

SCANNING ELECTRON MICROSCOPY/1979/III
SEM Inc., AMF O'Hare, IL 60666, USA

SOME EXAMPLES OF SCANNING ELECTRON MICROSCOPY IN FOOD SCIENCE

R. J. Carroll and S. B. Jones

Eastern Regional Research Center
Agricultural Research, Science and Education Administration
U. S. Department of Agriculture
600 East Mermaid Lane
Philadelphia, PA 19118

Abstract

The scanning electron microscope was applied to a variety of problems involving agricultural research at our Research Center. The investigations described include: (a) host-pathogen interactions in potato, (b) cracking of maturing cherries, (c) changes in meat structure as a consequence of thermal and mechanical stress.

Each study required specific fixation, dehydration and drying procedures depending on the physical characteristics and the nature of the structural information desired.

The study of fungal interaction with potatoes required freeze drying as well as chemical fixation to preserve fungal-potato cell relationships. The studies on meat depended on maintaining fiber-connective tissue orientations that resulted from heating and tensile stresses. A minitensile stage stressed the samples, and a special clamping holder maintained the specimens in a stress position through all stages of sample preparation and observation. The cherry tissue integrity was maintained by use of freeze-drying techniques.

Application of scanning microscopy to food and food products often requires ingenuity and expertise to retain structural relationships and thus gain an insight into functional properties of these commodities.

Research supported in part by U. S. Army Natick Development Center Project AMXRED 73-161.

KEY WORDS: Potato, *Phytophthora infestans*, Cherry Cracking, Ethyl Oleate, Meat, Tensile Stress, Connective Tissue, Meat Tenderness, Muscle

Introduction

The use of the scanning electron microscope (SEM) in the determination of structural changes in a wide variety of food and food products is finding increased applications. This use is governed for the most part by the availability of reliable procedures for adequate preservation of structures of the food and food products under investigation. This preservation of food structure depends on (a) methods of isolation of specimens, (b) fixation, (c) dehydration, (d) physical characteristics of the specimen, (e) structure of interest, and (f) instrumentation conditions.

For most studies, the fixation of choice is glutaraldehyde or glutaraldehyde-paraformaldehyde (after Karnovsky)¹ at the temperature, osmolarity, pH, and buffer type appropriate for each food sample. Dehydration, usually with ethyl alcohol, followed by critical point drying with carbon dioxide, results in preservation of structure. At times, better structure retention is obtained by freeze drying, depending on the food specimen.

The cryofracture technique developed by Humphreys *et al.*² is one of the better methods which can be used to obtain internal surfaces with good structural features and which minimizes cutting artifacts and eliminates ice crystal damage.

As an introduction to this Workshop on "The Scanning Electron Microscope in the Food Sciences," we would like to present the results of our SEM research which give information on the structures of selected foods. These investigations include: (1) host-pathogen interactions in potato tuber and leaf involving the fungus *Phytophthora infestans*, (2) use of ethyl oleate to reduce cracking of sweet cherries, and (3) effects of thermal and mechanical stresses on meat texture.

Each of these studies required a different approach to preserve food structures to obtain the desired information with the scanning electron microscope.

Instrumentation and Methods

Details of each sample preparation procedure are discussed under each subject. Some samples were freeze-dried, others were fixed, dehydrated in ethyl alcohol and critical point dried; then the samples were mounted on copper stubs. A JEOL 50-A* scanning electron microscope, operating at 10-20 kV, was used in these investigations.

Fungal Interaction with the Potato

The fungus Phytophthora infestans causes Late Blight in potato plants. In the field, fungal spores fall onto the soil and infect tubers through wounds or natural openings. Tubers then rot while in storage or in market. Late Blight is thus both a field and a post-harvest agricultural problem.

Certain varieties of potato are resistant to one or more races of the fungus. Our research has been directed toward investigating the ultrastructural differences between the fungus interacting with susceptible and resistant cultivars and complements other research in our Center on the biochemistry of stress metabolites produced in cells of potato plants. We have documented structural aspects of the growth and development of P. infestans on both susceptible and resistant tubers.³ Our current work utilizes both transmission electron microscopy (TEM) and SEM for the examination of leaves and tubers.

