

# Food Structure

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# FOOD MICROSTRUCTURE

**An  
International Journal  
on the  
Microstructure and Microanalysis  
of  
Foods, Feeds and their Ingredients**

**Guest Editors**

**Muscle Foods:** R.J. Carroll and C.A. Voyle

**Plant Foods:** W.J. Wolf and K. Saio

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# FOOD MICROSTRUCTURE

*An International Journal on the Microstructure and Microanalysis  
of Foods, Feeds and their Ingredients*

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AN ANALYSIS OF MICROSTRUCTURAL FACTORS WHICH INFLUENCE THE USE OF MUSCLE AS A FOOD

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Abstract

Study of structure of muscle provides information on the location and arrangement of various components and the changes which may be inflicted upon them. The structural features of muscle have been described in detail down to the molecular level, but regarding its use as a food, special interest centers on the connective tissue component and on myofibrillar proteins. Muscle comprises about 1/3 of the live weight of the animal and is not static, but rather is subject to major changes in properties associated with growth, repair and senescence. It is apparent that the nervous and hormonal systems exert a strong influence on the properties of muscle and great potential exists for regulation of muscle via control of these systems. Major advancement has been made in relating properties of meat to state of contraction, and current effort is centered on relating properties of meat to changes resulting from post-mortem degradation of muscle proteins. The microstructure of emulsions has been described with interest concentrated on the protein membrane surrounding the lipid globules, but as of yet, utilization of this information by the industry has not occurred. The challenge for the future is to utilize the enormous amount of structural information known about muscle to improve its use as a food.

Introduction

Knowledge of structure is an obvious prerequisite to successful study of or research on muscle. It follows then that almost any organized consideration of muscle has at least one presentation dealing with general structural factors. This discussion of meat microstructure is no exception. The exception, however, is that we do not intend to repeat what can be found in detail in numerous textbooks and countless reviews. Our objective is to analyze what is known and discuss how the information might be used to improve the properties of fresh meat and its use as a processed product.

Study of structure results in knowledge about (1) location and arrangement of various components and (2) changes in the location and arrangement as a result of some procedure. While such descriptive information is relatively easy to obtain, the key is to devise a means of utilizing it to improve something. Lewis (1981) has discussed this and pointed out that morphological results should have application and lead to control of manufacturing conditions.

Structural Features

Even though skeletal muscle by sheer volume alone is the overwhelming source of meat, the other types (cardiac and smooth) may be present in or incorporated into meat. Cardiac muscle, for example, may influence color because of its relatively high content of cytochrome c (Lozano and Cassens, 1984). Structural features of the three types of muscle together with information about protein composition, role of membrane components, neuromuscular function, contraction, etc., are readily available (Bourne, 1972; Cassens, 1984; Weiss and Greep, 1977). For our purpose here, however, only two figures will be used. Figure 1 is a diagrammatic cross-section of skeletal muscle. Most apparent is the highly organized connective tissue. An individual muscle is encased within a layer of connective tissue known as the epimysium. The muscle is divided into segments or groups of muscle fibers known as bundles by a level of organization of connective tissue termed the perimysium. And, finally, each individual muscle fiber is surrounded by a thin

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layer of connective tissue known as the endomysium. The connective tissue serves as a scaffold to hold the muscle in place and as a pathway for the circulatory and nervous systems to distribute themselves throughout the muscle, eventually making contact with each individual cell. While the connective tissue does a marvelous job of organizing the muscle and keeping components in place, it also presents a formidable barrier to breaking up the muscle or to selectively extracting specific components from it. Note should be made that intramuscular fat cells are also embedded in connective tissue (i.e. perimysial planes), and lipid also occurs intracellularly as a component of membranes or as droplets of free lipid.

Figure 2 presents in diagrammatic form the spectrum of structure from whole muscle to the ultrastructural level. The "bellied" shape of the whole muscle is the simplest example and is termed fusiform. The muscle is composed of individual muscle fibers which vary in size and appear striated when viewed with the light microscope. They are multinucleate with the nuclei being placed in a subsarcolemmal or peripheral location. Each muscle fiber is fitted with a motor end plate giving it communication with the central nervous system which signals the fiber to contract. The sarcoplasm or non-structured portion of the cell is composed primarily of soluble proteins (myoglobin and enzymes functioning in metabolism) and contains organelles such as mitochondria, and inclusions such as glycogen. The major components of the fibers are the subunits termed myofibrils. These are in register giving rise to the striated appearance and are, in fact, the actual contractile units of muscle. The myofibrils are in turn composed of smaller subunits shown as thick and thin filaments. The substructure of the filaments is known -- they are built up of protein molecules which have been relatively well characterized. In brief, an enormous amount of detail is now known about structure of muscle. We are, in fact, aware of structure to the molecular level. Therefore, new information appears slowly and most often is a refinement which allows further conclusion regarding the mechanisms of function.

### Biological Properties

When muscle is considered as meat, the role of the live animal is all too often overlooked. It is well to recall that simple management practices (genetics, nutrition) can influence the amount and properties of muscle produced by the animal. From a biological viewpoint, the function of skeletal muscle is contraction which translates into movement of the animal and the associated advantages. Muscle comprises about 1/3 of the live weight of a typical meat animal and, therefore, from the simple aspect of mass alone, is an important metabolic component. Finally, muscle gives shape to the animal. It is suggested that the serious student of muscle structure must devote some time to gross anatomy (see, for example, Getty, 1975; Swatland, 1984)

not only because of the above points but also because different muscles are used for very different functions and, therefore, have very different properties. For example, Kauffman and Safanie (1967) determined looseness of the fascicular organization and found that it paralleled fat content of the muscle. In high fat content muscles, the fascicular organization was distinct and orderly with highly separated large fasciculi.

### Active Processes

Muscle is not a static entity. It grows and matures and finally enters a stage of senescence. If damaged or injured, it may undergo some degree of repair. This also is another area in which an enormous amount of information is available, and the meat technologist should have an awareness of the processes in order to better understand the tissue (meat) with which he or she works.

Embryonic development of muscle occurs when mononucleated myoblasts fuse to form multinucleated myotubes. These myotubes accumulate muscle-cell-specific proteins, show the characteristic cross-banded pattern, are innervated and have contractile activity. At a given time, the number of myotubes is stabilized, they begin to grow, and the centrally placed nuclei migrate to the periphery of the cell. Most information indicates that the number of muscle fibers is essentially constant and that growth of the muscle occurs from then on by hypertrophy rather than by hyperplasia. Depending on the species and the muscle, the fiber also begins to differentiate and display the characteristics of fiber type during fetal development. During early development of the animal, the fiber type proportions change until an adult condition is approximated (Suzuki and Cassens 1980, 1983). Aside from an increase in mass of muscle by hypertrophy, the other general change as the animal reaches adulthood is compositional in that the amount of fat increases. See Swatland (1984) for a complete account of muscle fiber differentiation and growth and development of the animal.

Special mention is made of the concept of fiber type and the reader is referred to several early reviews on the topic (Needham, 1926; Denny-Brown, 1929; Cassens and Cooper, 1971). The important point is that muscle fibers are not homogeneous but differ greatly in properties. In the most general sense, these are red and white and the proportions present in a given muscle are responsible not only for the gross appearance (i.e. red or white) of the muscle but also for its function. In mammalian muscle, both types are twitch but the white are physiologically faster than the red. The red are designed for more aerobic type metabolism having more myoglobin, more lipid, and less glycogen than the white.

Aging is a progressive development in the muscle of living animals which is recognized first as a loss of strength and endurance. In humans and various laboratory animals, senile muscular atrophy is well recognized and the degenerative changes accompanying it have been described



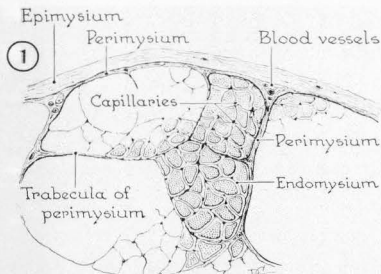


Figure 1: A diagrammatic cross-section of muscle which illustrates the arrangement of connective tissue (taken from Ham, 1965).

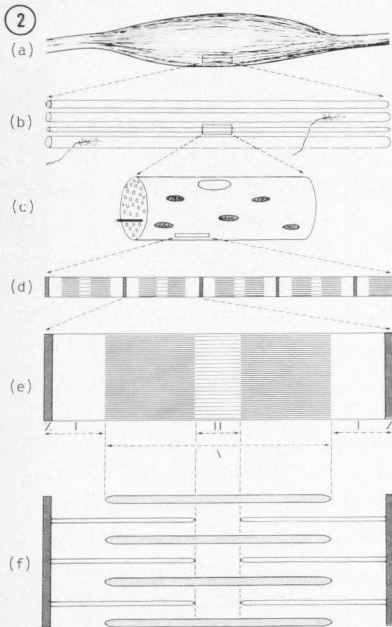


Figure 2: Diagrammatic representation of the levels of organization in muscle. (a) the entire muscle, (b) muscle fibers showing different sizes and innervation, (c) single fiber showing myofibrils and location of nuclei and mitochondria, (d) striation pattern of a myofibril, (e) a single sarcomere, and (f) the arrangement of thick and thin myofilaments (taken from Huxley, 1958).

(Caccia et al., 1979; Gutmann, 1977; Kaldor and DiBattista, 1978). Little has been done with meat animals, but it is an area deserving of investigation.

Muscle may undergo regeneration following injury (Mauro, 1979) and this is probably dependent on the presence of satellite cell nuclei. These nuclei are distinguished by their location outside of the sarcolemma but within the basement membrane of the muscle fiber (Lipton and Schultz, 1979).

#### Potential Regulation Methods

It is recognized that the nervous and hormonal systems exert a strong regulatory influence on muscle and must be intimately involved in the growth and senescence processes just discussed. It is further recognized that muscle has the ability to change or to adapt to its environment or to the demands placed on it by actually changing its properties.

The work of Buller et al. (1960) proved that the properties of muscle are under neural control. Their classical cross-reinnervation studies demonstrated that when nerves to red and white muscles are surgically severed and reimplanted on the muscle of opposite properties, the properties of the muscle change to those of the nerve. Other recent observations have given evidence for the strong role the thyroid hormones have in influencing the expressed properties of skeletal muscle. Ianuzzo, et al. (1977) reported that thyroid hormone caused a slow-twitch muscle to become more fast-twitch.

Cassens et al. (1983) have discussed how the above information can be used to regulate properties of muscle to improve its utilization as either fresh or processed meat. The concept of using regulation in the live animal to produce muscle with specific properties is an area deserving increased research effort.

#### Post-mortem Factors

The conversion of muscle to meat occurs post-mortem and the changes associated with rigor mortis have been described (Bendall, 1973). From a structural viewpoint, most emphasis has been placed on describing changes in morphology associated with rigor mortis. For example, Greaser et al. (1969), published detailed work on sub-cellular fractions from normal and pale, soft exudative porcine muscle prepared at various times post-mortem. Myofibrils from the PSE samples at 24 hr post-mortem had more granular appearing filaments and wider Z lines than normal muscle. Several other structural differences were observed.

A significant finding arose from the observation that the Z-line was degraded in aging of some muscle; the responsible enzyme known as CAF was isolated (Dayton et al., 1976).

At present, considerable emphasis is being focused on the so-called cytoskeleton of muscle. It is thought that the protein connectin, in some way, attaches A filaments of adjacent sarcomeres while the protein desmin plays a role in transverse linking at the level of the Z-line. The pertinent information as it affects properties of

meat is reviewed by Locker (1982) in a complete account of his theory of tenderness based on gap filaments.

It seems obvious, although there is no relevant hard evidence to cite, that during post-mortem change, permeability characteristics of the cells are drastically altered. This could be an important aspect for better understanding of conversion of muscle to meat and one where careful morphological investigation could produce needed and useful information.

#### Fresh Meat

Early morphological investigations of fresh meat were limited largely to measurements of fiber dimensions. This work was, for the most part, correlated with growth of the animal and little attention was paid to association with meat. However, as pointed out by Stanley and Swatland (1976), variations in microstructure of muscle can cause rather large changes in rheological properties. The goal of the morphologist working on fresh meat should be to identify such. The observation by Locker (1960) that contraction state of muscle affected tenderness ushered in a new era. Numerous investigations followed in which sarcomere length was shown to be associated with tenderness of the meat. The objective of our present work is to describe in a more refined way this association especially as related to aging (see previous section).

Major reviews (Voyle, 1979, 1981; Howgate, 1979) have been written which relate structural characteristics of muscle to its properties as food. Other than the already described structural changes occurring post-mortem and the association of contraction state with tenderness, it should be mentioned that surface texture is a reflection of the bundle arrangement. Color is due in part to the proportion of fiber types.

#### Processing

Another area of morphological study has been the description of the effect of various processing procedures on meat. Birkner and Auerbach (1960) reported that during heating, collagenous fibers swelled, shrank, and then disintegrated, and that fat translocated. Classical work on the morphology of muscle during freezing has been published by Rapatz and Luyet (1959). They described several ice patterns which occur in frozen muscle and cited evidence that the cell favors development of longitudinal spears rather than lateral growth of ice.

Morphological study of processed meats has concentrated mainly on emulsion type products and two early papers are mentioned as being significant. Hansen (1960) demonstrated that a protein membrane surrounded the lipid globules in meat emulsions and Borchert et al. (1967) used electron microscopy to confirm the Hansen finding and also to reveal the extremely small lipid droplets present in meat emulsions (down to 0.1  $\mu$ m diameter). Since then, numerous papers have appeared which have attempted to relate this emulsion

structure to properties of the meat. Very recently, Kempton and Trupp (1983) have used a very refined image analysis system to study morphology in wiener batters. They found no relationship of any feature of microstructure to firmness of the product.

Two areas for study deserve comment. The first is compartmentalization. Structural (and histochemical) studies reveal where components (such as fat) are concentrated and located. The investigator should be constantly aware of this situation as it may influence the procedure for preparation of a meat product and also how it might influence properties and storage life of the finished product. The second area of interest is interfaces (Cassens et al., 1979) in which polar and non-polar groups are aligned. These are present in muscle (where lipid of a fat cell contacts the surrounding sarcoplasmic protein, for example) or may be found in processed meats (where lipid globules contact surrounding protein membrane), and they may influence chemical reactions.

#### Specific Identifications

One final aspect of morphology is identification. The trained person may distinguish the different types of muscle in a product purely on the basis of structural characteristics. Similarly, components such as connective tissue, organs, glands or foreign bodies can be identified. Staining techniques such as for connective tissue or the mucopolysaccharides of salivary glands can be used to increase sensitivity and give very specific identification. Such identification procedures have the greatest application in regulation and do lend themselves to semi-quantitation. European scientists have been especially active in this area of morphology (Prandl, 1961).

The rapidly emerging immunological methods offer great potential for specific identification. Species identification by immunology has been available for some years and enzyme-linked-immunosorbent-assay (ELISA) procedures now offer precision and automation. When antibodies are coupled to markers, they can be used microscopically to relate presence to specific location. For example, antibody to myosin heavy chains from red and white muscle may be used to distinguish fiber types (Carpenter et al., 1984).

#### Conclusions

An enormous amount of detail is known about the structure of muscle. Major morphological accomplishments have been made regarding muscle as a food. These are (1) relating state of contraction to tenderness, (2) characterizing aging associated morphological changes with properties of the meat, and (3) description of the structure of meat "emulsions". Significant work is ongoing in the area of relating degradation of muscle proteins to properties of meat. The challenge to significant progress in the future is substantial but the rewards are likewise major. The era of description without utilization of the information is drawing to a close. The most likely opportunities lie in

# Structural Properties of Meat

regulating biological processes to produce custom made meat, in devising morphological control procedures for manufacturing processes and in utilizing the now available immunological procedures.

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#### Discussion with Reviewers

J. G. Sebranek: When one considers various equipment designs for meat comminution, claims are often made of improvement in particle definition, better visual differentiation between fat and lean, etc., in one system vs. another. Is a microstructural difference likely to result from use of different types of equipment for relatively coarse products like salami or summer sausage? Authors: We cannot visualize that there would be any microstructural difference in such large chunks unless a "cleaner" cut were actually made at the surface by sharper cutting edges.

J. G. Sebranek: How important is the microstructural arrangement at the interfaces of sectioned and formed or restructured products for particle adhesion or binding strength?

Authors: Theno et al. (*J. Food Sci.* 1978, 43, 493-498) showed that in the emulsion-like area which binds pieces of meat together in these products, optimal binding was associated with a high degree of junction alignment. It should be noted that these interfaces in sectioned formed products are not the same as the interface between polar and non-polar groups as described in the body of our text.

D. W. Stanley: Have the authors any thoughts on the relatively recent findings that attribute a major role in meat quality to the state of water, and has consideration been given to a possible role for a muscle cell cytoskeleton as a microstructural factor?

Authors: Because of the large amount of water present in meat and because of its effect on physical properties due to hydration, etc., we are not surprised that it plays a major role in determining meat quality. Work on the so-called cytoskeleton is progressing rapidly now and as more is learned about the basic properties, then

undoubtedly, further explanation will be forthcoming regarding the cause of various meat quality attributes. Reference is made to the discussion of gap filaments by Locker in this Food Microstructure volume.

C. A. Voyle: Is it possible to monitor the effect of biological processes in producing "custom made meat" by analysis of biopsy samples? What parameters, in addition to fibre size, should be determined? Is there a preferred sampling site which would provide an index of the whole?

D.F. Lewis: Do you envisage meat manufacturers using biopsy techniques on every cut of meat they process?

Authors: The biopsy technique can be used to follow changes occurring in muscle of live animals over time but it is not without its problems. The surgical procedure may influence the animal and thereby subsequent results. Sampling site is a major problem. No one muscle reflects the properties of the total muscle mass of the animal. Also a given muscle may not be large enough to allow serial biopsies required to plot a change. Fiber typing, probably with the ATPase method, is in our opinion the best way to determine changes which occur in the muscle.

H. J. Swatland: It has recently been suggested that the epimysium and perimysium are more or less mechanically independent of the endomysium. (Moore, M. J., (1983). Muscle and Nerve 6:416-422). What are the authors' opinions on this idea?

Authors: We have no argument with this idea. We would point out a reference by Ramsey and Street (1940), *J. Cell. Comp. Physiol.* 15:11-34 wherein single muscle fibers, the resting length tension relationship is essentially identical whether the fiber is left intact or injured in such a way as to disrupt the contractile proteins but not the sarcolemma and remnants of remaining connective tissue. This would be proof that resting tension of the muscle fibers is not due to the contractile component but rather to the sarcolemma and remnants of remaining connective tissue.

H. J. Swatland: Much of the early literature on the effect of cooking on meat structure was obtained by the examination of paraffin embedded sections which sometimes introduced drastic changes to the microstructure, such as the shrinking of fibers during hot-wax embedding. Are the classical descriptions of Birkner and Auerbach (1960) compatible with results obtained by the more recent methods of EM fixation and frozen sectioning?

Authors: The early work on changes in fiber size due to heating, must be viewed with some question for the reason you suggest. The observations on changes in connective tissue are in our opinion still correct. Careful use of frozen sections reveals much more information reflective of the in vivo state and electron microscopy, obviously, gives much more structural detail.

D. F. Lewis: You indicate that there is a vast amount of knowledge available on the structure of muscle and imply that the responsibility for applying this knowledge lies with the meat technologist. To what extent do you think the microscopist should act as a bridge between the animal scientist and the meat technologist?

Can you suggest how a meat technologist might modify his processes to cope with:

- i) Meat with a high red fibre content?
- ii) Meat with a high white fibre content?
- iii) Meat from an animal in which a state of muscular senile atrophy has developed?
- iv) Meat from an animal which has recovered from muscle injuries?

Authors: The microscopist can be most effective if he is aware of the influence the live animal exerts on the sample of muscle to be examined and if he maintains an awareness of the biochemistry of muscle. We believe progress is much easier with an integrated approach.

D. F. Lewis: Attempts to prove a concrete link between fibre type and meat behaviour on processing have been largely inconclusive. How would you suggest that such a link could be established?

Authors: Sair et al. (J. Food Sci. 1972, 37, 659-663) demonstrated that muscle from stress-susceptible pigs have large numbers of intermediate and white fibers compared to normal animals. It is well recognized that the PSE (pale, soft, exudative) muscle from the stress-susceptible animals is undesirable for manufacture into hams or processed meats.

D. F. Lewis: What effect does growth by hypertrophy rather than hyperplasia have on meat performance? Is it alterable?

Authors: See answer above -- muscle from stress-susceptible animals generally has hypertrophied fibers.

D. F. Lewis: Does the fact that nuclei migrate from the centre to the edge of the cell during embryonic development of muscle hold any practical significance to the behavior of meat on processing?

Authors: None that we know of at this time.

D. F. Lewis: Could you give practical details of your myosin antibody technique including availability of materials and applicability to processed meats?

Authors: The method is published in complete detail in the Proceedings of the 30th European Meeting of Meat Research Workers, Bristol, U.K. (see Carpenter et al., 1984). With proper specimen fixation and appropriate antibody labelling, we see no reason why myosin heavy chain could not be immunohistochemically localized at the light or electron microscope level in a processed meat product.

D. F. Lewis: It is possible that changes in the permeability of the cell membrane will affect distribution of salts in meat. Can you suggest ways in which the membrane permeability changes may be controlled? Which do you think has the greater effect of salt distribution -- the state of the connective tissue or the cell membranes?

Authors: We cannot suggest means to control membrane permeability. Several years ago we noticed during work on staining of myoglobin in skeletal muscle that if fixation did not occur immediately post-mortem, the differential localization of myoglobin in red and white fibers was lost. We

interpreted this as meaning that cell permeability changed rapidly allowing the soluble myoglobin to diffuse. If this is in fact the case, then the connective tissue may present a more effective barrier to diffusion of salt than does the cell membrane.

Editor: Following references were suggested by reviewers as relevant to the subject of this paper:

Carroll RJ, Lee CM. (1981). Meat emulsions -- fine structure relationships and stability. Scanning Electron Microsc. 1981; III: 447-452.

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STUDIES ON THE MICRODISTRIBUTION OF AEROBIC ENZYMES AND MYOGLOBIN IN PORK

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Abstract

Techniques were developed to study the microdistribution of aerobic enzymes and myoglobin directly on the surfaces of slices of meat. Despite its solubility, the in vivo distribution of myoglobin was preserved post mortem and, in pork, the distribution of myoglobin followed the distribution of aerobic muscle fibers. Aerobic fibers were grouped centrally in their fasciculi. Differences in oxidative enzyme activity between central and peripheral fibers within fasciculi were detected by staining with methylene blue in an atmosphere with controlled levels of oxygen and nitrogen. Within individual aerobic fibers, there was a radial gradient of SDH (succinate dehydrogenase) activity with more intense activity in the subsarcolemmal region. However, the magnitude of the slope of the gradient in each fiber was a function of reaction time. Within individual aerobic fibers, myoglobin was distributed evenly across the fiber. By fiber optic spectrophotometry, myoglobin had an absorbance peak at 560 nm and a low absorbance at 490 nm. When converted to nitrosylhemochrome in hams cured with sodium nitrite, there was a marked increase in absorbance at both 490 and 560 nm.

Introduction

The histochemical analysis of frozen sections of pork muscles obtained immediately post mortem shows that, in vivo, myoglobin is concentrated in muscle fibers with strong aerobic enzyme activity (Morita et al., 1969). Thus, muscles with a greater proportion of aerobic fibers appear darker or more red in color (Beecher et al., 1965). In the present study, observations were made directly on the surfaces of slices of meat to determine if the microdistribution of myoglobin maintains its original pattern with respect to the distribution of oxidative enzyme activity post mortem. Fiber optic spectrophotometry was used to measure the absorbance spectrum of nitrosylhemochrome formed from myoglobin in cured pork.

By light microscopy, the intracellular distribution of SDH activity within skeletal muscle fibers can be mapped with a scanning stage after the outlines of a fiber have been delineated by the observer (Swatland, 1983). However, the information contained in such maps is difficult to evaluate statistically unless it is simplified and standardized in some way. One solution is to calculate the slope of a radial gradient of enzyme activity within the fiber on the assumption that muscle fibers are essentially cylindrical in nature. Research was undertaken to investigate the effect of incubation time on the development of radial gradients of enzyme activity. The long term objective of these studies is to improve the spatial resolution of meat colorimetry.

Materials and Methods

Optical Equipment

The apparatus is shown diagrammatically in Figure 1. On one side (components C to E in Figure 1) was a fiber optic system while on the other side was a Zeiss Universal light microscope with attachments (components H to L in Figure 1). Both systems were linked to the same motor-driven interference filter monochromator with a range from 400 to 700 nm

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(B in Figure 1) and to the same photomultiplier (A in Figure 1). The monochromator and the photomultiplier were operated from a microcomputer (Zeiss Zonax; component M in Figure 1) from which data could be sent to a matrix printer (N in Figure 1) or to another microcomputer (Horizon NorthStar) for storage and transmission to a mainframe computer. Details of the sample chambers for the microscope are shown in Figure 2.

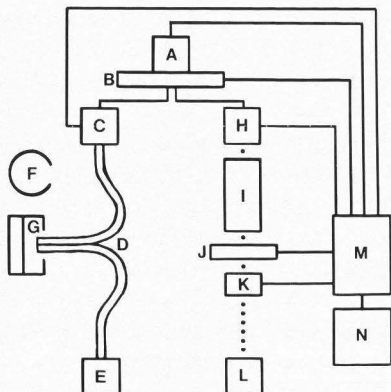


Figure 1. Apparatus used for fiber optic spectrophotometry (components C to F) and for light microscopy (components H to L). Photomultiplier, A; monochromator, B; shutters, C, H and K; light guide, D; illuminators, E and L; standardization cells, F and G; microscope, I; scanning stage, J; microcomputer, M; and printer, N.

The fiber optic system was composed of a Zeiss solenoid-operated shutter (C in Figure 1), a bifurcated non-coherent light guide (PBL Electro-optics, New London, NH; part BLGS-1875-36-M; component D in Figure 1), and a 12 V, 100 W halogen light source (E in Figure 1). To investigate the degree of spatial resolution that was possible with fiber optic spectrophotometry, the non-coherent light guide (D in Figure 1) was replaced by another guide (adapted from Model FS100 Industrial Fiberscope, AO Fiber Optics, Southbridge, MA) in which the light from

the sample to the photometer was carried by a coherent fiber optic bundle in the axis of the light guide. A coherent fiber optic bundle is one in which the relative positions of the optical fibers are maintained along the bundle so that an image can be generated. In a non-coherent bundle, relative positions are not maintained. The light from the illuminator to the sample was carried non-coherently by fibers around the axial bundle. The photometer branch of the coherent light guide was bolted into a flat metal plate with the same dimensions as a glass microscope slide and this was held by the scanning stage (a link from D to J in Figure 1). The microscope system was composed of a photometer head with an automatic shutter (Zeiss MPM-03; component H in Figure 1), a Zeiss Universal microscope (I in Figure 1), a 0.5  $\mu\text{m}$ -step scanning stage (J in Figure 1), a solenoid operated field stop aperture (K in Figure 1), and a 12 V 100 W halogen illuminator (L in Figure 1).

#### Measurement of SDH gradients

The terms "site", "area", and "run" are used here in a particular manner. A site is a unique position of the scanning stage with respect to the optical axis of the microscope, and is defined by its X:Y

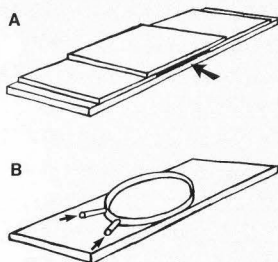


Figure 2. Sample chambers (parts not to scale). Chamber A was used for measuring changes in absorbance in a histological section during the course of a histochemical reaction. Cover slips glued to each end of the slide made a fluid-filled chamber over the muscle section (arrow). Chamber B was used to examine 1-mm thick slices of pork. On top of the spacer ring was placed a microscope cover slip or a coherent fiber optic plate (not shown). The arrows indicate entry ports for nitrogen or oxygen gas.

coordinates. An area is a collection of adjacent sites through which the stage moves and at which optical measurements are made. A run is a series of measurements at different areas. In a run, no area is measured more than once, and no measurements are made as the stage moves between areas. A series of runs allows areas to be measured repeatedly for the study of dynamic (time-based) changes in optical absorbance such as those that occur progressively during the course of a histochemical reaction.

Frozen transverse sections (10  $\mu$ m thick) of porcine adductor muscles (from 3 sows at about 150 kg live weight) were collected on microscope slides coated with egg albumen which was dried onto the glass. Cover slips were glued directly to the glass at each end of the slide with rubber cement as shown in Figure 2. The section was examined with the scanning stage of the microscope under manual control from the microcomputer keyboard. Figure 3 shows how the areas to be measured were defined. First, the stage was moved so that the optical axis of the microscope corresponded to the central axis of a particular muscle fiber and the coordinates of this position were stored. Second, the stage was moved eastwards until the edge of the fiber was reached and the coordinates of this position were stored. These two operations were repeated for each of the areas to be examined in the run.

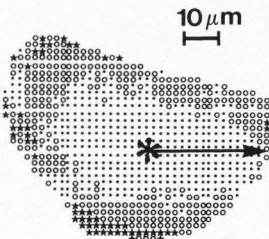


Figure 3. Computer display of the absorbance of diformazan in a single muscle fiber reacted for SDH. The plotting symbols are: (.) absorbance  $\leq 0.5$ ; (o) absorbance  $> 0.5$  and  $\leq 1.0$ ; and (\*) absorbance  $> 1.0$ . The large asterisk marks the central axis of the muscle fiber and the large arrow indicates the radius along which measurements were made as the histochemical reaction was in progress.

To set the photometer to 100% transmittance, the section was then moved under keyboard control so that a blank area between two muscle fibers was in the optical axis. A drop of medium for the enzyme reaction (0.54 g sodium succinate and 5 mg nitroblue tetrazolium in 10 ml 0.2 M phosphate buffer at pH 7.4) was placed on the section and zero time was set for a series of runs using the internal clock of the microcomputer. A cover slip was placed over the section. Because of the two cover slips already glued to the slide on each side of the section, a depth of approximately 0.15 mm of incubating medium remained over the section to allow the reaction to continue freely. The high voltage power supply and the gain of the photomultiplier were then set at zero absorbance on the blank area flooded with incubation medium and the first run was initiated immediately after the photometer was set. Runs were repeated at intervals. Data were converted to absorbance units and radial gradients were calculated from least-squares linear regressions (Swatland, 1983).

#### Fiber optic spectrophotometry

One of two methods was used to set the photometer to zero absorbance for non-coherent fiber optic spectrophotometry. The first used a test cell that held the tip of the fiber optic probe at a distance of 3 mm from a newly-pressed barium sulfate test plate (G in Figure 1). The second used a 7 cm diameter integrating sphere with a white interior and a single port (Labsphere, North Sutton, NH, part IS030-WR, component F in Figure 1). The common trunk of the bifurcated non-coherent light guide used in the present study was coated with a flat layer of epoxy resin that modified its optical properties. The light guide was tested as follows. The guard above the barium sulfate test plate (component G in Figure 1) was removed and the tip of the common trunk of the light guide was placed in contact with the plate. The trunk was moved away from the test plate in increments of 1 mm with a micromanipulator and, in a dark room, the amount of light returned to the photometer was measured at different wavelengths. The amount of light returned was almost constant from 5 to 12 nm. The proportion of light returned was independent of wavelength. When the photometer was set at 100% reflectance against the flat barium sulfate plate, the proportion of light returned from an integrating sphere (component F in Figure 1) was the same at all wavelengths.

#### Curing of hams

The hams from six pigs (live weights



79.5 to 90.9 kg) were pumped with curing brine (75.6 g/l NaCl, 15 g/l sugar) which contained a different level of sodium nitrite for each ham (from 0 to 2.2 g/l in 0.2 g/l increments). The hams were kept individually in plastic bags covered with pickle for 24 h. After being smoked, the hams were transected at the midlength of the femur and the color of the main part of the biceps femoris was measured with the non-coherent fiber optic probe directly on the face of each ham.

### Results

#### SDH gradients

In some fibers of the adductor muscle, SDH activity was greater in the subsarcolemmal region than in the central axis of the muscle fiber and the absorbance increased as the stage moved radially in steps of 2  $\mu$ m (Figure 3). Radial gradients increased with the length of the incubation time (Figure 4). The data in Figure 4 are grouped by runs and are shown in histogram form because time is not measured exactly on the x-axis. The runs were started at regular intervals of 6 min but the number of areas and sites within the runs was variable.

#### Microdistribution of Myoglobin

Thick (1 mm) transverse slices of longissimus dorsi and adductor muscles were placed in an optical chamber under a controlled atmosphere (Figure 2). The microdistribution of myoglobin was observed directly on the surface of the muscle slice (Figure 5).

A continuous flow of gas was necessary to stop the cover slip from fogging over. The gas escaped between the spacer ring and the cover slip. Despite the solubility of myoglobin and the translocation of fluids that probably occurred when the pork was sliced, the myoglobin was still contained with the aerobic fibers in the centers of fasciculi. The identity of the aerobic fibers was determined by flooding the face of the muscle slice with either the nitroblue tetrazolium incubation medium used previously for SDH or with 0.05% methylene blue in physiological saline. Both tetrazolium salts and methylene blue act as electron acceptors in enzyme-catalyzed oxidations (Pearse, 1972). The fibers that contained myoglobin were stained with tetrazolium salts or methylene blue (Figure 6).

The level of staining with methylene blue could be controlled by altering the flow rates of oxygen and nitrogen. When the chamber was flushed with oxygen the muscle fibers became intensely stained. Staining was initiated in fibers located centrally within fasciculi but it later

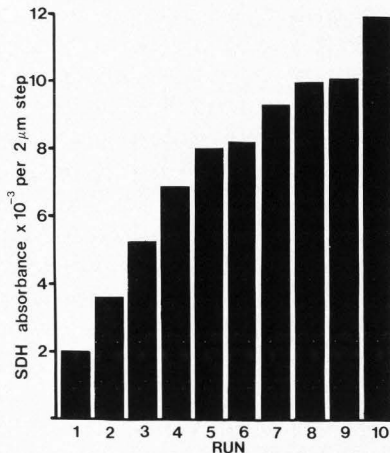


Figure 4. Effect of reaction time on the radial gradient of absorbance of diformazan produced in the SDH reaction. The radial gradient is expressed in terms of absorbance units per 2- $\mu$ m step of the scanning stage. The interval between the start of each run was 6 min. These cumulative data (n = 29) include fibers with a wide range in overall SDH activity.

spread to peripheral fibers. When the chamber was flushed with nitrogen, muscle fibers did not become deeply stained and those that had been stained after earlier exposure to oxygen lost their stain under nitrogen. Fibers in the periphery of fasciculi were more rapidly destained under nitrogen than fibers located centrally.

Figure 7 shows the appearance of fibers containing myoglobin and stained with methylene blue when they were viewed through a coherent fiber optic plate directly on the meat surface. A section stained with methylene blue is shown so that cellular boundaries and the grouping of fibers in fasciculi are more easily

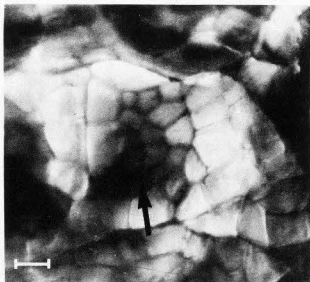


Figure 5. The microdistribution of myoglobin seen on the surface of a slice of pork. Myoglobin is restricted to fibers located centrally within their fasciculi, as indicated by an arrow. Illumination is brighter in the center of the figure than at the edge. Bar scale = 100  $\mu$ m.

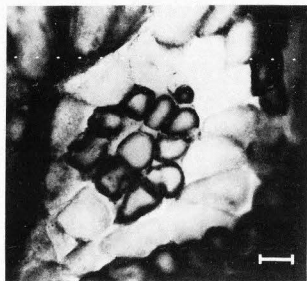


Figure 6. The surface of a slice of pork washed with methylene blue and maintained under a controlled balance of nitrogen and oxygen gas. Myoglobin-containing fibers located centrally within their fasciculi were reversibly stained with methylene blue as a result of their oxidative enzyme activity. Illumination is brighter in the center of the figure than at the edge. Bar scale = 100  $\mu$ m.

seen in the figure. The corresponding distribution of myoglobin in unstained preparations was detectable subjectively and spectrophotometrically but was difficult to demonstrate clearly in black and white photomicrographs. The resolution obtained with a coherent fiber optic

light guide was somewhat less (areas of about 0.25 mm<sup>2</sup>) than the resolution of the fiber optic face plate but the light guide could be linked to the microscope system (from C to J in Figure 1). Thus, the scanning stage was used to position individual optical fibers in the optical axis of the microscope for spectrophotometric measurement. The testing of this technique is still in progress.

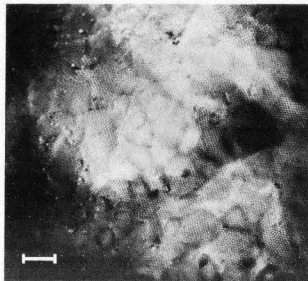


Figure 7. The appearance of methylene blue stained pork when seen through a coherent fiber optic plate. Illumination is brighter in the center of the figure than at the edge. Bar scale = 100  $\mu$ m.

#### Spectral properties of myoglobin

The ham that received no nitrite was a dull gray color. All the nitrite-treated hams had formed nitrosylhemochrome and there was no obvious relationship between the degree of pigment formation and the level of nitrite in the ham. Thus, the formation of nitrosylhemochrome behaved as an all or none reaction in this study. The hams were measured with a noncoherent light guide so that measurements were based on the light returned from an illuminated cone of tissue (about 10 cm<sup>2</sup>).

The extreme spectral range from the raw color (before being pumped) to the darkest cured color is shown in Figure 8. Raw pork had an absorbance peak at 560 nm and low absorbance at 490 nm. Conversion to nitrosylhemochrome was accompanied by a marked increase in absorbance at 490 nm and an elevation of the 560 nm peak. Spectra from all the nitrite-treated hams fell somewhere between the limits of the two spectra shown in Figure 8.

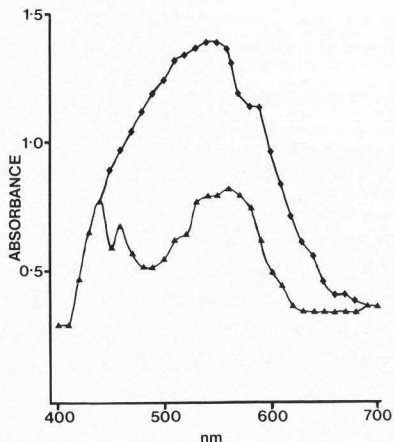


Figure 8. Absorbance spectra of raw pork (lower line) and cured pork (upper line) obtained by fiber optic spectrophotometry.

#### Discussion

The results reported here indicate that quantitative measurements of differences in enzyme activity made between two or more sites in a section are dependent on incubation time. Incubation time must, therefore, either be kept constant or else taken into account when comparing data from different sections. This principle also extends to section thickness and to any factors that affect the rate of enzyme activity. The use of methylene blue as a histochemical reagent was eclipsed by the advent of tetrazolium salts with a stable final reaction product (Pearse, 1972). However, as shown in the present study, the unstable indicator-like properties of methylene blue can be used advantageously to examine metabolically active meat surfaces. Thus, with further development of the system described here it may be possible to study oxidative metabolism on the meat surface as well as to measure meat color at the microstructural level.

