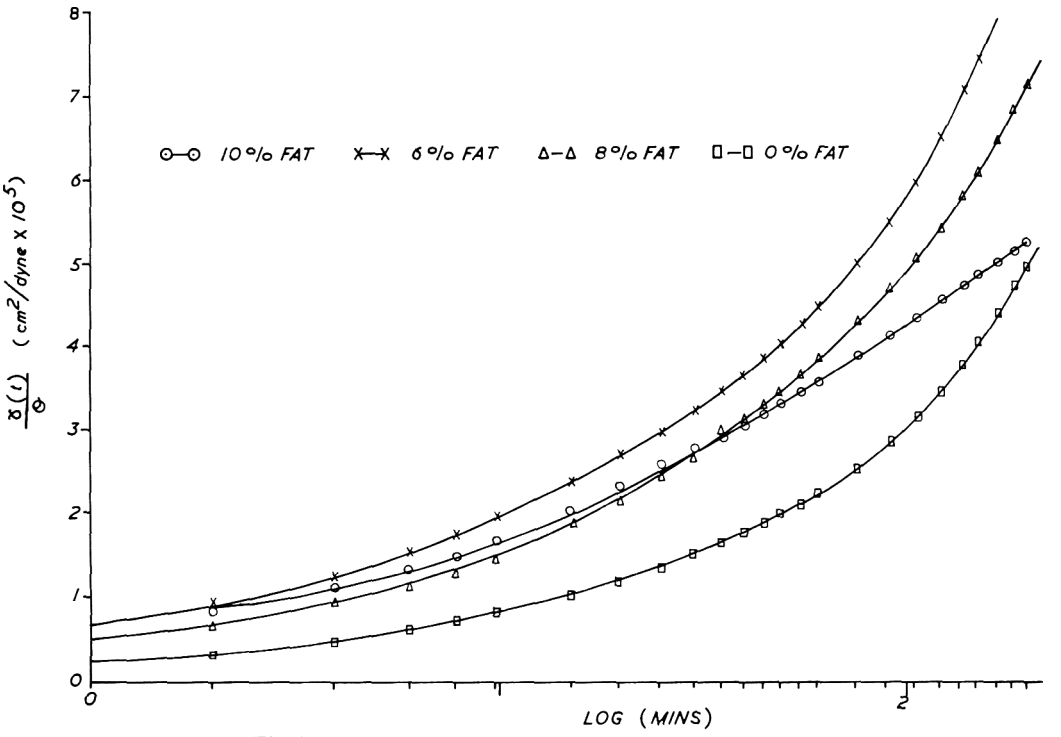


Fig. 7. Influence of time on creep compliance at −11°C.

as described in Appendix 1 of Part 3. Detailed analyses by this method indicate that only two retardation times are involved. The values of τ_3, τ_4, etc. were far too small to be of significance. Creep compliance can be defined therefore by

$$J(t) = J_0 + J_1 (1 - e^{-t/\tau_1}) + J_2 (1 - e^{-t/\tau_2}) + \frac{t}{\eta_N} \qquad [2]$$

Theoretical curves derived using eq. 2, and introducing the calculated values of all relevant parameters, showed excellent agreement with the experimental creep-compliance time curves (examples given in Fig. 6).

The influence of fat content, overrun, and temperature on E_1, E_2, η_1, η_2, η_N, τ_1, τ_2, are shown respectively in Figs. 9, 10 and 11. With increasing fat content, E_1, E_2, and η_2 do not change, whereas η_1 increases. When the fat content exceeds 7–8%, η_N shows a

Table 3. General analysis of creep-compliance time curve.

t (mins)	$\frac{J(t)}{J_a}$	$\frac{J_o + J_a}{J_a}$	$\frac{t}{\eta_N J_a}$	$\left[\frac{-J(t) + J_a + J_o + t/\eta_N}{J_a}\right]$	$\ln\left[\frac{-J(t) + J_a + J_o + t/\eta_N}{J_a}\right]$
1	0.203	1.172	0.0034	0.971	−0.029
2	0.301	"	0.0078	0.879	−0.129
4	0.387	"	0.0136	0.799	−0.224
8	0.519	"	0.0272	0.680	−0.386
10	0.601	"	0.034	0.605	−0.502
15	0.713	"	0.051	0.510	−0.673
20	0.815	"	0.078	0.435	−0.832
25	0.907	"	0.085	0.350	−1.050
30	1.008	"	0.102	0.266	−1.324
35	1.029	"	0.119	0.262	−1.339
40	1.110	"	0.136	0.198	−1.619
45	1.162	"	0.153	0.163	−1.814
50	1.216	"	0.170	0.126	−2.071

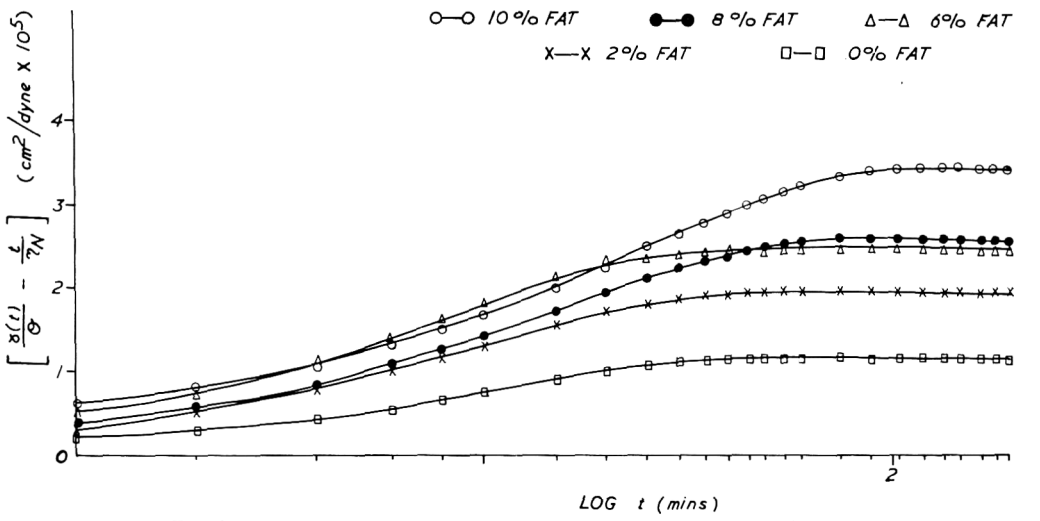


Fig. 8. Influence of time on the elastic component of creep compliance.

large increase. On the other hand, when the overrun is raised from 2% to 110%, η_N decreases slightly whereas E_1, E_2, η_1, and η_2 all increase. Lowering the temperature from -11 to -15°C produces little change in E_1 and η_N, but E_2, η_1, and η_2 increase quite appreciably. The low values of E_1 and E_2 suggest that bonds broken during shear are relatively weak bonds.

Comparison of creep data for frozen ice cream and ice. The values of E_1, E_2, η_1, η_2, and η_N are several orders of magnitude lower for ice cream than for pure ice. For ice at -10°C, $E_0 \sim 10^{11}$ dynes /cm^2, $E_1 \sim 10^{11}$ dynes/cm^2, $\eta_1 \sim 10^{13}$ poise, and $\eta_N \sim 10^{14}$ poise (Jellinek and Brill, 1956). If we regard frozen ice cream as an ice-crystal network which has been modified primarily by introducing fat and air, then air content causes by far the greater change in the magnitude of the ice crystal parameters. At -11°C, $\sim 75\%$ of the water in ice cream is frozen, so that this degree of supercooling cannot itself explain the difference. When ice cream contains no fat its parameters are several orders of magnitude lower than for ice irrespective of overrun content, and the introduction of up to 10% fat produces a rise in the former values of less than one order of magnitude. With increasing overrun the air cells, ice crystals, and fat cells become smaller (Sherman, unpublished data). Their number per unit volume increases as result of this change, and more bonds are established, so that the structure becomes stronger. This is reflected in the rise in E_1, E_2, η_1, and η_2 (Fig. 10). The apparent lack of change in η_N with increasing overrun is more difficult to explain.

Table 4. Influence of fat content on creep compliance of frozen ice cream (110% overrun) at -11°C.

% fat content	creep compliance $J(t) - t/\eta_N$ (cm^2/dyne)	
	low values of t	$t \longrightarrow \infty$
10	5.8×10^{-6}	3.4×10^{-5}
8	3.6×10^{-6}	2.6×10^{-5}
6	5.0×10^{-6}	2.4×10^{-5}
2	2.6×10^{-6}	1.9×10^{-5}
0	2.1×10^{-6}	1.1×10^{-5}

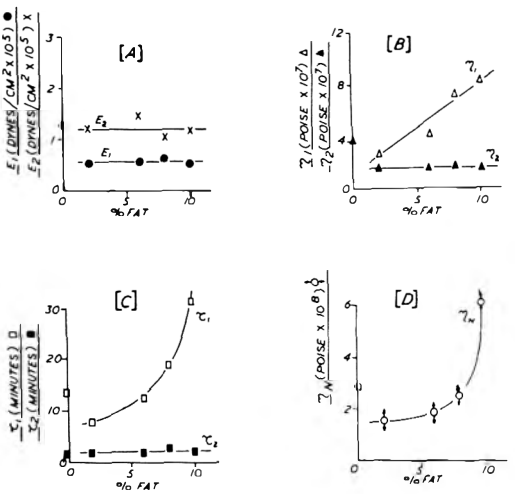


Fig. 9. Variation of the viscoelastic parameters of ice cream with fat content.

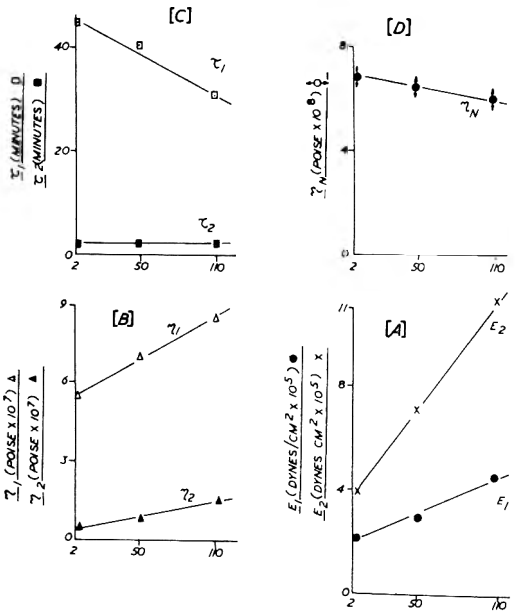


Fig. 10. Variation of viscoelastic parameters of ice cream with overrun.

The viscous component of creep can be represented by an equation of the form

$$\eta_N = Ge^{E/RT} \qquad [3]$$

where G is a constant, E is the activation energy for plastic flow, R is the gas constant, and T is temperature according to the absolute scale. Over the temperature range −11 to −15°C, where the transformation rate of water to ice is low, $E = 5{,}200$ cals/mole for a 10% fat ice cream with 110% overrun. The corresponding value for pure ice is ~ 16,100 cals/mole (Jellinek and Brill, 1956).

A mechanical model can be derived for eq. 1 using the convention described in Appendix 2, Part 3. It can be represented (Fig. 12) by a spring in series with a dashpot (Maxwell body), and two units each comprising a spring in parallel with a dashpot (Kelvin-Voigt body). By examining the relative effect of fat, overrun, and temperature on the rheological parameters associated with this model, one can deduce which components of ice cream structure are likely to be the principal factors influencing these parameters.

The present investigation supports the view (Sherman, 1965) that frozen ice cream is essentially an aerated ice crystal structure which is modified in textural properties by a superimposed fat network. The response of this structure when sheared depends on the strength of the lamellae between the irregular air cells. Within the protein coated lamellae are the ice crystals, and a fat crystal network is enmeshed in the unfrozen material, viz, a concentrated sugar solution-stabilizer gel.

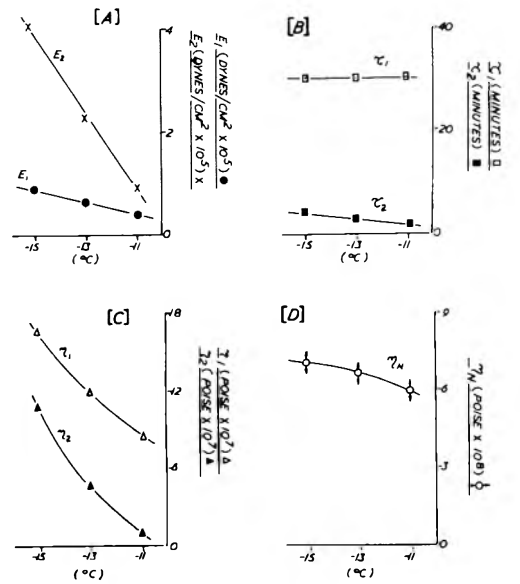


Fig. 11. Influence of temperatures on the viscoelastic parameters of ice cream.

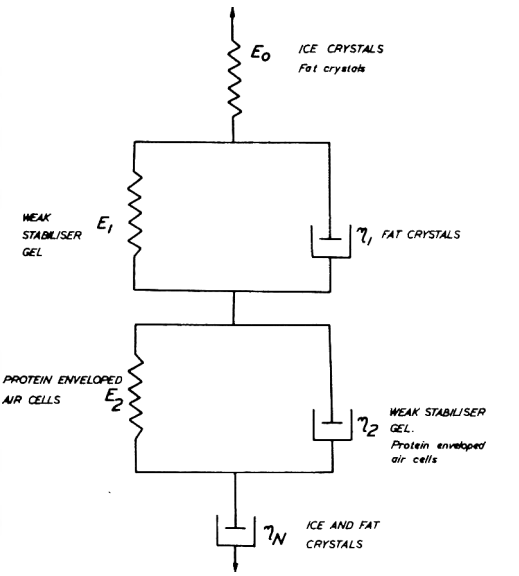


Fig. 12. Six element model for frozen ice cream showing rheological associations with structural components.

In general, there are few points of contact between ic crystals, the intervening distance being several microns, so there is little possibility of bonds forming between them. The main interactions must be between fat crystals situated in the spaces between the ice crystals and around the air cells, and possibly between ice crystals and fat crystals in their immediate vicinity. The moduli E_1 and E_2 are probably associated with the gel structure and the air cell-protein interface. E_2 is very much more influenced by overrun than E_1, so E_1 may be associated with the retarded elastic properties of the stabilizer gel whereas E_2 would be associated with the air cell-protein interface.

The viscosity η_1 is influenced to a greater extent by fat content than by overrun. This parameter is associated with the onset of flow following bond rupture in the fat crystal network. The value of η_2 is affected by overrun but not by fat content, thus suggesting a viscous damping effect which arises from the foam structure and surrounding gel. Since the increase in η_2 with increasing overrun is not very large, the gel structure may be the main contributory factor.

The effect exerted by increasing fat content on η_N arises from increased interaction between fat crystals during viscous flow. Fat crystal size is very much smaller than ice crystal size—less than 5 μ as opposed to an average size of 20–40 μ.

E_0 decreases when either fat content or overrun is increased, fat content exerting the much greater effect. The ice-crystal contribution to E_0 must be of primary importance therefore, since the ice crystal content decreases as fat content increases.

The components of ice cream structure which appear to exert major and minor effects on the six rheological parameters are shown by large and small type, respectively, in Fig. 12. If this representation is substantially correct, creep tests on ice cream of "good" and "poor" texture should indicate which parameters, and their related structural components, contribute most to texture.

In an ice-crystal lattice, instantaneous elastic deformation when sheared is attributed to distortion of the lattice structure (Jellinek and Brill, 1956). With increasing strain, flow occurs within the individual crystallites, giving rise to a retarded elastic response. At still higher strain, flow between grain boundaries is responsible for the linear Newtonian viscosity region of the creep-compliance curve. The strain per unit area developed in the ice cream samples was within the range 10^{-2} to 10^{-1}. This is much greater than the strain observed when flow occurs within ice crystallites. On the other hand, the energy of activation E, for viscous flow between grain boundaries has been shown to be much greater than E for viscous flow in ice cream at the same temperature. It is possible that the shear developed in the present tests causes flow within the ice crystallites; it is unlikely that viscous flow occurs between grain boundaries.

REFERENCES

Fredrickson, A. G. 1964. "Principles and Applications of Rheology." Prentice-Hall, New York.

Holde, E. K. van, and J. W. Williams. 1953. Study of the viscoelastic behavior and molecular weight distribution of polyisobutylene. *J. Polymer Sci.* **11**, 243.

Inokuchi, K. 1955. Rheology of surface films. IV. Viscoelastic properties of 6-nylon films at air/water interface. *Bull. Chem. Soc. (Japan)* **28**, 453.

Jellinek, H. H. G., and R. Brill. 1956. Viscoelastic properties of ice. *J. Appl. Phys.* **27**, 1198.

Shaw, D. J. 1963. The physical structure of ice cream. *In*: "Rheology of Emulsions." (P. Sherman, ed.) Pergamon Press, London.

Sherman, P. 1965. The texture of ice cream. *J. Food Sci.* **30**, 201.

Ms. rec'd 5/13/65.

The authors are indebted to Unilever Ltd. for permission to publish this paper. The project was initiated in the laboratories of Messrs. T. Wall and Sons (Ice Cream) Ltd., to whom due acknowledgement is made for the facilities provided.

P. SHERMAN

Unilever Research Laboratory, Welwyn, Hertfordshire, England

The Texture of Ice Cream
3. Rheological Properties of Mix and Melted Ice Cream

SUMMARY

Fat coagulation begins when ice cream mix is frozen and whipped. The effect is hindered by the simultaneous conversion of water to ice. When frozen ice cream thaws, the ice crystals disappear so that coagulation is able to continue. It leaves a melt with a weak structure which differs from the fat network present in the original mix. This is substantiated by the comparative creep behavior of mix and melt at low rates of shear. The creep behavior of melted ice cream is represented by a mechanical model containing 4 elements: instantaneous elastic compliance, J_0; a retarded elastic compliance, J_1, comprising an elastic modulus, E_1 and a viscosity, η_1; and a Newtonian compliance, t/η_N, where t is time and η_N is the Newtonian viscosity. Ice cream mix requires 6 elements since an additional elastic modulus, E_2, and a second viscosity, η_2, are associated with retarded elasticity. These additional elements are attributed to globules of 0.5 μ or less, which are separated by only a few Angstroms after flocculation rather than the 50 Å or more separating larger globules. Structure recovery in mix after it has been subjected to high shear indicates pronounced flocculation in the static emulsion. Weak forces of attraction hold the globules together within the flocculate.

INTRODUCTION

Part 2 of this series (Shama and Sherman, 1966) showed that fat globules coagulated to a limited extent during the whipping and freezing of ice cream mix. The restriction is imposed by simultaneous conversion of water to ice crystals. Coagulation results from the rupture of the protective protein-emulsifier membrane around the fat globules.

If frozen ice cream is allowed to melt at room temperature, or on the palate, the ice crystals melt and fat coagulation can now continue. The subjective assessment of ice cream on the palate is presumably related to the degree of fat coagulation in a melt of this kind.

The rheological properties of melted ice cream have been examined over a wide range of shear conditions, and compared with the rheological properties of unfrozen mix.

EXPERIMENTAL

Ice Cream mix recipe

		% composition (W/W)
Oil phase	Vegetable fat	10.0
	Commercial-grade glyceryl monostearate	0.53
Water phase	Water	60.6
	Milk	13.1
	Sugar	15.6
	Vegetable gum	0.18

The mix was pasteurized 15 sec by the HTST process at 175°F, homogenized at 2,500 psi, and cooled to 4.5°C.

Influence of temperature on mix viscosity. Viscosities were measured with a Haake Rotovisko coaxial-cylinder viscometer over a wide range of shear rates (14.1–1142 sec^{-1}) and temperatures (1.7–12.8°C).

Stress development in mix and melted ice cream at constant rate of shear. The change in shearing stress with time at −5.5°C as a function of constant low rate of shear was studied with the Rotovisko at 0.133 sec^{-1} and 1.197 sec^{-1}. The frozen ice cream was allowed to melt at 20–21°C for a few hours before cooling to the test temperature.

Structure recovery in mix after subjecting to high rate of shear. The rate of viscosity recovery at 4.5°C was followed in the Rotovisko after subjecting the mix to one of two high rates of shear. The shear conditions employed were:

1) 5.44 sec^{-1} after shearing for 5 min at 440.6 sec^{-1}
2) 7.05 sec^{-1} after shearing for 5 min at 1142.1 sec^{-1}
3) 14.1 sec^{-1} after shearing for 5 min at 1142.1 sec^{-1}

Creep compliance of ice cream mix and melted ice cream at very low rate of shear. A specially designed coaxial-cylinder viscometer (Fig. 1) was used at 20.0±0.1°C. It operated at much lower rates of shear than the lower limit of the Rotovisko. The cylinders were roughened to prevent slippage. The radius of the inner cylinder was 1.80 cm; the radius of the outer cylinder was 2.20 cm, thus providing a gap width of 0.40 cm. For creep measurements the outer cylinder was kept stationary and the inner cylinder was rotated slowly by a pulley-and-

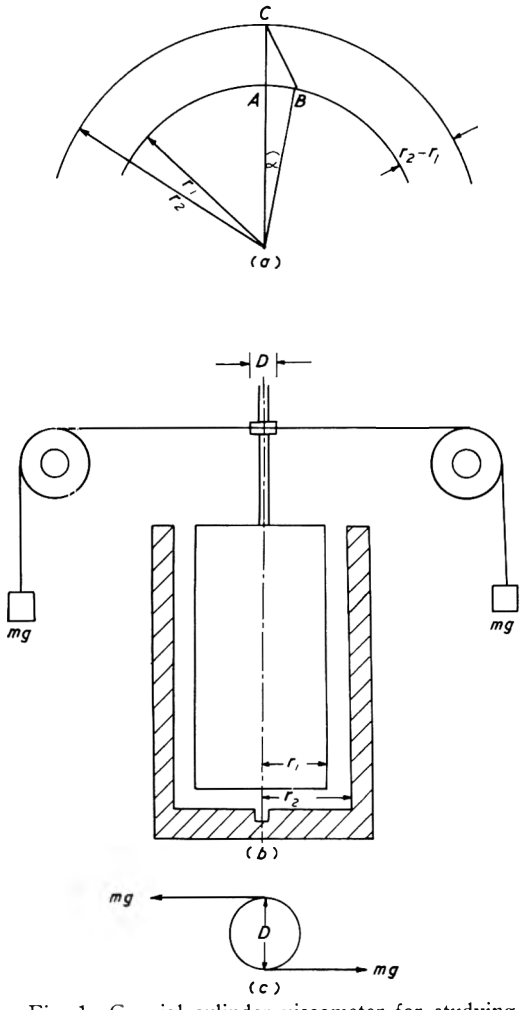


Fig. 1. Coaxial cylinder viscometer for studying the creep compliance of ice cream emulsions.

weight mechanism. The displacement was measured optically. Shear strain (ψ) was calculated from the angular rotation (ω)

$$\psi = \frac{r_1\omega}{r_2 - r_1} \qquad [1]$$

where r_1 and r_2 are the radii of the inner and outer cylinders respectively. The shear stress (S) is given by

$$S = \frac{mgD}{\pi (r_1 + r_2) l} \qquad [2]$$

where D is the pulley diameter, and l is the length of inner cylinder immersed in the test sample. Creep compliance was calculated as shown in Appendix 1.

Ice cream mix was introduced directly into the gap between the two cylinders. Frozen ice cream was melted at room temperature and then transferred to a separating funnel. It was allowed to stand in this condition for about 1 hr so that any remaining air bubbles could rise to the surface. Test liquid was drawn off from the lower part of the separating funnel.

In the early tests with melted ice cream it was noticed that the inner cylinder showed little or no recovery when the shear stress was removed. This phenomenon was due to the formation of a skin of coagulated protein at the air-liquid interface. No such skin formed when mix, from which the frozen ice cream was made, was tested. When a thin layer of benzene was deposited on the surface of the melted ice cream a protein skin did not form during the test period.

RESULTS AND DISCUSSION

Temperature dependence of ice cream mix viscosity. Viscosity-temperature relationships can be represented by an equation of the Arrhenius form:

$$\eta = \alpha_e^{-E/kT} \qquad [3]$$

where η is viscosity, E is the potential energy of interaction between fat globules, α is a constant, k is the Boltzmann constant, and T is absolute temperature.

Replacing E/kT by B,

$$\log \eta = \log \alpha + B/T \log e \qquad [4]$$

At all four rates of shear employed, the $\log \eta - 1/T$ graphs were linear (Fig. 2). This indicates that within the range 1.7–12.8°C the type of association between the fat globules does not alter. When similar measurements were made at 4.0 to −5.0°C, the slope of the graph rose steeply when the temperature fell below −2°C. because of ice formation and fat crystallization.

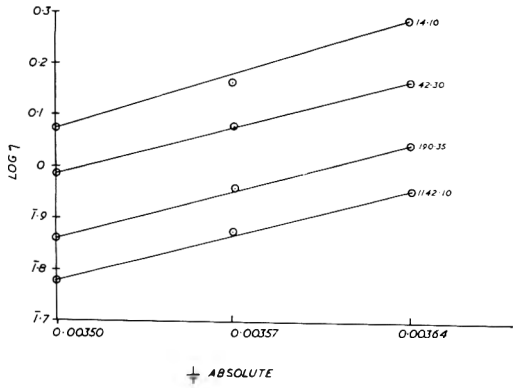


Fig. 2. Influence of temperature on ice cream mix viscosity.

The average value derived for B was 1.24×10^3, which is not very different from the value for water. The value obtained for E was 4.5 kT, or 1.7×10^{-13} erg, which is also very much lower than values for highly associated systems.

These observations support the view that the attraction forces between fat globules in ice cream mix are weak (Sherman, 1965).

Stress development at low shear rate. At rates of shear less than about 1 sec^{-1} a short time elapses before steady viscosity readings are obtained. The lag increases as the rate of shear decreases. For a constant rate of shear, the stress rises rapidly to a maximum, and then it falls away to an approximately steady value (Fig. 3). Bonds between flocculated globules stretch elastically at low rates of shear so that stress increases. A point is reached when the stress can be supported no longer and bonds rupture to an extent which depends on the applied rate of shear. Steady, but lower, stresses are established when equilibrium is reached between bond rupture and bond restoration.

The stress development curve for frozen ice cream which has been thawed shows that both the maximum and equilibrium stresses are lower than for ice cream mix which has not been frozen. Microscopic examination shows that globule size is much larger in the thawed ice cream so its lower viscosity must result from a weakening of the fat structure during freezing and thawing.

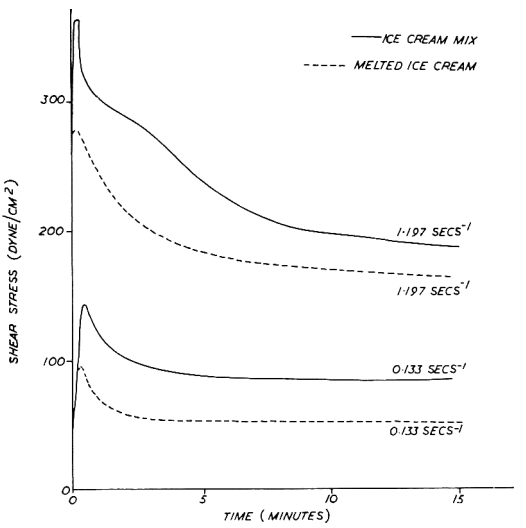


Fig. 3. Stress development in ice cream mix at low shear rate.

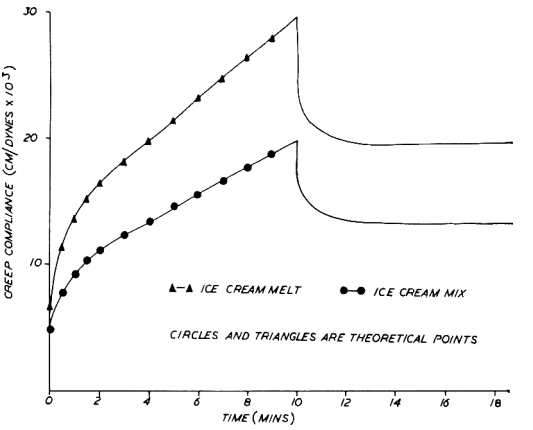


Fig. 4. Creep compliance v. time for ice cream mix and melted ice cream.

Creep compliance of ice cream mix and melted ice cream. Both ice cream mix and melted ice cream give a creep-compliance time curve indicative of viscoelastic behavior (Fig. 4). Very little instantaneous or retarded elasticity is shown, however, and Newtonian flow constitutes the main contribution to the overall compliance. Consequently, there is no substantial recovery when the shearing stress is removed.

Melted ice cream shows a greater creep compliance at any given time than unfrozen ice cream mix. The creep curves were analyzed by a graphical method (Inokuchi, 1955). Table 1 gives the rheological parameters—instantaneous elastic modulus (E_0), retarded elastic moduli (E_1, E_2), viscosities

Table 1. Rheological parameters for ice cream mix.

Rheological parameter	Sample		
	Mix at 20°C	Melted ice cream at 20°C	Frozen ice cream at −11°C
E_0(dynes/cm²)	200	143	10^5–10^6
E_1(dynes/cm²)	301	156	4.5×10^4
E_2(dynes/cm²)	1.43×10^3		11.2×10^4
η_1(poise)	13.4×10^3	5.2×10^3	8.5×10^7
η_2(poise)	8.3×10^3		1.6×10^7
τ_1(sec)	44.8	33.4	1866
τ_2(sec)	5.8		144
η_N(poise)	5.4×10^4	3.7×10^4	6.0×10^8

associated with retarded elasticity (η_1, η_2), retardation times (τ_1, τ_2), and viscosity associated with Newtonian flow (η_N)—relevant to ice cream mix and melted ice cream. It is clear that both mix and melted ice cream have very weak structures compared with frozen ice cream, and that mix has a stronger fat globule network than melted ice cream.

A very significant point which emerges from these analyses is that the creep behavior of mix is defined in terms of a six-component mechanical model as for frozen ice cream (Shama and Sherman, 1966), whereas the model for melted ice cream requires only four components. The behavior of mix is represented by a spring and dashpot which are connected in series with two units (Kelvin-Voigt body), each consisting of a spring and dashpot in parallel (Fig. 5). In the model for melted ice cream only one Kelvin-Voigt body is involved.

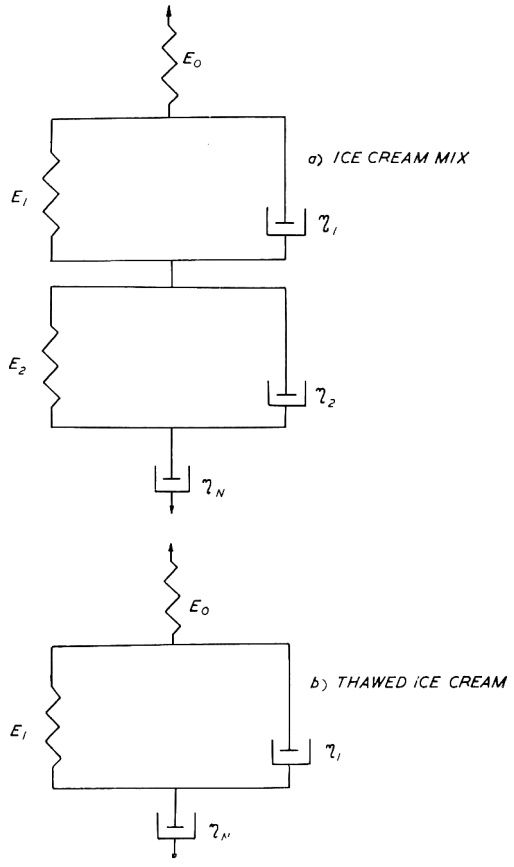


Fig. 5. Rheological models for ice cream mix and melted ice cream.

The equation defining the creep-compliance time curve for ice cream mix is:

$$\frac{\gamma(t)}{\sigma} = J_0 + J_1(1 - e^{-t/\tau_1}) + J_2(1 - e^{-t/\tau_2}) + \frac{t}{\eta_N} \quad [5]$$

where γ is the shear strain, σ is the shear stress, J_0 ($=1/E_0$) is the instantaneous elastic compliance, and J_1 ($=1/E_1$) and J_2 ($=1/E_2$) are the compliances associated with retarded elastic behavior.

For melted ice cream eq. 5 reduces to:

$$\frac{\gamma(t)}{\sigma} = J_0 + J_1(1 - e^{-t/\tau_1}) + \frac{t}{\eta_N} \quad [6]$$

Eq. 5 and 6 give theoretical values for the creep compliance which are in good agreement with the experimental findings (Fig. 4).

Structure recovery in ice cream mix following high shear. In an unsheared emulsion the fat globules flocculate and form aggregates. These aggregates grow progressively larger with time, and they immobilize part of the aqueous phase between the constituent globules. As a result, the ratio disperse phase/effective free volume aqueous phase increases, and so does the viscosity of the emulsion.

In polar media, the volume of continuous phase which is held within the globule aggregates depends to some extent on the magnitude of the charge on the globules. Globules pack more closely when the electrical double-layer thickness is reduced. In ice cream mix, the aggregated globules are separated by an average distance of about 50 Å (Sherman, 1965).

Globule size is not uniform in ice cream mix, so the attraction forces—which are directly proportional to globule diameter—will cover a range of bond strengths. At very low rates of shear, the weak bonds, mainly inter-aggregate, rupture but may reform. Higher rates of shear break the stronger bonds, mainly intra-aggregate, also. The data in Table 1 indicate that there is no really strong bonding between the fat globules in ice cream mix. This is confirmed by the recovery rate of relative viscosity (η_{rel}, where $\eta_{rel} = \eta/\eta_0$, and η is the viscosity of

the emulsion and η_0 is the viscosity of the aqueous phase) after shearing at 440.6 sec^{-1} or 114 sec^{-1}. The time interval for complete recovery is 20–30 sec at 5.4–14.1 sec^{-1} (Fig. 6).

From these data one can calculate the apparent increase in volume fraction disperse phase due to globule aggregation at the lower shear rates. The apparent volume fraction (φ_a) is related to the theoretical volume fraction (φ) by

$$\varphi_a = \varphi \left[w - \frac{w - 1}{(1 + bt)^2} \right] \qquad [7]$$

where w is a swelling factor defined by

$$w = \frac{v_n}{nv} \qquad [8]$$

and v_n is the volume of an aggregate containing n globules, v is the average volume of each globule, $b = GN_t$ where G is the rate of flocculation, and N_t is the number of oil globules per unit volume of mix at time t (Mooney, 1946).

When the mean globule size (D_m) in ice cream mix is 1.9 μ, the potential energy barrier to flocculation (V_{max}) is $\sim 6\ kT$ (Sherman, 1965) so that not all collisions between globules lead to flocculation. As a result

$$G = 4\pi \mathcal{D} D_m W \qquad [9]$$

where $\mathcal{D}$ is the diffusion constant, and W is the stability ratio, or factor by which flocculation is retarded.

Following Einstein (1905)

$$\mathcal{D} = \frac{kT}{3\pi\eta_0 D_m} \qquad [10]$$

so that

$$\varphi_a = \varphi \left[w - \frac{w - 1}{\left(1 + \frac{4N_t kT\ Wt}{3\eta_0}\right)^2} \right] \qquad [11]$$

The value of W cannot be determined easily for emulsions because flocculation is followed by coalescence. An approximate value (Verwey and Overbeek, 1948) is derived as follows

$$W = 2\int_0^\infty \exp\ (V/kT)\ \frac{ds}{s^2} \simeq \frac{1}{\chi D_m}\ (\exp\ V_{max}/k_1) \qquad [12]$$

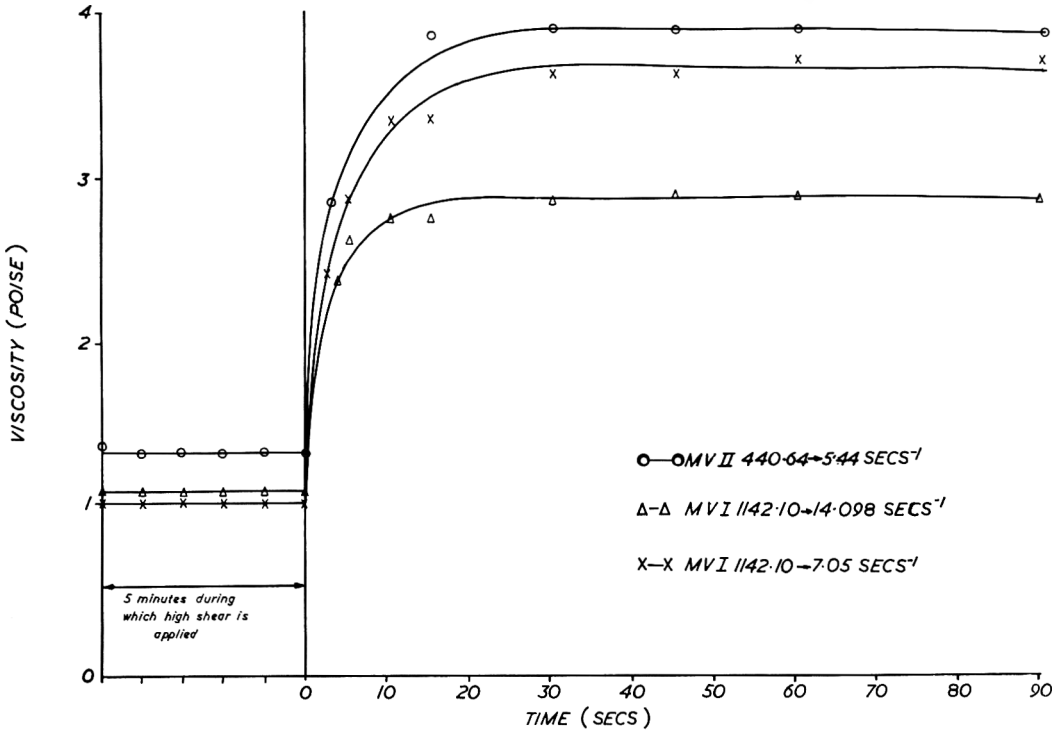


Fig. 6. Structure recovery in ice cream mix after subjection to high shear.

where $s = 2H_0/D_m$, H_0 is the distance between flocculated oil globules, and χ is the thickness of the diffuse part of the electrical double layer. Verwey and Overbeek (1948) quote data for solid dispersions where $W = 5.4\times10^4$ when $V_{max} = 15kT$. In conjunction with eq. 10 this leads to $W = 10^{0.8}$ when $V_{max} = 6\ kT$, i.e. 1 in every 6 collisions results in flocculation. To determine φ_a, one uses an equation relating φ to η_{rel} at high rate of shear ($v \rightarrow \infty$) such that all aggregates have been broken down and the globules move independently of each other. An empirical equation proposed for this purpose (Mooney, 1946) is

$$\underset{v\rightarrow\infty}{\eta_{rel}} = \frac{\sqrt{1 + 0.5\varphi}}{1 - \varphi} \exp\left(\frac{1.25\varphi}{1 - \varphi}\right) \quad [13]$$

The value of φ derived by introducing the experimentally determined η_{rel} on to the η_{rel}- φ curve calculated according to eq. 13 represents φ_a.

Eq. 11 suffers from one serious defect. It does not account for the influence exerted by globule size on viscosity. For example, η_{rel} increases at high shear rates as D_m decreases (Sherman, 1963) while keeping φ constant, but applying eq. 13 it would appear that φ increases. Consequently eq. 13 is applicable only to emulsions showing the same globule size ($\sim 2.7\ \mu$) as the emulsions for which it was originally designed. All the mixes studied in the present investigation had values of $D_m < 2\ \mu$.

A semi-empirical equation which represents viscosity data more accurately (Sherman, 1963) is

$$\underset{v\rightarrow\infty}{\log\ \eta_{rel}} = C\left[\frac{1}{D_m\left(\sqrt[3]{\frac{\varphi_{max}}{\varphi}} - 1\right)}\right] - 0.15 \quad [14]$$

where C is a constant related to D_m and φ_{max} is the maximum volume of disperse phase which can be incorporated in the emulsion (~ 0.74); when D_m exceeds 1 μ, $C \sim 0.036\ D_m^2$.

Applying eq. 13 to the present data, φ_a is 0.34, while eq. 14 suggests that φ_a is 0.47. If one allows for the fact that at $v \rightarrow \infty$ each globule behaves as if it is surrounded by a hydration layer of 0.3 μ thickness (Sherman, 1961) the ratio φ_a/φ is 1.55 or 2.14. Introducing these values into eq. 11 leads to $w = 2.2$ or 3.2. In either case, a greater volume of aqueous phase appears to be held within the globule aggregates than would be anticipated from a mean distance of separation between the globules of 50 Å.

The calculated w values are probably larger than the true values. If water is bound to the protein emulsifier layer around the fat globules by weak electrostatic and/or weak hydrogen bonds, the hydration sheath around each globule will be thicker at low rates of shear than at high rates of shear. Furthermore, eq. 11 applies only for globule sizes, or for D_m values, greater than 1 μ. Little is known about the relationship between viscosity and globule size for smaller globules. The only reliable data on this point (Saunders, 1961) suggest a progressively greater effect as size decreases from 1 μ to 0.1 μ. Studies on the stability to coalescence (Sherman, 1962, 1964) indicate that freshly prepared ice cream mix undoubtedly contains globules of 0.5 μ diameter or less. These globules will exert a greater effect on η_{rel} than the globules exceeding 1 μ in diameter, thus increasing the value of φ_a.

The components of frozen ice cream structure which are associated most directly with its six rheological parameters (Shama and Sherman, 1966) are E_0, ice crystals; fat crystals to a lesser extent; E_1, vegetable gum stabilizer gel; E_2, protein enveloped air cell interface; η_1, fat crystals; η_2, stabilizer gel and air cell interface; and η_N, fat crystals and ice crystals.

When ice cream melts, the ice crystals disappear, the overrun is lost, and the fat crystals melt, leading to further coagulation. The anticipated net effect is that E_0 and η_N will be reduced substantially; E_1 will decrease appreciably because the vegetable gum stabilizer is now dispersed in four times the quantity of water as in frozen ice cream, thus drastically weakening the gel structure. The viscosity η_2 will show an even greater reduction for the same reason since it also depends to some extent on the now nonexistent foam structure. E_2 should disappear completely. The viscosity η_1 will decrease because the

attraction forces between flocculated fat globules in an aqueous medium are weaker than in a solid ice medium. In frozen ice cream, the fat crystals are bound together by strong attraction forces in the primary minimum of the potential energy curve (Sherman, 1965). During melting, sufficient energy presumably enters the system, as latent heat of fusion, for the fat globules to pass over V_{max}—which is not large—and flocculate in the secondary minimum, as in the original unfrozen mix. The decrease in the ionic concentration of the aqueous phase when ice cream melts will also promote flocculation at the greater distance of separation.

The 4-component rheological model for melted ice cream therefore follows logically from the 6-component model for frozen ice cream. Pursuing the same line of reasoning, one would expect a 4-component model to define the rheological behavior of ice cream mix, although each parameter would now be larger. This assumption is not supported by the experimental data. Nevertheless, E_2 and η_2 for ice cream mix cannot be ascribed the same origin as for frozen ice cream, because of their structural differences.

The essential difference between ice cream mix and thawed ice cream should be that the fat globules are larger in the latter. However, ice cream mix also contains globules which are 0.5 μ in diameter or less. The potential energy curves for these very small globules will have a V_{max} no greater than $\sim 2kT$, so that they are able to flocculate in the primary minimum at a distance of separation which does not exceed a few Angstroms. A flocculate of this kind would show a greater degree of elasticity than a flocculate in the secondary minimum, where the globules are separated by 50 Å or more. If globules of 0.5 μ diameter flocculate with larger-size globules, V_{max} will increase proportionately.

E_1 and η_1 for both ice cream mix and melted ice cream can be attributed to the retarded elastic properties of globules which flocculate in the secondary minimum. E_2 and η_2 reflect properties of those globules which flocculate in the primary minimum. Since the latter constitute a smallish proportion of the total number of globules/cc, E_2 and η_2 are not as large as E_1 and η_1.

Creep-compliance data for melted ice cream at very low rates of shear have been offered as evidence for a structure in which fat globules are linked together by protein molecules (Shaw, 1963). Protein bridges are formed only when the protein concentration is so low that a reasonably condensed monolayer cannot be formed around the fat globules (Kragh and Langston, 1962; La Mer and Healey, 1963). Protein concentration in ice cream mix is sufficient to allow the formation of an adsorbed layer which is several molecules thick (Sherman, 1965). Furthermore, it has recently been shown that the protein-bridging mechanism may not operate even when the protein concentration is low. Charge stabilization can arise by adsorption of nonpolar groups of the protein molecules onto the fat globule surface so that the charged polar groups project outward into the aqueous phase (Gutoff *et al.*, 1963).

Even if one assumed that protein bridges are formed during emulsification of the ice cream ingredients, they would be very weak. It is unlikely that they would be able to withstand the vigorous agitation and whipping to which the mix is subjected, leading to partial coagulation, during the freezing process.

When frozen ice cream melts, the fat crystals melt, thus promoting further coagulation. The residual material retains little of the fat network structure present in the original ice cream mix. The rate, and degree, of coagulation depend on the extent to which the adsorbed emulsifier-protein layers around the fat globules are ruptured during freezing, and the efficiency of fat dispersion during the initial emulsification and homogenization of the mix.

REFERENCES

Einstein, A. 1906. The energy content dependence of the inertia of a body. *Ann. Phys. Lpz.* **19**, 289.