SEM is ideal for the examination of surfaces of leaves and tuber slices that have been inoculated with sporangia or zoospores of P. infestans. Inoculated leaves were incubated at 12 C and sampled at time intervals ranging from a few hours to four days, at which time growth at the leaf or tuber-slice surface was easily visible without magnification. Samples of leaf were removed and fixed in 3% glutaraldehyde in 0.025M sodium cacodylate, pH 6.0, for microscopic investigations.

Specimens for SEM examination were dehydrated in ethanol and critical point dried. Fig. 1 shows the surface of a tuber slice with fungal hyphae growing across the cells. Starch granules can be seen on the floor of the tuber cells. Most of the starch was washed out before inoculation to make visualization of the fungal structures easier.

Both zoospores and sporangia of P. infestans can germinate, depending on conditions of temperature and humidity. Fig. 2 shows a sporangium which has germinated on a leaf surface. The germinating hypha extends across an open stomata. This germination structure will not necessarily infect the leaf, because the ability of the structure to penetrate tuber cells depends on a specific interaction between host and pathogen. On the leaf surface, hyphae emerging from sporangia have been observed to grow across the surface. We have not observed any hyphae entering

* Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature.

stomata or any conclusive observations up to this time of direct penetrations of the cuticle. Infections must occur in leaves thus inoculated because after approximately four days the zoosporangioophores (the sporangia-bearing hyphae) emerge from stomata (Fig. 3). Each zoosporangioophore terminates in a characteristic bulb-like structure. Sporangia easily disengage and can be seen resting on the leaf surface in the micrograph. Only after the fungus has ensured the completion of its life cycle does the destruction of the leaf commence. Then the leaf "withers and dies."

Cherry Surface Alteration

SEM provides a way to view the surfaces of raw agricultural products. One example of such an application is a study in which cherries were examined for alterations in surface wax.

Sweet cherries in the orchard often crack just before harvest. The cause has been shown to be water absorbed through the cuticle,⁴ with much damage occurring within a few hours after a rain-storm.

Incidence of cracking can be reduced by spraying the fruit with a water emulsion of ethyl oleate (EO).⁵ Fruit treated with EO has been examined to determine what structural changes have occurred. Tangential sections (5x5x2) of untreated and EO-treated cherries were cut with a razor blade. Specimens were rinsed with deionized water to remove juice, frozen in liquid nitrogen, and dried overnight in a vacuum evaporator. Fixation was omitted because the fixative containing glutaraldehyde and paraformaldehyde produced artifacts in the waxy coating of the cherries. Surface wax in untreated fruit formed a continuous layer over the cuticle which sometimes partially or wholly obstructed stomata (Fig. 4). Cherries which had been dipped in 4% EO appeared to have little or no wax on the cuticle or around stomata (Fig. 5). The surface was similar to that of a cherry which had been dewaxed with chloroform. Cherries sprayed with EO in the field showed no visible effects over most of the surface examined. However, on some sprayed samples, surface wax appeared to have been moved about and formed into flow patterns clearly different from the usual appearance of the wax (Fig. 6). The effect of the EO may be to redistribute the wax on the surface, permitting enhanced vapor exchange in certain areas of the cuticle. It is unlikely that spraying with EO removed wax clogging stomatal pores because, even after the cherries were dipped in EO, wax in stomatal opening often remained and appeared to be more resistant to solubilization than cuticular wax.

Meat Structure

An important desired characteristic of meat is tenderness, a quality easy to determine at mealtime but difficult to predict by any objective measurement. Much information is now available concerning the factors contributing to toughness or tenderness. Toughness can be separated into two components: one arising from

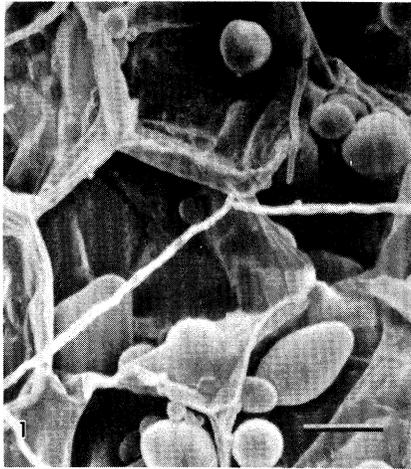


Fig. 1 Potato tuber cells with hyphae of *Phytophthora infestans*. Some starch granules are present. Scale marker (S.M.) = 40 μ m.