Both the macroscopic reflectance colorimetry of sliced meat and the analysis of the microstructure of meat are common techniques in meat research. The intention of the studies reported here was to link together these two

techniques. As shown, it is possible to increase greatly the degree of microstructural resolution in meat colorimetry. In practical examples such as, (1) comminuted meat products, (2) pink spots in cooked non-cured meat due to nitrite contamination from spices, (3) gray spots in cured ham due to incomplete penetration of nitrite, (4) surface spoilage, or (5) pathological lesions in meat, the affected areas may have a small size that makes them difficult to measure with existing methods of colorimetry. As demonstrated here, spatial resolution at the level of muscle fasciculi is attainable by fiber optics and would be appropriate for the study of these problems.

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#### Discussion with Reviewers

**C.A. Voyle:** Have you any information on the correlation between microdistribution of myoglobin and of oxidative enzyme activity post-mortem, as implied in your Introduction?

**Author:** Yes. On an intercellular basis, fibers with strong aerobic enzyme activity (SDH) had the highest levels of myoglobin. On an intracellular basis, no correspondence was detected in the relationship between aerobic enzyme activity and myoglobin distribution.

**C.A. Voyle:** How do the results obtained by the described method of fibre optic spectrophotometry compare with those currently used to measure colour at meat surfaces?

**Author:** Fiber optic spectrophotometry gives similar results to macroscopic reflectance except in the low wavelengths (< 460 nm).

**C.A. Voyle:** Is it not possible to produce uniform illumination for Figures 5 and 7?

**Author:** The problem is due to the use of

an ordinary substage condenser with thick slices of meat. The problem could be avoided with some other type of substage system.

**R.J. Carroll:** The spectral measurements were made using the non-coherent light guide; the muscle morphology micrographs were made using the coherent light guide. Would you explain the rationale for using the non-coherent and the coherent light guides in each situation?

**Author:** The earliest observations (absorbance spectra) were made with the non-coherent light guide. I then started to investigate coherent systems and obtained the image shown in Figure 7 using a plate of coherent optical fibers. I then built a coherent fiber optic system with a flexible light guide and coupled it to the scanning stage. At the present time, I am just about to write the software to link the scanning stage to the coherent fiber optic system so as to make spectrophotometric measurements on the light from individual optical fibers.

**R.G. Cassens:** The author comments on the problems solubility of myoglobin causes in doing histochemical analysis. Were any time course studies conducted to determine how long of a time period post-mortem was safe before diffusion became evident?

**Author:** In the peroxidase histochemical reaction for myoglobin, the solubility of myoglobin can be overcome by combining sulfosalicylic acid in the first step of the reaction (Swatland, H.J. 1979. Stain. Tech. 54: 245-249). In the present study using thick slices of meat, the localization of myoglobin to certain muscle fibers was evident at least several days postmortem. Thus, although myoglobin is very soluble, it does not appear to leave the muscle fiber in the fresh product. However, I have not investigated this topic with any time course studies.

**R.G. Cassens:** In regard to hams, the conclusion was made that level of nitrite used was not related to degree of pigment formation. Does the author wish to qualify this statement in any way to take into account the possibility of fading of color levels of nitrite?

**Author:** I am an absolute novice at producing nitrosylhemochrome in a piece of pork. I was very suspicious of the industrial-type calculations used to estimate the nitrite levels required in

the brine, and then very surprised to find that the reaction appeared to be an all-or-nothing response. In fact, where the control ham with no nitrite touched the next ham with the lowest level of nitrite by accident in the smokehouse, sufficient nitrite diffused across to create nitrosylhemochrome to a depth of several millimeters in the control ham. The point that you make (that pigment may have been unstable at low nitrite levels) may well be correct, and is something that I will certainly bear in mind in any future studies on this topic.

**E.A. Davis:** What are the reflectance maxima of myoglobin and nitrosylhemochrome? How are they influenced by the different oxidation states of myoglobin? Would the different oxidation states of myoglobin cause one to misinterpret the nitrosylhemochrome data?

**Author:** With the system I have at present, wavelength discrimination is only possible at about 10 nm intervals. This is partly because of the relationship between aperture size and the length of the continuous interference filter, and partly because of a difficult interaction between the software and the stepping motor that drives the monochromator. This makes the accurate definition of spectral peaks and the resolution of spectral shifts rather difficult. For example, sometimes it is possible with fiber optics to detect the dent that appears in the myoglobin peak when the pigment is converted to oxymyoglobin, but it depends how deep into the meat that the formation of oxymyoglobin has occurred. A similar situation occurs with oxidation to form metmyoglobin. When working with isolated pigments in solution, one can obtain a homogeneous system in the light path. When working with macroscopic reflectance colorimetry, one is dealing mainly with pigments in the surface layer of the meat. With fiber optics, however, measurements are based on an illuminated cone of tissue that includes the interior of the sample as well as the sample surface. This is both the strength and the weakness of this new system. Its strength is that it is very good for detecting pH-dependent light scattering and separating this from the overall concentration of myoglobin derivatives. One would not, however, choose to use a fiber optic probe on a solid meat sample as a method to investigate the spectral properties of pigments.





## THE ROLE OF GAP FILAMENTS IN MUSCLE AND IN MEAT

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### Abstract

The neglected "gap filaments" are now beginning to receive close attention in several centers, in terms of organisation, composition and function. The author's model for their connections gave them a role as cores to A-filaments. This review attempts to weigh the implications of such a role, and relevant evidence, old and new. New ideas arising largely from PAGE\* and immunochemical studies on candidate proteins, and from the developing concept of the cytoskeleton, are considered.

The author's theory of meat tenderness, based on G-filaments, has been tested by PAGE studies on changes in the large structural proteins (particularly titin and nebulin) during tenderising treatments. The results, together with those from parallel work elsewhere, are in some conflict with the theory.

\*Polyacrylamide Gel Electrophoresis

### GAP FILAMENTS IN MUSCLE

#### Introduction: sliding filaments are not the whole story

In 1954, two classic papers in "Nature" laid down the sliding filament theory of muscular contraction, which proved to be a landmark in muscle biology. In one of these papers, H.E. Huxley and Hanson (1954) felt bound to discuss some observations which the theory could not explain. They noted that when the myosin was extracted from their myofibrils, the center of the sarcomere appeared empty under phase contrast. This was an illusion, since as the authors themselves said: "The ghost fibrils are however still structurally continuous; stretched fibrils shorten spontaneously to a little less than rest length during extraction --- they may be reversibly extended again --- with great ease, and it is apparent that only weak forces oppose such a stretch; the gap elongates in the process but the length of material from the Z-line to the gap remains constant ---". They concluded that the gap was bridged by an elastic component: "The S-filaments provide continuity between the set of actin filaments associated with one Z-line and that associated with the next ---". The S-filaments were last heard of when Huxley and Hanson (1957) assessed the "S-substance" in the gap as 3% of the myofibril by interference microscopy (note that the gap was only a third of the sarcomere, and if the S-filaments extended from one Z-line to the other they would amount to 9%). The definitive electron microscopy study of Huxley (1957) did not substantiate the presence of extra filaments, and S-filaments were shelved.

#### The gap filaments and other "extra" filaments

The other paper associated with the sliding filament theory was the first to record gap filaments. A.F. Huxley and Peachey (1961) were investigating the ability of stretched fibres to contract. A micrograph of a fibril stretched well beyond the point where A-and I-filaments over-lap, led them to comment: "The suggestion of fine filaments connecting the ends of the two major sets of filaments may be a result of super-position in the section or may represent an additional component of the myofibril". So, in a sentence, they dismissed this important observation. It was Sjostrand (1962) who finally described and named the "gap

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**Key Words:** Gap filaments, G-filaments, muscle structure, meat tenderness, A-filament, core, titin, connectin, nebulin, N-lines

filaments". Carlsen *et al.* (1965) gave them further attention, but there the gap-filaments (G-filaments) rested, having no role in the new theory which was then stimulating muscle biology.

The idea of extra filaments received fresh attention with the "superthin" filaments (McNeill and Hoyle, 1967; Walcott and Ridgway, 1967). These have recently been discussed again (Hoyle, 1983) but their relationship, if any, to the G-filaments remains obscure.

The presence of residual filaments after extraction of actin and myosin has been recorded by Guba *et al.* (1968a), dos Remedios (1969) and by dos Remedios and Gilmour (1978). These "extra" filaments will be further discussed below.

In 1973, I found by chance that beef fibers could be stretched by 4-5 times (up to 12  $\mu$ m) and that G-filaments showed great advantage in such material. A series of subsequent papers described the morphology of the G-filaments in some detail (Locker and Leet, 1975, 1976a, 1976b). A model for their connections (Fig. 1) was proposed (Locker and Leet, 1976a). It was based on the curious behaviour of A-filaments at these extreme degrees of stretch (stretching and dislocation), counts on the sparse but well defined array of filaments in the gap, and the lack of strain in I-filaments (summarised in Locker, 1982a). According to this model each G-filament forms a core to an A-filament, emerging at one end only, passing independently of the I-filaments through the Z-line, between the I-filaments of the next sarcomere and into a second A-filament, to terminate as a second core. The symmetry is based on the Z-line, with half the A-filaments in any A-band linked to A-filaments in one adjacent sarcomere and half to the other side. This model may seem odd, but it appeared to offer the only explanation of the evidence. The author will be happy to see it modified if satisfactory alternative explanations emerge, as in fact seems inevitable. The model will be discussed further in relation to the difficulties it raises, and to newer evidence.

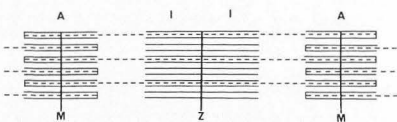


Figure 1. Model for the connections of G-filaments (dotted). Each acts as a core to two A-filaments in adjacent sarcomeres linking these A-filaments through the Z-line and independently of I-filaments.

#### The protein of G-filaments

While we were attempting to characterise the protein of G-filaments, candidates for this role were emerging elsewhere. It seemed most probable that the "connectin" of Maruyama *et al.* (1976) was the protein of the G-filaments (Maruyama *et al.*, 1980; Locker and Daines, 1980; King and Kurth, 1980). A better

characterised material emerged in titin (Wang *et al.*, 1979; Wang, 1982a). Connectin and titin appear to be essentially identical (Maruyama *et al.*, 1981a, b).

An interesting development with apparent potential for exploring the physiological role of connectin is its isolation in a "native" state and in high yield by a very mild extraction procedure (Kimura and Maruyama, 1983a). These authors have already demonstrated a weak interaction of this protein with both myosin and actin (Kimura and Maruyama, 1983b). However Wang (pers. comm.) considers this "native" form to be titin-2, a partly degraded titin, which has become salt-soluble in the process (Wang and Ramirez-Mitchell, 1983b).

The link between titin and G-filaments seems to have been finally established (La Salle *et al.*, 1983; Robson and Huiatt, 1983; Robson *et al.*, 1983). Antibody to bovine skeletal titin labelled the filaments in the gap of beef fibers, over-stretched after Locker and Leet (1975).

There is of course no reason why G-filaments should be composed of a single strand or of a single protein. King (1984) has suggested that they may be compound and we have raised the possibility that nebulin might be involved with titin on the basis of changes in PAGE patterns during ageing of meat (Locker and Wild, 1984a). The idea of the involvement of both proteins in G-filaments has been developed in detail by Wang (1983b) on the basis of his immunochemical studies (see later).

#### How much titin is there in muscle?

This is an important preliminary question in seeking a role for titin and G-filaments. Twenty years ago the balance sheet for structural proteins seemed more or less complete. It has proved remarkably elastic with the discovery of a host of new myofibrillar proteins. "Stroma" has been a convenient "throw-away" label for the largest untapped reservoir of new proteins. While this must contain connective tissue and membranes, there is clearly a great deal more. Indeed washed beef sternomandibularis myofibrils leave a residue of 16-20% of the protein nitrogen after extraction with M.KI, and all but 1-2% of this is soluble in 5M guanidine hydrochloride -DTT (Locker and Daines, 1980). This dissolved material, when made water-soluble by maleylation, gave a fraction unabsorbed by DEAE-cellulose (5-8% of the myofibril) which appeared to be connectin. Maruyama *et al.* (1976) estimated connectin in rabbit psoas at 5% of the myofibrillar protein, but the drastic treatments used in the preparation make this figure suspect. This criticism also applies to their estimate of 18% in cardiac muscle (Maruyama *et al.*, 1977), indeed we found the content in heart and skeletal muscle to be much the same (Locker and Daines, 1980), an assertion borne out by later experience of four different cardiac muscles (Locker and Wild, unpublished results).

Wang *et al.* (1979) estimated titin-1, -2 and -3 in chicken breast at 10-15% of myofibrillar protein, based on the Lowry estimation of 20 eluates from agarose columns (stain intensity of gels gave only half these values). Titin-3, a distinct protein, has since been renamed "nebulin" (Wang and Williamson, 1980). Our own gel staining for beef (Locker and

Wild, 1984a) shows nebulin to be about half as abundant as titin (also the conclusion of Wang, 1982a). This means that the above column data, probably the best available, translate to 7-10% titin.

King (1984) has estimated titin in sheep semi-membranous muscles in much the same way as Wang et al. (1979) and found it to be 12% of the myofibril (dry weight).

Our measurements of stain intensity in gels (Locker and Wild, 1984a) show the titin content of beef to be a third that of myosin, equivalent to 14% of myofibrillar protein, if 43% is taken as the best value for myosin (Yates and Greaser, 1983). This method must be suspect due to uncertainties about staining. Curiously the value is high, in contrast to Wang's low value from this method.

Finally, in our survey of the occurrence of titin across the animal kingdom (Locker and Wild, unpublished results), visual comparison of myosin and titin bands, suggest their relative amounts to be remarkably constant in skeletal and cardiac muscles, suggesting a consistent role for titin in a structure common to a wide range of vertebrate and invertebrate muscles.

Considering all the above data together it seems that the round figures of Wang (1982a) for titin and nebulin (10% and 5% respectively of the myofibril) are a very reasonable choice. The figure mentioned earlier of 9% for through-running S-filaments makes an interesting comparison. Titin is therefore a major component of the myofibril, lying between actin and tropomyosin in abundance. On these grounds alone it must be the leading candidate for the substance of an extra set of filaments. There would clearly be enough for the G-filaments of my model (Fig. 1).

It may also be noted that nebulin, about as abundant as tropomyosin, is also a major constituent. There seems to be more than would be required for an N<sub>2</sub>-line (Wang and Williamson, 1980).

#### The G-filament as a possible core to the A-filament

The manner in which A-filaments are dragged one way or the other and are themselves stretched in over-stretched beef fibers, shows a strong tensile link between A- and G-filaments, but this does not prove that G-filaments continue inside the A-filament. However the various extraction studies described earlier, indicate that they do extend into the A-band, and there seems to be no place in this region, other than the A-core, in which to hide G-filaments, which are about as thick as I-filaments (Locker and Leet, 1975). Huxley (1957) found the H-band devoid of fine filaments and this has been the general conclusion since. But it should be noted that McNeill and Hoyle (1967) disagreed. Their Fig. 5b appears to show many "superthin filaments" between the thick filaments in the H-band.

Our micrographs clearly show continuity of A- and G-filaments in sarcomeres with A-bands either dislocated (Locker and Leet, 1975, Fig. 15) or undislocated (Locker and Leet, 1976a, Fig. 3). Moreover, when myosin is extracted from undislocated sarcomeres, the fine filaments left in the A-band still appear to emerge from the A-filament stubs, preserved within the M-line (ibid., Fig. 11). When dislocated sarcomeres are extracted the residual

pattern of fine filaments mimics that of the original A-filaments, even to the surviving overlap of about 0.6  $\mu$ m at the centre (ibid. Fig. 8). This suggests a core running right through the A-filament.

#### There is room for a core

The accumulation of evidence for the structure of the vertebrate A-filament derived from X-rays, biochemical studies, quantitative mass measurements or direct morphology in the electron microscope, now lies heavily in favour of the 3-stranded model (see Kensler and Stewart, 1983; Ip and Heuser, 1983). This was the preferred model of Squire (1973), who discussed its implications for the core of the A-filament. He favoured a 3-stranded core for vertebrate muscle and a 6-stranded core for insect flight muscle, based on his detailed X-ray diffraction studies of frog sartorius and *Leucotherus* flight muscle. These models have internal spaces of clear diameter about 30A and 100A respectively. The vertebrate "hole" is actually triangular and would best accommodate a 3-stranded core of 20A filaments, while the insect "hole" would accommodate best a hexagonal array of 18 such filaments. The "hollowness" of A-filaments is visible in many electron micrographs. It is not uncommon in vertebrate muscle (e.g., Huxley, 1957) and is very striking in insect flight muscle (e.g., Ullrick et al., 1977). As pointed out by Squire it should not be regarded as emptiness, but as a central region of a lower staining capacity. His estimate of the diameter of this region, from direct observation of micrographs is 50A and 100A in the vertebrate and insect filaments respectively. While there is no evidence from his work for a core, he estimates that a vertebrate core, as above, should comprise not more than 4% of the myosin mass. This could not accommodate the titin estimated above at 10% of the myofibrillar protein, although nebulin at 5% comes close.

#### A-Filament cores do exist

There is precedent for A-filament cores in the case of molluscan adductors where paramyosin definitely fills the role (Szent-Gyorgyi et al., 1971). However these A-filaments differ markedly from the mammalian ones in having much larger and variable diameters of 300-1200A. Paramyosin may account for half the structural protein in some cases. This protein occurs in the musculature of various arthropod classes, annelids and nematodes, although its location is not known (Waterston et al., 1974). Paramyosin has proved abundant in some insect flight muscles (Bullard et al., 1973). Assuming a core location, that is an A-filament composed of myosin and paramyosin, the paramyosin content was equivalent to about 11% of the A-filament in *Lethocerus* (close to the predicted 12-13% of core permitted in the Squire 6-strand model), 18% in the rosechafer beetle, but only 2% in the blowfly. Since bumble bee flight muscle contains titin and nebulin (Locker and Wild, unpublished results) the possibilities for cores in insect flight muscles become complex.

#### Filament counts in the I-band

In the I-band, G-filaments cannot be hidden and the evidence is conflicting on whether they can be

counted with the actin filaments. Huxley (1960) found in serial cross sections of rabbit psoas, a mean ratio of 1.9 for I-filaments: A-filaments, close to the 2.0 predicted for the double hexagonal array. Again using serial sections of rabbit psoas (and taking pains to employ an impartial counter), dos Remedios (1969) obtained a ratio of 2.8, intermediate between the prediction of his model (3.0 for through-running residual filaments) and mine (2.5). Guba *et al.* (1968b) using the same material (but not serial sections), partly extracted for myosin, found a ratio of 3.1. Ullrick *et al.* (1977), using serial sections, found a ratio of 2.1 in frog sartorius and 2.2 for chameleon tongue. In short the present situation is unsatisfactory, but hardly excludes a model requiring a ratio of 2.5.

The classic electron micrographs of Huxley (1957) defined the hexagonal packing of the interdigitated thick and thin filaments in the A-band. His very special sections, cut at about 150A, were only one layer of filaments thick. Those lying exactly in the 1120 plane of the lattice, had in the A-band two thin filaments between each pair of thick filaments. But my counts of filaments in the I-bands, using his best plates, bear a ratio to thick filaments well in excess of 2.0, and closer to the 2.5 predicted by my model. This excess appears to be due to filaments emerging from thick filaments (see next section). Although counts on longitudinal sections are suspect, in these special sections they should be more reliable than usual.

#### Thin extensions to A-filaments

Numerous papers from 1962 onwards, leave no doubt that in insect flight muscle there are thin extensions of the A-filament to the Z-line (C-filaments). These are seen clearly in a break which opens up at the I-Z junction when *Lecotherus* flight muscle is stretched in rigor by 10% (White and Thorson, 1973, their Fig. 11). In transverse sections each thick filament gives way precisely to a thin filament in an equivalent hexagonal array (their Fig. 12). The ratio of filaments in the I-Z region to A-filaments was found by Ullrick *et al.* (1977) in this muscle to be 4.1, as to be expected with a set extra to the actin filaments (which in this muscle are three times as numerous as A-filaments). The C-filaments have been shown beautifully in honey bee flight muscle (Trombitas and Tigyi-Sebes, 1974, their Fig. 7).

The presence of C-filaments in these highly specialised muscles is no guarantee of their presence in vertebrate muscle. One of the best indications that equivalent structures may be present comes from the special one-layer sections of Huxley (1957) referred to above. I wish to point out that most thick filaments there have a thin extension at one end and some at both ends. These extensions appear indistinguishable from other I-filaments. In ordinary sections, about four times thicker, this could be dismissed as superposition of thick and thin filaments, but it is harder to dismiss so many occurrences in one-layer sections, especially as the thin filaments in the next layer up or down in the lattice do not overlie the thick filaments.

There is also evidence from isolated A-filaments. In a paper claiming to disprove the existence

of an A-core, Morimoto and Harrington (1973) show micrographs of A-filaments and neglect to point out the stumps of thin filament protruding at one end of three out of four A-filaments, in their Plate V. These somewhat resemble the "end-filaments" of Trinick (1981) (see below).

It is interesting that Morimoto and Harrington could not isolate A-filaments at an early stage when dilute tris-buffer had already removed the M-line (but not the Z-line) from their myofibrils. After a week, when Z-lines had gone, A-filaments were released by gentle agitation. I believe they had remained moored by their G-filaments until released by slow proteolysis. This problem has not really been solved up to the latest methods for the preparation of A-filaments. Trinick (1982) claims only 5-10% of A-filaments are liberated on homogenisation of freshly glycerinated rabbit muscle, and that A-segments can be prepared in the same way, but best if the muscle is first aged for several days. Our own experience is that ageing is necessary to get significant yields of A-filaments from beef sternomandibularis muscle.

Morimoto and Harrington (1973) found by PAGE that myosin and C-protein were the only significant components of the A-filament. The apparent necessity to age A-filaments during preparation leaves a nagging doubt about such studies. I have previously argued strongly (Locker, 1982a, b) that an A-core would be fully protected from autolytic change, and indeed will show later that titin is resistant (although nebulin is not). While I still hold to this argument, I have to concede that an element of doubt exists. If the G-filament passes through the A-filament in any location other than as a central core, then the question of its vulnerability to proteolysis during A-filament preparation becomes a serious one.

Some recent disassembly of A-filaments reveals no sign of a core. Maw and Rowe (1980) found that rat (but seldom rabbit) A-filaments frayed into three strands when rinsed with water on a grid. These presumably correspond to the three myosin strands of the favoured A-filament model (Kensler and Stewart, 1983). Trinick (1981) found that rat or rabbit A-filaments frayed more reliably in 2 mM imidazole at pH 7.3. Again there was no sign of a core but he observed at each end a single "end-filament" (85 x 5 nm) with a period of 4.2 nm and sometimes a globular head. He considered these end-filaments were normally folded back inside since they were not visible in unfrayed A-filaments (although he quotes Craig as having seen them in unfrayed frog filaments). Pepe (1982, his Fig. 4) has observed a similar fraying in 10 mM tris-citrate buffer, pH 8.0.

The apparently core-less frayed A-filament suggests a possible alternative to a central core: that each of the three myosin strands might incorporate a strand of titin as a "mini-core", the three mini-cores uniting to form a triple-stranded G-filament. Such a concept would be difficult to reconcile with the withdrawal of a core under stretch or retraction during contraction (see below).

With regard to a multistrand G-filament we have on one occasion observed an unusually large number of filaments in the gap (Locker *et al.*, 1977). This was on a sample of beef sternomandibularis, glycerinated at 100% stretch and then incubated 24 h

at 25°C with a crude muscle protease preparation and calcium at pH 7. Over most of the sample, A- and I-bands remained just in contact, but in a few places (Fig. 2) had separated leaving an abundance of beautifully defined filaments in the gap. Instead of the usual sparse array seen there (usually half the number of A-filaments) the filaments in the gap are here in excess (by about 1.2 times). This could be interpreted as the unravelling of a two- or three-stranded G-filament, possibly due to proteolytic removal of some cementing substance. On the other hand it could be taken as evidence for a G-filament emerging from every A-filament, on a different model (see later).

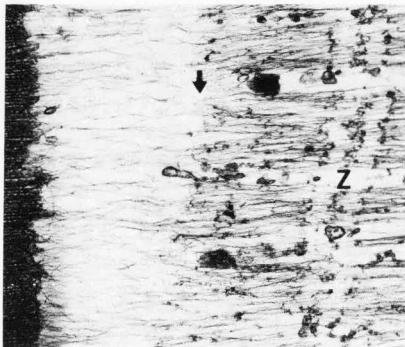


Figure 2. Beef muscle, glycerinated at twice excised length and incubated 24 h at 25°C in a crude muscle protease preparation (+Ca, pH 7). I-filaments touched the A-band except in a few places (as here) where a gap had opened up containing an unusually large number of sharply defined filaments. (Arrow shows edge of I-band.)

#### Shortening and stretching of G-filaments

If G-filaments exist in parallel array in the I-band, where do they go when the I-band shortens and disappears? There is no evidence for fine filaments coiling up around the Z-line. The thickening of the Z-line into "contraction bands" seems adequately accounted for by a pile-up of A-filament ends, bending or penetrating the Z-line (Marsh and Carse, 1974). I suggest that the G-filaments retract completely inside the A-filaments, reaching their minimum length of 1.5  $\mu\text{m}$ . (Note that this length, as used here and below, is the length within one sarcomere. In my model, each G-filament extends into the next sarcomere, so that the total length is double that quoted.) G-filaments must then be withdrawn from the A-core on stretching muscle. In excised beef muscle the equilibrium sarcomere length is 2.1  $\mu\text{m}$  (ibid.) which implies a G-filament length of 1.8  $\mu\text{m}$ . Over-stretched sarcomeres may reach 12  $\mu\text{m}$

(Locker and Leet, 1975) with filaments clearly surviving in the gap. Recently, observing over-stretched fibers in the light microscope, I have frequently seen 12  $\mu\text{m}$  sarcomeres, 13  $\mu\text{m}$  not uncommonly and once a record 14  $\mu\text{m}$ . In the latter case the G-filament should be 7.3  $\mu\text{m}$  long (allowing for residual overlap of 0.6  $\mu\text{m}$  in the dislocated A-filaments). This means a stretch of five times minimum length, rather high for a biological filament. This alone should be enough to dismiss claims that G-filaments are wisps of myosin or actin.

If the G-filament merely joined the A-filament but did not penetrate, it would have a length in an equilibrium sarcomere (2.1  $\mu\text{m}$ ) of only 0.3  $\mu\text{m}$ . In a 14  $\mu\text{m}$  sarcomere the G-filament would then be stretched about thirteen times. Such a degree of stretch becomes hard to believe, and is a good argument either for a core or at least for continuation into the A-filament in a stretchable form. In short, there has to be something to stretch.

In pre-rigor muscle the G-filaments must have a high extensibility since a load of only 0.1  $\text{kg}/\text{cm}^2$  is necessary to extend the muscle by 80%. This, in terms of the model, means stretching G-filaments to about 1.5 times excised length. A high extensibility appears to be preserved into the rigor state, since when a muscle "yields" by fracturing of actin filaments (Locker and Wild, 1982a), the G-filaments survive in the I-band and stretch without further loading. It is also possible to over-stretch muscle that has gone into rigor at maximum stretch (100%), that is with no actin-myosin overlap (Locker and Leet, 1976a).

As discussed above, the Squire model has a central space in the A-filament with a clear diameter of 30A, but actually triangular and best suited a three stranded core (3 x 20A). Direct observation of micrographs suggested a core of about 50A diameter. These estimates appear to impose a maximum diameter of 30-50A to a G-filament core at the fully retracted length of 1.5  $\mu\text{m}$ . This is in serious conflict with our observation that the filaments seen in the gap of overstretched muscle were of about the same diameter as actin filaments (60-70A). In these sarcomeres of 9-10  $\mu\text{m}$  the G-filament length would be about 5  $\mu\text{m}$ : i.e., stretched by 3.3 times. The maximum allowable diameter should then be 30-50A divided by  $\sqrt{3.3}$ , that is 16-27A. The gap filaments reported by Sjostrand (1962) were considered to be 30A in diameter, and being less stretched than ours are not too far from Squire's prediction. Guba et al. (1968a) found the filaments remaining after KI extraction to be 40A in diameter.

In the Squire model any core is likely to be a tight fit, and its extension might pose problems, accentuated by the predicted 3° helical twist. The central space resembles the bore of a rifle with three grooves of gentle pitch. The problem of fitting a core to a helically-grooved internal space is not affected by the bipolar arrangement of the myosin molecules. Given the same "handedness" of the twist, the internal grooves can pass uninterrupted through the whole A-filament. Extension of the core might at first be difficult and impose a twist, but as extension progressed the fit would rapidly become looser and twisting might cease.

The photo width for each of the three micrographs, Figs. 2, 4 and 5 is 3 micrometers.



The concept of retractable or stretchable A-core implies firstly that core and cortex are not firmly bound together, and secondly that the core must be composed of material which because of its nature or organisation is unusually elastic. It seems possible that the equilibrium length of the G-filament, whether core or not, could determine the equilibrium length of the resting sarcomere (2.1  $\mu$ m). It is not the meeting of opposing I-filaments which determines this, since at 2.1  $\mu$ m these are already overlapped by 0.3  $\mu$ m (beef I-filaments are 1.2  $\mu$ m long).

This idea is supported by the previously quoted observation of Huxley and Hanson (1954): "stretched myofibrils shorten spontaneously to a little less than rest length during extraction" of myosin. I have confirmed this for fibers using beef sternomandibularis, glycerinated at 2.0 x and 1.5 x excised length. The muscle was then chopped into fiber pieces or fiber bundles and extracted with Hasselbach-Schneider (H-S) solution. The 1.5 x sample reverted to a sarcomere length of  $2.04 \pm 0.29$   $\mu$ m (S.D.), while sarcomeres of the 2.0 x sample retracted less completely, to  $2.77 \pm 0.28$   $\mu$ m (104 fibers each). An attempt to achieve the same result on extracting long sarcomere beef myofibrils produced a lesser degree of retraction. However extraction of chicken myofibrils from muscle stretched only 22% during rigor, produced a rather precise retraction to excised sarcomere length.

The spring-back of fibers, cut after over-stretch and H-S extraction, has been recorded by Locker and Leet (1975, Figs. 23, 24,  $S=2.4$   $\mu$ m). I have repeated this experiment by over-stretching fibers directly into H-S solution. When cut after measurement and again measured under phase contrast, the sarcomeres were found to have contracted from  $11.8 \pm 0.9$   $\mu$ m to  $2.9 \pm 0.4$   $\mu$ m (20 fibers), shorter in fact than fibers cut after stretching into isotonic neutral solution ( $3.6 \pm 0.5$   $\mu$ m).

#### The symmetry of the G-filament connections

If G-filaments pass from the center-filled hexagonal array of the A-filaments to the tetragonal array at or near the Z-line, they are likely to do so in a systematic way.

If the G-filaments emerge from both ends of the A-filaments there is no problem. However to link half the A-filaments in each direction (as in my model), while preserving a hexagonal symmetry is not possible. This requires one third of the cores to emerge in one direction and two thirds in the other (Fig. 3a). However the same number of links in both directions can be achieved by selecting alternate A-filaments along two of the directions parallel to the sides of the hexagons, but the third direction then has all its connections the same way (Fig. 3b).

At the Z-line, the only feasible place for a G-filament to pass seems to be through the centres of the "Z-squares". If G-filaments emerged at both ends of A-filaments the ratio of I-filaments to G-filaments would be 2:1. This can be achieved symmetrically with the arrangement in Fig. 3c, using all centres on alternate diagonals. The 4:1 ratio required by the model can also be achieved by using alternate centres in alternate rows (Fig. 3d).

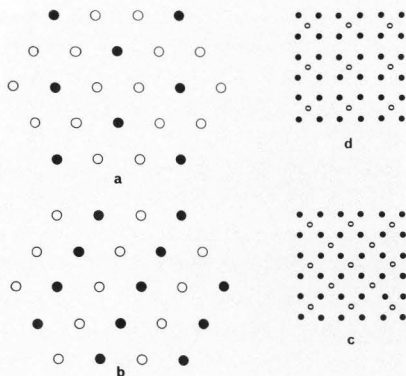


Figure 3. The possibilities for symmetrical connections of G-filaments within A-band and Z-line.

- The two kinds of circles represent A-filaments linked to a Z-line one way or the other by G-filaments. Only this arrangement preserves hexagonal symmetry, but it produces a 2:1 ratio of the directional links.
- This arrangement produces a 1:1 ratio, but is not fully symmetrical. It alternates directions along two sides of the hexagons but not along the third.
- A symmetrical arrangement of I-filaments (solid) and G-filaments (open centers) at the Z-line, which produces the 2:1 ratio required by a modified model with G-filaments emerging from both ends of an A-filament.
- A similar symmetrical arrangement which produces the 4:1 ratio required by the model of Fig. 1.

#### Extension of A-filaments during over-stretch

In the original paper on over-stretched beef-fibers (Locker and Leet, 1975) it was found that the A-filaments not only slid in one direction or the other, thus dislocating the A-band, but that the A-filaments themselves stretched. The dislocated A-band in a 10.6  $\mu$ m sarcomere was 4.6  $\mu$ m long (ibid., Fig. 12). Allowing for an 0.6  $\mu$ m central overlap of A-filaments this means that each was 2.6  $\mu$ m long (or stretched by 73%). It seems doubtful that an A-filament consisting entirely of packed myosin molecules would survive such stretch without

breaking, and I suggest that its survival was due to an intact elastic core. For the A-filament to have stretched, the core must have been attached initially to the cortex of myosin. It must have retained some attachment, with slippage, since the A-filaments continued extending up to the longest sarcomeres recorded in the cited figure, but to a lesser degree than the G-filaments.

Some extremely curious fibers were observed which, having been over-stretched with dislocation of the A-band, had then apparently lost tension and sprung back (Locker and Leet, 1976a). Except in the central overlap the A-filaments had totally lost their characteristic rigidity. They were thrown into waves and had apparently dissociated into strands (*ibid.*, Figs. 20, 21). This effect suggests to me the violent recoil of a core.

A minority of over-stretched fibers did not dislocate (Locker and Leet, 1976a). Sarcomeres of over 7  $\mu$ m were obtained with an A-band more or less intact. The M-line was sometimes intact, sometimes not (*ibid.*, Figs. 2, 3). In these fibers the A-filaments were not stretched, so if there is a G-filament core, it must have been freely withdrawn. Again the G-filaments appear to be definitely continuous with the A-filaments.

Why the difference in behaviour of different fibers? Is it a question of fiber type in this "mixed" muscle? It appears not. Orcutt and Dutson (1984) have repeated the over-stretch technique with the same muscle and always obtained non-dislocated sarcomeres. The only difference between their procedure and ours appears to be that they stretched the muscle immediately post mortem, whereas ours was stretched generally several hours later (when the muscle is still however, far from rigor). Another difference is that A-filament:G-filament ratios are close to 2:1 in our fibers (either kind), but they found near 1:1. On the other hand La Salle *et al.* (1983) obtained both kinds of over-stretched beef fibers, closely resembling ours (personal communication).

This seems an appropriate point to comment on a peculiarity of my model: the notion that half the A-filaments in any A-band are connected by G-filaments through one Z-line and half through the other. This seemed necessary to account for the dislocation. The only reasonable alternative explanation ever offered to me was by Prof. K. Maruyama, who suggested that A-filaments are connected at both ends by G-filaments and that under stress there is random breakage of these, one side or the other, and the A-filaments slide accordingly. I take this suggestion seriously and admit that it would eliminate one of the oddest features of my model. Recent evidence that "ageing" of muscle begins at death (Marsh *et al.*, 1981) may be relevant. If ageing is indeed a factor so early, and G-filaments are certainly weakened by ageing (Locker *et al.*, 1977), Maruyama's explanation seems possible. It could be argued in the light of Orcutt and Dutson's experience that when over-stretch occurs very early, G-filaments remain intact at both ends of the A-filament, and no dislocation occurs. However even in our non-dislocated fibers, only half the A-filaments were connected in any one direction, so it would have to be concluded that random breakages had occurred there too.

#### A core in relation to the assembly of A-filaments

The in-built ability of the myosin molecule to self-assemble into thick filaments argues against the need for a template. Purified myosin solutions can be induced, merely by lowering the ionic strength, to produce "synthetic A-filaments" which closely resemble the real thing in all respects but length (for a review see Pepe, 1983). However Pepe found that a carefully programmed two-step reduction on ionic strength produced the correct length. This is, of course, not an option open to the cell.

Davis (1981a, b) found the rate of association to be independent of length, while the rate of dissociation increased rapidly with length. He therefore concluded that a kinetic equilibrium defined the length of the filament, and that this basic mechanism could be fine tuned by various factors in the cell. One of the factors he also suggested was co-polymerisation of other thick-filament proteins, an idea which others have also explored (see Pepe, 1983). A core filament with an equilibrium half-length of 1.8  $\mu$ m could be a very suitable regulator, if its effective length was reduced to 1.5  $\mu$ m by an N-line structure.

A length determining role is distinct from that of template for assembly. The former does not demand such a close association of myosin molecules with the core, and would be more compatible with its withdrawal or retraction.

Quite apart from either role it should be easier to assemble the A-filament around a core than to thread it in afterwards. The only relevant evidence on myogenesis published so far appears to be that of Gruen *et al.* (1982), who found that myosin heavy chains appear in fetal lambs some weeks earlier than connectin (which appears at about 7 weeks). They conclude, very reasonably, that this "argues against a possible role for connectin as a framework for the assembly of the sarcomere in vivo and that this result, together with the known susceptibility of connectin to hydrolysis, suggests "an exposed environment". It might however be asked to what extent the organisation and genetic expression of contractile proteins at this early stage resembles that in adult muscle. Histological data on embryonic lamb muscle are scarce, but at 45 days (about the time at which connectin appears) myotubes with just perceptible striations are present (Joubert, 1956, his Fig. 28).

I am aware of other work, as yet unpublished, which does not support the lag of titin behind contractile proteins during development and it therefore seems too early to make judgements in this area.

#### A role for G-filaments in contraction?

The constancy of the A-filament in length and structure is basic to the sliding filament theory. There have however been a number of reports of changes in the length of A-filaments or in their packing, most of which are suspect. Some of these have already been discussed (Locker and Leet, 1976a, Locker *et al.*, 1976). Some unorthodox theories of contraction have been proposed, including cyclic changes in the backbone of the A-filament. For example, the "quantum" contraction theory of Davydov (1974) envisages excitation of the peptide groups that form three parallel chains of hydrogen bonds down the myosin helix. A wave of compression

travels down the molecule shifting the myosin heads relative to actin. The highly original theory of dos Remedios (1969), involves dissociation of I-filaments, changes in the packing of myosin molecules, the movement of A-filament cores and the re-making of I-filaments.

A much more plausible theory, also from Australia, came recently from Obendorf (1981). He suggests that the problems of matching the helically arranged myosin heads and binding sites on the I-filament during cross-bridge formation would be solved if the A-filament rotated during contraction. This novel idea retains the essentials of the conventional theory, and is not in conflict with its basic premises. So far it has drawn no fire.