Gutoff, E. B., P. H. Roth, and A. E. Steigmann. 1963. Coagulation of silver halide suspensions in the presence of gelatin. *J. Phys. Chem.* **67**, 2366.

Inokuchi, K. 1955. Rheology of surface films. IV. Viscoelastic properties of 6-nylon films at

air/water interface. *Bull. Chem. Soc. (Japan)* **28**, 453.

Kragh, A. M., and W. B. Langston. 1962. The flocculation of quartz and other suspensions with gelatine. *J. Colloid Sci.* **17**, 101.

La Mer, V. K., and T. W. Healy. 1963. Adsorption-flocculation reactions of macromolecules at the solid-liquid interface. *Rev. Pure and Appl. Chem.* 112.

Mooney, M. 1946. A viscometer for measurements during thixotropic recovery; results with a compounded latex. *J. Colloid Sci.* **1**, 195.

Saunders, F. L. 1961. Rheological properties of monodisperse latex systems. 1. Concentration dependence on relative viscosity. *J. Colloid Sci.* **16**, 13.

Shama, F., and P. Sherman. 1966. Texture of ice cream. 2. Rheological properties of frozen ice cream. *J. Food Sci.* **31**, 699.

Shaw, D. J. 1963. The physical structure of ice cream. *In:* "Rheology of Emulsions." (P. Sherman, ed.) Pergamon Press, London.

Sherman, P. 1961. Rheological methods for studying the physical properties of emulsifier films at the oil-water interface in ice cream. *Food Technol.* **15**, 394.

Sherman, P. 1962. The colloidal stability of ice cream mix. Presented at 1st. Intern. Congr. Food Sci. and Technol., London.

Sherman, P. 1965. An equation for the Newtonian contribution to pseudoplastic flow in concentrated dispersions. *Proc. 4th. Intern. Congr. Rheology* **3**, 605.

Sherman, P. 1964. The coagulation of oil-water emulsions containing small-sized globules. Presented at 4th Intern. Congr. Surface Activity, Brussels.

Sherman, P. 1965. The texture of ice cream. Part 1. *J. Food Sci.* **30**, 201.

Verwey, E., and J. Th. G. Overbeek. 1948. "Stability of Lyophobic Colloids." p. 168. Elsevier Publishing Co.

Ms. rec'd 5/13/65.

The author is indebted to Unilever Ltd., for permission to publish this paper. He thanks Mr. J. Whitehead for his assistance with some of the experimental work. Part of this project was carried out in the laboratories of Messrs. T. Wall & Sons (Ice Cream) Ltd., London, W.3 to whom due acknowledgment is made for the facilities provided.

APPENDIX 1.

Creep compliance of frozen ice cream.

The creep curve for viscoelastic materials has an overall creep compliance $J(t)$ at any time t, where J is the ratio of strain $\gamma(t)$ to stress (σ).

Strain is given by

$$\gamma(t) = \frac{\beta f}{h} \qquad [a]$$

where β is the displacement observed with a travelling microscope having a reciprocal magnification factor f, and h is the thickness of each sample.

The stress is given by

$$\sigma = \frac{mg}{2A} \qquad [b]$$

where m is the weight applied, g is the acceleration due to gravity, and A is the cross-section area of each sample.

Therefore,

$$J(t) = \frac{2\beta f A}{mgh} \qquad [c]$$

The creep compliance curve can be subdivided into three distinct regions:

a) A region of instantaneous elastic compliance J_0 (in the region A-B of Fig. 1 in Part 2) in which bonds are stretched elastically. If shear does not continue beyond this point the recovery is complete when stress is removed.

$$J_0 = \frac{1}{E_0} = \frac{\gamma_0(t)}{\sigma} \qquad [d]$$

b) A time-dependent retarded elastic compliance J_R (B-C), associated with a retarded elastic modulus E_R, viscosity η_R and retardation time τ $(= \eta_R/E_R)$ during which bonds break and reform. All bonds do not break and reform at the same rate, so that τ should be replaced by a spectrum of retardation times. Consequently, E_R and η_R should be replaced by a distribution spectrum of retarded elastic moduli E_1, E_2, E_3 ..... E_i, and viscosities η_1, η_2, η_3 ..... η_i, assuming i components.

In its simplest form

$$J_R = \frac{1}{E_R} = J_a\,(1 - e^{-t/\tau}) = \frac{\gamma_R(t)}{\sigma} \qquad [e]$$

where J_a is the average retarded elastic compliance.

In the expanded form—

$$J_R = \sum_{i=1}^{n} J_i\,(1 - e^{-t/\tau_i}) = \sum_{i=1}^{n} J_i\,(1 - e^{-t/\eta_i J_i}) \qquad [f]$$

where η_i is the number of viscosity components associated with retarded elastic compliance.

Applying the graphical procedure of Inokuchi (1955) to eq. f, let

$$Q = \sum_{i=1}^{n} J_i - \frac{\gamma_R(t)}{\sigma} \qquad [g]$$

This corresponds to the distance between the extrapolated straight line DCP and the curve DCB at any time t, because of the exponential decay or relaxation of compliance.

In addition, therefore,

$$Q = \sum_{i=1}^{n} J_i e^{-t/\tau_i} \qquad [h]$$

When ln Q is plotted against t a straight line should be obtained at large values of t, from which a single retardation time (τ_1) and creep compliance (J_1) can be calculated. These values are inserted in eq. e, and if it does not adequately define the form of the experimental curve, a second plot is required of ln $(Q - J_1 e^{-t/\tau_1})$ against t to determine the magnitude of the second retardation time (τ_2) and the second creep compliance J_2. Eq. h indicates that this second plot should also be linear at large values of t provided $\tau_1 > \tau_2$. Similarly, if it is necessary a third retardation time τ_3 can be determined by plotting ln $(Q - J_1 e^{-t/\tau_1} - J_2 e^{-t/\tau_2})$ against t. This procedure is repeated until such time that sufficient retardation times and compliances have been calculated for eq. f to represent adequately the form of the experimentally determined retarded elastic compliance.

c) A region of Newtonian flow (C-D) with compliance J_N. Once the bonds have ruptured, i.e. the time for them to reform is much longer than the test period, individual particles or units flow past one another.

Newtonian flow is proportional to the time of loading in this region of the curve, so that

$$J_N = \frac{\gamma_N(t)}{\sigma} = \frac{t}{\eta_N} \qquad (i)$$

where γ_N is the shear strain in the linear region of the creep curve. Thus, the gradient of this region equals $1/\eta_N$.

When the stress is removed recovery follows a similar pattern to compliance. An instantaneous elastic recovery (D–E) is followed by a retarded elastic recovery (E–H). Since bonds were broken in the C–D region of the compliance curve a part of the structure is not recovered. This is represented by F–G, which is equivalent to D–H.

The total creep compliance is defined as

$$J(t) = J_0 + \sum_{i=1}^{n} J_i (1 - e^{-t/\tau_i}) + J_N \qquad [j]$$

when $t \to \infty$, eq. j reduces to

$$J(t) = J_0 + \sum_{i=1}^{n} J_i + J_N \qquad [k]$$

When $t \to 0$

$$J(t) = J_0 \qquad [l]$$

From eq. k and l the value of J_a in eq. e can be determined.

Eq. j can be written in the simpler form:

$$J(t) = J_0 + J_a (1 - e^{-t/\tau}) + \frac{t}{\eta_N} \qquad [m]$$

if one is interested only in the average value of the retarded elastic compliance.

Rearranging eq. m leads to

$$e^{-t/\tau} = e^{-t/J_a\eta_a} = -\left[\frac{J(t) - \dfrac{t}{\eta_N} - J_0 - J_a}{J_a}\right] \qquad [n]$$

so that if the right hand side of eq. n is plotted against t, the slope of the line represents $1/J_a\eta_a$. Thus η_a and τ can be calculated since J_0 can be derived from eq. k.

APPENDIX 2.
Mechanical model analogs for viscoelastic behavior.

The most generally used convention represents instantaneous elastic compliance under a shearing stress by an elastic spring, which stretches immediately when a force is applied to an extent depending on the magnitude of the force. No time interval is involved. Similarly when the force is removed the spring returns instantaneously to its unstretched condition. Pure viscous flow is represented by a dashpot consisting of a cylindrical container filled with a fluid of high viscosity in which a plunger is loosely fitted so that it can move up or down. The *rate* of movement of the plunger depends on the applied force. These two elements in series, referred to as a Maxwell body, represent regions *(a)* and *(c)* of the creep-compliance time curve discussed in Appendix 1.

The retarded elastic compliance, i.e. region *(b)* of the creep-compliance curve, can be represented by a spring and dashpot in parallel, which is known as a Kelvin-Voigt body, when defining eq. e in Appendix 1. This latter model depicts the behavior of a solid since the maximum extension depends on the force applied and is independent of the time for which it is applied. The viscosity component depicted by the dashpot is a solid viscosity which damps the extension and contraction of the elastic spring. To represent eq. f in Appendix 1, and to derive more detailed information of retarded elastic compliances, the single Kelvin-Voigt body is replaced by i Kelvin-Voigt bodies in series.

NOMENCLATURE

A	area of ribbed plate between ice cream samples in parallel plate viscoelastometer (cm)
B	$= E/kT$
C	constant related to mean globule size
D	diameter of pulley in coaxial cylinder viscometer (cm)
D_m	mean fat globule diameter (μ)
E	potential energy of interaction between fat globules (ergs or kT)

E_0 instantaneous elastic modulus (dynes/cm²)

E_1, E_2 elastic moduli associated with retarded elasticity (dynes/cm²)

E_a mean retarded elastic modulus (dynes/cm²)

G rate of flocculation

H_0 distance between flocculated globules (Å)

J_0 instantaneous elastic compliance (cm²/dyne)

J_1, J_2 compliances associated with retarded elastic compliance (cm²/dyne)

J_R mean retarded elastic compliance (cm²/dyne)

J_N Newtonian compliance (cm²/dyne)

$J(t)$ total compliance at time t (cm²/dyne)

N_t number of fat globules per unit volume of ice cream mix at time t

Q distance between extrapolated linear portion of creep curve and curved part (cm²/dyne)

R gas constant (=1.9865 cals/°C, or 8.3144×10^7 ergs/°C)

S shear stress in coaxial cylinder viscometer (dynes/cm²)

T absolute temperature

V_{max} potential energy barrier to flocculation (ergs, or kT)

W fraction of collisions leading to flocculation

b $= G\ N_t$

f reciprocal magnification factor of travelling microscope

h thickness of each ice cream sample inserted in parallel plate viscoelastometer (cm)

l length of inner cylinder of coaxial cylinder viscometer which is immersed in test sample

k Bolzmann constant (1.38047×10^{-16} ergs/°C)

m total weight applied as shearing stress (g)

r_1, r_2 inner and outer cylinder radii in coaxial cylinder viscometer

S $= 2H_0/D_m$

t time (sec)

v average volume of each fat globule (cc)

v_n volume of aggregate containing n globules (cc)

w swelling factor

α constant in Arrhenius equation

β displacement of ribbed plate in parallel plate viscoelastometer (cm)

γ shear strain (cm²/dyne)

η_1, η_2 viscosity components associated with retarded elasticity (poise)

η_a mean viscosity component associated with retarded elasticity (poise)

η_0 viscosity of continuous phase in ice cream mix (poise)

η_{rel} relative viscosity

τ_1, τ_2 retardation times associated with retarded elasticity (sec)

τ mean retardation time associated with retarded elasticity (sec)

ϕ theoretical volume fraction of fat in ice cream mix, or thawed ice cream

ϕ_a apparent volume fraction of fat in ice cream mix, or thawed ice cream

χ thickness of diffuse part of electrical double layer (cm)

ψ strain developed in coaxial cylinder viscometer

ω angular rotation of inner cylinder in coaxial cylinder viscometer (°)

D diffusion constant

M. A. KATZ,[a] L. R. DUGAN, JR., and L. E. DAWSON
Food Science Department, Michigan State University
East Lansing, Michigan 48823

Fatty Acids in Neutral Lipids and Phospholipids from Chicken Tissues

SUMMARY

Lipid material from skin, depot fat, and dark and white meat from broiler-type male chickens was fractionated into neutral lipids and phospholipids by column chromatography. The fatty acids of these fractions were analyzed by gas-liquid chromatography.

Muscle tissues contained relatively larger quantities of phospholipids than did skin and depot fat. Neutral lipids and phospholipids had similar percentages of unsaturated fatty acids. Some 18 different fatty acids were found in the neutral lipids, and 22 fatty acids were found in the phospholipid fraction. The composition of fatty acids in the neutral lipids was similar in the four tissues. Phospholipids from muscle tissues contained more long-chain fatty acids than phospholipids from skin and depot fat. Arachidonic acid was found to be one of the major fatty acids in the phospholipid fraction.

INTRODUCTION

An increasing quantity of poultry meat is being processed and used as boneless and cooked meat, much of which is used in comminuted or freeze-dried products. In addition to problems related to the use of accumulated poultry fat *per se*, the remaining lipids in the meat are often responsible for problems related to product utilization and stability. The main problem in processed and stored meat is flavor stability. Since oxidative rancidity is a major cause of flavor changes, the lipids in both fatty and muscle tissue may affect flavor quality and product stability.

The metabolism of fatty acids and lipids in chickens has received considerable research attention. Major analysis, however, has been related to laying hens, eggs, body fluids and organs, and depot fat. Detailed fatty acid analysis of broiler muscle tissue has received little research attention. Reiser (1951) found that in chicken the fat reserves are much more similar to general body fats than is the case in swine and cattle. Fingenbaum and Fisher (1959) and Marion and Woodroof (1962) showed that the dietary fat affects and reflects body fat composition. Chang and Watts (1952), in agreement with others, found that poultry fat is much more unsaturated than beef, lamb, and even pork fat. Hilditch *et al.* (1934) reported that 65% of the fatty acids present in poultry were unsaturated.

Of the workers who have studied fatty acids in poultry meat, Marion and Woodroof (1963) identified the largest number of fatty acids. They reported fatty acids with chain length up to 20 carbon atoms and five double bonds in skin and adipose fat, and fatty acids with chain lengths of 24 carbon atoms with 6 double bonds in lipids from thigh and breast meat. They did not, however, separate the neutral lipids from the phospholipids.

Polyunsaturated fatty acids are synthesized in the chicken body, and therefore the body fat composition may vary from that of the dietary fat. Machlin and Gordon (1961) suggested that arachidonic acid can be synthesized from linoleic acid, which is an essential fatty acid for the chicken. Reiser (1951) found that linoleic acid can be converted to a number of polyunsaturated fatty acids with 2–6 double bonds.

Miller *et al.* (1962) observed most of the arachidonic to be in the phospholipids. Widmer and Holman (1950) found phospholipids to be more unsaturated than neutral lipids, and to be the cause of rancidity. El-Gharbawi (1964) showed that the unsaturated fatty acids in the phospholipids oxidize more rapidly than the fatty acids in the neutral lipids in freeze-dried beef.

The fatty acids of the phospholipids and other long-chain fatty acids seem to play a major role in lipid oxidation. Yet little work has been done, to our knowledge, to compare specifically how much and what kind of fatty acids are present in the phospholipids and the neutral lipids of poultry tissues. This study was conducted to determine the amounts and percentages of fatty acids pres-

[a] Present address: Hebrew University, Rehovot, Israel.

ent in neutral and phospholipid fractions in lipids from chicken skin, muscle, and depot fat.

EXPERIMENTAL METHODS

Twelve 14-week-old male fryers were obtained from a commercial source for this study. These birds were reared under a program in which the composition of the diet was altered four times. During the last two weeks (finishing stage) the diet contained a calculated 3.8% fat. Four uniform 2-kg birds (eviscerated weight) were selected from the 12 for this experiment, and their dark meat, white meat, skin, and depot fat (taken from the abdominal cavity after the removal of the viscera) were analyzed to determine the fatty acid composition of the neutral lipids and the phospholipids from these tissues.

The lipids were extracted by the method developed by Folch *et al.* (1957). Each lipid sample was separated into neutral lipids and phospholipids by the method suggested by Borgstrom (1952) (modified by using a silicic acid column system described by Katz and Dawson, 1965) and dried for gravimetric measurement of these lipids. A second sample was separated into neutral and phospholipid fractions, and the fatty acids of each fraction were methylated and used for analysis of the fatty acids by gas-liquid chromatography.

The silicic acid (80–100-mesh) was washed with distilled water to remove the fines. Water was removed under vacuum on a Buchner funnel. The silicic acid was then washed with methanol and activated overnight at 105°C. After packing the column, 2 cm of dehydrated sodium sulfate were placed on top of the silicic acid–chloroform slurry. Total lipids dissolved in chloroform were added to this column. The sample was eluted from the column under nitrogen pressure at the rate of 2 drops per second, into drying flasks. The chloroform eluant of the sample was taken as neutral lipids, and the methanol eluant as phospholipids. Neutral lipids were eluted by chloroform and monitored by the Salkowski test (Kuchmack and Dugan, 1963) until negative, and the elution of phospholipids was followed by the ninhydrin test until negative (Rouser *et al.*, 1961). Double check for purity was accomplished by checking the neutral lipids with ninhydrin, and the phospholipids were checked by micro-plate thin-layer chromatography. New column packing was used for each sample, and the nonlipid material present in the lipid fraction was discarded.

The eluant for gravimetric analysis was dried at 40°C in a round-bottom flask on a Rinco rotary vacuum evaporator. After vacuum drying, the sample was placed in a 105°C oven for 10 min and brought to a constant weight by holding overnight in a desiccator at room temperature.

Fatty acids were esterified by the method developed by McGinnis and Dugan (1965). This method involves hydrolysis and methylation of the lipids from H_2SO_4 and methylation of the fatty acids at −60°C. Recovery of the methyl esters was achieved by extraction with 30–60° petroleum ether. The ether solution containing methyl ester was evaporated to 0.2 ml (or 0.1 in the case of phospholipids) over a 60°C bath and under nitrogen. The concentrate was transferred with a syringe to a tube made out of No. 4 capillary glass tube to minimize the surface area and to aid in handling such minute quantities. These tubes were stored at 0°C in the dark for not more than 12 hr before GLC analysis, and were checked by thin-layer chromatography for complete esterification.

Fractionation of the methyl esters was accomplished on a dual column F & M 810 GLC, utilizing a flame ionization detector. Helium was used as a carrier gas at 35 ml/min. A 72 × ¼-inch column was used isothermally at 190°C with detector temperature of 260°C and injector temperature of 250°C. Acid-washed chromosorb W, 80–100-mesh, was used as solid support with 15% DEGS plus 3% phosphoric acid as liquid phase. The phosphoric acid helped to obtain well defined and symmetrical peaks, as was suggested by Metcalf (1963).

Fatty acids were identified by comparison with standards from the Hormel Institute, and by comparing the unknown peaks to the semilog plots of retention time vs. carbon number to that of the known fatty acid esters. To improve separation of the fatty acids, the DEGS column was used at different temperatures and gas flow rates.

The combined weight of the neutral lipids and phospholipids was taken as the total lipid content of the tissue. Neutral lipids and phospholipids were calculated as a percent of this total. The fatty acids were calculated as a percent of the total fatty acids in the neutral lipid or the phospholipid fractions. This was done by calculating the peak area with the help of the disc chart integrator, and expressing it as a percent of total area from all peaks of the methyl esters. At least two replicate analyses were determined from each of four samples.

RESULTS AND DISCUSSION

The total lipids, phospholipids and neutral lipids found in each tissue are reported in Table 1 and the fatty acid composition of each lipid in each tissue is given in Tables 2 and 3. The fatty acid composition of lipids from different tissues depends both on com-

Table 1. Total lipid, phospholipid and neutral lipid content (%) of poultry tissues.

Tissue	Total lipids[a]	Phospho-lipids[b]	Neutral lipids[b]
White meat	1.0	48	52
Dark meat	2.5	21	79
Skin	25	2.0	98
Depot fat	60–80	0.9	99.1

[a] As percentage of raw tissue.
[b] As percentage of total lipids.

position of diet (Marion and Woodroof, 1962) and on synthesis within the bird (Machlin and Gordon, 1961). The rate and extent of development of oxidative rancidity in meat (or separated fat) during extended storage is influenced markedly by the degree of unsaturation of those fatty acids present, so additional data on composition of lipids in each major portion of the chicken should be of value in the manufacturing of meat-containing products and controlling the stability of such products.

As expected, total lipid content was lowest in the white meat, followed by dark meat and skin, and was highest in depot fat. The values for white and dark muscles are relatively low. However, the muscle tissues were excised and all apparent fatty tissues removed prior to analysis. The 1:2 ratio of lipids in white and dark meat was as expected. This type of information has been used in the past to indicate that rancidity development should occur primarily in depot fat and skin, with less chance that it will occur in the white or dark meat. When the lipids were separated into phospholipids and neutral lipids, however, a different relationship was apparent. The present results show that although white meat and dark meat contained only 1 and 2.5% total lipids, 48% and 21% respectively of that total was in the phospholipid fraction. As previously pointed out (El-Gharbawi, 1964), fatty acids in phospholipids oxidized more rapidly than the fatty acids in neutral lipids. On a weight percentage basis, all tissues contained about the same amount of phospholipid material. Therefore the high percentage of phospholipids in muscle tissues, may make muscles as susceptible to oxidative rancidity as are depot and skin fat.

Since oxidation depends on the presence of unsaturated fatty acids, an analysis of individual fatty acids was made for each tissue. The percentage of each fatty acid was similar among the four tissues, with the

Table 2. Fatty acids of the neutral lipids (calculated as a percentage of the fatty acids in the neutral lipids).

Fatty acid[a]	Dark meat	White meat	Skin	Depot fat
8	tr	tr	tr	tr
10	tr	tr	tr	tr
12	tr	0.1	tr	0.1
14	0.8	1.0	0.7	0.7
14:1	0.3	0.3	0.3	0.2
15	0.2	0.2	0.2	0.2
16	22.4	24.2	23.1	22.8
16:1	6.8	5.6	5.7	5.7
16:2	0.5	0.5	0.5	0.4
17	0.2	0.3	0.2	0.2
18	6.4	6.0	5.9	6.5
18:1	34.6	34.6	37.0	37.0
18:2	24.7	24.7	24.1	23.7
18:3	1.5	1.3	1.4	1.3
20:1	0.5	0.5	0.6	0.7
20:2	0.3	0.3	0.2	0.2
20:3	0.2	0.3	0.1	0.1
20:4	0.5	0.5	0.2	0.2

[a] Number of carbons: number of double bonds.

Table 3. Fatty acids of the phospholipids (calculated as a percentage of the fatty acids in the phospholipids).

Fatty acid[a]	Dark meat	White meat	Skin	Depot fat
12	tr	tr	tr	tr
14	3.2	1.8	3.5	1.9
14:1	0.3	0.1	0.5	1.1
15	0.7	0.8	0.7	0.9
16	14.6	23.0	22.1	19.0
16:1	1.6	0.9	3.1	4.8
16:2	0.5	0.3	0.7	0.7
17	0.4	0.5	1.0	0.6
18	16.6	9.9	11.8	9.3
18:1	13.5	16.3	19.5	25.8
18:2	19.8	17.0	14.8	21.7
18:3	0.6	0.5	1.0	1.8
20:1	0.6	0.6	0.8	1.1
20:2	0.8	0.8	0.9	0.7
20:3	1.2	1.5	1.6	0.9
20:4	16.8	15.1	11.4	4.7
22:2	0.4	1.0	1.1	0.9
22:3	0.3	0.7	0.6	0.5
22:4	2.4	3.0	2.7	1.3
24:1	0.9	1.0	1.7	1.6
22:5–24:2	1.5	1.7	0.9	0.3
22:6–24:4	3.4	3.9	1.4	1.3

[a] Number of carbons: number of double bonds.

greatest variation in arachidonic acid (20:4) in the phospholipid fraction, as reported by Miller *et al.* (1962). The percentage of this acid was highest in the low-fat tissues (white and dark meat) and lowest in the high-fat tissue (depot fat). Arachidonic acid is synthesized from linoleic acid (Machlin and Gordon, 1961). The lipid fraction in the diet of the birds in this study (Table 4) contained only traces of arachidonic acid but approximately 50% linoleic acid. This indicates that these birds probably synthesized arachidonic acid, and deposited a lower percentage of it in the phospholipid fraction of the lipid rich tissues (skin and depot fat) than in the white and dark meat.

In the past, when only whole birds were stored, oxidation occurred first in the skin and depot fat tissues, mainly because they were more exposed to the air. Many present-day products are cut-up parts or boneless meat tissues, and their lipids were found, in this study, to contain sufficient polyunsaturated fatty acids to be of major concern in maintaining the stability of such poultry meat products.

REFERENCES

Borgstrom, B. 1952. Investigation on lipid separation methods. Separation of phospholipids from neutral fat and fatty acids. *Acta Physiol. Scand.* **25**, 101.

Chang, I., and B. M. Watts. 1952. The fatty acids of meat and poultry before and after cooking. *Am. Oil Chemists Soc.* **29**, 334.

El-Gharbawi, M. I. 1964. Chemical and physiochemical changes in lipids and other constituents of freeze-dried raw beef during storage under modified atmosphere. Ph.D. thesis. Michigan State University.

Fingenbaum, A. S., and H. Fisher. 1959. The influence of dietary fat on the incorporation of fatty acids into body and egg fat of the hen. *Arch. Biochem. Biophys.* **79**, 302.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497.

Hilditch, T. P., E. C. Jones, and A. J. Rhead. 1934. The body fat of the hen. *Biochem. J.* **28**, 786.

Katz, M. A. 1965. Fatty acids in neutral lipids and phospholipids from chicken tissues. M.S. thesis. Michigan State University.

Katz, M. A., and L. E. Dawson. 1965. A pressure control valve as an aid in column chromatography. *J. Chromatog.* **18**, 589.

Kuchmak, M., and L. R. Dugan. 1963. Phospholipids of pork muscle tissues. *J. Am. Oil Chemists Soc.* **40**, 734.

Machlin, L. J., and R. S. Gordon. 1961. Effect of dietary fatty acids and cholesterol on growth and fatty acid composition of the chicken. *J. Nutrition* **75**, 157.

Marion, J. E., and J. G. Woodroof. 1962. The influence of dietary fat on lipid composition of broiler tissues and the development of rancidity during storage. *Poultry Sci.* **42**, 1287 (Abstr).

Marion, J. E., and J. G. Woodroof. 1963. The fatty acids composition of breast, thigh, and skin tissues of chicken broilers as influenced by dietary fats. *Poultry Sci.* **42**, 1202.

Metcalf, L. D. 1963. The gas chromatography of fatty acids and related long chain compounds on phosphoric acid treated column. *J. Gas Chromatog.* **1**, 7.

McGinnis, G. W., and L. R. Dugan, Jr. 1965. A rapid low temperature method for preparation of methyl esters of fatty acids. *Am. Oil Chemists Soc.* (in press).

Miller, E. C., H. Menge, and C. A. Denton. 1962. A comparison of fatty acids content of skin of chickens and turkeys. *Poultry Sci.* **41**, 1667 (Abstr.).

Reiser, R. 1951. The synthesis and interconversion of polyunsaturated fatty acids by the laying hen. *J. Nutrition* **44**, 159.

Rouser, G., J. O'Brien, and D. Heller. 1961. The separation of phosphotidyl ethanolamine and phosphotidyl serine by column chromatography. *J. Am. Oil. Chemists Soc.* **38**, 14.

Widmer, C., and R. T. Holman. 1950. Polyethenoic fatty acid metabolism, deposition of polyunsaturated fatty acids in fat deficient rats upon single fatty acid supplementation. *Arch. Biochem.* **25**, 1.

Table 4. The main fatty acids in the phospholipids and the neutral lipids in the diet.

Fatty acid	Lipid fraction	
	Phospholipids	Neutral lipids
14:0	0.3	0.3
16:0	20.4	12.4
16:1	0.8	0.7
18:0	3.5	2.9
18:1	15.4	26.9
18:2	49.5	53.3
18:3	9.5	3.0
20:4	0.5	tr.

Ms. rec'd 6/24/65.

Journal Article No. 3658, Michigan Agricultural Experiment Station.

J. O. RODIN, DALE M. COULSON, ROBERT M. SILVERSTEIN
Stanford Research Institute, Menlo Park, California 94025
and
ROBERT W. LEEPER
Pineapple Research Institute of Hawaii, Honolulu, Hawaii 96822

Volatile Flavor and Aroma Components of Pineapple III. The Sulfur-Containing Components

SUMMARY

Methyl β-methylthiopropionate and ethyl β-methylthiopropionate were isolated and identified from pineapple flavor concentrate. These are the major volatile sulfur-containing compounds present. Also detected were two minor sulfur-containing compounds. The total volatile sulfur content of pineapple flavor concentrate was found to be 0.4–1.0% (13–32 ppb based on whole pineapple).

Methyl β-methylthiopropionate $CH_3SCH_2CH_2COOCH_3$ (I) was identified in Hawaiian pineapple (Haagen-Smit *et al.*, 1945) by oxidation of an impure sulfur-containing fraction to the corresponding sulfone; a mixture melting point determination with an authentic sample of the sulfone served for confirmation of identity.

For a number of years, these results were treated with some reservation because preliminary gas chromatography investigations on crude instruments failed to confirm the presence of I, and taste-tests on a synthetic sample elicited very unfavorable responses ("foreign" taste) down to the lowest detectable level (unpublished results, Pineapple Research Institute).

In the course of our work on Hawaiian pineapple (Rodin *et al.*, 1965; Silverstein *et al.*, 1965), we isolated, in pure form, and identified two sulfur-containing compounds, I and ethyl β-methylthiopropionate $CH_3SCH_2CH_2COOCH_2CH_3$ (II). Their infrared and NMR spectra were congruent with those of authentic samples (Figs. 1, 2). This work was done on a 1963 winter crop . A Carius sulfur microdetermination gave 1.0% sulfur in the distillate from the flavor concentrate (32 ppb based on whole pineapple).

A "sulfur profile" was run on a distillate of a flavor concentrate, representing one-tenth of a pineapple from a 1964 summer crop, by passing the gas chromatography effluent directly and continuously into an electrolytic conductivity sulfur detector (Coulson, 1965). The sulfur detector recording is shown in Fig. 3. The peaks at 3.2 min and 5.4 min matched those of authentic I and II run under identical conditions. Besides the methyl (1.1 μg S) and ethyl (1.4 μg S) peaks, there are two smaller sulfur-containing peaks (0.3 μg S). This corresponds to a total of 0.4% sulfur in the distillate (13 ppb based on whole pineapple), in fair agreement with the Carius determination on the winter crop.

The presence of I and II in Australian pineapple was reported recently by Connell (1964), who matched gas chromatography retention times of peaks, which may not have been completely resolved, against those of authentic samples. His value of 8.6% sulfur in his "pineapple oil" differs from ours by an order of magnitude.

Taste tests were carried out on pure authentic samples of both esters. The source of the "foreign" taste in the early sample of the methyl ester I was traced to appreciable contamination by methyl β-mercaptoproprionate. The purified samples were acceptable as additives to "stripped" pineapple juice. In high concentrations, they imparted an "overripe" taste.

In this connection, we might speculate on the biogenesis of the sulfur-containing compounds. Methionine, a likely precursor, has been found to be generally absent in pineapple until the onset of ripening, but present in considerable amounts thereafter (Gortner and Singleton, 1965). Fully mature, but not overripened, fruit was used in the present work.

ISOLATION AND IDENTIFICATION

This study was carried out on a flavor concentrate prepared from 250 freshly-picked pineapples (winter crop 1963). Preparation of the concen-

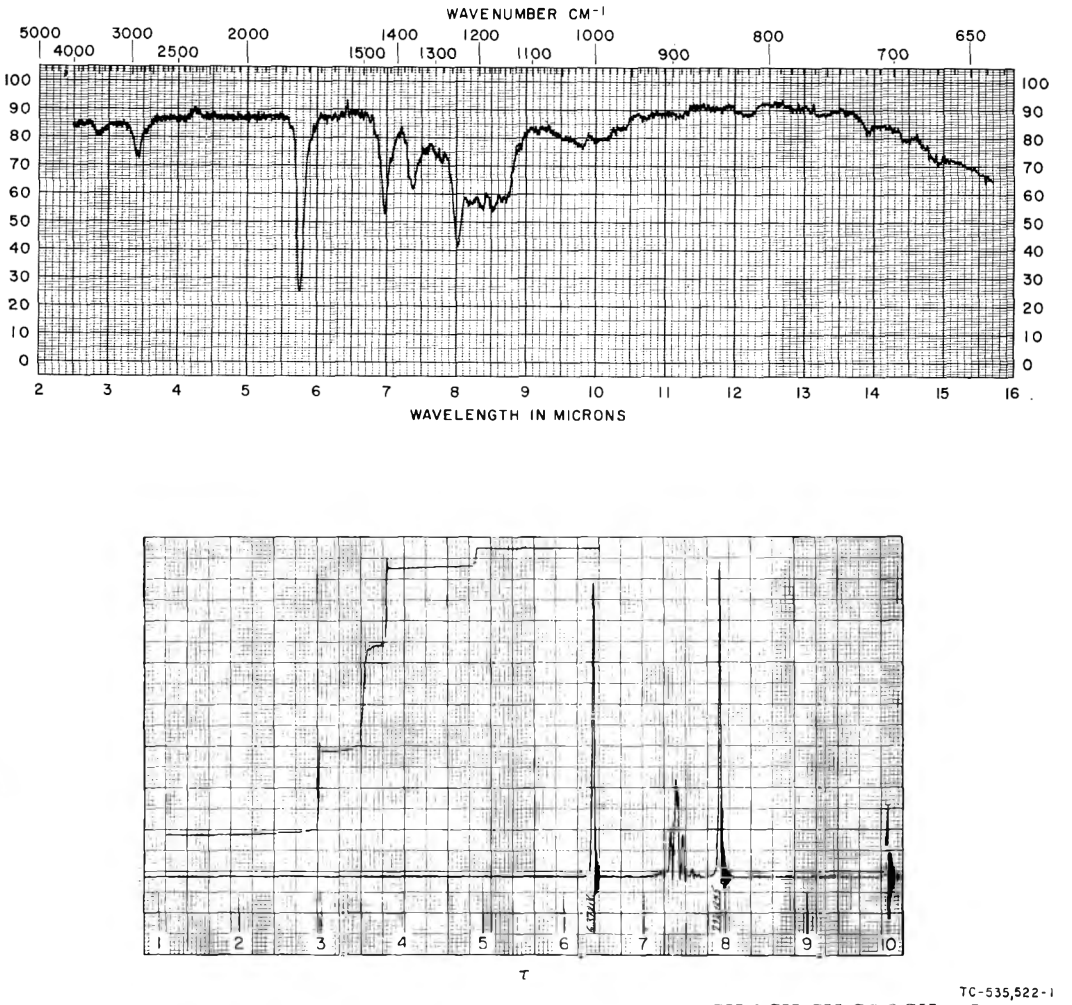


Fig. 1. Infrared (isolated) and NMR (synthetic) spectra of $CH_3SCH_2CH_2COOCH_3$ (I).

trate was described in paper No. I (Rodin *et al.*, 1965). Preparation of the gas chromatography fractions was described in paper No. II (Silverstein *et al.*, 1965); the same numbering system for fractions is used in this paper.

Gas chromatography fraction 3D (41 mg) was collected from 23 to 31 min from a Silicone QF1 column as described in paper No. II. Fraction 3D was rechromatographed on a LAC 446 column (15% on Chromosorb W 30/60-mesh, 12 ft × 5 mm ID, 161°C, 34 cc helium/min, on-column injection) to give fraction 3D3 (8 mg, retention time 19 to 22 min). The IR spectrum and retention time of this fraction agreed with those of an authentic sample of I.

Fraction 3E (40 mg) was collected from the Silicone QF1 column from 32 to 41 min, and rechromatographed on a LAC 446 column (25% on Chromosorb W, 30/60-mesh, 6 ft × 5 mm ID, 130°C, 30 cc helium/min, on-column injection) to give fraction 3E3 (4 mg, retention time 32–36 min). This fraction was identified as II by comparative retention times and IR and NMR spectra.

The NMR Spectrum of I (Fig. 1) shows the CH_3S— protons as a singlet at τ 7.92, the CH_3O— protons as a singlet at τ 6.37, and the $—CH_2CH_2—$ protons as two closely-spaced, distorted triplets centered at about τ 7.3 and τ 7.5. The NMR Spectrum of II (Fig. 2) shows the CH_3S— protons as a singlet at τ 7.93, the CH_3CH_2O— protons as a triplet at τ 8.77, the $CH_3\overrightarrow{CH_2O}$— protons as a quartet at τ 5.88, and the $—\overleftarrow{CH_2}CH_2—$ protons as

two closely-spaced, distorted triplets centered at about τ 7.3 and τ 7.5. In addition to distortion, the outer peaks of the triplet show second-order splitting because of the small difference in shift positions.

SULFUR PROFILE

A Coulson Instruments Company gas chromatograph, model 1, containing a CIC temperature programmer, model 30, was used for the selective electrolytic conductivity detection of sulfur-containing organic compounds. The column was a coiled Pyrex tube 4 ft × 2 mm ID, packed with 80/100-mesh Gas Chrom-Q coated with 10% DC 200 oil (12,500 centistokes). The helium carrier gas flow rate was 35 cc/min, and the oxygen flow rate into the 800° combustion zone was approximately 100 cc/min. The temperature program for the sulfur component analysis was 10 min isothermal at 80°C followed by a linear increase of 9°/min for 18 min. The attenuator setting on the detector bridge was ×200, which represents a sensitivity of 1.3 μg of sulfur per square inch on a strip-chart recorder with a chart speed of 0.5 in./min and a sensitivity of 0.1 mv/in.

Most of the smaller peaks, including the peak between 1 and 2 min and the group between 22 and

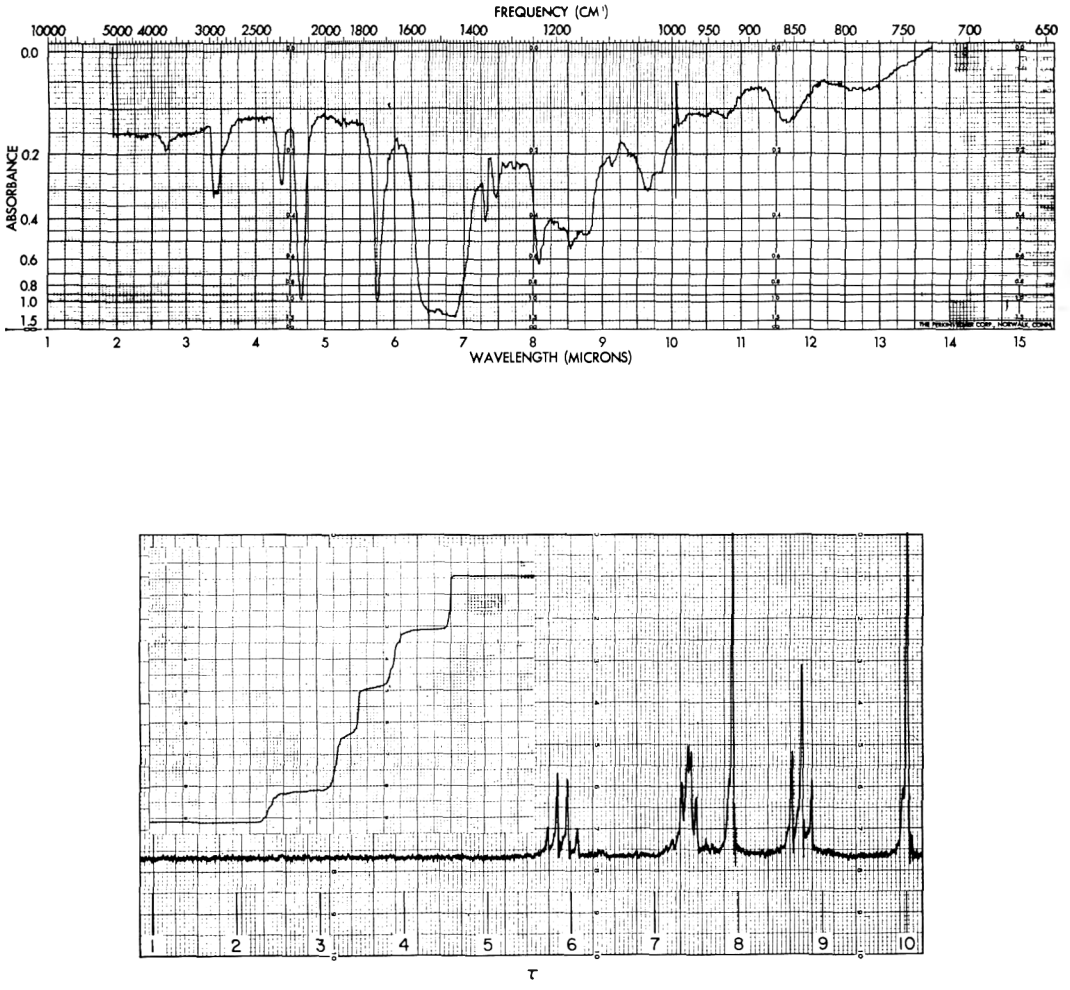


Fig. 2. Infrared (synthetic) and NMR (isolated spectra of $CH_3SCH_2CH_2COOCH_2CH_3$ (II).

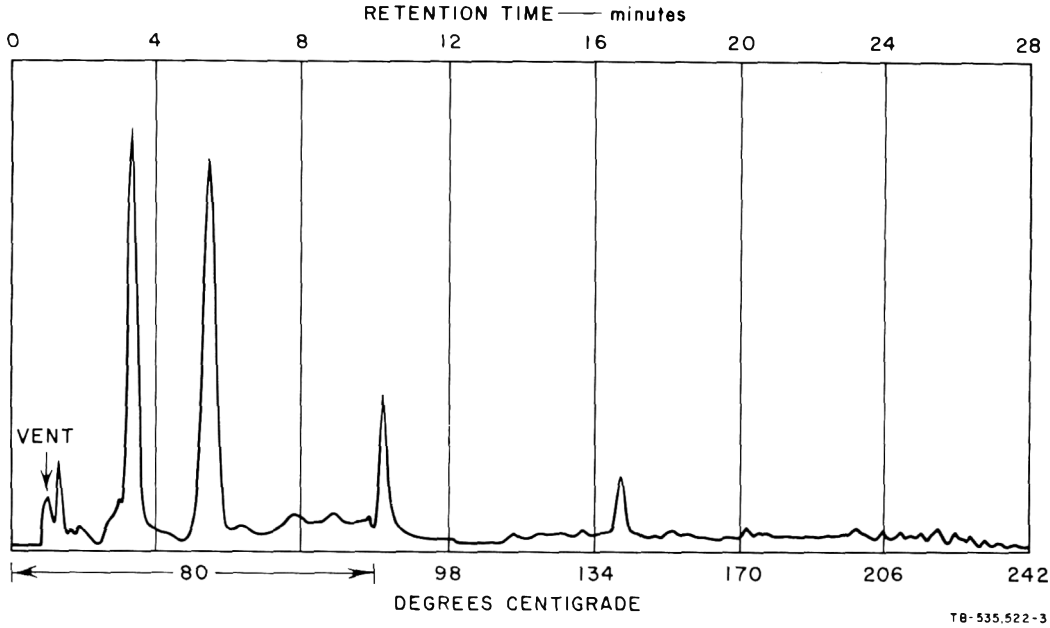


Fig. 3. "Sulfur profile" of distillate.

28 min, were halogen-containing contaminants, based on a separate analysis under conditions specific for halogen detection.

The flavor concentrate used to obtain the "sulfur profile" was prepared from 10 pineapples from a summer 1964 crop, translucence 4.4 (see papers Nos. I and II of this series). This concentrate was transferred to a short-path distillation apparatus (distance from pot to cold-finger cooled with dry ice was 20 mm) and distilled at 60°C/0.01 mm for 1 hr. The distillate was rinsed from the cold-finger with 1 ml of freshly-opened tetrahydrofuran (Mallinckrodt AR). A 10-μl portion of the solution was used for the sulfur profile shown in Fig. 3.

PREPARATION OF AUTHENTIC SAMPLES

Methyl-β-methylthiopropionate (I) was prepared by treating the sodium salt of methyl-β-mercaptopropionate with methyl iodide. The product was distilled at 105–107°C/35 mm (lit. bp 69°C/10 mm, Haagen-Smit *et al.*, 1945) and purified by gas chromatography through an Apiezon L column (20% on Chromosorb W, 30/60-mesh, 6 ft × 8 mm ID) at 166° and 30 cc helium/min, retention time 13.5–16.5 min.

Ethyl-β-methylthiopropionate (II) was prepared by the method of Protiva *et al.* (1957). The product was distilled at 192–197°C (lit. bp 192°C, Barger and Coyne, 1928); it showed a symmetrical peak on gas chromatography at 12.5 min and a minor peak at 5 min (< 1%) on a Carbowax 20 M column (10% on Chromosorb W, 60/80, 5 ft × 2 mm ID) at 100°C and 13 cc helium/min.

SPECTRA

The infrared spectrum of I was obtained as a film with a Beckman IR5. The infrared spectrum of II was obtained in carbon disulfide solution with a Perkin-Elmer 221.

The NMR spectra of I and II were obtained in carbon tetrachloride solution, with a Varian HR60.

REFERENCES

Barger, G., and F. P. Coyne. 1928. The amino acid methionine; constitution and synthesis.

Connell, D. W. 1964. Volatile flavoring constituents of the pineapple. *Australian J. Chem.* **17**, 130.