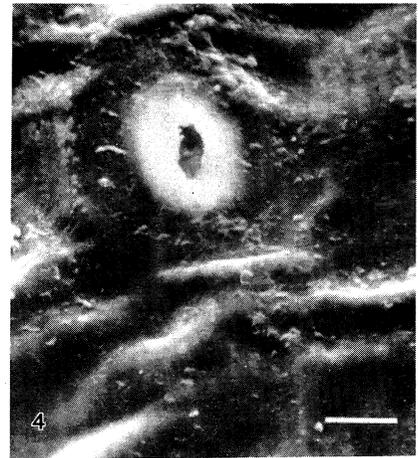


Fig. 4 Surface of cherry fruit. Wax covers the cuticle surface nearly closing stomata. S.M. = 10 μ m.

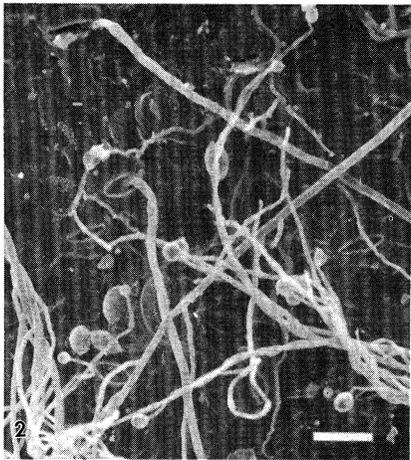


Fig. 2 Germinating hypha of *Phytophthora infestans* on potato leaf. S.M. = 12.5 μ m.

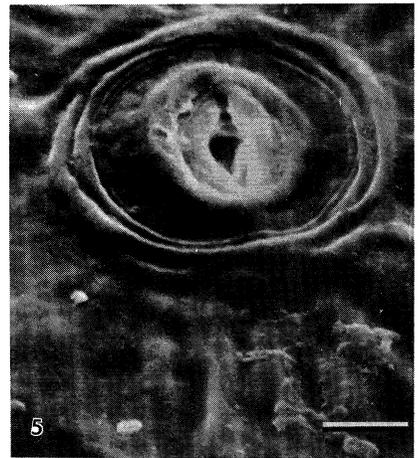


Fig. 5 Cherry treated with 3% EO in laboratory. Most wax removed. S.M. 15 μ m.

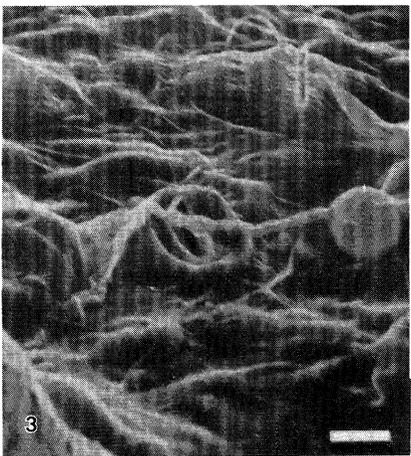


Fig. 3 Potato leaf surface with hyphae exiting from stomata. S.M. = 33 μ m.

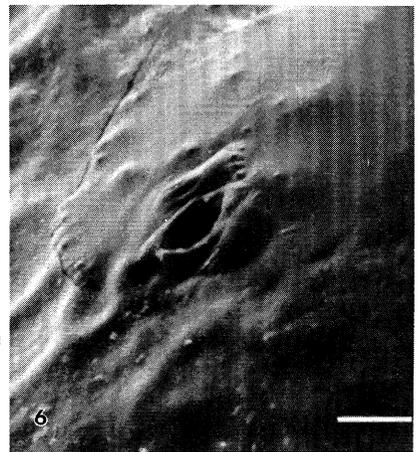


Fig. 6 Surface of cherry sprayed with 3% EO in the field. Wax formed flow pattern. S.M. = 20 μ m

actomyosin interaction, and the other from background or connective tissue contributions.⁶

In an effort to elucidate the structural bases of tenderness and/or toughness and, hopefully, to find a foundation for an objective measurement of tenderness, scanning electron microscopy was used to examine alterations of meat structure as influenced by heating⁷ and applied tensile stress.⁸ Bovine semitendinosus (eye-of-round) was obtained either commercially or from aged hanging carcasses. A standard sample preparation procedure was developed to minimize introduction of surface artifacts.⁹ The preferred fixative was a solution which contained 2.5% glutaraldehyde and 2% paraformaldehyde buffered in 0.07M phosphate to the pH of the sample.¹ Meat samples were clamped to prevent contraction during fixation. Ethyl alcohol dehydration, coupled with the cryofracture technique of Humphreys,² followed by critical point drying, gave the best preservation of meat structures. For cooked meat studies, 6 mm thick slices of the meat were placed in water within a polyethylene bag, and heated in a water bath at the specified temperature for 45 minutes.