This theory has interesting implications for G-filaments. If these are in fact the A-core, then the A-filament cortex might rotate on the core as on an axle. It might be argued that the tight M-line structure would prevent this, but it must be remembered that the center of the A-filament is the smoothest part, that isolated M-proteins do not show any binding affinity to myosin *in vitro* (Woodhead and Lowey, 1983) and that A-filaments under the stress of over-stretch can slide through M-lines (Locker and Leet, 1975).

The G-filaments seem much too elastic to be effective as transmitters of tension (as proposed by dos Remedios, 1969) unless they were transformed in some way by stimulation.

#### Localisation of titin in the myofibril

The original paper on titin used fluorescent antibody to locate the protein in chicken myofibrils (Wang *et al.*, 1979). They conclude that "titin is present in M-lines, Z-lines, the junction of A and I bands, and perhaps throughout the entire A-band". This conclusion needs re-examination. The myofibrils (a-d) in their Fig. 4 appear to be artefacts of the kind we have described in some detail (Locker *et al.*, 1976). They are the usual product of blending pre-rigor muscle, and are seen less commonly on blending rigor or glycerinated muscle. A-bands with their material apparently concentrated at the outer edges, as in (a-d) are typical. It seems significant that these are the ones staining most intensely across the A-band. Myofibrils (f-h) appear to be normal and stain strongly only in the H-zone and at the A-I junction. The relative intensity in these two regions varies. The Z-line is barely stained at all, and only heavily in short sarcomeres (j), where the line is in fact a contracture band, representing a pile-up of A- and I-band material against the Z-line. Apart from H-zone staining, these results seem not incompatible with a core role for titin: that is exposure in the outer I-band, and in the A-bands only when there has been damage.

The location of titin by the antibody-peroxidase method in the electron microscope is the subject of a preliminary communication by La Salle *et al.* (1983). The results were "complex and somewhat variable" for myofibrils (artefact problems?) but "always demonstrated labelling in the region of the A band-I band junction with some labelling extending into the A band." In fibers over-stretched after Locker and Leet (1975), the G-filaments were clearly labelled. This is the only direct link so far between titin and

G-filaments. Often the outer A-band and the outer half of the I-band were also labelled, which seems to agree entirely with the light micrographs of Wang *et al.* (1979). Various models for the A-filament suggest a looser packing of myosin at the ends, and it is possible a core could be more accessible to antibody there. The failure of the inner I-band to label might be due to N-line structures there (see later).

Having criticised the early results of Wang *et al.* (1979) I must now acknowledge that the far more sophisticated approach which the group has since undertaken, using four distinct monoclonal antibodies to rabbit titin (Wang and Ramirez-Mitchell, 1984), bears out the earlier claims. These antibodies collectively indicate that titin passes from the M-line through the A-band and just into the I-band (Wang *pers. comm.*). The results suggest a degree of exposure not compatible with location in a central core. The results of Greaser's group (S.M. Wang *et al.*, 1983) using a single monoclonal antibody to bovine cardiac titin are in agreement. Their staining occurred at the A-I junction, (but also at M-line and Z-line after extraction of major proteins).

#### Wang's third filament

When this review was almost complete, I received a paper I had not seen (Wang, 1982b) and another in press (Wang, 1983b). These offer a quite new alternative to G-filaments in terms of "an elastic filamentous matrix consisting of titin and nebulin as additional sarcomere constituents." The new concept, derived from his immunochemical studies, sees the filament as continuous from Z-line to Z-line. In the A-region it is composed of titin, wound spirally on the outside of the A-filament (and therefore able to react with antibody) and extending into the I-band on to an I-filament (or two I-filaments?). Here nebulin, in series, takes over, enveloping the I-filament in a manner unspecified. No extra filaments are needed, except of course where the titin thread bridges the A- and I-filaments. On contraction, the titin section in the A-band remains unchanged, but the nebulin section bunches up on the I-filament.

The model is speculative and has problems of its own, but brings an exciting new concept. It will be of great interest to see how it develops with new information.

#### The N-lines and the G-filaments

The N-line was first seen over a century ago and has been as much neglected as the G-filaments. It is now believed that there are two: the  $N_1$ - and  $N_2$ -lines. Curiously, studies on G-filaments and N-lines have converged recently in two laboratories. We claimed that  $N_1$ -lines are suspended on the G-filaments (Locker and Leet, 1976b), while in Austin, Texas, titin and nebulin have been studied together, and the latter located on the  $N_2$ -line (Wang and Williamson, 1980).

The disappearance of nebulin, but not titin, during tenderisation of beef by ageing (Locker and Wild, 1984a) led us to suggest that nebulin might be an essential part of G-filaments (which largely determine the myofibrillar contribution to meat tenderness). We queried the nebulin antibody work on the same grounds as the titin antibody experiments

(i.e., damaged myofibrils), and suggested that the nebulin antibody distribution was just as compatible with a location of nebulin in the G-filaments.

The elusive nature of the N-lines has defied systematic study. However we have just completed a survey of micrographs made here and elsewhere for other purposes (Locker and Wild, submitted for publication). My conclusion is that there are at least seven N-lines: an  $N_1$ -line always close to the Z-line; a group of four  $N_2$ -lines in the mid I-band, sometimes sharp, sometimes diffuse and often embedded in an "extra density"; and an  $N_3$  doublet, seen near the edge of the I-band in fully stretched sarcomeres. It seems that both the  $N_2$  and  $N_3$  group may be suspended on the G-filaments. These groups were apparently confused in Locker and Leet (1976b), that is in one case it was the  $N_2$ -lines and in the other the  $N_3$ -lines which were found to be suspended on the G-filaments.

This new classification of N-lines into three distinct groups should provide a base for new research on these intriguing structures and on their relationship to the G-filaments. It may be noted that nebulin antibody labelled more than just a line in the I-band, and, if it is an N-line protein, could have attached to the whole  $N_2$  and perhaps the  $N_3$  groups. It is also possible that the failure of the inner part of the I-band to label with titin antibody (as noted in the previous section) could be due to the interference of N-structures.

#### G-filaments in moderately stretched muscle

While G-filaments can be reliably demonstrated in over-stretched fibers, we have never found oriented G-filaments after extraction of A- or I-filaments from muscle at excised length or stretched by up to 100%.

Extraction of beef sternomandibularis, glycerinated at 100% stretch, with Hasselbach-Scheider solution (1 h) left a drifting M-line (Fig. 4). At the ends of the residual A-filament stubs was a tangle of fine filamentous material with only a few slack filaments spanning across to the I-band. This is in contrast to the taut filaments emerging from such stubs in extracted over-stretched sarcomeres (Locker and Leet, 1976a, Figs. 9, 11). It is possible that some proteolysis may have occurred during glycerination (2-3 weeks at 2°C in EDTA, pH 7), but the same drifting M-lines were seen in thin strips of fresh muscle extracted at the same stretch for 24 h at 2°C in H-S solution.

When the same stretched glycerinated muscle was extracted for 1 h with a KI solvent (after Guba *et al.*, 1968a) the sarcomere was reduced to a mass of disorganised fine filaments largely out of the plane of the section, but with some tending to longitudinal orientation (Fig. 5). The previously observed pile-up of material on the Z-line was evident.



Figure 4. Beef muscle glycerinated at twice excised length and extracted 1 h in H-S solution. Continuity has been lost in the center of the sarcomere, but filamentous material is bunched up at the ends of the thick filament stubs preserved by the M-line.

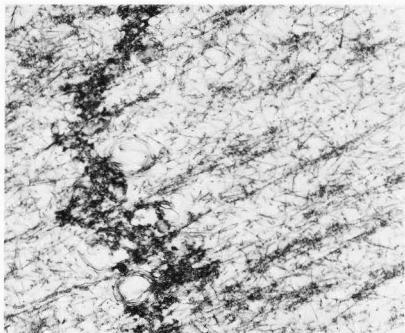


Figure 5. The same glycerinated muscle as in Figure 4, but extracted with strong KI solution (Guba *et al.*, 1968a). Many fine filaments survive, but largely in random array. Only patches of longitudinal orientation remain. A pile-up of dense material on the Z-lines has occurred.

In short, there is no difficulty in demonstrating residual fine filaments (which I believe to be G-filaments) within the myofibril when muscle that has not been extended beyond its natural limit is extracted with H-S or KI solution. However

organisation is lost. Of course on the basis of my model, solution of myosin from the A-filament should leave both extremities of each G-filament free to retract and tangle. However the filament should be held taut between the M-line (where this survives) and the Z-line. This does not appear to be the case in Figure 4.

If it is a question of anchorages, why do these survive so well in over-stretched fibers, but not at lesser extensions? When A-filaments dislocate they never completely lose their overlap for reasons unknown. Why does this overlap of 0.6  $\mu$ m survive, and why does it still provide anchorage for G-filaments after H-S extraction (Locker and Leet, 1976 a, Fig. 8)? These questions have intrigued me for years, but I still have no answers.

#### The cytoskeleton and the G-filaments

The concept of the cytoskeleton is now as fashionable in muscle as in other cells. It began in muscle with the observation of the Z-line "scaffold" of desmin by Grainger and Lazarides (1978) and has been further explored by Richardson *et al.* (1981). It has been reviewed by Stanley (1983), Robson and Huiatt (1983), Robson *et al.* (1983) and by Wang (1982b, 1983a, 1983b).

Wang and Ramirez-Mitchell (1983a) extracted myofibrils on a grid with KI and concluded that there is both a transverse and a longitudinal network of desmin, lying outside the myofibril but closely associated with it, so closely that it may survive partial disintegration of the fiber during homogenisation. Tokuyasu *et al.* (1983) have confirmed this external longitudinal network in intact tissue, using ferritin-labelled antibody to desmin.

Wang and Ramirez-Mitchell consider the longitudinal "residual filaments" of dos Remedios and Gilmour (1978) and others are not internal elements of the sarcomere at all, but merely these external intermediate filaments. This explanation could account for our failure to find organised filaments after extraction of moderately stretched muscle (previous section).

They note a heavy aggregation against the Z-line of material which they consider to be titin. This may well be so, since titin is the dominant electrophoretic component of their KI-residue, while desmin is present only as a trace. We have seen a similar accumulation on the Z-line after KI-extraction of both muscle at 100% stretch (Fig. 5) and over-stretched fibers (Locker and Leet, 1976a, Figs. 14-18). In both these cases enough filaments remain through the sarcomere to account for titin (in disorder in the first case and in parallel array in the second). I suspect that the Z-line aggregate may here be largely N-line substance or the associated "extra density".

#### The Ghosts Become More Ghostly

Coming back to the point at which this review began, we must now ask why, if no ordered, through-running filaments remain within sarcomeres after H-S or KI extraction, the ghosts of stretched myofibrils still shorten to near original rest length? If the G-filaments are no longer capable, is it a surviving longitudinal sheath of intermediate filaments that causes retraction? For fibers (which I used earlier to

verify the effect) this is quite possible. But I suspect that isolated myofibrils are normally quite nude. I have noted earlier that my attempts to induce beef myofibrils of long sarcomere length to shorten by H-S extraction were less successful than those of Huxley and Hanson (1954) using rabbit myofibrils, and certainly less effective than experiments with beef fibers.

A new candidate for the ghostly spring has recently appeared in the "covalently cross-linked matrix" of Loewy *et al.* (1983). Exhaustive extraction of skeletal, cardiac or smooth muscle with 6M guanidine. HCl (+ thiol) leaves only a 1% residue (in good agreement with our earlier results; Locker and Daines, 1980). Treatment with collagenase reduces the residue to only 0.2% of original protein, but the shape of the fiber is perfectly preserved and the matrix extends throughout its volume. Although the protein is distinct in composition from titin, it cross reacts, in spite of the fact that the antibody was made from the residue of chicken gizzard, which has no titin. These authors did not observe filaments, but Ozaki and Maruyama (1980) found a residue in the slime mould *Physarum polycephalum*, after extraction with 6M guanidine. HCl (+ thiol) or 1% SDS, which contained filaments less than 5 nm thick. In the same slime mould Gassner *et al.* (1983) also found a residue resistant to 4% SDS + 7M urea, which contained filaments 2-3 nm in diameter. This recalls the "superthin" filaments of Hoyle (1983).

It now seems that we may need to contemplate within a fiber not only a third longitudinal filament, but a fourth of desmin (Wang, 1983b) and even a fifth of "covalently cross-linked matrix".

### MEAT TENDERNESS AND THE G-FILAMENTS

#### A new theory of tenderness

My interest in G-filaments arose from a chance observation of the ability of beef fibers to stretch to an extraordinary degree, far beyond previous reports for vertebrate muscle. This observation was pursued purely from an academic interest in this neglected avenue of muscle biology, which seemed to me potentially important. It was several years before I realised that G-filaments were also relevant to meat tenderness. The first convincing demonstration came from my colleagues Davey and Graafhuys (1976), who found in beef neck muscle, cooked at maximum stretch, that G-filaments survived well in the "gap", but not if the muscle had previously been aged. This and other observations led to the beginning of a theory (Locker *et al.*, 1977). The crucial subsequent observation was that in cooked meat the A- and I-filaments disperse, leaving an actomyosin coagulum with a through-running array of fine filaments, which we identified as G-filaments (Locker and Wild, 1982b). Thus tensile continuity becomes dependent on G-filaments. As other evidence accumulated, a more comprehensive attempt at a tenderness theory was published (Locker 1982a, b). Some special attention was given to cold shortened meat (Locker, 1984). Now, several papers on, some foundations of the theory appear to have crumbled a little, and a reassessment is due. The detailed argument behind the theory has been presented (Locker, 1982a). The following are its main points.



In the normal cooking of meat, only the G-filaments survive within the myofibril. As the temperature rises from 60° to 70°C, the A-filaments and I-filaments disintegrate, leaving G-filaments embedded in an actomyosin gel, while the collagen net denatures, becoming tensioned and elasticised. These two elements, heat modified myofibril and perimysium, stretch in unison under stress. They give cooked meat its essential character. The G-filaments denature slowly at 60°C, and more rapidly at higher temperatures. They survive with strength and elasticity for several hours at 100°C (whereas collagen does not).

Treatments which improve the myofibrillar component of tenderness, work by weakening the G-filaments. This is true of ageing and of pressure-heat treatment (Locker and Wild, 1984b), where the filaments are vulnerable to proteases and to pressure respectively.

The toughening due to cold shortening is attributed to an increasing incidence of sarcomeres without I-bands. The latter, having only half the number of C-filaments present in the A-band, constitute the weak link in cooked sarcomeres.

The failure of cold shortened meat to age is claimed to be due to retraction of G-filaments into the A-core, where they become completely protected against proteolytic attack.

#### Conflict between morphological and protein studies

The theory was based largely on morphological studies, which still appear valid. However study of changes in structural proteins during tenderising treatments and cooking has raised difficulties. Since the antibody work of La Salle *et al.* (1983) has shown that G-filaments, as expected, contain titin, its behaviour during such treatments is important to the theory.

In a series of papers, Australian workers had already shown that titin was rapidly degraded by the proteases in muscle at elevated temperatures, within its denaturation range (see King, 1984). Most recently King (1984) has shown that it is also degraded at 0° and at 15°C in beef and sheep muscles (using light scattering and PAGE). His result is contrary to our recent PAGE study of the fate of the large myofibrillar proteins during ageing, pressure-heat treatment and cooking (Locker and Wild, 1984a). We found that in beef sternomandibularis, titin appeared resistant to ageing at 15°C for 10 days and even 20 days whereas most of the tenderness improvement occurs in 2 days in the muscle. More in line with this time scale of tenderisation was the disappearance of nebulin and the appearance of a new band B midway between titin and nebulin. The rate of disappearance of nebulin varied somewhat between animals (as indeed does the rate of ageing), although generally it was largely gone at two days whereas band B appeared reliably in that interval. While the position of band B suggested titin as a source, densitometry showed insufficient loss of titin, but an adequate loss of material from the gel top. This top protein might be an aggregated form of titin. There was little evidence of titin doublets or conversion of titin-I to titin-. To sum up, there was no significant change in the titin content during the period of tenderisation, but a closely parallel appearance of

faster band B, apparently derived from material unable to penetrate the gel. The disappearance of nebulin also followed tenderisation, more or less.

We have since found (unpublished results) that ageing of lamb longissimus followed the same pattern very consistently. On the other hand chicken leg muscle aged one day at 15°C retained titin and nebulin, but produced band B (the sharpest and densest we have seen) while chicken breast also retained titin but lost nebulin and did not generate band B. The patterns for the same muscles of a thrush were different again.

A variety of rabbit muscles was also aged one day at 15°C. In every case the titin band survived, and in all but gluteus minimus, the nebulin band. The disappearance of protein from the gel top was a consistent feature, while the appearance of band B was seen in multifidus dorsi, vastus intermedius, soleus and gluteus medius, but not in psoas, gluteus minimus or longissimus. However when a sample of the longissimus was aged 3 days at 15°C, nebulin disappeared and B appeared. These particular muscles represent a range of essentially "white", "pure red" and "mixed" muscles (Locker and Hagyard, 1968) but their ageing behaviour cannot be so divided. It is clear that the pattern of ageing varies widely between muscles.

At the same time, and quite independently, the Iowa group has been involved in a similar study of ageing in beef longissimus (Lusby *et al.*, 1983) using three storage temperatures 2°, 25° and 37°C for 1, 3 and 7 days. In the intact muscle they claim a graded conversion of titin-I to titin-2 with increased time and temperature, although the photographs of their gels are not convincing, showing no resolution of these bands. Titin remained up to 7 days at 2° and 25°C but vanished within 3 days at 37°C.

They also found that nebulin disappeared, apparently faster (1 day at 2°C) than in our muscle, and they record breakdown products just below the original band (as we did). They also report a breakdown product apparently corresponding to our band B, but only after severe ageing (7 days at 25°C, or 3 days at 37°C).

Considering only ageing conditions of practical significance, it seems that our results and theirs are in substantial agreement: conservation of titin, and rapid loss of nebulin. Their ageing differs in the claimed conversion of titin-I to titin-2, and in the rather sluggish appearance of band B. It is possible that our gels did not resolve titin-I and titin-2, and we propose to investigate this further.

If titin is not substantially degraded, but G-filaments are during ageing, some other component, essential to their integrity, may be suffering. We have suggested that nebulin may have such a role (Locker and Wild, 1984a). The new model of Wang (1983b), which has nebulin in series with titin in the G-filament, fits well with this idea. Attack on nebulin would inevitably weaken the filament.

It may be noted that Maruyama *et al.* (1981a) found that when chicken myofibrils were prepared in the presence of EGTA, both titin and nebulin were conserved. In the absence of EGTA there was some loss of titin and nebulin had disappeared. The equivalent of band B was present. If mM Ca was added, titin was much depleted and nebulin absent.

These workers also subjected myofibrils, prepared with EGTA, to purified calcium activated neutral protease (CANP) and serine protease (both at pH 8.0). In the case of CANP, nebulin disappeared rapidly, but the titin doublet remained (although becoming distinctly more mobile on the gel with time). Serine protease destroyed both proteins at the same rate.

#### Heart muscle

We have subjected heart muscle from lamb, rabbit, chicken and thrush to PAGE and find in all cases a level of titin comparable to that in skeletal muscle, but no nebulin at all. The material at the gel top is also missing. Ageing in every case had little apparent effect, and did not generate band B.

If, as suggested, nebulin is part of the G-filament we must conclude that either heart has no G-filaments, or they differ in a major respect. We have in fact cooked fully stretched papillary muscle and find filaments in the "gap". Recently I have also managed to over-stretch papillary muscle within a few minutes of death, but the few samples obtained so far have not reached the electron microscope due to mishap. However I conclude that G-filaments are an essential feature of cardiac as well as skeletal muscle.

#### Cold shortening and ageing

King (1984) reported that cold shortening did not influence the rate of ageing in terms of survival of titin. Our study has also shown that the gel patterns are the same whether the muscle has been cold shortened, set at excised length or stretched by two times. The only differences seen were at longer times in the fully stretched muscle, when myosin heads appeared to have become vulnerable due to loss of interdigitation allowing better access of proteases (Locker and Wild, 1984a).

Of course if titin is not attacked at all, it is not possible to demonstrate its protection by contraction. But there has definitely been no protection of nebulin nor suppression of band B. It can only be concluded that the present results offer no support for the attractively simple idea of protection of G-filaments by retreat into the A-core.

#### Survival of the G-filament proteins on cooking

The Australian group has repeatedly claimed that titin degrades in cooking at 80°C, and cannot therefore be significant to meat tenderness. This is in contrast to the undoubted survival of the G-filaments themselves (Locker et al., 1977; Locker and Wild, 1982b). We have pointed out in discussing King's work, that although little of the original titin survives, most of the "smeared" product represents material with a molecular weight of over half a million, and could therefore still be capable of contributing to structural strength, particularly with the "healing" possibilities of cross-linking during heat denaturation (Locker and Wild, 1984a). At 100°C the degradation becomes more serious, although after an hour, half the material is still larger than  $10^3$  daltons. We have not investigated the degradation after another  $3\frac{1}{2}$  hours at 100°C when the shear force falls by only a quarter (Davey and Niederer, 1977). It may be noted that most of the myosin heavy chain has also disappeared after an hour at 100°C, so that

any theory of residual strength based on this protein is likewise in difficulty.

Whether meat is tenderised by ageing (where the titin survives), or by pressure-heat treatment at 60°C (where it is largely degraded), the gels derived from the cooked material (80°C, 40 min) differ remarkably little from each other or from untreated cooked controls. However the remnant of the titin band seen in the cooked controls is absent in the cooked treated samples.

With respect to the possibility that nebulin may be part of the G-filaments, it may be noted that this protein generally (but not always) survives cooking at 80°C better than titin. The variation may be due to variable degrees of ageing during cooking. However the band disappears entirely on cooking at 100°C.

#### Denaturation point of G-filaments

A feature of the theory was that G-filaments denature slowly at 60°C, with a sharp decrease in their elasticity. However they remain strong and extensible. Numerous pieces of circumstantial evidence were listed in support of denaturation (Locker, 1982a). More direct evidence has now been obtained from beef fibers, over-stretched in 0.15 M KCl + 5 mM K phosphate, pH 7.0, and then heated for 1 hour at 60°C in that medium. On cutting, the fibers ( $n = 13$ ) shortened from  $11.3 \pm 0.5 \mu\text{m}$  to  $9.5 \pm 0.5 \mu\text{m}$  (S.D.), that is to 84% of stretched length. When this is compared with shortening from  $11.4 \pm 0.8 \mu\text{m}$  to  $3.6 \pm 0.5 \mu\text{m}$  (31% of stretched length) in unheated samples it is clear that denaturation of G-filaments must have occurred within an hour at 60°C.

#### More basic information on G-filaments is needed

The present dilemma arises from the failure of what appears to be the major constituent of G-filaments to suffer during ageing periods which damage G-filaments, and the failure of this protein to survive severe cooking, which does not destroy G-filaments, nor greatly decrease the tensile strength of meat. The relationship between G-filaments and tenderness now seems much less tidy in the light of PAGE studies than it did when the theory was put together. The revision of the theory must wait for more basic knowledge about the nature of G-filaments, for firm answers on their components and assembly. If indeed they prove to be compound in structure, some of the difficulties may be resolved. The question of the strength of the anchorages of G-filaments, and how this survives tenderising treatments and heat, must not be overlooked and may be as important as the strength of the filaments themselves.

If my model (Fig. 1) were revised on Maruyama's suggestion of G-filaments emergent at both ends of the A-filament, the G-filament count would be the same in both A-band and I-band and the concept of the I-band as the weak link in cooked meat is itself weakened. However the reinforcement of the A-band with actomyosin gel should still provide a significant margin of strength.

Whatever the nature and properties of G-filaments, it is hardly possible to deny the accumulated evidence that they are there and that they are survivors in cooked meat.

Previously I have emphasised that my theory is over-simple in considering only longitudinal strength in meat, and that lateral strength must also be important. This is emphasised by the recent review paper of Stanley (1983) on the cytoskeleton. His own results confirm for beef earlier observations that emptying of the sarcomere becomes possible only after an ageing period. This indicates that decay of the cytoskeleton could be an important part of tenderisation.

### CONCLUSIONS

This review has of necessity been speculative, raising more questions than answers. However it represents an attempt to gather many scattered pieces of information and to weigh honestly the evidence, inadequate as it may be, for and against the concept of G-filaments as A-cores. This may prove to be effort wasted on an erroneous idea, but I still feel it is one which should not be discarded without adequate consideration. A heavy dependence on this laboratory for work on G-filaments is regretted, but over most of the last decade little else has come forward. The existence of G-filaments has been challenged only once in the literature (Ullrick et al., 1977) but otherwise has been ignored, until quite recently. The situation reminds me of that in the sixties when I was busy characterising the light chains of myosin, which then tended to be dismissed as impurities. It is pleasing that a number of highly capable groups are now busily studying the relationship between the myofilaments and the new large proteins. Papers have begun to appear in rapid succession, bringing important new evidence and stimulating ideas. It seems that a new chapter in muscle biology will soon be written. It will be concerned with the "extra" filaments, their composition and disposition in relation to A- and I-filaments; a more detailed picture of the architecture of the I-band, and the relationship within it of longitudinal filaments, transverse structures such as the N-lines and the "extra density". Some important and basic evidence on G-filaments has come from meat-oriented research. Those who work in meat research institutions tend to do experiments which scientists in university biology departments would not dream of. The use of these unorthodox materials and treatments sometimes pays dividends, and opens new vistas.

My model for G-filament connections, and tenderness theory based upon them, already have some dents and more are in store. Both are bound to be remodelled before many years have passed, as indeed are the bold and welcome ideas of Wang (1983b). I have given some attention in various places to Maruyama's suggestion that the G-filaments may emerge at both ends of the A-filament and run right across the sarcomere. I consider this a real possibility, which would bring my ideas somewhat closer to those of Wang.

The role of G-filaments in muscle function must remain for the moment an entirely open question, but in meat seems already better defined. That G-filaments are significant in both fields appears well enough established. They are not likely to go away. I was going to say, like S-filaments, but

the G-filaments are in my view, the rightful successors to that extinct species.

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Discussion with Reviewers

D.W. Stanley: Considering the concept of self-assembly and your hypothesis about the location and length of G-filaments, how do you conceive of the formation of these elements in vivo?

Author: I am aware of the work going on in embryological development (still unpublished) and it is too early to speculate in this direction.

D.W. Stanley: A recent paper by Koohmaraie et al., 1984 (Koohmarai M., Kennick W.H., Anglemier A.F., Elgasim, E.A. and Jones T.K. Effect of Post Mortem Storage on Cold-shortened Bovine Muscle: Analysis by SDS-Polyacrylamide Gel Electrophoresis, J. Food Science, 49, 290-291) also explores cold-shortened muscle.

Author: Thank you.

## PROCESSING EFFECTS ON MEAT PRODUCT MICROSTRUCTURE

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### Abstract

Animal species, meat ingredient properties, comminution equipment, mechanical action, product composition, type and level of non-meat ingredients, and thermal processing greatly affect the structural and organoleptic properties of meat products. However, additional research in the area of restructured meat products, meat protein functionality and lipid properties in meat products remains to be done. The interaction of meat proteins with lipids, water and ions should be further investigated. Considerable control of raw materials, mechanical action and heat processing is essential to make research applicable to product and process development.

### Introduction

Meat processing affects meat microstructure. However, meat processing is a very complex and diverse technology for modifying properties of meat to fulfill perceived needs of consumers. Meat products may be affected by properties of the meat, method of comminution, addition of ions, mechanical action, and thermal treatment. Meat products are sold either frozen, refrigerated or heat processed. The level of heat processing is minor for some dried products, more severe for pasteurized products and extensive for commercially sterile shelf-stable products. These factors have an effect on the microstructure, cook yield and rheological properties of the product.

The purpose of this paper is to depict the many properties of meat products that may affect microstructural characteristics. The review of literature is selective to prevent excessive length. Recommendations are presented for future work and precautions to be taken in designing experiments are offered.

### Factors Relevant to Processed Meat Microstructure

#### Meat Source

Meats derived from many sources are processed for human consumption. The source and treatment of animal tissue prior to further processing affects many product characteristics, including microstructure. Beef, pork, poultry, fish, and lamb are the primary species used for processed products. These tissues can be fresh, aged, fresh frozen or mechanically deboned prior to use in processed meat products.

#### Products

The products produced are diverse in several traits. Meat may be utilized as intact muscles or comminuted to various extents (Huffman and Cordray, 1982). In some meat products various organs such as livers and hearts are included (Chyr et al., 1980; Ray et al. 1981). The degree of comminution has a great impact upon the microstructure of the finished product (Cross and Stanfield, 1976; Costello et al., 1981). Addition of various ions, alteration of pH and degree of mechanical action will affect the level of disruption of tissue microstructure.

Mechanically separated chicken and turkey, including skin, is widely used to make processed, finely comminuted poultry products. The type of machine used as well as the properties

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of the poultry affect the chemical and structural properties of mechanically separated poultry meat (Baker et al., 1969; Angel et al., 1974). In Europe, pork skin is frequently included in meat products, but it is not widely utilized in the United States (Schut, 1978a, 1978b).

Tradition and consumer acceptance play important roles in determining the composition of various products. Certain hams and beef roasts contain little added water and less than five percent fat. Other meat products contain up to 20 percent added water or up to 70 percent fat. The wide disparity in chemical composition of various meat products indicates that research results from a specific experiment may be applicable to a relatively narrow group of products.

Heat treatment determines many of the ultimate properties of a meat product. Therefore it is often necessary to examine the product prior to and subsequent to heat application. The heat applied at the site of manufacture as well as the heat treatment applied by the consumer alters product properties.

Packaging itself affects appearance, yield and palatability of a meat product. Some products are processed in a metal mold or casing which is removed after heat processing and chilling. Product is then repackaged in a moisture and oxygen impermeable film. Other products may be processed in a heat stable film or metal can and sold directly to the consumer. The interaction between the package and the product may affect yield as well as the convenience that the consumer has in preparing the product for consumption.

Light microscopy can be used effectively to observe properties of packaging films. Ham (Fig. 1) and turkey breast (Fig. 2) are seen with their packaging films. Samples were embedded in paraffin, sectioned, deparaffinized with xylene, hydrated to water, fixed in picric acid, rinsed in water, and stained with Mayer's Hematoxylin for 15 min. at pH 3.4.

### Comminution

Products are manufactured from whole meat cuts with or without subcutaneous fat. Other products have muscles removed whole and further sectioned into large portions to manufacture sectioned and formed products or sections are reduced in size by knife, dicer or grinder to be processed to produce chunked and formed products which have a texture similar to intact roast or steak. An alternative to grinding is flaking using a Comitrol. The method and extent of particle reduction as well as the sharpness of the machine parts can have an effect on the appearance of the particles as well as other properties (Chesney et al., 1978; Hermanson, 1980; Berry, 1980).

Extremely fine comminution is accomplished using a bowl chopper or an emulsion mill. These machines break down the fine structure of muscle and fat to create a homogeneous texture for a finished meat product such as a wiener or bologna. A scanning electron micrograph (Fig. 3) shows the presence of large fat globules and smaller fat droplets embedded in a protein matrix. This photo is similar to results shown by Basgall et al. (1983). Caution must be exercised in sample fracturing, glutaraldehyde fixation and osmium tetroxide fixation to prevent the formation of artifacts. In addition, samples should be processed fresh to prevent excessive contamination with bacteria which may be visualized on micrographs as spheres with diameters of approximately 1  $\mu$ m. Bacteria are clearly seen in Fig. 4.

Fig. 5 is a transmission electron micrograph of a freeze fractured frankfurter. The sample was frozen in liquid Freon 12 at  $-150^{\circ}\text{C}$ , fractured in a Balzer 301 unit, etched for 5 minutes at  $-100^{\circ}\text{C}$  and coated with carbon-platinum to form a replica. The tissue was digested from the replica with chlorine bleach. The replica was examined with a Philips 400T operated at 80 kV.

The ability of machines to reduce particle size is highly dependent upon sharpness and setting of knives as well as temperature of product. The greatest amount of comminution, in the case of the bowl chopper, takes place with very sharp knives set in close tolerance to the bowl. In the mill, knives and plates must be very carefully sharpened and matched for maximum contact. One property that may be varied in a bowl chopper is whether vacuum is applied. Use of vacuum during comminution affects the ability of the knives to disrupt tissue microstructure. In addition, vacuum excludes air from the finished product. Absence of air produces a very dense product which has different textural and structural properties than if air were included (Solomon and Schmidt, 1980; Wiebe and Schmidt, 1982; Tantikarnjathep et al., 1983).

A treatment widely applied in Europe is to pre-emulsify fat and water with emulsifying protein prior to blending these materials into the final batter (Schmidt et al., 1982). In this case the fat, water and non-meat proteins such as dried milk, modified wheys or soy proteins are utilized to form an emulsion in the bowl chopper. Salt is often included in the pre-emulsion to prevent spoilage. Non-meat proteins in a pre-emulsion bind water and fat during severe heat treatment needed for commercial sterility of canned products. In products which have little added fat and water this treatment may not be necessary.

### Mechanical Action

Preblending is widely used to incorporate sodium nitrite, sodium chloride and water with some of the meat tissues for 24 to 48 hours prior to the manufacture of the final product (Acton and Saffle, 1969). Preblending gives meat time to combine extensively with added ions and water prior to final mechanical action. Connective tissue and sarcolemma membranes inhibit movement of ions within meat. By allowing additional time after preblending for ions to equilibrate throughout tissue, the effect of ions on tissue is more homogeneous. Sodium chloride and sodium nitrite bind to meat proteins, but migrate through meat slowly which requires adequate time and mechanical action (Solomon et al., 1980).

Blending or mixing is widely used to disrupt tissue sufficiently to allow salt, nitrite and phosphates to interact with myofibrillar proteins at the molecular level. The combination of ionic strength, pH and mechanical action assist in disrupting the microstructure of meat tissue. This disrupted microstructure interacts with the ions to cause a swelling of meat tissue (Offer and Trinick, 1983). Swelled meat tissue has an enhanced ability to retain fat and water during heat processing.

If large sections of meat are to be cured and processed, mechanical action is applied by tumblers or massagers subsequent to injection of pickle containing water and ions. Injection is essential since ions will not migrate extensively throughout a large chunk of meat. Subsequently, tumbling and massaging disrupt internal structure of meat tissue allowing ion migration and enhancing water binding capacity (Theno et al., 1978b). In

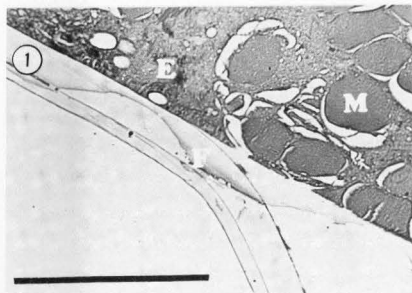


Fig. 1. Light micrograph of commercially prepared ham. The bilayer plastic laminate package is designed to cohere to the product. The plastic film (F) coheres to the exudate (E) which surrounds muscle fibers (M). Bar = 1 mm.

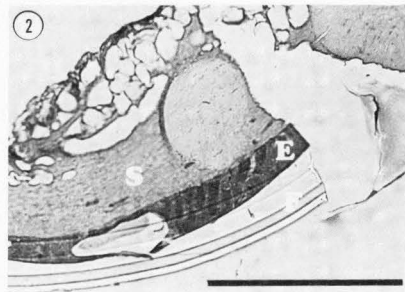
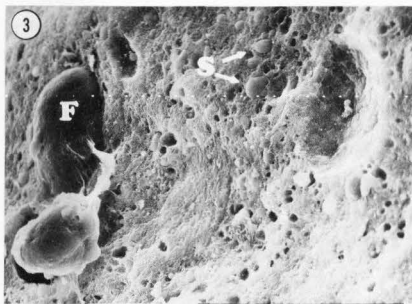


Fig. 2. Light micrograph of commercially prepared turkey breast. The four layer plastic laminate package is designed not to cohere to the skin. The skin (S) is covered with a heat coagulated exudate (E). The plastic film (F) has been dislodged during preparation. Bar = 1 mm.

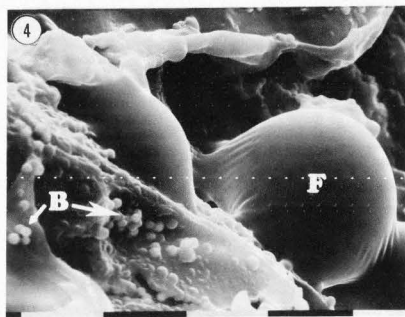


Fig. 4. Scanning electron micrograph of commercially prepared frankfurter. Sample was frozen in liquid Freon 12; fractured; fixed in 1% osmium tetroxide in ethanol at dry ice temperature,  $-56.6^{\circ}\text{C}$ ; critical point dried and sputter coated. Medium size fat globules (F) are visibly coated with some material. This product was stored for some time and bacteria (B) are visible as  $1\text{ }\mu\text{m}$  spheres. Bar =  $10\text{ }\mu\text{m}$ .

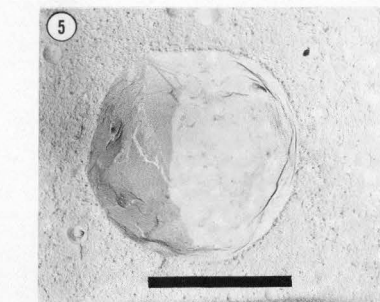


Fig. 5. Transmission electron micrograph of a carbon-platinum replica of a freeze fractured surface of a frankfurter. The major feature is a fat droplet. Bar =  $1\text{ }\mu\text{m}$ .

addition, abrasion of the surface of meat chunks produces an exudate of meat proteins. Upon heating, this exudate acts as a heat set gel to bind one meat particle to another (Theno et al., 1978a,c; Siegel and Schmidt, 1979b).

The proper combination of mechanical action, ionic environment, pH, and temperature are essential for maximum tissue swelling and heat gelling ability (Shultz et al., 1972; Shultz and Wierbicki, 1973; Siegel et al., 1978a; 1978b; Siegel and Schmidt, 1979a). An ionic strength of about 0.6, pH of 6.0 and temperature of less than 7°C contribute to maximizing protein extraction and subsequent heat set gel formation (Gillett et al., 1977; 1982; Trout and Schmidt, 1983).

### **Composition**

The appearance and palatability of a meat product is greatly affected by the level and degree of saturation of the incorporated fat (Lee et al., 1981). Beef fat is often comminuted to 20°C during processing. Poultry fat may be processed successfully at or below 0°C. The amount of fat dispersion during comminution affects the ability of protein to retain fat in the product during heat processing. The interaction of the levels of fat, myofibrillar protein, non-meat ingredients, (Kempton et al., 1982; Lauck, 1975) and the melting temperature of the fat all play an important role in flavor and texture of the finished product (Helmer and Saffle, 1963; Jones and Mandigo, 1982).