Coulson, D. M. 1965. Electrolytic conductivity detector for gas chromatography. *J. Gas Chromatog.* **3**, 134.

Gortner, W. A., and V. L. Singleton. 1965. Chemical and physical development of the pineapple fruit. III. *J. Food Sci.* **30**, 24.

Haagen-Smit, A. V., J. G. Kirchner, A. N. Prater, and C. L. Deasy. 1945. Chemical studies of pineapple. *J. Am. Chem. Soc.* **67**, 1951.

Protiva, M., V. Mychajlyszyn, L. Novák, M. Borovicka, J. D. Jílek, E. Adlerová, and V. Hach. 1957. Synthetic antispasmodics. *Ceskoslov. farm.* **6**, 425. *Chem. Abstr.* **52**, 9944 g (1958).

Rodin, J. O., C. M. Himel, R. M. Silverstein, R. W. Leeper, and W. A. Gortner. 1965. Volatile flavor and aroma components of pineapple. I. *J. Food Sci.* **30**, 280.

Silverstein, R. M., J. O. Rodin, C. M. Himel, and R. W. Leeper. 1965. Volatile flavor and aroma components of pineapple. II. *J. Food Sci.* **30**, 668.

Ms. rec'd 2/25/66.

This phase of the study was supported by SRI and PRI. Published with the approval of the Director of PRI as Technical Paper No. 310.

The authors are indebted to Dr. Willis A. Gortner, Human Nutrition Research Division, U. S. Dept. of Agriculture, Beltsville, Md., for his assistance and encouragement; to Dr. G. C. Bassler for preliminary experiments with the sulfur detector; and to Mr. W. A. Anderson, Jr., for the NMR spectra.

A. PINTO and C. O. CHICHESTER
Department of Food Science and Technology
University of California, Davis, California 95616

Changes in the Content of Free Amino Acids During Roasting of Cocoa Beans

SUMMARY

The major changes noted in the roasting of cocoa beans are the destruction of amino acids and reducing sugars, followed by the production of volatile carbonyl compounds. The relation between these reactions was demonstrated by means of amino acid analysis, titrimetric determination of the reducing sugars, and gas chromatography. It is postulated that the majority of the carbonyl compounds produced during the course of the roasting process are the result of the oxidative deamination of free amino acids.

INTRODUCTION

Changes in the composition of the volatiles of cocoa beans during roasting were analyzed, since this operation induces the greatest changes in aroma during the usual processing of beans for chocolate manufacture. Experiments were also conducted to determine the nature of the precursors of the aroma developed during roasting.

The formation of low-temperature-boiling aldehydes from free amino acids is not unknown. In a research note, Casey *et al.* (1965) analyzed the factors influencing the production of low-boiling volatiles from foods. Linko and Johnson (1963) studied the changes in amino acids and formation of carbonyl compounds during the baking of bread. Herz and Shallenberger (1960) obtained chocolate aroma by heating leucine, glutamine, and threonine at 100°C, and valine at 180°C with glucose.

Bailey *et al.* (1962) analyzed the chemical composition of the aroma present in the roasted cocoa beans and postulated that the aldehydes present are the result of a Strecker degradation of the free amino acids.

DeWitt (1955–56) observed an increase in free amino acid concentration during fermentation, and Rohan (1964) found that the amount of free amino acids present in cocoa beans increases during fermentation, although the increases were confined principally to leucine, phenylalanine, alanine, and valine.

Rohan (1963) extracted and concentrated the aroma precursors present in fermented cocoa beans. This extract was made up of amino acids, sugars, and flavonoid compounds.

MATERIALS AND METHODS

Materials. Four types of cocoa beans were used in this investigation. The following descriptions of each type are given in the literature:

Bahia (good quality) has a sharp, acid flavor. Its aromatic quality is very poor, or hardly aromatic.

Lagos has a basic chocolate flavor, sometimes inclined toward a trace of fruitiness and nuttiness. Not aromatic.

Puerto Cabello is used for making high-quality products; its flavor is sharp and penetrating. It has a nutty flavor and is considered one of the best-quality cocoa beans.

Arriba has an unusual floral aroma; a typical aromatic type of cocoa bean.

The samples were obtained from different manufacturers of chocolate and cocoa, but the majority were supplied by the Guittard Chocolate Company.

Methods. *Roasting.* A modified Yabez-Burns roaster was used. The equipment was set up following the procedure of Little *et al.* (1959) with the following modifications: a) The diameter of the tube feeding the gas to the burner controlled by the proportioning Robertshaw-Fulton valve was reduced. This was done to lower the heat output of the burner. The modified burner was then regulated to maintain the temperature near 130°C. b) The heat supply was controlled by changes in the temperature of the input air entering the roasting chamber. The temperature-measuring thermocouple controlling the burner, operated by a General Controls valve, type B650, was placed in the hot air flow entering the roasting chamber. In this way the temperature of the air was kept constant at 130±0.5°C. This temperature was selected following the recommendation of Cook (1963), although it is somewhat lower than that used commercially.

The roaster was loaded with approximately 300 g of cocoa beans. A 100-g sample of the same lot of raw beans was stored in a hermetically sealed glass jar for other analyses.

After 15, 30, and 45 min of roasting, the chamber was opened and a sample of approximately 100 g

was taken out. The samples were stored in glass jars similar to the one used for the unroasted sample, and allowed to cool at room temperature.

Samples for the gas chromatographic analysis. The method used was basically that of Bailey *et al.* (1962). After the shell and germ were removed, 30 g of the roasted or unroasted beans were ground in a small laboratory blender and immediately placed in a 250-ml filtering flask. The mouth of the flask was sealed with a rubber cork wrapped with aluminum foil. The lateral tube was sealed with a small serum cap.

After remaining at least 2 hr at room temperatures to reach equilibrium, 2.0-ml samples of the headspace over the ground cocoa beans were removed through the serum cap with a gas-tight Hamilton syringe, model 1002, and introduced into the gas chromatography instrument.

Extraction of water-soluble substances for analysis of free amino acids. One gram of the ground roasted or unroasted cocoa beans (shell removed) was mixed in a small laboratory Waring blender with 100 ml of distilled water, and blended for 5 min. The blender was stopped at least two times, so the walls of the flask could be washed with small amounts of distilled water, to dislodge particles adhering to the walls. The suspension obtained was then poured into a 250-ml Erlenmeyer flask. The blender was washed with distilled water, and the wash water added to the original extract.

The suspension was heated to boiling in 10 min to coagulate the proteins and peptides and after cooling the suspension was sterile filtered. A clear and brilliant solution was obtained, whose volume was adjusted to 250 ml.

Photometric determination of total free amino acids. The method of Moore and Stein (1954) for photometric determination of amino acids and related compounds was used to estimate the amount and variation in quantity of the free alpha-amino acids present in roasted and unroasted cocoa beans. Samples of the aqueous extract of roasted and unroasted beans were used (0.5 ml), and the results are expressed as leucine.

Quantitative determination of free amino acids. The sample was adjusted to pH 1.9 with citrate, and a 0.5-ml sample of a concentrated aqueous extract (concentrated from 250 ml to 27 ml by lyophilization) was analyzed in the Technicon Auto Analyzer, using an internal standard of norleucine, and compared against a Technicon 20 amino acid standard.

Gas chromatographic method. Two-ml samples of vapor from the headspace over the roasted or unroasted ground material were injected into a Perkin-Elmer gas chromatograph, model 226, equipped with a flame ionization detector. After many assays with different column materials, temperatures, and flow rates, the following conditions were selected and used in all analyses.

Column: Packed column. Coating (liquid phase) polypropylene glycol. Support material chrom. W. Particle size 80–100-mesh. Length 15 ft. Outside diameter ⅛ in. Coating wt. 15%.

Block temperature 160°C
Column temperature 100°C
Detector temperature 190°C
Chart speed 30 inches/hr
Helium pressure (carrier) 30 psi
Hydrogen pressure 12 psi
Compressed air pressure 45 psi

Identification of compounds in the aroma. Volatiles were identified on the basis of retention times compared with those of samples of known aldehydes, using the standard conditions given above. Compounds for comparison were selected initially on the basis of Bailey (1962).

Determination of reducing sugars. The extract was prepared for analysis by the method of Hassid (1936), with the following modifications: 1) the extraction was made with 80 ml of 80% alcohol over 1 g of ground cocoa beans by using a Waring blender, and letting the extract stand overnight. The next day the extract was filtered (Whatman No. 1 paper) in a Buchner funnel. Afterward it was concentrated by evaporation under vacuum to 20 ml. Then, after washing, the volume was adjusted to 30 ml, and a 10-ml portion was used for analysis. In the final clarification, 0.5 g of decolorizing carbon was used in order to obtain an extract devoid of any coloring matter. The extract was then adjusted to 50 ml, and 5.0 ml was used for determination of the reducing sugars, using the method of Hassid (1937).

RESULTS

Variations in composition of aroma during roasting. Fig. 1 is an example of the gas chromatograms obtained when the aroma of unroasted and roasted (15, 30, and 45 min) cocoa beans of four different kinds were analyzed.

Table 1 gives the values of the areas under each peak of the gas chromatograms.

Figs. 2, 3, 4, and 5 show the change in surface areas of the peaks undergoing the greatest changes with roasting time.

1) Peaks 18, 12, and 10 increase rapidly as roasting time increases. In the first 30 min the increase is almost linear with time.

2) The increase in area of peak 18 is three times that of peak 12, and many times greater than that of peak 10 or 6.

3) With roasting, peak 6 increased rapidly at

first, but the increase was only for the first 15 or 18 min.

4) Peak 14 increased with roasting up to 15 min, and then decreased.

5) The other peak slowly decreased with roasting.

6) The aromatic types of cocoa beans (Arriba and Puerto Cabello) do not have peak 14, and have only very small amounts of peak 13.

Variations in the total amount of free amino acids during roasting. Table 2 gives the total amounts of free amino acids, expressed in mg of leucine per g of beans, found in unroasted and roasted Bahia, Puerto Cabello, Lagos, and Arriba cocoa beans.

1) As the roasting time increases, the total amount of free amino acids decreases and the rate of change is almost linear with roasting time.

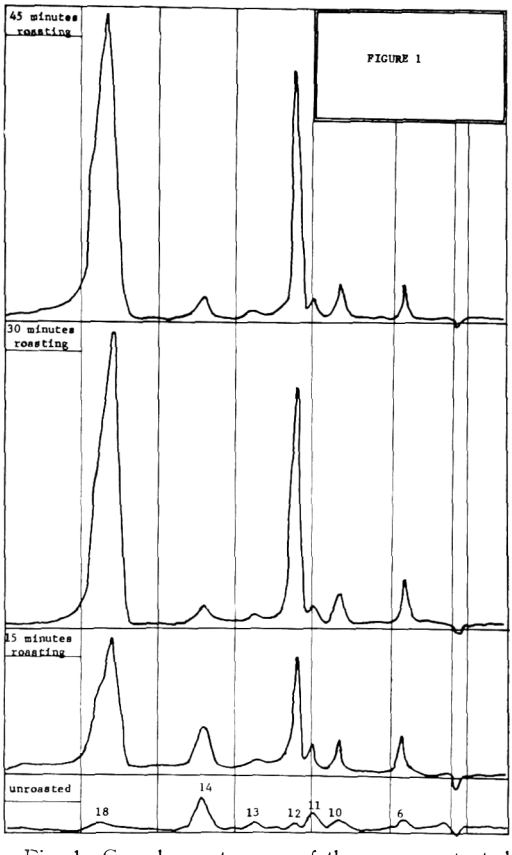


Fig. 1. Gas chromatogram of the unconcentrated flavor from unroasted and roasted Bahia cocoa beans (good quality).
6 Acetaldehyde
10 Propionaldehyde
11 Methyl alcohol
12 Isobutyraldehyde
13 Unknown
14 Diacetyl
18 Isovaleraldehyde

2) Lagos cocoa beans have a lower concentration of free amino acids, but the rate of change is the same as found in the other types.

Changes in the concentration of the individual free amino acids during roasting. Table 3 and Fig. 6 show the concentration of the individual free amino acids found in unroasted and roasted Bahia (good-quality) cocoa beans.

1) Except for methionine (almost no change), the amount of each free amino acid present became less as roasting time lengthened.

2) Glutamic acid, leucine, phenylalanine, and the unknown X_2 decreased markedly with roasting.

3) The decreases in unknown X_1, threonine, alanine, lysine, tyrosine, proline, valine, arginine, isoleucine, and histidine with roasting were significant, but not as large as those in 2, above.

4) Aspartic acid, serine, and glycine showed only small decreases.

5) The increase in the content of ammonia in cocoa beans due to roasting is of appreciable magnitude. The reactions of the ammonia with the cocoa butter on other compounds present in the bean are probably important in the generation of chocolate flavor.

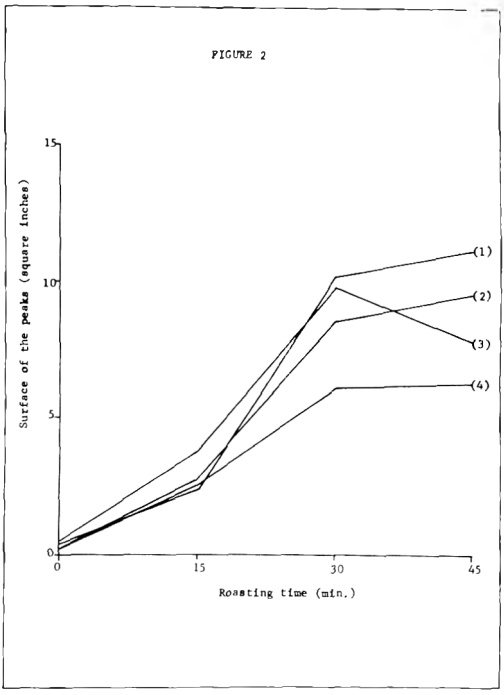


Fig. 2. Changes in surface area under Peak 18 (isovaleraldehyde) during roasting of four different varieties of cocoa beans.
(1) Lagos
(2) Puerto Cabello
(3) Arriba
(4) Bahia

Table 1. Surface areas (sq in.) under the peaks obtained in the gas chromatographic analysis of the aroma from unroasted and roasted cocoa beans.

Variety	No. of peak	Roasting time (min) 0	15	30	45
Arriba	6	0.165	0.213	0.243	0.151
	10	0.071	0.161	0.142	0.165
	11	....	....	....	....
	12	0.283	1.146	3.236	2.575
	13	....	....	....	....
	14	....	....	....	....
	18	0.567	3.858	9.862	7.826
Lagos	6	0.118	0.319	0.307	0.252
	10	0.094	0.170	0.203	0.203
	11	0.330	0.217	0.217	0.217
	12	0.247	0.954	3.527	3.873
	13	0.252	0.312	0.283	0.283
	14	0.165	0.634	0.551	1.017
	18	0.472	2.394	10.267	11.212
Puerto Cabello	6	0.038	0.113	0.165	0.165
	10	0.040	0.176	0.236	0.280
	11	0.070	0.142	0.115	0.154
	12	0.094	0.831	2.116	3.120
	13	....	....	....	....
	14	0.055	0.118	0.189	0.276
	18	0.227	2.764	8.563	9.574
Bahai (good quality)	6	0.087	0.198	0.187	0.154
	10	0.094	0.161	0.189	0.205
	11	0.198	0.236	0.189	0.154
	12	0.101	0.945	1.953	2.001
	13	0.129	0.132	0.071	0.079
	14	0.340	0.419	0.189	0.252
	18	0.251	2.645	6.141	6.299

Identification of the compound in the aroma. Table 4 gives the tentative identification of some of the peaks found in the gas chromatograms.

1) The same chemical compounds were found in varying amounts in the aroma of unroasted and roasted cocoa beans.

2) Peak 13 was not N-butyraldehyde, as was thought (Bailey, 1962), because the retention time differed slightly from that measured when the pure chemical compound was injected in the gas chromatograph, and could be separated when both were injected simultaneously.

Table 2. Total amount of alpha-amino acid present in unroasted and roasted cocoa beans (mg of leucine per g of beans).

Variety	Roasting time (min) 0	15	30	45
Bahia (good quality)	15.80	14.50	12.15	11.40
Puerto Cabello	15.16	14.70	11.69	10.40
Lagos	9.15	6.50	6.18	5.45
Arriba	14.38	14.30	12.65	12.00

Table 3. Amino acid present in unroasted and roasted Bahia (good-quality) cocoa beans (μm/g beans).

Amino acid	Roasting time (min) 0	30	45
Lysine	14.15	12.54	10.50
Histidine	4.93	4.55	3.43
Arginine	14.76	12.68	12.39
Aspartic acid	9.54	10.03	8.38
Threonine	15.57	13.28	10.41
Serine	13.10	12.94	11.96
Glutamic acid	16.76	9.50	6.55
Proline	15.78	12.38	12.38
Glycine	8.16	7.77	7.36
Alanine	32.82	32.38	28.30
Valine	17.94	17.02	15.37
Methionine	2.04	1.99	1.94
Isoleucine	11.97	11.74	9.78
Leucine	39.01	36.84	30.91
Tyrosine	13.88	12.13	10.58
Phenylalanine	26.24	23.87	20.10
Unknown X_1	8.72	5.22	3.93
Unknown X_2	23.44	19.60	16.07
Ammonia	30.08	29.18	37.77

Table 4. Identity of the peaks present in gas chromatograms of the aroma of roasted and unroasted cocoa beans and of chocolate liquor.

Peak no.	Chemical compound
6	Acetaldehyde
10	Propionaldehyde
11	Methyl alcohol
12	Isobutyraldehyde
14	Diacetyl
18	Isovaleraldehyde

Changes in reducing sugars during roasting. Table 5 gives the average reducing sugar contents, expressed as dextrose equivalent, in unroasted and roasted Bahia cocoa beans (good quality). It also shows the degrees Brix of the extract and Fig. 7 shows these changes as a function of roasting time.

With roasting, the amount of reducing sugars present in the cocoa beans decreased. The rate of decrease was constant and linear, and the amount of soluble solids present in the aqueous extract (measured in degrees Brix) remained constant for the first 30 min, and then decreased.

Table 5. Changes in the reducing sugars during roasting of Bahia cocoa beans (good quality).

	Roasting time (min)			
	0	15	30	45
Reducing sugars (mg of dextrose per g of cocoa beans)	17.62	12.22	8.04	3.94
Degrees Brix of extract at 20°C	3.0°	3.0°	3.0°	1.8°

DISCUSSION

During roasting the free amino acids are transformed into structurally related aldehydes by a process of deamination and decarboxylation. These volatile compounds are important constituents of the aroma present in the roasted cocoa beans and chocolate liquor, and are partially responsible for the final chocolate aroma. The aldehydes cause the pungent odor present in the freshly roasted beans.

The chemical reactions involved in this process are not completely understood. Two possibilities exist: 1) A thermal degradation (deamination and decarboxylation) of the free amino acids induced by the heating under roasting temperatures; or, more likely,

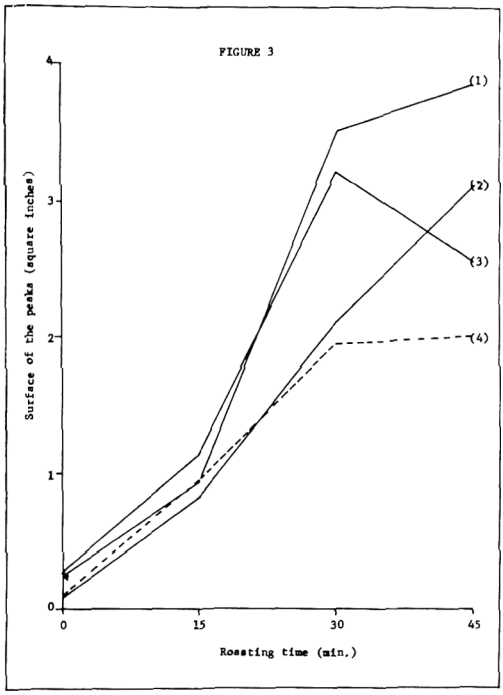


Fig. 3. Changes in surface area under Peak 12 (isobutyraldehyde) during roasting of four different varieties of cocoa beans.
(1) Lagos
(2) Puerto Cabello
(3) Arriba
(4) Bahia

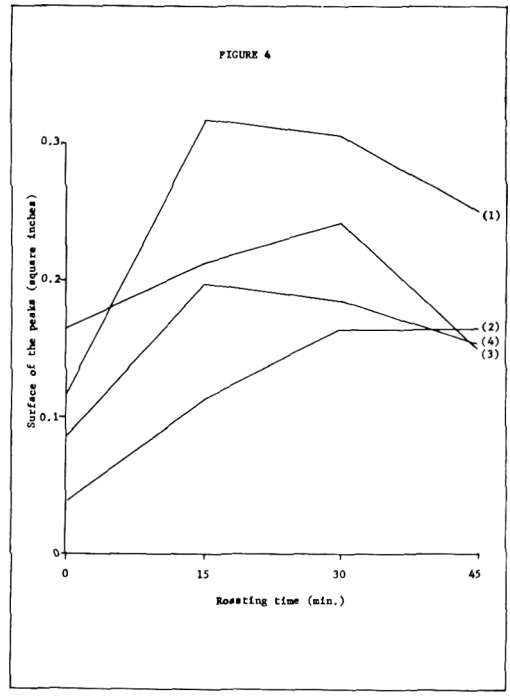


Fig. 4. Changes in surface area under Peak 6 (acetaldehyde) during roasting of four different varieties of cocoa beans.
(1) Lagos
(2) Puerto Cabello
(3) Arriba
(4) Bahia

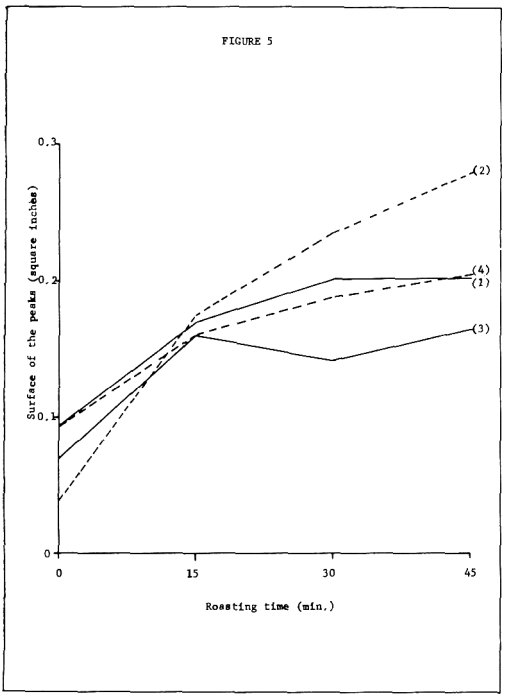


Fig. 5. Changes in surface area under Peak 10 (propionaldehyde) during roasting of four different varieties of cocoa beans.
(1) Lagos
(2) Puerto Cabello
(3) Arriba
(4) Bahia

2) the reactions of the amino acids with reducing sugars to form Amadori rearrangement products (Anet, 1957), which upon continued heating will decompose to aldehydes and other compounds derived from the sugar moiety. A common feature in the formation of these volatiles from the amino acids is ammonia production as a consequence of the deamination process. The ammonia liberated in this reaction can react with reducing sugars (Schonberg *et al.*, 1948) and with other substances present in the cocoa beans to yield products which may influence the aroma and flavor of the chocolate. The Dutch process of alkalization, although somewhat more severe in its action, gives evidence of the flavor and color changes which can result from basic treatment of cocoa liquor.

The fact that this increase in ammonia appears at between 30 and 45 minutes roasting times can be interpreted either that after this much time the compounds reacting with the ammonia become saturated, or that the rate of ammonia release is so large that it appears as free ammonia. It is also possible that the deamination to produce ammonia does not occur until the critical concentration of intermediate is produced, e.g., late in the roasting process.

The large release of ammonia after long roasting would explain the formation of off-flavors when the cocoa beans are roasted longer than the conventional times.

The characteristics of the cocoa lipids must have a definitive influence in the retention of the aldehydes produced; for example, the amount of acetaldehyde produced is probably equal to or larger than isovaleraldehyde, but its retention is smaller because of its volatility, or poorer adsorption.

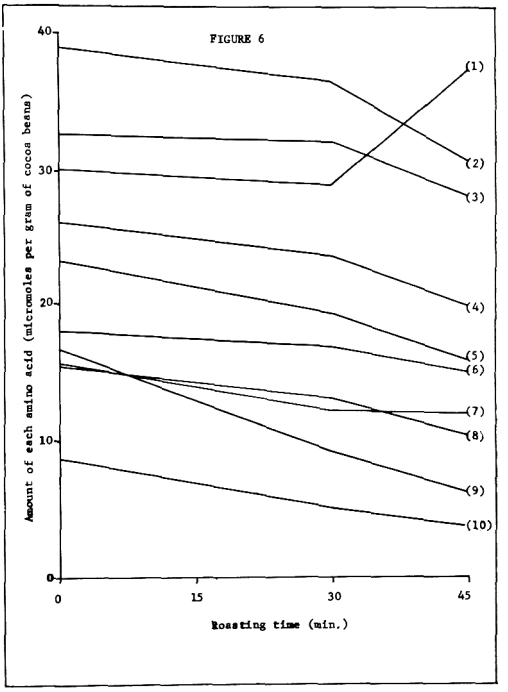


Fig. 6. Change in the amount of each amino acid during roasting of Bahia (good-quality) cocoa beans.
(1) Ammonia
(2) Leucine
(3) Alanine
(4) Phenylalanine
(5) Unknown X_2
(6) Valine
(7) Proline
(8) Threonine
(9) Glutamic acid
(10) Unknown X_1

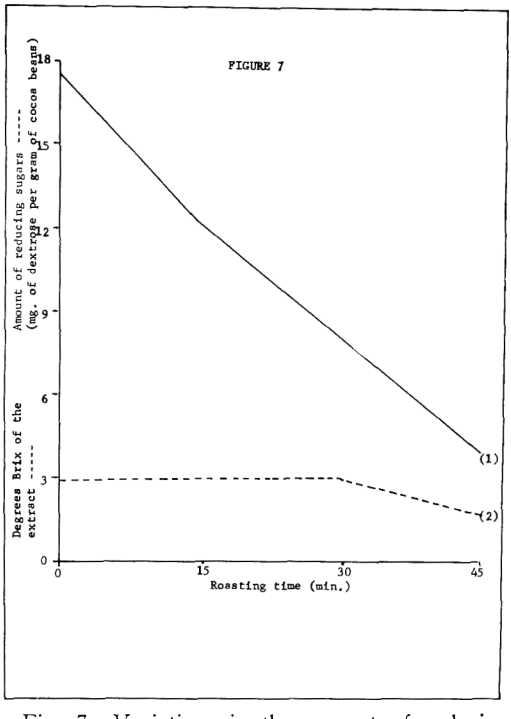


Fig. 7. Variations in the amount of reducing sugars during the roasting of Bahai cocoa beans.
(1) Reducing sugar expressed as mg of dextrose.
(2) Degrees Brix of the extract.

It is interesting that during the fermentation of cocoa beans there is an increase in the amount of those free amino acids that form isobutyraldehyde and isovaleraldehyde in roasting.

The simple techniques used in the present investigation for the determination of the amounts of free amino acids and reducing sugars can be useful in evaluating the quality of unroasted cocoa beans in order to specify chocolate blends at least partially.

REFERENCES

Anet, E. F. L. J. 1957. Chemistry of non-enzymic browning. II. Some crystalline amino acid-deoxy-sugars. *Australian J. Chem.* **10**, 193.

Anet, E. F. L. J., and T. M. Reynolds. 1957. Chemistry of non-enzymic browning. I. Reactions between amino acids, organic acids, and sugars in freeze-dried apricots and peaches. *Australian J. Chem.* **10**, 182.

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

Casey, J. C., R. Self, and T. Swain. 1965. Factors influencing the production of low boiling volatiles from foods. *J. Food Sci.* **29**, 456.

Cook, L. R. 1963. Chocolate production and use. Catalog and Book Division Magazines for Industry, Inc., New York.

DeWitt, K. N. 1955–56. *Imp. Coll. Trop. Agr.* (Trinidad), Rept. Cacao Res. 1955–56, **54**.

Hassid, W. Z. 1936. Determination of reducing sugars and sucrose in plant materials. *Ind. Eng. Chem. Anal.*, ed. 8, 138.

Hassid, W. Z. 1937. Determination of sugars in plants by oxidation with ferricyanide and ceric sulfate titration. *Ind. Eng. Chem. Anal.*, ed. **9**, 228.

Herz, W. J., and R. S. Shallenberger. 1960. Some aromas produced by simple amino acid, sugar reaction. *Food Research* **25**, 491.

Linko, Y. Y., and J. A. Johnson. 1963. Changes in amino acids and formation of carbonyl compounds during baking. *Agr. Food Chem.* **11**, 150.

Little, A. C., C. O. Chichester, and G. Mackinney. 1959. On the color of coffee. III. *Food Technol.* **8**, 684.

Moore, S., and W. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**, 907.

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

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

Schonberg, A., R. Moubasher, and A. Moustafa. 1948. Degradation of alpha amino acids to aldehydes and ketones by interaction with carbonyl compounds. *J. Chem. Soc.* **163**, 176.

Ms. rec'd 5/19/65.

B. K. NORTJÉ
Fruit and Food Technology Research Institute, Stellenbosch, South Africa

Some Catechins and Proanthocyanidins in the Cores of Bartlett Pears

SUMMARY

Two catechins and two proanthocyanidins were isolated from the cores of Bartlett pears. The catechins, (+)-catechin, and (−)-epicatechin were obtained in crystalline form. Proanthocyanidin P-1 was found to be identical with the major proanthocyanidin of cacao beans. Proanthocyanidin P-2 was found to consist of at least three flavan units. Treatment of proanthocyanidin P-2 with sulfurous acid yielded proanthocyanidin P-1.

INTRODUCTION

The discoloration of Bartlett pears during canning was investigated by Luh *et al.* (1960). The pigment isolated from such fruit was found to resemble cyanidin. Polyphenolic compounds which are converted to anthocyanidins by acid treatment are usually referred to as "leucoanthocyanins." Since these compounds can be classified in different groups, the term "proanthocyanidin," which has no explicit structural implication, has been proposed by Freudenberg and Weinges (1960).

The group of proanthocyanidins, of which the known examples are C_{30} compounds formed by coupling two flavan units, have been investigated recently, but there seems to be uncertainty as to the exact chemical structure of these compounds (Forsyth and Roberts, 1960; Freudenberg and Weinges, 1962; Hsia *et al.*, 1964; Geissman and Dittmar, 1955). Recently, Mayer *et al.* (1966) presented evidence that the two units of the proanthocyanidin present in the seeds of *Aesculus hippocastanum* are joined at two positions.

Polymeric proanthocyanidin material has previously been found present in perry pears (Williams, 1960). The present study provides details of the isolation and characterization of two proanthocyanidins and two catechins from the cores of the fruit of *Pyrus communis* L. cv. Bartlett.

EXPERIMENTAL

Reagents and materials. Whatman no. 3 MM paper sheets (46 × 57 cm) were used for preparative separations. Whatman no. 1 paper sheets (46 × 57 cm) were used for analytical purposes. *n*-Butanol–acetic acid–water (20:5:11, by vol.), hereinafter referred to as BAW, and aqueous 2% acetic acid were used as solvent systems. All chromatograms were developed by downward migration. Ammoniacal silver nitrate reagent and alcoholic 3% toluene-*p*-sulfonic acid were used as spray reagents (Roux and Maihs, 1960).

Spectrophotometry. Absorption spectra in the visible region of the spectrum were recorded with a Carl Zeiss PMQ II spectrophotometer. Infrared spectra were recorded with a Beckman IR-9 infrared spectrophotometer by the KBr pellet technique.

Distribution of catechins and proanthocyanidins in Bartlett pears. The peels, flesh, cores, and seeds of Bartlett pears were examined by extracting 5 g of each part of the fruit with 95% ethanol (25 ml) and spotting different concentrations of the extracts on Whatman no. 1 paper sheets. After two-way chromatography with BAW and aqueous 2% acetic acid as solvent systems, the major polyphenolic constituents were detected under ultraviolet light and by treatment of different chromatograms with ammoniacal silver nitrate and ethanolic 3% toluene-*p*-sulfonic acid.

Isolation of the catechins and proanthocyanidins. Bartlett pear cores (3 Kg) were macerated with acetone (5 L) in a blender and filtered through Büchner funnels. The material on the filters was washed with aqueous 50% (v/v) acetone until nearly colorless. The combined extracts (10 L) were evaporated under reduced pressure to approximately 1.5 L and continuously extracted with chloroform for 12 hr. The aqueous solution was then extracted with ethyl acetate (5 × 300 ml) to yield a total of 4.3 g extractables. This material was dissolved in acetone (50 ml) and applied in 4-cm bands on the shorter sides of 24 Whatman no. 3 MM paper sheets, which were developed with BAW. After drying in a current of air at 30–35°C, the individual bands were located by cutting a strip from the side of one paper and treating it with alcoholic 3% toluene-*p*-sulfonic acid. Four brownish-red bands were produced by this treatment. Bands I, II, III, and IV (numbered in descending order of mobility) were eluted

separately with aqueous 70% ethanol. Each eluate was concentrated under reduced pressure and rechromatographed on 20 paper sheets with aqueous 2% acetic acid as solvent system. The final eluates were concentrated to small volumes and extracted with ethyl acetate (6 × 50 ml). This solvent was removed under reduced pressure.

Identification of the catechins. The materials from bands I and II crystallized as white needles from water. After recrystallization from water the respective isolates were identified as (+)-catechin and (−)-epicatechin by melting point, specific rotation, infrared spectra, and chromatographic properties of the free compounds and of their penta-O-acetyl derivatives (Weinges, 1964).

Purity of the proanthocyanidins. The light-brown amorphous powders obtained from bands III and IV were respectively named proanthocyanidins P-1 and P-2. When chromatographed with BAW and aqueous 2% acetic acid as solvent systems, both substances migrated as single spots, with a slight degree of tailing.

Hydrolysis of the proanthocyanidins with hydrochloric acid. The isolated proanthocyanidins were hydrolyzed by heating 15 mg of the material with 2N HCl (10 ml) for 60 min on a boiling-water bath. After cooling, the solution was extracted with a small quantity of *n*-butanol and the pigment in the organic layer was compared with authentic cyanidin by paper chromatography. A small quantity of the butanol extract, suitably diluted with ethanol containing 0.1% HCl, was used for determination of the absorption spectrum over the range 360–570 mμ. Alcoholic $AlCl_3$ (5%, 0.5 ml) was added to this solution (2.5 ml), and the shift in the position of the absorption maximum at 546 mμ was noted to establish the presence of a free *o*-dihydroxyl grouping in the molecule (Geissman *et al.*, 1953; Harborne, 1958).

The aqueous phase, after extraction of the pigment, was neutralized and desalted electrolytically. Chromatographic analysis of the resultant solution failed to reveal the presence of sugars.

Treatment with sulfurous acid. Oxidative condensation of the proanthocyanidins during acid hydrolysis was counteracted by the use of sulfurous acid (Forsyth and Roberts, 1960). Proanthocyanidin material (100 mg) was dissolved in water (10 ml), and sulfur dioxide was bubbled through for several minutes, followed by heating on a boiling-water bath with continued bubbling of sulfur dioxide. After heating for 45 min, about 5 ml of the solution was withdrawn and cooled to room temperature. The remainder of the solution was heated for a total time of 2 hr before cooling. The two fractions were extracted separately with ethyl acetate (6 × 10 ml). The ethyl acetate and aqueous fractions were examined by paper chromatography using BAW and aqueous 2% acetic acid as solvent systems. The major components present in the ethyl acetate fractions were then separated on Whatman no. 3 MM paper sheets, using BAW as solvent system, and identified by paper chromatography and infrared spectroscopy.

The major proanthocyanidin of cacao (cacao leucocyanidin 1) was treated in the same way for comparison.

RESULTS AND DISCUSSION

The preliminary examination of the peels, flesh, cores, and seeds of Bartlett pears revealed two catechins and two proanthocyanidins in all these parts. These components were present in relatively high concentrations in the peels and cores. Because of the presence of flavonol glycosides in the peels (Nortjé and Koeppen, 1965), the cores were considered more suitable for isolation of the catechins and proanthocyanidins.

Though catechins have been reported present in the fruit of various plants on the basis of their detection by paper chromatography, (+)-catechin and (−)-epicatechin have been isolated from only a limited number of fruits. (+)-Catechin has been isolated from the fruit of *Castanea vesca* (Schmidt and Hüll, 1947), *Cola acuminata* (Freudenberg and Oehler, 1930), *Gleditschia triacanthos, Vaccinium vitis-idaea, Rosa* spp., *Prunus amygdalus,* and *Wisteria sinensis* (Weinges, 1964). (−)-Epicatechin has been isolated from the fruit of *Theobroma cacao* (Freudenberg *et al.*, 1932), *Malus* spp. (Nakabayashi, 1952), *Cola acuminata* (Freudenberg and Oehler, 1930), *Crataegus oxyacantha, Crataegus monogyna, Crataegus lavallei, Pyracantha coccinea, Prunus amygdalus,* and *Wisteria sinensis* (Weinges, 1964). The isolation of (+)-catechin and (−)-epicatechin in crystalline form from Bartlett pears is significant since these compounds have so far been found to occur together in the fruit of only three other species.

The two proanthocyanidins isolated from the cores of Bartlett pears appeared on chromatograms as separate spots free from impurities. Proanthocyanidin P-1 had R_f values of 0.47 in BAW and 0.51 in aqueous 2% acetic acid. Proanthocyanidin P-2 had

R_f values of 0.37 in BAW and 0.41 in aqueous 2% acetic acid. Differences in the infrared spectra of the two compounds were observed, particularly in the region of 840 to 760 cm^{-1}.

Proanthocyanidin P-1 was found to be chromatographically identical with the major proanthocyanidin of cacao beans (cacao leucocyanidin 1), which has been found to be a dimer consisting of a 5,7,3′,4′-tetrahydroxyflavan-3,4-diol unit and an (−)-epicatechin unit (Forsyth and Roberts, 1960).

The ethyl acetate extract obtained after treatment of proanthocyanidin P-2 with sulfurous acid for 45 min showed the presence of four components when examined by paper chromatography. After purification, the individual substances were identified as (+)-catechin, (−)-epicatechin, proanthocyanidin P-1, and unchanged proanthocyanidin P-2. Treatment of proanthocyanidin P-2 with sulfurous acid for 120 min results in an ethyl acetate extract which contained mainly (+)-catechin and (−)-epicatechin, present in approximately equal concentrations. Examination of the aqueous phase, after extraction with ethyl acetate, revealed the presence of only a single spot, which corresponds to substance A, described by Forsyth and Roberts (1960). This indicated that the proanthocyanidin P-2 molecule consists of at least three flavan units which can be separated by treatment with sulfurous acid. At present it is uncertain how the different units of proanthocyanidin P-2 are linked, but further investigation of the structural details of this compound is needed in view of the proposed mechanism of dimer formation (Geissman and Dittmar, 1965).

REFERENCES

Forsyth, W. G. C., and J. B. Roberts. 1960. Cacao polyphenolic substances. 5. The structure of cacao "leucocyanidin 1" *Biochem. J.* **74**, 374.

Freudenberg, K., and L. Oehler. 1930. Die Catachine der Colanuss. *Liebigs Ann.* **483**, 140.

Freudenberg, K., and K. Weinges. 1960. Systematik und Nomenklatur der Flavonoide. *Tetrahedron* **8**, 336.

Freudenberg, K., and K. Weinges. 1962. Catechins and flavonoid tannins. *In:* "The Chemistry of Favonoid Compounds." T. A. Geissmann, ed.) Pergamon Press, Oxford.

Freudenberg, K., R. F. B. Cox, and E. Braun. 1932. The catechins of the cacao bean. *J. Am. Chem. Soc.* **54**, 1913.

Geissman, T. A., and H. F. K. Dittmar. 1965. A proanthocyanidin from avocado seed. *Phytochemistry* **4**, 359.

Geissman, T. A., E. C. Jorgenson, and J. B. Harborne. 1953. The effect of aluminum chloride on the absorption spectra of Anthocyanins. *Chem. & Ind. (London)* **1953**, 1289.

Harborne, J. B. 1958. Spectral methods of characterizing anthocyanins. *Biochem. J.* **70**, 22.

Hsia, C., L. L. Claypool, J. L. Abernethy, and P. Esau. 1964. Leucoanthocyan material from immature peaches. *J. Food Sci.* **29**, 723.

Luh, B. S., S. J. Leonard, and D. S. Patel, 1960. Pink discoloration in canned Bartlett pears. *Food Technol.* **14**, 53.

Mayer, W., L. Goll, E. M. Von Arndt, and A. Mannschreck. 1966. Procyanidino-(−)-epicatechin, ein zweiarmig verknüpftes, kondensiertes proanthocyanidin aus *Aesculus hippocastanum*. *Tetrahedron Letters* **4**, 429.

Nakabayashi, T. 1952. Browning of apple fruit. 1. Isolation and estimation of chlorogenic acid and L-epicatechin. *J. Agr. Chem. Soc. Japan.* **26**, 813; cited in *Chem. Abstr.* **48**, 5943.

Nortjé, B. K., and B. H. Koeppen. 1965. The flavonoid glycosides in the fruit of Pyrus communis L. cultivar Bon Chrétien. *Biochem. J.* **97**, 209.

Roux, D. G., and A. E. Maihs. 1960. Selective spray reagents for the identification and estimation of flavonoid compounds associated with condensed tannins. *J. Chromatog.* **4**, 65.

Schmidt, O. T., and G. Hüll. 1947. Über das Vorkommen von Catechin in den Fruchtschalen der Edelkastanie. *Chem. Ber.* **80**, 509.

Weinges, K. 1964. The occurrence of catechins in fruits. *Phytochemistry* **3**, 263.

Williams, A. H. 1960. The distribution of phenolic compounds in apple and pear trees. *In:* "Phenolics in Plants in Health and Disease." (J. B. Pridham, ed.) Pergamon Press, Oxford.

Ms. rec'd 2/28/66.

The author is indebted to Dr. B. H. Koeppen for helpful discussions, and to Dr. V. C. Quesnel for a gift of cacao proanthocyanidin. The technical assistance of Mr. G. L. Wium is gratefully acknowledged.

TERUHIKO MORIMOTO

Tsukaguchi Division Laboratory of Confectionery Institute, Morinaga Seika Co., 400 Kamisakake, Amagasaki City, Hyogo, Japan

Studies on Free Amino Acids in Sponges, Doughs, and Baked Soda Crackers and Bread

SUMMARY

Free amino acids decreased markedly during an 18-hr cracker sponge fermentation or during a 6-hr straight dough bread fermentation. No commercial proteases were used. However, the free amino acids increased after 48 and 72 hr of cracker sponge fermentation, respectively. This suggested that the amino acids were metabolized by microorganisms during active growth at a faster rate than they were being formed from the protein by native proteases. Later, the amino acids accumulated as the rate of active fermentation and growth subsided. Proteolytic activities did not change with fermentation time, suggesting that microorganisms did not release additional proteases. The amino acid content of soda crackers made with a long sponge fermentation decreased during baking. Likewise, the free amino acid content of bread crust decreased during baking, while the free amino acid content of the crumb remained essentially constant.

When a commercial protease was added to a soda cracker sponge and the sponge fermented for 18 hr, the free amino acids in the baked products increased to a level equivalent to that of the soda crackers made with a 72-hr sponge fermentation without the use of commercial protease.

INTRODUCTION

The chemistry of browning as related to the Maillard reaction (Maillard, 1912) has been reviewed by Hodge (1953, 1955). The relationship of the Maillard reaction to the browning of baked products has been studied by Baker *et al.* (1953), Kielly *et al.* (1960), and Johnson and Miller (1961). Linko and Johnson (1963) reported on the changes in amino acids and the formation of carbonyl compounds during bread baking. Wiseblatt and Zoumut (1963) reported that a bread flavor constituent was produced by the reaction of proline and dihydroxyacetone. The above studies were made from the viewpoint that amino acids serve as precursors for bread flavor produced by the baking process.

Since amino acids are precursors of the Maillard reaction, Horvat *et al.* (1962) studied the free amino acids in a bread dough and concluded that browning of bread might be related more highly to the insoluble protein fraction than to the presence of free amino acids. Kretovich and Ponomareva (1961), studying the amino acid change in rye and white breads, concluded that free amino acids changed appreciably in rye doughs but only slightly in white bread doughs. No study has been made concerning amino acid changes in soda cracker.

The present investigation was made to study changes in the free amino acids during the making of soda crackers. The amino acids were of interest because of their relation to the Maillard reaction during baking of the soda crackers. The effect of commercial protease was included because of the creation of free amino acids during fermentation as a function of proteolysis of the protein. Also included were changes in the amino acids of a French bread formulation.

EXPERIMENTAL METHODS

Sponge and dough preparation of soda cracker. The sponge of soda crackers was made by mixing of 700 g of wheat flour, 30 g of shortening, 2 g of compressed yeast, 2 g of yeast food, and 270 ml of water. The dough was made by mixing the above sponge with 300 g of wheat flour, 70 g of shortening, 15 g of salt, 16 g of sucrose, and variable amounts of sodium bicarbonate.

Rhozyme A-4 (Rohm and Hass Co.) was used in the sponge in experiments requiring greater amounts of proteolysis.