A special tensile stage was designed and built to carry out dynamic stress studies. Stressed samples were placed in a clamping holder for observation in the SEM. All of the additional specimen preparation steps (fixation, dehydration, and critical point drying) were performed with the stressed tissue in the clamping holder. All stress experiments were monitored by a stereo microscope equipped with a video recording system. Specific areas of the stressed tissue were compared with similar areas of tissue examined with the SEM.

Fig. 7 shows in cross fracture the main components of meat tissue. The fiber bundle is surrounded by a perimysial connective tissue (P). The endomysium (E) is the sheath-like connective tissue surrounding the individual muscle fibers.

Structural changes in meat tissue heated to 50, 60, and 90 C were determined.⁷ The structures examined included the sarcomere, endomysial connective tissue, and the sarcolemma. In Fig. 8 the tissue which had been heated to 50 C appears similar to unheated meat, with the fibers well separated and the endomysium free. The tissue which had been heated to 60 C became compact (Fig. 9). The fracture plane passed through and around the fiber, and the endomysium was in close contact with the muscle fibers accompanied with the deposition of particulates. Heating to 90 C produced most drastic changes. The endomysium was quite congealed, and the sarcolemma was granular.

Heating to 50 and 60 C did not alter the sarcomere length as compared to that of unheated tissue. The sarcomeres were about 1.9 μ m long. In tissue heated to 60 C, congealed material was observed, particularly at the Z-line region. In tissue heated to 90 C, however, the sarcomeres were shortened to about 1.4 μ m, the Z-line material was detached, and particulates were observed at the Z-line (Fig. 10). Denaturation of the connective tissue, accompanied by the formation of rigid protein blocks, corresponds respectively to the connective tissue and the actomyo-

sin complex as they influence meat tenderness. The changes in the endomysium at 60 C (denaturation of connective tissue) probably help to make the meat more tender. Conversely, the shortening of the sarcomeres and the "rigid blocks" of the actomyosin complex probably make the meat less tender. The ultimate tenderness in meat is in part the result of opposing changes in the two major meat components. These observations of the changes induced in meat tissue structures may help in the elucidation of their effects on meat tenderness.

Tensile stress, both parallel and perpendicular to the meat fiber axis, was applied to unheated and heated meat tissue, to ascertain the weakest and the strongest structures in meat.

The muscle tissue samples were stressed on a ministage (Fig. 11) and observed with a stereo microscope. Applications of tensile stress parallel to the fiber axis caused initial rupture of the endomysium muscle fibers accompanied by strand formation. Fig. 12A shows the strands as observed with a light microscope, and Fig. 12B shows a similar area observed with the SEM. In the lower part of the micrograph, the muscle fibers have ruptured and the strands appear taut, while some of the strands have broken and coiled back. The strands appear to originate from the perimysial connective tissue.

Raw and heated (90 C) meat samples were stressed either parallel or perpendicularly to the fiber axis almost to rupture. Interior surfaces of the tissue adjacent to the rupture point were obtained by the cryofracture technique. Fig. 13 shows the alignment of the perimysial connective tissue fibers in uncooked meat after parallel stress was applied. Similar results were obtained with heated tissue. Fig. 14 shows the effect of stress perpendicular to the fiber axis in tissue heated to 90 C. The initial rupture occurred at the junction of the endomysial-perimysial connective tissue.

Orientation of the strands originating from the perimysial connective tissue took place after application of considerable stress. The apparent elasticity of the strands resulted from the orientation of the random connective tissue fibers which took up the stress after rupture of the endomysial-perimysial connective tissue junction.¹⁰ When perpendicular stress was applied, the muscle fiber-endomysium complex was the stronger structure. These strands were identified as collagen with light microscopy staining techniques. The strands were birefringent when viewed under polarized light. Small amounts of elastin were also observed in association with blood vessels.