The major classes of proteins in meat are myofibrillar, stromal and sarcoplasmic. A number of model system studies investigating the fat binding ability of the three classes of meat proteins have been completed (Grabowska and Sikorski, 1976; Randall and Voisey, 1977; Samejima et al., 1969). Studies that do not include cooking of the emulsion are of questionable value. Myofibrillar proteins play a major role in entrapping fat and water in cooked meat products (Hansen, 1960; Borchert et al., 1967; Theno and Schmidt, 1978). The myosin molecules act to form a heat set matrix to entrap both water and fat (Tsai et al., 1972; Ishioroshi et al., 1979; 1980; Samejima et al., 1981). Little is known of the role of stromal and sarcoplasmic proteins in cooked meat products (Macfarlane et al., 1977). Heat breaks down the major stromal protein collagen to gelatin. If higher levels of collagenous material are utilized in meat products, pockets of gelatin are formed during cooking. This is especially true in high fat, finely comminuted meat products. However, little is known of the use of higher levels of stromal proteins in low fat meat products where the level of myofibrillar protein is more than sufficient for optimal fat and water binding.

Sarcoplasmic proteins do not form a rigid heat set gel at pH, ionic strength and temperature conditions generally used in processed meat. Upon heating, sarcoplasmic proteins tend to form a flocculent. However, sarcoplasmic proteins are important in contributing to the color, flavor and aroma of meat products.

Ions cause swelling of meat tissues during product preparation. Sodium chloride and alkaline phosphates at the proper level extract myofibrillar proteins from postmortem muscle. A lower ionic strength can be utilized in prerigor meat. If inadequate ionic strength and too low a pH is utilized, there will be a large cook loss of both fat and water during heat processing (Trout and Schmidt, 1983). In addition, the product will have a soft texture. A high ionic strength applied to lean meat during extensive mechanical action may result in excessive extraction of

myofibrillar proteins. The resulting product will form a tough skin during heat processing and be rubbery in texture. Therefore, the optimization of product composition, mechanical action, ionic environment, and heat treatment are all necessary to produce a palatable meat product.

Little is known of the effect of levels of stromal proteins on optimizing meat product texture (Saffle, 1969; Puolanne and Ruusunen, 1981; Jones et al., 1982a,b). In low fat meat products it may be desirable to include additional stromal protein to substitute for reduced fat levels so as to inhibit the formation of a rubbery texture. Non-meat proteins function in finely chopped (Cassens et al., 1975; Schmidt et al., 1982), as well as sectioned and formed meat products (Siegel et al., 1979a; 1979b; 1979c). Microstructure research in the area of stromal and non-meat proteins in meat products is certainly warranted.

### **Future Research**

Other than the work of Swasdee et al. (1982), little research has centered on the role of cooking temperature on the texture and microstructure of meat products. Our laboratory is currently investigating the role of binders to cause meat particles to cohere prior to thermal processing. Restructured meat products must either be cooked or sold in the raw frozen state to prevent structural disruption. It would be useful to develop a binder that would bind meat particles together in the raw refrigerated state. As heat would be applied another binder could function to form a heat set gel to bind the cooked meat particles together. There is virtually no research on the role of non-meat binders in raw refrigerated meat products. Gelatin may be useful in this regard and there is some microstructure research in this area (Lewis, 1981).

Most research has been done on meat products cooked to about 80°C. As heat treatment becomes more severe (commercial sterilization) additional microstructure alteration takes place (Schmidt et al., 1982). The effect of heat on meat protein gelation has not been adequately investigated. Research in the area of thermal alteration of structure of myofibrillar, stromal and sarcoplasmic proteins is needed.

Dried sausage production is extensive in many parts of the world. Additional research is needed to understand the microstructural changes that take place during this process. The role of case hardening in blocking moisture release from products should be investigated. New techniques of understanding the microstructural changes in the product during drying could assist processors in more uniform drying of sausage products.

There are certain questions that must be asked before embarking on microstructure research of meat products. One of the most basic questions is whether one should work on a complete commercial product or isolate components and work on them in a model system. Both approaches are probably valid if caution is taken in interpreting results. It is important to understand that an isolated protein may not act the same in a product when it is interacting with physiological ions, other proteins, lipids and other biochemical constituents. There are many structural components of meat products that inhibit the free movement of ions within tissue. When model systems utilize purified proteins, these inhibitors to ionic movement are removed. It may be wise to utilize various isolated components and then do additional research by combining several of these components.



## Processing effects on meat product microstructure

Research should be performed in such a way as to subject the material being investigated to as many of the mechanical, ionic and thermal treatments as are anticipated in the manufacture of the finished product. Ionic environment should be investigated within the range utilized in the product. Mechanical energy should be applied in such a way as to mimic existing or anticipated blenders or comminutors.

Additional research is needed to develop methods to accurately identify components of processed meat products (Froning et al., 1970; Coomaraswamy and Flint, 1973; Cassens et al., 1975; 1977; Ray et al., 1979; Bisgall et al., 1983). The localization of protein, lipid, water, and ions within meat products would be useful in determining functionality of these components. Transmission and scanning electron microscopy as well as light microscopy to accurately localize meat product components would assist in developing new products and optimizing composition of existing products.

## Conclusions

Research of the microstructure of meat products must consider the diversity of meat products. Species of meat utilized, product produced, degree of comminution, application of mechanical energy, composition desired, and thermal treatment of the product affect basic properties of raw and finished material. Well designed factorial experiments should be utilized to determine basic effects as well as interactions. Basic research tools should be utilized to determine which treatment effects are compatible with industrial production practices. Basic research on the components of processed meat products is useful to produce background material for application in product testing. Integration of modern research tools to determine which factors affect meat product properties should be a continuing effort of researchers.

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### Discussion with Reviewers

**D.F. Lewis:** In the "Mechanical Action" section, you mention "... The combination of ionic strength, pH ... assist in disrupting the microstructure of meat tissue." What combination of ionic strength, pH and temperature is optimal?

**Author:** A pH of 6.0; ionic strength of 0.6 with pyrophosphate present and heating from 65°C to 87°C are optimal.

**D.F. Lewis:** Are you sure sarcoplasmic proteins do not form a rigid heat-set gel?

**Author:** I have not observed this during my work at pH 5.0 to 7.0.

**R.J. Carroll:** What is severe heat treatment of pre-emulsion product as discussed under "Comminution"? How does the severe heat treatment help to improve binding of water and fat? What happens at lower temperatures?

**Author:** Since severe heat treatment for commercial sterility causes the greatest cook loss, lesser heat treatment would result in even less loss.

**K.W. Jones:** One of the basic problems with meat microstructural studies is the lack of accurate, quantifiable techniques. What's being done in this area?

**Author:** I know of no work in this area. In fact, a thorough understanding of distortion of material due to fixative techniques is not yet available.

**K.W. Jones:** Fred Ray and coworkers (see Ray et al., 1979) have developed a procedure for identifying fat in comminuted meat systems using serial sections and both SEM and LM procedures. How might other specific components (i.e., specific proteins) be identified on the SEM in meat systems without serial sectioning which may produce surface artifacts?

**Author:** The use of labeled antibodies specific to a protein may aid in their locations. Stains specific for collagen may be used in light microscopy.

**K.W. Jones:** What is being done in the area of fluorescence microscopy and specific fluorescence antibody stains in meat products?

**Author:** As mentioned in the answer to your last question, this is a possibility. However, I know of no research in this area on processed meat. Numerous references exist on the topic for fresh muscle.

**F.K. Ray:** Does sodium chloride and alkaline phosphates extract or solubilize myofibrillar proteins? I have heard this explained both ways.

**Author:** The combination of pH, ionic strength and phosphate type cause muscle tissue to swell. Mechanical action may be necessary to disrupt tissue sufficiently to cause protein solubility.

**F.K. Ray:** Is there an interaction between myosin and gelatin which affects the final bind strength of restructured meat products?

**Author:** I do not have any information on this. It is worth future research.

**F.K. Ray:** What is the difference between an emulsion and the final batter of processed meat?

**Author:** The final batter contains much more than emulsified fat. The heat set protein matrix binds emulsified fat, fat particles, connective tissue and water. The emulsion of fat may not be of much importance in cooked meat batters.

the 1990s, the number of people in the world who are undernourished has increased from 600 million to 800 million, and the number of people who are malnourished has increased from 1.2 billion to 1.5 billion (FAO 1996).

There are a number of reasons why the world's population is becoming increasingly malnourished. First, the world's population is growing rapidly, and this is putting increasing pressure on the world's food resources. Second, the world's food resources are becoming increasingly scarce, as a result of a number of factors, including the depletion of the world's fisheries, the depletion of the world's forests, and the depletion of the world's soil. Third, the world's food resources are becoming increasingly expensive, as a result of a number of factors, including the depletion of the world's fisheries, the depletion of the world's forests, and the depletion of the world's soil.

There are a number of ways in which the world's food resources can be made more sustainable. First, the world's fisheries can be managed more sustainably, by reducing the number of fish that are caught and by protecting the world's fisheries from overfishing. Second, the world's forests can be managed more sustainably, by reducing the number of trees that are cut and by protecting the world's forests from deforestation. Third, the world's soil can be managed more sustainably, by reducing the number of trees that are cut and by protecting the world's soil from erosion.

There are a number of ways in which the world's food resources can be made more affordable. First, the world's food resources can be made more affordable by reducing the number of people who are undernourished. Second, the world's food resources can be made more affordable by reducing the number of people who are malnourished. Third, the world's food resources can be made more affordable by reducing the number of people who are obese.

There are a number of ways in which the world's food resources can be made more available. First, the world's food resources can be made more available by reducing the number of people who are undernourished. Second, the world's food resources can be made more available by reducing the number of people who are malnourished. Third, the world's food resources can be made more available by reducing the number of people who are obese.

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There are a number of ways in which the world's food resources can be made more secure. First, the world's food resources can be made more secure by reducing the number of people who are undernourished. Second, the world's food resources can be made more secure by reducing the number of people who are malnourished. Third, the world's food resources can be made more secure by reducing the number of people who are obese.

# X-RAY MICROANALYSIS OF HOLLOW HEART POTATOES

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## Abstract

Electron microprobe and X-ray fluorescence techniques were used to study elemental gradients associated with the physiological disorder hollow heart in potato tubers. Gradients were found along the length and across the width of mature tubers. These were not related to the disorder, however. Tubers with advanced symptoms of the disorder had elemental levels and gradients similar to those in healthy, control tubers. The results suggest that if the disorder is initially caused by an elemental deficiency, as has sometimes been proposed, the deficiency is temporary and no longer exists in mature tubers with advanced hollow heart. Radial gradients were associated mainly with two contrasting tissues, the central pith and the surrounding perimedullary zone. Tissue differences are critical in microprobe studies involving small samples. Microprobe studies of developing tubers containing incipient stages of hollow heart, employing strip samples restricted to the central pith where the disorder originates and taken so as to traverse the small lesions, showed a dramatic increase in Mg in lesion areas. It is suggested that a nutrient imbalance may trigger the onset of the cell necrosis that characterizes the initiation of hollow heart in potato. A localized Mg toxicity or Ca deficiency due to high Mg:Ca ratio is implicated.

## Introduction

Hollow heart in potato is a physiological disorder that causes serious economic losses some years. It has been associated with sudden, very active vine growth following cool temperatures shortly after tuber set (Van Denburgh et al. 1979; Timm, 1981). Such growing conditions apparently alter the physiological balance of the plant so that foliar increases are at the expense of tuber growth. A popular theory is that certain important nutrients may become limiting, leading to the death of some pith cells (Krantz and Lana, 1942; Levitt, 1942; Kallio, 1960; Arteca et al. 1980). The histological events associated with hollow heart development, from a few necrotic cells in the pith of the young tuber to the well-known advanced stages in the mature tuber, have been described in detail (Levitt, 1942). Some results on elemental gradients in potatoes showing advanced hollow heart symptoms compared with healthy tissue have been reported (Levitt, 1942; Macklon and De Kock, 1967; Arteca et al. 1980). The present work extends these studies and takes into account the large compositional differences between pith and perimedullary tissue, a point often overlooked. Incipient as well as advanced stages of hollow heart were studied, the former being particularly important to an understanding of any nutrient imbalances associated with the disorder's inception. Electron microprobe and X-ray fluorescence techniques employed at the histological level were used for most of the analyses.

## Materials and Methods

### Plant Material

Early phases of the work were carried out on mature potatoes. These were obtained from the University of California, Davis, coordinated varietal trials. The tubers were cut in half longitudinally along the central pith and those revealing hollow heart were retained. In these mature tubers the hollow heart was nearly always of the advanced type (Figs. 1b and 1c). For each hollow heart tuber found, a healthy tuber (no hollow heart symptoms) of the same cultivar and comparable in size, shape, and pith dimensions was also retained.

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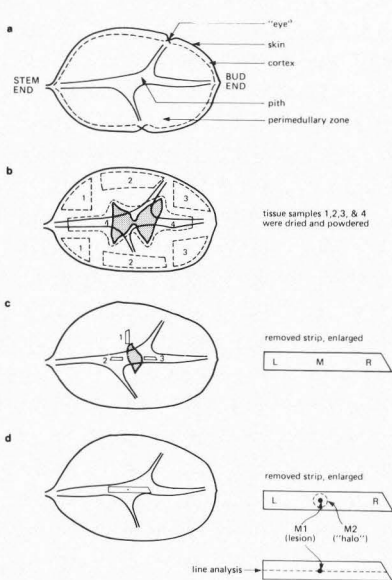


Figure 1. Schematic of potato tubers cut longitudinally along pith showing (a) major tissue parts; (b) zones (tuber with advanced hollow heart) sampled for X-ray fluorescence analysis; (c) location of strips taken (tuber with advanced hollow heart) for electron microprobe analysis, and test sites on strips; and (d) location of strips taken (tuber with incipient hollow heart) for electron microprobe analysis, and test sites on strips. Tissue samples were also taken from tubers with no visually-detectable hollow heart ('controls') at locations comparable to those shown in (b), (c), and (d).

Later phases of the work were carried out on young, developing potato tubers, cultivars Red La Soda and Pontiac. In early May, 1981, the Bakersfield area of California experienced a sudden change to higher day-time temperatures (25–30°C) following a cool period (15–20°C), during which time the tubers were still at a relatively early stage of enlargement. Tubers hand-harvested two weeks before commercial harvest operations from these naturally-stressed potato plants revealed, on cutting, a considerable number showing very early symptoms of hollow heart. For each hollow heart tuber found, a

corresponding control was selected, i.e., a tuber of the same cultivar, grown in the same location, and of comparable size, shape, and internal tuber anatomy. Typical internal anatomy of a potato tuber is shown in Figure 1a.

#### Tissue Samples for X-Ray Fluorescence Analysis

Blocks of tissue from the four main tuber zones (Fig. 1b) were removed from each tuber, utilizing both halves. Care was taken during this and subsequent steps to avoid elemental contamination of the cut tissue surfaces from hands, scalpels and other objects. After recording fresh weights, the tissue samples were dried at 70°C, weights recorded, and ground to a fine powder using a mortar and pestle. Circles 6 mm in diameter of powdered sample were attached to mylar film (untouched by hands) held in place in plastic photographic slide holders. Clear protective coating aerosol spray was used to fasten the powder to the mylar. Sheets of paper with circular cutouts placed over the mylar-slide facilitated precise application of the spray, followed immediately by application of the powdered sample. For each sample, two successive applications of spray and powdered sample ensured a uniform distribution of sample over the 6 mm circular area. Powdered samples of National Bureau of Standards Orchard Leaves were similarly prepared for use as standard reference material. Blanks consisted of 2 coatings of the aerosol spray on mylar film. The samples were analyzed using a Kevex 7000 energy dispersive spectrometer employing rhodium tube source and direct excitation mode, secondary targets Gd, Sn, Ag, Ge, Fe, and Ti and a Si(Li) detector. Data were recorded in counts per second.

To confirm the X-ray fluorescence results, powdered samples were also analyzed for the elements of interest using standard atomic absorption methods.

#### Tissue Samples for Electron Microprobe Analysis

An approximately 25 mm cube of tissue from the middle part of the tuber, containing the region of interest, was sectioned on a sliding microtome to produce slices 400 µm thick. Before sectioning the slice used for subsequent sampling, the knife blades were cleaned with distilled water and wiped dry. The slice was placed on a clean sheet of hard filter paper. Using a clean scalpel, strips 2 mm wide and 12 or 20 mm long were removed. The 12 mm long strips were taken at the sites shown in Figure 1c. These were: (1) adjacent to the hollow, in the case of the hollow heart tuber, extending radially outward into the perimedullary region; (2) along the central pith in the direction of the stem end; and (3) along the central pith in the direction of the bud end. Strips were also taken at comparable sites in healthy, control tubers. The 20 mm long strips were taken at the sites shown in Figure 1d. These were taken along the longitudinal length of the central pith traversing the small dark lesion(s) characterizing the first visible symptom of hollow heart. The entire strip was taken carefully from within the central pith core, avoiding the surrounding perimedullary tissue. Strips were also taken at comparable sites in healthy, control tubers.

To assist in identifying and orienting the strips, one end was taper-cut. Any handling, by means of forceps, was done at that end. The tissue strips were allowed to air-dry while being prepared. They were mounted on 25 mm diameter round glass microprobe slides, using electrical circuit copper print as the adhesive. The mounted samples were placed in a 30°C oven for final drying and held in a desiccator until analyzed.

Elemental spectra at a few sites on the strips (Fig. 1c and upper strip in Fig. 1d) were obtained using an ARL EMX/SM electron microprobe, with the operating voltage 15kV, beam current 300 nA, and beam diameter 35  $\mu$ m. X-ray counts for 100 sec live time were collected using a Kevex 7000 energy dispersive spectrometer. This gave preliminary information on the elements present and their relative levels at selected sites on the strips.

The entire length of some tissue strips (lower strip in Fig. 1d) was analyzed for selected elements using the more critical line analysis method. This was done with the electron microprobe with wavelength dispersive crystal spectrometers utilizing RAP (Mg K $\alpha$ ), ADP (Cl K $\alpha$ ), and LiF (Ca K $\alpha$ ) crystals.

### Results

#### Major Parts of Tuber

The dry matter content of pith tissue was much lower than that of the surrounding perimedullary tissue (Fig. 2). It changed little

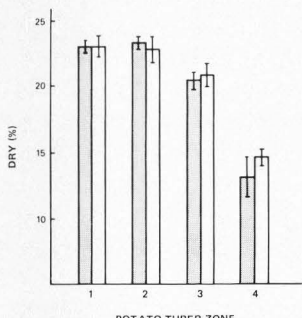


Figure 2. Dry weight content of major tuber zones of hollow heart (advanced type, Figs. 1b and 1c) and control potato tubers. Shaded bars represent hollow heart tubers; empty bars represent control tubers. Tuber zones 1-4 represent, respectively, the stem end perimedulla, mid-tuber perimedulla, bud end perimedulla, and central pith (Fig. 1b). Each bar is the mean of 9 tuber replicates, with standard error shown at top of bar.

from stem to bud end of the tuber's perimedulla, although it was slightly lower in the bud end than in the stem or mid-tuber zones. The dry matter content of hollow heart tubers was similar to that of tubers without hollow heart.

X-ray fluorescence analysis of dried powdered tissue samples from the different zones of the tuber detected the elements Cl, K, and Ca in significant quantities (Fig. 3). From stem to bud end of the tuber's perimedulla, there was a decrease in Cl and an increase in K. Calcium tended to be lower in the stem end than elsewhere. Atomic absorption analyses (not reported here) confirmed these gradients.

Elemental content of the pith was considerably higher than that of the perimedullary tissue when expressed on a dry weight basis. On a fresh weight basis, this difference was reduced considerably.

Differences in levels of detectable elements between tubers with and without hollow heart were

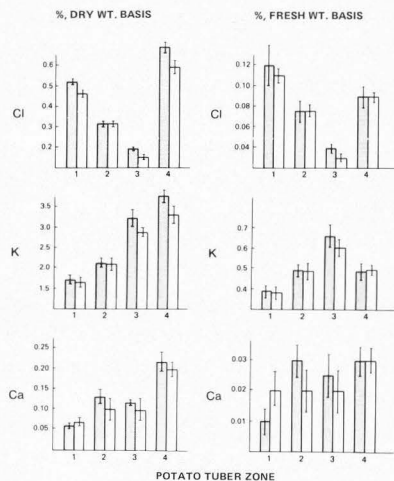


Figure 3. X-ray fluorescence analysis for elements in tissue from major potato tuber zones of hollow heart (advanced type, Fig. 1b) and control tubers. Shaded bars represent hollow heart tubers; empty bars represent control tubers. Tuber zones 1-4 represent, respectively, the stem end perimedulla, mid-tuber perimedulla, bud end perimedulla, and central pith (Fig. 1b). Each bar is the mean of 9 determinations using 9 different tubers, with standard error indicated at top of bar.

relatively minor and not significant in most cases.

#### Advanced Hollow Heart in Mature Tubers

Elements detected in significant quantities by electron microprobe analysis of tissue strips were K, Cl, S, P, and Mg. The results are shown in Figure 4. The "1" strips, taken radially from the pith region towards and extending into the perimedulla, showed a considerable decrease in level of all elements along the strip. In "2" and "3" strips, taken entirely within the central pith and extending from the middle of the tuber towards the stem and bud ends, respectively, there were no such gradients along the length of the strips. Test sites restricted to pith tissue (L, M, and R in "2" and "3" strips) or very close to pith tissue (L in "1" strips) had much higher elemental levels than those in the high starch, lower moisture perimedullary tissue (M and R in "1" strips).

Differences in elemental levels between hollow heart and control tubers were not considered significant, although levels of some elements (Cl, Mg, and K) tended to be higher in tissue samples of hollow heart tubers.

#### Incipient Hollow Heart in Developing Tubers

Amongst these field-stressed, Red La Soda and Pontiac cv. developing tubers, many were found on cutting to have hollow heart symptoms. No symptoms were detectable in tubers weighing less than 80 g and they occurred most frequently in large (>150 g) tubers. Most of these were in the early stages of the disorder. A few contained a single very small (2-3 cells in diameter) dark spot characteristic of the first visible symptom. Others had a slightly more advanced, but still early, stage consisting of a patch of small dark lesions. They were always contained within the central pith, usually about half way between stem and bud ends of the tuber. Scanning electron micrographs (Fig. 5) showed that the cells forming a lesion were distorted and had thickened walls. These and surrounding cells were deficient in starch, while cells further away from the lesion had the normal complement of starch granules for pith cells.

Preliminary elemental analyses were carried out at selected points along each strip using the electron microprobe's energy dispersive X-ray spectrometer. The same samples were then analyzed along the entire length of each strip, using the wavelength dispersive method. Results obtained by the two methods were essentially the same, therefore only wavelength dispersive results are reported. One element in particular, Mg, was present in much higher levels in and around the incipient lesions than in healthy tissue further away from the lesion area or in comparable sites of control tubers (Figs. 6, 7). Moreover, the 'halo' zone immediately surrounding the lesion (M2 in Fig. 6 depicting a single lesion sample), and areas between lesions in a multi-lesion sample (M2 in Fig. 7), had much higher Mg levels than the lesion itself (M1 in Figs. 6, 7). Other tissue strips of the same cultivar, and of certain other cultivars tested, revealed similar gradients for Mg, leaving no question as to the validity of the examples presented here.

Potassium and Cl gradients (not shown here) in the region of the lesion resembled those for Mg in that the 'halo' zone gave higher counts than the inner part of the lesion. However, there was little or no gradient in K or Cl between the lesion and healthy tissue at either end of the strip, as was the case with Mg. Calcium levels were below detectable limits using either the energy dispersive or wavelength dispersive methods of analysis with the electron microprobe.

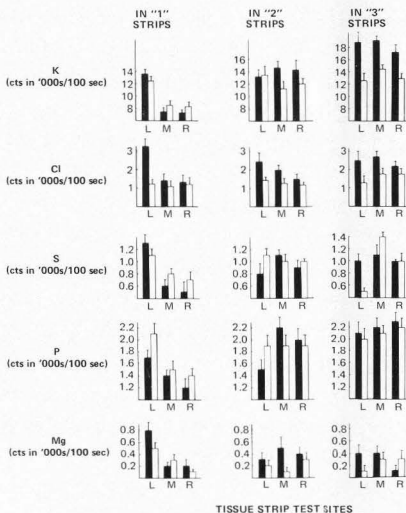


Figure 4. Electron microprobe analysis for the localization of elements in potato tuber tissue from hollow heart (advanced type, Fig. 1c) and control tubers. Tissue strips 1-3 were taken adjacent to the hollow (and comparable sites in control tubers) extending, respectively, radially into the perimedulla, along the pith in the direction of the stem end, and along the pith in the direction of the bud end (Fig. 1c). Left, middle, and right (L, M, R) sites on each strip were analyzed. Shaded bars represent hollow heart tubers; empty bars represent controls. Each bar is the mean of 11 tubers for "1" strips, 7 tubers for "2" strips, and 3 tubers for "3" strips, with standard error indicated at top of bar.

Tissue strips from comparable sites of control tubers, similarly analyzed, gave results (not shown here) almost identical to those of the healthy L and R portions of strips containing hollow heart. Not only were their levels of Mg, K, and Cl the same as the healthy ends of tissue strips containing hollow heart, but no gradients were evident along the length of the strips. As with strips containing lesions, no Ca was detected in strips from control tubers.

### Discussion and Conclusions

The dried and powdered samples used for X-ray fluorescence analysis represented relatively large areas of the fresh tuber. This method is well suited to compositional study of the tuber's major zones. The results, confirmed by atomic absorption analysis, were similar to those reported for mature potato tubers by other workers using other analytical methods (Macklon and DeKock, 1967; Johnston et al. 1968; Arteca et al. 1980). Potato tubers have elemental gradients along their length and width in response to metabolic changes that occur during their development and maturation on and off the plant (Weaver et al. 1978). Lengthwise gradients probably reflect to a large extent these metabolic changes in the tuber relative to total plant needs at a particular period in the life of the tuber. Radial gradients, while also varying with the tuber's metabolism, are mainly associated with two contrasting tissues (Mohr, 1972). The central pith (high moisture, low starch) and the surrounding perimedulla (low moisture, high starch) contain very different elemental levels, the extent of the difference depending on whether results are expressed on a dry or fresh weight basis. These fundamental tissue differences must be taken into account in any meaningful study of elemental gradients associated with an internal disorder.

Mature tubers displaying advanced hollow heart had essentially the same elemental composition as mature tubers without hollow heart. This was indicated by both analytical methods - the X-ray fluorescence method in which the tissue samples represented fairly large areas of the tuber (Fig. 3) and the electron microprobe method employing localized strip samples (Fig. 4). The gradients obtained in the tissue strips extending radially from the edge of the hollow ("I" strips in Fig. 4) are probably gradients between tissue types, and not related to specific tissue disorder. It is felt that the inclusion of more than one tissue in microanalytical studies of this kind is a source of error that has not always been fully appreciated. From the preliminary work which dealt with mature tubers, we concluded that (1) by the time tubers reach the mature stage, those with and without hollow heart do not differ appreciably in elemental composition, (2) any further attempt to establish elemental imbalances associated with hollow heart should be focused on incipient rather than mature stages of the disorder, (3) the microprobe technique should be used, with the tissue strips carefully restricted to the central pith and taken so as to include affected (lesion) and non-affected tissue, and (4) it was important to continue to work with paired tuber samples - with and without hollow heart, each pair being of the same cultivar, grown under the same conditions, and comparable in size and internal anatomy.

Our observations of the histological origin and development of hollow heart in the tuber correspond with those reported by others. The discoloration of necrotic hollow heart cells is believed to be due to melanin discoloration of

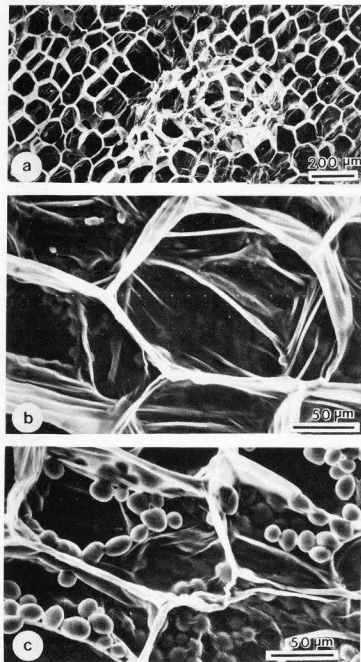


Figure 5. Scanning electron micrographs of potato tuber pith tissue with incipient hollow heart showing (a) thickened walls of a group of distorted cells forming a small lesion in middle of picture, (b) starch-deficient cells in and immediately surrounding the lesion, and (c) normal cells containing starch granules in tissue further away (4-5 mm) from the lesion.

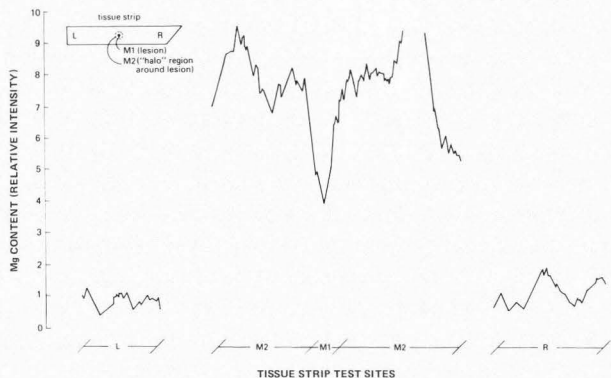


Figure 6. Segments of an electron microprobe line analysis for magnesium along the pith traversing a small lesion (Fig. 1d) characterizing incipient, potential hollow heart of potato tuber tissue (cv. Red La Soda).

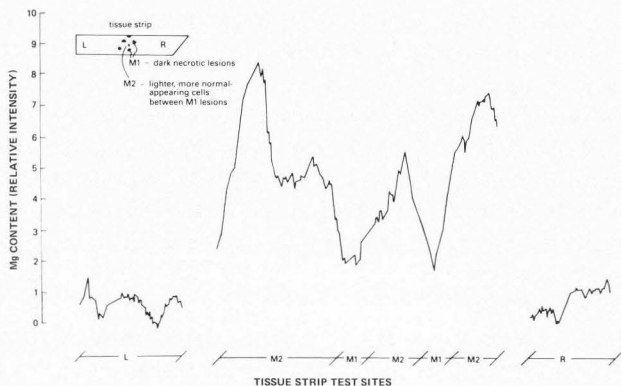


Figure 7. Segments of an electron microprobe line analysis for magnesium along the pith traversing a patch of several small dark necrotic lesions characterizing an early, potential stage of hollow heart of potato tuber tissue (cv. Red La Soda).

the cellular protoplasmic contents and cell walls, and perhaps also to accumulation of polyphenols such as chlorogenic acid and tyrosine (Reeve, 1968). The thickening of lesion cell walls results from their suberization and increased hemicellulose and pectin content (Reeve, 1968). Starch depletion in the lesion area, particularly in the cells immediately around the lesion, seems to be related to the formation of wound tissue (Ilker *et al.* 1977). It is logical that any finding of mineral imbalances in these regions could be associated with one or more of these histochemical changes.

That a mineral imbalance may be the cause of the initial necrosis which eventually leads to hollow heart has been suggested frequently in the

literature. Levitt (1942) found a lower content of all elements tested (Ca, Mg, K, Cu, Fe, and Mn) in hollow heart tubers than in controls. Arteca *et al.* (1980) found a lowered Ca content in hollow heart tubers. However, both of these studies dealt only with mature tubers. In our work with mature tubers, there was no evidence of a lowered mineral content of tissue in hollow heart tubers. This variance with the results of the above authors could be due to the particular combination of tuber growth and maturation properties involved. Our work with mature tubers suggests that if any mineral imbalance existed at an early stage of the disorder's inception, it no longer exists in mature tubers showing advanced hollow heart symptoms.



In young, developing tubers containing incipient hollow heart, the increase in Mg in the immediate vicinity of the small, forming lesions was dramatic. It seems likely that a nutrient deficiency associated with possible Mg toxicity triggers the onset of the cell necrosis in the pith; rapid growth expansion of the tuber then results in the familiar large hollow regions. Because of the well-known interaction between Mg and Ca, it may be a Ca deficiency that causes the initial cellular breakdown and necrosis. The role played by Ca in membrane and cell wall integrity is well known. In a study of bitter pit in apple, Hopfinger and Poovaiah (1979) also found a Mg:Ca imbalance and concluded that localized Mg toxicity or localized Ca deficiency causes this physiological disorder. However, it has also been documented that mineral elements and other constituents tend to accumulate in the vicinity of injured or necrotic tissue (Yarwood, 1967; Faust et al. 1968; Ford, 1979). It is possible that the observed mineral imbalances are coincidental to, rather than the cause of, the development of hollow heart. The fact that our study included lesions only a few cells in diameter lends some support to, but does not prove, the theory that mineral imbalances initiate the disorder.

Experiments involving the addition of Ca and other elements as soil fertilizers to field-grown potatoes, cv. Red La Soda, are now in progress. These may help to resolve the above question. Hopfinger and Poovaiah (1979) were able to show that bitter pit symptoms in apple fruit could be induced by vacuum-infiltrating the fruit with Mg and that they could be totally prevented by adding Ca, lending support to their conclusion that mineral imbalances caused the disorder.

Potatoes contain only about 0.002 - 0.012% Ca on a fresh weight basis, with most of that being contained in the outer parts of the tuber (Johnston et al. 1968; Bretzloff, 1971). The low levels in the pith are below the 0.05 - 0.1% range needed for detection by the electron microprobe technique used in our work thus far, which has emphasized analyses at the histological level. In future work it may be possible to follow Ca levels associated with specific cellular structures such as cell walls (using a microprobe interfaced with an electron microscope, and appropriate tissue preparation) in order to verify the role of Ca in the initiation of hollow heart.

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#### Discussion with Reviewers

N.S. Wright: If an imbalance in Mg and Ca initiates hollow heart, would you not expect some control tubers (those predisposed to develop hollow heart) to show abnormally high Mg and

abnormally low Ca in the M2 region?

**Authors:** Yes, but there is the problem of finding it. The M2 ('halo') region around the incipient lesion is small in area relative to the total region of the pith in which the lesion could develop. Furthermore, the incidence of hollow heart in a given tuber population may not be high when one wants to study it, so that even exhaustive analysis of the pith of some tubers does not necessarily prove anything. The tubers tested might not be the ones to develop hollow heart, or might not be at the stage of tuber development to show it. We did not detect an imbalance between Mg and Ca in any of our analyses of control tubers, but these analyses were limited in number. The approach mentioned in your question is sound and should be investigated further. By testing all parts of the central pith of developing tubers of susceptible cultivars that have received stress conditions usually resulting in hollow heart, it may be possible to detect a Mg:Ca imbalance corresponding with a potential M2 (or M1) region if, in fact, the imbalance precedes the lesion.

**D.A. Walker:** Why would the relatively high Mg level be restricted to the region immediately surrounding the lesions?

**Authors:** The 'halo' region immediately surrounding the lesion probably reflects gradients in relation to the necrotic cells, with the lesion as the locus. For example, the lesion could change the pH and, therefore, the solubility of various elements (Ca being very susceptible to pH changes).

**D.A. Walker:** What would account for the mineral imbalance in the early stages of the disorder being rectified in the mature tuber?

**Authors:** The large split or hollow region present in the mature tuber is believed to be due to continued expansion of the tuber after the dead cells of the incipient lesion in the developing tuber are no longer capable of enlarging. The disorder tends to develop in large-sized tubers. Expansion of the tuber would tend to deplete, or physically 'dilute', a previously existing mineral imbalance.

**D.A. Walker:** What factors would contribute to possible Mg toxicity or Ca deficiency?

**Authors:** Any number of stress factors, especially those affecting root growth activity, could affect Mg and Ca source-sink relationships. Temperature stress or water stress (deficiency or flooding) are among these. Also, because of its smaller atomic weight, Mg is more mobile than Ca and, when Ca is deficient, Mg may readily substitute for it.

**D.A. Walker:** Would a difference in water content between pith and perimedullary region account for the dissimilarity in elemental composition when measured by dry versus fresh weight?

**Authors:** The water component of plant tissues such as potato tuber is normally low in Mg and Ca, these minerals generally being present in association with cellular structures. Therefore,

the water component per se should have little or no direct bearing on the pattern of results. The other major component of potato tubers is the starch granules which make up a large part of the dry weight. Perimedullary tissue contains considerably more starch than does pith tissue. The mineral content of starch granules from several plant sources tends to be rather low (Gracza, 1965). Presumably, the Mg and Ca content of potato starch is low too. This would tend to lower the levels of these minerals present in the perimedulla as compared with the pith, especially when calculated on a dry weight basis. Variations in mineral levels of the starch granules from the pith versus perimedulla might also play a role in affecting the results.

**N.S. Wright:** Can you envisage your methods being used to determine the predisposition to hollow heart of potato seedlings?

**Authors:** Not at this stage of the work. Before considering this application, it should be confirmed that the mineral imbalance exists before, and is therefore the probable cause of, the formation of the lesion. One way of investigating the latter point might be to treat susceptible pith tissue with added Mg and Ca, as Hopfinger and Poovaiah (1974) did in their study of the physiological disorder bitter pit in apple fruit. These authors showed that bitter pit symptoms could be induced by vacuum-infiltrating the fruit with Mg and that they could be totally prevented by adding Ca, lending support to their conclusion that mineral imbalances caused the disorder.

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DETERMINATION OF ELEMENT CONCENTRATIONS IN FRESH AND PROCESSED  
VEGETABLES BY QUANTITATIVE X-RAY MICROANALYSIS

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Abstract

Within the cell walls and cytoplasm of fresh, blanched, boiled and rehydrated carrots and green bean pods the element concentrations were measured by energy dispersive X-ray microanalysis in the scanning transmission electron microscope. Based on a set of calibration standards which had been prepared before, the element concentrations were calculated in  $\text{mg/kg}$  wet weight. Fresh green bean pods and fresh carrots do not differ in the kind, but in the concentration of the elements they contain. In the course of blanching and boiling, elements are gradually removed from the tissues. The concentrations measured in the rehydrated tissue correspond to those found in the blanched or boiled samples. That means, a further leaching of elements during rehydration did not take place, except for potassium which is leached from the tissue to a very large extent into the rehydration medium.

Introduction

Since carrots and green bean pods have recently been put on the European market more and more in the form of dried vegetables, e.g., as ingredients of bag soups, the influence of hot-air drying technology on the quality of the rehydrated, edible product has attracted the increasing attention of food technologists and biologists. It has become evident that during the processing of vegetables (blanching, boiling, dehydration and rehydration) alterations take place within the samples that concern both the morphological and chemical constitution of tissues and cells. An investigation of the basic changes in morphology on an ultrastructural level (maceration of the tissue, cell wall swelling, clumping and dislocation of cytoplasm) was performed as previously described by Grote and Fromme (4,5).

The present paper deals with alterations in the chemical constitution of processed vegetables as expressed by differences in the elemental composition. Advances in the electron microscopic method of X-ray microanalysis, together with the development of techniques such as freeze-drying at very low temperatures, have made it possible in the last few years to determine elements qualitatively and quantitatively with an atomic number  $>10$  in very small areas (approx. 100 nm, analyzing semi-thin sections by STEM). A synopsis of these techniques is given by Van Steveninck and Van Steveninck (18) as well as by Ingram and Ingram (9).

As an important prerequisite for quantitative evaluation of the elemental content one has to use calibration tools. One of these could be the preparation of suitable standards which allow the establishment of calibration curves for the elements of interest.