Straight dough preparation of bread. A kind of French bread dough was made by mixing 1000 g of wheat flour, 20 g of sugar, 20 g of salt, 10 g of shortening, 20 g of compressed yeast, and 625 ml of water.

Fermentation. The soda cracker sponge was fermented at 27°C for various periods. The dough was fermented at 32°C for 5 hr before sheeting. The bread dough was given a primary fermentation of 6 hr, followed by 30 min of final proof. All fermentation of bread was done at 26°C.

Extraction and concentration of free amino acids. The cracker sponge, cracker or bread dough, or baked products thereof were broken into small

pieces, and 100 g were extracted three times with 500 ml of 70% ethanol at 65°C. The extracts were recovered by centrifugation. The supernatant was concentrated at 65°C under vacuum of a water pump. The concentrate was dialyzed against 40 times volume of distilled water for 24 hr at 10°C. The dialysate was concentrated at 65°C under vacuum of a water pump.

Quantitative analysis of amino acids. The concentrated amino acids solution was analyzed by an amino acid autoanalyzer KLA-Type II (Hitachi Co.). Ion-exchange resin Amberlite CG-120 Type III (Moore *et al.*, 1958; Spackman *et al.*, 1958) was used. For chromatography of the acidic and neutral amino acids, a 0.2*N* citrate buffer, pH 3.25 and pH 4.25, was used. For alkaline amino acids, a 0.35*N* citrate buffer, pH 5.28, was used.

Replication determinations of the amino acids were made in most instances, and although the results varied slightly, the trends of changes in amino acid composition with fermentation and baking were consistent.

Proteolytic activity of sponges. A preliminary experiment indicated that the proteolytic activity of sponge was stronger at pH 3 than at pH 4 and pH 7. The proteolytic activity of sponges, therefore, was estimated by extracting the proteases from 100 g of sponge with 100 ml of 0.1*N* lactate buffer at pH 3 for 1 hr and allowing the extract to act on a casein substrate (pH 3). The extract was diluted 5 times with the lactate buffer before it was added to the casein. A digestion time of 3.5 hr at 38°C was allowed. The assay of protease activity was followed by Folin's method (Folin and Ciocalteu, 1927). Proteolytic activity was expressed in arbitrary units as optical density according to Folin's method.

Viable counts of microorganisms. One hundred grams of sponge were added to 500 ml of sterilized phosphate buffer (0.025*M*, pH 7) and homogenized in a homo-mixer for 5 min. After dilution of the homogenized suspension to 10^4 or 10^5 times, 1 ml was used for plate cultures. To determine viable counts of yeast, a medium consisting of 1.5% glucose, 1.0% peptone, 0.5% yeast extract, and 2.5% agar-agar was used. Used to determine viable counts of bacteria was the medium "Daigo" (Takeda Chemicals Co.), containing the antibiotic Eurocidin, which depresses growth of fungi and yeast. By keeping the plate cultures at 37°C for 2 days, using both kinds of media, counts of colonies of both yeast and bacteria were possible.

RESULTS AND DISCUSSION

Change in amino acids during sponge and dough fermentation of soda cracker. In the cracker sponge, all amino acids except cystin and cystein were found at the beginning of fermentation (Table 1). It was established by a preliminary experiment that the free amino acids were derived from the flour and not the yeast. Naturally, the compressed yeast contained a small amount of free amino acids, but this amount was negligible in the sponge compared to those provided by the flour. Only 0.2% of yeast was used in the sponge of soda cracker.

Serine, alanine, and aspartic acid were present in comparatively large amounts in the 0-hr sponge. Table 1 shows, however, that the amino acids decreased after 18 hr of fermentation. Certain amino acids could not be expressed quantitatively, because they were present in too small a quantity to be estimated by the analyzer. The tendency of the amino acids to decrease with fermentation time suggests that free amino acids were metabolized by microorganisms in the cracker sponge. As Micka (1955) showed, the soda cracker fermentation is a symbiosis rather than a fermentation by a single organism. The changes in the bacteria and yeast population in soda cracker sponge are illustrated in Fig. 1. Viable yeast counts in the sponge could be estimated at the beginning of the fermentation, but the bacteria counts could not be estimated until after 10 hr of fermentation. The number of yeast cells in

Table 1. Change of free amino acids during fermentation of cracker sponges and doughs.

	μM/g (dry basis)			
	Sponge		Dough	
Amino acids	0 hr	18 hr	0 hr	5 hr
Aspartic acid	0.215	0.020	0.050	0.050
Threonine	0.075	0.020	0.040	0.045
Serine	0.525	0.045	0.105	0.095
Glutamic acid	0.195	0.055	0.033	0.085
Proline	0.135	0.108	0.060	0.080
Glycine	0.145	0.090	0.060	0.055
Alanine	0.535	0.155	0.130	0.145
Valine	0.150	0.025	0.038	0.035
Methionine	0.025	.....	.....	.....
Isoleucine	0.108	.....	0.018	0.020
Leucine	0.127	0.015	0.022	0.025
Tyrosine	0.074	.....	0.010	0.010
Phenylalanine	0.062	.....	0.010	0.010
Tryptophan	0.150	.....	0.020	0.020
Lysine	0.030	0.055	0.015	0.030
Histidine	0.026	0.008	.....	.....
Arginine	0.040	0.005	0.010	0.012

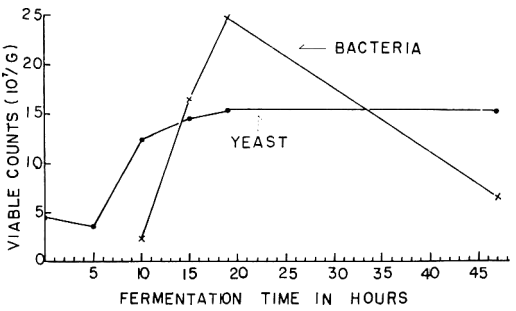


Fig. 1. Change of viable counts of microorganisms during fermentation of soda cracker sponge with fermentation time. Viable counts were expressed per g of sponge.

the cracker sponge increased up to 19 hr of fermentation and thereafter remained essentially constant. The number of bacteria increased markedly between 10 and 19 hr of fermentation and then decreased sharply. These data agree with those of Micka (1955). The bacteria were derived from both the yeast and the flour. These bacteria were of the genus *Coccus* as shown microscopically. They were gram-positive and produced lactic acid in a liquid medium containing arabinose, xylose, glucose, lactose, sucrose, maltose, or glycerol. These bacteria did not develop in a liquid medium containing 6.5% sodium chloride. Based on these characteristics and those given by Bergey's Manual, it was assumed that they belonged to the genus *Pediococcus* and were neither *Streptococcus faecalis* nor *Leuconostoc mesenteroides*.

Because of these results, it was assumed that the free amino acids in the sponge decreased as a result of metabolism by the microorganisms which continued to increase until after 18 hr of fermentation.

The data in Table 1 indicate that the amino acid content of the dough after mixing with the sponge was essentially the same as for the 18-hr sponge. Likewise, the amino acid content of the dough did not change even after 5 hr of fermentation in the dough stage.

Since the evidence indicated that the free amino acid in cracker sponges decreased after 18 hr of fermentation and did not increase in the dough, it is likely that the amino acids would not contribute greatly to the flavor of soda cracker. In an attempt to increase the amount of free amino acids at the end of the dough stage, fermentation of the sponges was extended to 48 and 72 hr, respectively. As shown by the data in Table 2, fermentation of the sponge for 48 or 72 hr increased the amino acid content. Alanine content increased the most. Serine, proline, glycine, valine, leucine, lysine, and arginine increased significantly. Seventy-two hours of fermentation increased the amino acid content more than did 48 hr of fermentation.

Table 2. Increase of free amino acids of soda cracker sponge during long fermentation.

	μM/g (dry basis)	
Amino acids	48-hr sponge	72-hr sponge
Aspartic acid	0.079	0.090
Threonine	0.188	0.392
Serine	0.565	0.890
Glutamic acid	0.395	0.408
Proline	0.650	0.782
Glycine	0.615	0.920
Alanine	2.100	3.292
Valine	0.290	0.660
Methionine	0.050	0.170
Isoleucine	0.100	0.280
Leucine	0.320	1.020
Tryosine	0.150	0.288
Phenylalanine	0.190	0.505
Tryptophan	0.080	0.140
Lysine	0.510	0.755
Histidine	0.080	0.165
Arginine	0.390	0.695

It appeared that, after the active growth period of 18 hr, the free amino acids could accumulate in the sponge because the organisms no longer metabolized the amino acid at a rapid rate.

Amino acids in soda cracker. The amino acid content of the baked soda cracker after 18 and 72 hr of sponge followed by 5 hr of dough fermentation was compared. The data are summarized in Table 3. Comparing the amino acid content of the dough made with an 18-hr sponge fermentation (Table 1) with the amino acid content of the baked soda cracker (Table 3), it is evident that there was little or no change. Apparently, the amino acid content of the dough after 18 hr of fermentation was so small that little remained to react during the baking process. With 72 hr of sponge fermentation, the amino acid content of the soda cracker decreased significantly during the baking

Table 3. Comparison of free amino acids content in soda crackers made from 18 and 72 hr fermented sponge.

Amino acids	μM/g (dry basis) 18 hr	72 hr
Aspartic acid	0.110	0.020
Threonine plus Serine	0.170	0.345
Glutamic acid	0.110	0.050
Proline	0.120	0.248
Glycine	0.090	0.080
Alanine	0.230	0.720
Valine	0.085	0.165
Methionine	0.025	0.025
Isoleucine	0.030	0.070
Leucine	0.045	0.142
Tyrosine	0.025	0.048
Phenylalanine	0.030	0.068
Tryptophan	0.060	0.070
Lysine	0.050	0.082
Histidine	0.012	0.025
Arginine	0.050	0.032

process. Even so, the amino acid content was more than that of the crackers made with 18 hr of sponge fermentation. It was also significant that the flavor of the cracker after 72 hr of sponge fermentation was much improved over the flavor of crackers produced with only 18 hr of sponge fermentation.

Activities of proteases in the sponge. The exact reason for the increase in the free amino acids in the 48- and 72-hr cracker sponge is not clear. It could have been due to a release of proteases either by the yeast or bacteria, or to proteases normally present in the flour. Measurement of the proteolytic activity in the sponges at various stages of fermentation, summarized in Table 4, indicated that the activity remained essentially constant. The proteolytic activity in flour doughs when yeast was eliminated was the same as for 0-hr cracker sponges. It was concluded, therefore, that release of proteases in the sponge by microorganisms was likely not an important factor. It would appear that the free amino acids increased in the 48- and 72-hr sponges because the organisms metabolized less of the amino acids, whereas greater amounts of the amino acids were being produced by flour proteases with the extended fermentation time.

Table 4. Relationship between fermentation time and protease activity in soda cracker sponge.

Fermentation time (hr)	Protease activity
0	0.205
24	0.196
48	0.176
72	0.193

Effect of commercial protease enzymes on amino acid content of crackers. Previous experiments had demonstrated that free amino acids increase in 48- or 72-hr sponges. However, it is not economical or practical to use such long fermentation times. Accordingly, an experiment was designed in which a commercial protease (Rhozyme A-4) was added to the cracker sponge to hasten the release of free amino acids during fermentation. The data of Table 5 indicate that the addition of 200 mg of protease per 700 g of flour caused the free amino acids content of the 18-hr sponge to be equivalent to the free amino acids content of the cracker made with 72 hr of fermentation. In contrast, the addition of 10 mg of the protease to 700 g of flour did not increase the free amino acids content of the cracker made with 18 hr of fermentation.

It is of interest that addition of 200 mg of Rhozyme A-4 with 18 hr of fermentation, or 72 hr of fermentation without addition of the protease, caused a particular increase

Table 5. Free amino acids in soda cracker produced by using a commercial protease with 18 hr of sponge fermentation.

Amino acids	μM/g (dry basis) 10 mg protease	200 mg protease
Aspartic acid	0.040	0.117
Threonine	0.015	0.110
Serine	0.100	0.270
Glutamic acid	0.100	0.175
Proline	0.160	0.540
Glycine	0.062	0.190
Alanine	0.310	1.090
Valine	0.040	0.208
Methionine	.....	0.030
Isoleucine	0.020	0.120
Leucine	0.025	0.320
Tyrosine	0.015	0.075
Phenylalanine	0.010	0.126
Tryptophan	.....	0.098
Lysine	0.035	0.225
Histidine	.....	0.070
Arginine	0.035	0.390

in alanine and proline. The increased intensity of the flavor of the soda crackers made with the protease or the extended fermentation time may be due, in fact, to the increased amount of proline which is known to produce a specific cracker flavor (Wiseblatt and Zoumut, 1963; Morimoto and Johnson, 1966). According to those workers, the cracker flavor produced from proline is highly polar and may be bound to any sites in the cracker or the bread. It is believed that cracker flavor is somewhat different from bread flavor. Naturally, aldehydes also may be produced in soda cracker baking by Maillard-type reaction, but these products may escape from crackers readily during baking at a high temperature, even though the reaction occurs. In this connection, the specific flavor produced from proline should be studied further in the future.

Changes in the amino acids during bread dough fermentation and baking. Since bread dough fermentation and baking are different from those in cracker production, a few experiments were performed to follow changes in the amino acid content of bread dough and the baked bread. The data are summarized in Table 6. Exception for proline and glycine, the amino acids decreased during a 6-hr fermentation of the French-type bread. The situation may be different from that with other types of bread using different formulation and fermentation times that do not exceed 4 hr.

Table 6. Changes of free amino acids during fermentation and baking of a French-type bread.

	μM/g (dry basis)			
	Dough		Bread	
Amino acids	0 hr	6 hr	Crumb	Crust
Aspartic acid	0.550	0.050	0.067	0.028
Threonine }	1.075	0.060	0.075	0.027
Serine }		0.065	0.100	0.042
Glutamic acid	0.577	0.108	0.280	0.098
Proline	0.078	0.102	0.132	0.075
Glycine	0.172	0.258	0.320	0.142
Alanine	1.075	0.065	0.177	0.078
Valine	0.205	.....	0.050	0.022
Methionine	0.040	0.008	0.010	0.002
Isoleucine	0.100	.....	0.025	0.012
Leucine	0.140	0.040	0.052	0.022
Tyrosine	0.077	.....	0.015	0.005
Phenylalanine	0.090	.....	0.017	0.008
Tryptophan	0.288	0.048	0.032	0.015
Lysine	0.128	0.112	0.177	0.075
Arginine	0.142	0.100	0.060	0.052

In baked bread, the amino acids decreased in the crust compared to the crumb. This might be expected since the Maillard reaction is much more pronounced in the crust (Linko and Johnson, 1963). Kretovich and Ponomareva (1961) observed little changes in the free amino acids in white bread doughs fermented 3 hr, but they did observe that the amino acids decreased more in the crust than in the crumb during baking of both white and rye breads.

REFERENCES

Baker, J. L., H. K. Parker, and K. L. Fortman. 1953. Flavor of bread. *Cereal Chem.* **30**, 22.

Folin, O., and V. Ciocalteu. 1927. On tyrosine and tryptophan determination in proteins. *J. Biol. Chem.* **73**, 627.

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

Hodge, J. E. 1955. The Amadori rearrangement. *Advances in Carbohydrate Chem.* **10**, 169.

Horvat, R. J., H. Ng, and J. W. Pence. 1962. Amino acids in fermented bread dough. *Food Sci.* **27**, 583.

Johnson, J. A., and B. S. Miller. 1961. Browning of baked products. *Baker's Dig.* **35**. October 52.

Kielly, P. J., A. C. Nowlin, and J. H. Moriarity. 1960. Bread aromatics from browning systems. *Cereal Sci. Today* **5**, 273.

Kretovich, V. L., and A. N. Ponomareva. 1961. Participation of amino acids in the reaction of melanoidine formation in bread-making. *Biochemistry* **26**, 213.

Linko, Y. Y., and J. A. Johnson. 1963. Changes in amino acids and formation of carbonyl compounds during baking. *J. Agr. Food Chem.* **11**, 150.

Maillard, L. C. 1912. Action des acides aminés sur les sucres; formation des melanoidines par voie methodique. *Compt. Rend.* **154**, 66.

Micka, J. 1955. Bacterial aspects of soda cracker fermentation. *Cereal Chem.* **32**, 125.

Moore, S., D. H. Spackman, and W. H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins, an improved system. *Anal. Chem.* **30**, 1185.

Morimoto, T., and J. A. Johnson. 1966. Studies on flavor fraction of bread crust adsorbed by cation exchange resin. Presented 51st Annual Meeting of American Association of Cereal Chemists. New York.

Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**, 1190.

Wiseblatt, L., and H. F. Zoumut. 1963. Isolation, origin and synthesis of a bread flavor constituent. *Cereal Chem.* **40**, 162.

Ms. rec'd 3/14/66.

The author thanks Mr. T. Morinaga, President of Morinaga Seika Co., for permission to publish this paper; Mr. Y. Hayakawa for technical assistance; and Dr. J. A. Johnson, Kansas State University, for kind advice in preparation of this manuscript. Presented at the 26th Annual Meeting of the IFT, Portland, Oregon, May 1966.

KIN WA CHENG [a] and WENDELL W. KILGORE
Agricultural Toxicology and Residue Research Laboratory, University of California, Davis, California 95616

Gas Chromatographic Determination of Pentachlorophenol and Sodium Pentachlorophenate Residues in Fruits

SUMMARY

A sensitive procedure for the determination of pentachlorophenol and sodium pentachlorophenate residues on fruits by electron-capture gas chromatography is described. In this method sodium pentachlorophenate is converted to pentachlorophenol in acid solution prior to analysis. The pentachlorophenol is then methylated with diazomethane, and is analyzed as the methyl ether by electron-capture gas chromatography. As little as 0.01 ppm can be detected, with recovery values ranging from 76 to 90%.

INTRODUCTION

Pentachlorophenol (PCP) and sodium pentachlorophenate (SPCP) are used on a variety of food crops to control various fungus diseases. These chemicals, particularly PCP, are also used in some areas as preplanting herbicides. Several colorimetric procedures are available for detecting PCP and SPCP residues (Monsanto Chemical Co., 1961), but these methods are not particularly sensitive and it is difficult to adapt them in the laboratory for large numbers of samples.

Chlorophenols are readily converted to methyl ethers by reacting them with diazomethane. These methyl derivatives are then easily detected by thermal-conductivity gas chromatography (Kanazawa, 1963). The present paper describes a simple but sensitive procedure for the extraction and gas chromatographic determination of PCP and SPCP residues on a variety of fruits. In this method the water-soluble SPCP is first converted to PCP in an acid solution. The resulting PCP residues are extracted with benzene, methylated with diazomethane, and analyzed by electron-capture gas chromatography. PCP residues are extracted directly with benzene, followed by methylation and analysis by electron-capture gas chromatography.

[a] Present address: Department of Agricultural Engineering, University of California, Davis, California 95616.

MATERIALS AND EQUIPMENT

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 vacuum bottle to avoid extreme temperature fluctuations. The signal from the detector was supplied to a 1-mv Brown recorder having a 1-second pen response. Recorder speed was 1 inch/min.

Column and operating conditions. Spiral-shaped Pyrex glass, ⅛-inch-OD × 5-ft-long column purchased from Wilkins Instrument and Research Inc., was packed with 5% Dow 11 (w/w) silicone oil on acid-washed 60/80-mesh Chromosorb W.

Column temperature was maintained at 180°C, the injection port at 195°C. The nitrogen carrier gas was passed through a small molecular-sieve filter (Wilkins Instrument and Research, Inc.) and was regulated to provide a flow rate of 25 cc/min through the column.

Planimeter. A compensating polar planimeter (Keuffel and Esser Co., model No. 620015) was used to measure the area within the boundaries of the recorder response curve.

Reagents. Reagents were redistilled reagent-grade benzene; redistilled, reagent-grade hexane; sodium pentachlorophenate standard; pentachlorophenol standard; diazomethane—prepared by the method described by Marquardt *et al.* (1964).

PROCEDURE

Preparation of standard curve. Prepare a standard stock solution containing 5 nanograms/ml of PCP in benzene. A 1-ml portion is transferred to a test tube and evaporated to dryness in a water bath at 60–70°C. If necessary, direct a fine stream of pure dry air into the tube to accelerate evaporation.

Add diazomethane, drop by drop, to the test tube until the yellow color persists. Shake the tube gently and then evaporate to dryness again in the water bath. Make separate injections of 1, 2, 3, 4, and 5 μl respectively containing 50, 100, 150, 200, and 250 pcg of the methylated PCP. The area under the curve is then determined and the average area of three injections for each aliquot is plotted against the concentration. The methylation of PCP with diazomethane is quantitative (Kanazawa, 1963).

A PCP methyl ether standard can also be prepared from SPCP. This can be accomplished as follows: Prepare a solution containing 5 nanograms/ml of SPCP in deionized water. Transfer 10 ml of the solution to a 125-ml separatory funnel, and add 1 ml of 0.1*N* sulfuric acid. Shake well. In this step SPCP is converted to PCP. Extract the PCP thus formed from the solution with 10 ml of benzene. Remove and discard the lower layer (water). Decant the benzene (upper phase) into a small Erlenmeyer flask and dry the solvent extract with about 5 g of anhydrous sodium sulfate. Transfer 1 ml of the solution to a test tube and evaporate to dryness. Subsequent treatment of the standard is the same as described above for PCP.

In preparing a PCP methyl ether standard from SPCP, some loss may occur during the extraction process. If this method of preparing the standard is used, the solution should be compared directly to a known sample of PCP methyl ether to determine if any loss due to the extraction process does occur. However, repeated comparisons in this laboratory have shown that any such loss is usually insignificant.

Extraction method. Macerate a representative sample of approximately 500 g in a food chopper. In this laboratory, crop samples are macerated in a Hobart food chopper, although any type of homogenizing or chopping equipment would probably be satisfactory. Transfer 100 g of the chopped sample to a 1-gallon tin can equipped with a metal baffle for mixing. Wet the sample thoroughly with acidified distilled water (0.1 ml H_2SO_4 in 100 ml distilled water), followed by the addition of 2 ml benzene for each gram of sample in the can. A ratio as great as 4 ml benzene to 1 g of sample can also be used. After the addition of benzene, seal the can tightly and roll on a mechanical roller (35 rpm) for 30 min.

For recovery studies, fortify the samples with SPCP (or PCP) before wetting them with the acidified distilled water. After the rolling procedure the cans should not be opened for at least 10 min, in order to permit dispersion of any emulsion which may be formed during the extraction process. After the 10-min waiting period, decant the mixture into a beaker containing 200 g of anhydrous sodium sulfate and mix thoroughly. Filter the mixture through fluted filter paper and store the eluate in a tightly sealed bottle until analyzed.

Removal of interfering substances. Since extracts of apples do not contain any substances which interfere in the gas chromatographic analyses of the samples, no cleanup procedure is required prior to analysis. However, almond extract has presented a somewhat greater problem. For these samples it is necessary to use sulfuric acid for the removal of interfering substances.

Sulfuric acid treatment. Transfer a portion of the extract equivalent to 10 g of crop to a 120-ml separatory funnel and add 3 ml of concentrated reagent grade sulfuric acid. Stopper and shake gently for 1 min. Allow ample time, about 10 min, for the phases to separate, and then remove and discard the lower (acid) layer. The emulsified upper layer is retained in the funnel. Repeat this step twice using 3 ml of sulfuric acid for each washing. Then wash the upper phase successively with four 10 ml-portions of deionized water. Shake the funnel vigorously and discard the water which collects at the bottom after each addition. Decant the upper phase into a 125-ml Erlenmeyer flask containing 15 g of anhydrous sodium sulfate and mix thoroughly.

Evaporation and determination. Transfer 1 ml of the benzene extract to a test tube and evaporate to dryness in a water bath held at 60–70°C. If necessary, direct a fine stream of clean dry air into the solvent to accelerate the evaporation. Add the diazomethane ethereal solution drop by drop into the test tube until the yellow color persists. Shake the test tube gently and then evaporate the solvent again in the water bath. When the evaporation is complete, add 1 ml of hexane and shake well. Inject 1–5 μl of the resulting hexane solution into the gas chromatograph. The peak area for each sample is measured and compared with the standard curve.

RESULTS AND DISCUSSION

In this procedure the water-soluble salt of pentachlorophenol (PCP), sodium pentachlorophenate (SPCP), is converted in acid solution to pentachlorophenol prior to its extraction with benzene. This step was necessary to avoid using water as the extracting solvent, which would have removed large quantities of interfering substances. Thus, the conversion of SPCP to PCP, followed by extraction with benzene, reduced the amounts of interfering plant substances in the final extracts. The loss of SPCP residues in the conversion and extraction process was less than 5%.

The response of the electron capture detection system to the methyl ether of PCP was measured over a wide range and exhibited a linear curve (area vs. concentration plot) from 50 to 350 picograms.

Use of the electron capture rather than the thermal conductivity detector extended the quantity of PCP methyl ether which

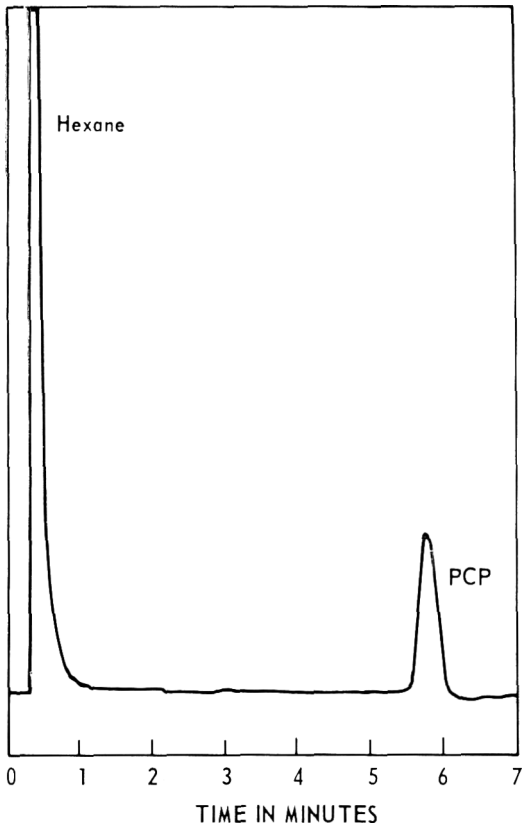


Fig. 1. Chromatogram of PCP methyl ether standard. The response represents 10 picograms of PCP methyl ether.

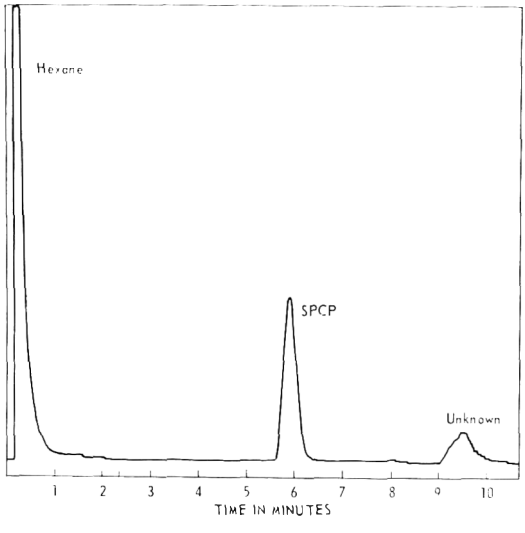
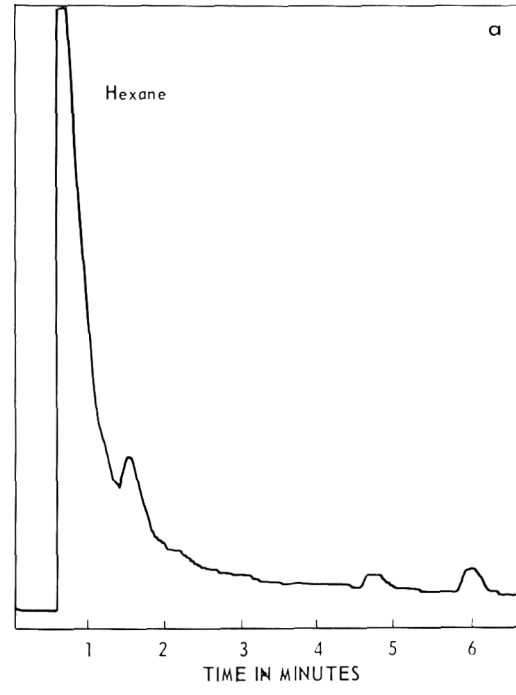


Fig. 2. Chromatogram of PCP methyl ether in apple extract. Sample fortified with SPCP and converted to PCP methyl ether.

could be detected from several micrograms to a few picograms. This reduced the quantity of material which had to be extracted,



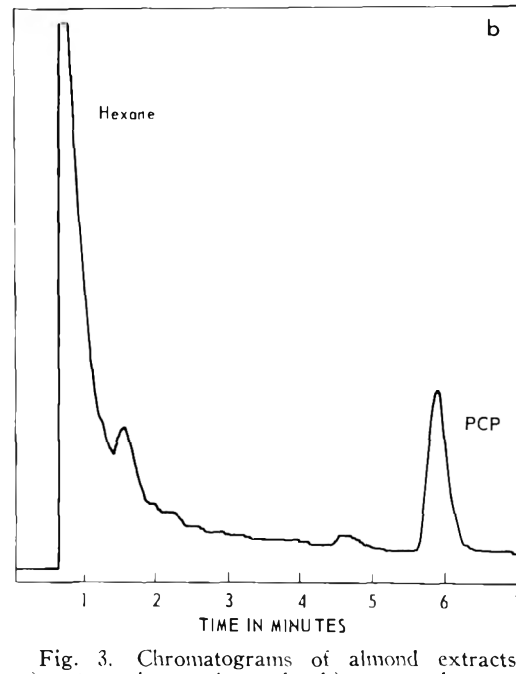


Fig. 3. Chromatograms of almond extracts: a) untreated control sample; b) untreated control sample fortified with 0.04 ppm of PCP. A 1-μl aliquot of each extract, equivalent to 500 μg of almonds, was chromatographed.

and increased the overall sensitivity of the method.

Recoveries of SPCP residues ranged from 76 to 90%, when fortified control samples were analyzed (Table 1). The overall average recovery for all fruits (almonds, cherries, peaches, prunes, apricots, and apples) was 84%. As little as 0.01 ppm was detected with considerable reliability. Fig. 1 shows the response given by 10 picograms of PCP after conversion to its methyl ether.

Table 1. Recovery study of SPCP from various fruits.[a]

Fruit	ppm Added	ppm Found	Recovery (%)	Av. rec. (%)
Drake almond[b]	1) 0.50	0.40	80	
	2) 0.50	0.42	84	80
	3) 0.50	0.38	76	
Bing cherry	1) 0.50	0.44	88	
	2) 0.50	0.46	92	89
	3) 0.50	0.44	88	
Peach	1) 0.50	0.42	84	
	2) 0.50	0.43	86	84
	3) 0.50	0.41	82	
French prune	1) 0.50	0.45	90	
	2) 0.50	0.44	88	88
	3) 0.50	0.43	86	
Royal apricot	1) 0.50	0.41	82	
	2) 0.50	0.42	84	81
	3) 0.50	0.39	78	
Apple	1) 0.50	0.41	82	
	2) 0.50	0.41	82	83
	3) 0.50	0.42	84	
Overall av.				84%

[a] SPCP determined as PCP methyl ether by gas chromatography.
[b] Required sulfuric acid cleanup procedure.

A negligible amount of interfering substances was found in untreated control samples of cherries, peaches, prunes, apricots, and apples. These samples were analyzed directly following the methylation step without additional cleanup. The response curve of an apple extract, fortified with 0.2 ppm SPCP is shown in Fig. 2. The aliquot injected into the gas chromatograph contained an equivalent of 20 picograms of SPCP.

Additional cleanup was required for the almond extracts since they contained large quantities of interfering substances. The sulfuric acid washing procedure was adequate to remove these substances (Fig. 3a). The small peak which has the same retention time (6 min) as PCP methyl ether represents less than 0.004 ppm of interfering substances. Fig. 3b shows the results obtained with a fortified control sample treated with sulfuric acid.

REFERENCES

Kanazawa, J. 1963. Gas-liquid chromatographic separation of chlorophenols and determination of pentachlorophenol as methyl ethers. *Agr. Biol. Chem.* **27** (3), 153.

Marquardt, R. P., H. P. Burchfield, E. E. Storrs, and A. Bevenue. 1964. 2,4-Dichlorophenoxyacetic acid. In "Analytical Methods of Pesticides, Plant Growth Regulators, and Food Additives." Vol. IV, page 113. G. Zweig, Ed. Academic Press, New York and London.

Monsanto Chemical Co. 1961. Analytical methods for pentachlorophenol and its salts. Tech. Bull. No. SC-8. St. Louis, Missouri.

Ms. rec'd 1/7/66.

EDWARD H. COHEN
Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118

Protein Changes Related to Ham Processing Temperatures I. Effect of Time-Temperature on Amount and Composition of Soluble Proteins

SUMMARY

Knowledge of protein changes produced by heat is basic to the development of a needed analytical method for determining whether canned meats have been pasteurized at high enough temperatures. Ground cured uncooked ham was heated, with different programming, to temperatures up to 165°F; and the influence of the treatments on the amount and composition of proteins soluble in 0.9% NaCl solution was determined. Rate of heating as well as the temperature reduced the amount of extractable protein. Fractionation of soluble proteins by disc electrophoresis showed five components to be relatively resistant to heat coagulation. One of these components was identified as an acid phosphatase.

INTRODUCTION

In quality control and regulatory work there exists a need for an easily applied simple laboratory technique for determining the temperature to which a heat-treated meat product has been processed. This is very important with products imported into the United States, to ensure that the temperatures applied are high enough to meet requirements. With domestic products also, there are regulations which require heating to, or above, specified temperatures. Quality-control laboratories in industry should often make similar determinations, both to assure compliance with regulations and to maintain quality standards.

A method of determining maximum temperature attained in heat processing has been devised in this country, although not published, and applied both here and in some European laboratories. It depends on heating a solution prepared by extracting the meat product with 0.9% NaCl and observing the temperature at which a flocculent precipitate is formed. Within limits, this temperature corresponds to the highest temperature reached internally when the product is processed. Improvement of the above method, or the development of a new method, to obtain closer correlation between analysis and actual processing temperatures is highly desirable.

The components of meat muscle have been described by Smith (1934, 1937), Szent-Györgyi (1951), Dubuisson (1954), and Mommaerts (1950, 1958). Rabbit myosin is reported to denature in solution when heated to 105–115°F (Smith, 1937), and actomyosin when heated to 113–127°F for 3–12 min (Locker, 1956). Globulin X has been reported to denature on standing at room temperature (Smith, 1937). Myogen, which contains many of the enzymes of glycolysis and of the tricarboxylic acid cycle, denatures, in part, upon standing. However, some of these enzymes are known to be relatively heat-stable. Myoalbumin, which has not been studied to any great extent in the last 30 years, exhibited a series of denaturation "points" at 120, 132, 145, 161, 176, and 194°F (Smith, 1937; Jacob, 1947). In these investigations the solution was filtered free of coagulum (denatured protein) after each temperature was reached, and the heating was continued to higher temperatures until a further coagulum was reached. The multiplicity of denaturation temperatures could possibly be explained by the fact that the myoalbumin used was contaminated with proteins, possibly those generally referred to as myogen.

The above background indicates that myosin, actomyosin, globulin X, and part of the myogen group are denatured at lower temperatures than those used in heat-processing meat. A more accurate knowledge of the heat denaturation of the relatively heat-stable proteins would be useful in developing an index of thermal processing. In preliminary experiments, results showed that temperature and content of proteins soluble in low ionic strength (0.15) were closely related. If not these proteins collectively, identification of one which denatures on heating in the tem-

perature range of processing should be a possibility. For example, phosphatase activity indicates the processing time-temperatures applied to milk (Sanders and Sager, 1947). Data available on the acid phosphatase of meat suggest that it is similarly denatured as a function of time and temperature (Körmendy and Gantner, 1960). Accordingly, an investigation of the heat denaturation of proteins soluble in 0.9% NaCl has been conducted, of which the presently reported study was the initial phase. The present paper deals with changes in the solubility of proteins on heating cured uncooked ham to different temperatures with various rates of heating. The electrophoretic analysis of uncoagulated (relatively heat-stable) components was conducted. One of these components was identified as a muscle acid phosphatase.

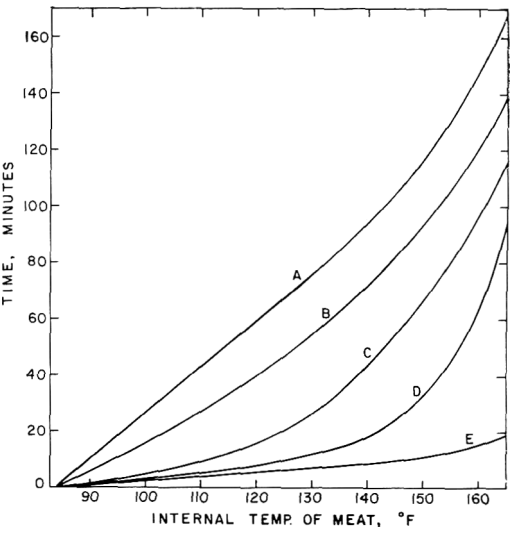


Fig. 1. Time required to obtain selected internal temperatures in cured meat samples by varying initial bath temperatures. Heating for programs A, B, C, D, and E, respectively begun at 84, 100, 120, 140, and 170°F.

EXPERIMENTAL PROCEDURE

Sample preparation. A 15-lb boneless ham was cured at a local processing plant for five days. The arterial pumping method was used to inject 10% by weight of 68° pickle; this was also the strength of the covering pickle. The meat was trimmed free of all but the interstitial fat, ground in a meat grinder in a cold room at 37°F, and then thoroughly mixed. Ground meat was used to minimize the differences in composition and variation of heat penetration that could be expected if samples for analysis were taken from either the whole ham or the individual muscles.

Twenty-five-gram samples, in duplicate, were placed in individual stainless-steel centrifuge cups (approximately 1 × 6 inches), packed tightly, and covered with aluminum foil to minimize evaporation. These samples were heated to an internal temperature of 165°F and samples were withdrawn for analysis at 120, 130, 140, 150, 160, and 165°F. These temperatures correspond to the range that would produce undercooked, properly cooked, and slightly overcooked products in processing. Five heating programs were obtained by starting the cooking bath at 84, 100, 120, 140, and 170°F (Programs A–E, respectively, in Fig. 1), then increasing the bath temperatures to a maximum temperature of 170°F by setting the temperature-control unit to maximum, and heating until the above-mentioned internal temperatures were obtained. The cooked samples were ambient-air-cooled (room temperature 84°F) for 10 min, removed from the tubes, and placed in a 500-ml-capacity two-speed blender with an equal weight of 0.9% NaCl. Each mixture was homogenized for 20 sec (10 sec high speed, and 10 sec low speed), transferred to a beaker, covered with polyethylene film, and held at room temperature for a 30-min extraction. The pH was recorded at this time with a single-probe electrode calibrated at pH 6.5 and 84°F. The mixtures were then centrifuged at 32,800 × G in a refrigerated centrifuge for 30 min. The supernatant fractions, which contained some fat floating on the surface, were removed with a long cannula, and then further clarified in a refrigerated centrifuge at 32°F at 130,000 × G for 30 min. The clear extracts were used for protein determinations and disc electrophoresis.

Protein determination. One-ml fractions were used for protein determinations by the biuret method (Gornall *et al.*, 1949). The biuret standard was a solution of bovine serum albumin (BSA) calibrated by micro-Kjeldahl analysis (Ogg, 1960). The protein was determined by spectrophotometry at 540 mμ after 30 min of color development. Two different concentrations of the BSA, in duplicate, and a biuret blank were read against a distilled-water blank. The color was stable for several hours. The calibration curve (containing six points) was linear to 0.650 absorbance units on an instrument calibrated to be linear from 0.000 to 3.000 absorbance units. Each fraction of meat extract, containing 3–26 mg protein, was run in duplicate, and the results are reported as the mean, with standard deviation of ±0.03 mg.

Disc electrophoresis. Extracts of several samples which had been heated to various temperatures were fractionated by disc electrophoresis on polyacrylamide gels according to techniques of Ornstein (1964) and Davis (1964). These included extracts from unheated samples and samples heated to 150°F at different programs (A,D,E,); also included were samples heated to different temperatures: 120, 150, and 160°F at the same program (D).

Lower gels 2 inches long were used in place of the standard 1½-inch gels, to obtain better separation. The extracts, each containing 200 μg protein, were mixed with upper gel solution at 1:1 instead of the standard 1:50 dilution. This was done because the protein solutions were already dilute and it was necessary to restrict the volume of extracts added to the top of the electrophoretic tube. The 1:1 mixtures were placed on top of the spacer gels and then photopolymerized for 20 min. Electrophoresis was conducted for 30–50 min, depending on the progress of a tracking dye which preceded the fastest-moving protein. To minimize diffusion, the gels were immediately removed from the electrophoretic bath upon completion of the run, placed in an ice bath, removed from the tube with a probing needle, and stained for 1 hr. For complete destaining, the gels were soaked in a large test tube containing 7.5% acetic acid which was changed three times in 36 hr, and were then destained electrophoretically at 10 ma per tube for 40 min. The upper chamber contained a small amount of staining dye to prevent excessive destaining. This destaining method produced a background almost as lightly colored as that found in the area in front of the tracking dye. The absorbance of stained gels was scanned with a recording microdensitometer.

In addition to staining gels for protein (aniline black 0.5% w/v in 3.5% acetic acid), a specific stain for phosphatase was used on duplicate gels of unheated samples and samples heated to 150°F (program E). A modification of the simultaneous-coupling azo-dye method (Barka, 1961) was used. After the gels were removed from the glass tubes, the gels were immersed twice in a pH-5.0 acetate buffer for 15-min periods. The gels were then placed into tubes containing Fast Garnet GBC salt, 1 mg/ml, in pH-5.0 acetate buffer for 30 min. This allowed the dye to penetrate to the center of the gel. Finally, the gels were placed into tubes containing 2 mg/ml of α-naphthol phosphate salt, made in pH 5.0 acetate buffer, for 24 hr at 35°F. The reaction was stopped by placing the gels in 7.5% acetic acid. The sites colored dark red were compared with the gels stained with aniline black to determine the position of the phosphatase. These gels were photographed by transmitted light with a Mazda lamp through a series B filter. High-contrast film and standard development was used.

RESULTS AND DISCUSSION

Fig. 1 shows the time in minutes required to attain various internal temperatures in the heated samples. The curves plotted indicate that the procedure used in heating produced five different programs of heating: A (slow)–E (rapid).

Fig. 2 relates to temperature the amounts of protein that remained soluble in 0.9% NaCl when the samples of ham were heated at the five different programs. Heating reduced the content of soluble proteins from 26.75 mg/ml of extract to 5.10–9.24 mg/ml at 120°F and to 2.60–4.48 mg/ml at 150°F. The proteins remaining soluble after heating were a small fraction of those originally soluble. However, more significantly, the rapid rates of heating tended to increase the amount of protein insolubilized. This is indi-

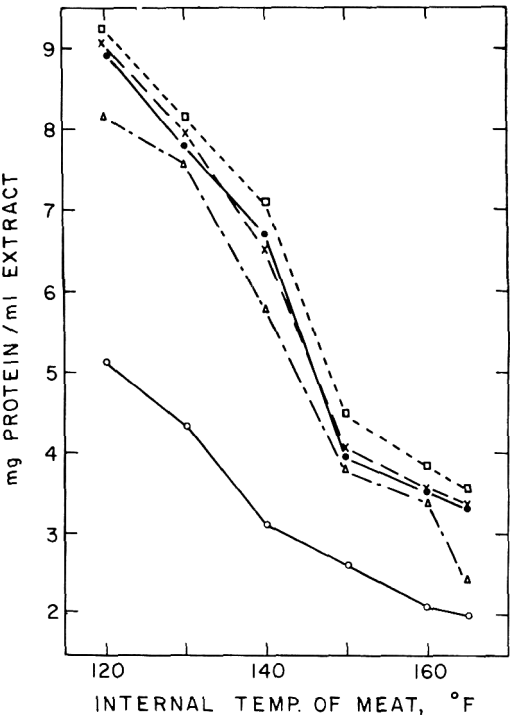


Fig. 2. Soluble protein in meat as affected by heating at different rates and to different temperatures (*cf.* Fig. 1). The protein content of the extracts from the uncooked meat samples at 84°F was 26.75 mg/ml extract. Heating curve: A = □---□; B = x--x; C = ●——●; D = △---△; E = ○——○.

cated by comparison of the protein remaining soluble at corresponding sampling temperatures on curves A–E (Fig. 2). The results show that the amount of protein extractable after heating to a given terminal temperature—for example, 150 or 160°F—varied with the time-temperature combination rather than internal temperature. For example, 3.8 mg soluble protein/ml sample was extractable from samples heated with the five different programs at temperatures of 133, 152.5, 153.5, 154.5, and 161°F. These results indicate that simply determining the protein content of extracts of heat-processed meats can indicate approximate processing temperature, but not with high accuracy.

The pH values of the ham samples investigated were within the normal range of cured heated ham; otherwise, adjustment would have been advisable. The effect of time and temperature on the change of pH of the meat extracts (Table 1) shows that pH increased on heating, as previously reported and explained by Hamm (1960) and Hamm and Deatherage (1960). The observed pH change was not more than 0.3 unit, not as large as reported previously (0.4 pH). This may be accounted for by differences in the buffering capacity of cured and uncured meat, since Hamm's studies were, primarily, on uncured meats. Over 65% of the change occurred on heating to 120°F, and differences produced by increasing temperature above 120°F were 0.07 unit or less. The results indicate that the effects of varying temperature 10–30°F would be expected to produce small pH changes that measurably influence protein solubility only under unusual circumstances.