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Key words: Carrots, green bean pods, X-ray microanalysis, fresh and processed vegetables, analytical electron microscopy.

Materials and Methods

$$R = I_{sp} / C_{sp} \cdot I - Cf \quad (1)$$

Preparation of standards.

The preparation of calibration standards was described elsewhere in detail (6). For most standards, we used the method introduced by Spurr (17) but had to modify the procedure to a considerable extent (12).

In short: 1:1 stoichiometric quantities of a suitable salt, e.g. KSCN (Merck No.5125) or NaSCN (Baker No. 1778) and a macrocyclic polyether, dicyclohexyl-18-crown-6 (Fluka No. 36665), were dissolved in absolute methanol. For obtaining Mg- and Ca-standards we dissolved  $Mg(SCN)_2$  (Riedel-de-Haen No. 13146) and  $Ca(SCN)_2$  (Riedel-de-Haen No.12061), respectively in the bicyclic complexing agent Kryptofix 221 (Merck No. 16646). These standards could serve as well as sulphur standards.

After evaporation of the methanol in a rotation evaporator, a highly viscous substance was left. Since it was difficult to add defined quantities of this complex to appropriate amounts of Spurr's medium we prepared a series of graded dilutions of this complex with Spurr's resin without knowing the exact concentration of the element content in this mixture (see below and (6)). For obtaining phosphorus standards different quantities of triphenylphosphine (Baker No. 14852) were directly dissolved in chlorine-free Spurr's resin according to Roomans (12). Chlorine standards were prepared by varying the amount of the flexibilizer DER 736 in the complete resin mixture.

Moreover, we had to modify the composition of the medium by not adding the accelerator, S1, before the complex had been completely dissolved (after stirring for several hours at 312 - 323 K). Otherwise, by using the complete resin mixture right from the beginning of the dissolution process, polymerization would take place before the complex had been completely and uniformly dissolved (see also (12)). This would result in inhomogeneous preparations which could not be used for calibration purposes.

Since the exact element concentrations of the resin blocks prepared in this way were unknown, we had to carry out chemical analyses of each block. The chemical determination of the element concentrations was done by flame photometrical methods (6,10). From the remainder of the block dry sections of approx. 1µm were cut and placed for X-ray analysis on Pioloform® coated one-hole copper grids (hole diameter 1 mm).

Calibration curves were established using a formula developed by Hall (7):

( $I_{sp}$  = characteristic radiation of the specimen after background subtraction,  $C_{sp}$  = continuum radiation of the specimen and the supporting film in a selected "white window",  $Cf$  = continuum radiation of the supporting film in the "white window").

Results were expressed in mM/kg cell wall and cell cytoplasm fresh weight, respectively.

Preparation of tissues.

Fresh, blanched, boiled and dried samples of the roots of carrots (cv."Zino") and the pods of green beans (cv."Cascade") were prepared at the Institute of Food Technology, Dept. of Fruit and Vegetables Technology, Berlin, as part of the correlated AIF research project No. 3664 (Bielig et al.(1), Bielig and Schwaiger (2)). Carrots and green bean pods were harvested from vegetable gardening where they had been grown to edible maturity for purposes of food industries. The vegetables were chopped, blanched for different times and dried in a hot-air drying device (1). They were rehydrated by cooking to "doneness" according to reports from the Berlin Institute of Food Technology where the optimum cooking time for each sample had been previously determined by sensory and texture tests.

For determination of the elements cubes (1 mm<sup>3</sup>) of tissue (pods of green beans and phloem of carrot roots) were rapidly frozen by immersion in nitrogen slush (approx. 63 K) and transferred to the precooled cooling plate of a low-temperature freeze-drying system (GT 1, Leybold-Heraeus Company, FRG). Freeze-drying was extended under vacuum ( $5 \times 10^{-4}$  Pa) over a period of 7 days at 173 K. Still under vacuum the samples were embedded in Spurr's low-viscosity medium (16) which was made chlorine free by replacing the original flexibilizer DER 736 by dibutylphthalate (8).

After a two days impregnation under vacuum at room temperature polymerization was carried out under normal pressure at 343 K. Dry sections of 1µm thickness were cut on an ultramicrotome (OM U3, Reichert Company, Austria) and placed upon one-hole copper grids as had been done with the sections from the calibration standard resin blocks.

Analytical evaluation.

The dry sections were analyzed in a H 500 transmission electron microscope (Hitachi Company, Japan) equipped with the scanning attachment H 5010, and the energy dispersive X-ray microanalysis system PGT 1000 XCEL (Princeton Gamma

Tech, USA) operated at 75 kV in the STEM mode. The integrated beam current was 25  $\mu\text{A}$ . The beam diameter was estimated to be  $< 10 \text{ nm}$  because in a lateral resolution test in the STEM it was possible to resolve details  $< 10 \text{ nm}$  operating with the same beam diameter, used for analysis. That means the analysed area of the tissue should be in the order of approx.  $100 \text{ nm}$  (see "Discussion").

The Si(Li)-detector of 145 eV energy resolution has an active area of  $30 \text{ mm}^2$  and is positioned at a distance of  $13 \text{ mm}$  to the specimen with a take-off angle of  $68^\circ$ .

For X-ray microanalysis the width of the autodefined elemental windows were preset for 1.2 times the full width at half peak maximum (FWHM-value) regardless of the respective X-ray energy. 20 eV were chosen for channel width. Each analysis took 300 sec live time. Background subtraction was carried out manually using pre-stored background markers.

As the detection of Ca in the presence of large quantities of K is difficult the calcium content was determined by correction using the empirically established formula:

$$\text{Caconc} = \text{Ca}(K\alpha) - K(K\alpha) / 12 \quad (2)$$

Element concentrations were measured separately within each of the two main compartments of the cell, the cell wall and the cytoplasm. There is no danger of interference from cytoplasmic element content to that of the cell wall since the cytoplasm in rehydrated samples as well as in boiled and blanched specimens is separated from the cell wall and clotted within the cell lumen. Analyzing fresh carrot and green bean pericarp tissue, cytoplasmic compartments which were situated in a thin layer near the cell wall were omitted. Since it proved difficult in fresh material to differentiate between cytoplasmic organelles in  $1 \mu\text{m}$  dry sections even if they could be recognized in ultra-thin sections, the points of analyses were randomly chosen. In processed material cytoplasmic details were generally destroyed (4).

From both vegetables (carrot roots and green bean pods), fresh, blanched, boiled and rehydrated samples were analyzed in at least 10 parallel measurements. The amounts of elements were determined as described by Grote and Fromme (6). The results are given in mM/kg of cell wall wet weight and cytoplasm wet weight, respectively, assuming that Spurr's resin has a density of approx. 1. The concentrations of elements in the blanched, boiled and rehydrated samples are expressed as percentages of those found in fresh tissues.

## Results

Qualitative analyses had shown the presence of potassium, calcium, and magnesium within the cell wall, whereas the cytoplasm was characterized by the presence of potassium, calcium, chlorine, sulphur and phosphorus. The concentrations of sodium were too low to be detected by our X-ray analysing system.

### Cell walls of carrot roots.

The concentrations of elements measured in the cell walls of fresh and processed carrot tissues are shown in Table 1.

The values demonstrate a gradual decrease in elemental concentration as a function of prolonged blanching. The boiled tissue contains approximately half the concentration (potassium, magnesium) of the fresh tissue. The loss of calcium, however, is more distinct. Rehydration after drying leads to a drastic reduction of the potassium concentration. Calcium and magnesium, on the other hand, do not show an additional loss of concentration after rehydration. Their concentrations range between those of the blanched samples (6 min) and the boiled tissues. As the values demonstrate, prolonged blanching cannot be clearly correlated with the decrease of elements.

### Cytoplasm of carrot root phloem cells.

Elemental analyses within the cytoplasmic compartment of carrot root phloem cells showed the results listed in Table 2.

As within the cell wall, the concentrations of the elements present in the cytoplasm are gradually reduced as a function of prolonged blanching. Boiled tissue has the same (calcium, sulphur) or a reduced concentration (potassium, chlorine, phosphorus) in comparison with the samples blanched for 15 min. The rehydrated tissues show concentrations that may considerably range over those measured in the boiled samples (potassium, calcium, sulphur: blanching time 10 min; calcium, phosphorus: blanching time 6 min). On the whole, the concentrations of elements in the cytoplasm of the rehydrated cells correspond with those found in the tissue after short blanching. There is no obvious correlation between blanching and elemental concentrations in the rehydrated tissues.

### Cell walls of the pericarp of green bean pods.

The elemental concentrations determined within the cell walls of fresh and processed green bean pods are given in Table 3.

As demonstrated by the results, the concentrations of potassium, calcium and magnesium generally decrease during blanching and boiling of the tissue. The



**Table 1.** Concentrations of elements in the cell walls of fresh, blanched, boiled and rehydrated carrot roots (in mM/Kg of cell wall wet weight).

Tissue type	Blanching time(min)	K %	Ca %	Mg %	Cl %	S %	P %
Fresh	-	326	100	117	100	12	100
Blanched	6	267	82	93	79	11	92
	10	246	75	86	74	6	50
	15	233	71	68	58	6	50
Boiled	-	134	41	25	21	5	42
Rehydrated	6	12	4	64	55	10	83
	10	26	8	47	40	6	50

**Table 2.** Concentrations of elements in the cytoplasm of fresh, blanched, boiled and rehydrated carrot root phloem cells (in mM/Kg of cytoplasm wet weight).

Tissue type	Blanching time(min)	K %	Ca %	Cl %	S %	P %	
Fresh	-	69	100	28	100	47	100
Blanched	6	17	25	12	43	4	100
	10	33	48	12	43	3	75
	15	20	29	11	40	2	50
Boiled	-	13	19	11	40	1	25
Rehydrated	6	10	14	19	68	2	50
	10	20	29	15	54	2	50

**Table 3.** Elemental concentrations in the cell walls of fresh, blanched, boiled and rehydrated green bean pods (in mM/Kg of cell wall wet weight).

Tissue type	Blanching time(min)	K %	Ca %	Mg %	Cl %	S %	P %
Fresh	-	273	100	117	100	230	100
Blanched	6	97	36	36	31	117	51
Boiled	-	46	17	30	26	133	58
Rehydrated	6	13	5	79	68	79	34
	9	12	4	63	54	85	37
	12	19	7	50	43	100	43

**Table 4.** The concentrations of elements within the cytoplasm of fresh, blanched, boiled and rehydrated green bean pod cells (in mM/Kg of cytoplasm wet weight).

Tissue type	Blanching time(min)	K %	Ca %	Cl %	S %	P %
Fresh	-	92	100	50	100	30
Blanched	6	43	47	30	60	6
Boiled	-	30	33	13	26	10
Rehydrated	6	5	5	7	14	7
	9	5	5	6	12	10
	12	15	16	8	16	7

decrease of potassium, however, is greater than that of calcium or even magnesium. Drying and rehydration once more reduces the potassium concentration, whereas the concentrations of calcium and magnesium range considerably above (calcium) or somewhat below (magnesium) those measured in the boiled tissue. As in carrots, there is no correlation of the concentration of elements in the rehydrated samples with different blanching times.

#### Cytoplasm of green bean pod cells.

Concentrations of elements determined in the cytoplasm of fresh and processed green bean pods are shown in Table 4.

As within the cell wall, the concentrations of elements are reduced within the cytoplasm of blanched and - still more - boiled tissue. The sulphur content of the boiled samples, however, ranges above that of the fresh tissue. Rehydrated vegetables show a drastically reduced potassium concentration, whereas the calcium and chlorine concentrations correspond to those found in the boiled tissue. Sulphur and phosphorus, however, are characterized by quite variable concentrations, which may even range (sulphur and phosphorus: rehydrated tissue, blanched for 6 min) above that of the fresh tissue. A distinct relationship of elemental content with blanching time cannot be established.

#### Discussion

##### Analytical processing.

The use of crown-ether complexes dissolved in epoxy resin was first proposed by Spurr (17). With the above described modifications of the procedure, it has proved in our case a successful approach to the quantitation of element concentrations. The main difficulty seems to be the low solubility of the salt complexes within the Spurr's resin as other authors (8,12) mentioned at nearly the same time. For dissolving the complex the temperature of the mixture has to be increased but in order to prevent an undesired prepolymerization one has to add the accelerator S 1 after the complete dissolution. Thus one can obtain very homogeneous blocks from which analytical data could be established with standard deviations of 4.9 to 7.7 %.

The determination of the actual molarity of the calibration resin blocks by an independent analytical method has also been practised by other authors (8,14). The problem in this procedure concerns the disintegration process, e.g. for flame-photometric analyses since there is the possibility of loss of volatile elements. In our case we used a special disintegration method (10) by which the preservation of all elements is rather good. The reliability of our

calibration curves is documented by regression coefficients of 0.9886 (potassium) to 0.9981 (calcium).

Concerning the analysed volumina of the vegetable tissues and - in this context - the spatial resolution of the analytical results we assumed a beam broadening within the 1  $\mu$ m thick sections of about 10 times. From experimental observations of Reimer and Pfefferkorn (11) and Gentsch et al. (3) it seems to be more realistic to calculate a 6 to 8 time broadening. In our own attempts to estimate the diameter of the beam used for X-ray analysis we established a similar model (3,11). Evaporated gold islets covering the surface of a 1  $\mu$ m Epon section could laterally be resolved in the range of <10 nm (see above). The gold islets on the reverse surface could be imaged with a resolution power of <50 nm, thus indicating that the incident beam had a diameter of <10 nm whereas the penetrating beam showed a <50 nm diameter. Monte Carlo calculations by Russ (13), however, show a significant stronger broadening of the beam, but using an acceleration voltage of 40 kV, whereas the analytical results presented in this paper were obtained in STEM mode operated at 75 kV. Moreover, the analysed areas in the respective cell compartments were chosen so far from one another that there is no danger of interference of the analytical values.

#### Analytical results.

When comparing the analytical concentrations found in the present study in fresh carrots and green beans pods with those from overall chemical analyses reported in literature (15), one has to take several specific aspects into consideration. Firstly, there is no possibility for separate determination of the ion content of the vacuole and the cytoplasm, respectively. The sublimation of ice during freeze drying of fresh tissues will cause a precipitation of salts - formerly dissolved in the vacuole fluid - onto the tonoplast and there is no reason to assume that some of the re-precipitated salts will be re-dissolved by the infiltrating embedding medium. Similar considerations apply to the preparation of boiled and/or blanched samples. By these procedures the cytoplasm is denatured and clotted within the cell lumen formerly occupied by the vacuole fluid. Thus one has to assume that the elemental concentrations measured in cytoplasmic regions may consist to a certain degree of elements from the vacuole fluid (if some of the elements should remain after boiling and/or blanching). Secondly, the concentrations from overall chemical analyses have to be lower than those found in the cytoplasm and the cell wall, respectively, namely in a dilution proportional to the relation of the whole cell volume to the volume of the vacuole.

Thus, if one assumes that the volume of the vacuole is usually more than five times bigger than that of cytoplasm and cell wall in parenchymatous plant cells and that it predominantly consists of water, a dilution factor of at least five of the element concentrations measured in cytoplasm and cell wall has to be taken into consideration. As preliminary calculations showed the values then obtained are in rather good agreement with those from chemical overall analyses reported in literature. So Souci and Bosch (15) reported potassium concentrations in fresh carrot root tissues of 72 mM/kg while our results showed 326 mM/kg K in the cell wall and a potassium concentration of 69 mM/kg within the cytoplasm. In this context it is certainly desirable to analyse the elemental content of the vacuole fluid e.g., by X-ray microanalysis of frozen hydrated sections in order to get a more adequate comparison between microanalytical and overall chemical results. The microanalytical approach, however, has the advantage of correlating morphological with analytical information thus enabling a deeper and more refined understanding of the mechanisms involved.

So the above results show that there are differences in the concentration of elements, not only between carrots and green beans, between the fresh, blanched and boiled tissue, between cell walls and cytoplasm on the one hand and the rehydrated tissue on the other, but also in the reaction of the elements to the influence of technological procedures.

Firstly, there are differences between the two vegetables concerning the concentration of certain elements. Thus fresh carrots contain only 5 % of the magnesium (cell wall), 13 % of the chlorine and 50 % of the calcium concentrations (cytoplasm) found in the corresponding compartments of fresh green bean pods. The concentrations of potassium, sulphur and phosphorus largely agree in both vegetables.

Secondly, the two types of tissues, fresh and dried, react differently to the ion-solubilizing influence of (hot) water.

Whereas fresh tissue shows increasing loss of elements due to a prolonged action of hot water, dried tissue, apart from the initial loss of elements by blanching, does not suffer an additional decrease in elemental concentration by the action of hot water during rehydration (but see potassium below). Concentrations in boiled or rehydrated tissue that exceed those of the fresh samples must be considered as misleading. They may result from precipitation of salts occasionally occurring within the cytoplasm of processed plant cells. It is probable that by the evaluation of a great number of measurements throughout the cytoplasm these variations in the chemical

composition would be reduced.

The morphological and/or biochemical basis of this "drying phenomenon" is not yet clear, but it seems possible that during the drying process fibrillar and membranous components of cell wall and cytoplasm become so firmly attached that they form barriers to the migration of ions during rehydration. Thus, from the nutritional point of view, the drying of vegetables as means of food conservation must be considered as a favourable process.

Finally, the results show that the elements determined may be divided into two groups, potassium on the one hand and the rest of the elements on the other. In contrast to calcium, magnesium, chlorine phosphorus and sulphur, potassium to a very large extent disappears during the rehydration of the dried tissues. Thus, concerning potassium, drying does not seem to establish a barrier against the migration of potassium ions into the boiling medium.

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ELECTRON MICROSCOPIC INVESTIGATIONS OF THE CELL STRUCTURE IN FRESH AND PROCESSED  
VEGETABLES (CARROTS AND GREEN BEAN PODS)

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Abstract

The cell structure of fresh, blanched, boiled, dried and rehydrated tissues from carrot roots and green bean pods was examined in the scanning and/or transmission electron microscope. The secondary phloem of carrot roots represents a typical plant storage parenchyma characterized by a high starch and lipid content. Green bean pods show many characteristics of assimilation tissue (e.g. chloroplasts), but they also contain a considerable amount of starch. Blanching, boiling, de- and rehydration affect the texture of both vegetables in a similar way: swelling of cell walls, maceration of tissue during blanching and boiling coupled with a granular denaturation of cytoplasm. Drying leads to a shrinkage and twisting of the cells and clumping of the cytoplasm. Rehydrated tissue is characterized by strong cell wall swelling, maceration, and clumping of cytoplasm. Morphometric measurements of cell wall thicknesses after rehydration showed that various food technological process parameters may strongly influence the appearance of the rehydrated product.

Introduction

Dried carrots and green beans are traditional ingredients of bag soups which have become more and more popular on the European market. In comparison with genuine instant products these bag soups are characterized by comparatively long cooking times which can be influenced - within certain limits - by varying process conditions during the production of such dried vegetables. The aim of industrial research now is to find suitable process conditions in order to produce dried vegetables which need extremely short cooking times and are similar to well cooked fresh vegetables as regards taste and consistency. In this sense we tried to evaluate the relationship of process conditions to cell ultrastructure during the production of dried vegetables and the ultrastructural alterations which occur during the rehydration process (i.e. cooking) by comparing them with the ultrastructural morphology of well cooked fresh products. It is hoped by understanding these interrelationships that process conditions can be optimized to obtain dried and rehydrated vegetables of high quality.

There exists large literature on the morphological structure of both vegetables, carrots and green beans. Most of them were carried out with the optical microscope, but in recent times the improved resolution power of the electron microscope was also utilized. Thus optical microscopic studies in the developmental anatomy of the root of *Daucus carota* were carried out by Havis (14), Esau (9) and Drake and Selstam (7, 8). Reck (18) published a detailed paper on the optical microscopical structure of the plastids in the root.

Scanning electron micrographs of raw and cooked carrot tissue are found in the studies by Davis et al. (3 - 6). Transmission electron microscopical papers on the roots of *Daucus carota* chiefly deal with some cytological problems such as the structure of chromoplasts (11), nuclei (15) or the in vitro culture of carrot cells and tissues (28). (For a more detailed list of literature see the above mentioned papers).

Most of the morphological studies in green bean pods that have so far been carried out were also done with the optical microscope. The first to examine the histological structure of the legumen were Kraus (16) and Steinbrink (25). Like many of the following authors, some of whom applied modern techniques such as polarisation microscopy or X-ray diffraction, they put their emphasis on the morphology of mature pericarps, especially on the structure of the vascular bundles and fibres in

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order to investigate the dehiscence mechanism of the pods (10, 13, 17).

Roth (22) treats the anatomy of the legumen as part of her comprehensive work on the anatomy and histology of the fruits of angiosperms. Gassner (12), who deals with green beans within the scope of a general optical microscopical investigation of vegetable foodstuffs, also gives some histological information.

This is even more the case with the detailed studies by Reeve and Brown (21), who provide a survey of the developmental sequence and differentiation of pod tissues and their structure and composition at edible maturity.

Optical microscopic studies of the morphological changes occurring in plant tissues during preparation processes, such as blanching, cooking, and dehydration/rehydration, have been published by a number of authors.

Thus Simpson and Halliday (24) studied the disintegration of membrane material in vegetables during the cooking procedure. Reeve and Leinbach (20) and Sterling (26) examined the effect of moisture and high temperature on carrot, potato, and apple tissues. Reeve (19) published a microscopical study on textural structural changes in fresh and processed fruits and vegetables.

The effects of drying and rehydration on cellulose crystallinity of carrots were investigated by Sterling and Shimazu (27). (For more detailed literature see the above mentioned papers).

Transmission electron microscopy has so far not been employed in a systematic fine-structural investigation of prepared and processed vegetables.

Likewise, the application of morphometrical techniques in combination with transmission electron microscopy of vegetables has so far not been reported in literature.

In the present study we first want to present the basic morphological changes involved in the blanching, cooking, dehydration and rehydration of carrots and green beans without correlating them to special food technological process conditions. The ultrastructural appearance of the fresh, unprocessed cells and tissues serves as a baseline for structure.

In the second part of the present paper, we want to analyse systematically the influence of special process conditions on the degree of cell wall swelling, which – in our opinion – is the most conspicuous feature of dried and rehydrated carrots and green beans.

#### Materials and Methods

Two varieties of green beans ("Koralle", "Cascade") and of carrots ("Bauer's Kieler Rote", "Zino") were analysed. Blanching, drying and storing of the vegetables were carried out at the Institute of Food Technology – Fruit and Vegetables Technology – in Berlin as part of a correlated research project (AIF project No. 3664). Some of the results of this project were published by Bielig and Schwaiger (1). For drying under defined and reproducible conditions, a hot-air drying system previously designed was used (Bielig et al., 2). The dried samples were rehydrated by cooking to "doneness" according to reports from the Berlin Institute of Food Technology where the optimum cooking time for each sample had been previously determined

by two different methods: by a sensory test of doneness and by measuring the shear strengths of the samples mechanically (For further details cf. Bielig and Schwaiger [1]).

For transmission electron microscopy (TEM), from fresh, blanched and rehydrated tissues (rehydration by boiling) cubes of approx. 1 x 1 mm size (from the secondary phloem of the carrot root and the outer and inner part, respectively of the green bean pod) were cut with a sharp razor blade and fixed in 2% phosphate buffered glutaraldehyde (pH 7.3) for two hours at room temperature. After a short washing with the buffer solution, the samples were postfixed for two hours in buffered 1% OsO<sub>4</sub> solution. They were dehydrated in a graded series of ethanol. In 70% ethanol, block staining with phosphotungstic acid/uranyl acetate was carried out. After embedding the samples in Epon resin, ultrathin sections were cut with a diamond knife using a Reichert ultramicrotome OmU3. Sections were stained with lead citrate and examined in a Siemens Elmiskop I.

The specimens dried by hot air were briefly immersed in ethanol and then embedded in Epon resin, because it had proved impossible to obtain sections from specimens directly embedded in the resin.

For scanning electron microscopy (SEM), fixation and dehydration were carried out as described above. After the 100% ethanol stage, the samples were critical-point-dried in Freon 13 using Freon 11 as a transitional fluid. The dried specimens were mounted on specimen holders with "Leit-C", coated with gold by sputtering and observed in a STEREO-SCAN Mk I.

For morphometric measurements of cell wall diameters in TEM the semi-automatic image analysis system MOP AM/03 (Kontron, Germany) was used. In each test 10 - 15 separate measurements were made on at least three parallel samples from three different experiments, and the mean values were calculated. For obtaining comparable results, the points of measurement on cell walls were defined as follows: midway between two intercellular spaces taking only those cell walls into consideration which were not (yet) separated along their middle lamella.

Since the morphological appearance of the boiled raw material was considered to be the "ideal" for a rehydrated vegetable, the measured cell wall thickness of the boiled raw tissue was put at 100. Cell wall alterations in dried and rehydrated samples were expressed as percentages of this value.

#### Results

##### Ultrastructural aspects of fresh and processed vegetables

SEM of fresh carrot tissues. The critical-point-dried carrot exhibits a very good preservation of its histological structure (Fig. 1). The tissue consists of polygonal parenchymatous cells and shows many intercellular spaces.

At higher magnification, details of the cytoplasmic layer become visible. The rather homogeneous cytoplasm includes particles of variable shape and size, which by comparison with transmission electron microscopic analyses can be identified as different cell organelles. Within the cells of fresh carrots, roundish-shaped inclusions of approximately 5 µm diameter are seen, which have to be interpreted as starch grains or big chromoplasts. Round particles of much smaller size (1 - 2 µm

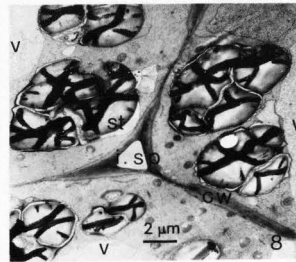
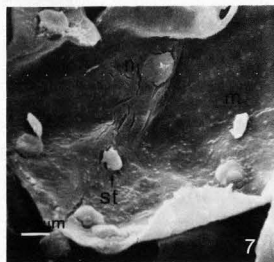
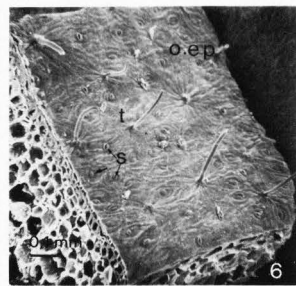
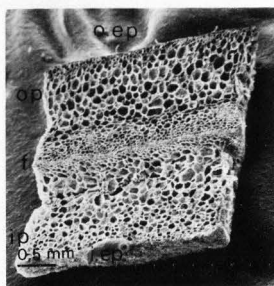
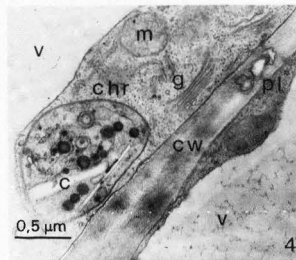
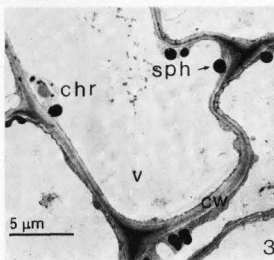
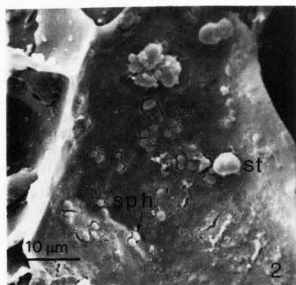
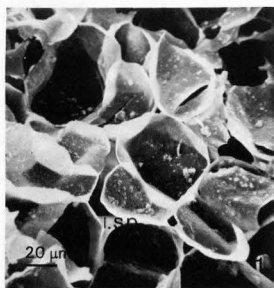


Fig. 1 – 4: Electron micrographs of fresh carrot tissue

Fig. 1: Phloem parenchyma cells at low magnification in SEM (i.sp=intercellular space)

Fig. 2: Cytoplasmic layer of a single cell in SEM (sph=spherosome, st=starch grain)

Fig. 3: Survey of phloem parenchyma tissue in TEM (sph=spherosome, chr=chromoplast, v=vacuole, cw=cell wall)

Fig. 4: Detail from cell wall and cytoplasm in TEM (v=vacuole, m=mitochondrion, g=golgi apparatus, chr=chromoplast, c=carotin pigment, cw=cell wall, pl=plasmodesm)

Fig. 5 – 8: Electron micrographs of fresh green bean pod tissue

Fig. 5: Cross section through one entire valve of the pod in SEM (o.ep=outer epidermis, i.ep=inner epidermis, op=outer parenchyma, ip=inner parenchyma, f=fibre layer)

Fig. 6: Outer epidermis of the valve with trichomes and stomata in SEM (o.ep=outer epidermis, t=trichome, s=stomata)

Fig. 7: Fracture through the outer parenchyma showing the cytoplasmic layer of a single cell in SEM (n=nucleus, m=mitochondrion, st=starch granule)

Fig. 8: Detail of parenchyma cells in TEM (v=vacuole, st=starch granule, i.sp=intercellular space, cw=cell wall)



diameter) represent lipid containing spherosomes (Fig. 2).

**TEM of fresh carrot tissues.** A survey of carrot phloem parenchyma tissue is shown in Figure 3. Each cell possesses a big central vacuole, whereas the cytoplasm appears as a thin layer along the walls. Numerous round particles heavily stained with osmium represent lipid droplets (spherosomes).

At higher magnification (Fig. 4), numerous plasmodesmata as plasmic connections between neighbouring cells become visible. Within the cytoplasm itself we find parts of the endoplasmic reticulum, mitochondria of the tubuli type and golgi apparatuses.

The most remarkable morphological feature of the carrot phloem parenchyma cell, however, is represented by the chromoplasts bearing the typical red or yellow pigment. On cross sections they show manifold shapes and structures. Within the fresh carrot cells, chromoplasts in most cases are round or oval and include starch grains. They often contain numerous plastoglobuli, irregularly shaped thylacoids and the outlines of pigment inclusions, whereas most of the carotin pigment itself is extracted during preparation procedures. Besides these typical chromoplasts amyloplasts also occur.

Nuclei in general show an irregular shape, with many protrusions, and two nuclear bodies within the nucleoplasm. Closely associated with the nucleolus ( $1.5 - 2 \mu\text{m}$ ) there is another nuclear body with a size of approximately  $0.6 \mu\text{m}$ .

**SEM of fresh green bean tissues.** Cross sections through one entire valve of the pod show an outer and inner surface tissue (epidermis) and a bisection of the enclosed parenchyma tissue into an outer and inner parenchyma ("seed-cushion") by a middle layer of cells with very small lumina (Fig. 5). The outward epidermis reveals a pattern of trichomes, stomata and a characteristically ridged cuticle (Fig. 6), whilst the inner surface tissue adjoining the seed consists of small papillate cells.

Fracturing of the parenchyma tissue allows the observation of the cytoplasmic layer inside the single cells. Especially conspicuous are round particles of approximately  $10 \mu\text{m}$  diameter, which are preferably located within the cells of the outer parenchyma. These organelles may occur in large numbers within the cells.

As a comparison with transmission electron microscopical observations shows, they represent starch grains or chloroplasts with extensive starch inclusions. Flat-roundish bodies of approximately  $15 \mu\text{m}$  diameter might represent nuclei whereas the numerous very small particles within the cytoplasm (approximately  $0.6 \times 0.8 \mu\text{m}$ ) must be regarded as mitochondria (Fig. 7).

**TEM of fresh green bean tissues.** In contrast to the inner parenchyma, the outer parenchyma tissue of the immature pod (Fig. 8) shows many intercellular spaces. The single cells possess big central vacuoles and parietal cytoplasmic layers of varied thicknesses. The cell walls themselves are frequently traversed by plasmodesmata. A detailed analysis of the cytoplasm shows many small vacuoles, flat-shaped nuclei, mitochondria of the tubuli-type, golgi apparatus and frequently a richly developed rough endoplasmic reticulum.

The morphological appearance of the plastids is remarkably heterogeneous. Besides well developed starch grains (amyloplasts) we find chloroplasts with small starch granules and chloro-amyloplasts with considerable starch inclusions, but there are still clearly visible stroma and grana thylacoids.

Whereas these types of plastids occur in the outer parenchyma tissue, the inner parenchyma possesses only small lens-shaped chloroplasts with apparently little or no starch grains.

**TEM of processed carrot tissues.** After blanching, carrot parenchyma cells show a slight swelling of their cell walls. They tend to separate along their middle lamellae. The cytoplasmic compounds still adhere to the inner side of the walls in the form of small granules (Fig. 9). Boiled tissue (Fig. 10) exhibits still thicker cell walls. The cytoplasmic granules are coarser, but on the whole they still retain their marginal position. Always lipid droplets of various sizes can be observed in blanched and boiled tissues. Blanched and dried carrot root tissue is characterized by an enormous shrinkage and twisting of the cells, but no rupture occurs. The cytoplasm fills the remaining lumina of the cells as a dense, clumpy material enclosing large lipid droplets (spherosomes) which seem to stem from the fusion of several small ones (Fig. 11).

The morphological appearance of blanched, dried and rehydrated cells and tissues (Fig. 12) strongly depends on the drying conditions (temperature, atmospheric moisture, drying time etc., [see below]) but, as a rule, cell walls are thicker after blanching, drying and rehydration than they are after cooking alone. Intercellular spaces seem to be enlarged and increased in number after drying and rehydration. The cytoplasm is more strongly condensed and clumped after rehydration and often does not regain its original marginal site, but remains in the centre of the cells. The degree of cytoplasm clumping and the localization of the cytoplasm within the cell can also be strongly influenced by the kind of dehydration employed.

**TEM of processed green bean tissues (inner parenchyma).** Blanched pod cells show cell walls which are slightly thickened. Cell wall junctions are slightly enlarged, their electron density being decreased at the same time. The cytoplasm being denatured by heat forms a marginal layer of fine granules still closely lining the cell walls (Fig. 13).

After boiling the tissue, cell walls appear increased in thickness. Sometimes larger intercellular spaces are formed. The cytoplasm consists of granules coarser than those in blanched cells. The strictly marginal arrangement of the cytoplasmic layer is disturbed but, on the whole, the cytoplasmic granules are still assembled along the cell walls (Fig. 14).

As in carrot tissue, blanched and dehydrated pod tissue is built up of shrunk and twisted, but still intact and clearly distinguishable individual cells. The small lumina of the cells are more or less filled up with clumped cytoplasmic material (Fig. 15).

As to the morphological picture of the blanched, dried and rehydrated tissue (Fig. 16), many of the characteristics observed in the rehydrated carrots are found in the rehydrated beans as well. Here, too, we observe a striking dependence of the morphological appearance on the influence of the preceding food technological processing (see below).

#### Morphometric measurements of cell wall swelling in processed vegetables

Since swelling of the cell walls seemed to be one of the most striking phenomena occurring in rehydrated tissues (see above), we carried out systematic measurements of cell wall thicknesses in rehydrated carrots and green beans in order to

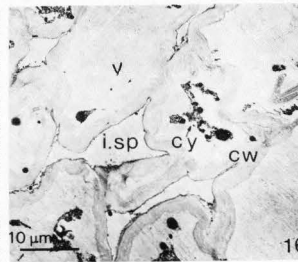
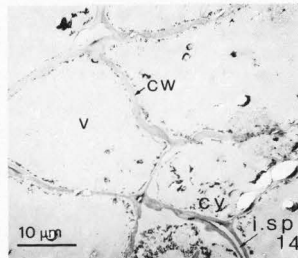
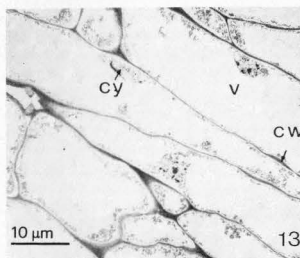
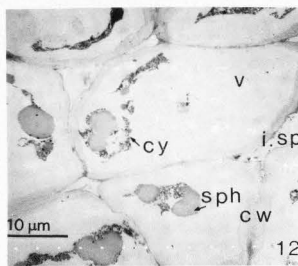
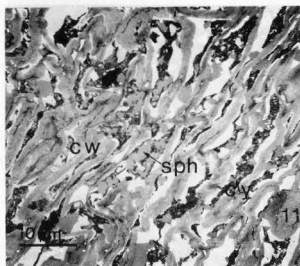
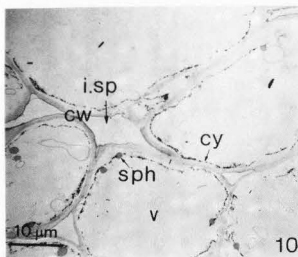
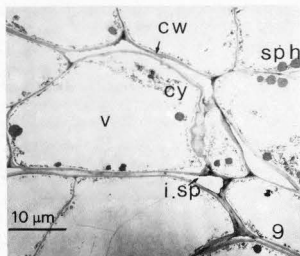


Fig. 9 – 12: TEM micrographs of processed carrot phloem parenchyma tissue

Fig. 9: Blanched tissue (v=vacuole, cy=cytoplasm, cw=cell wall, sph=spherosome, i.sp=intercellular space)

Fig. 10: Boiled tissue (v=vacuole, cw=cell wall, i.sp=intercellular space, sph=spherosome, cy=cytoplasm)

Fig. 11: Dried tissue (cw=cell wall, sph=spherosome, cy=cytoplasm)

Fig. 12: Rehydrated tissue (v=vacuole, cy=cytoplasm, i.sp=intercellular space, cw=cell wall, sph=spherosome)

Fig. 13 – 16: TEM micrographs of processed green bean pod tissue

Fig. 13: Blanched tissue (cy=cytoplasm, v=vacuole, cw=cell wall)

Fig. 14: Boiled tissue (v=vacuole, cw=cell wall, cy=cytoplasm, i.sp=intercellular space)

Fig. 15: Dried tissue (cw=cell wall, cy=cytoplasm)

Fig. 16: Rehydrated tissue (v=vacuole, cy=cytoplasm, i.sp=intercellular space, cw=cell wall)

Table 1. Influence of different blanching periods upon the degree of cell wall swelling in rehydrated green beans (var. "Cascade")

Sample No.	Blanching time min	Cell wall swelling %
B1	3	135
B2	6	167
B3	9	190
B4	12	192

Table 2. Influence of different blanching periods upon the degree of cell wall swelling in rehydrated carrots (var. "Zino")

Sample No.	Blanching time min	Cell wall swelling %
C5	6	99
C6	10	119

Table 3. Influence of blanching in different blanching media upon cell wall swelling in carrots (var. "Bauer's Kieler Rote") after drying and rehydration

Sample No.	Blanching time min	Blanching medium	Cell wall swelling %
C1	2	H <sub>2</sub> O	212
C2	5	H <sub>2</sub> O+3%NaCl	287
C3	10	H <sub>2</sub> O	244
C4	10	H <sub>2</sub> O+5%NaCl	293

Table 4. Influence of the relative humidity of the drying air upon cell wall swelling in rehydrated green beans (var. "Koralle")

Sample No.	Blanching time min	Relative humidity %	Cell wall swelling %
B5	6	21	189
B6	6	3	125

Table 5. Influence of the residual water content of dried carrots (var. "Bauer's Kieler Rote") upon cell wall swelling after rehydration

Sample No.	Residual water content %	Cell wall swelling %
C7	6	122
C8	13	200
C9	18	173
C10	22	184
C11	6	126

Table 6. Influence of the drying temperature upon cell wall swelling in rehydrated green beans (var. "Cascade")

Sample No.	Blanching time min	Drying temperature °C	Cell wall swelling %
B7	6	50	160
B2	6	70	167
B8	6	90	178

Table 7. Influence of the drying temperature upon cell wall swelling in rehydrated carrots (var. "Zino")

Sample No.	Blanching time min	Drying temperature °C	Cell wall swelling %
C12	6	70	99
C13	6	90	90
C14	10	80	104
C15	10	90	119

Table 8. Influence of the storing temperature upon cell wall swelling in carrots (var. "Bauer's Kieler Rote") after rehydration

Sample No.	Storing temperature °C	Cell wall swelling %
C16	4	217
C17	20	273
C18	4	198
C19	20	261

more closely analyze the influence of some process parameters. All samples were blanched before drying and rehydration (The rehydration periods are not shown in the tables).

Influence of different blanching times. Blanching in water for different periods led to different degrees of cell wall swelling after rehydration (Tables 1 and 2). The values in these tables show that cell wall swelling in the rehydrated tissue is correlated with different blanching times: the longer the blanching time, the greater the cell wall swelling. Different rehydration periods do not seem to influence much the alterations caused by different blanching periods. This means that samples with short blanching periods which require long rehydration times for reaching the point of doneness do not show the same morphological appearance (expressed by the degree of cell wall swelling) as those with long blanching times and – consequently – shorter rehydration periods.

Influence of the addition of NaCl to the blanching medium. Blanching with variable concentrations of NaCl led to a general increase in cell wall thickness and maceration of the tissue in the rehydrated carrots as compared with blanching in pure water (Table 3). It is remarkable that the addition of 3% NaCl causes almost the same amount of cell wall swelling in 5 min as the addition of 5% in 10 min. Blanching with NaCl reduced the cooking times of the samples considerably.

Dehydration in dried air. If the relative humidity of the drying air was reduced to 3%, the samples exhibited less cell wall swelling than parallel samples dried in air with a relative humidity of 21% (Table 4). Lowering the relative humidity also led to a reduction of the cooking times and a better conservation of chlorophyll.

Residual water content of the dried vegetables. As Table 5 shows, the morphological structure of the rehydrated carrots is adversely affected by a comparatively high water content (>10%) of the dried product. Reducing the residual water content to 6% by prolonged drying reduces cell wall swelling considerably. There were no significant differences with regard to the cooking times.

Drying temperature. If the blanching time is kept constant, whereas the drying temperature is varied, morphological differences in the rehydrated sample were observed (Tables 6 and 7). As the figures from Table 6 show, in green beans less cell wall swelling was observed at low drying temperatures. In carrots, however, (Table 7), this is only true for samples blanched for 10 min. The short blanching time (6 min) gives better results with the high drying temperature. In green beans, drying at 90° C reduced the cooking time considerably. Reduction of cooking time was also observed in carrots after blanching for 6 min and drying at 90° C. Carrots after blanching for 10 min, however, showed shorter cooking times at low temperatures.

Influence of storing. Dried carrots were stored at different temperatures (4° C, 20° C) for 5 weeks. As the figures in Table 8 show there is less cell wall swelling in rehydrated samples after storing the dried vegetables at the lower temperature. Apart from a general prolongation of the cooking time due to the storing process itself, no difference between the two storing temperatures as regards the cooking time could be observed. However, there was a tendency to a better conservation of pigments in carrots stored at 4° C.

## Discussion

The SEM and TEM micrographs show that fresh carrot root parenchyma is a typical plant storage parenchyma, characterized by a high starch content and numerous lipid droplets. Green bean pod parenchyma, on the other hand, represents another type of plant tissue, i.e. that of assimilating tissue although the relatively high starch content within the chloroplasts and the occurrence of intermediate forms between typical chloroplasts and typical amyloplasts point out that it has storage functions as well.

As expected, structural changes involved in the processing of both vegetables affect cytoplasm and cell wall in a specific way.

Cytoplasmic changes are expressed by a variable degree of clumping and by translocation of cytoplasmic substance from its marginal site into the vacuole space.

After being transformed into a fine (blanched) or coarse (boiled) granular substance the cytoplasm keeps its place even in boiled tissue. The strong clumping and translocation of cytoplasmic substance into the cell lumen which can be observed in dried specimens is in general not compensated by water absorption during rehydration. So within the cytoplasm, dehydration seems to bring with it physical and chemical changes that can only partially be made reversible during rehydration.

As far as the cell wall is concerned two aspects seem important: (1) the degree of swelling and (2) the separation of the single cells along the middle lamellae.

As the electron micrographs of raw, blanched and boiled tissues show, the effects of moisture and heat lead to a swelling of the walls and to a beginning of separation of the individual cells. This "thermal maceration" has long been observed in prepared tissues by optical microscopists, and has been attributed to the extraction of protopectin from the middle lamella.

In the present results, however, it is remarkable that not only short blanching, but also boiling to "doneness" preserves the histological structure of the tissue so well. Apart from moderate cell wall swelling, only occasional cell separation and genuine "maceration" take place.

Like Davis and Gordon (3) we found no submicroscopic evidence that any rupture of cell walls ("holes") occurs during cooking or dehydration and rehydration, as some authors reported (23, 24). This would indicate a degradation of the cellulose skeletal network. The observed swelling of the cell walls, which is accompanied by a loss of electron density, surely indicates a loosening of the cellulose fibril network. If this loosening is caused by a breakdown of matrix material (pectin, hemicelluloses, etc.) and/or by a loosening of chemical bonds between the cellulose fibrils or by some other factor must be decided by cytochemical analysis.

The observed shrinkage of dehydrated cell is compensated by water absorption during rehydration by boiling. Rehydrated samples often show more voluminous cell walls than boiled raw tissue. As other experiments demonstrated, this swelling may lead to cell walls being five to ten times thicker than those in raw tissue. In most cases these tissue samples also exhibited a higher degree of maceration. It is obvious that such enormous structural deviations from the appearance of the boiled raw tissue must be regarded as undesirable from the morphologist's

point of view.

Another structural alteration occurring during dehydration which was observed by Sterling and Shimazu (27) is the increase in cellulose crystallinity in dehydrated samples. According to these authors this phenomenon may account for the often observed toughness of rehydrated vegetables and the prolongation of their cooking times, because a high amount of crystalline cellulose would prevent the cell wall from taking up water during swelling.

The present results clearly demonstrated the tendency of an increased swelling of cell walls during dehydration/rehydration so that cellulose crystallinity might seem to be rather decreased.

The results of our morphometrical measurements of cell wall swelling in processed vegetables show that in the production of dried food the process parameters may exert a decisive influence on the ultrastructural appearance of the rehydrated product.

Some of these parameters (short blanching without NaCl, low relative humidity of the drying air, low residual water content of the dried product and storage at low temperatures) produced samples that after rehydration showed a morphological status more like that of the boiled raw tissue than samples treated in the opposite way. Whereas the influence of blanching upon cell wall swelling was remarkably distinct, even in the rehydrated product, the choice of the drying temperature did not seem to be very critical within the above limits.

In some cases (e.g. relative humidity of the drying air) favourable morphological and favourable food technology results (e.g. short cooking times) agreed. In other cases (most prominent: long blanching times) a discrepancy was observed between the morphologist's claim for a good preservation of structural features and the food technologist's claim for short cooking times.

Some parameters (e.g. storage temperature) did not have any influence upon the cooking times of the dried samples, whereas – from the morphological point of view – distinct differences in cell wall swelling were observed. There was, however, a better conservation of pigments in carrots at low storage temperatures.

### Conclusion

Although the present investigation answers some questions dealing with the morphological consequences of food technological procedures, the goal pursued by both the morphologist and the food technologist ("The rehydrated product should be similar in all aspects to the boiled raw tissue") has not yet been fully reached. For the food scientist this means further improvement of process engineering in close correlation with morphological investigations.

Beside cell wall swelling, these future morphological studies should examine further characteristics of cell wall alterations, e.g. changes in the degree of crystallinity of the cellulose fibres (Sterling and Shimazu (27)), which seems to be quite significant for a deeper understanding of the process involved in the drying and rehydration of plant tissues.

### Acknowledgement

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#### Discussion with Reviewers

S. Jones: One of your basic assumptions is that the dried, rehydrated product should resemble as closely as possible the fresh cooked vegetable with respect to morphology of the cell wall. Has this assumption been tested?

Authors: In most cases foodstuffs during preservation (drying, canning, freezing) lose some of their qualities which they had in the fresh or fresh cooked state. In this sense preservation can be regarded as a kind of expedient, which is dictated by seasonal or commercial needs. The aim of food scientists is to keep these negative influences of preservation as minimal as possible as regards taste, consistency and chemical composition of the preserved food. Our morphological investigations must be regarded as part of these efforts in minimizing deviations from the original material which occur during preservation. In this sense the morphology of fresh or freshly cooked, i.e., unpreserved and unaltered tissue, seems to be the reasonable standard for estimating the influences of processing conditions.

S. Jones: What are the processing parameters?

Authors: Detailed information of the processing parameters are given in literature references 1,2.

S. Jones: How reproducible are your swelling data, given that a subjective estimation of doneness is (probably) used? Are your data from single or multiple runs? What are the standard errors? Authors: Since sensory as well as shearing tests were used for the estimation of doneness, the reproducibility of our morphometric results must be considered as good. Our data are from multiple runs extending over a total period of three years. This means that the influences of three different annual growth conditions on the structure of the raw material are included. Nevertheless, standard deviations ranged only between 10 – 15 %.

S. Jones: Are all the dried, rehydrated samples first blanched? What is blanching, specifically? If the vegetable is dried and rehydrated without blanching, is the only effect a longer cooking time?

Authors: All samples were blanched before drying, only blanching periods and blanching media were varied (s. Tables 1 – 3). Drying of vegetables without previous blanching is—from the view of food conservation—undesirable, because blanching inhibits enzyme activity and therefore leads to a better conservation of the colour, taste, and chemical composition of vegetables. We did not check whether drying without previous blanching leads to a prolongation of the cooking time although this might well be the case.

K. Saio: Many starch grains are observed in fresh green bean tissue (Fig. 8), but not in processed green bean tissues (Figs. 13 to 16). The authors do not refer to the changes in starch grains during processing. It is also considerable in carrot tissue, although the number of starch grains are fewer than in green beans. Please explain.

Authors: Micrograph No. 8 was taken from the outer part of the green bean pod (see text) which is characterized by a high starch content within the cells. All micrographs of processed green beans were taken from the inner part where there are only few amyloplasts occurring in the cytoplasm. We did not particularly focus on the fate of starch grains during processing in this study since the degradation of starch seems to be a complex phenomenon which surely requires separate investigations. But as far as we noticed there did not seem to be big differences between carrots and green beans with regard to these organelles.

K. Saio: To resolve the "hard-to-cook" phenomenon of dried beans, soaking in salt solutions prior to cooking (quick-cooking) have been reported by many researchers (Rockland and Jones, J. Food Sci. **39**, 342, 1974, Sefa-Dedeh et al., J. Food Sci., **43**, 1832, 1978, Jackson and Variano-Mortston, J. Food Sci., **46**, 799, 1981 etc.). They pointed out that the quick-cooking was rendered by the mechanism of ion exchange and possibly by chelation, as the middle lamella which cements the individual cells together consists of a calcium salt of polymers of galacturonic acid. The cell wall swelling in these experiments seems to be essentially the same as the phenomenon above, in view of the acceleration of swelling with NaCl. The TEM micrographs show that after processing the microstructure is largely unchanged



except for enlarged intercellular spaces and lower density. But, what happens to the actual texture of the vegetables on processing? Aren't the lowering of the crispness and hardness dependent on the treatments? And how is the degree of cell wall swelling related to such textural properties?

Authors: We agree with you that cell wall swelling and maceration of tissue during processing is surely connected with reactions taking place within the pectin/protopectin component of the cell wall, especially the middle lamella. Dissolution of pectin/protopectin is not only promoted by salt addition, but also by the action of hot water which might explain the swelling and maceration of cell wall material in the experiments without the addition of salts. We suppose that the lowering of the density of cell walls which is visible in our TEM micrographs is at least partially due to the extraction of matrix material such as pectin resulting in a loosening and broadening of the remaining cellulose fibre network. The actual composition and texture of the walls (e.g. crystallinity of cellulose fibres) cannot be elucidated by purely morphological methods, but their analysis seems to be the next step towards a deepened understanding of the effects of processing.

MICROSTRUCTURAL CHANGES IN WINGED BEAN AND SOYBEAN  
DURING FERMENTATION INTO MISO

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Abstract

Miso was prepared from winged bean, being substituted for soybean. Microstructural changes of winged bean miso at various stages of the manufacturing processes were studied by means of light and transmission electron microscopies. Soybean was also studied for comparison.

After steaming, winged bean and soybean cells were shrunken and intracellular spaces enlarged and cell wall structure was degraded, showing layered structures and aggregated lumps in intraspaces between cell walls during fermentation into miso. At the end of the fermentation, the PAS reaction with cell walls almost disappeared. Surprisingly the thick cell walls of winged bean degraded as completely as those of soybean. However, seed coat tissues were not digested. Lipid bodies were broken and fused into oil drops located inside and outside of cells and then degraded, showing wavy and scalpy patterns during fermentation.

Protein bodies lost their membranes and coagulated after steaming, and then were degraded as aging progressed. Generally, degradation of the gross cellular structures seemed to be faster in soybean miso than in winged bean miso. Partly degraded gross structures of raw beans remained in the miso after two months of aging.

Introduction

The green pods and leaves of winged beans have long been eaten by a few tropical people who used them in a minor way. The potentiality of this crop as a food resource, which is adapted to the wet tropics where protein deficiency in the human diet is most serious, was pointed out by Pospisil et al. (1971) and Masfield (1973). The National Academy of Sciences (1975) has called for an experimental survey of winged beans throughout the world. As a result, worldwide attention has been increasing and the 1st and 2nd International Seminars on winged bean took place at Los Baños, the Philippines in 1978 and at Colombo, Sri Lanka in 1981, respectively.

Microstructural studies of ripe winged bean seed (Varriano-Marston et al., 1983, Saio et al., 1983) have revealed that contents of protein and lipid bodies in winged bean are high and comparable to those in soybean. However, cell walls of winged bean are much thicker than those of soybean. The hard tissues of winged bean, which can be cooked only with difficulty, may be caused by this thick cell wall structure.

Winged bean has traditionally been used in an immature form as a vegetable but is also deep-fried or fermented into tempeh (Gandijar, 1978). Recently, the preparation of products analogous to soymilk (Shurtleff, 1978) and tofu (Omachi et al., 1983, Shurtleff, 1978) from ripe winged beans was reported.

In the present paper, miso making from winged bean was studied with primary interest in changes of the seed microstructure, especially the digestion of the thick cell walls during fermentation.

Materials and Methods

Winged bean, 1981 crop, was provided by the Okinawa Branch of Tropical Agricultural Research Center, Japan. It was a mixture of cultivars produced by the Center. Soybean, which was used for comparison, was a U.S. crop (Harosoy).

Miso making

Four kinds of miso were prepared by the processes shown in Figure 1; they are designated as WB miso, WB-R miso, SB miso and SB-R miso as shown in the figure.

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**Key words:** Winged bean, Soybean, Miso, Cell wall, Protein body, Lipid body, Fermentation, Light microscopy, Transmission electron microscopy, Microstructure

Preparation of specimens for microscopy

Cotyledonary tissues of raw and steamed beans were cut into small pieces with a razor blade, fixed with 5% glutaraldehyde solution and then with 1% osmium tetroxide solution (both in phosphate buffer containing 5% sucrose, pH 6.7), dehydrated with a graded alcohol series (40 to 100%), exchanged with propylene oxide-Epon resin series (50 to 100%) and finally embedded in Epon resin.

With miso paste, a small piece (2 to 3 mm<sup>3</sup>) on the tip of a stainless steel needle was dipped into a lukewarm agar sol and the beaded specimen was then processed for electron microscopy in the same way as bean tissues described above.

For light microscopy (LM) the Epon block was sliced to about 5 to 10 µm thickness. The slices were affixed to glass slides and stained with the PAS reaction for polysaccharides as described in a previous paper (Saio et al., 1983). The same Epon block used for LM was ultrathin-sliced and stained with saturated uranyl acetate solution and then with saturated lead acetate solution in ethanol for the transmission electron microscope (TEM, JEM EX-1200).

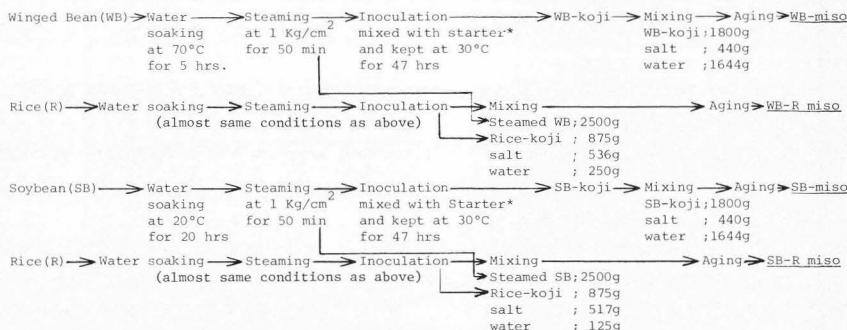
Results

LM micrographs of winged bean, soybean, WB miso and SB miso at various stages of miso manufacturing processes are shown in Figure 2. The characteristic winged bean cell wall structure with pit-pairs (Fig. 2A1) became indistinct after steaming; the steamed cells were shrunken and the intracellular spaces were enlarged as compared to the raw seeds (Fig. 2B1). On the other hand, the cells of steamed soybean were plasmolyzed and lipid bodies were broken and fused into large oil drops located inside and outside of the cells (Figs. 2A2, 2B2). Lipid body fusion into oil drops in steamed winged beans was usually minor (Fig. 2B1), but was clearly observed in later stages of processing,

such as after inoculation with starter (Figs. 2C1, 2D1) and in the first period of aging (Fig. 2E1). After inoculation, mycelia from koji (cereal grains on which abundant conidiospores of *Aspergillus oryzae* have been grown; rice is most popularly used) were recognized in WB miso and SB miso (Figs. 2D1, 2D2). The PAS reaction with cell walls gradually weakened during processing and appeared in network spaces between degraded cellular substances (Fig. 2G2). The seed coat tissues remained undigested in both WB miso and SB miso (Figs. 2G1, 2F2). Generally, the degradation of the gross cellular structures seemed to be faster in SB miso than in WB miso.

LM micrographs of WB-R miso and SB-R miso at major stages of processing are shown in Figure 3. Some lipid body fusion was noted in the steamed winged beans (Fig. 3A1) but not to the extent seen in soybeans (Fig. 3A2). Almost the same changes found in WB miso and SB miso were recognized in the miso pastes fermented after mixing with rice koji. Because SB miso after one to two month aging is widely sold in the Japanese market, LM micrographs of one month aging SB-R miso (Figs. 3C1, 3C2) and two month aging WB-R miso (Figs. 3D1, 3D2) are shown. At lower magnification (Figs. 3D1, 3D2), heterogeneity of miso paste is clearly observed and it is noted that gross cell structures were still retained.

TEM micrographs of WB miso and SB miso at different steps of miso making are shown in Figures 4 and 5. In the latter figure, the micrographs are at higher magnification than those in Figure 4 and were selected to show the changes in cell wall structure and degradation process of the oil drops. The protoplasts in raw beans (Figs. 4A1, 4A2) shrank and plasmolyzed on steaming (Figs. 4B1, 4B2). As miso making progressed, cracks between cell walls and cell membranes became indistinct (Figs. 4C1, 4G1) and layered structures (Figs. 4G2, 5A2) and aggregated lumps in intraspaces between cell walls (Figs. 4C1, 4G2, 5A1, 5A2, 5B1) were often



\* Starter used was conidiospores of mycelia of *Aspergillus oryzae* (trade name: Yamazaki)

Figure 1 Processes of preparation of four kinds of miso

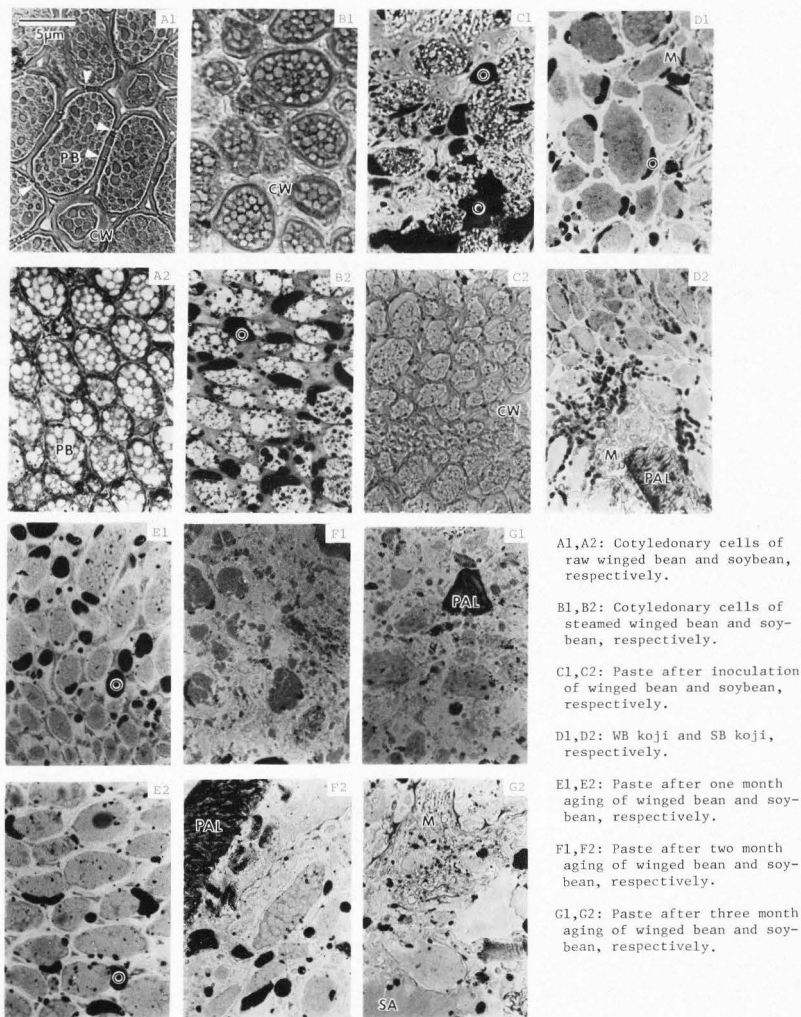


Figure 2. LM micrographs of winged bean, soybean, WB miso and SB miso at various stages of miso making. PB, protein body; CW, cell wall; O, oil drops; M, mycelia of koji; PAL, palisade cells of seed coat; SA, area which is strongly positive to PAS reaction; White arrows, pit-pairs. Magnification in all micrographs is the same as in Fig. A1.

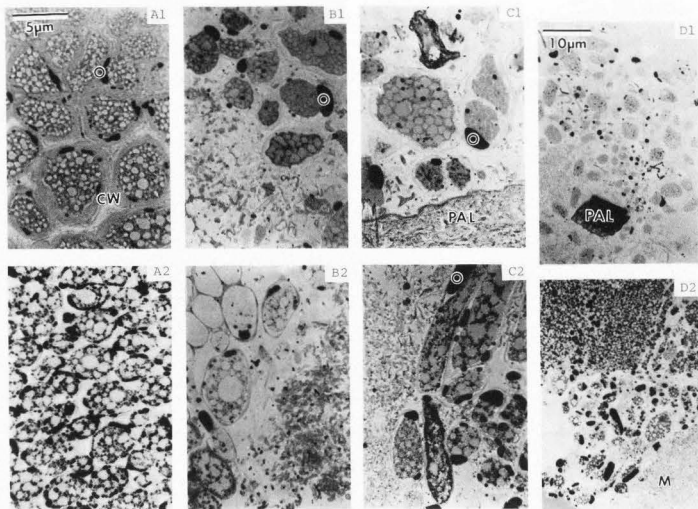


Figure 3. LM micrographs of winged bean, soybean, WB-R miso and SB-R miso in miso making.

A1,A2: Cotyledonary cells of steamed winged beans and soybean, respectively.  
 B1,B2: Paste of winged bean and soybean after mixing with rice koji, respectively.  
 C1,C2: Paste after one month aging of winged bean and soybean, respectively.  
 D1,D2: Paste after two month aging of winged bean and soybean, respectively.  
 CW, cell wall; O, oil drops; PAL, palisade cells of seed coat; M, mycelia of koji;  
 SA, area which is strongly positive for PAS reaction.  
 Magnifications of B and C are the same as A. Those of D1 and D2 are the same.

found. These layers and lumps may be intermediate structures of degrading cell walls.

After steaming, lipid bodies were extensively broken and fused into oil drops in soybean and only slightly in winged bean. In the comparison of lipid bodies in winged bean (Figs. 4A1, 4B1) with those in soybean (Fig. 4A2), the latter were electron dense when stained by osmium tetroxide, whereas the former were electron translucent in spite of being fixed in the same way at the same time. After fusing, the oil drops in winged bean at first seemed to be electron dense, but degraded portions of oil drops became electron translucent (Figs. 4F1, 4H1, 5C1, 5D1), showing wavy or scalpy patterns (Figs. 4F1, 4F2, 4H1, 5C1, 5D1, 5D2) and finally disappeared. In soybean, small fused oil drops (Fig. 5C2) were found up to two to three months of aging, located around coagulated protein bodies. The membranes of protein bodies were broken after steaming and coagulated protein parts were also degraded (Figs. 4D1, 4D2, 4E1, 4G1, 4H2). As shown in Fig. 4E2 hyphae of koji mycelia were sometimes observed in cells.

#### Discussion

Miso in Japan has many variations depending on regions and people just as is found for cheese in Europe, but can be classified into three groups based on the starting materials, namely, miso prepared only with soybean, miso made with soybean and rice and miso manufactured from soybean and wheat. The production and consumption of miso made from soybean and rice is the largest in the Japanese market, even though this type has a variety of minor deviations in formula, salt concentration, heating conditions for raw materials and degree of mashing of the mixture of soybean and rice. In our studies winged bean was substituted for soybean in the all-soybean and soybean plus rice types of miso.

Because the thick cell walls of winged bean became negative to the PAS reaction, they apparently are utilized effectively as a carbohydrate source in miso making as is true with soybeans. However, the seed coats were not digested even after three months of aging. Cell wall structure seems to be less lignified than seed coat tissues

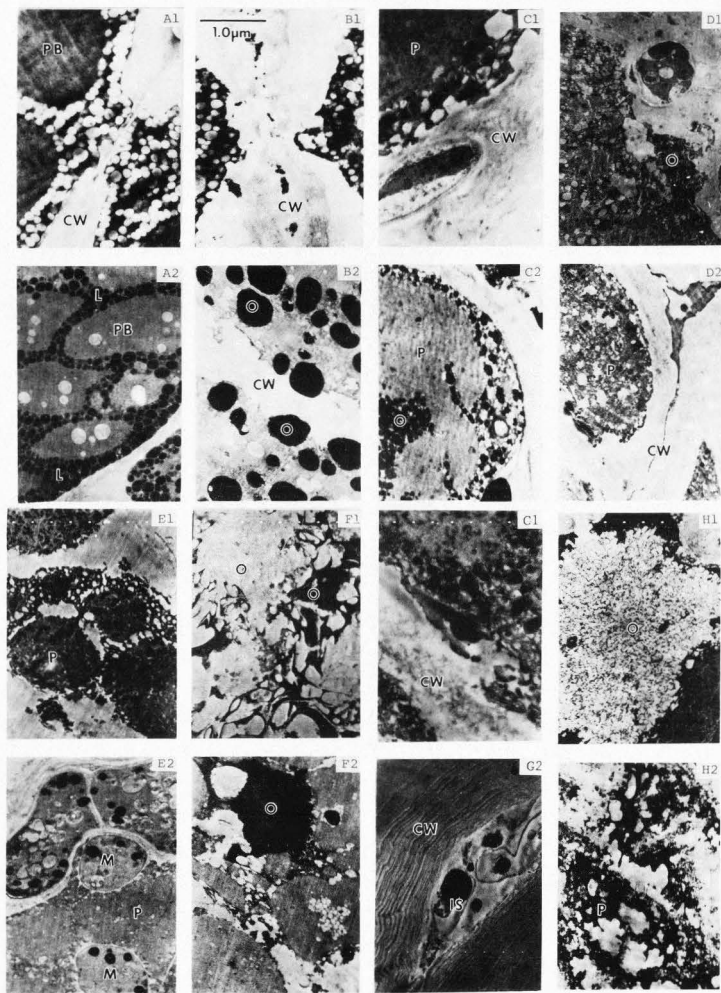


Figure 4. TEM micrographs of winged bean, soybean, WB miso and SB miso making.

A1,A2: Cotyledonary cells of raw winged bean and soybean, respectively. B1,B2: Steamed WB and SB cotyledonary cells. C1,C2: WB and SB paste after mixing with koji starter. D1,D2: WB and SB paste after inoculation. E1,E2: WB-R koji and SB-R koji. F1,F2: WB and SB paste after one month aging. G1,G2: WB and SB paste after two month aging. H1,H2: WB and SB paste after three month aging. CW, cell wall; O, oil drops; P, protein body; L, lipid body; M, hypha of koji mycelia; IS, intracellular space; Magnification of all micrographs as indicated in Fig. B1.



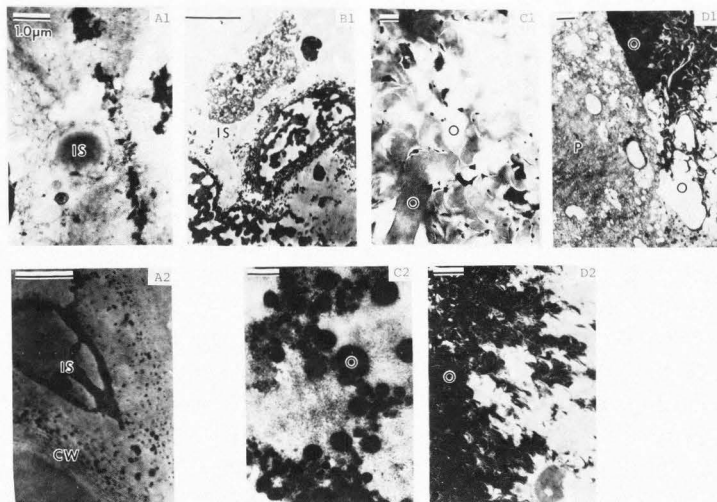


Figure 5. TEM micrographs of WB miso and SB miso at high magnification.

A1,A2: Intracellular space adjacent to cell walls after steaming of winged bean and soybean, respectively. B1: Intracellular space adjacent to cell walls after two month aging of winged bean. C1,C2: Degrading oil drops after two month aging of winged bean and soybean, respectively. D1,D2: Degrading oil drops after three month aging of winged bean and soybean, respectively.

IS, intracellular space; O, oil drops; CW, cell wall; P, coagulated protein.

Bars shown in each micrograph equal 1 µm.

as judged from the microstructure of the latter (Saio and Watanabe, 1973). The digestion is probably caused by hydrolytic enzymes such as cellulase, pectinase and amylases present in the koji.

Measurements of pH, acidity, protein and carbohydrate digestibilities and lightness of color of miso paste were carried out by the laboratory of Nagano Miso Co. Ltd., using the same samples (Kobayashi et al. in preparation). Results on protein and carbohydrate digestibilities showed that SB miso digested faster than WB miso but both types of miso reached almost the same level of digestion after three months of aging. The Y values of lightness were much higher for SB miso than WB miso in the beginning but became somewhat higher for WB miso after three months of aging, which seemed mainly due to nonenzymatic browning between liberated amino acids and sugars.

WB miso and WB-rice miso prepared in these experiments, were acceptable in taste, texture of body, and color, according to testing by Nagano Miso Co.Ltd. and the National Food Research Institute. Their flavor was somewhat different from that of usual SB miso, but the preference depends on individual tastes, as Japanese are used to eating soybean foods, whereas people of other countries such as Southeast Asia, Latin

America and Africa prefer other pulses. Trials to make WB miso as a special product of Okinawa are progressing.

Microstructural changes in steamed soybeans for miso making (Shibazaki and Asano, 1968, Saio and Watanabe, 1973) and chopped soybeans in miso paste (Shibazaki and Asano, 1968) by LM were reported, but there are no reports on soybean paste by LM and TEM. Consequently, our studies provide new information on the microstructural changes in soybeans and winged beans that occur during the complex fermentation involved in manufacturing miso.

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### Acknowledgement

The authors thank Dr. Abe S, Okinawa Branch of Tropical Agricultural Research Center for his gift of winged beans and helpful advice.

### Discussion with Reviewers

**W.J.Wolf:** You state that winged beans are more difficult to cook than soybean, yet the same cooking conditions were used for both legumes. Does this cause overcooking of soybeans and could such overcooking be responsible for the greater fusion of the lipid bodies in soybeans as compared to winged beans? It would be interesting to measure tenderness and structure of the seeds as a function of cooking time and determine whether lipid body fusion parallels tenderness.

**Authors:** In this experiment, the temperature and time of soaking beans were different but cooking time was the same in both. As the cooking condition used is normal for SB-miso making, it may be under-cooking for winged bean, which might result in the delay of microstructural changes as compared to soybean. I also think that the experiment you suggested would be interesting.

**D.J.Gallant:** In a recent work yet unpublished on cytochemical studies of lupine seeds, we have found a negative correlation between the content of storage components and the thickness of cotyledon cell walls. It might be the same in your case. Could you explain why ripe winged

beans which have comparable content of storage components as soybean have much thicker cell walls?

**Authors:** We have no answer to your question. We also found thick cell walls in some lupine seeds but not in wild type soybean seeds. We are now working on other legume seeds.

**D.J.Gallant:** What kind of polysaccharides and possible antinutritional components such as the alpha-galactosides exist in winged bean cell walls? Is miso from winged bean nutritionally as good as soybean miso?

**Authors:** We reported in a previous paper (Saio et al., 1983) that winged bean contains extraordinarily high levels of hemicellulose. It might serve a role as dietary fiber, but we did not determine the nutritional value of winged bean miso.

**D.J.Gallant:** Could you explain why protein bodies were clearer in soybean cotyledons than in winged bean cotyledons (Figure 2 A1-A2, B1-B2)?

**Authors:** The original micrographs are color prints of sections stained by the PAS reaction. The intensity of PAS staining varies with minor changes in conditions, so that we can not say that the protein bodies of winged bean contain more polysaccharides than those of soybean.

**D.J.Gallant:** Were the raw materials soaked or hydrated before fixation?

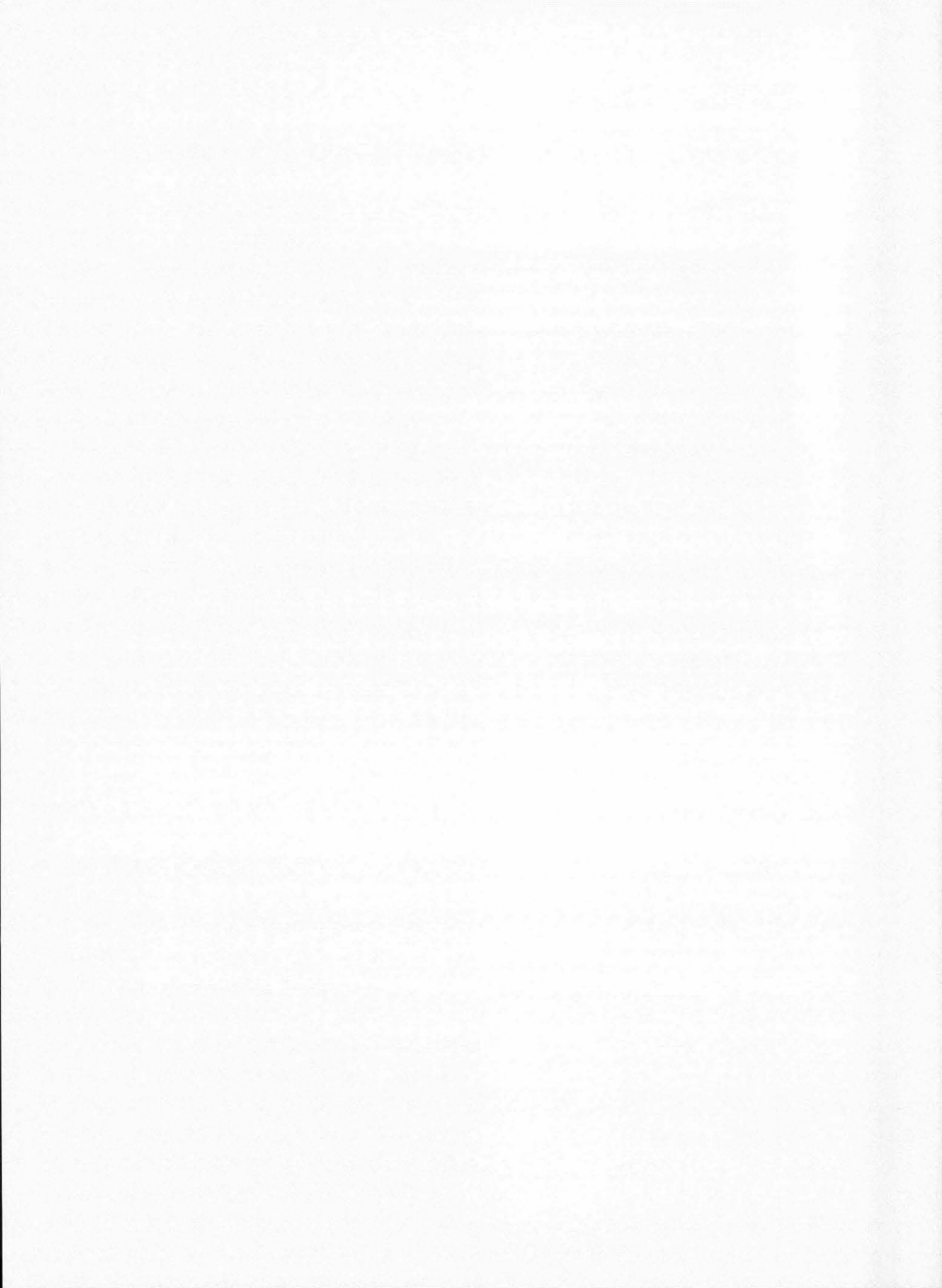
**Authors:** No, they were not.

**D.J.Gallant:** In the TEM studies, you noted differences in contrast between lipids in the winged bean and soybean cells. Could they be related to less saturation of storage soybean lipids as compared to winged bean lipids?

**Authors:** We also think that is one of the reasons for differences in the contrast of lipid bodies between soybean and winged bean, but are still not sure.

**D.J.Gallant:** You noticed remnant cell walls in winged bean miso after three month aging. Are they organoleptically detected by Japanese people?

**Authors:** We found remnant seed coats after fermentation. They were darker in color than the paste part and felt rough to the tongue.