Fig. 3a shows a disc electrophoretic scan of proteins extractable with a solution having an ionic strength of 0.15 from uncooked cured ham, while Fig. 3b shows scans indicating the proteins that remained in samples that were cooked to 150°F with different rates of heating. These electrophoretic patterns show that protein components that were a major fraction of the extract from unheated ham were not present in the heated samples to any appreciable extent. In contrast, there is an increase in the peaks designated as 1–5 after heating to the 150°F range. Each pattern represents fractionation of 200 μg protein. The heat-stable proteins represent an increasingly larger portion of the proteins remaining as heating removed that portion of protein which was heat-coagulable.

The relative size of peaks 1–5 diminished in the order A, D, E, reflecting the tendency of rapid heating rates to heat-coagulate more protein than comparatively slower rates.

Fig. 4 shows the effect of increasing sampling temperature on heating at the same rate (Program D). The scans indicate that peaks 2 and 4 increase to a maximum with heating to 150°F and then decrease at 160°F, thus indicating a heat lability in the 150–160°F range. Peak 5 increases with increased heating, indicating a heat-stable protein, which, because of its fast mobility, may

Table 1. Time and temperature effects on the change of pH of meat samples.[a]

Program of heating[b]	pH of meat at given temperatures (°F)					
	120	130	140	150	160	165
A	5.96	5.96	6.02	6.02	6.02	6.04
B	5.95	5.95	5.95	6.02	6.04	6.05
C	5.95	5.98	6.02	6.02	6.05	6.05
D	5.95	5.95	5.98	6.00	6.02	6.02
E	5.95	5.95	6.02	6.02	6.02	6.02

[a] pH of uncooked meat was 5.75.
[b] *cf.* Fig. 1.

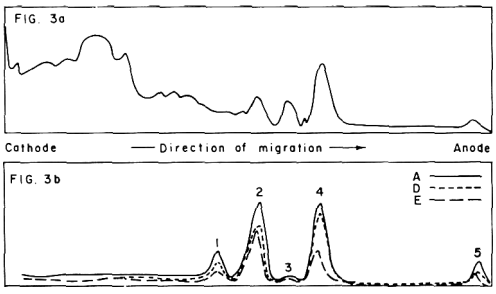


Fig. 3a. Disc electrophoretic pattern of proteins in extract from uncooked meat.
Fig. 3b. Disc electrophoretic patterns of proteins in extracts from meat heated to 150°F at different rates, programs A, D, and E.

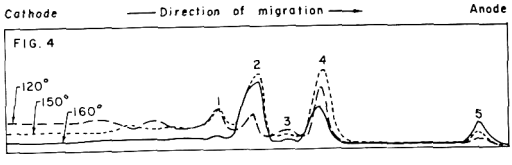


Fig. 4. Disc electrophoretic patterns of proteins in extracts from meat heated to 120, 150, and 160°F at the heating rate of program D.

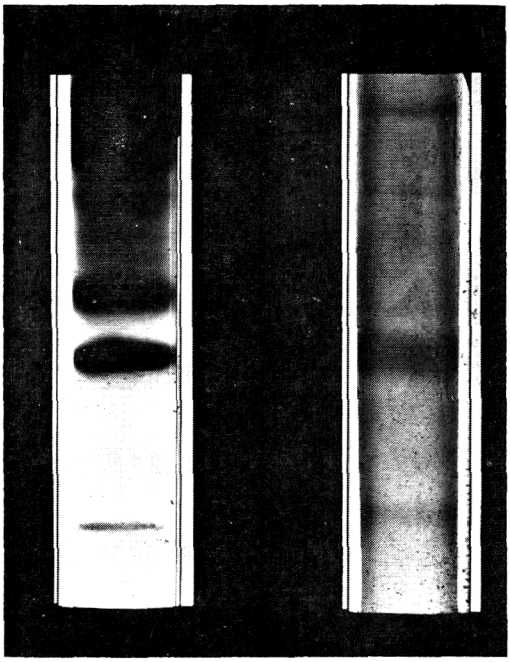


Fig. 5. Gels prepared by disc electrophoresis of meat extract stained (left gel) with aniline black to show location of protein, and (right gel) with Fast Garnet GBC-α-naphthol to show location of acid phosphatase.

be an albumin or a low-molecular-weight fragment derived from one of the proteins initially present. The comparative size of peaks 1 and 3 reduced as temperature increased.

Peaks 1–5 contain proteins apparently of relatively high heat stability.

Fig. 5 is a photograph of disc electrophoretic gels of unheated extracts stained to locate protein or phosphatase. The location of the area reacting positively for phosphatase activity corresponds to peak 4 in Figs. 3a, 3b, and 4. Owing to the relative insensitivity of the dye for locating acid phosphatase (Barka, 1961), the stained areas of the heated samples were barely discernible and not intense enough for photography. Further characterization may indicate the muscle phosphatase identified in the present work to be identical to the preparations investigated by Körmendy.

Further investigation is concerned with the identity and properties of the remaining heat-stable components fractionated by electrophoresis and the correlation of their heat lability with time-temperature conditions in heat processing.

REFERENCES

Barka, T. 1961. Studies of acid phosphatase. I. Electrophoretic separation of acid phosphatases of rat liver on polyacrylamide gels. *J. Histochem. Cytochem.* **9**, 542.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**, 404.

Dubuisson, M. 1954. "Muscular Contraction." Charles C. Thomas, Springfield, Ill.

Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751.

Hamm, R. 1960. Biochemistry of meat hydration. *Advances in Food Research* **10**, 355.

Hamm, R., and F. E. Deatherage. 1960. Changes in hydration, solubility and charges of muscle proteins during heating of meat. *Food Research* **25**, 587.

Jacob, J. J. C. 1947. The electrophoretic analysis of protein extracts from striated rabbit muscle. *Biochem. J.* **41**, 83.

Körmendy, L., and Gy. Gantner. 1960. Über die saure Phosphatase des Fleisches. *Z. Lebensmitt.-Untersuch.* **113**, 13.

Locker, R. H. 1956. The dissociation of myosin by heat coagulation. *Biochim. et Biophys. Acta* **20**, 514.

Mommaerts, W. F. H. M. 1950. "Muscular Contraction." Interscience, N. Y.

Mommaerts, W. F. H. M. 1958. Chemical investigation of muscular tissues. *Methods in Med. Research* **7**, 1. Year Book Publishers, Chicago, Ill.

Ogg, C. L. 1960. Determination of nitrogen by the micro-Kjeldahl method. *J. Assoc. Offic. Agr. Chemists* **43**, 689.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.* **121**, 321.

Sanders, G. P., and O. S. Sager. 1947. Time-temperature conditions required to inactivate phosphatase in different dairy products. *J. Dairy Sci.* **30**, 518.

Smith, E. C. B. 1934. A scheme for the approximate determination of the proteins of muscle. *J. Soc. Chem. Ind.* **53**, 351T.

Smith, E. C. B. 1937. Native and denatured muscle proteins. *Proc. Roy. Soc.* **124B**, 136.

Szent-Györgyi, A. 1951. "Chemistry of muscular contraction." 2nd ed. Academic Press, N. Y.

Ms. rec'd 3/29/66.

Mention of commercial names does not imply endorsement by the U. S. Dept. of Agriculture.

Research Note

R. L. HOFFMANN, G. R. LIST, and C. D. EVANS
Northern Regional Research Laboratory, Peoria, Illinois 61604

Enrichment of Volatile Samples by Syringe Collection

SUMMARY

Volatile samples for gas chromatographic analysis can be collected and enriched by slow passage through a gas-syringe barrel packed with solvent-dampened cotton. Then the condensate can be injected into a gas chromatograph directly from the same syringe.

Volatile odor constituents from autoxidizing fats are difficult to collect. Although vacuum distillation into traps cooled with dry ice or liquid nitrogen is effective for sample collection and enrichment, this method requires extensive equipment (Lawrey and Cerato, 1959; Nawar and Fagerson, 1960; Rhoades, 1958). Also, during transfer the sample may become degraded, and, often, some is lost. To achieve economy and simplicity, direct headspace sampling is preferable even though odorous-sample constituents are usually present in low concentrations. Large injections of headspace gas either upset chromatograph baseline stability intolerably or cause broad, poorly resolved peaks.

We have developed a collecting technique that combines the advantages of enrichment trapping with the simplicity and economy of syringe sampling. Details of the collecting apparatus are diagrammed in Fig. 1.

A few drops of liquid oil from which volatiles are to be collected are absorbed on a Whatman filter accelerator held in the center of a close-fitting Teflon annulus. The annulus rests inside a 40-mm-OD Pyrex filter funnel sealed with a polyethylene milk-bottle cap. Nitrogen sweep gas is admitted to the funnel through a 1-in. × 20-gauge needle that pierces a septum in the bottle cap. Volatiles are swept from the funnel, through a 2-in. × 20-gauge delivery needle, into a 2.5-cc gas syringe barrel. The syringe is supported in a paper cup filled with dry ice. Nitrogen-borne volatiles are condensed on a small tuft of diethyl-ether-dampened cotton loosely packed in the needle end of the barrel. A flow rate of 10 cc/min is sufficient to sweep volatiles from the filter disc to the syringe. Enrichment time varies for different concentrations of volatiles in the sample. It is difficult to specify accurately the length of time necessary to collect a sample of useful concentration without preliminary trials.

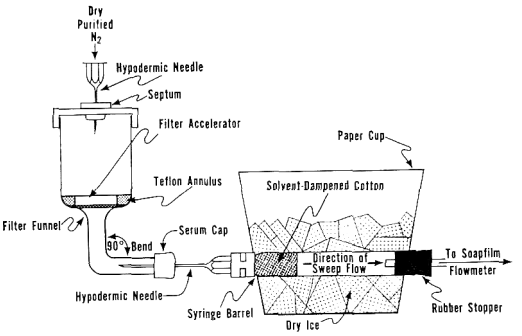


Fig. 1. Collecting apparatus for autoxidative volatiles from oils.

When a sufficient sample has been collected, as determined by trial, the syringe and attached needle are quickly withdrawn from the filter-funnel serum cap and paper cup. With the plunger in place the collected sample is injected from the syringe directly into the gas chromatograph. The cotton is not removed for the injection. Rapid depression of the syringe plunger during injection warms the cotton and releases the condensed odor constituents.

Blank analyses made with the same collecting apparatus but without a sample will account for any chromatographic peaks caused by the solvent or contamination from the cotton. With clean apparatus and pure solvent, no extraneous peaks were evident.

Fig. 2 is a chromatogram of volatile hydrocarbons from autoxidized methyl linolenate that were collected with the apparatus. Direct headspace injection without syringe enrichment yielded no peaks. The chromatogram was produced from a 6-ft × ¼-in. activated alumina column operated isothermally. No solvent or polar volatiles were evident, because alumina irreversibly adsorbs these materials. Between sample injections, the column was relieved of its solvent overload by purging it with carrier gas at 300°C for 0.5 hr.

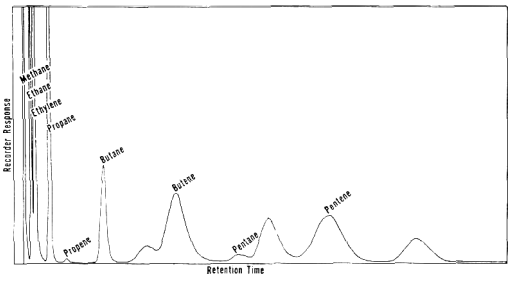


Fig. 2. Chromatogram of volatile hydrocarbons from autoxidized methyl linolenate. The various steps included a sample collection time of 5 min at a flow rate of 10 cc/min; diethyl ether as the solvent; an activated alumina column; and a flame ionization detector.

Chromatograms from gas-liquid chromatographic columns, free of solvent peaks, may be obtained from all volatiles (polar included) if carbon disulfide is used to dampen the cotton and if the column effluent is monitored by a flame ionization detector (FID). This solvent is not detected by FID. Volatile constituents from liquid materials other than oils may be collected similarly. Solid samples loosely packed into the filter funnel yield equally convenient chromatograms.

This collecting apparatus, assembled from inexpensive materials readily available in a laboratory, can be used for routine aromagram determinations.

REFERENCES

Lawrey, D. M. G., and C. C. Cerato. 1959. Determination of trace amounts of methane in air. *Anal. Chem.* **31**, 1011.

Nawar, N. W., and I. S. Fagerson. 1960. Technique for collection of food volatiles for gas chromatographic analysis. *Anal. Chem.* **32**, 1534.

Rhoades, J. W. 1958. Food research sampling method for analysis of coffee volatiles by gas chromatography. *Food Research*, **23**, 254.

Ms. rec'd 5/16/66.

The Northern Laboratory is headquarters for the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Mention of firm names or trade products does not constitute endorsement.

CHIEN-MEI CHIU and Y. POMERANZ

Department of Flour and Feed Milling Industries Kansas State University, and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Manhattan, Kansas 66502

Changes in Extractability of Lipids During Bread-Making

SUMMARY

Free lipids were extracted with petroleum-ether, and total lipids with a chloroform-methanol mixture from flour, dry milk solids, yeast, dough, fermented dough, bread crumb, and bread crust. Dough formulations used in bread-making included—in addition to a basic formula of flour, water, yeast, and sodium chloride—either sugar, commercial vegetable shortening, and dry milk solids, or their combinations. The extracted lipids were fractionated by thin-layer chromatography (TLC). Petroleum-ether-soluble flour lipids were reduced to one-third during dough mixing or fermentation; subsequent baking lowered the residual free lipids to half. Petroleum-ether-soluble free lipids were affected little by dough composition. Only small amounts of hydrogenated vegetable shortening were bound during dough-mixing, but about 1/3–1/2 of the added shortening lipids became bound during baking. Processing flour into bread had no effect on the amounts of total lipids extractable by the chloroform-methanol mixture. Fractionation of extracted lipids by TLC showed that much more polar wheat flour lipids than nonpolar components were bound during dough mixing.

INTRODUCTION

Lipids play an important role in determining the rheological properties of dough and in the improver action of oxidants (Fisher, 1962; Daniels, 1963; Mecham, 1964). Bread baked with 2–4% shortening has a larger loaf volume. The finished bread has a more uniform crumb grain structure and is more tender; the flavor is richer and freshness retention is improved. Recent studies (Pomeranz *et al.*, 1965, 1966b) show that polar wheat flour lipids are highly effective in improving loaf volume, crumb grain, and freshness retention of bread. Nonpolar wheat flour lipids have no improving effect.

The reasons for differences in bread-improving effects of various wheat flour lipids, and between liquid oil and vegetable shortening, continue to intrigue many cereal chemists. Yet, little is known of changes that may occur in flour lipids during fermentation and baking (Mecham, 1964). Olcott and Mecham (1947) found that the amount of free flour lipids (ether-extractable) was reduced to one-third as a result of dough mixing. Phospholipids (determined from the amount of extracted lipid phosphorus) were bound preferentially. Most of the bound lipid was associated with the glutenin fraction in the gluten. In a later study, Mecham and Weinstein (1952) reported on the effects of bread ingredients on lipid binding and the formation of lipoprotein during dough mixing. Baldwin *et al.* (1963, 1965) studied lipid binding in doughs and bread made by the conventional and continuous process. After submission of our paper for publication we learned about the studies of Daniels *et al.* (1966), who investigated the distribution of lipids in bread produced by three mixing methods, using bread formulae which included low levels of added shortening and full-fat soy flour.

This investigation was made to study the effects on lipids of dough mixing, fermentation, and baking. Lipids were extracted with petroleum ether and a polar solvent (chloroform-methanol mixture). Extracts were fractionated by thin-layer chromatography (TLC).

MATERIALS AND METHODS

Flour. Untreated flour, used for the baking tests, was milled experimentally on an Allis mill from a composite grist of several hard winter wheat varieties grown in 1964 at a number of locations throughout the Great Plains. Expressed on a 14% moisture basis, the flour had an ash content of 0.42% and protein (N × 5.7) content of 13.1%. The bromate requirement of bread baked by the complete formula was 3.0 mg per 100 g flour, and without nonfat milk solids was 1.5 mg. Water absorption was 61.8%, and mixing time was 3¼ min.

Bread-making. The basic bread formula included 100 g flour, 1.5 g salt, 2 g yeast, water as needed, and optimum potassium bromate. This

formula was varied to include 3 g commercial vegetable shortening, 4 g nonfat dry milk solids, 6 g sucrose, and combinations of the optional ingredients. An optimum mixing time with the straight-dough procedure and a 3-hr fermentation time at 30°C were employed. Punching and panning were performed mechanically. Baking time was 24 min at 218°. Loaf volumes were measured with a National Manufacturing Co. volumeter, employing seed displacement. After the loaves had cooled, they were cut and their crumb grains evaluated. The following code was employed: S = satisfactory, Q = questionable, U = unsatisfactory.

Lipid extraction. Samples of dough were removed at the end of mixing and of fermentation, and frozen immediately on a block of dry ice. The frozen dough and baker's yeast were lyophilized. One hour after baking, bread was separated into crumb and crust, divided into small pieces, and dried under vacuum below 60°. All samples were ground to pass a 40-mesh sieve on a micro-Wiley mill and stored at −20° in dark bottles until analyzed.

Lipids were extracted exhaustively with petroleum ether (bp 35–60°) in a Goldfish extractor. In addition, lipids were extracted with a chloroform-methanol mixture by the method of Bligh and Dyer (1959) as modified by Tsen *et al.* (1962). Yields of lipids were equal to or slightly lower than yields obtained by direct extraction with water-saturated butanol or by the acid hydrolysis method. Results of lipid extractions are means of at least two determinations.

Thin-layer chromatography. Extracted lipids were fractionated by thin-layer chromatography (TLC) as described previously (Daftary and Pomeranz, 1965a,b). The solvents used for one-dimensional ascending development of 100 μg lipids were: chloroform for nonpolar, and a mixture of chloroform-methanol-water (65:25:4) (chloroform mixture) for polar lipids.

The spots were visualized by exposure to iodine vapor, or by spraying with a saturated solution of $K_2Cr_2O_7$ in 70% volume of aqueous sulfuric acid. More specific spraying methods included ninhydrin, modified Dragendorff reagent, and molybdenum spray. In addition, TLC plates were sprayed with α-naphthol for identification of glycolipids (Feldman *et al.*, 1965). For identification of polar lipids separated by TLC, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine (from Applied Science Labs., State College, Pa.), and mono- and digalactosyl glyceride (gift from Dr. D. H. Hughes, Procter and Gamble Co., Cincinnati, Ohio) were used as standards.

RESULTS AND DISCUSSION

Table 1 gives the effects on loaf volume and bread quality of adding milk solids, sucrose, and shortening to the basic formula. Crust color depended primarily on adding sucrose; the contribution to crust color of milk solids was small. The largest loaf volume increase resulted from adding sucrose. Determinations of proof height and examinations of crust color indicated that dough compositions 1, 2, and 3 contained inadequate amounts of sugar for production of carbon dioxide during the proof and baking periods. They are included for comparative purposes. Adding shortening to the basic dough had little effect; loaf volume was increased from 675 to 740 cc by adding shortening to a dough which contained 6 g sugar. The best bread in terms of crumb grain was obtained only after both sugar and shortening were added.

The lipid contents of the dough ingredients are summarized in Table 2. In view of the relatively large amount of wheat flour and small levels of milk solids and yeast in the dough formulation, wheat flour contributed much more than the other ingredients to the lipid contents of the dough or bread. Only the contribution of shortening was larger

Table 1. Effects of dough composition on bread quality.

Dough composition	Proof height (cm)	Loaf volume (cc)	Crumb grain	Crust color
1) Basic [a]	below 5.8	345	U	Pale
2) Basic + shortening	below 5.8	355	U	Pale
3) Basic + milk solids	below 5.8	360	U	Light brown
4) Basic + sugar + shortening	6.5	740	Q-S	Brown
5) Basic + milk solids + sugar	7.1	695	Q	Rich brown
6) Basic + milk solids + shortening + sugar	7.0	765	Q-S	Rich brown
7) Basic + sugar	6.5	675	Q	Brown

[a] Flour, water, sodium chloride, yeast, and potassium bromate.

Table 2. Lipid contents of dough ingredients.

Ingredient	Petroleum-ether extract (%)	Chloroform-methanol extract (%)
Flour	0.88	1.25
Nonfat dry milk solids	0.10	0.84
Yeast	0.58	3.44

than of wheat flour lipids. The polar chloroform-methanol solvent system extracted much more lipids than petroleum ether. The increase was respectively 8- and 6-fold in milk solids and in yeast. Bound lipids composed 30% of the total flour lipids. This is in agreement with the findings of Daniels *et al.* (1966). Baldwin *et al.* (1963) reported that 62% of flour lipids were in a bound form. Extensive studies in our laboratories showed that only ⅓ of flour lipids were not extracted with petroleum ether (Pomeranz *et al.*, 1966a).

Petroleum ether and chloroform-methanol extracts showed little difference in amounts and kinds of nonpolar lipids fractionated by TLC with chloroform (Fig. 1). Clearly shown is the presence of two kinds of triglycerides in milk lipids, as described by Blank and Privett (1964) and Cerbulis and Zittle (1965). Fig. 2 compares TLC of chloroform-methanol extracts of flour and dough with standard polar lipids. The lipids

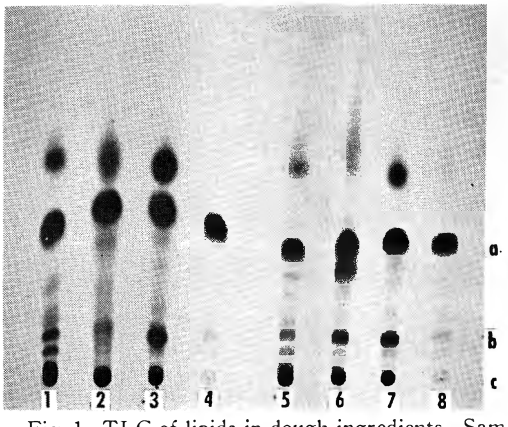


Fig. 1. TLC of lipids in dough ingredients. Samples 1–4 extracted with petroleum ether, 5–8 with chloroform-methanol. Samples 1 and 5, flour; 2 and 6, nonfat dry milk solids; 3 and 7, yeast; and 4 and 8, shortening lipids. Developed with chloroform; spots visualized by charring with sulfuric acid. Picture taken under UV light. a) Triglycerides; b) Mono- and diglycerides; c) Unfractionated polar lipids.

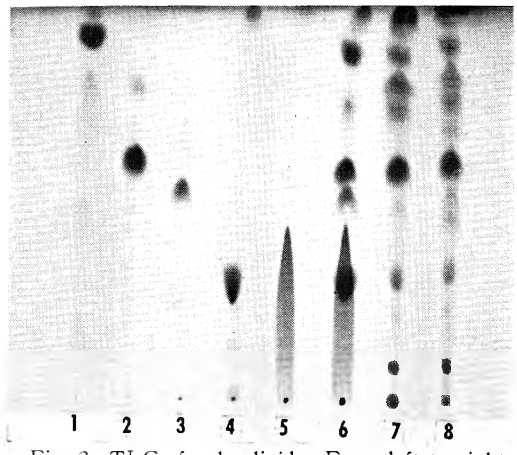


Fig. 2. TLC of polar lipids. From left to right: monogalactosyl glyceride, digalactosyl glyceride, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, mixture of all standard lipids, flour lipids, and dough lipids. Flour and dough lipids were extracted with chloroform-methanol. Standard and mixture of all standard lipids contained 25 μg of each lipid. Flour and dough lipids were applied at 100-μg levels. TLC plate developed with chloroform mixture.

were fractionated by TLC with the chloroform mixture. Petroleum ether extracted less polar lipids from flour than did chloroform-methanol. There was, however, no difference in the kinds of polar lipids extracted from flour by petroleum ether or by chloroform-methanol (Fig. 3). Only flour contained substantial amounts of polar components (phospholipids and glycolipids) in a free and in a bound form. In the case of milk

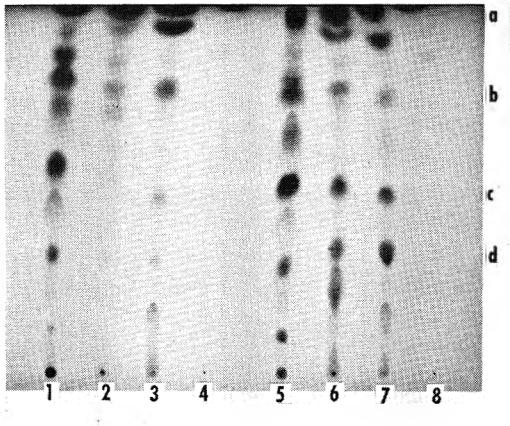


Fig. 3. TLC of lipids in dough ingredients. Samples as in Fig. 1 but developed with chloroform mixture. a) Nonpolar lipids; b) Monogalactosyl glyceride; c) Digalactosyl glyceride; d) Phosphatidyl choline.

Table 3. Lipid contents [a] of dough and bread.

Dough composition	Petroleum-ether extract				Chloroform:methanol extract			
	Dough	Fermented dough	Bread crumb	Bread crust	Dough	Fermented dough	Bread crumb	Bread crust
Basic [b]	0.28	0.25	0.12	0.09	1.30	1.40	1.12	1.35
Basic + shortening	2.87	2.95	1.47	1.81	3.52	3.90	3.74	3.94
Basic + milk solids	0.20	0.16	0.09	0.09	1.35	1.31	1.33	1.33
Basic + sugar + shortening	2.66	2.83	1.44	1.79	3.51	3.50	3.60	3.81
Basic + milk solids + sugar	0.18	0.16	0.12	0.10	1.29	1.35	1.42	1.48
Basic + milk solids + shortening + sugar	2.57	2.82	1.45	1.62	3.60	3.55	3.58	3.68
Basic + sugar	0.29	0.30	0.13	0.18	1.20	1.23	1.37	1.33

[a] In lyophilized sample, %.

[b] Flour, water, sodium chloride, yeast, and potassium bromate.

solids and yeast, little polar lipids were extracted with petroleum ether, and most of the polar lipids with low R_f values (presumably phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl serine) were present only in the chloroform-methanol extract. The major components of the polar fraction of milk lipids are, in decreasing order, phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, and phosphatidyl serine (Morrison *et al.*, 1965). Yeast lipids contain triglycerides, small amounts of sterols and hydrocarbons, a number of phospholipids (mainly phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine) and sphingolipid-like compounds (Eddy, 1958). Glycolipids, mono- and digalactosyl glyceride, were present in relatively high concentrations in flour lipids only. The vegetable shortening contained no measurable amounts of polar lipids fractionated by the chloroform mixture.

Table 3 summarizes the effects of dough-mixing and fermentation, and of bread-baking, on the extractability of lipids. Dough-mixing lowered the amounts of flour lipids soluble in petroleum ether to one-third the value in untreated flour. Flour lipids contain a 1:1 ratio of nonpolar-polar components (Pomeranz *et al.*, 1966a). Triglycerides compose the major nonpolar component. Reduction in amounts of free lipids as a result of dough mixing indicates, therefore, partial insolubilization of nonpolar flour lipids. There was no significant difference in amounts of petroleum-ether-soluble lipids in the dough between the beginning and the end of fermentation. Baking the fermented dough, however, reduced the amount of petroleum-ether-soluble flour lipids to half the amount in the dough, indicating additional binding of nonpolar flour lipids. Unlike the flour lipids, only less than 15% of the shortening lipids were bound during dough-mixing and fermentation. At the baking stage, about ⅓–½ of the shortening lipids were inextractable in petroleum-ether. The amounts of bound lipids in doughs made by the complete formula were 26% of the total, agreeing with results of Baldwin *et al.* (1963) and Daniels *et al.* (1966). Both our data and those of Baldwin *et al.* (1963) show substantial increases in lipid binding during the baking stage. Daniels *et al.* (1966) found that only 10% of the dough lipids were bound during baking. Our findings seem to support the concept that shortening lipids are not bound by proteins and carbohydrates during dough-mixing and fermentation, and can function advantageously by melting in the oven and strengthening the cell structure (Baker and Mize, 1942; Baldwin *et al.*, 1963, 1965).

Adding nonfat dry milk solids (but not sugar) to the basic formula seems to reduce the levels of petroleum-ether-extractable lipids somewhat, indicating a possible binding of flour lipids by milk proteins.

Binding of flour or shortening lipids during mixing, fermentation, or baking seems to result from relatively weak bonding forces between lipids and flour proteins and carbohydrates. There was no consistent effect of dough-mixing, fermentation, or baking on

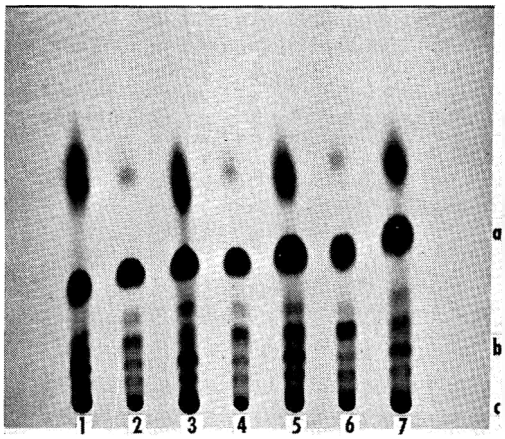


Fig. 4. TLC of petroleum-ether-soluble lipids in bread crumb. From left to right, from crumb of bread baked by formulae 1 to 7 (Table 1). Developed with chloroform.

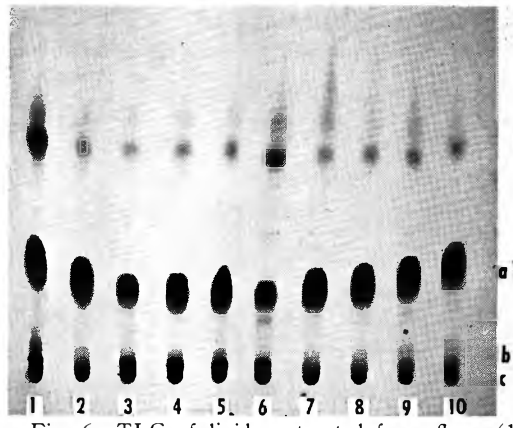


Fig. 6. TLC of lipids extracted from flour (1 and 6), complete dough formula (2 and 7), fermented dough (3 and 8), bread crumb (4 and 9), and crust (5 and 10). Samples 1–5 extracted with petroleum ether, 6–10 with chloroform-methanol. Developed with chloroform.

the amounts of chloroform-methanol-extractable total lipids. Small differences are explicable by variations in moisture contents and relative concentrations of lipids in the dough. In keeping with baking practices, shortening was added on a flour basis and kept uniform at 3 g per 100 g flour. Consequently, the level of shortening was smaller in rich formulae than in lean formulae. Fermentation and baking losses make it difficult to express the results with precision on a common basis. Fig. 4 shows TLC of petroleum-ether-soluble lipids in bread crumb. The nonpolar lipids were fractionated with chloroform. Samples 2, 4, and 6, from bread baked with shortening, differ from the odd-numbered samples, baked without shortening. It should be stressed that in all chromatographs the total lipid concentration was kept constant. Consequently, in the crumb that contained shortening the relative amounts of flour lipids are reduced. For similar reasons, TLC of chloroform-methanol-extracted lipids, in which fractionation was made with the chloroform mixture (Fig. 5), showed higher relative concentrations of polar flour lipids in bread baked without shortening than with it.

A comparison of Fig. 6 with Fig. 7 shows clearly that whereas the polar wheat flour lipids are bound during mixing and baking of dough containing shortening, the nonpolar components are affected little during process-

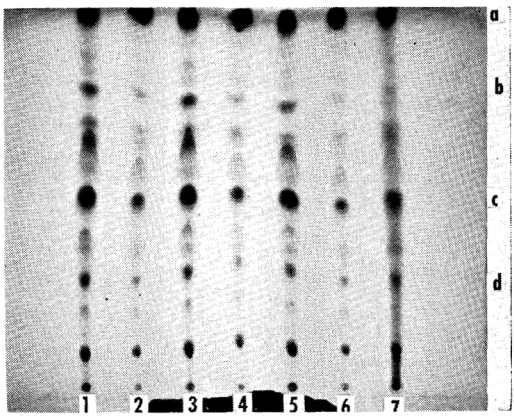


Fig. 5. TLC of chloroform-methanol-soluble lipids in bread crumb; samples as in Fig. 4 but developed with chloroform mixture.

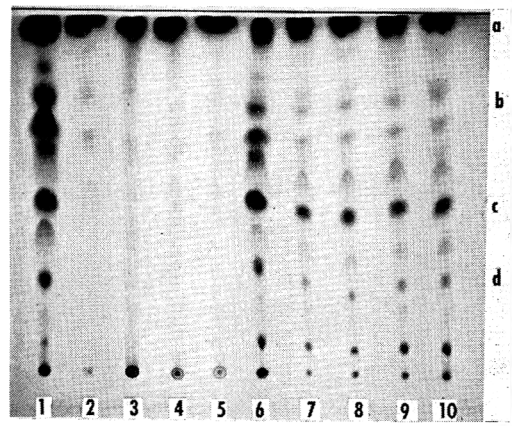


Fig. 7. TLC of lipids described in legend of Fig. 6, but developed with chloroform mixture.

ing of flour into bread. Results were similar when lipids were extracted at various stages of processing from dough containing no shortening. Changes in various wheat flour lipids and commercial fats and oils during the bread-making process are being studied.

REFERENCES

Baker, J. C., and M. D. Mize. 1942. The relation of fats to texture, crumb and volume in bread. *Cereal Chem.* **19**, 84.

Baldwin, R. R., R. G. Johansen, W. J. Keogh, S. T. Titcomb, and R. H. Cotton. 1963. The role that fat plays in continuous bread-making. *Cereal Sci. Today* **8**, 273.

Baldwin, R. R., S. T. Titcomb, R. G. Johansen, W. J. Keogh, and D. Koedding. 1965. Fat systems for continuous mix bread. *Cereal Sci. Today* **10**, 452.

Blank, M. L., and O. S. Privett. 1964. Structure of milk fat triglycerides. *J. Dairy Sci.* **47**, 481.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911.

Cerbulis, J., and C. A. Zittle. 1965. Identifizierung von Milchfett in anderen Fetten mit Hilfe der Dunnschicht-Chromatographie. *Fette, Seifen, Anstrichmittel* **67**, 273.

Daftary, R. D., and Y. Pomeranz. 1965a. Changes in lipid composition in maturing wheat. *J. Food Sci.* **30**, 577.

Daftary, R. D., and Y. Pomeranz. 1965b. Changes in lipid composition in wheat during storage deterioration. *J. Agr. Food Chem.* **13**, 442.

Daniels, N. W. R. 1963. The lipids of wheat and wheat products. *In:* "Reports on the Progress of Applied Chemistry." **48**, 165.

Daniels, N. W. R., J. W. Richmond, P. W. R. Eggitt, and J. B. M. Coppock. 1966. Studies on the lipids of flour. III. Lipid binding in bread-making. *J. Sci. Food Agr.* **17**, 20.

Eddy, A. A. 1958. Aspects of the chemical composition of yeast. *In:* "The Chemistry and Biology of Yeasts." (A. H. Cook, ed.) Academic Press, N. Y.

Feldman, G. L., L. S. Feldman, and G. Rouser. 1965. Occurrence of glycolipids in the lens of the human eye. *J. Am. Oil Chemists' Soc.* **42**, 742.

Fisher, N. 1962. Wheat lipids. *In:* "Recent Advances in Food Science." Butterworths Publ. Co., London.

Mecham, D. K. 1964. Lipids. *In:* "Wheat Chemistry and Technology." American Assoc. Cereal Chem., Minneapolis, Minn.

Mecham, D. K., and N. E. Weinstein. 1952. Lipid binding in doughs. Effects of dough ingredients. *Cereal Chem.* **29**, 448.

Morrison, W. R., E. L. Jack, and L. M. Smith. 1965. Fatty acids of bovine milk glycolipids and phospholipids and their specific distribution in the diacylglycerophospholipids. *J. Am. Oil Chemists' Soc.* **42**, 1142.

Olcott, H. S., and D. K. Mecham. 1947. Characterization of wheat gluten. I. Protein-lipid complex formation during doughing of flours. Lipoprotein nature of the glutenin fraction. *Cereal Chem.* **24**, 407.

Pomeranz, Y., G. L. Rubenthaler, and K. F. Finney. 1965. Polar vs. nonpolar wheat flour lipids in bread-making. *Food Technol.* **19**, 1724.

Pomeranz, Y., O. Chung, and R. J. Robinson. 1966a. The lipid composition of wheat flours varying widely in bread-making potentialities. *J. Am. Oil Chemists' Soc.* **43**, 45.

Pomeranz, Y., G. L. Rubenthaler, R. D. Daftary, and K. F. Finney. 1966b. Effects of lipids on bread baked from flours varying widely in bread-making potentialities. *Food Technol.* in press.

Tsen, C. C., I. Levi, and I. Hlynka. 1962. A rapid method for the extraction of lipids from wheat products. *Cereal Chem.* **39**, 195.

Ms. rec'd 3/11/66.

Financial support of Cargill Inc., Minneapolis, Minnesota, to Miss Chien-Mei Chiu is gratefully acknowledged. The authors are indebted to K. F. Finney, Federal Hard Winter Wheat Quality Lab., Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, for baking the samples.

Mention of a trade product does not imply endorsement. Contribution No. 551, Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan.

A. LAURENCE CURL

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

The Carotenoids of Muskmelons

SUMMARY

The carotenoid mixture of orange-fleshed muskmelons consisted predominantly of β-carotene. ζ-Carotene was the next most abundant constituent. Small amounts of phytoene, phytofluene, α-carotene, lutein, zeaxanthin, violaxanthin, luteoxanthins, and apparently neoxanthin were found. Monols and monoepoxide diols were found in small amounts, and a fraction, perhaps monoepoxide monols, in very small amount. In the ripening of the fruit there is a considerable buildup of β-carotene, and to a lesser extent of ζ-carotene.

INTRODUCTION

Orange-fleshed muskmelons (commonly called cantaloups in the United States) are known to be a good source of carotene (Fraps, 1947). Vavich and Kemmerer (1950) found that β-carotene amounted to at least 94% of the total carotenes, and reported the presence of one additional unidentified pigment. Rabourn and Quackenbush (1953) reported the presence of phytoene and phytofluene in the cantaloups. A systematic study has now been made of the carotenoids of orange-fleshed muskmelons, using countercurrent distribution to achieve a preliminary fractionation.

EXPERIMENTAL

The muskmelons used, which were the common orange-fleshed variety, were obtained in September at a local market. Two 1000-g batches of the edible portion were each blended with a mixture of 1 L of water, 1 L of methanol, and 10 g of magnesium carbonate. Filter aid (Celite, 10% by weight of the fruit used) was added, and the mixture filtered on a Buchner funnel. The filter cake was worked up essentially as previously described (Curl, 1961), including saponification by potassium hydroxide in a one-phase system of ether-methanol.

Countercurrent distribution runs were carried out in a Craig apparatus, using solvent systems I, II (Curl, 1953), and IV (Curl, 1960). Solvent system I is hexane and 99% methanol, 1.8:1 by volume; II is benzene, hexane, and 87% methanol, 1:1:1.15 by volume; IV is hexane and 73½% methanol, 1:1 by volume. The fractions obtained were chromatographed on columns of magnesia (Sea Sorb 43), 14 by *ca.* 90 mm, and the various constituents eluted by a graded series of eluants (Curl, 1959). Spectral data for the various bands eluted were obtained in a Beckman DK-2 recording spectrophotometer.

RESULTS AND DISCUSSION

The total carotenoid content of the edible portion of the fruit used was found to be equivalent to 20.2 mg/kg (as β-carotene).

During evaporation of the acetone extract of the filter cake, a relatively large amount of crystals of β-carotene separated.

Countercurrent distribution. One-fourth of the carotenoid extract was used in a 100-transfer countercurrent distribution run with solvent system I. The results (Table 1) show the great preponderance of the hydrocarbon fraction. The diol-polyol fraction from this run was used in a second distribution with system IV (Table 1). The hydrocarbon fraction as obtained above was examined chromatographically; the other fractions were not, since they occurred in such small amounts.

Table 1. Composition of the muskmelon carotenoids as determined by countercurrent distribution.

Fraction	Approximate % of total carotenoids [a]	N_{100} [b]
System I		
I (Hydrocarbons)	96.5	91
IIA (Monols)	0.7	59
IIB (Monol epoxides ?)	0.07	38
III (Diols and polyols)	2.7	8
System IV		
IIIA (Diols)	1.3	90
IIIB (Monoepoxide diols)	0.16	64
IIIC (Diepoxide diols)	1.1	23
IV (Polyols)	0.21	1

[a] Color measured at 440 mμ in an Evelyn photoelectric colorimeter.

[b] Position of maximum per 100 transfers.

Table 2. Carotenoid constituents of muskmelons as obtained by countercurrent distribution and chromatography.

Fraction and constituent	Spectral absorption maxima (mμ[a])	Approx. %
I Phytoene	(297), 286, 276	1.5
Phytofluene	367, 348, 331	2.4
α-Carotene	472, 441, (417), 332	1.2
β-Carotene	477, 447, (423), 339	84.7
ζ-Carotene	422, 398, 376	6.8
IIA (Monols)[b]		0.7[c]
IIB (Monoepoxide monols ?)		0.07[c]
IIIA Lutein	484, 453, 428, 338	1.0
Zeaxanthin	485, 457, (432), 342	0.08
IIIB (Monoepoxide diols)[b]		0.16
IIIC Violaxanthin	481, 449, 423, 336, 320	0.9
Luteoxanthin a	457, 428, 404	0.07
Luteoxanthin b	455, 427, 404	0.08
IV (Polyols)[b]		0.23
Neoxanthin-like	479, 447	0.20
Sinensiaxanthin-like	424, 402, 376	

[a] Fraction I constituents in hexane, others in benzene
[b] Not chromatographed, because too small amount of material available.
[c] From countercurrent distribution.

The remaining three-fourths of the carotenoid extract was used in a 100-transfer countercurrent distribution run with solvent system II, in order to obtain a sufficient quantity of the diol and diepoxide diol fractions for chromatography.

The composition of the cartoenoid mixture, as far as could be determined chromatographically, is given in Table 2.

Hydrocarbons (I). Chromatography of this fraction showed the great preponderance of β-carotene, which was accompanied by a much smaller amount of ζ-carotene, and still smaller amounts of phytoene, phytofluene, and α-carotene.

Monols (IIA). The monol fraction was not chromatographed, because of the small quantity of material available. The N_{100} value of this fraction was near that previously found for cryptoxanthin (56–57).

Fraction IIB. This very small fraction may have been monoepoxide monols; fractions with similar N_{100} values have been found in a number of fruits.

Diols (IIIA). This fraction contained lutein and zeaxanthin, in the approximate ratio of 12:1; this relative proportion is similar to that found in this fraction from green leaves.

Monoepoxide diols (IIIB). This fraction occurred in quite small amount, so that no chromatography was attempted.

Diepoxide diols (IIIC). This fraction was found to consist of violaxanthin (zeaxanthin-5,6,5′,6′-diepoxide), together with a much smaller amount of the 5,6,5′,8′-diepoxide isomers, the luteoxanthins. On treatment of an aliquot of the solution in ethanol of violaxanthin with HCl, the spectral absorption maxima changed to those of the corresponding 5,8,5′,8′-diepoxide isomers, the auroxanthins (423, 398, 377 mμ), confirming the identification as violaxanthin.

Polyols (IV). This fraction was present in too small amount to chromatograph, so the spectral absorption of the entire fraction was measured. Maxima in benzene were present at 479, 447, 424, 402, and 376 mμ, indicating the presence of at least two components, the longer-wavelength one probably being neoxanthin. The other maxima were

similar to those of sinensiaxanthin, a pigment of unknown structure found originally in oranges (Curl and Bailey, 1954). On treatment of the solution in ethanol with a drop of hydrochloric acid, the wavelength of the spectral absorption maxima shifted from 466, 437, 415, and 394 mμ to 445, 418, 394, and 370 mμ, indicating that the major component, at least, was a 5,6-monoepoxide such as neoxanthin.

Aside from the hydrocarbon fraction, the fractional composition of the carotenoids was rather like that of green leaves or green fruit. The components obtained on chromatography were also similar to those to be expected from green leaves or fruit. On ripening and turning orange of the interior, there is a very great buildup of the carotenoid content, mainly β-carotene, and to a lesser extent ζ-carotene. The other hydrocarbon constituents usually occur as minor constituents in green leaves or fruit, and there may have been some increase here on ripening.

REFERENCES

Curl, A. L. 1953. Application of countercurrent 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. 1961. The xanthophylls of tomatoes. *J. Food Sci.* **26**, 106.

Curl, A. L., and G. F. Bailey. 1954. Polyoxygen carotenoids of Valencia orange juice. *J. Agr. Food Chem.* **2**, 685.

Fraps, G. S. 1947. Vitamin A and carotene in human foods. *Texas Agr. Expt Sta. Bull.* 690.

Rabourn, W. J., and F. W. Quackenbush. 1953. The occurrence of phytoene in various plant materials. *Arch Biochem. Biophys.* **44**, 159.

Vavich, M. G., and A. R. Kemmerer. 1950. The carotenes of cantaloupes. *Food Research* **15**, 494.