THE EFFECTS OF MICROWAVE ENERGY AND CONVECTION HEATING ON WHEAT STARCH GRANULE TRANSFORMATIONS

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Abstract

Wheat starch-water dispersions at 1:1, 1:2, 1:4, and 5:95 (w/w) starch:water ratios, representing systems with varying levels of water availability, were heated under static conditions to 75°C by microwave energy at two power settings and by convection heating. Starch granule swelling was evaluated by light and scanning electron microscopy. Six stages in swelling were identified on the basis of swelling of small and large granules and development of an extragranular matrix. The range of swelling stages found in different locations within a sample decreased as water became more available and less limiting. At each starch:water ratio, the range of stages of swelling and matrix development was smaller in convection-heated samples than in microwave-heated samples, but the convection-heated samples were at more advanced stages of gelatinization than were the comparable microwave-heated samples. Each starch:water ratio and heating combination resulted in characteristic patterns of gelled and non-gelled regions that could be observed visually. The microscopic and macroscopic characteristics are explained on the basis of differences in interaction with microwave radiation and subsequent heat and mass transfer.

Introduction

Use of microwave energy for processing and cooking foods has increased greatly in recent years. Heating that occurs as a result of microwave irradiation is caused by molecular friction resulting from the dielectric coupling of molecules as they orient themselves with frequencies in the microwave region of the radiation spectrum and as molecules change their orientation with the oscillating microwave field in order to maintain their dielectric coupling. The heating of an object depends on its dielectric constant which determines how a material couples with microwaves, and on the dielectric loss factor which expresses the ability of the material to absorb microwave energy and transform it into heat. Moisture content is an important factor in determining each of these parameters.

Although many acceptable food products are produced by microwave heating, less satisfactory results are found in some starch-based foods. Development of acceptable structures in batter and dough systems is one example of such problems. The reasons for these differences may be related to fast heating rates, differences in heat and mass transfer mechanisms, or specific interaction of the components of the formulation with microwave radiation.

Starch is one component of formulated food systems for which the sequence of transitions when it is heated in the presence of water is known to be affected by the level of available water and the rate of heating. Collison and Chilton (1974) found that microwave-heated samples of potato starch were damaged more rapidly than forced air convection-heated samples, but the starch:water ratio was more important than heating rate in determining extent of damage.

In the present study, starch granule swelling was investigated over a range of water levels commonly found in starch-based food systems. Two heating rates were used in the microwave mode, one in the convection mode. A reference system sequencing granule swelling was developed for purposes of classifying the stages of granule swelling present at each combination of heating rate and starch:water ratios.

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**KEY WORDS:** Wheat starch, Microwave heating, Starch granule swelling, Gel formation, Heat and water transport-starch:water dispersions.

### Materials and Methods

Wheat starch (Aytex-P, General Mills) and distilled water dispersions with four starch:water ratios, 1:1, 1:2, 1:4, and 5:95 (w/w basis), were prepared by mixing the appropriate weights of starch and water. Fifty gram samples of each ratio were weighed into 100 ml beakers (Teflon \*PFA, diameter 50 mm; height 68 mm). Each sample was heated to 75°C in a household microwave-convection oven (Sharp Carousel Model R-8310) using either the convection mode at 177°C or the microwave mode at Low (20% power) or Medium (50% power) settings. The microwave oven operated at 2450 MHz, 650 watts nominal, and 400 watts effective power (method of Van Zante, 1973). Each beaker containing a sample was placed at the center of the carousel. Temperature at the center of each sample was measured with a fiber optic probe (Luxtron temperature probe, Model 1000A). The probe was placed at the center axial position of the beaker and 10 mm from the bottom of the beaker. Temperature was recorded every 10 seconds during the first minute and then every 30 seconds until 75°C was reached. Samples were cooled immediately to 22°C in an ice bath to minimize further temperature increases after removal from oven.

Total weight loss and change in height were recorded for all samples. The 1:1 and 1:2 starch:water samples, which formed firm gels, were removed from beakers and cut in half. Tracings were made of the gelled and chalky appearing areas. Total areas of the cross-sections were measured with a planimeter. Proportions of gelled and chalky regions were determined by weighing total tracings and the cut-out sections of the gelled regions. Cross-sections of the 1:4 and 5:95 starch:water samples were prepared by cutting and examined, but the extent of the gelled and chalky areas was not quantified.

Five replications of each starch:water ratio heating rate series were prepared. The order of preparation was randomized within each series.

#### Microscopy:

##### Light and polarized light microscopy

Gelled and chalky areas were sampled for

microscopic evaluation. A single layer of granules was observed with a polarizing microscope (Unitron, Model MPS-2) using both ordinary or polarizing light after some distilled water had been added to the microscope slide.

##### Scanning electron microscopy (SEM)

Samples were taken from the same sample areas as were used for light microscopy. No water was added, however. A thin layer approximately 1.0 mm thick of the starch preparation was placed on a cover slip which had been cut to fit the stub. The cover slip was attached to the stub with silver conducting paint. The stubs were dried over calcium sulfate in a desiccator overnight and then coated with gold/palladium. Samples were examined in a Philips Model 500 SEM at 6 kV.

### Results

#### Temperature Profiles

The time-temperature profiles for the microwave heated samples are shown in Fig. 1. As would be expected, shorter times were needed to reach 75°C at the medium power setting (200 watts, effective) than at the low power setting (80 watts, effective). At both power settings, the times to reach 75°C increased as the amount of water present in the samples was increased. The time-temperature profiles for equivalent amounts of water heated in the absence of starch (Fig. 2) show the same trend of longer times with increasing amounts of water. The actual times, however, were not necessarily equivalent (Table 1). At the medium setting, the times for starch:water and the equivalent amounts of water were quite similar, but at the low setting, the times for the starch:water samples were shorter than those for the equivalent amount of water in the absence of starch. In addition, the temperature at the center of the starch:water samples heated at the higher setting continued to rise above 75°C, by as much as 15°C, before cooling was sufficient to begin to reduce the center temperature. This temperature rise was less than 5°C in starch:water samples heated at the lower wattage or in water heated in the absence of starch. These results

Table 1. Starch heating time measurements.

Starch:water ratio	Weight of H <sub>2</sub> O (g)	Time to reach 75°C (min) <sup>a</sup>					
		Medium microwave				Low microwave	
		Starch:water		Water only		Starch:water	Water only
		X	s.d.	X	s.d.	X	s.d.
1:1	25.0	0.6 ± 0.02		0.8 ± 0.14		2.4 ± 0.16	3.5 ± 0.06
1:2	33.0	0.9 ± 0.12		1.0 ± 0.58		3.4 ± 0.88	4.6 ± 0.20
1:4	40.0	1.0 ± 0.25		1.1 ± 0.20		3.5 ± 0.26	5.3 ± 0.27
5:95	47.5	1.3 ± 0.24		1.4 ± 0.13		4.9 ± 0.64	5.6 ± 0.42
	50.0			1.3 ± 0.16			6.2 ± 0.45

<sup>a</sup>Means (X) and standard deviations (s.d.) based on 5 measurements.

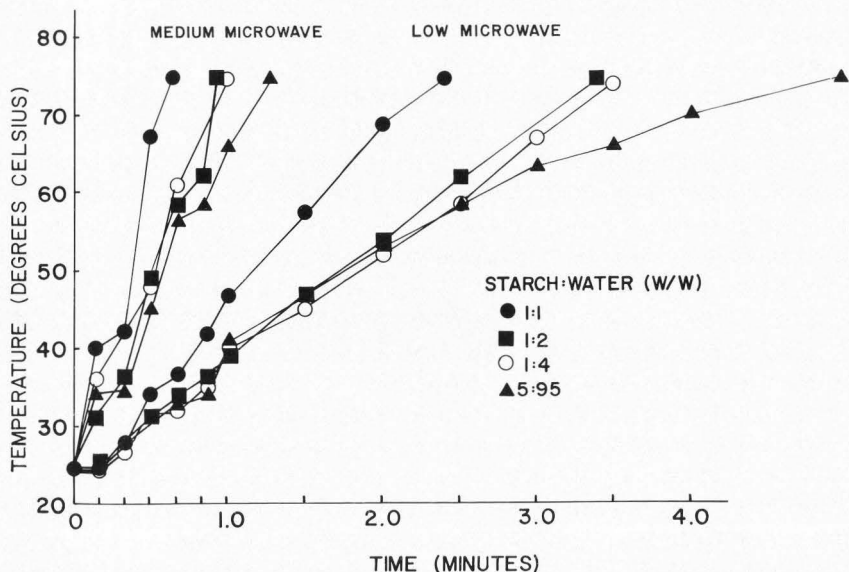


Fig. 1 Time-temperature relationships using microwave (low and medium settings) for four starch:water ratios.

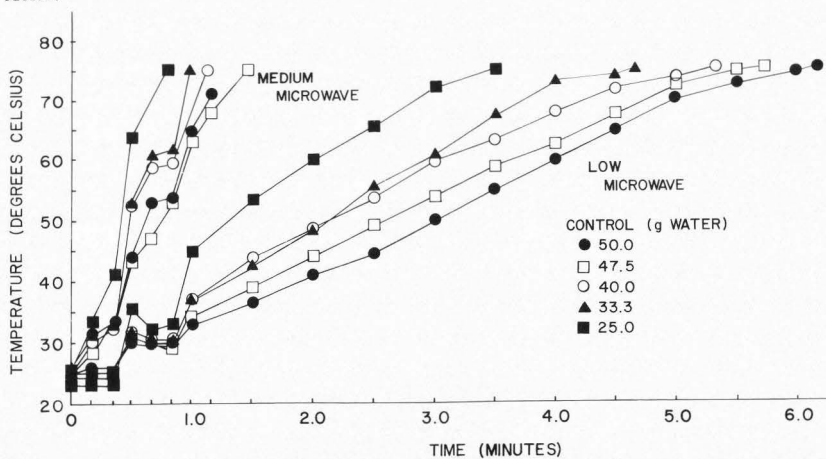


Fig. 2 Time-temperature relationships using microwave heating at low and medium settings for weight of water equivalent to water present in the starch:water systems (25 g equivalent to 1:1; 33.3 g to 1:2; 40 g to 1:4; 47.5 g to 5:95. 50 g equivalent to total sample volumes).



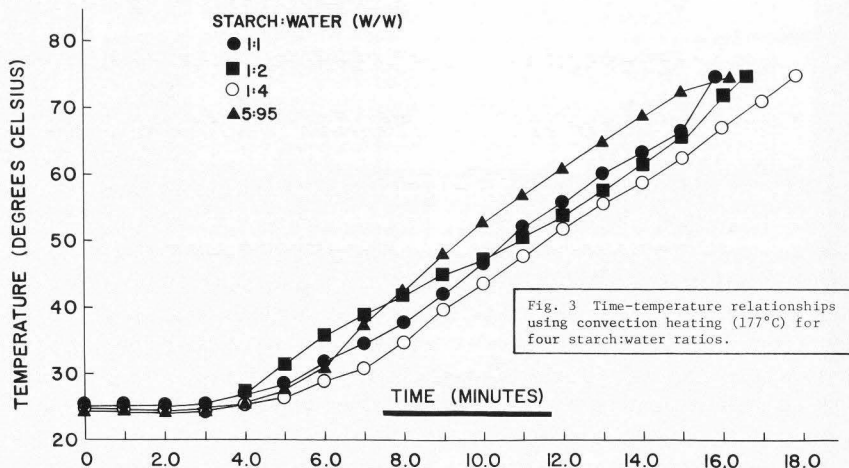


Fig. 3 Time-temperature relationships using convection heating (177°C) for four starch:water ratios.

Table 2. Height and weight losses and distribution of gelled and chalky areas in starch:water samples heated by microwave irradiation and convection.

Starch:water ratio	Heating mode	Height loss (mm)	Weight loss (g)	Cross-sectional area		
				Total area (cm <sup>2</sup> )	Gelled area <sup>b</sup>	Chalky area <sup>b</sup>
1:1	Microwave - medium	0.5 ± 0.5	0.5 ± 0.2	11.0 ± 0.6	63	37
	Microwave - low	0.7 ± 0.3	0.6 ± 0.7	11.3 ± 0.3	61	39
	Convection	2.6 ± 0.7	2.6 ± 0.5	10.1 ± 0.3	49	51
1:2	Microwave - medium	0.3 ± 0.5	0.9 ± 0.2	11.1 ± 0.4	88	12
	Microwave - low	0.7 ± 0.4	0.7 ± 0.1	11.5 ± 0.5	85	15
	Convection	3.1 ± 0.3	2.9 ± 0.6	9.4 ± 0.2	84	16
1:4 <sup>a</sup>	Microwave - medium	0.4 ± 0.5	0.9 ± 0.3			
	Microwave - low	0.3 ± 0.4	0.8 ± 0.2			
	Convection	1.5 ± 0.5	4.0 ± 0.5			
5:95 <sup>a</sup>	Microwave - medium	0	0.8 ± 0.4			
	Microwave - low	0	1.1 ± 0.2			
	Convection	1.4 ± 0.6	3.3 ± 1.2			

<sup>a</sup>Measurements of cross-sectional areas were not possible due to fragility of the heated samples.

<sup>b</sup>% of total area.

suggest that water is important in heating samples by microwave irradiation, but it is not the sole molecular species that interacts with microwave irradiation.

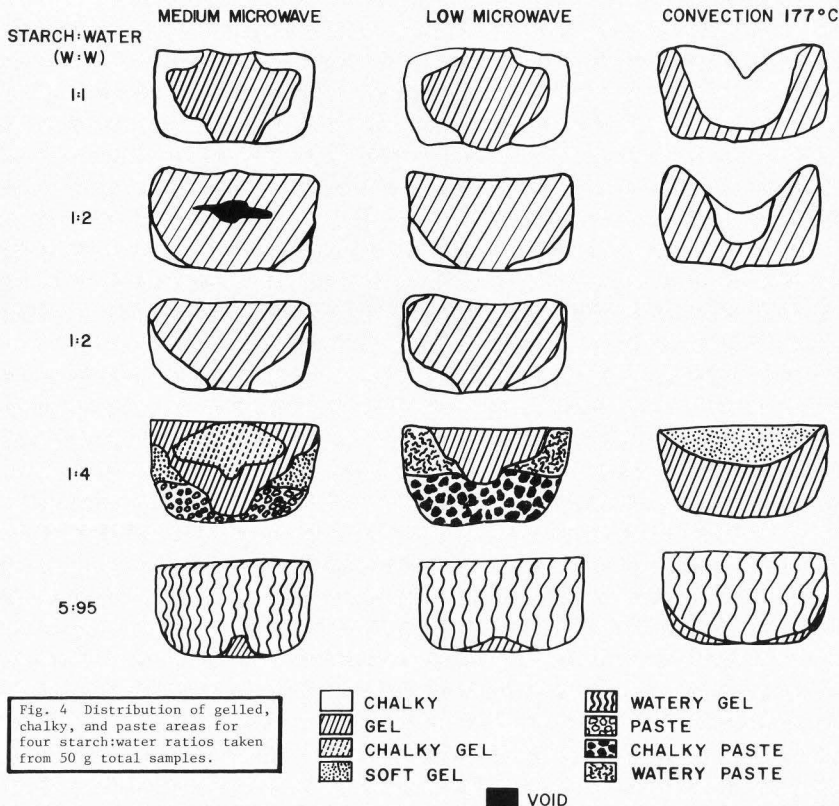
The time-temperature heating profiles for convection-heated samples are shown in Fig. 3. The times to reach 75°C ranged from 15.9 min for 1:1 ratio to 17.8 min for 1:4 ratios with the times for 1:2 and 5:95, 16.7 and 16.2 respectively, falling within this range. They were closely related to water content than was the case for microwave-heated samples.

#### Macrostructural Characteristics

Both weight loss and shrinkage as measured by the change in height were greater in samples

heated by convection mode than in samples heated by either power setting using the microwave mode (Table 2). Weight loss and shrinkage for samples heated at the two microwave power settings were similar to each other although heating rates were not the same.

The locations of the gelled and chalky areas (1:1 and 1:2 starch:water ratios), the various gel and paste areas (1:4 starch:water ratios) and watery gel and gel areas (5:95 starch:water ratios) were determined by the heating mode as shown in Fig. 4. The gelled regions were the inner regions of the microwave samples and the outer regions of the convection samples.



The 1:1 and 1:2 starch:water ratios showed two distinct regions, one gelled, the other chalky. Voids were found at the center of some of the 1:2 ratio samples. The proportions of gelled to chalky areas increased as the starch:water ratio was increased from 1:1 to 1:2 and more water was available. At the lower ratio, approximately 60% of the microwave sample and 50% of the convection-heated sample were gelled. At the 1:2 level, 85% of the sample was gelled under either microwave or convection heating conditions.

Patterns for the 1:4 ratio samples heated by microwave energy were more complex than those for the lower ratios. A soft gel region was at the top of the outer region, and a paste region was at the bottom of the outer region. The central gel region varied from an opaque gel at the top to a clear gel at the bottom. The patterns for the convection-heated samples consisted of a soft paste at the top and a gel region at the bottom.

The 5:95 samples, whether heated by microwave or convection, consisted almost entirely of a watery gel area with a small amount of a gel region (possibly 5% of the total) located at the center bottom of microwave samples and the outer bottom side of the convection-heated samples.

#### Microstructural Characteristics

Six stages in the swelling patterns were identified on the basis of changes in small and large granules and the development of an amorphous-appearing matrix. Representative areas from each stage are shown in Fig. 5 and the characteristics of each stage summarized in Table 3. In the first three stages, (Figs. 5a-c) the small granules which are approximately 2 to 10  $\mu$ m in diameter, are seen to progressively clump and then swell at the edges to form a swollen, dimpled granule. The large granules, which ranged in diameter from approximately 10 to 40  $\mu$ m, swell

radially to disc shapes and then fold. Development of matrix material which is defined as any material other than discrete granular structures, cannot be observed. Birefringence is retained through the first three stages. In the last three stages (Figs. 5d-f), matrix development, which is first observed at stage 4, advances until it reaches a stage where small amounts of the sample material also contain a fibrous state in stage 6 as shown in Fig. 5f. The small and large granules can be clearly identified in stage 4, but begin to flow together by stage 5. Small granules are less frequently seen as discrete entities; presumably they have become part of the matrix by stage 5. Some granule structure is retained, in stage 5 and 6, however, as can be seen by examination of washed samples by light microscopy (Inserts 5d-f).

The range in microstructural characteristics within a sample decreased as the starch:water ratio was increased and water became less limiting (Table 4). For each starch:water ratio, structural differences were also associated with the heating mode. In the 1:1, 1:2, and 1:4 starch:water systems, the inner chalky or soft gel regions of the convection mode were more advanced than their counterparts in samples heated by the microwave mode (the outer chalky or soft gel regions). For example, at the 1:1 starch:water ratio and at either the medium or low microwave power, the outer regions were essentially unchanged in appearance from that of the unheated starch. The inner regions had reached stages 5 and 4, respectively, with small granules swollen and dimpled and the large granules disc-shaped and folded. The matrix had developed, and in the case of medium power, the small and large granules had flowed together. The convection-heated samples at this ratio of starch:water had reached a similar degree of swelling in the outer gelled regions, but degree of granule swelling in the inner chalky region was more advanced (stage 2) than the chalky area of the microwave samples (stage 1).

A similar contrast between microwave and convection-heated samples was present at the 1:2 starch:water ratio, except that for each heating mode the chalky area had structures that were one stage more advanced than the comparable regions of the 1:1 starch:water ratio samples. The same contrast between microwave and convection-heated samples was found at the 1:4 starch:water ratio, but the structures in the gelled region of the medium power samples were also at a more advanced stage than the gelled regions in the low power samples and showed a fibrous rather than amorphous matrix (stage 6). This fibrous matrix was also found in the gelled region of convection-heated samples.

In the 5:95 starch:water systems, the difference between the microwave and convection-heated samples was not as great as for the other starch:water systems. The watery gel areas in which starch apparently was not concentrated enough to form a gel had structures that were similar to the gelled area, except that fibrous development in the matrix was not as extensive in the watery gel area. These watery gel areas may or may not contain small amounts of the chalky, limited water areas discussed earlier. Although

Table 3. Stages in swelling of starch granules.

Stage	
1	Granules - essentially unchanged.  Birefringent.
2	Small granules - clumped.  Large granules swelling to disc shapes, appear plastic prior to folding.  Birefringent.
3	Small granules-cluster swell, dimple.  Large granules - disc shaped, folding beginning.  Birefringent.
4	Small granules - swollen, dimpled.  Large granules - disc shaped, folded.  Matrix present.  Some birefringent granules.
5	Small granules no longer visible.  Flowing together of small and large granules.  No birefringence.
6	Granules as in stage 5.  Matrix - fibrous.

the watery gel area appeared to be quite advanced on the basis of the SEM examinations, it retained a small amount of birefringence, and some granule retention could be seen in washed samples examined by light microscopy.

In summary, microscopic observations showed that the convection-heated samples were structurally more uniform in the sense that a narrower range and, therefore, fewer stages of starch granule swelling and matrix development were observed for samples at each starch:water ratio. This was attributed to the chalky areas in the convection-heated samples being further along in the gelatinization process than the comparable areas in the microwave mode, yet the gelled areas were at similar or less advanced stages in the gelatinization process. Within the microwave mode, differences in the rate of heating (medium vs low power) did not result in major differences in swelling patterns.

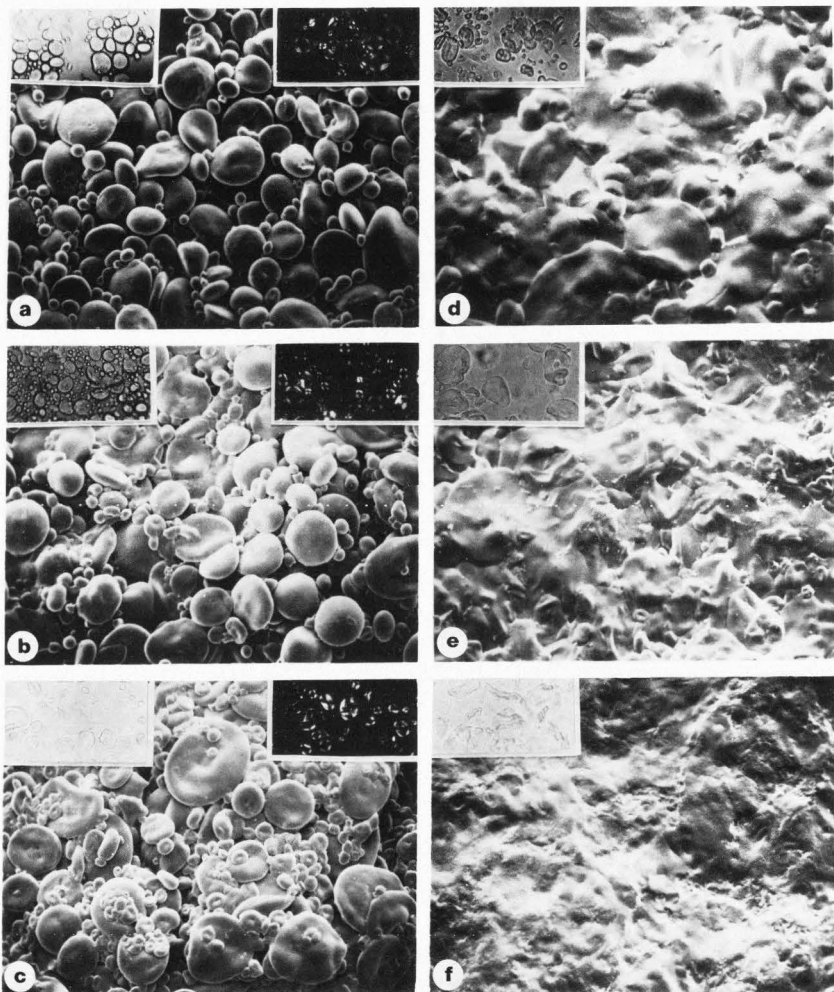


Fig. 5 Stages in granule swelling. Larger photos are SEM micrographs; upper lefthand inserts are light micrographs; upper righthand inserts are polarized light micrographs (when birefringence was observed in (a), (b), and (c)). SEM 30  $\mu\text{m}$ . Light and polarized 40  $\mu\text{m}$ . (a) stage 1, (b) stage 2, (c) stage 3, (d) stage 4, (e) stage 5, (f) stage 6.

Table 4. Stages of swelling in various regions in starch:water samples heated by microwave irradiation and convection.

Starch:water ratio	Heating mode	Area <sup>a</sup>	Stage of swelling <sup>b</sup>
1:1	Microwave-Medium	Gel	5
		Chalky	1
	Microwave-Low	Gel	4
		Chalky	1
	Convection	Gel	5
		Chalky	2
1:2	Microwave-Medium	Gel	5
		Chalky	2
	Microwave-Low	Gel	5
		Chalky	2
	Convection	Gel	5
		Chalky	3
1:4	Microwave-Medium	Gel	6
		Chalky paste	3
	Microwave-Low	Gel	5
		Chalky paste	3
	Convection	Gel	6
		Soft gel	4
5:95	Microwave-Medium	Gel	6
		Watery gel	5
	Microwave-Low	Gel	6
		Watery gel	5
	Convection	Watery gel	5
		Watery gel	5

<sup>a</sup>For locations of areas, see Fig. 4.<sup>b</sup>For descriptions of stages, see Table 3 and Fig. 5.

### Discussion

The specific roles that the water content and mode of heating play in the range of granule swelling observed microscopically are difficult to separate from one another, since they are interre-

lated. A number of researchers (Burt and Russell, 1983; Donovan, 1979; Eliasson, 1980; Eliasson et al. 1981; Chiassi et al. 1982; Wootton and Bamunuarachchi, 1979; Lund, 1983; Biliaderis et al. 1980) have shown, in differential scanning calorimetry experiments, that the effect of decreasing water content is to suppress the initial gelatinization endotherm (as shown by a decrease in enthalpy) until it disappears at limited water contents. The water content at which this occurs varies somewhat with the experimental conditions but is usually placed at about 30%. When water content is above this critical level and the gelatinization endotherm is present, the onset temperature of the endotherm for wheat starch is relatively constant. Since the water contents in our experiment were above this critical level, the advancement in the stage of swelling with increasing water content which we observed microscopically is not likely to be due to a change in onset temperature of starch phase transitions. The enthalpy may be different and this could contribute to changes in the swelling as observed microscopically. The starch:water ratio may also affect other properties of the system that are more nearly related to the manner in which microwave energy couples with localized areas within the sample. In microwave-heated samples, the time to reach the predetermined final temperature increased as the amount of water was increased. Thus, the more advanced stages of starch gelatinization as water became less limiting in microwave-heated samples might be partly due to longer heating times once a certain critical stage of swelling had taken place. Also, in contrast to the gelled region where gelatinization was more advanced, there were chalky regions that were unaffected by exposure to microwave radiation as seen in the 1:1 and 1:2 ratios. Since the dielectric loss factor is much less in the chalky region because there are rigid, more crystalline structures within the unchanged granules, less heating will result in these regions as long as less crystalline or amorphous structures also exist in other parts of the samples. This is analogous to the boiling of water when ice is present, as is seen in heating frozen materials by microwave energy. Callaghan et al. (1983) concluded from nuclear magnetic resonance measurements that about 60% of the starch molecules in extensively heated pastes are in a "mobile liquid-like" polymer form and 40% in an "immobile crystalline" state. Exchange of water occurs between the free water and mobile polymer form and not with the crystalline form. If this is also true under the heating conditions of the present study, interaction with microwave radiation in the crystalline form would be restricted as compared with interactions in the liquid mobile polymer fraction. In the convection mode in the absence of interactions between microwave irradiation and crystal structures, the advancing stages, as water content was increased, might be more closely related to the differences seen on the microscale by differential scanning calorimetry studies as summarized above.

The total sample size of 50 g, although smaller than often used in microwave applications, was large enough so that some heat transfer by convection within the sample would be expected.

This could lead to localized regions of high and low water content with free water being immobilized as starch transformations occur. The patterns of the gelled and chalky areas for the 1:1 and 1:2 ratios were reproducible, suggesting that heat transfer within the sample vessel also followed consistent patterns.

The patterns in stages of starch transformations observed at both the macrostructural and microstructural level within a given sample probably contribute to the non-uniformity in texture and other properties seen in microwave-heated starch-based products. For example, Evans et al. (1984) found that the cracking patterns and surface appearance of cakes were related to heating modes (microwave vs convection) and the time during baking at which the heating mode was interchanged.

Excessive and rapid hardening is also often observed in microwave-heated starch-based products. If development of staling depends on recrystallization of starch, and recrystallization of partially melted crystallites is faster than that of completely melted crystallites, uneven staling and moisture redistribution could occur.

The stages of swelling that were identified suggest that the same overall sequence of swelling was occurring in the various samples. The stages for the large granules were similar to those identified by Bowler et al. (1980) for radial swelling and the initial stages of tangential swelling. When samples differed as to stage, it was not possible to isolate a single cause such as water being limiting, a heating rate effect, or a microwave radiation coupling effect. Each of these factors interacted, so that a direct effect on starch transformation could not be identified. Further research with smaller samples and more rapid heating rates in microwave and convection/conduction modes are in progress in an attempt to isolate these heating rate effects from effects inherent in microwave interactions.

#### Implications for Microwave Heating

1. Water in part controls the rate of heating in microwave heating, but external temperature controls the rate of sample heating in the convection mode within the water content normally found in food products. Therefore, overall water content as well as localized movement of water will affect rate or kinds of starch granule transitions seen in microwave heating. Greater water content means that there are fewer localized differences in water content and fewer structural differences will be observed.
2. When a material such as starch absorbs more water as in gel formation, increased heating rates will occur in that location due to the interaction of microwave radiation with water. Therefore, phase transitions can continue to advance. When a component is more crystalline or latticed, as might be expected in the less advanced stages of starch granule transformations, microwave interaction is smaller and fewer changes occur, especially when the water is more limited.
3. When non-uniformity in the stage of granular transformation develops within a sample, non-uniformity in other characteristics that

depend on the stage of gelatinization will be present also.

#### Acknowledgements

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#### Discussion with Reviewers

**E.M. Snyder:** Since it takes 12-14 minutes for the starch-water mixtures to reach 75°C when heated by convection (Figure 3), does the starch settle out in the higher moisture systems, i.e., 1:4 and 5:95, or at 80% and 90% moisture? Was any effort made to keep water levels constant by covering beakers?

**D.D. Christianson:** Did the granules settle during convection heating in the 5:95 starch:water system? Thermal histories in Figure 3 would indicate this.

**Authors:** Settling could occur, and might be responsible for the small gel regions at the bottom of the 5:95 samples whether heated by convection or microwave. It could also occur in the 1:4, but the positions of the swollen and less swollen granules are also consistent with the differences in heat transfer, so the two effects, settling and specifics of heat transfer may be superimposed. The beakers were not covered because the relative water losses during heating by different methods were of interest in the experiments.

**E.M. Snyder:** In your polarized light microscopy work, were samples suspended in a non-aqueous media as well as water for comparative purposes? (Partially hydrated starch and granules damaged by excessive heat may swell in water at room temperature.)

**Authors:** No, we did not do this.

**E.M. Snyder:** Does the amorphous appearing matrix stain blue to iodine? If not, what could it be due to?

**Authors:** We did not try this because the matrix was not obvious in the light micrographs and was differentiated only by SEM and we do not stain for SEM. However, it would be useful to try some staining with iodine in the future.

**E.M. Snyder:** Sample preparation of high moisture specimen for SEM has plagued those of us in the food industry for years. We are all too familiar with the various artifacts that are introduced during dehydration procedures. Do you think the fibrous material in stage 6 from preparations originally at 80% and 95% moisture are due to shrinking and shrivelling of starch material as it dries to a tight film? If it is not an artifact, do you have any theory or ideas as to the cause? Was any other dehydration method attempted?

**Authors:** We have been concerned also that the fibrous material may be an artifact arising during drying. However, the artifact is different in the variously heated samples. In freeze-etch studies (Clove et al. 1982, Freeze-etch of emulsified cake batters during baking, *Food Microstructure* 1:177-187) we have seen some "beading" close to the granule surface that might be related to the fibrous matrix. We are extending our freeze-fracture studies, although we recognize that the technique also has potential for development of artifacts (Davis, E.A. and Gordon, J. 1984.

Microstructure analyses in gelling systems. *Food Technol.* (in press); Davis, E.A. and Gordon, J. 1983. Freeze-fracture, freeze-etch techniques. In *New Frontiers in Food Microstructure*. Ed. D.B. Bechtel. American Association of Cereal Chemists, Inc., St. Paul, Minnesota pp. 241-268).

**D.D. Christianson:** It is interesting to speculate that at a higher microwave temperature even more solubilization would occur. Microscopically, the gel should show some correlation with Callaghan's NMR work.

**Authors:** We have not attempted to go beyond the upper limits of the gelatinization ranges (approximately 85°C), but we are trying, in experiments currently underway in our laboratory, to increase the rates of heating as compared to those used in this study. To do this, we have used smaller samples and placed the samples directly in the waveguide of a specially designed hybrid convection:microwave oven (Wei, C.K., Davis, H.T., Davis, E.A., and Gordon, J. 1984. Heat and mass transfer in water-laden sandstone: microwave heating. *AIChE J.* (Submitted.)) We are comparing these samples with samples heated to the same temperature in the same time by conduction in an oil bath. Callaghan et al (1983) reported that they heated their samples for 1 hr at 95°C and then held them at 30°C for 1 hr before making measurements. These conditions are much more drastic than those that we used.

**P. Resmini:** Have you attempted to support your observations on starch structure by light and scanning electron microscopy with chemical and physical analysis?

**Authors:** We have used differential scanning calorimetry to determine whether endotherms are found in the various regions to confirm whether starch phase transitions have occurred.

**P. Resmini:** Are the starch ultrastructure modifications comparable to those found by other authors?

**Authors:** Relatively few studies of the effects of microwave irradiation on starch granule transformations have been made. The overall sequence of ultrastructure modifications appear similar to those reported by other authors working with a variety of heating methods. No structures that resulted uniquely from microwave heating appeared to be present.

**D. Bechtel:** In the "Results" section, you mention that "...small granules...progressively clump and then swell at the edges..." Do the granules clump and then swell to form a "Swollen, dimpled granule" or does the center collapse?

**Author:** This is a possibility.

MICROSTRUCTURE OF SET-STYLE YOGHURT MANUFACTURED FROM COW'S MILK FORTIFIED BY  
VARIOUS METHODS

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Abstract

Five different batches of skim milk were prepared and fortified by the addition of skim milk powder (SMP) or sodium caseinate (Na-cn) or by concentration using a vacuum evaporator (EV), ultrafiltration (UF), or reverse osmosis (RO) to contain similar levels of protein (5.0-5.5%). Yoghurts were made by inoculating the milks with one of 3 commercial yoghurt starter cultures and by incubating the mixes at 42°C for 2.5 h. The following factors were found in this study to affect firmness of the yoghurts:  
(a) Lactic acid production (acidity) - Yoghurts containing 1.02% of lactic acid or more (pH 4.54 or less) were firmer than yoghurts having a lower lactic acid content and a higher pH value.  
(b) Casein to non-casein protein ratio - Firmer yoghurts were obtained at a ratio of 4.62 than at 3.20-3.40.

Microstructure of the yoghurts as examined by electron microscopy was affected by the method of fortification of the milk. SMP-fortified yoghurt had the most dense matrix composed of short micellar chains and small micellar clusters. This was the softest yoghurt. Na-cn-fortified yoghurt had the most open matrix consisting of robust casein particle chains and large clusters. This was the firmest yoghurt.

"Appendages" or "spikes" formed by heat-denatured  $\beta$ -lactoglobulin or by a complex consisting of  $\beta$ -lactoglobulin and  $\kappa$ -casein were attached to casein micelles in all the yoghurts except the one fortified by the addition of Na-cn.

Void spaces (cavities) around lactic acid bacteria and filaments of mucous or slimy material produced by a "ropy" bacterial culture and attaching the bacterial cells to the protein matrix were additional microstructural features observed in the yoghurts under study.

Introduction

The process of yoghurt making dates back thousands of years, and the acidification of milk by fermentation, e.g. with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, is one of the ancient crafts which is used to preserve milk. Over the past two decades, the popularity of yoghurt has spread from the Balkans and the Middle East to other parts of the world, and the consumption has increased significantly in all these countries in recent years. Consequently, the science and technology of yoghurt production, including the microbiology of the starter culture and quality aspects of yoghurt, have been studied and reviewed in great detail (10-12, 16, 17).

The level of total solids in the yoghurt milk plays an important role in the quality of the product during manufacture (16), and various methods of fortification are widely used in the industry. Examples of such methods may be summarised as follows: The addition of milk powder (whole milk, skim milk, or buttermilk), or the concentration of milk [(by boiling, evaporation (EV), ultrafiltration (UF), or reverse osmosis (RO)), and/or the addition of other miscellaneous ingredients which may include whey powder and caseinate.

Microstructure studies by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of foodstuffs including dairy products are modern techniques which may have an increasing application in assessing various aspects of food quality and characteristics. The application of such techniques to different types of yoghurt has been used to a limited degree, and up-to-date information in this field has recently been reviewed (7).

The objectives of this study were to assess and evaluate the microstructure of set-style plain yoghurt manufactured from cow's milk fortified with skim milk powder (SMP) or sodium caseinate (Na-cn), and from concentrated milk (EV, UF, and RO). In addition, the effects of 3 different strains of yoghurt starter cultures on the microstructure of the yoghurts were studied.

Materials and Methods

Preparation of the yoghurt milk

Whole cow's milk (antibiotic-free, which is regularly tested according to the quality schemes

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of the Scottish Milk Marketing Board) was obtained from the College Farm. The milk fat was separated using a centrifugal separator (Alfa-Laval type 29AE/1963) and the resultant skim milk was divided into five batches for the production of the basic mix as follows: The first two batches were fortified by the addition of SMP or Na-cn to produce two milk bases containing approximately 14.5% or 11.0% solids-not-fat (SNF), respectively. The remaining three batches of skim milk were concentrated by EV, RO, and UF. Later, both the fortified and concentrated skim milks were standardised with cream (49% fat) to give a milk base containing 1.5% fat. It is important to note that the batches of skim milk fortified by the addition of Na-cn and skim milk concentrated by UF contained lower levels of SNF as compared with other batches, but the protein content in all the five different basic mixes ranged between 5.0 and 5.5%.

The preparation of the UF, RO, and EV milks was as follows: UF-skim milk was concentrated by ultrafiltration in an Alfa-Laval model UFP pilot plant fitted with a Hollow Fibre membrane cartridge with PM50 membranes. The milk was slightly overconcentrated and then standardised with permeate to 10.3% SNF. During the concentration, the temperature of the milk increased from 10°C to approximately 40°C.

RO-skim milk was concentrated by reverse osmosis using a DDS type 20-1.8-0 module (Danske Sukkerfabrikker, DDS RO Division, DK-4900 Nakskov, Denmark). The concentrate was standardised with permeate to 14.5% SNF. At the end of the processing, the temperature of the concentrated milk was around 20°C.

EV-skim milk was concentrated in a QVF glass climbing film evaporator at 70-75°C and the concentrate was standardised to 14.5% SNF using the condensate.

#### Production of yoghurt

The procedure for the production of yoghurt is outlined in Figure 1. In order to minimise the effect of day to day variation of the milk composition and quality, all the yoghurts were made from the same batch of milk. The heating and cooling of the yoghurt milks took place in a water bath; during the heat treatment, steam was injected into the water and during the cooling stage, mains water was circulated.

Three yoghurt starter cultures consisting of different *L. bulgaricus* and *S. thermophilus* strains were used and in this present study coded as CH-4, Boli-3, and RR. The former two starter cultures were supplied by Chr. Hansen's Laboratory Ltd., Reading, U.K., and the RR culture was obtained from the Netherlands Dairy Research Institute (NIZO), Ede, the Netherlands.

#### Chemical analysis

Total solids, SNF, protein, fat, ash, and moisture contents were determined according to the British Standards Institution (BSI) and AOAC methods (3-5).

Titrate acidity was determined by titrating 10 g yoghurt samples with N/9 sodium hydroxide using 1 mL of a phenolphthalein (0.5% in 50% ethanol) solution as an indicator.

#### Preliminary treatment of milk (standardisation/fortification)

Pre-warm basic milk mix to 60°C

↓

Homogenise at 200 kg/cm<sup>2</sup>

↓

Heat the milk to 90°C  
for 5 minutes

↓

Cool to 45°C and inoculate with  
starter culture at a rate of  
3% (v/v)

↓

Dispense the inoculated milk into  
150 ml plastics cups and incubate  
at 42°C for 2½ hours

↓

Refrigerate for 48 hours  
at 6°C

Fig. 1. Flow chart for the production of set-style yoghurt.

The duration between prewarming and inoculation stages did not exceed 30 min.

pH was determined using a model 290 pH meter (Pye Unicam Ltd., Cambridge, U.K.) fitted with a standard combined glass electrode.

Chemical composition of the ingredients used to make the yoghurts is presented in Table 1.

#### Rheological analysis

A penetrometer (Universal Model 1700, Stanhope-Seta, Surrey, U.K.) was used to assess the firmness of the coagulum according to the method of Robinson and Tamime (12).

The coagulum was broken with a spoon and a part of it was lifted on the back of the spoon to visually assess the texture characteristics of the yoghurt.

#### Microscopic analysis

The yoghurts were sampled after 2 days of storage. For SEM, sections 3 x 3 x 1 mm were excised from the yoghurt approximately 1 cm below the surface and were fixed in an aqueous 1.4% glutaraldehyde solution for 7 days. The sections were then cut into prisms 1 x 1 x 3 mm, dehydrated in a graded ethanol series, frozen in Freon 12 at -150°C, and freeze-fractured in liquid nitrogen. The fragments were melted in absolute alcohol (+20°C), critical-point-dried from carbon dioxide, mounted on SEM stubs, coated with gold by vacuum evaporation, and examined in a Cambridge Stereoscan Mark II scanning electron microscope operated at 20 kV.

For TEM, fixed yoghurt particles less than 1 µm<sup>3</sup> in volume were postfixed in a 2% buffered OsO<sub>4</sub> solution (pH 6.75) for 1 h, dehydrated, and embedded in a low-viscosity Spurr's medium (14). Sections (90 nm) were stained with uranyl acetate and lead citrate solutions and examined in a Philips EM 300 electron microscope operated at 60 kV.

### Results and Discussion

Chemical composition of the five various yoghurt mixes (SMP, EV, UF, RO, and Na-cn) following the addition of the starter culture is shown in Table 2. It is evident that the protein level in the milk was maintained between 5.09 and 5.49%, whereas the total solids contents fluctuated between 11.79 and 16.01%; the SMP yoghurt milk was at the upper limits and the UF yoghurt milk was at the lower limits of both the protein and total solids contents, the relative differences being 7.4 and 26%, respectively, related to the SMP yoghurt milk as the reference (100%). The lactose content was lowest in the UF yoghurt milk (4.27%), higher in the Na-cn (5.13%) and EV (7.11%) yoghurt milks, and highest in the RO and SMP yoghurt milks (7.53 and 7.64%, respectively).

Firmness of the experimental yoghurts was affected considerably by the starter culture used (Table 3), when incubation at 42°C was maintained for a predetermined period of 2.5 h. All the five yoghurt variants made with the RR starter culture were softer than the corresponding variants made with the other two starter cultures; the softer yoghurts had weaker bodies. The softness (high penetrometer readings as listed in Table 3) may be associated with low acidity (0.92-1.02% lactic acid, pH 4.54-4.73) in the yoghurts made with the RR starter culture as opposed to a higher acidity (1.08-1.39% lactic acid, pH 4.21-4.43) in the firmer yoghurts made using the CH-4 and Boll-3 starter cultures.

Within the individual starter culture variants, the yoghurts made with Na-cn were firmest and the firmness decreased in the RO, UF, EV, and SMP yoghurts in that order. Such results are in agreement with the work reported by Abrahamson and Holmen (1, 2), with the UF yoghurt as an exception. Abrahamson and Holmen (1, 2), however, standardised their yoghurt samples to similar levels of total solids (14.13 to 14.57%), thus achieving a high protein content in the UF yoghurt, whereas in this study, the yoghurt milks were adjusted to similar protein levels; consequently, the total solids content of the UF yoghurt was the lowest of all the experimental yoghurts (11.79-11.86%; Table 2).

In general, the microstructure of all the fifteen yoghurt samples as examined by electron microscopy was similar in that the yoghurts had protein matrices composed of casein micelle chains and clusters. The ways in which the casein micelles were linked to each other, however, differed noticeably. SEM revealed that in the SMP, RO, and EV yoghurts (Figure 2 a-c), the micellar chains were short and simple and the clusters were small. The resulting matrices appeared to be relatively more compact (denser) than the matrices of the UF and Na-cn yoghurts (Figure 2 d and e). The more open matrices were due to larger interstitial spaces ("pores") resulting from a different distribution of the casein micelles in the UF and Na-cn yoghurts. This was more clearly visible in the TEM micrographs (Figure 3): the chains were considerably longer than in the SMP, RO, and EV yoghurts and were either complex in the UF yoghurt or robust in the Na-cn yoghurt. As opposed to simple chains (Figure 3 a), complex chains were

Table 1.  
Chemical composition (%) of dairy ingredients used to prepare yoghurt milks

Ingredient	Total Solids	Protein	Fat	Lactose*	Ash	Moisture
Skim milk	9.16	3.39	0.10	4.94	0.73	90.84
Cream	53.59	n.d.	49.00	n.d.	n.d.	46.41
SMP	96.97	36.50	0.97	51.8	7.88	3.03
Na-caseinate	95.50	89.30	1.17	n.d.	3.68	4.50
RO permeate	0.40	n.d.	n.d.	n.d.	n.d.	99.60
UF permeate	3.41	0.12	n.d.	n.d.	n.d.	96.59
EV condensate	0.00	n.d.	n.d.	n.d.	n.d.	100.00

\* Lactose determined by difference.

n.d.: not determined.

Table 2.  
Chemical analysis (%) of various types of low-fat milks used in the manufacture of yoghurt\*

Starter + Yoghurt Milk	Total Solids	Protein	Fat	Ash	Lactose**
<b>1. CH-4</b>					
SMP	15.89	5.49	1.53	1.21	7.66
EV	15.03	5.22	1.62	1.15	7.04
UF	11.81	5.09	1.57	0.84	4.31
RO	15.81	5.44	1.60	1.15	7.62
Na-cn	13.19	5.34	1.53	0.85	5.47
<b>2. Boll-3</b>					
SMP	15.99	5.61	1.58	1.21	7.59
EV	15.14	5.24	1.61	1.16	7.13
UF	11.79	5.12	1.55	0.84	4.28
RO	15.74	5.54	1.60	1.15	7.45
Na-cn	12.67	5.38	1.52	0.85	4.92
<b>3. RR</b>					
SMP	16.01	5.56	1.58	1.21	7.66
EV	15.17	5.26	1.62	1.14	7.15
UF	11.86	5.21	1.54	0.84	4.22
RO	15.82	5.55	1.60	1.15	7.52
Na-cn	12.74	5.36	1.53	0.85	5.00

\* The milks were analyzed following the addition of the starter culture.

\*\* Lactose was determined by difference.

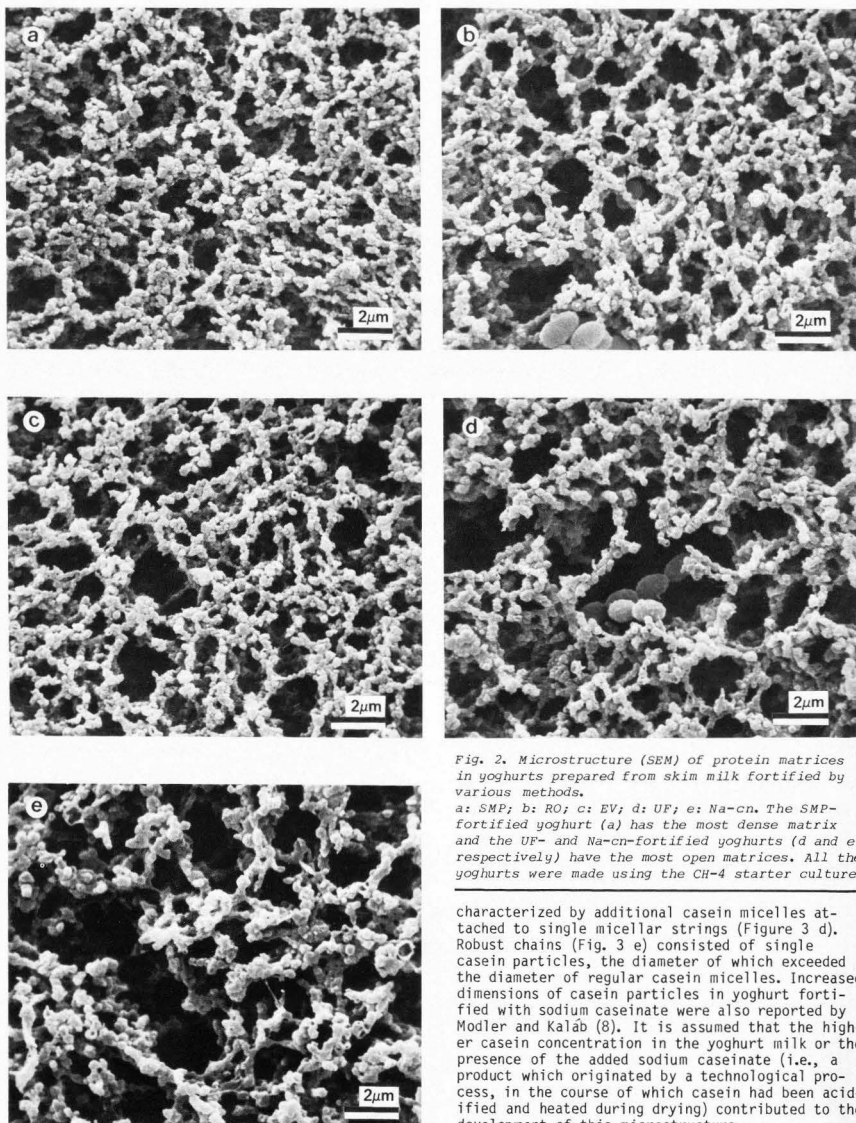


Fig. 2. Microstructure (SEM) of protein matrices in yoghurts prepared from skim milk fortified by various methods.  
a: SMP; b: RO; c: EV; d: UF; e: Na-cn. The SMP-fortified yoghurt (a) has the most dense matrix and the UF- and Na-cn-fortified yoghurts (d and e, respectively) have the most open matrices. All the yoghurts were made using the CH-4 starter culture.

characterized by additional casein micelles attached to single micellar strings (Figure 3 d). Robust chains (Fig. 3 e) consisted of single casein particles, the diameter of which exceeded the diameter of regular casein micelles. Increased dimensions of casein particles in yoghurt fortified with sodium caseinate were also reported by Modler and Kalab (8). It is assumed that the higher casein concentration in the yoghurt milk or the presence of the added sodium caseinate (i.e., a product which originated by a technological process, in the course of which casein had been acidified and heated during drying) contributed to the development of this microstructure.

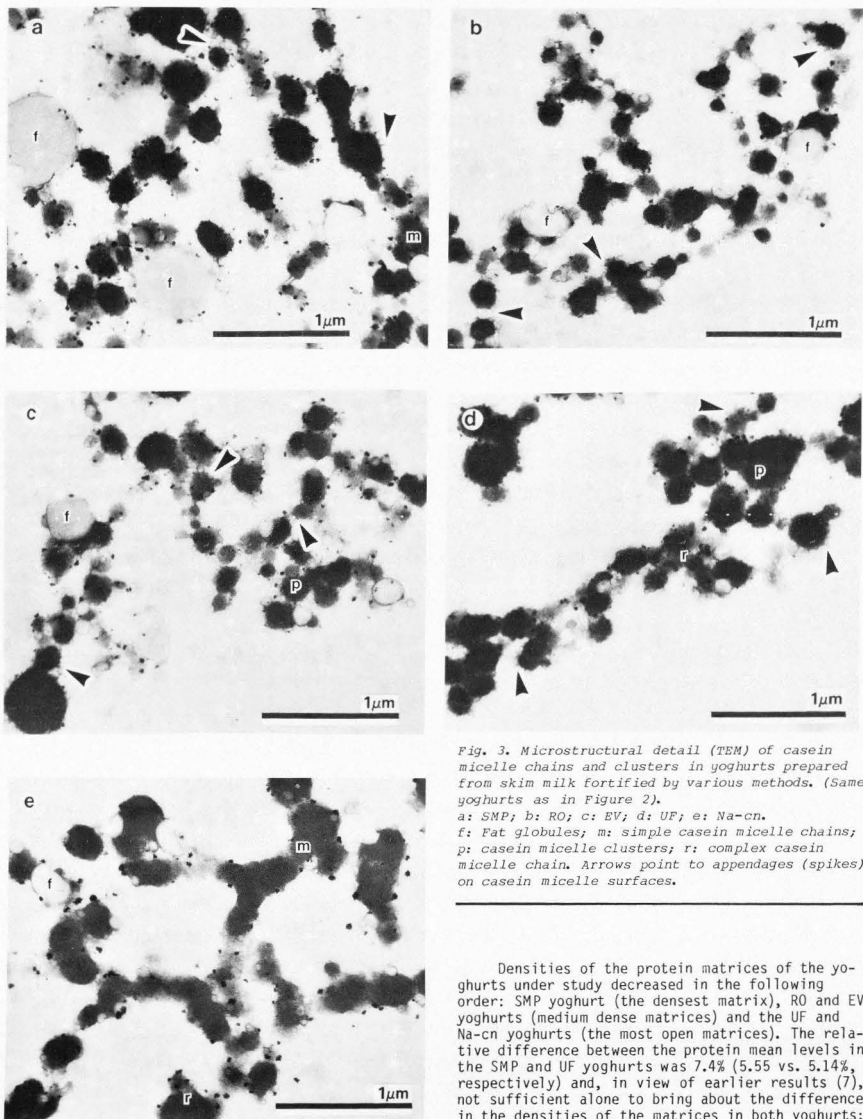


Fig. 3. Microstructural detail (TEM) of casein micelle chains and clusters in yoghurts prepared from skim milk fortified by various methods. (Same yoghurts as in Figure 2).

a: SMP; b: RO; c: EV; d: UF; e: Na-cn.  
f: Fat globules; m: simple casein micelle chains; p: casein micelle clusters; r: complex casein micelle chain. Arrows point to appendages (spikes) on casein micelle surfaces.

Densities of the protein matrices of the yoghurts under study decreased in the following order: SMP yoghurt (the densest matrix), RO and EV yoghurts (medium dense matrices) and the UF and Na-cn yoghurts (the most open matrices). The relative difference between the protein mean levels in the SMP and UF yoghurts was 7.4% (5.55 vs. 5.14%, respectively) and, in view of earlier results (7), not sufficient alone to bring about the difference in the densities of the matrices in both yoghurts;



Table 3.  
Firmness, lactic acid content (%), and pH  
of the yoghurts under study

Starter Culture	Yoghurt	Penetro-meter Reading* (1/10 mm)	Lactic Acid %	pH**
CH-4	SMP	95.3	1.25	4.37
	EV	87.3	1.39	4.28
	UF	84.0	1.22	4.32
	RO	82.6	1.29	4.39
	Na-cn	82.0	1.04	4.21
Boll-3	SMP	94.0	1.21	4.43
	EV	91.7	1.24	4.32
	UF	87.0	1.20	4.36
	RO	86.0	1.24	4.38
	Na-cn	79.0	1.08	4.25
RR	SMP	142.0	0.92	4.73
	EV	119.3	0.99	4.69
	UF	108.7	0.94	4.63
	RO	111.0	0.91	4.71
	Na-cn	90.0	1.02	4.54

\* The higher the penetrometer reading, the softer the yoghurt. No syneresis was observed in any of the yoghurts 3 days old.

\*\* Soft yoghurts had their pH values between 4.63 and 4.73; firmness was increased as pH approached the isoelectric point of casein or decreased below it.

the ratios of casein to non-casein proteins were very close to each other for the SMP and UF yoghurts, 3.38 and 3.40, respectively (Table 4). The other yoghurt with the most open protein matrix was made with Na-cn; its protein content was only 3.4% lower than the protein content of the SMP yoghurt, but the casein to non-casein protein ratios differed significantly (4.62 vs. 3.38, respectively); interestingly, the Na-cn yoghurt was the firmest of all the yoghurts under study.

TEM also revealed differences in the superficial features of the casein micelles forming the protein matrix. The casein particle chains and clusters in the Na-cn yoghurt had a smooth surface (Figure 3 e) whereas "appendages" or "spikes" were noticeable on the surface of the casein micelle structures in the other yoghurts (Figure 3 a-d). Appendages or spikes were found by Davies *et al.* (6) to be formed by heat-denatured  $\beta$ -lactoglobulin or by a complex consisting of  $\beta$ -lactoglobulin and  $\kappa$ -casein which develops in heated milk. "Spiky" casein micelles in the SMP yoghurt are compared in greater detail with the relatively "smooth" robust chains of casein particles in the Na-cn yoghurt in Figure 4. The reason for the absence of the spikes in the latter yoghurt is not known; it may only be hypothesized that their absence is associated with the high casein to non-

Table 4.  
Protein and non-casein nitrogen content (%)  
in yoghurt milks fortified by various methods

Yoghurt Milk	Total Protein	Non-Casein Nitrogen	Casein	Ratio of Casein to Non-Casein
SMP	5.34	1.22	4.12	3.38
EV	5.13	1.22	3.91	3.20
UF	5.02	1.14	3.88	3.40
RO	5.40	1.24	4.16	3.35
Na-cn	5.23	0.93	4.30	4.62

\* The protein content in the milk before heat treatment was determined by the Kjeldahl method.

casein protein ratio and/or the presence of added sodium caseinate in the yoghurt milk.

The open matrix and the robust casein particle chains and clusters may also be the factors contributing to the appearance of the protein coagulum as assessed visually after having been broken with a spoon. Only the Na-cn yoghurt had a coarse texture whereas all the other yoghurts were smooth. The detrimental effect of sodium caseinate on the texture of yoghurt was also observed by Modler *et al.* (9).

In addition to the protein level in the yoghurt milk, casein to non-casein protein ratio, bacterial starter culture, and acidity, there are other factors which are known to affect the firmness and microstructure of yoghurt, e.g. the heat treatment of the milk (temperature and duration of heating)(7), the homogenisation pressure (16), the level of minerals etc.

Lactic acid bacteria not only coagulated the yoghurt milk, but their cells became one of the structural components of the yoghurt matrix. At a low magnification (250 x), void spaces (cavities) were noticeable by SEM in the protein matrix in all the yoghurt samples. A higher magnification (5,000 x) revealed that the void spaces were occupied by streptococci or lactobacilli alone (Figure 5 a and b, respectively) or by their combination (Figure 5 c). The exact mechanism for the formation of the void spaces has not yet been established, but it is assumed that this phenomenon could primarily be attributed to the microbial activity of the starter culture (production of lactic acid, proteolysis, or liberation of carbon dioxide) and not due to artifacts arising from the preparation of the sample for microscopic examination (7).

No particular attention was paid to the dimensions of the void spaces, but within any one yoghurt in these experiments, smaller void spaces were observed around *L. bulgaricus* than around *S. thermophilus* alone or the combination of both, provided that the combined volumes of the microbial cells in each case were similar. Because the symbiotic relationship, that exists between these lactic acid bacteria, is well recognized (16), it can be hypothesized that the metabolic activity is increased by their mutual proximity.

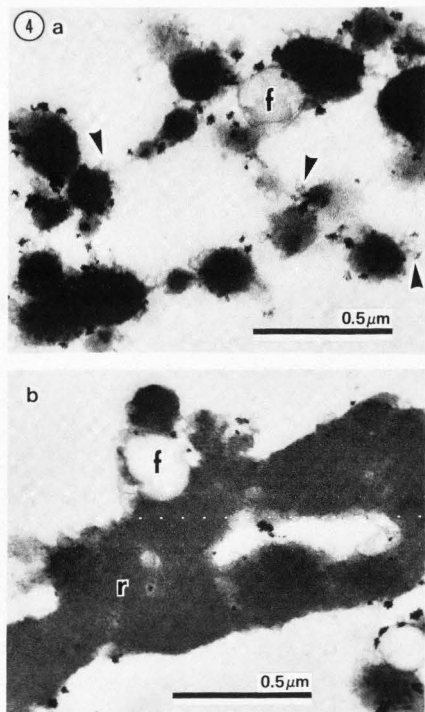


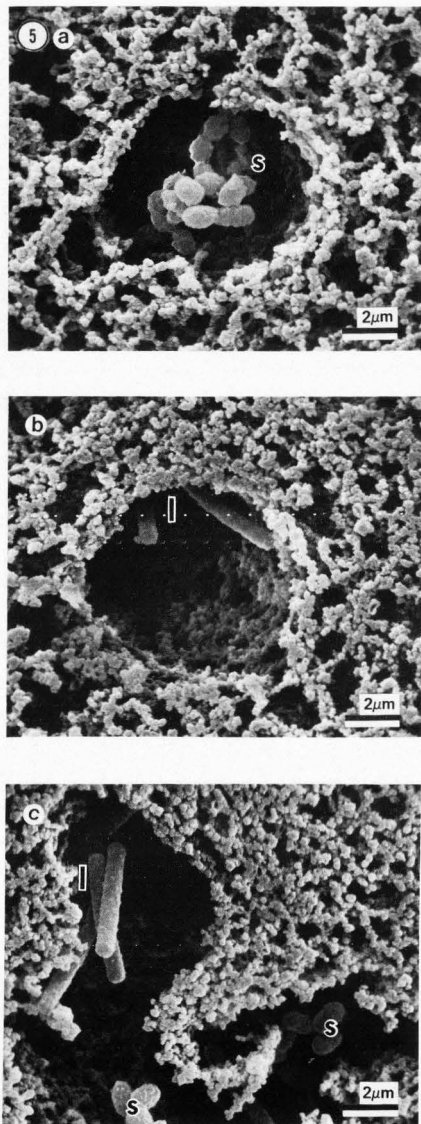
Fig. 4. Detail (TEM) of casein micelles in the matrix of an SMP-fortified yoghurt (a) and in a yoghurt fortified by the addition of sodium caseinate (b).

f: Fat globules; r: robust casein particle chain. Arrows point to appendages (spikes). Both yoghurts were made using the CH-4 starter culture.

Another observation is related to the compactness of the protein matrix (Figure 6). Provided that other factors such as the amount of the starter culture used and the age and pH of the yoghurts were similar, larger void spaces developed in a less compact matrix of UF yoghurt than in the more compact matrix of the SMP yoghurt. However, because even small void spaces were more easily detected in a compact matrix than in a highly porous matrix and because no measurements were carried out, such an observation may be misleading.

Fig. 5. Void spaces (cavities) in UF (a), SMP (b), and RO (c) yoghurts made using the RR starter culture.

l: *Lactobacilli*; s: *Streptococci*.



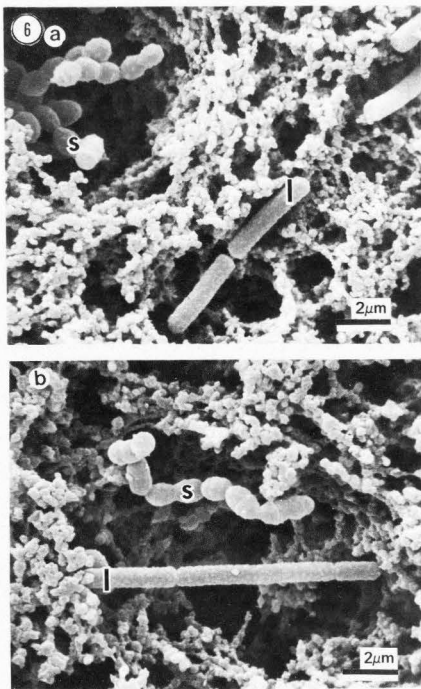
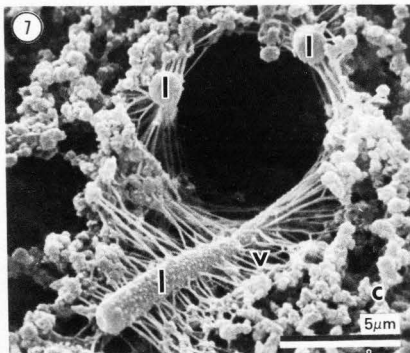


Fig. 6. Void spaces (cavities) occupied by lactic acid bacteria in SMP (a) and UF (b) yoghurts made using the CH-4 starter culture.  
l: Lactobacilli; s: streptococci.



The type or strain of the bacterial starter culture can play a role in the microstructure of the yoghurt, because so-called "slime", "ropy", or "mucogenic" bacteria have the ability to produce polysaccharide material (13, 15). Figure 7 shows a typical example, i.e. slime production, of such a yoghurt. Here the filaments are attaching the lactobacilli to the protein matrix. Mucogenic starter cultures are widely used in the industry to produce a viscous yoghurt without the addition of stabilisers and to minimise reduction in viscosity, which may occur during the mechanical handling of the coagulum.

### Conclusion

Fortification of skim milk by the addition of skim milk powder (SMP) or sodium caseinate (Na-cn) influenced the overall microstructure and texture of the resulting yoghurts. The use of Na-cn was the most effective means of increasing yoghurt firmness. The milk base prepared by this form of fortification contained the highest casein level of all the experimental variants (fortification by vacuum evaporation, reverse osmosis, and ultrafiltration, and by the addition of SMP). Whereas the firmness of the Na-cn yoghurt was highest, the texture was inferior compared to the other yoghurts. This was probably associated with the large casein particle clusters and robust micellar chains resulting in the most open protein matrix of all the yoghurts studied. It is assumed that the casein to non-casein protein ratio was the major contributing factor; this ratio was high (4.6) in the inferior Na-cn yoghurt and was lower (3.4 or less) in the other yoghurts of acceptable quality.

The other yoghurts had their protein matrices denser than the Na-cn yoghurt and were softer and smooth.

### Acknowledgments

The authors thank Dr. R.J.M. Crawford and Dr. H.W. Modler for useful comments, and Mr. C. Kelly, Miss M. McDougall, and Mrs. P. Allan-Wojtas for skilful technical assistance. Electron Microscope Centre, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 569 from the Food Research Institute in Ottawa.

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Fig. 7. Slime production by lactobacilli of the Boll-3 starter culture in the SMP yoghurt. The slime (v) in this micrograph is in the form of filaments attaching the lactobacilli (l) to the protein matrix of the yoghurt (c).

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# Discussion with Reviewers

R.K. Abrahamsen: The RO- and UF-treatments are highly dependent on the membranes used. Please give more specific information about the membranes and the filtration temperature.

Authors: Information about filtration temperatures has now been included in the Materials and Methods section. Concerning reverse osmosis, the D05 type 20-1.8-0 module had previously been used for trials on membrane performance and was fitted with an assortment of membranes. In a total of 50 membrane pairs there were 2 pairs of type 865 membranes, 15 pairs of 930, 16 pairs of 990, 15 pairs of 995, and 2 pairs of type 999 membranes. The presence of the more open membranes led to some loss of dissolved solids but this would be mainly lactose and dissolved salts with little or no loss of protein.

R.K. Abrahamsen: To my knowledge, the pH of sodium caseinate is at the level of 6.6-7.0. Can you provide some additional information regarding the sodium caseinate used in this experiment?

Authors: According to the information obtained from the supplier, sodium caseinate was manufactured by precipitating skim milk with hydrochloric acid, washing the acid casein, and treating the wet curd (50% total solids) with sodium hydroxide and water. The caseinate solution (30% solids and pH around 6.5) was then dried in a conventional spray drier at an outlet temperature of 95-98°C.

D.E. Carpenter: What effect might the void spaces in the microstructure, which are produced by the bacteria, have on subsequent yoghurt syneresis? Authors: Presumably, the void spaces are filled with whey, although the possibility that they are occupied by gas bubbles [see the question by M. Rügge in (7)] has not been excluded. If it is whey which constitutes the contents, void spaces could indeed affect susceptibility of the yoghurt to syneresis. The extent of syneresis would depend on the dimensions and numbers of the void spaces in addition to other factors such as low total solids content and inadequate heat treatment of milk, pH of the finished product, handling of the coagulum during manufacture, storage, and distribution etc. (19). In this present study, syneresis was not observed in any of the 15 yoghurts produced.

Reviewer 2: If no particular attention was paid to the void space dimensions around the bacteria, how can any conclusions be drawn?

Authors: The overall impression of the distribution and dimensions of the void spaces is evident during the microscopical examination of several freeze-fractured particles of the same yoghurt. By examining different specimens at the same low magnification it is possible, at least subjectively, to compare the incidence and dimensions of the void spaces among samples. For this reason, subjective terms such as "smaller" and "larger" were used to describe the void spaces observed, without any reference to numerical data, because measurements and analyses of these have not as yet been made. The problem of the void spaces is quite complex and should be the subject of a separate study.

R.K. Abrahamsen: From Figure 5 it is difficult to conclude anything with regard to the connection between a greater metabolic activity of the starter bacteria and the volume of the void spaces, because this figure illustrates void spaces from yoghurts fortified by different methods. Your hypothesis is very interesting, however, I feel that you would have come closer to an answer if you had in the same type of yoghurt observed the volume of a number of void spaces around separate chains (cells) of *S. thermophilus* and of *L. bulgaricus*, and of void spaces where both organisms were present close to each other. Have you made such observations?

Authors: Figure 5 has been used to illustrate rather than prove that the close proximity of cocci and bacilli will result in larger void spaces. Driessen et al. (18) provided evidence that *L. bulgaricus* in yoghurt is stimulated by carbon dioxide produced by *S. thermophilus*. Problems associated with establishing the origin of void spaces around lactic acid bacteria in yoghurt appeared to be more complicated than was initially anticipated (20). Three-dimensional reconstruction of the void spaces from serial thin sections (TEM) would be a reasonable approach for determining their dimensions, shapes, and contents but would require a prohibitively large number of sections because the void spaces are large with respect to the thickness of the sections. SEM shows void spaces and the bacteria present in them randomly fractured and to a limited depth and has been used here to illustrate this interesting phenomenon.

N.F. Olson: It is interesting that the samples in this study can be divided into two sets: one in which the calcium and calcium to casein ratio is lower than normal (UF and Na-cn), and one in which the calcium concentration is higher than in normal milk (RO, EV, SMP). Is it possible that the competition for calcium by Na-cn in milk before acidification affected the calcium "bridging" within the micelle and thereby produced a larger, more porous micelle in the acid gel? I have always been surprised that casein micelles seen by SEM are similar in acid gels and rennet gels since the acidified micelles presumably should have been substantially depleted of colloidal calcium.

Authors: You have asked a very interesting question, to which we have no answer because there are no experimental data on this subject. It would be necessary to use microscopical techniques other than SEM to study casein micelle ultrastructure. For example, carbon and platinum replication of freeze-fractured and freeze-etched unfixed micelles could be employed. Changes to be investigated would include the distances between casein sub-micelles, presumably increased due to partial removal of calcium phosphate, and the dimensions of the casein micelles, presumably increased in size due to their higher porosity. Neither conventional SEM, which requires a fixed, dried sample, nor conventional TEM, which requires sections of embedded fixed and dehydrated material, would be suitable for this purpose, because of the probable development of artifacts during the many preparatory steps.

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TECHNICAL TIP

TRANSPORTATION OF FRAGILE FOOD SPECIMENS  
SUCH AS MILK GELS  
DESTINED FOR ELECTRON MICROSCOPY

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Collaboration between an electron microscopist and a food scientist over a distance frequently involves some kind of transportation of food specimens for electron microscopy. This poses no problems provided that the specimens being sent are resistant to damage resulting from handling of the containers. Dry food specimens such as milk powders, and compact fixed specimens such as cheese belong in this category. However, fragile specimens such as gelled milk completely disintegrate when transported in containers (glass vials) partially filled with the fixative or other fluid medium, because the movement of the liquid encountered during transport breaks the specimens.

In this laboratory, excellent results have been obtained by placing these specimens in screw-cap glass vials which are subsequently filled with fluid to the top, thus avoiding the presence of any air bubble. Such specimens transported by couriers arrive at the point of destination without damage. However, because most specimens are sent by mail and yet postal regulations do not allow mailing of liquids (fixatives), the specimens can alternatively be immobilized in the vials using agar gel. In this technique, lukewarm agar sol (2%) made in the same liquid as the one impregnating the specimen, is added to the specimens in the vial and allowed to gel. The vial is tightly closed (screw-cap vials are best), wrapped in paper tissue, sealed in a plastic bag, and mailed. Upon arrival and after unpacking, a score mark is made on the vial close to the bottom with a diamond scribe, the screw cap is removed, and a Pyrex glass rod heated to almost white glow with a torch is used to touch the score mark. The glass cracks and the bottom is separated from the rest of the vial. The gel with the embedded specimens is pushed out through the bottom into liquid of the same composition as the one originally impregnating the specimens. The specimens are then retrieved from the agar gel with a scalpel and are processed for scanning or transmission electron microscopy as required.

LITERATURE ABSTRACTS

The past four issues of Food Microstructure have included related abstracts of papers from other publications. These abstracts have been and are being prepared for publication by Dr. Milos Kalab (address on the inside cover page) with help from our readers and editorial board members. The abstracts section will be included in the next issue of Food Microstructure, please continue to send abstracts to Dr. Kalab.

Managing Editor



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## FOOD MICROSTRUCTURE 1985

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**FOOD MICROSTRUCTURE** will be the subject of a program scheduled during **March 31–April 5, 1985** at the Riviera Hotel in Las Vegas, Nevada. Five special programs — on Meat Foods, Plant Foods, Dairy Foods, Fats and Specimen Preparation of Food Samples, as well as several tutorials are being planned.

**All types of foods**, including *vegetables, grains, sea foods, meat, dairy products and others* will be covered. Papers on the *fundamental aspects of food microstructure such as the molecular and colloidal forces which determine it, and the practical relationship between food microstructure and processing, ingredient changes, shelf life, consumer acceptability*, and other food-related areas are invited. *Techniques may include transmission electron microscopy, scanning electron microscopy, light microscopy, x-ray microanalysis or related microscopy/microanalytical methods.*

The organizers of the "Meat Microstructure" program are: **Prof. G.R. Schmidt**, Colorado State University, Fort Collins, CO; **Dr. A.-M. Hermansson**, The Swedish Food Res. Inst., Goteborg, Sweden. The organizers of the "Microstructure of Foods from Plant Origin" program are: **Dr. W.J. Wolf**, U.S.D.A., Peoria, IL; **Dr. D.J. Gallant**, INRA, France; **Dr. K. Saio**, National Food Research Institute, Ibaraki, Japan, and **Dr. D.B. Bechtel**, U.S.D.A., Manhattan, KS. The organizers of the "Microstructure of Milk and Milk Products" program are: **Dr. W. Buchheim**, Bund. Milchlorschung, Kiel, W. Germany; **Dr. M. Kalab**, Agriculture Canada, Ottawa, and **Prof. Z. Saito**, Hiroasaki University, Japan. The organizers of the "Microstructure of Fat" program are: **Prof. J.M. deMan**, University of Guelph, Ontario, Canada; **Prof. E.A. Davis**, University of Minnesota, St. Paul, MN, and **Dr. N. Krog**, Grindsted Products, Brabrand, Denmark. The "Specimen Preparation of the Food Samples" program will be organized by **Dr. D.N. Holcomb**, Kraft R&D, Glenview, IL.

Papers offered for this program will be published in the semi-annual Journal, "**FOOD MICROSTRUCTURE**". The editors of this Journal are **Dr. S.H. Cohen**, U.S. Army R&D Command-Food Research Laboratory, Natick, Mass. (Telephone 617-651-4578); **Prof. E.A. Davis**, Food Science Department, University of Minnesota, St. Paul (Telephone 612-373-1158); **Dr. D.N. Holcomb**, Kraft R&D, Glenview, IL (Telephone 312-998-3724); and **Dr. M. Kalab**, Food Research Institute, Agriculture Canada, Ottawa (Telephone 613-995-3700 Ext. 272).

The Food Microstructure program in Las Vegas will be held in conjunction with the **SCANNING ELECTRON MICROSCOPY/1985** meetings from **March 31–April 5, 1985**. **Registrants at the Food Microstructure program will be able to attend all of the activities of the SEM meeting at no additional charge.** Of particular interest should be: several tutorials, and programs on *Analytical Electron Microscopy (including STEM), Microprobe Surface Analytical Techniques, Particulate Characterization, Plants, and many areas of physical, biological and biomedical application.* A **comprehensive equipment exhibition** will take place during the meetings also. Complete details of SEM meetings are available on request.

The **registration fee** for this program is **\$40.00**, if paid before Jan. 31, 1985, and **\$55.00** after that. For subscription to the journal include an additional \$50.00 (for U.S. delivery) or \$55.00 (for elsewhere).

**A Call for Papers, Registration Form, details of travel support and Hotel information are available on request.** For more information on this program and the Journal, "Food Microstructure" contact Dr. Om Johari or one of the editors.

## FOOD MICROSTRUCTURE INSTRUCTIONS TO AUTHORS

Papers for publication in Food Microstructure can be offered at any time. Papers intended for oral presentation at the Annual Food Microstructure meeting in April are due January 15th. Only papers acceptable for publication are allowed oral presentation.

In a letter accompanying the paper, authors must provide names and complete addresses of at least four persons competent to review their paper. Please note: a. Suggested reviewers must neither be from authors current or recent affiliations, nor coworkers; b. preferably suggested reviewers should be amongst active researchers in the field (e.g., whose work is being extensively referred); and c. Authors are neither expected to personally know nor required to contact the suggested reviewers. From the names suggested by the authors and SEM's advisors, editors will select the most suitable reviewers irrespective of their geographical location. Each paper will be intensely reviewed by at least three, and often more, reviewers.

The initial paper (hereafter referred to as 'paper') should conform to these Instructions. However, to be published after reviewing, the final manuscript (hereafter referred to as 'manuscript'), must be submitted on model sheets conforming to the Manuscript Submission Guidelines; these guidelines and model sheets (a sample model sheet is available on request) will be mailed along with the reviewers' comments. In addition to all the text, the manuscript must also contain author's publishable responses to questions raised by the paper's reviewers. (See, e.g., discussion with reviewers in this issue.)

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# FOOD MICROSTRUCTURE

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