Ms. rec'd 4/23/66.

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

C. B. DENNY, P. L. TAN, and C. W. BOHRER
National Canners Association, Research Foundation, Washington, D. C. 20036

Heat Inactivation of Staphylococcal Enterotoxin A

SUMMARY

A large batch of enterotoxin A from *Staphylococcus aureus* strain 196-E was prepared in casamino acid medium and concentrated 13.5 times by dialysis under polyethylene glycol. Concurrent tests on heat inactivation of this toxin were conducted with monkeys and cats. The heat inactivation curve based on the cat emetic reaction to intraperitoneal injection can be expressed as a straight-line semi-logarithmic curve with a slope *(z)* of 48°F to traverse one log cycle of time and a resistance at 250°*F* of 11 min (F_{250}^{48} = 11 min). The heat inactivation curve based on the monkey emetic reaction to oral feeding was F_{250}^{40} = 8 min. The heat resistance tests based on the two test animals are in remarkable agreement. Cats were found to be considerably more susceptible to the toxin by injection than were monkeys by feeding. The implication of these tests in relation to heat processing of foods is discussed, and preliminary indications are that the processes given commercially canned foods in which the organisms will grow are sufficient to destroy the toxin.

INTRODUCTION

Enterotoxin is a substance elaborated by the growth of certain coagulase-positive staphylococci in a suitable growth substrate. If the enterotoxin is ingested, a severe but non-lethal food poisoning can occur. It is probably the most common cause of food intoxication in this country. Unlike the lethal toxin of *Clostridium botulinum,* which is destroyed rapidly at temperatures below the boiling point, *Staphylococcus* enterotoxin possesses some degree of heat stability (Dack, 1956). The extent of this heat stability has been clouded by the fact that various test animals and types of enterotoxin have been used in diverse substrates without adequate documentation of the amount of heat actually applied. Casman *et al.* (1963) reported that several serological types of enterotoxin exist and that most food poisoning from enterotoxin occurs with the enterotoxin A (196-E prototype strain). Because enterotoxin B is easily produced in quantity, most reported work has been with enterotoxin B. Recently, Read and Bradshaw (1966) defined heat inactivation of enterotoxin B, with both cats and serological means used to determine the endpoint. To our knowledge, their work is the first thorough study of the heat inactivation of enterotoxin B reported in the literature, and the authors state that caution should be exercised in attempting to use the data in predicting the fate of enterotoxin A in a food during processing.

Definite knowledge of the time and temperature necessary to inactivate enterotoxin A by heat should be valuable to all food handlers, processors, and public health authorities in the control of food poisoning. The test animals that can be used in such a study are limited to man, monkeys, and cats. The use of nematodes was tested by the authors, and rejected because of false positive results. Preliminary work in this laboratory was done using intraperitoneal injection of cats (Denny and Bohrer, 1963). During the investigation, which was initiated in 1959, it was found that cats may develop an emetic reaction within 2 hr of intraperitoneal injection of a variety of materials. Some of these materials are staphylococcal hemolysins, cold liquids, by-products of growth produced by *Escherichia coli* and by *Proteus* strains, and a combination of trypsin and egg white inhibitor. Preliminary work in this laboratory indicated that enterotoxin had a heat resistance which could be expressed as F_{250}^{35} = 100 min, but this resistance appeared so high that the cat test was questioned as to its suitability for use in such a test, and preliminary monkey feeding tests did not confirm this extremely high resistance. Monkey feeding tests, previously reported, also did not confirm this high heat resistance for enterotoxin (Laing and Shinn, 1940). Human feeding tests (Dack and Surgalla, 1953) also contradicted this high heat resistance.

At about this time, Manohar *et al.* (1964) indicated that staphylococcal hemolysins were reactivated by strong heat. Subsequently, both alpha- and beta-hemolysins were found to be present in the samples giving the high

heat-resistance results by cat injection tests. Methods were investigated for eliminating the hemolysins, and a colony of *Staphylococcus aureus* 196-E was isolated that produced little or no hemolysins when grown and dialyzed under specific conditions. Also, reduction of the incubation period from 96 hr to 18 hr helped eliminate the hemolysins. Since monkeys are affected by enterotoxin but not by hemolysins when fed, this experiment was designed whereby duplicates of the heated samples injected into cats were fed to monkeys.

MATERIALS AND METHODS

A culture of *Staphylococcus aureus* No. 196-E was obtained from Dr. M. S. Bergdoll of the Food Research Institute of the University of Chicago. This strain is known to produce enterotoxin of the type A variety only. A single colony isolate which was found to produce very little hemolysin was chosen for study. An 18-hr 98°F culture in brain heart infusion was added to sterile bead-like insulators (part no. 40882-005, Minneapolis-Honeywell Regulator Co.) in a sterile container and dried under vacuum and silica gel at 38°F. Two of these inoculated dried insulators were placed in a sterile vial with a cotton stopper, and this vial was placed in a screw-capped tube containing silica gel. All the beads were stored in this manner at 38°F until used. Cultures were activated by adding two beads to a tube containing 6 ml of sterile brain heart infusion and incubating at 98°F for 18 hr. Then, 4 ml of sterile water was added to the growth material, and the contents were mixed (on a Vortex Jr. Mixer). Viable counts have ranged from 30 million to 3.4 billion per ml. One ml of the growth material was inoculated into 500 ml of completely dialyzable sterile casamino acid medium (Casman, 1958) in a 2-L Erlenmeyer flask. Eight flasks (4 L) were prepared each time. The flasks containing the inoculated medium were attached to a mechanical shaker (Burrell Side Arm) at a 45° angle and shaken at 320 strokes per min during incubation at 98°F for 18 hr.

At the completion of the incubation period, the growth from each flask was examined microscopically for contamination and discarded if contaminated. The liquid was centrifuged at 9,000 rpm for 60 min in a refrigerated (34°F) centrifuge. Because this treatment cleared the growth material but did not completely remove all the staphylococci, the centrifuged growth liquid was passed through an HA Millipore filter. The filtered liquid was then placed in washed dialysis sacs (average pore radius of 24 Å) and both ends knotted. The sacs were placed in plastic buckets and covered with a hydrophilic colloid (polyethylene glycol, 20,000). The buckets were stored at 38°F for 48 hr or until the sacs were essentially without liquid. The outside of the sacs was washed thoroughly with distilled water, and the material inside the sacs was removed by washing with two rinses, using the smallest amount of sterile water possible (about 10 to 15 ml). The concentrated material represented a 13.5-fold concentration, with a final volume of 1,850 ml. The concentrated toxin contained very little alpha- or beta-hemolysin as determined by hemolysin assay with washed sheep cells.

A volume of 2.25 ml of the enterotoxin was sealed in each Pyrex thermal-death-time tube (7 mm ID, 9 mm OD). Heating of the tubes was accomplished in an oil bath controlled by a Thermocap relay adjusted for accuracy with a thermometer standardized (at each temperature used) against a mercury-in-glass thermometer calibrated by the National Bureau of Standards. Variation was ±0.2°F from the desired temperature, and the time was controlled with a stopwatch. Lag in heating time was determined by thermocouple measurements, and all times mentioned are corrected for the lag time. Tubes were immersed in cold water immediately upon removal from the bath and stored under refrigeration until used.

The treatment, care, and method of injection of cats or kittens were similar to those described by Matheson and Thatcher (1955). In brief, the cats were quarantined for 21 days, during which time they were wormed, given immune serum, pneumonitis vaccine, and distemper vaccine (twice), and checked for ringworm. At the time of the test, the cat injection area was sponged with 70% alcohol and the test material injected with a 21-gauge needle just below the rib cage in the right intraperitoneal section. The cat test was accepted as positive for enterotoxin if decisive vomiting occurred between 10 min and 2 hr after injection. Individual cats were used only 4 times at 10-day intervals. No immunity was detected with this procedure, and no cats died.

Monkey feeding was done at the Food Research Institute of the University of Chicago under the supervision of Dr. M. S. Bergdoll. Known enterotoxin-sensitive young rhesus monkeys were administered the enterotoxin preparation in 50 ml of water through a rubber catheter directly into the stomach. The monkeys were observed for 5 hr for emesis.

It was found that 2 ml of a 1:6 dilution of the concentrated stock enterotoxin A elicited emetic response when injected intraperitoneally in cats. However, 6.75 ml of the unheated toxin was re-

quired to obtain a 100% emetic response in monkey feeding. Consequently, the contents of three heated tubes (6.75 ml) were fed to each monkey, whereas a 1:6 dilution of a single heated tube was made and 2 ml of the dilution injected intraperitoneally into each cat. This was equivalent to feeding about 47 μg to each monkey or injecting about 2.3 μg into each cat, based on the serological assay (7 μg/ml) reported by Bergdoll (1965b). This assay also positively identified the enterotoxin used in this study as type A.

RESULTS AND DISCUSSION

Endpoints of heat inactivation of enterotoxin A were determined at five temperatures: 212, 220, 230, 240, and 250°F. Fig. 1 shows the results of the heat inactivation of enterotoxin A as indicated by the cat emetic reaction (135 cats tested). The endpoints were confirmed by determining negative reactions of five cats at each of three consecutive time intervals above the last time interval of heating which showed at least one positive cat reaction. Any single positive cat at any time interval was recorded as positive even though there was the slight possibility of a false positive reaction. On semi-log paper, a straight-line curve was drawn above all positive points and below as many negative points as possible. The heat inactivation curve based on the cat emetic reaction can be expressed as a straight-line semilogarithmic curve with a slope (z) of 48°F to traverse one log cycle of time and a resistance at 250°F of 11 min ($F_{250}^{48} = 11$). This method of interpreting results is commonly used in expressing heat resistance of bacterial spores in the derivation of heat processes for canned foods (Townsend *et al.*, 1938). Results on this enterotoxin A batch as indicated by monkey feedings are shown in Fig. 2. Because monkey-feeding is expen-

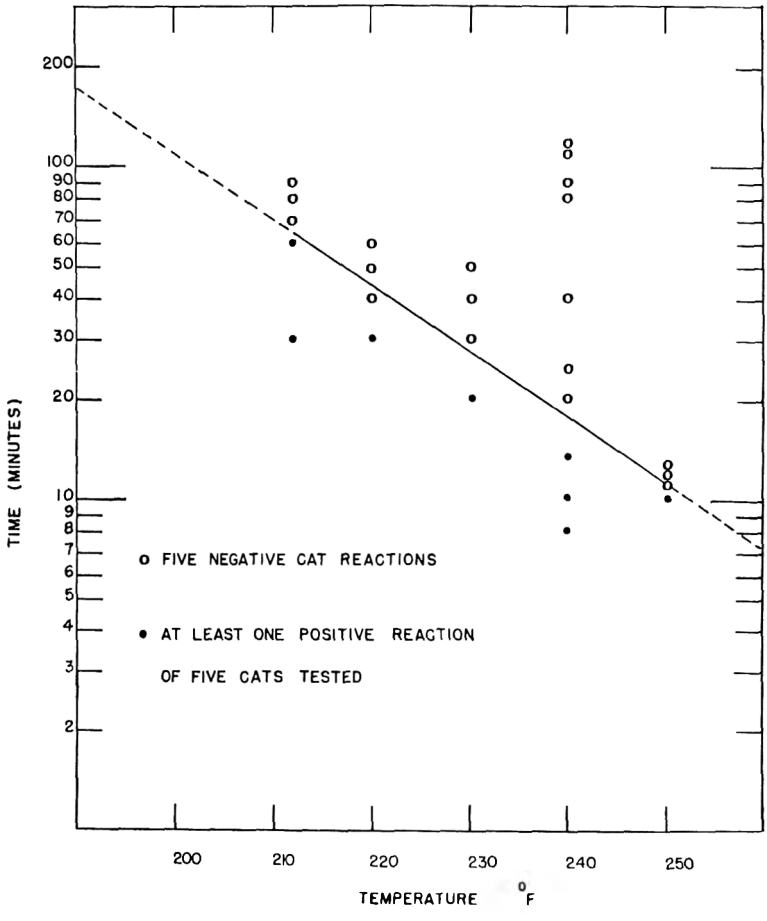


Fig. 1. Thermal inactivation curve of enterotoxin A based on the cat emetic reaction.

sive, only a three-monkey emetic reaction at each of the five temperatures was tested (93 monkeys). In other words, the endpoints were confirmed by obtaining negative reactions of three monkeys at each of three consecutive time intervals above the last time interval of heating which showed one positive monkey-reaction. The heat-inactivation results in this monkey test show close agreement with the results of the cat test. The heat inactivation curve based on the monkey emetic reaction can be expressed as a straight-line semilogarithmic curve with a slope (z) of 46°F to traverse one log cycle of time and a resistance at 250°F for 8 min ($F_{250}^{46} = 8$). The final positive reaction at 212°F was found only after testing 6 monkeys. The authors conducted the additional testing at 212°F because the monkeys were much more inclined to give negative responses than were the cats, and all other results, with the possible exception of those at 230°F, indicated that the 40-min interval at 212°F should give a positive test. One of 6 cats at the interval of 40 min at 212°F was positive, indicating that the straight-line semilogarithmic curve could be used. Testing a large number of monkeys with enterotoxin A heated for 15 min at 230°F would quite likely produce a positive reaction. The extensive series of time intervals tested at 240°F indicated that toxin was definitely reduced in strength by heating and that the curve is very near the maximum which might be achieved by additional monkey tests.

The cat results (Fig. 1) on enterotoxin A show substantially less heat resistance than the preliminary cat results mentioned in the Introduction, where large amounts of hemolysins were present. The validity of the latter cat tests with a preparation free of

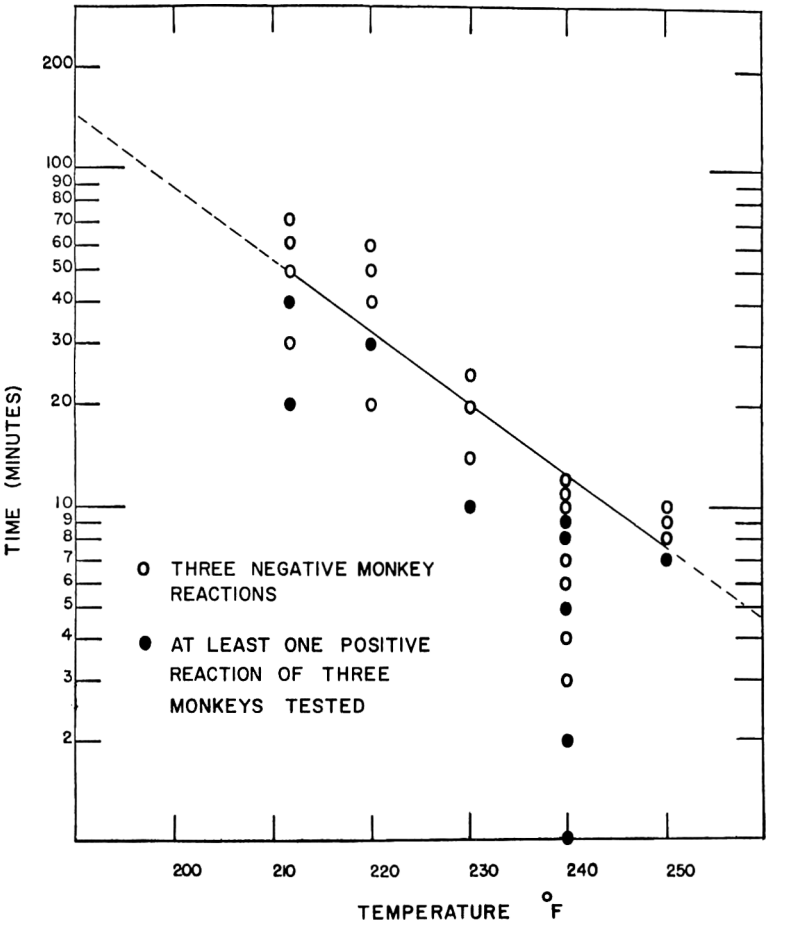


Fig. 2. Thermal inactivation curve of enterotoxin A based on the monkey emetic reaction.

hemolysins is confirmed by the monkey tests because the monkeys do not react to the staphylococcal hemolysins.

This work was complete when Read and Bradshaw (1966) published their heat inactivation figures on enterotoxin B determined by serological assay (a comparison with the cat emetic test was made only at one temperature). Their results can be expressed as a straight-line semilogarithmic curve with a slope (z) of 58°F and a resistance at 250°F of 16.4 min ($F_{250}^{58} = 16.4$). Enterotoxin B is not a common cause of food poisoning, whereas enterotoxin A has been reported in almost all human food poisoning cases (Bergdoll, 1965a).

Initial testing with cats and monkeys with the batch of enterotoxin used in these studies demonstrated that most cats were sensitive to unheated toxin when the concentration reached about 2.3 μg, whereas most monkeys were sensitive to the unheated toxin only after 47.25 μg were fed. In other words, 20.8 times as much unheated toxin was needed for monkeys as for cats. This enterotoxin A was concentrated by a factor of 13.5, and it took 6.75 ml for most monkeys to give an emetic reaction. The concentration of this toxin necessary to elicit a response in man is not known. However, if a man were affected to the same degree as a monkey, about 91 ml of the original growth material, formed under conditions most favorable for enterotoxin production, would have to be consumed to produce a reaction.

These results indicate that enterotoxin A is fairly resistant to heat. However, if interpreted against the commercial heat processes used in still retorts for low-acid canned foods, the preliminary indications are that even high concentrations of enterotoxin A would be destroyed unless afforded protection by the foods. If enterotoxin A were present in a high concentration in a raw low-acid food (which is extremely unlikely under commercial canning conditions), it is conceivable that some toxin might survive high-temperature short-time processing, because of the slope of the heat inactivation curve. However, only one or two cats or monkeys of those tested reacted to enterotoxin A given the higher heat treatments. In canned acid foods, such as tomatoes (pH 4.3) and peaches (pH 3.7), Segalove *et al.* (1943) showed that food-poisoning staphylococci could not grow, and that these canned foods therefore could not contain enterotoxin.

The heat inactivation results are on enterotoxin A heated at a pH of 7.8, which resulted after growth in the culture medium. Tests are currently under way on toxin adjusted to lower pH levels. Because acid denatures protein and enterotoxin is a simple protein, the heat required for toxin inactivation at lower pH levels might logically be less. Heat inactivation studies in food products at their natural pH levels are also contemplated.

REFERENCES

Bergdoll, M. S. 1965a. Part II. Research achievements. The enterotoxins. Chemical-physical properties, purification, production, and identification. Annual Report for 1964, The Food Research Inst., Univ. of Chicago, 1.

Bergdoll, M. S. 1965b. Personal communication.

Casman, E. P. 1958. Serological studies of staphylococcal enterotoxin. *U.S. Public Health Repts.* **73**, 599.

Casman, E. P., M. S. Bergdoll, and J. Robinson. 1963. Designation of staphylococcal enterotoxins. *J. Bacteriol.* **85**, 715.

Dack, G. M. 1956. Food Poisoning. Univ. of Chicago Press, Chicago.

Dack, G. M., and M. J. Surgalla. 1953. Thermal destruction of Staphylococcus enterotoxin experimentally inoculated into fresh pork. *Food Technol.* **8**, 307.

Denny, C. B., and C. W. Bohrer. 1963. Improved cat test for enterotoxin. *J. Bacteriol.* **86**, 347.

Laing, M. L., and B. M. Shinn. 1940. Preliminary studies in heat resistance of bacterial toxins. *Canner* **90** (12), Part 2, 85.

Manohar, M., S. Kumar, and R. K. Lindorfer. 1964. Heat reactivation of the alpha hemolytic, dermal necrotic, and lethal activities of crude and purified staphylococcal alpha toxin. *Bacteriol. Proc.* **61** (M 95).

Matheson, B. H., and F. S. Thatcher. 1955. Studies with staphylococcal toxins. 1. A reappraisal of the validity of the kitten test as an indication of Staphylococcus enterotoxin. *Can. J. Microbiol.* **1**, 372.

Read, R. B., Jr., and J. G. Bradshaw. 1966. Thermal inactivation of staphylococcal enterotoxin B in veronal buffer. *Appl. Microbiol.* **14**, 130.

Segalove, M., E. Davison, and G. M. Dack. 1943. Growth of a food poisoning strain of Staphylococcus experimentally inoculated into canned foods. *Food Research* **8**, 54.

Townsend, C. T., J. R. Esty, and F. C. Baselt. 1938. Heat resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. *Food Research* **3**, 323.

Ms. rec'd 2/23/66.

This investigation was supported in part by U. S. Public Health Service research grant EF 00124 from the Environmental Engineering and Food Protection Division.

ROBERT E. LEVIN [a] **and REESE H. VAUGHN**
Department of Food Science and Technology, University of California, Davis, California 95616

Desulfovibrio aestuarii, the Causative Agent of Hydrogen Sulfide Spoilage of Fermenting Olive Brines

SUMMARY

***Desulfovibrio aestuarii* was found in concentrations as great as 2 x 10^6 per ml in fermenting malodorous sulfureted olive brines. Of 268 commercial brine tanks randomly sampled, 46 (17.6%) yielded positive enrichments for *D. aestuarii*.**

INTRODUCTION

The use of microbiologically selective concentrations of solar salt (5–8%) for the storage and fermentation of olives is well established in the California olive processing industry. The presence of sulfate in the impure salt (Shewan, 1951), the ensuing formation of lactic acid, and the resulting anaerobiosis with an initial pH above 5.5 create ideal conditions for the development of hydrogen sulfide from the growth and metabolism of the halophilic *Desulfovibrio aestuarii*. Soriano (1955) was the first to isolate members of the genus *Desulfovibrio* from olive brines which had undergone the malodorous fermentation termed "zapatera." The odor of hydrogen sulfide, however, is not pronounced in "zapatera" spoilage. Furthermore, spoilage is avoided completely only when the pH of the brine is decreased by careful control to a value of about 3.8. Growth of all of the cultures described herein failed at less than pH 5.5. Therefore, it would appear to be very doubtful that the sulfate-reducing bacteria are capable of producing the foul, fecal stench found by Kawatomari and Vaughn (1956) to be caused by the clostridia associated with "zapatera" spoilage of olives in brine. The present investigation was initiated folllowing a severe outbreak of hydrogen sulfide spoilage of brined Sicilian-style fermented olives involving all storage brines in an industrial plant in the lower Sacramento Valley. Subsequently, isolated examples of this spoilage were detected in other processing plants throughout the major olive growing regions of California in the upper as well as the lower Sacramento Valley and throughout the San Joaquin Valley.

EXPERIMENTAL METHODS AND RESULTS

Culture media. Cultures were grown at pH 7.0 in a medium containing: yeast extract (Difco), 0.50%; $(NH_4)_2HPO_4$, 0.30%; sodium lactate, 0.30%; Na_2SO_4, 0.20%; K_2HPO_4, 0.17%; and sodium thioglycollate, 0.05%; in distilled water, with and without 1.5% agar as required.

H_2S determinations. Hydrogen sulfide was determined according to Standard Methods of Chemical Analysis (Scott, 1925) except that 5–10 ml of a broth culture were transferred by hypodermic syringe to a 250-ml ground-glass-stoppered Erlenmeyer flask containing 5 ml of 0.1*N* iodine, 25 ml of distilled water, and 1 ml of 1*N* HCl. The flask was stoppered and allowed to stand for at least 2 min before titrating with 0.1*N* sodium thiosulfate. Two drops of 1% soluble starch indicator were added just before the end point was reached.

Phase microscopy. Dark-phase microscopy was performed with a Carl Zeiss standard model GFL microscope with a neofluor 100x dark-phase objective. Photomicrographs were taken with Kodak panatomic X film with a Nikon camera back at a magnification of 1,200× and developed in Kodak DK60A for 7 min.

Electron microscopy. Electron micrographs were made with an RCA electron microscope model EMU-3E.

Dry cell weight. Cell densities were determined with a Bausch and Lomb spectronic 20 at 620 mμ using 13 × 100-mm tubes. The dry cell weight of cultures and cell suspensions was based on a standard curve prepared by plotting the OD (optical density) of cell concentrations against dry cell weight.

Enrichment cultures. One-ml brine samples were transferred to 9 ml of sterile broth containing 0.05% ferrous ammonium sulfate and 4% NaCl in screw-capped test tubes. Of 268 brine samples obtained from seven processing plants, 46 yielded positive enrichments for *D. aestuarii* (Table 1) from which pure cultures were obtained.

Enumeration of sulfate reducers in fermenting brines. To obviate the possibility that positive enrichments resulted from the chance occurrence of a floral minority, most-probable-number determinations were performed on malodorous brines. Tubes containing 9 ml of broth with 4% NaCl and 0.05% ferrous ammonium sulfate were inoculated

[a] Present address: Department of Food Science and Technology, University of Massachusetts, Amherst, Massachusetts 01003.

Table 1. Incidence of *Desulfovibrio aestuarii* in commercial olive fermentation brines.

Processing plant	Brine process	No. of tanks sampled	No. of positive enrichments
A	Salt stock for black ripe	35	0
	Spanish style	10	0
B	Salt stock for black ripe	26	8
	Spanish style	6	0
C	Salt-stock for black ripe	44	0
D	Salt stock for black ripe	12	0
E	Salt stock for black ripe	26	18
F	Salt stock for black ripe	60	1
G	Sicilian style	50	19
	Total	268	46
	%	100	17.2

with 1 ml of serial dilutions of brines. The formation of H_2S and black FeS accompanied by the microscopic demonstration of vibrio cells was regarded as indicative of the presence of *Desulfovibrio*. The results (Table 2) indicate that members of the genus *Desulfovibrio* can constitute a major population in fermenting brines.

Isolation of pure cultures. Enrichment cultures were serially diluted in broth, and 1 ml of several dilutions was transferred to tubes containing 20 ml of melted agar media at 48°C and then poured into petri plates. The plates were then placed in brewer anaerobic jars along with a mixture of 6 g of pyrogallic acid and 20 ml of 20% Na_2CO_3. The jars were sealed with plastacine clay and alternately evacuated and filled with nitrogen three

Table 2. Enumeration of *Desulfovibrio aestuarii* in fermenting brines by three-tube MPN.

Brine sample	Dilution	No. of positive tubes	No. of *D. aestuarii* per ml of brine
L6-J	10^{-7}	0	
	10^{-6}	3	2×10^6
	10^{-5}	3	
L6-31	10^{-7}	0	
	10^{-6}	3	2×10^6
	10^{-5}	3	
VR-189	10^{-7}	1	
	10^{-6}	2	2×10^6
	10^{-5}	3	
VR-170	10^{-5}	0	
	10^{-4}	1	4×10^4
	10^{-3}	3	
TK-77	10^{-5}	2	
	10^{-4}	2	2×10^4
	10^{-3}	3	

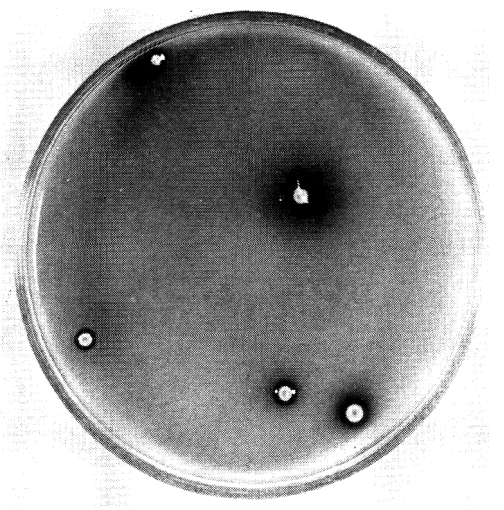


Fig. 1. Isolated colonies of *D. aestuarii* grown in agar medium containing 0.003% ferrous ammonium sulfate under anaerobic conditions in brewer jar.

times. Typical colony growth is shown in Fig. 1, and characteristic cell shape in Fig. 2. Pure cultures were obtained by picking well isolated colonies from plates free of microscopic satellite colonies and immediately inoculating into broth. The entire purification procedure was again repeated twice. Since pure cultures failed to utilize dextrose, final purity was established by lack of aerobic or anaerobic growth at pH 7.0 in a medium consisting of yeast extract (Difco), 0.50%; dextrose, 0.30%; and distilled water. A number of presumably pure cultures produced growth in this medium, which was always found to be due to contaminants. Cultures were identified as members of the genus *Desulfovibrio* on the basis of cell morphology, gram-negative staining, obligate an-

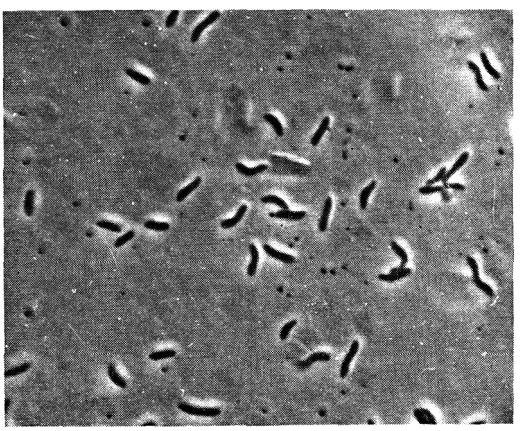


Fig. 2. Typical field of *D. aestuarii* taken with dark-phase microscopy. ×4,500.

Table 3. Effect of NaCl on H_2S production. H_2S determinations performed after 30 days of incubation at 30°C.

Culture	% salt												
	0	.5	1	2	4	6	8	10	12	14	16	18	20
	H_2S mgm/L												
138	484	464	396	350	86	41	36	0	0				
F6	43	46	420	–	570	377	372	412	0	0			
30	10	114	95	432	433	394	302	204	0	0			
BR10	0	0	0	0	333	337	258	0	0				
Tk14	0	0	0	61	455	477	212	0	0				
L36	0	0	0	272	444	–	395	57	0	0			
MAD	0	0	0	312	262	244	163	0	0				
P51-2	0	0	391	367	437	423	400	405	20	0	0		
L35	0	0	0	405	465	374	174	240	0	0			
VR133	0	0	242	44	452	435	408	278	78	0	0		
VR189	0	0	442	417	442	173	11	0	0				
H8	0	0	463	436	449	432	383	194	0	0			
A1	0	0	218	261	310	409	153	49	0	0			
OB249	0	0	102	300	316	298	–	243	252	0	0		
Br2	0	0	446	423	415	291	289	0	0				
RHS	0	534	549	–	425	418	–	–	495	388	346	54	0
P51-1	0	364	364	260	233	242	–	168	220	188	65	46	0
VR48	0	148	427	431	423	414	405	383	170	0	0		
1	0	413	451	452	465	435	102	112	0	0			
19	0	316	398	427	457	428	416	291	0	0			
48	0	380	409	442	446	430	428	385	0	0			

– Tube lost.

aerobiosis, polar flagellation, and formation of H_2S from lactate plus sulfate (Breed *et al.*, 1957).

Tolerance to NaCl. One ml of each actively growing culture was inoculated into 80 ml of broth of various NaCl concentrations contained in 25 × 250-mm tubes. The tubes were sealed aseptically with rubber stoppers and incubated for 30 days at 30°C. After incubation, H_2S determinations were performed (Table 3). Culture 138 was obtained from black ripe olives which had been allowed to remain at room temperature for several weeks in the final wash water. This culture showed a preference for a salt-free environment, growing only marginally in 6 and 8% NaCl, thus identifying it as a representation of the non-halophilic species *Desulfovibrio desulfuricans*. The remaining 20 cultures were all obtained from fermenting brines. From Table 3, the organisms studied may be placed into two general groups: 1) optimum growth without NaCl (Fig. 3); and 2) obligately halophilic growth, requiring at least 0.5% NaCl (Fig. 4). The low H_2S concentration formed by strains F6 and 30 in the absence of NaCl appears anomalous since no detectable turbidity was observed in the tubes. To preclude obligate halophily being due to osmotic shock when cells were transferred to salt-free media, progressive training at low salt concentrations was attempted. Cultures 19 and 48 were allowed to grow in continually decreasing increments of 0.50% NaCl at each transfer until both cultures failed to grow, at concentrations below 0.50% NaCl.

Osmotic sensitivity. Halophilic strain 30 was grown in 4 L of broth with 4.0% NaCl. After one day's incubation, the cells were centrifuged at 6,000 × G for 20 min and suspended in 40 ml of *2M* NaCl containing 0.01% K_2HPO_4 at pH 7.0 and allowed to equilibrate for 30 min. The concen-

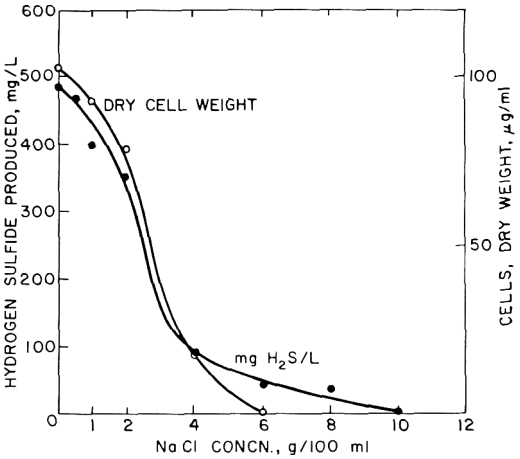


Fig. 3. Effect of NaCl on cell yield and H_2S production of *D. desulfuricans* strain 138 after 30 days of incubation at 30°C.

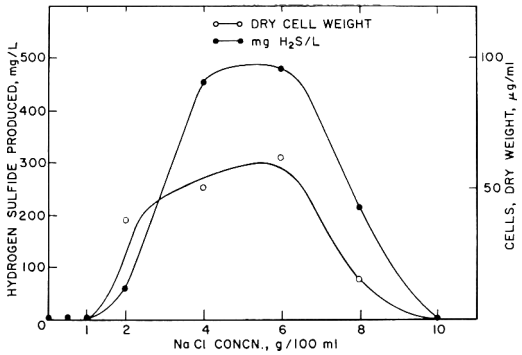


Fig. 4. Effect of NaCl on cell yield and H_2S production of *D. aestuarii* strain Tk14 after 30 days of incubation at 30°C.

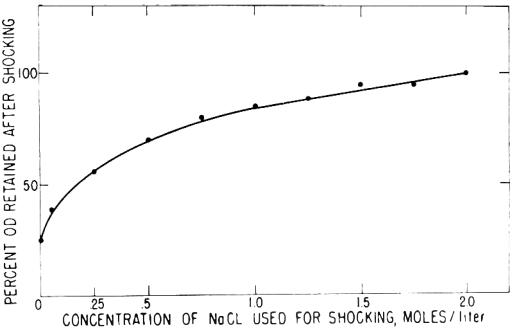


Fig. 6. Effect of osmotic disruption of *D. aestuarii* strain 30 on optical density of cell suspension. Cells were equilibrated in *2M* NaCl, then rapidly diluted 1:100 with varying concentrations of NaCl.

tration of suspended cells was then adjusted to yield an optical density of 0.200 on dilution to 1:100 with buffered *2M* NaCl. One ml of the adjusted cell suspension was then shocked by the rapid addition of 99 ml of buffered solutions of various NaCl concentrations. The results (Figs. 5 and 6) indicate pronounced sensitivity to osmotic shock. These observations are in contrast to the failure of Littlewood and Postgate (1957) to observe lysis of halophilic and non-halophilic cultures of *Desulfovibrio* when the cells were suspended in distilled water after growth in 10 and 4% NaCl, respectively.

DISCUSSION

There is considerable evidence to date indicating that members of the genus *Desulfovibrio* can no longer be regarded as autotrophic in their metabolism (Postgate, 1953; Kadota and Miyoshi, 1961; Mechalas and Rittenberg, 1960). The requirement for organic growth factors, low redox potential (Postgate, 1959; Starkey, 1960), ability to produce H_2S from lactate and sulfate, and the high population in H_2S-containing olive brine implicates *D. aestuarii* as the causative organism responsible for H_2S formation. Since the growth of sulfate reducers is favored by a neutral pH (Zobell, 1958) and since growth below pH 5.5 failed with all cultures reported herein, a faltering or slow fermentation which fails to lower pH rapidly serves as an ideal environment for the ascendency of *D. aestuarii*.

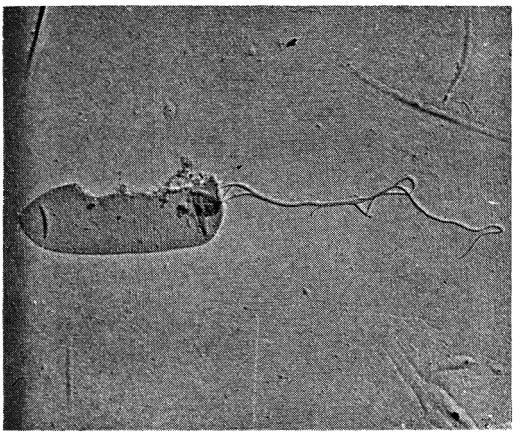


Fig. 5. Cell of *D. aestuarii* strain 30 subjected to osmotic disruption. Uranium-shadowed preparation. Cells were harvested from 4% NaCl broth culture, washed, and equilibrated in *2M* NaCl, then rapidly diluted 1:100 with distilled water. ×11,600.

Unfortunately, there is, to our knowledge, no authenticated record of the first hydrogen sulfide fermentation occurring in California olive brines, although they were first observed by the junior author during the 1937–38 harvest season in the upper Sacramento Valley. The fact that one of us (Vaughn) and his associates were able, between 1940 and 1948, to maintain sulfate-reducing enrichment cultures of bacteria from samples of highly sulfureted olive brines in carefully controlled "mineral" enrichment media for up to three transfers but were never able to produce pure autotropic cultures serves to emphasize this conclusion. The "mineral" media used in the earlier studies lacked essential nutrients now recognized as necessary for perpetuating the growth of most, if not all, strains of *Desulfovibrio*.

The seventh edition of Bergey's manual recognizes just two species of *Desulfovibrio*: the nonhalophilic *D. desulfuricans* and the halophilic *D. aestuarii*. The abundance of the halophilic species in distinctly sulfureted

olive brines and its failure to grow in the absence of NaCl appears to lend credence to the taxonomic distinction between the two species, based on ecology and salt tolerance. This differentiation is augmented by the failure in this study to train halophilic strains 48 and 49 to grow in the absence of NaCl. Littlewood and Postgate (1957), in studying the salt tolerance of several strains of both types, found a complete gradation of behavior toward NaCl within the genus *Desulfovibrio*. They were able to train a nonhalophilic culture that was originally capable of growing only below 5% NaCl to grow above 9% NaCl, and questioned the taxonomic distinction based solely on salt tolerance. Saunders *et al.* (1964), in a study of the base deoxyribonucleic acid (DNA) composition of sulfate-reducing bacteria, established three groups based on the percent of guanine + cytosine. Although both marine and freshwater strains appear in their groups I and II, all strains in group III are obligate halophiles. These results indicate a fundamental genetic difference distinguishing certain halophilic strains from nonhalophilic and intermediate types. The nonhalophilic strain 138 isolated from the final wash water in the absence of NaCl is capable of marginal growth from 4 to 8% NaCl (Fig. 3) and could be expected to persist throughout the processing, being favored by low salt concentrations. That such a nonhalophilic strain might arise from a halophilic progenitor is doubtful in view of the stable DNA base ratios observed when a nonhalophilic strain was adapted to salt (Saunders *et al.*, 1964), indicating a stable genotype with respect to salt tolerance.

The origin of *D. aetuarii* in fermenting brines remains uncertain since numerous attempts to isolate sulfate reducers from fresh olives, olive orchard soil, and solar salt failed. However, positive enrichments were obtained from mud along the banks of solar salt ponds; and, considering the halophilic nature of the implicated organisms, the most likely source would appear to be solar salt. This conclusion is particularly tenable from a consideration of the 10^5 halophilic bacteria per gram reported for solar salts (Shewan, 1951). The origin of the nonhalophilic strain 138 may also be solar salt since numerous nonhalophilic cultures were also isolated from shore mud adjoining the solar salt ponds in the San Francisco Bay area, the prime source of salt for the California olive industry.

REFERENCES

Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed. Williams and Wilkins Co., Baltimore. 1094 p.

Kadota, H., and H. Miyoshi. 1961. Organic factors responsible for stimulation of growth of Desulfovibrio. *Bacteriol. Proc. Soc. Am. Bacteriologists* **61**, 43.

Kawatomari, Toshio, and Reese H. Vaughn. 1956. Species of Clostridium associated with zapatera spoilage of olives. *Food Research* **21**, 481.

Littlewood, D., and J. R. Postgate. 1957. Sodium chloride and the growth of Desulphovibrio desulphuricans. *J. Gen. Microbiol.* **17**, 378.

Mechalas, B. J., and S. C. Rittenberg. 1960. Energy coupling in Desulfovibrio desulfuricans. *J. Bacteriol.* **80**, 501.

Postgate, J. R. 1953. On the nutrition of Desulphovibrio desulphuricans. A correction. *J. Gen. Microbiol.* **9**, 440.

Postgate, J. R. 1959. Sulphate reduction by bacteria. *Ann. Rev. Microbiol.* **13**, 505.

Saunders, Grady F., L. Leon Campbell, and John R. Postgate. 1964. Base composition of deoxyribonucleic acid of sulfate-reducing bacteria deduced from buoyant density measurements in cesium chloride. *J. Bacteriol.* **87**, 1073.

Scott, W. W. 1925. Hydrogen sulphide and soluble sulphides. p. 509–510. *In:* Standard methods of chemical analysis. 4th ed. D. Van Nostrand Co., New York. 1 vol.

Shewan, J. 1951. Common salt: its varieties and their suitability for fish processing, p. 1–9. *In:* World Fisheries Year Book. British Continental Trade Press, Ltd., London.

Soriano, S. 1955. La bacteria causante de la aceituna "zapatera" y su control. p. 397–401. *In:* Primera conferencia national de olivicultura. Ministerio de Agricultura y Canaderia. Buenos Aires, Argentina.

Starkey, R. L. 1960. Sulfate-reducing bacteria—physiology and practical significance. *Published by University of Maryland*, pp. 1–32. See *New Jersey Agr.* **44** (1) 14, 1962.

Zobell, C. E. 1958. Ecology of sulfate reducing bacteria. *Producers Monthly* **22** (7), 12.

Ms. rec'd 4/14/66.

The research reported here was supported in part by a contract with the Olive Advisory Board under authority of the Director of Agriculture of the State of California.

W. W. SADLER, R. E. CORSTVET, H. E. ADLER, and R. YAMAMOTO
Departments of Public Health and Avian Medicine, School of Veterinary Medicine, University of California, Davis, California 95616

Relative Prevalence of Bacterial Agents and Histopathology in Passed and Condemned Poultry Carcasses and Livers

SUMMARY

Although postmortem inspection (organoleptic examination by seeing, feeling, and smelling) of market poultry contributes significantly to the assurance of wholesomeness of the meat, such assurance is relative and not absolute. A significantly higher incidence of salmonellae in the cecal area of the intestines of condemned carcasses than in passed carcasses was found in chicken fryers but not in chicken hens or turkeys. Although no single genus of bacteria in the liver was correlated with disposition, the presence of "one or more species" of bacteria in livers was significantly correlated with gross pathology sufficient to cause condemnation. Each of eight separate species of organisms in livers was significantly correlated with condemnation of the carcass for septicemia-toxemia or inflammatory process. Certain isolates were found in a significantly greater percent of normal livers, suggesting that these are "normal" flora and not associated with disease. Livers that were grossly normal were found to have significantly fewer histopathologic lesions than livers condemned for gross pathology, which, in turn, had fewer lesions than livers from condemned carcasses. No single histopathologic process in the liver was correlated with its condemnation, whereas the category of "one or more lesions" was so correlated. The presence of each of three histopathologic processes as well as "one or more lesions" in the liver was also significantly correlated with condemnation of the carcass. The presence of at least one or more species of bacteria was correlated with at least one or more forms of histopathology, but no specific species of bacteria was correlated with specific histopathologic lesions. Inspection failed to detect numerous livers with histopathologic lesions of varying degrees of unwholesomeness, and, conversely, resulted in condemnation of livers in which no histopathologic evidence of unwholesomeness was detected. Inspection did not detect livers harboring a variety of bacteria of unknown significance.

INTRODUCTION

In 1957, the Congress of the United States enacted a law stating that "It is hereby declared to be the policy of Congress to provide for the inspection of poultry and poultry products by the Inspection Service—to prevent the movement in interstate or foreign commerce or in a designated consuming area of poultry products which are unwholesome, adulterated, or otherwise unfit for human food" (U.S. Congress, 1957). This law defines the term wholesome as being sound, healthful, clean, and otherwise fit for human food. It defines unwholesome, in part, as being unsound, injurious to health, or otherwise unfit for human food, and consisting, in whole or in part, of any filthy, putrid, or decomposed substance. These terms presumably cover such abnormalities as pathogenic or other abnormal bacterial agents in the tissues as well as abnormal morphologic and physiologic changes. A search of the literature has not revealed any study of the extent to which postmortem inspection of poultry by organoleptic methods (i.e. seeing, feeling, and smelling) detects microbial agents or abnormal morphological structures in the tissues of carcasses at slaughter or an abnormal physiological state prior to slaughter.

EXPERIMENTAL PROCEDURE

This report is based on data accumulated during a random sampling of market poultry for salmonella infection (Sadler *et al.*, 1961; Sadler and Corstvet, 1965a), a random sampling of market poultry livers for the presence of bacterial agents (Sadler *et al.*, 1965), and a study of the relationship of husbandry practices and disease problems to condemnation rates of selected flocks at slaughter (unpublished data). Although neither the flock itself nor the individual carcasses were selected randomly in the last study, most of the carcasses and tissues from which the data for this report were drawn, were chosen on a commercial slaughter line regardless of gross pathology or condemnation status.

All samplings were made in large processing plants of birds examined by qualified personnel of the United States Department of Agriculture. Immediately after an inspector had made his decision as to the wholesomeness of the carcass or tissue, samples were taken for bacteriological and/or histopathological examination. The liver was chosen

Table 1. Relative frequency of isolation of *Salmonella* spp. or *Paracolobactrum arizonae* from intestines of passed and condemned turkeys, chicken fryers, and chicken hens.

	Status of carcass	Turkeys		Chicken fryers		Chicken hens	
		Number	%	Number	%	Number	%
Total sampled	Passed	1572		1351		806	
	Condemned	158		485		329	
Salmonella spp.	Passed	199	12.7	14	1.0[a]	14	1.7
isolated	Condemned	26	16.5	28	5.8[a]	4	1.2
Salmonella spp. or *Paracolobactrum*	Passed	209	13.3	55	4.1	14	1.7
arizonae isolated	Condemned	26	16.5	29	6.0	4	1.2

[a] Difference is significant (chi-square = $P<.01$).

for tissue examination because it is an important gauge of disease for the inspector and is the edible tissue most likely to contain endogenous bacteria. Of condemned livers, only those condemned because of a disease process rather than because of contamination, etc., were taken for sampling. Because of the recognized role of fecal contamination in disseminating *Salmonella* spp. and *Paracolobactrum arizonae*, the cecal area of the intestine was excised with sterile instruments, placed in a half-pint ice cream carton and sealed for bacteriological examination. Livers for bacteriological culture were also placed in half-pint ice cream cartons and sealed. When histopathology was to be examined, a small segment of the liver was first excised with sterile instruments from the area exhibiting gross pathology, if any was present, and dropped into a 10% formalin solution. Cartons containing the cecal area of the intestines and/or the livers were covered with ice and taken to the laboratory. Intestinal samples were opened with sterile instruments, and portions of cecal tonsils and intestinal contents were placed in a tube of selenite enrichment broth. The largest surface of the liver was seared with a hot spatula, a stiff inoculating loop was inserted through the seared surface, and a piece of tissue of approximately 2–3 cubic mm was removed for culture. Cultural procedures for both intestines and liver have been described (Sadler and Corstvet, 1965a; Sadler *et al.*, 1965). Sections for microscopic examinations were prepared by standard procedures, stained with hemotoxylin-eosin, and examined by a pathologist,

Table 2. Frequency of isolation of salmonella serotypes from intestines of passed and condemned turkeys, chicken fryers, and chicken hens.

	Turkeys		Chicken fryers		Chicken hens	
	Passed	Condemned	Passed	Condemned	Passed	Condemned
Serotypes isolated						
S. anatum	27	2				
S. bredeney	29	3	3	11		
S. chester-san diego	21	8				
S. derby	16	5				
S. give	4	1				
S. heidelberg	11	1	0	1		
S. infantis	5	0	3	4	13	3
S. newport	45	2				
S. oranienberg	3	0				
S. san diego	3	0				
S. schwarzengrund	2	1				
S. senftenberg	1	0				
S. st paul	1	0				
S. thompson			6	2		
S. typhimurium	25	3	2	9	1	1
S. worthington			0	1		
Salmonella species	6	0				
Paracolobactrum arizonae	10	0	41	1		
Total isolates	209	26	55	29	14	4

who recorded his findings in a series of categories chosen in an attempt to develop sufficiently objective observations for meaningful analysis. Histopathological and bacteriological findings were recorded qualitatively rather than quantitatively.

RESULTS AND DISCUSSION

Table 1 shows the relative frequency of isolation of *Salmonella* spp. or *Paracolobactrum arizonae* from the intestines of 4701 turkeys, chicken fryers, and chicken hens classed individually as passed or condemned. The isolation of *Salmonella* spp. from the intestines of chicken fryers significantly correlated with the "wholesomeness" status of the carcass. This suggests one of three possibilities with this class of market poultry: 1) *Salmonella* spp. in the intestinal tract are associated with disease processess which render the carcass unwholesome; 2) chicken fryers debilitated by disease are more prone to infection with *Salmonella* spp.; or 3) *Salmonella* spp. proliferate more readily in the intestines of chicken fryers debilitated by disease and thus are easier to detect. Neither the *Salmonella* spp. nor the *Paracolobactrum arizonae* appeared to be correlated with disease processes in either the turkeys or chicken hens at slaughter. The serotypes of salmonellae isolated from the passed and condemned birds are listed in Table 2. In chicken fryers, each serotype found, except *S. thompson*, was consistently more prevalent in condemned carcasses than in passed carcasses. Obviously, the numbers of any one serotype are insufficient to make meaningful statistical analysis of the relationship of its presence to the condemned or passed status of the carcasses.

Livers of 5524 turkeys, chicken fryers, and chicken hens were examined bacteriologically. Tables 3, 4, and 5 show the rela-

Table 3. Percentage of turkey livers from which bacterial agents were isolated.

	Status of livers from passed carcasses		Cause of condemnation of carcasses			
	Liver passed	Liver condemned	Sept.[a] tox.	Inflm. proc.	Leucosis	Other or unknown
Total number sampled	1160	673	64	51	6	46
Percent of total yielding one or more isolates	39.1	40.7	54.7	49.0	50.0	28.3
Isolate	Percent yielding each isolate					
Achromobacter spp.	0.8	0.4	0	0	0	0
Bacillus spp.	4.4	3.9	7.8	0	0	2.2
Clostridium spp. (not perfringens)	0.08	0.3	0	0	0	0
Coliform group	9.5	8.4	17.2	11.8	16.7	6.7
Diphtheroids	2.8	3.0	3.0	2.0	16.7	2.2
Lactobacillus spp.	5.9	6.4	9.4	7.8	16.7	2.2
Paracolobactrum spp. (not arizonae)	0.7	0.4	1.6	0	0	4.3
Pasteurella spp. (gallinarum)	0.08	0.1	0	0	0	0
Pasteurella spp. (hemolytica)	0.5	0.1	0	2.0	0	0
Pasteurella spp. (multocida)	0.08	0.1	1.6	5.9	0	0
Proteus spp.	0.4	0.6	1.6	2.0	0	2.2
Pseudomonas spp.	0.5	1.3	0	2.0	0	8.7
Salmonella spp. (typhimurium)	0	0.1	1.6	0	0	0
Staphylococcus aureus (coagulase-positive)	2.1	1.8	4.7	3.9	0	4.3
Staphylococcus aureus (coagulase-negative)	12.0	9.7	7.8	2.0	0	4.3
Streptococcus spp. (beta hemolytic)	2.2	1.3	0	0	0	0
Streptococcus spp. (alpha & gamma hemolytic)	8.4	10.0	3.1	11.8	0	4.3
Streptococcus spp. (anaerobic)	1.2	1.0	3.1	2.0	0	2.2

[a] Sept. tox. = septicemia or toxemia; Inflm. proc. = inflammatory process.

Table 4. Percentage of chicken fryer livers from which bacterial agents were isolated.

	Status of livers from passed carcasses		Cause of condemnation of carcasses			
	Liver passed	Liver condemned	Sept.[a] tox.	Inflm. proc.	Leucosis	Other or unknown
Total number sampled	1520	25	275	170	24	134
Percent of total yielding one of more isolates	66.6	56.0	76.0	59.4	66.7	70.3
Isolate			Percent yielding each isolate			
Achromobacter spp.	1.3	0	1.8	1.2	4.2	0
Bacillus spp.	4.3	4.0	4.4	1.2	8.3	4.5
Clostridium spp. (perfringens)	0	0	0.4	0	0	0
Coliform group	40.7	44.0	54.5	34.7	29.2	35.0
Diphtheroids	3.9	4.0	2.2	2.4	0	0
Lactobacillus spp.	8.4	0	5.1	2.4	0	0
Paracolobactrum spp. (not arizonae)	0	0	1.1	2.9	0	0
Pasteurella spp. (gallinarum)	0.1	0	1.1	0	0	0
Pasteurella spp. (hemolytica)	0.1	0	0	0	0	0
Proteus spp.	1.1	4.0	1.1	4.7	0	1.5
Pseudomonas spp.	0.6	0	1.8	3.5	0	0
Salmonella spp. (typhimurium)	0	0	1.1	0	0	0
Staphylococcus aureus (coagulase-positive)	3.6	8.0	4.0	1.8	0	2.2
Staphylococcus aureus (coagulase-negative)	21.9	32.0	18.5	4.1	0	14.9
Streptococcus spp. (beta hemolytic)	3.5	0	1.8	0.6	4.2	0.7
Streptococcus spp. (alpha & gamma hemolytic)	24.1	4.0	14.9	11.8	16.7	12.7
Streptococcus spp. (anaerobic)	0.3	0	0	0.6	0	0

[a] Sept. Tox. = septicemia or toxemia; Inflm. Proc. = inflammatory process.

Table 5. Percentage of chicken hen livers from which bacterial agents were isolated.

	Status of livers from passed carcasses		Cause of condemnation of carcasses			
	Liver passed	Liver condemned	Sept.[a] tox.	Inflm. proc.	Leucosis	Other or unknown
Total number sampled	913	49	148	105	50	111
Percent of total yielding one or more isolates	45.1	49.0	60.1	45.7	58.0	54.1
	Percent yielding each isolate					
Achromobacter spp.	3.2	0	8.1	7.6	2.0	1.8
Bacillus spp.	4.1	6.1	2.0	0	2.0	1.8
Coliform group	7.9	20.4	19.6	13.3	18.0	28.0
Diphtheroids	2.8	2.0	2.7	0.9	4.0	0.9
Lactobacillus spp.	7.8	8.2	6.8	3.8	0	2.7
Pasteurella spp. (hemolytica)	1.0	0	2.0	1.9	0	0.9
Proteus spp.	1.2	2.0	0.7	1.9	4.0	3.6
Pseudomonas spp.	1.0	2.0	1.4	0	0	2.7
Staphylococcus aureus (coagulase-positive)	3.6	2.0	1.4	1.0	6.0	3.6
Staphylococcus aureus (coagulase-negative)	19.4	24.5	12.2	5.7	14.0	22.5
Streptococcus spp. (beta hemolytic)	2.0	0	1.4	0	0	3.0
Streptococcus spp. (alpha & gamma hemolytic)	12.9	6.1	14.9	13.3	26.0	9.9
Streptococcus spp. (anaerobic)	0	0	0	1.0	0	0.9

[a] Sept. tox. = septicemia or toxemia; Inflm. proc. = inflammatory process.

tionship of the presence of the indicated bacteria in the livers to either the disposition of the livers from passed carcasses or to the cause of condemnation of condemned carcasses. The categories of livers taken from each of the market classes of poultry were: 1) normal (passed) livers from carcasses passed as wholesome; 2) condemned livers from such carcasses; and 3) livers, of unknown pathological state themselves, from carcasses condemned for one of the indicated conditions. In the latter categories, there was no information as to whether the livers would or would not have been condemned on the basis of their own gross pathology if they had not come from condemned carcasses. There was no evidence of a significant difference in the incidence of any of the organisms isolated from the various categories.

To provide adequate numbers for statistical evaluations, data from all three market classes of poultry were combined in three categories of liver or carcass disposition: 1) passed liver from passed carcass; 2) condemned liver from passed carcass; and 3) liver from carcass condemned for septicemia-toxemia or inflammatory process. Since certain bacterial agents are associated most often with either septicemia-toxemia or inflammatory process (which cause condemnation of the carcass), the combined data from these categories are compared with the passed-carcass passed-liver category for each isolation. Since certain bacterial agents have been found to be associated with pathological processes of the liver, combined condemned-liver passed-carcass data are also compared with passed-liver passed-carcass data. These data and the results of tests for significance of observed differences are presented in

Table 6. Bacterial isolations from selected categories of livers from turkeys, chicken fryers, and chicken hens.

	Number yielding isolate from liver of indicated status		
	Passed liver from passed carcass	Condemned liver from passed carcass	Liver from carcass condemned for septicemia-toxemia or inflammatory process
Total livers in selected categories	3593	747	813
Total yielding one or more isolate	866	298[a]	197
Achromobacter spp.	57	3	27[b]
Bacillus spp.	153	30	22
Clostridium spp. (not perfringens)	1	2	1
Coliform group	801[a]	78	269[b]
Diphtheroids	118	22	18
Lactobacillus spp.	268	47	42
Paracolobactrum spp. (not arizonae)	14	3	9[b]
Pasteurella gallinarum	3	1	3[b]
Pasteurella hemolytica	17	1	6
Pasteurella multocida	1	1	4[b]
Proteus spp.	32	6	16[b]
Pseudomonas spp.	24	10	14[b]
Salmonella spp. (typhimurium)	0	1	2
Staphylococcus aureus (coagulase-positive)	111	15	22
Staphylococcus aureus (coagulase-negative)	649[a]	85	88[b]
Streptococcus spp. (beta hemolytic)	96[b]	9	8
Streptococcus spp. (alpha & gamma hemolytic)	640[a, b]	71	105
Streptococcus spp. (anaerobic)	19	7	5

[a] Chi-square test indicates significantly higher ($P < .01$) number of isolations, comparing passed livers from passed carcasses and condemned livers from passed carcasses.

[b] Chi-square test indicates significantly higher ($P < .01$) number of isolations, comparing passed livers from passed carcasses and livers from carcasses condemned for septicemia-toxemia or inflammatory process.

Table 7. Occurrence of histopathology in passed and condemned chicken and turkey livers.

Histopathology	Percent of category with lesion		
	Passed livers from passed carcasses	Condemned livers from passed carcasses	Livers from condemned carcasses
Fibrosis	1.4	0	4.0
Inflammation granulomatous	1.4	4.7	1.0
Inflammation nonsuppurative	7.6	11.6	37.7[a]
Inflammation suppurative	1.4	1.1	4.0
Lipidosis (degeneration or excessive infiltration)	14.1	21.6	7.9
Necrosis	3.8	7.4	20.8[a]
Neoplasia	0.3	0.5	5.9[a]
One or more lesions	22.2	47.4[b]	67.3[a]
Total examined	370	190	101

[a] Significantly greater frequency than in either of the other two categories of livers (chi-square = $P < .01$).
[b] Significantly greater frequency than in passed liver category (chi-square = $P < .01$).

Table 6. As would be expected, significantly relatively more condemned livers than passed livers yielded at least one or more isolates. There was no evidence of a higher rate of isolation of any single genus from the condemned livers than from passed livers. Three organisms—Coliform group, *Staphylococcus aureus* (coagulase-negative), and *Streptococcus* spp. (alpha and gamma hemolytic)—were each isolated significantly more often from passed livers than from condemned, suggesting that these may be "normal" inhabitants of the liver. This is not surprising, since these are all ubiquitous in either the intestinal tract or the external environment. Eight bacteria were isolated significantly more often from livers from carcasses condemned for septicemia-toxemia or inflammatory process than from passed livers from passed carcasses. These findings are not surprising, in view of the genera and species of bacteria involved. As in the previous comparison, there were organisms—alpha, beta, and gamma hemolytic *Streptococcus* spp.—that were found proportionately more often in normal livers than in livers from condemned carcasses, suggesting that these are "normal" flora.

Histopathological examination was made of 661 livers: 370 passed livers from passed carcasses, 190 condemned livers from passed carcasses, and 101 livers from carcasses condemned because of disease processes. Again, there was no information as to whether the livers in this last category would have been condemned on their own characteristics if they had not come from condemned carcasses. Table 7 presents the relationship of the various categories of histopathologic change in the three disposition categories of livers. The histopathologic categories were chosen arbitrarily as representative of the type of pathology observed in these market classes of poultry. The descriptiveness of the recorded pathology had to be limited so that enough observations could be made in each category to permit even a cursory analysis of the data. No attempt was made to quantitate the histopathology, since the numbers in each category were so small. In comparing passed livers with condemned livers from passed carcasses, the only significant difference found was the greater frequency of "one or more lesions" in the condemned than in the passed livers. There was no significant difference for any one type of lesion. However, when livers from condemned carcasses were compared with each of the other two categories, several differences were significant. Non-suppurative inflammation, necrosis, and neoplasia were each found significantly more often in livers from condemned carcasses than in either passed or condemned livers from passed carcasses. As would be expected, the category of "one or more lesions" applied significantly more often to livers from condemned carcasses than to either of the other two groups. Histopathological and bacteriological findings in the 661 livers are compared in Table 8. No single bacterial agent was significantly correlated with a specific histopathologic change.

Table 8. Relation of presence of bacterial agents to presence of histopathology in chicken and turkey livers.

	Total yielding each isolate	Total with one or more pathological lesions	Fibrosis	Inflammation granulomatous	Inflammation nonsuppurative	Inflammation suppurative	Lipidosis (degeneration or excessive infiltration)	Necrosis	Neoplasia
Total with each pathological process	661	240	9	15	88	11	101	49	8
		Number with both lesion and isolate							
Total yielding one or more isolate	237	103[a]	3	6	38	6	35	23	4
Achromobacter spp.	7	4	0	0	3	0	0	0	1
Bacillus spp.	28	15	1	0	4	0	8	2	0
Clostridium spp. (not perfringens)	2	0	0	0	0	0	0	0	0
Coliform group	97	36	1	1	18	1	8	8	2
Diphtheroids	14	10	1	2	2	2	2	1	0
Lactobacillus spp.	66	19	0	1	6	1	8	2	1
Nocardia spp.	1	1	0	0	1	0	0	0	0
Paracolobactrum spp. (not arizonae)	3	1	0	0	0	0	1	0	0
Pasteurella multocida	2	2	0	0	1	1	0	1	0
Proteus spp.	6	4	1	0	1	0	1	1	1
Pseudomonas spp.	14	4	0	0	1	0	1	2	0
Salmonella spp. (typhimurium)	1	0	0	0	0	0	0	0	0
Staphylococcus aureus (coagulase-positive)	23	9	0	2	4	1	1	1	0
Staphylococcus aureus (coagulase-negative)	73	32	1	2	9	3	11	7	2
Streptococcus spp. (beta hemolytic)	15	4	0	1	1	0	0	2	0
Streptococcus spp. (alpha & gamma hemolytic)	66	26	1	1	10	0	8	6	0
Streptococcus spp. (anaerobic)	2	1	0	0	0	0	1	0	0

[a] Significant correlation (chi-square = $P < .01$).

However, the presence of one or more bacterial agents was significantly correlated with the presence of one or more histopathologic lesions.

Obviously, the results of the histopathological observations are weighted in favor of a high correlation between gross observations and microscopic findings. The portion of the liver usually selected had the greatest gross pathologic change, so the pathologist examining the tissue microscopically had a marked advantage in detecting cellular alterations. Although the correlation between isolation of a bacterial agent from a liver and microscopic lesions was significant at the 1% level (chi square = 8.22), the lack of a stronger correlation may be explained on the basis of the paucity of acute inflammatory lesions observed in the tissue sections; noninflammatory categories of liver pathology would not be expected to yield organisms upon bacteriological culture. Even granulomatous inflammation of the liver, a lesion seen most commonly in turkey livers, would

have viable organisms since these lesions are usually the result of parasitic invasion. Possibly, the significance of the data, favoring the isolation of bacteria where histopathologic changes were observed, may be related to the relatively high incidence of necrotic lesions in the condemned livers (Table 7).

Inspection passed livers of which 22.2% had varying degrees of histopathologic processes, condemned livers of which 52.6% were without evident histopathologic processes, and condemned carcasses (because of disease processes) from which 32.7% of the livers were without evident histopathologic processes. The fact that histopathologic processes were detected in 22.2% of the passed livers and were not detected in 52.6% of the condemned livers suggests either that there is no strong correlation between gross and histopathologic processes in this organ or that discoloration or other apparent abnormalities are easily mistaken for pathologic lesions in the liver. Because only a very small portion of each liver was examined microscopically, histopathologic lesions may have been present in other areas. However, as previously mentioned, all tissues for histopathological examination were taken from the area exhibiting gross pathology, if present, thus weighting the results in favor of a correlation between gross and microscopic lesions. Certain histopathologic processes found in passed livers (i.e. inflammation, necrosis, neoplasia) render tissue unwholesome by the accepted definition. Thus inspection can, apparently, only assure a relative degree or level of wholesomeness of the liver, and probably of other tissues as well. This conclusion also applies to detection of livers harboring genera of bacteria with varying associations with abnormal conditions and to detection of carcasses harboring *Salmonella* spp. either in their intestinal tracts or on their surfaces. *Erysipelothrix insidiosa* was not isolated in the studies reported here, but, with experimentally infected turkeys, this organism has been isolated from bone marrow and liver of lesion-free carriers in which the livers yielding the organism were themselves without gross lesions (Sadler and Corstvet, 1956b). In market poultry, "absolute" assurance of wholesomeness is an unattainable goal, particularly in view of the subjectivity of the very definition of wholesomeness. Each term used in its definition is subject to various definitions by different ethnic, social, and economic groups as well as by personal taste (esthetics). However, the significant contribution that inspection makes to the assurance of wholesomeness is indicated by the findings that histopathologic processes were found in only 22.2% of passed livers, but were found in 47.4% of condemned livers, and in 67.3% of livers from carcasses condemned because of disease processes. The contribution of inspection is further indicated by the findings that a significantly greater number of condemned livers yielded "one or more" bacterial isolates than the passed livers and that livers from condemned carcasses yielded 8 species of organisms significantly more often than did livers from passed carcasses.

REFERENCES

Sadler, W. W., and R. E. Corstvet. 1965a. Second survey of market poultry for salmonella infection. *Appl. Microbiol.* **13**, 348.

Sadler, W. W., and R. E. Corstvet. 1965b. The effect of Erysipelothrix insidiosa (rhusiopathiae) infection on wholesomeness of market turkeys. *Am. J. Vet. Research* **26**, 1429.

Sadler, W. W., R. Yamamoto, H. E. Adler, and G. F. Stewart. 1961. Survey of market poultry for salmonella infection. *Appl. Microbiol.* **9**, 72.

Sadler, W. W., R. Yamamoto, and R. E. Corstvet. 1965. Bacteriological survey of market poultry livers. *Poultry Sci.* **44**, 993.

U. S. Congress. 1957. An act to provide for the compulsory inspection by the United States Department of Agriculture of poultry and poultry products. Public Law 85-172, 85th Congress, S. 1747.

Ms. rec'd 4/15/66.

This study was supported in part under basic memoranda of agreement nos. 12-25-020-2276 and 12-25-110-854 and contract no. 12-25-010-2654 between the U. S. Department of Agriculture and the Regents of the University of California.

The assistance of Drs. John Hendershott and Irvin Peterson, as well as personnel of the Inspection Branch, Poultry Division, C & MS-USDA, is greatly acknowledged.

Presented in abstract form at Second International Congress of Food Science and Technology, Warsaw, Poland, August 22-27, 1966.

Research Note

M. L. KAKADE [a] and ROBERT JOHN EVANS

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

Effect of Soaking and Germinating on the Nutritive Value of Navy Beans

SUMMARY

The effect of soaking or germination on the protein content, trypsin inhibitor activity, and hemagglutinin activity of navy beans was studied. Soaking navy beans for 1–4 days decreased the trypsin inhibitor activity, and especially the hemagglutinin activity. The decrease in protein content due to soaking did not account for all of the decrease in anti-trypsin and hemagglutinin activity. No significant changes were observed in hemagglutinin activity due to germination, although some decrease in the trypsin inhibitor activities of germinated beans was noticed. Protein content increased progressively in beans germinated 1–4 days, and decreased in beans soaked 1–4 days. Rats fed raw, soaked, and germinated navy beans as a source of dietary protein lost weight and died 8–16 days after the beginning of the experiment. It is concluded that soaking prior to heating is not necessary to eliminate the toxicity of navy beans.

INTRODUCTION

Peas and beans constitute an important source of dietary protein for a majority of the world's population (Patwardhan, 1962). Most beans contain "toxic" substance(s) (Liener, 1962). Besides the isolation and characterization of the toxic factors present in the edible beans their elimination appears to be of particular interest from the standpoint of public health and safety. Most of the toxic substances in beans can be inactivated or destroyed by proper heat treatment (Liener, 1962), but in some cases treatments such as soaking or germination as well as heating are necessary to eliminate the toxicity completely. Jaffé (1949) and Honavar *et al.* (1962) showed that soaking prior to heat treatment is an essential step to abolish the deleterious effects of beans (*Phaseolus vulgaris*). Various workers (Everson *et al.*, 1944; Desikachar and De, 1947; Chattopadhay and Banerjee, 1953) have demonstrated that germination improved the nutritive values of certain beans. The present investigation was undertaken, therefore, to determine the effect of soaking and germinating on the nutritive value of navy beans.

EXPERIMENTAL

Navy beans (*Phaseolus vulgaris*) were first soaked overnight in tap water at room temperature. Then they were soaked 1–4 days and the water changed every day.

Germination was carried out by spreading the soaked beans (soaked overnight) on the trays at room temperature for 1–4 days. At the end of the desired period the beans were dried before a fan and ground into fine flour.

Protein (AOAC, 1960), trypsin inhibitor activity (Kunitz, 1947), and hemagglutinin activity (Liener, 1953) were determined on germinated and soaked beans.

Rat feeding experiments were conducted to evaluate the growth-promoting property of soaked and germinated beans. Raw and heated navy beans were included in the diets to furnish a 10% protein level. The composition of the basal diet and the details of rat feeding experiments are described elsewhere (Kakade and Evans, 1965a).

RESULTS AND DISCUSSION

The trypsin inhibitor activity and hemagglutinin activity of raw, soaked, and germinated navy beans are presented in Table 1. Soaking for a longer time decreased these activities progressively. Soaking the beans for 4 days destroyed about 28% of the trypsin inhibitor activity and 75% of the hemagglutinin activity. The decrease in protein content could not account for all the observed decrease in these activities, since the trypsin inhibitor and hemagglutinin activity values per gram of protein gave essentially the same values as those per gram of beans. It appears therefore that the trypsin inhibitor and hemagglutinins were destroyed or inactivated or possibly leached out of the beans as a result of soaking.

Germination had the following effects: 1) increased the protein content of the beans; 2) first decreased and then increased the trypsin inhibitor activity; and 3) did not change the hemagglutinin activity.

[a] Present address: Department of Biochemistry and Nutrition, University of Nebraska, Lincoln, Nebraska 68503.

Table 1. Influence of soaking and germination on the trypsin inhibitor and hemagglutinin activities of navy beans.

Treatment	Protein (%)	Trypsin inhibitor activity		Hemagglutinin activity	
		TIU [a]/g bean	TIU/g protein	HU [b]/g bean × 10^3	HU/g protein × 10^3
None (raw beans)	23.60	23.25	98.5	8	33.9
Soaked 1 day	21.88	22.50	102.8	8	36.5
Soaked 2 days	21.63	22.00	101.7	8	37.0
Soaked 3 days	20.31	19.75	97.2	4	19.7
Soaked 4 days	20.00	16.75	83.7	2	10.0
Germinated for 1 day	24.88	21.25	85.4	8	32.1
Germinated for 2 days	25.31	21.50	84.9	8	31.6
Germinated for 3 days	25.63	24.00	93.6	8	31.3
Germinated for 4 days	26.31	24.50	93.1	8	30.4

[a] Trypsin inhibitor unit expressed in terms of tryptic units inhibited, the tryptic unit being defined as the increase of one unit of extinction at 280 mμ/min of digestion under the experimental conditions.

[b] Hemagglutinin unit defined as the least amount of hemagglutinin that will produce positive evidence of agglutination (1+) of 0.2 ml of 4% suspension of washed chicken red blood cells after 1 hr of incubation at 37°C.

The biological implications of soaking and germination as reflected in the decrease and/or increase in the trypsin inhibitor activity, and hemagglutinin activity of navy beans are very difficult to rationalize at present.

Table 2 shows the effect on the growth of rats of feeding soaked or germinated navy beans as a source of dietary protein. Rats fed raw, soaked, or germinated navy beans lost weight and died within the experimental period. Since heated and soaked beans gave essentially the same growth rate as heated beans (Kakade and Evans, 1965a), no growth studies were made in the present investigation involving the soaked and germinated beans which had received heat treatment. The fact that rats fed the heated navy bean diet supplemented with methionine grew as well as rats fed the casein diet (Kakade and Evans, 1965a) seems to indicate that heating alone could completely eliminate the toxicity. These results are in contrast to those obtained by Jaffé (1949) and Honavar *et al.* (1962), who observed that soaking beans (*Phaseolus vulgaris*) prior to heating was necessary to destroy or inactivate all the toxic effects of *Phaseolus vulgaris*. One explanation for these differences may be the use of different varieties of *P. vulgaris*.

According to Liener (1962), the causative factor for the toxicity of the *P. vulgaris* is hemagglutinin, which could be destroyed or inactivated only by preliminary soaking followed by heating. In the light of the present results, it appears that the hemagglutinin may not be the only active factor for the

Table 2. Effect on growth of rats of feeding soaked and germinated navy beans.

Protein source	Weight gain/day (g)	Food intake/day (g)	Mortality (days)
Heated beans[a]	1.50	8.75	
Raw beans	−0.92	2.95	9–14
Soaked 1 day	−1.00	2.90	11–15
Soaked 2 days	−1.11	3.00	10–16
Soaked 3 days	−1.06	2.92	12–16
Soaked 4 days	−1.10	3.10	10–14
Germinated for 1 day	−0.99	3.02	8–16
Germinated for 2 days	−0.96	2.94	8–16
Germinated for 3 days	−1.05	2.99	10–16
Germinated for 4 days	−1.09	3.10	10–16

[a] Autoclave at 121°C for 5 min.

toxicity of navy beans, a conclusion supported by work of Kakade and Evans (1956b).

REFERENCES

AOAC. 1960. Official Methods of Analysis, Assoc. Official Agr. Chemists. 9th ed., p. 12.

Chattopadhay, H., and S. Banerjee. 1953. Effect of germination on the biological values of protein and the trypsin inhibitor activity of some common Indian pulses. *Indian. J. Med. Research,* **41**, 185.

Desikacher, H. S. R., and S. S. De. 1947. Role of inhibitors in soybean. *Science* **106**, 421.

Everson, G. J., M. Steenbock, and D. Cederquist. 1944. The effect of germination, the stage of maturity, and variety upon the nutritive value of soybean protein. *J. Nutrition* **27**, 225.

Honavar, P. M., C. V. Shih, and I. E. Liener. 1962. Inhibition of the growth of rats by purified hemagglutinin fractions isolated from Phaseolus vulgaris. *J. Nutrition* **77**, 109.

Jaffe, W. G. 1949. Limiting essential amino acids in some legume seeds. *Proc. Soc. Exptl. Biol. Med.* **71**, 398.

Kakade, M. L., and R. J. Evans. 1965a. Nutritive value of navy beans (Phaseolus vulgaris). *Brit. J. Nutrition* **19**, 269.

Kakade, M. L., and R. J. Evans. 1965b. Growth inhibition of rats fed navy bean fractions. *J. Agr. Food Chem.* **13**, 450.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.* **39**, 291.

Liener, I. E. 1953. The effect of heat treatment on the nutritive value and hemagglutinating activity of soybean oil meal. *J. Nutrition* **49**, 609.

Liener, I. E. 1962. Toxic factors in edible legumes and their elimination. *Am. J. Clin. Nutrition* **11**, 281.

Patwardhan, V. N. 1962. Pulses and beans in human nutrition. *Am. J. Clin. Nutrition* **11**, 12.

Ms. rec'd 3/29/66.

This investigation was supported in part by Public Health Service Research Grant AM 06755 from the National Institute of Arthritis and Metabolic Diseases. Journal article No. 3805 from the Michigan Agricultural Experiment Station.

HERBERT STONE
Department of Food Sciences and Nutrition, Stanford Research Institute
Menlo Park, California 94025

Factors Influencing Behavioral Responses to Odor Discrimination—A Review

Numerous suggestions and hypotheses have been proposed to account for the mechanisms of olfactory detection and discrimination; however, no satisfactory theory has yet been formulated (Jones and Jones, 1954; Moulton and Tucker, 1964). One reason is the paucity of good, reliable experimental data on olfaction, considered both as a psychophysical experience and as a psychological one—that is, from the point of view of both quantitative and qualitative measurement—and the interaction between these two response modes. This is especially important in view of the more recent research findings relating to molecular properties and odor quality (Amoore, 1964; Beets, 1964; Davies, 1965; Schutz *et al.*, 1958).

In an attempt to meet this challenge, we have given some thought to the individual steps involved in olfaction: the stimulus, stimulus presentation, the discrimination process (perceptual task), and how the data are interpreted.

From the work of Thurstone, as reported by Guilford (1954), Torgeson (1958), Swets (1961), and others, it is recognized that stimulus detection and discrimination is confounded by numerous and unrelated factors. For example, it has been shown experimentally that in the resting state there will be indiscriminate firing of receptors from the olfactory mucosa as recorded by electrophysiological techniques. In olfactory threshold experiments with man, one continually encounters subjective comments of the presence of something different from the background, yet not identifiable as the stimulus. Thus it is not surprising that for any individual the threshold might show considerable variation. The stimulus might not elicit the same response from the same subject at different test sessions. Furthermore, the behavioral response at supra-threshold concentrations usually bears little relation to the response at the "threshold." While it is true that both are part of the same discrimination process, there are differences (in the form of background, frame of reference, and hedonic continuum) which vary in importance as one approaches the detection threshold. There also exists confusion as to which odor threshold one is considering: absolute or recognition and/or the mathematically derived 50, 67, or 75% value.

In discussing the significance of sensory thresholds, Swets (1961) emphasized the difficulties encountered with measuring the "threshold": the problem of the signal to noise ratio, the "likelihood-ratio criterion," and their effect on the discriminal process and data interpretation. According to Swets there may be a sensory threshold, but it is a very low value and probably directly related to the mean for the noise distribution. This is especially important in its application to study of the olfactory process. By defining the olfactory threshold mathematically, it is possible to use these data in developing a theory of olfaction. It also permits applications of these data to more sophisticated methods of analysis which have been developed as a part of information theory. As knowledge of the behavioral aspects of olfaction and means of transmitting this information into a useful form increases, it is possible to better utilize these data.

Several recent experiments (Stone, 1964; Stone and Bosley, 1965a) have demonstrated the value of including behavioral data from threshold and suprathreshold tests to establish a more realistic and accurate measure of the Weber fraction for olfaction. This relationship, shown in Fig. 1, is described by Eq. 1:

$$Y_e = -2.05 + .281x \qquad [1]$$

and was based on the Weber fraction, Eq. 2:

$$K = \frac{\Delta I}{I + I_t} \qquad [2]$$

Where ΔI is the difference threshold, I is the reference stimulus concentration, and I_t represents the experimentally determined

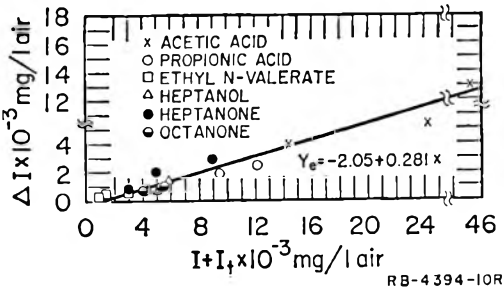


Fig. 1. Regression equation for difference threshold data, where ΔI is the difference threshold at I, the stimulus concentration, and I_t is the experimentally determined 50% threshold (Stone and Bosley, 1965a).

50% threshold. The 50% threshold is determined from probit analysis of data collected in a forced-choice, constant-stimulus test situation. These data have added advantage since you can obtain any threshold desired by reference to the plotted curve. This equation would permit one to predict either I_t or ΔI from knowledge of the other provided its validity can be tested. Experiments are now in progress with different stimuli to develop this information (Stone and Bosley, 1965a,b). However, the utilization of this information in developing a reliable theory of olfaction remains to be developed. As mentioned earlier, establishment of a jnd threshold represents a task not entirely comparable to the task in the 50% threshold test. For example, at suprathreshold concentrations, unpleasant stimuli may evoke a response different from pleasant stimuli which might influence discrimination; in some experiments, it has been shown that subjects were better able to discriminate differences between unpleasant stimuli, while pleasant stimuli seemed to cause greater difficulty in discrimination (Stone, 1963b). A recent experiment by Engen and McBurney (1964) indicated that the hedonic scale for odors was much greater than the intensity scale for different concentrations of the same odorants. In order to make better use of such information, it is important to know what factors influence odor discrimination and how behavioral data can best be used in developing a theory of olfaction. The following sections discuss these problems in detail.

The Stimulus

Presentation of a stimulus to a subject can evoke a number of different types of responses, dependent on stimulus concentration, hedonic properties, and the perceptual task. When we reduce the stimulus concentration and approach the 50% threshold, the subject soon loses the ability to recognize the characteristic quality associated with the stimulus. He then relies on a variety of devices and cues in order to detect a difference from the background, which brings up a number of problems associated with the discrimination process and the response matrix.

Typically, the stimulus is presented to the subject in a variety of ways: as a solute in a non-odorous solvent system, adsorbed on cotton-balls or in a controlled-air system. Each technique has certain limitations. The most important factor here is the experimenter's ability to control concentration for as long as the experiment is in progress. All three techniques are used, although there has been some recent evidence to suggest an air-dilution system as being more reliable (Benjamin *et al.*, 1965). It was also demonstrated that some solvent systems do not yield ideal solutions at concentrations approaching threshold (Stone, 1963a). These data indicated mineral oil–propionic acid mixtures were non-ideal even at threshold concentrations. Some of these data are shown in Table 1. Clearly, care should be used in the selection of an appropriate solvent system. If one is concerned with the quantitative aspects of olfaction, e.g., threshold measurements or the adaptive process, it is extremely important to control stimulus concentrations—a problem that has been given some attention.

It may be that only certain solvents lend

Table 1. Calculated and observed concentrations of propionic acid-1-C^{14} in the vapor of mineral oil–propionic acid solutions.[a]

Concentration (mg/L air)	
Calculated	Observed
.0003	.025
.00016	.0067
.000079	.00136
.000016	.00069

[a] Stone (1963a).

themselves to use in odor testing. This might be based on the polarity or non-polarity of the solvent and of the odorant. Many solvents have an odor, thus introducing another variable in the procedure. Engen *et al.* (1963) managed to circumvent this problem through the use of "Q-tips" set in test tubes containing a few cc of the purified odorant. The subject merely removes the stopper holding the stem of the "Q-tips" and sniffs the cotton ball containing the stimulus. This method permits a wider range of studies involving olfaction. However, if one is interested in studying the basic mechanisms of the olfactory process, the air-dilution olfactometer, with precise control of air flow and odor concentration, is necessary. This method provides rapid presentation and removal of the stimulus.

The Perceptual Task

In some situations, the perceptual task is relatively simple, e.g., a jnd test where the subject is required to indicate the sample of a pair having the most intense odor. In other situations, the subject might be required to describe a stimulus in relation to some identified standards, i.e., multi-dimensional scaling. Both odor quality and quantity are equally important in any sensory problem, whether we are dealing with a single stimulus or a complex food material. In most experiments, the degree of difficulty of the perceptual task will affect the amount of training and familiarization, the response and type of response, and the maximum number of tests the subject can effectively handle per test session. Unfortunately, our knowledge to date does not permit us to make specific rules for each test method. We must rely on informational feedback during the preliminary stages of the experiment. The theoretical aspects of this area of psychophysics have been adequately reviewed elsewhere (Garner and Hake, 1951; Guilford, 1954; Tanner and Swets, 1954; Torgeson, 1958). Recently, several studies have been published which have application here.

Ough *et al.* (1964) found that subjects could judge a quantity difference slightly better when a time limit was imposed, while taste quality was judged better when time was not a factor. The perceptual task is more difficult for a quality judgment and requires more information from the subject.

From another experiment, Ough and Baker (1964) reported that subjects, when required to scale odor differences, responded similarly as far as scale structure was concerned. Intensity ratings for differences yielded as much information as the paired-comparison method, but in a shorter time. Experiments in our laboratory (Stone and Bosley, 1965b), while not directly applicable, yielded opposing results. Our subjects were required to make intensity ratings during or after the jnd test. Results indicated that subjects were not using the entire scale and were not capable of discriminating differences as measured by the intensity ratings.

Hedonic Properties

Engen and McBurney (1964) studied magnitude and category scales of odor pleasantness. The study evolved from several earlier experiments by Engen (1956, 1961), who attempted to develop a psychophysical scale for odor intensity by the fractionation method. In that particular study it was found that the scale for odor pleasantness was much greater than the intensity scale (125:1 vs. 3:1, respectively). These data suggest that hedonic scales should be used for sensory problems, since this would permit the determination of much finer discriminations.

Adaptation

Adaptation to odors occurs rapidly in man and is dependent, at least in part, on stimulus concentration and exposure time. According to Stuiver (1958), adaptation is primarily a central phenomenon which results in an increase in the stimulus detection threshold but not in proportion to the concentration of the adapting stimulus. Recovery appears to occur in two phases: a fast central and a slow peripheral process. Studies by Schutz *et al.* (1961) indicated the adaptation rate was different for different stimuli.

Experiments in our laboratory, while not directly comparable, are pertinent to this discussion (Stone and Bosley, 1965b). The

procedure involved continuous stimulation with timed intervals for judgment of odor intensity in an attempt to investigate adaptation and recovery as a function of stimulus concentration and exposure time.

Fig. 2 shows results of some preliminary experiments to establish the appropriate time interval with n-propyl alcohol as the stimulus. In this experiment the subject was instructed to breathe normally, and at a pre-set time interval of 5, 10, or 15 sec a signal light would direct the subject to rate the stimulus intensity. The scale was 0 to 10, from no detectable odor to most intense odor. The subjects received identified warm-up samples of 10 and 0 intensities. Experiments were carried out for as long as 10 min; however, as these data show, adaptation was very rapid, especially during the first 1.5 min.

Fig. 3 shows the results of two additional experiments involving adaptation with propyl alcohol and serves to demonstrate the kinds of problems we are dealing with. The data with the solid circles were based on 9 replications by each of 2 subjects at the 10-sec timed interval. The open circles indicate responses obtained in a somewhat different experiment. The subjects were presented with the first sample after 70 sec of exposure to the stimulus, and then asked to rate the intensity; the second sample followed 100 sec later. The purpose of the experiment was to determine the reliability of the data from the set-interval test.

After the first few experiments, the subjects quickly realized that the stimulus concentration was decreasing with time. It would be possible for the subject to introduce a bias by indicating the presence of the stimulus when, in fact, he was adapted. In this particular experiment no such problem was encountered. However, Fig. 4 shows the results of an experiment on adaptation and recovery with another subject. Observation of these data indicated that adaptation occurred in 6 min, although the subject never indicated "no odor" (0 on the score sheet). The recovery experiment was carried out at 30-sec intervals for an additional 2.5 min, at which time the subject was approximately 80% recovered. Interestingly, when the subject was retested 5 min later at 70 sec and 100 sec, the scale values did not coincide with the previous results. It appeared that the subject experienced no adapting effects of the stimulus for the first minute, followed by adaptation much the same as in previous tests. While the line is displaced, it is quite obvious that the slope is steep, and equally so, for recovery.

These data are preliminary, and experiments are now in progress to study this problem more thoroughly with several stimuli, at high and low stimulus concentrations. To overcome the scaling error, a signal system has been installed to replace the score sheet, so subjects will not be confronted by their previous scores. Adapting at longer time intervals for spot checks is also programmed. However, from these preliminary data it seems that continuous exposure to a stimulus for longer than 30 sec will lead to adaptation.

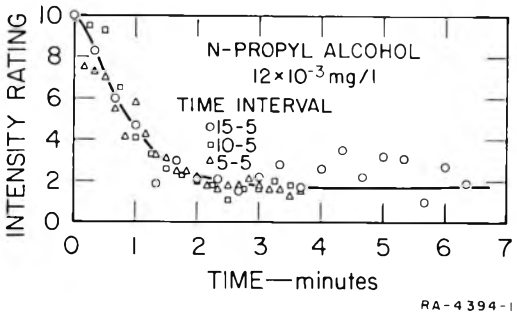


Fig. 2. Adaptation to *n*-propyl alcohol at three time intervals: △, 5 sec; □, 10 sec; ○, 15 sec; followed by a 5-sec test period.

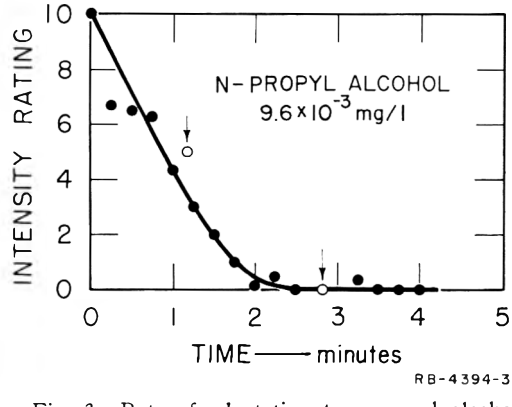


Fig. 3. Rate of adaptation to *n*-propyl alcohol for 2 subjects. The arrows refer to repeat experiments at more prolonged time intervals.

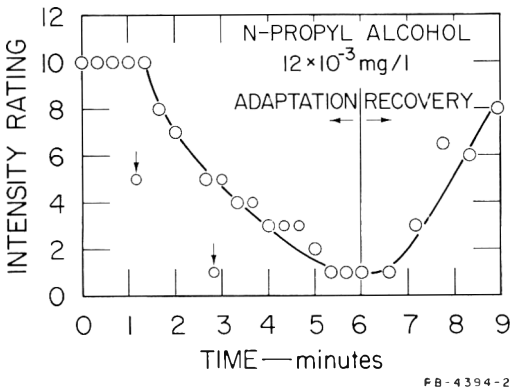


Fig. 4. Rate of adaptation and recovery to *n*-propyl alcohol. Arrows refer to repeat experiments at more prolonged time intervals.

Other types of studies involving adaptation and odor interaction have been carried out by several methods. One area of considerable interest is the phenomenon of cross-adaptation and background odor. Cheesman and Townsend (1956) reported that when adapting and test stimuli are the same or from the same chemical class, the threshold for the test stimulus is reduced by a factor of 0.7. When the stimuli are different, the reduction in threshold is not the same. While the data are not extensive, the results are suggestive.

Rosen *et al.* (1962) investigated the additive and synergistic effects produced by mixtures of butanol, p-cresol, and pyridine. Both additive and synergistic effects were found with these stimuli when tested at the threshold. Unfortunately, suprathreshold concentrations of the mixtures were not tested. More recently, Baker (1963) found similar results in a more extensive investigation involving a larger number of odorants. Binary mixtures were studied, as well as an eight-component mixture. In the latter study, the mixtures produced a significant olfactory response, with most subjects detecting the odor or requiring further dilution to reach threshold.

Schutz *et al.* (1958) studied the problem of cross-adaptation at suprathreshold concentrations, and concluded that there was no effect. They suggested that the odorants may have been too dissimilar or that adaptation may be a central phenomenon, and, with introduction of a new stimulus, a new pattern occurs in the brain.

Jones and Woskow (1964) also studied the intensity of odor mixtures at suprathreshold concentrations. Their data indicated neither an additive nor a simple average effect; instead, responses fell between these two limits. If one considers these data in line with conclusions reached by the previous workers, it is evident that our knowledge of odor interaction is quite incomplete. However, there are aspects of these data which need to be mentioned and which do give us a further insight into this problem. Baker's data were obtained from threshold experiments of aqueous solutions, while those by Schutz and Jones and Woskow were suprathreshold measurements using an air-dilution type of apparatus. Finally, the selection of stimuli can be very important in influencing the results.

Temperature and Humidity

Evidence for the influence of temperature and humidity on olfactory perception and discrimination has been reported elsewhere; however, there are questions which require additional investigation (Tucker, 1963; Stone, 1964). Tucker's experiments with the tortoise indicated that variations in relative humidity and temperature had little if any effect on recorded response. The data reported by Stone were from experiments using man in the olfactometer. Odor thresholds were determined for acetic acid from 12.5 to 35°C. No significant difference was found between the 50% thresholds at 12.5, 15, 20, 30, and 35°C for the panel. To support the behavioral information, temperature recordings were obtained at the inferior turbinate. The recordings revealed that the turbinates provided not only physical protection but maintained the olfactory region at body temperature. Thus, under normal physiological conditions, temperature does not have a significant role in odor detection. Fig. 5 is a summation of these temperature recordings.

The information on relative humidity is not as clearly defined; however, Tucker's data indicated no effect. It is reasonable to assume, for the moment, that, provided ex-

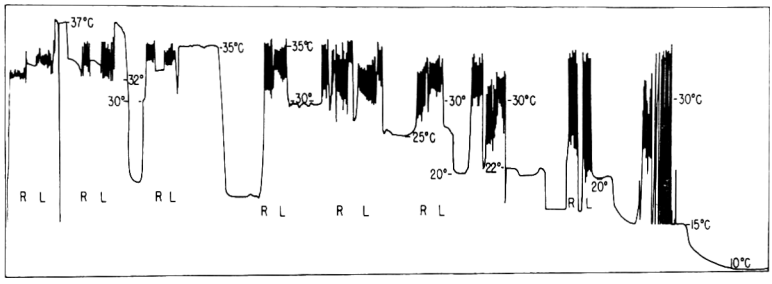


Fig. 5. Summated temperature recordings made in the nasal cavity for subjects sitting in the odor hood exposed to 15, 20, 22, 25, 30, 32, 35, and 37°C environments. *R* and *L* refer to the nostril tested. Chart speed was 30 in./hr, and each recording required 15 sec. The thermistor was located approximately ½ in. into the nares, just below the inferior turbinate (Stone, 1963a,b).

treme conditions are not encountered, no significant changes in olfactory sensitivity would occur. This also applies for the temperature experiments, since both Tucker's and Stone's studies went only from 10 to 35°C.

Summary and Conclusions

While it is evident that there are other factors which influence the behavioral response to odors, the aforementioned problems—stimulus presentation, perceptual task, the solvent system, stimulus concentration, temperature and humidity—were considered most important and timely. Increased research in olfaction, coupled with the use of more sophisticated experimental tools, has permitted us to better understand the olfactory process. However, much basic knowledge is needed if we are to develop a workable theory of olfaction. At present, there is a scarcity of quantitative data on odor thresholds, the relationship between threshold and suprathreshold concentrations, and the appropriate handling of behavioral data.

Certainly, the sense of smell has been considered as one of our less used sensory channels. It is becoming evident that man's olfactory sense is capable of very fine discriminations and is able to perform reliably over long periods.

REFERENCES

Amoore, J. E. 1964. Current status of the steric theory of odor. *Ann. N. Y. Acad. Sci.* **116**, 457.

Baker, R. A. 1963. Odor effects of aqueous mixtures of organic chemicals. *J. Water Pollution Control Federation* **35**, 728.

Beets, M. G. J. 1964. A molecular approach to olfaction. *In:* "Molecular Pharmacology." Vol. 11. E. J. Ariens, ed. Academic Press, N. Y. 3–51.

Benjamin, R. M., B. O. Halpern, D. G. Moulton, and M. M. Mozell. 1965. The chemical senses. *Ann. Rev. Psychol.* **16**, 381.

Cheesman, G. H., and M. J. Townsend. 1956. Further experiments on the olfactory thresholds of pure chemical substances, using the "sniff-bottle method." *Quart. J. Exptl. Psychol.* **8**, 8.

Davies, J. T. 1965. A theory of the quality of odours. *J. Theoret. Biol.* **8**, 1.

Engen, T. 1956. An evaluation of a method for developing ratio scales. *Am. J. Psychol.* **69**, 92.

Engen, T. 1961. Direct scaling of odor intensity. Report no. 106. Univ. of Stockholm. Psychological Laboratory.

Engen, T., and D. H. McBurney. 1964. Magnitude and category scales of the pleasantness of odors. *J. Exptl. Psychol.* **68**, 435.

Engen, T., L. P. Lipsitt, and H. Kaye. 1963. Olfactory responses and adaptation in the human neonate. *J. Comp. Physiol. Psychol.* **56**, 73.

Garner, W. R., and H. W. Hake. 1951. The amount of information in absolute judgments. *Psychol. Rev.* **58**, 446.

Guilford, J. P. 1954. "Psychometric Methods." 2nd ed. McGraw-Hill Book Co., New York.

Jones, F. N., and M. H. Jones. 1964. Modern theories of olfaction: a critical review. *J. Psychol.* **36**, 207.

Jones, F. N., and M. H. Woskow. 1964. On the intensity of odor mixtures. *Ann. N. Y. Acad. Sci.* **116**, 484.

Moulton, D. G., and D. Tucker. 1964. Electrophysiology of the olfactory system. *Ann. N. Y. Acad. Sci.* **116**, 380.

Ough, C. S., and G. A. Baker. 1964. Linear dependency on scale structure in differential odor intensity measurements. *J. Food Sci.* **29**, 499–505.

Ough, C. S., V. L. Singleton, M. A. Amerine, and

G. A. Baker. 1964. A comparison of normal and stressed-time conditions on scoring of quality and quantity attributes. *J. Food Sci.* **29**, 506.

Rosen, A. A., J. B. Peter, and F. M. Middleton. 1962. Odor thresholds of mixed organic chemicals. *J. Water Pollution Control Federation* **34**, 7.

Schutz, H. G., R. C. Overbeck, and R. S. Layman. 1958. Relationship between flavor and physicochemical properties of compounds. QMFC1 (Contract DA-19-129-QM-1141) 38 pp.

Schutz, H. G., V. G. Vely, R. B. Iden, and S. M. Soliday. 1961. Relation of odor to physicochemical properties of compounds. QMFC1, Contract No. DA-19-129-QM-1500. 51 pp.

Stone, H. 1963a. Technique for odor measurement: olfactometric vs. sniffing. *J. Food Sci.* **28**, 719.

Stone, H. 1963b. Influence of temperature on olfactory sensitivity. *J. Appl. Physiol.* **18**, 746.

Stone, H. 1964. Behavioral aspects of absolute and differential olfactory sensitivity. *Ann. N. Y. Acad. Sci.* **116**, 527.

Stone, H., and J. Bosley. 1965a. Olfactory discrimination and Weber's law. *Precept. Motor Skills* **20**, 657.

Stone, H., and J. Bosley. 1965b. Unpublished laboratory reports.

Stuiver, M. 1958. Biophysics of the sense of smell. Doctoral dissertation. University of Groningen, Holland.

Swets, J. A. 1961. Is there a sensory threshold? *Science* **134**, 168.

Tanner, W. P., Jr., and J. A. Swets. 1954. A decision-making theory of visual detection. *Psychol. Rev.* **61**, 401.

Torgeson, W. S. 1958. "Theory and Methods of Scaling." J. Wiley & Sons, New York.

Tucker, D. 1963. Physical variables in the olfactory stimulation process. *J. Gen. Physiol.* **46**, 453.

Ms. rec'd 5/26/65.

LINDA MAY BELE, HELEN H. PALMER, ALVIN A. KLOSE, and THEODORE F. IRMITER [a]
Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, California 94710

Evaluation of Objective Methods of Measuring Differences in Texture of Freeze-Dried Chicken Meat

SUMMARY

Breast and thigh meat of nine-week-old fryers from a commercial source was processed as frozen or freeze-dried raw or cooked meat. Objective methods were compared with sensory evaluation of texture and juiciness differences in the meat. The effect of freezing temperature (−23° and −191°C) on the quality of freeze-dried poultry meat and the differing effect of freeze drying on raw and cooked breast and thigh meat were also determined.

Freeze-dried chicken meat was readily distinguished from frozen. Freeze-dried meat had poorer texture and less juiciness, higher shear resistance, and more expressible liquid than frozen. Cooked chicken meat had significantly higher shear resistance (Allo-Kramer and Instron) after freeze-drying than after freezing. The shear resistance tests agreed well with panel judgments of texture and could be substituted for them. However, for meat that was frozen or freeze dried before cooking, the differences in shear resistance were not consistently significant. Freeze-dried breast meat had larger amounts of expressible liquid (Succulometer) than the frozen. For breast meat this objective test agreed well with panel judgments of juiciness and could be substituted for them. Although the panel consistently distinguished differences between frozen and freeze-dried thigh meat, little or no liquid could be expressed from any of the thigh meat; thus the test could not be substituted for the panel judgments. No significant differences in "water-holding capacity" (Grau-Hamm press) were found between frozen and freeze-dried meat; this test could not be substituted for the panel tests under the conditions used.

Freezing temperature (−23° and −191°C) had no significant effect on meat freeze-dried before cooking. The freezing temperatures resulted in detectable differences in meat freeze dried after cooking, but the differences were too small to be of practical importance.

Freeze drying adversely affected breast more than thigh. The breast meat was less tender and juicy, and had higher shear resistance, and more expressible liquid.

Meat cooked after freeze drying had better texture and juiciness, lower shear resistance, and less expressible liquid than meat cooked before freeze drying.

INTRODUCTION

At present, freeze-dried poultry meat is not uniform in rehydration, reconstitution, texture, juiciness, or flavor. To expedite the development of improved freeze-dried poultry products, means are needed to measure the effect of treatment variations on the meat quality. Since testing foods by panels is usually time-consuming and expensive, supplementation or replacement of sensory evaluation by objective methods of measurement is desirable, particularly for routine commercial control.

The main purpose of this investigation was to determine whether objective methods of measuring the texture, tenderness, and juiciness of freeze-dried poultry could be substituted for sensory methods. Also determined were the effects of freeze drying on the quality of cooked and raw breast and thigh meat and of freezing the meat at −23° and −191°C.

Research on freeze-dried meat has been reported by Auerbach *et al.* (1954), Burke and Decareau (1964), Doty *et al.* (1953), Harper and Tappel (1957), Tappel *et al.* (1955, 1957) Wang *et al.* (1953, 1954), and Yao *et al.* (1956). Connell (1957) defined rehydration as the total uptake of water by the dried sample, and reconstitution as the actual water-binding capacity of the dried sample such that it is indistinguishable from the original product. The rehydration level of freeze-dried beef has generally been above 80% of the original water content. Wang *et al.* (1954) found that all samples, regardless of freezing rate, rehydrated to 85–90% of the original moisture content, and that

[a] Present address: Michigan State University, East Lansing, Michigan 48823.

muscle fibers returned to 89–98% of their original diameter. Suden *et al.* (1964) reported that freeze-dried pork rehydrated to 73.8%.

In sensory evaluations the freeze-dried food could be differentiated from the frozen control and usually had a drier texture. Brooks (1958), Burke and Decareau (1964), Harper and Tappel (1957), and Tappel *et al.* (1955) reported that reconstituted freeze-dried meat was tougher and had a drier texture than frozen control meat. Hamdy *et al.* (1959) reported that the loss of tenderness and juiciness of rehydrated freeze-dried meat samples is apparently caused by a loss of the water-holding capacity of muscle proteins. Tappel *et al.* (1955), Harper and Tappel (1957), and Hamdy *et al.* (1959) pointed out that dry texture and poor rehydration are the principal problems in freeze-dried meat.

Rehydration levels for freeze-dried poultry have been low. Tappel *et al.* (1957) reported that pre-cooked freeze-dried poultry rehydrated poorly. Rehydration was 68% in 212°F water for 15 min at atmospheric pressure, and 63% under 690-mm vacuum for 2 min. For serving-size pieces (1½ × 2 × ⅝ in.) of pre-cooked freeze-dried poultry Yao *et al.* (1956) reported a low rehydration of 57–74% and a sensory quality comparable with that of controls which had not been freeze-dried.

Commercial cold-air blast and liquid immersion freezing (i.e., sodium chloride, liquid nitrogen, propylene glycol) have made possible very rapid freezing of meat. Both methods are being used with poultry (Barrie *et al.*, 1964; Marion and Stadelman, 1958).

Deatherage and Hamm (1960) showed that rapid freezing of beef muscle tissue neither decreases the rehydration of muscle nor denatures proteins. They concluded that the effect of freeze dehydration on the water-holding capacity of meat is due not to the freezing process but to the process of dehydration. Meat frozen quickly (−55°C) reconstituted better than meat frozen slowly (−15°C).

Barrie *et al.* (1964) reported that blast *vs.* liquid freezing made no difference to the general acceptability of turkey, and that the freezing method had no significant effect on the shear resistance of light or dark meat. Marion and Stadelman (1958) found that the method of freezing (liquid at 10°F, plate at −5°F, and moving air at −15°F) did not change percentage drip, percentage total cooking loss, or tenderness of turkey breast. Wang *et al.* (1954) found that rehydration of beef muscle does not depend on the freezing temperature (−150, −80, and −17°C).

PROCEDURE

Sample processing. Breasts and thighs from nine-week-old mixed-sex fryer chickens, commercially processed and chilled for 24 hr, were divided into two lots, half deboned raw and the other half steamed before deboning ("Steamcraft" steam cooker, Cleveland Range Co., Cleveland, Ohio, direct steam, two-compartment model). Breasts were cooked 15 min, and thighs 20 min. Skin and subcutaneous fat were removed from the muscles. Deboned meat was held on trays overnight at −7°C (20°F) to make it firm enough for dicing.

The meat was diced into ½ × ½ × ⅝-in. pieces with a Urschell dicer (model F, Urschell Labs., Inc., Valparaiso, Ind.) and weighed into polyethylene bags. Half of each lot was rapidly frozen in liquid nitrogen at −191°C (−320°F) and held at −34°C (−30°F); the other half was slowly frozen in an air flow of approximately 50 ft/min at −23°C (−10°F) and held at −23°C. Testing was completed within 2½ months.

Half of the frozen breast and thigh meat tested each day was dried in a freeze dryer (RePP Sublimator, RePP Industries, VirTis Co., Gardiner, N. Y., model 11-15) during the 20–24 hr immediately prior to use. Throughout the freeze-drying operation the condenser was at −51°C (−60°F), the chamber pressure 25–50 μ Hg, and the shelf heat was automatically shut off when the product temperature reached −15°C (5°F). Because some of the diced meat stuck together and

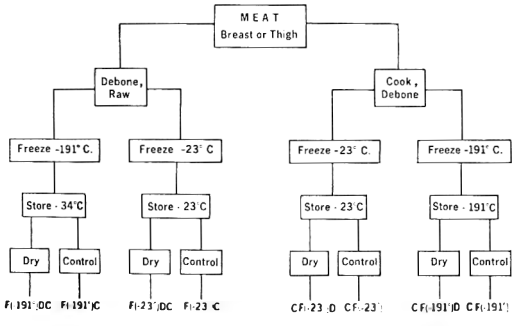


Fig. 1. Diagram of experimental design.

froze in large clumps, some raw samples required longer than 24 hr to dry. Fig. 1 is a diagram of the experimental design.

Sample preparation and rehydration. The frozen meat was thawed, or dried meat was cooked or rehydrated, according to the following plan:

FC: frozen (−23 or −191°C), cooked
FDC: frozen (−23 or −191°C), dried, cooked
CF: cooked, frozen (−23 or −191°C)
CFD: cooked, frozen (−23 or −191°C), dried

Based on pilot tests of rehydration time (10–30 min) and water temperature for rehydration (25, 47, 56, 76, 100°C), the freeze-dried samples were rehydrated in 6⅓ parts by weight of distilled water for 20 min with stirring at 5-min intervals. Rehydration ratios of freeze-dried meat were not affected by the length of time of reconstitution. The temperature of the rehydration water was 25°C (77°F) for raw meat (FDC) because higher temperatures reduced reabsorption of water due to coagulation of surface protein (case-hardening). The cooked, dried samples (CFD) were rehydrated in boiling water. The cooked, thawed meat (CF) was just covered with boiling water and held for 5 min, which brought the meat to serving temperature. The raw reconstituted (FDC) or thawed samples (FC) were steamed for 5 min, which was adequate to cook the raw diced samples. The samples were drained and reweighed, and the rehydration ratios calculated.

Samples for the panel were held in covered dishes in a water bath at 52°C (126°F) for a half-hour to ensure uniform serving temperature.

Sensory evaluation. Eight persons from the Poultry Laboratory with previous experience in judging texture and tenderness of poultry served as the panel. Panel members were blindfolded. Each day the panel had two triangle tests. The panelists removed the blindfold between the tests and indicated the odd sample on a score sheet (Fig. 2). They also indicated whether the selection was based on texture, juiciness or flavor.

Comparisons by the panel were made according to the following plan:

Frozen vs. freeze-dried meat
  Breast meat
    F(−23°)C vs. F(−23°)DC
    F(−191°)C vs. F(−191°)DC
    CF(−23°) vs. CF(−23°)D
    CF(−191°) vs. CF(−191°)D
  Thigh meat
    Same as for breast meat

Freezing at −23°C vs. at −191°C before freeze-drying
  Breast meat
    F(−23°)DC vs. F(−191°)DC
    CF(−23°)D vs. CF(−191°)D
  Thigh meat
    Same as for breast meat

Freeze-dried breast meat vs. freeze-dried thigh meat
  Breast CF(−23°)D vs. Thigh CF(−23°)D
  Breast CF(−191°)D vs. Thigh CF(−191°)D

Each comparison was repeated four times, and each triangle test was randomized independently. One complete set of tests (two triangle tests for each comparison) was followed by a second set of tests. Both sets were randomized independently.

Objective evaluation. *Allo-Kramer shear press.* A recording Allo-Kramer shear press (KSP, model SP-12 Imp), equipped with an automatic recorder (Varian strip-chart recorder, model G 11A-F1), measured shear resistance. The conditions for this

Each set of samples will contain 2 like and 1 odd sample.

Sample	Check Odd Sample	Please indicate basis for selection of odd sample			
		Flavor	Juiciness (dry, juicy, rapid moisture release)	Texture (soft, hard; stringy, rubbery; tender, tough, friable)	Comments
A					
B					
C					

Fig. 2. Score sheet, triangle test.

test were based on information by Kramer (1961) and on preliminary tests with the diced chicken. Use of a weighed sample rather than a full cell improved precision. Preliminary tests with samples weighing 30–100 g gave shear resistances which were high. As the sample size increased, the shear resistance (kg force per g sample) decreased. Samples of 120–140 g (wet weight) were used because they gave most consistent results.

Samples were poured into the standard cell with random orientation of the meat fibers with respect to the slots of the shear press. Meat samples cut with or against the fiber grain yield different values depending on orientation in the test cell. However, orientation of diced chicken is impractical; the product did not tend to pack or form cavities.

The faster the blade descended through the sample, the higher was the peak reading. For example, two 66-g samples, on 3000-lb recorder range, had shear resistance of 6.7 kg/g with a 15-sec downstroke and 5.5 kg/g with a 30-sec downstroke.

Samples were sheared only once, since repeated shearings on the same sample had lower readings for a constant sample weight (i.e., first pass: 5.4 kg/g; second pass: 4.7 kg/g; and third pass: 4.5 kg/g). Average of the successive passes is not a true indication of the texture of the test sample. Wells *et al.* (1962) sheared each sample several times, whereas other investigators report shearing the sample once.

A calibration of 3000-lb full-range scale, 3000-lb proving ring, and 15-sec downstroke was maintained for all tests on samples of 120–140 g. The maximum shear resistance was recorded and then divided by the weight of the sample to obtain the resistance per gram. This value was used as the mechanical index of shear resistance for the samples tested. Representative curves of shear resistance for the different test samples are presented in Fig. 3.

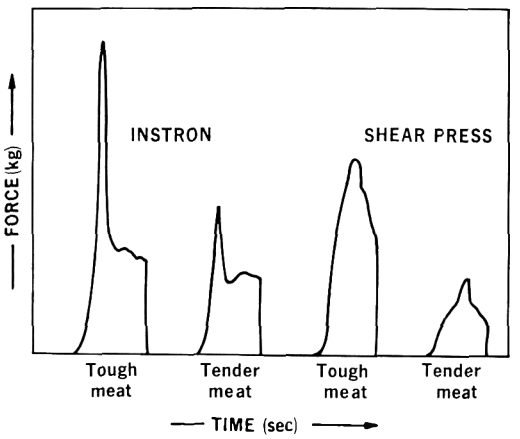


Fig. 3. Typical shear-resistance curves.

Instron. A standard test cell for holding the meat and a plunger adapted from the Allo-Kramer test cell were constructed and mounted on an Instron (Model TM, Instron Engineering Corp., Canton, Mass.). This instrument makes a continuous recording of the force-penetration curve. The plunger consisted of 20 uniformly spaced metal dowels (6.35 mm in diameter, 25 mm in length) which were forced down through the sample in a cell having holes 3 mm apart, spaced to match the metal dowels (Fig. 4).

Samples of 10 g wet weight were randomly oriented as to meat fibers in the cell, and sheared. Initial studies indicated that a calibration of 200-lb full scale, 5-lb full load, and downstroke of 2-in. per sec would be most suitable. Two samples of each treatment were sheared once. Calculation of shear resistance was the same as for the Allo-Kramer. Representative shear resistance curves are presented in Fig. 3.

Succulometer. Expressible liquid was determined in the Succulometer cell supplied with the Allo-Kramer. A 70-g sample, wet weight, was randomly orientated in the cell. A standard calibration of 3000-lb full scale, 3000-lb proving ring, and 30-sec downstroke was used. The full force was main-

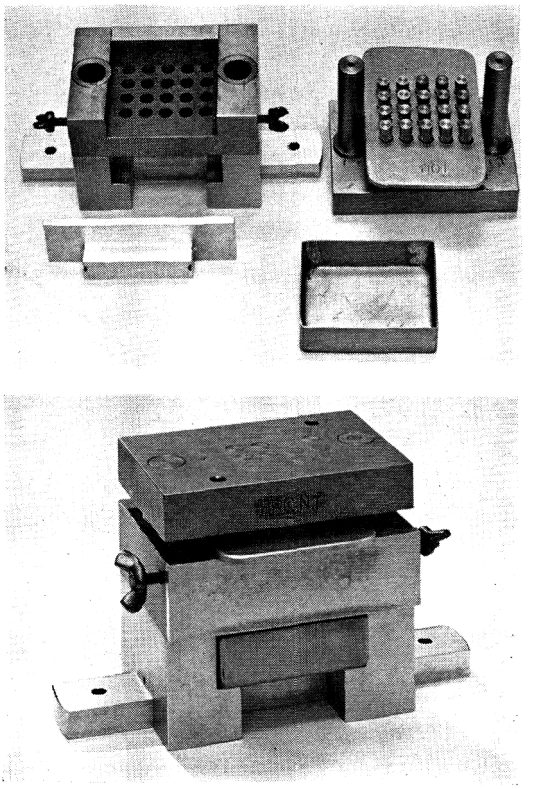


Fig. 4. Instron test cell: A) component parts; B) assembled.

tained on the sample for 30 sec, and liquid expressed through the sprout in the cell was collected in a graduated cylinder. Expressible liquid was calculated as the percent of the known weight of material squeezed out as liquid under established conditions of pressure and time.

Grau-Hamm press. In the method of Grau and Hamm (1957), 300 mg of meat tissue was placed on a piece of filter paper and compressed between two plexiglass plates. The pressure on the plates was exerted by turning down two hand screws. The pressure was applied to the plates for 5 min.

The method has been used in combination with the Carver press to determine expressible liquid in beef and turkey (Sanderson and Vail, *1963*; Barrie *et al.*, 1964). The method reported by Barrie *et al.* (1964) was modified for this study, on the basis of preliminary experiments, by using a pressure of 1000 lb instead of 10,000 lb for 5 min. The meat (300 mg) was pressed to a thin round film, and the expressed juice was absorbed by the filter paper. The area of the outer ring of expressed juice and the area of the inner meat disc were determined with a planimeter. The "water-holding capacity" was expressed as a ratio of the area of the outer circle to that of the inner circle.

Statistical analysis. Data from the Allo-Kramer, Instron, Succulometer, and Grau-Hamm press were subjected to analysis of variance (Guenther, 1964) using a one-way classification. Significant differences for the taste-panel triangle tests were determined by the method of Roessler *et al.* (1948). Correlations were calculated between the Allo-Kramer vs. Instron for frozen breast, freeze-dried breast, frozen thigh, and freeze-dried thigh.

RESULTS AND DISCUSSION

Correlation between taste-panel and objective tests. *Panel tests.* The taste panel was able to distinguish between frozen and freeze-dried meat under all conditions tested (Table 1). Differences were highly significant ($p < 0.01$) for all comparisons. The panel indicated that the frozen samples had better sensory properties than the freeze-dried samples. Texture and juiciness were usually the basis for distinguishing between the samples. This agrees with results of Brooks (1958) and Tappel *et al.* (1955, 1957) that reconstituted freeze-dried meat and poultry were tougher and had a drier texture than the control meat. A flavor difference was noted by ⅔ of the judges in one comparison of frozen and freeze-dried thigh meat (F-191°C vs. F-191°DC) (Table 1).

The texture of breast meat frozen before cooking (FC) was described by the panel as more cohesive, firm, tender, and better than the texture of the freeze-dried (FDC), which was generally described as hard and slightly more tough. Thigh meat frozen before cooking (FC) was described as tender, firm, and good or outstanding, compared

Table 1. Ability of panel to distinguish freeze-dried from frozen poultry meat.

Type of meat	Freezing temp. (°C)	Triangle test [a]: No. of correct odd sample selections out of 32 [b]	Triangle test [a]: % of correct odd sample selections having comments on Texture	Juiciness	Flavor
			(%)	(%)	(%)
Frozen and freeze dried before cooking					
Breast	−23°	29***	34	62	28
	−191°	27***	70	52	11
Thigh	−23°	18**	39	50	44
	−191°	28***	50	29	68
Frozen and freeze dried after cooking					
Breast	−23°	27***	81	63	30
	−191°	27***	67	67	11
Thigh	−23°	32***	84	63	34
	−191°	31***	90	65	35

[a] 8 judges, 4 replications of each test.
[b] **, *** respectively, $p < 0.01$ and 0.001.

with the freeze-dried (FDC), variously described as tough or tender, stringy, and slightly more rubbery.

Texture of meat frozen after cooking (CF) was usually described as softer, more tender, and better than the texture of the freeze-dried (CFD), which was described as dry, grainy, stringy, rubbery, tough, and hard.

Breast and thigh meat frozen before cooking (FC) was generally described as more juicy and having sustained moisture release during chewing compared with the freeze-dried (FDC), described as releasing water more rapidly and being soggy, watery, mealy, wet, or dry (a conflict probably based on the initial presence of loosely held water and its rapid release on chewing).

A similar difference in juiciness was found between the samples frozen or freeze-dried after cooking. The CF samples were generally described as more juicy and the CFD samples as more dry and as releasing water more rapidly. Szczesniak *et al.* (1963) observed that rehydrated freeze-dried beef releases most of its moisture quickly (on the first two chews).

The flavor of breast meat frozen before cooking (FC) was described as good-to-superior, compared with the freeze-dried (FDC), described as flavorless, flat, bland, off, stale, and watery. The FC thigh meat had typical desirable chicken flavor compared with the FDC meat, which was described as slightly off or rancid, cardboard, bland, and having less chicken flavor. However, since approximately equal numbers of favorable and adverse flavor comments were made on chicken meat frozen or freeze-dried after cooking, it was apparent that flavor was not responsible for the ease with which CF and CFD samples were differentiated.

Shear resistance. The shear resistance of the freeze-dried meat was consistently greater than that of the frozen meat (Table 2). For all comparisons of CF vs. CFD meat, the differences between Allo-Kramer values were significant at $p < 0.01$ or $p < 0.001$ level; differences between Instron values were significant at $p < 0.05$ or < 0.001. Differences between FC and FDC Allo-Kramer values were not significant except for the breast meat frozen at −191°C ($p < 0.01$). Difference between F(−23°)C vs. F(−23°)DC Instron values were significant ($p < 0.05$), but not those for F(−191°)C vs. F(−191°)DC.

The correlations between the shear values from the Allo-Kramer and Instron tests were high. The correlation coefficients between these two objective methods were for the frozen breast +0.975, freeze-dried breast +0.940, frozen thigh +0.995, and freeze-dried thigh +0.964. The shear values (kg/g) for frozen breast meat ranged from 2.8–6.3 on the Allo-Kramer and from 3.6–7.4 on the Instron; for freeze-dried breast, 2.9–9.6 (A–K) and 4.8–9.0 (Instron); for frozen thigh, 1.3–2.8 (A–K) and 2.8–4.8 (Instron); for freeze-dried thigh, 1.2–6.2 (A–K) and 3.0–6.8 (Instron).

Differences in texture detected by the panel were reflected in Allo-Kramer or Instron measurements at the same or nearly the same levels of statistical significance for CF vs. CFD meat. However, the instru-

Table 2. Shear resistance of frozen and freeze-dried meat.

Type of meat	Freezing temp. (°C)	Raw or cooked	Shear resistance [a] (Kg/g)			
			Kramer		Instron	
			Frozen	Dried	Frozen	Dried
Breast	−23°	Raw	3.2	3.9	4.6	6.1
		Cooked	4.1	8.1	5.4	8.4
	−191°	Raw	3.1	4.5	5.5	6.2
		Cooked	4.5	6.7	6.1	7.5
Thigh	−23°	Raw	1.7	1.9	3.1	3.6
		Cooked	2.1	5.0	3.7	5.4
	−191°	Raw	1.7	1.9	3.1	3.6
		Cooked	2.2	4.9	3.4	5.2

[a] Kg of force per g of sample; the lower the value the more tender the meat. Each value is an average of 3–4 samples of frozen meat, 7–8 samples of freeze-dried meat.

Table 3. Summary of significant differences[a] between frozen and freeze-dried poultry meat.

Type of meat	Freezing temp. (°C)	Panel [b]	Kramer [c]	Instron [d]	Succulometer [e]	Grau-Hamm [f]
Frozen and freeze dried before cooking						
Breast	−23°	0.001	N.S.	0.05	0.01	N.S.
	−191°	0.001	0.01	N.S.	0.05	N.S.
Thigh	−23°	0.01	N.S.	0.05	N.S.	N.S.
	−191°	0.001	N.S.	N.S.	N.S.	N.S.
Frozen and freeze-dried after cooking						
Breast	−23°	0.001	0.001	0.001	0.01	N.S.
	−191°	0.001	0.01	0.05	0.05	N.S.
Thigh	−23°	0.001	0.001	0.001	N.S.	N.S.
	−191°	0.001	0.01	0.001	N.S.	N.S.

[a] Expressed as probability levels.
[b] Triangle test: 8 judges, 4 replications of each test.
[c] No. of samples: 3–7
[d] No. of samples: 4–8
[e] No. of samples: 2–8
[f] No. of samples: 3–6

ments were not as consistent as the panel in recording texture differences in the FC vs. FDC meat (Table 3).

Good correlations between the Allo-Kramer and tenderness judgments of competent taste panels have been reported by Shannon *et al.* (1957) on cooked breast muscle, and by Sosebee *et al.* (1964) on freeze-dried chicken. However, Wells *et al.* (1962) reported that Allo-Kramer measurements contradicted taste-panel findings on the tenderness of freeze-dried chicken breast meat.

The shear resistance of meat freeze dried after cooking was 49–168% higher on the Allo-Kramer test and 21–52% higher on the Instron test than the shear resistance of meat freeze dried before cooking. Thus, freeze-drying the cooked meat was more damaging than freeze-drying the raw meat. Meat frozen after cooking also had higher shear values than meat frozen before cooking. These differences ranged 24–43% for the Allo-Kramer and 10–19% for the Instron tests. Similar results have been found for freezing of raw vs. pre-cooked turkey meat (Goodwin *et al.*, 1962) and for chicken meat (Mickelberry and Stadelman, 1962).

Processing losses. Three factors could contribute to the juiciness differences between frozen and freeze-dried meat detected by the panel: 1) differences in the moisture lost during processing; 2) the amount absorbed during rehydration; and 3) the expressible moisture of the cooked meat.

The cooking loss from diced FC breast meat was 25%; the decrease in yield after rehydration of diced FDC breast meat was 32% (Table 4). Comparable losses for FC and FDC thigh meat were respectively 37 and 39%.

The cooking losses from the CF and CFD breast and thigh meat were not determined, because the breasts and thighs were cooked before deboning. However, the yield of CFD breast meat after rehydration was 22% lower than that of the CF meat; the yield of CFD thigh meat was 30% lower than that of the CF meat.

Expressible liquid and "water-holding capacity." The differences in the fluid release of frozen and freeze-dried chicken breast meat that were detected by sensory judgments of juiciness were reflected in tests with the Succulometer. Much of the water absorbed by the freeze-dried meat was not firmly held. More water could be pressed from the freeze-dried breast meat than from the frozen breast meat. The difference was significant at $p < 0.001$ for meat frozen or freeze dried before cooking and for the meat frozen or freeze dried after cooking. Little or no measurable water could be pressed from either the frozen or freeze-dried thigh meat (Table 5).

Table 4. Meat weight after processing steps (percent of weight of frozen meat).

Condition of diced meat	Type of meat	Processing steps				Pressed meat (after Succulometer test)
		Frozen	Freeze dried	Rehydrated	Cooked	
Raw	Breast	100	.....	.....	75	69
			25	87	68	54
	Thigh	100	.....	.....	63	63
			25	89	61	61
Cooked	Breast	100	.....	.....	.....	85
			28	78	.....	62
	Thigh	100	.....	.....	.....	100
			30	70	.....	70

There were no significant differences in press fluid from frozen vs. freeze-dried meat tested by the Grau-Hamm press method (Table 5). Since the method did not show differences between frozen and freeze-dried samples that were detected by the taste panel and Succulometer, it was of little value in this study.

Effect of freezing method on quality of freeze-dried poultry. *Panel tests.* The taste panel detected no difference due to freezing temperature used for the chicken meat freeze dried before cooking (F-23°DC vs. F-191° DC). However, the freezing temperature did result in a difference in the properties of meat frozen vs. dried after cooking (CF-23°D vs. CF-191°D). The panel detected this difference at $p < 0.001$ for the thigh meat and $p < 0.01$ for breast meat. Texture and juiciness were the characteristics mentioned by the panel to distinguish between samples. However, since comments were comparable for samples frozen at the two temperatures, the difference between the freezing temperatures was judged to be unimportant.

Shear resistance. The freezing temperatures used did not have a significant effect on the shear resistance of the FDC meat. However, for CFD meat, breast meat samples frozen at −191°C had lower shear resistance than those frozen at −23°C. The difference was significant at $p < 0.01$ for the Allo-Kramer and $p < 0.05$ for the Instron; the difference in thigh meat was not significant (Table 2).

No panel comparisons were made of chicken meat frozen at the two temperatures (F-23° vs. F-191°); objective evaluations show no significant differences. These data agree with those of Marion and Stadelman (1958) and Barrie *et al.* (1964).

Table 5. Expressible liquid of frozen and freeze-dried meat.

Treatment before freezing or drying	Freezing temp. (°C)	Frozen or dried	Expressible liquid			
			Succulometer [a]		Grau-Hamm Press [b]	
			Breast	Thigh	Breast	Thigh
Raw	−23°	Frozen	6.4	0.0	4.4	3.5
		Dried	16.0	0.0	3.9	3.4
	−191°	Frozen	11.6	0.0	3.9	3.4
		Dried	21.2	0.4	4.6	3.4
Cooked	−23°	Frozen	17.1	0.7	4.9	3.6
		Dried	23.8	0.5	3.7	3.1
	−191°	Frozen	15.5	0.0	5.6	3.6
		Dried	19.5	0.0	9.6	3.3

[a] Percent of sample weight.
[b] Ratio of area of outer circle to area of inner circle. Each value is an average of 2–4 samples of frozen meat, 6–8 samples of freeze-dried meat.

Expressible liquid. About 4% more liquid based on percent of sample weight was pressed from the CF(−23°)D breast meat than from the CF(−191°)D meat during the Succulometer test. About 5% more liquid was pressed from the F(−191°)DC meat than from the F(−23°)DC meat. The differences were unimportant, however, since the panel's comments on juiciness did not indicate any consistent difference due to freezing temperature.

Little or no liquid could be pressed out of any of the freeze-dried thigh meat subjected to any of the processing variables (Table 5).

Differential effect of freeze drying. *Panel tests.* The panel distinguished ($p<0.001$) between breast and thigh meat freeze dried after cooking, primarily on the basis of texture and juiciness (Table 6). CF(−23°)D meat was correctly distinguished in 84% of the panel judgments, CF(−191°)D meat in 81% of the judgments. The freeze-dried thigh meat was more desirable than the breast meat. Although adverse comments were made about the texture and juiciness of both samples, the breast meat was generally considered to be more dry, powdery, stringy, hard, and tough, and to have more rapid moisture release than the thigh meat. No panel comparisons were made between breast and thigh meat freeze dried before cooking.

Shear resistance. Freeze-dried thigh meat had lower shear resistance than freeze-dried breast meat, whether the meat was freeze dried before or after cooking (Table 2). The shear values were significantly different at $p<0.001$ for all comparisons with both the Allo-Kramer and the Instron.

Expressible liquid. The Succulometer pressed little or no water from the thigh meat compared with that pressed from the breast meat, indicating that the liquid absorbed by the thigh was held more firmly by the muscle cell structure (Table 5). The difference was significant ($p<0.001$) for the FDC and for the CFD meat.

REFERENCES

Auerbach, E., H. Wang, N. Maynard, D. M. Doty, and H. R. Kraybill. 1954. A histological and histochemical study of beef dehydration. V. Some factors influencing the rehydration level of frozen-dried muscle tissue. *Food Research* **19**, 557.

Barrie, P. J., G. E. Goertz, and J. L. Fry. 1964. Acceptability of blast-frozen and liquid-frozen turkey hens and toms. *Food Technol.* **18**(4), 565.

Brooks, J. 1958. The structure of the animal tissues and dehydration. *In* "Soc. Chem. Ind.," Fundamental aspects of the dehydration of foodstuffs, p. 8.

Burke, R. F., and R. V. Decareau. 1964. Recent advances in the freeze-drying of food products. *Advances in Food Research* **13**, 1.

Connell, J. J. 1957. Some aspects of the texture of dehydrated fish. *J. Sci. Food Agr.* **8**, 526.

Deatherage, F. E., and R. Hamm. 1960. Influence of freezing and thawing on hydration and charges of the muscle proteins. *Food Research* **25**, 623.

Doty, D. M., H. Wang, and E. Auerbach. 1953. Chemical and histological properties of dehydrated meat. J. Agr. Food Chem. **1**, 664.

Goodwin, T. L., W. C. Mickelberry, and W. J. Stadelman. 1962. The effect of freezing, method of cooking, and storage time on the tenderness of precooked and raw turkey meat. *Poultry Sci.* **41**, 1268.

Grau, R., and R. Hamm. 1957. Ueber das wasserbindungsvermoegen des saeugetiermuskels. II. Mitt. Ueber die bestimmung der wasserbin-

Table 6. Ability of panel to distinguish cooked freeze-dried breast meat from cooked freeze-dried thigh meat.

Freezing temp. (°C)	Triangle test [a]			
	No. of correct odd sample selections out of 32 [b]	% of correct odd sample selections having comments on		
		Texture	Juiciness	Flavor
−23°	27***	67	44	26
−191°	26***	77	65	19

[a] 8 judges, 4 replications of each test.

[b] *** $p < 0.001$.

dung des muskels. *Z. Lebensm.-Untersuch. u-Forsch.* **105**, 446.

Guenther, W. C. 1964. "Analysis of Variance." Prentice-Hall, New Jersey. p. 31.

Hamdy, M. K., V. R. Cahill, and F. E. Deatherage. 1959. Some observations on the modification of freeze dehydrated meat. *Food Research* **24**, 79.

Harper, J. C., and A. L. Tappel. 1957. Freeze-drying of food products. *Advances in Food Research* **7**, 220.

Kramer, A. 1961. The shear-press, a basic tool for the food technologist. *Food Scientist* **5**, 7.

Marion, W. W., and W. J. Stadelman. 1958. Effect of various freezing methods on quality of poultry meat. *Food Technol.* **12**, 367.

Mickelberry, W. C., and W. J. Stadelman. 1962. Effect of cooking method on shear-press values and weight changes of frozen chicken meat. *Food Technol.* **16**(8), 94.

Roessler, E. B., J. Warren, and J. F. Guymon. 1948. Significance in triangular taste tests. *Food Research* **13**, 503.

Sanderson, M., and G. E. Vail. 1963. A method for determining press fluid in cooked beef. *J. Food Sci.* **28**, 596.

Shannon, W. G., W. W. Marion, and W. J. Stadelman. 1957. Effect of temperature and time of scalding on the tenderness of breast meat of chicken. *Food Technol.* **11**, 284.

Sosebee, M. E., K. N. May, and J. J. Powers. 1964. The effects of enzyme addition on the quality of freeze-dehydrated chicken meat. *Food Technol.* **18**, 551.

Suden, J. R., A. M. Pearson, and L. R. Dugan, Jr. 1964. Rehydration of freeze-dried pork as related to pH and protein denaturation. *J. Food Sci.* **29**, 192.

Szczesniak, A. S., K. Sloman, M. Brandt, and E. Z. Skinner. 1963. Objective measurement of texture of fresh and freeze-dehydrated meats. XIV. Proc. of the 15th Research Conference, American Meat Institute Foundation. p. 121.

Tappel, A. L., A. Conroy, M. R. Emerson, L. W. Regier, and G. F. Stewart. 1955. Freeze-dried meat. I. Preparation and properties. *Food Technol.* **9**, 401.

Tappel, A. L., R. Martin, and E. Plocher. 1957. Freeze-dried meat. V. Preparation, properties, and storage stability of precooked freeze-dried meats, poultry, and seafoods. *Food Technol.* **11**, 599.

Wang, H., F. Andrews, E. Rasch, D. M. Doty, and H. R. Kraybill. 1953. A histological and histochemical study of beef dehydration. I. Rate of dehydration and structural changes in raw and cooked meat. *Food Research* **18**, 351.

Wang, H., E. Auerbach, V. Bates, D. M. Doty, and H. R. Kraybill. 1954. A histological and histochemical study of beef dehydration. IV. Characteristics of muscle tissues dehydrated by freeze-drying techniques. *Food Research* **19**, 543.

Wells, G. H., K. N. May, and J. J. Powers. 1962. Taste-panel and shear-press evaluation of tenderness of freeze-dried chicken as affected by age and pre-slaughter feeding of ions. *Food Technol.* **16**(9), 137.

Yao, A., A. I. Nelson, and M. P. Steinberg. 1956. Factors affecting the rate of chicken meat dehydration under vacuum. Food Technol. **10**, 145.

Ms. rec'd 1/28/66.

Appreciation is expressed to Dr. Clifford L.Bedford and Dr. Lawrence E. Dawson, Michigan State University, and Dr. Hans Lineweaver, Chief of Poultry Laboratory, for their suggestions, criticisms, and encouragement; to members of the taste panel for their sensory evaluations; and other laboratory personnel whose assistance and interest contributed to bring this work to its completion.

This paper reports a portion of M.S. research by the senior author at Michigan State University. The work was conducted in the Poultry Laboratory at the Western Regional Research Laboratory.

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