Transmission electron microscopy of dispersed perimysial connective tissue showed a random network of collagen fibers with the typical banding pattern. Perimysial connective tissue heated to 90 C and viewed in the TEM was highly denatured as expected. Approximately 10% of the fibrils, however, still retained the banding pattern of native collagen. This result was unexpected since collagen denatures in the range of 58-63 C.¹¹

The tensile properties of meat tissue depend considerably on the nature and extent of the perimysial connective tissue and its reaction to

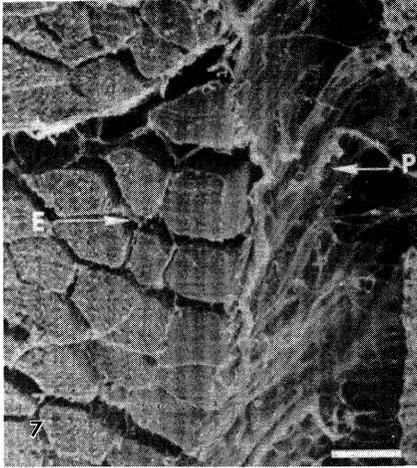


Fig. 7 Raw bovine semitendinosus. Fibers are surrounded by endomysial (E) collagen. The perimysium (P) encloses fiber bundle. S.M. = 33 μ m.

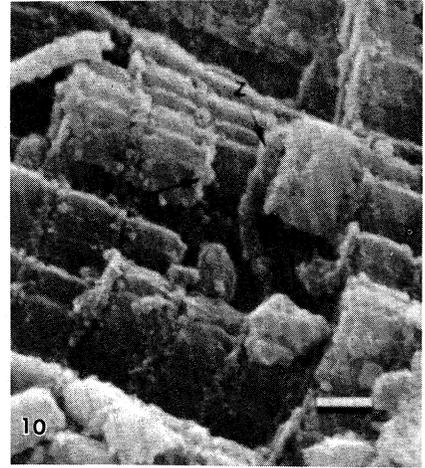


Fig. 10 Sarcomeres of meat heated to 90 C. Z-disc (Z) material detached from sarcomere. Protein block broken away at unmarked arrow. S.M. = 1 μ m.

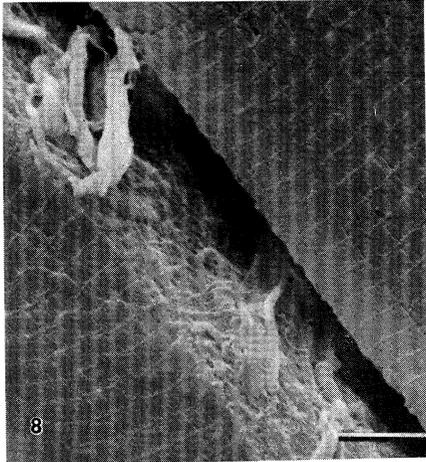


Fig. 8 Endomysial collagen between muscle fibers heated to 50 C shows no change from raw control. S.M. = 4.5 μ m.

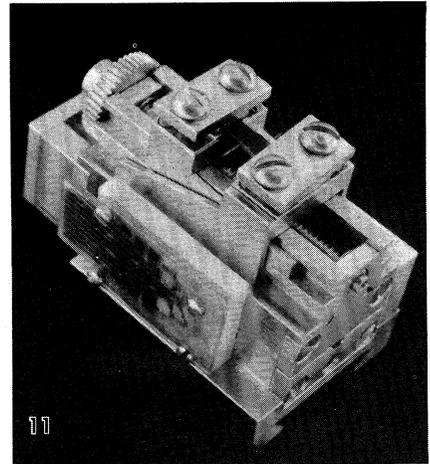


Fig. 11 Minitensile stage designed to operate outside the SEM.⁸



Fig. 9 Heating at 60 C endomysium (E) congealed and sarcolemma (S) became granular. Myofibrils (MF) remained intact. S.M. = 5 μ m.



Fig. 12 A - Light micrograph of connective tissue strands under stress.
B - SEM of area similar to A, strands under stress and ruptured muscle fibers.
S.M. = 45 μ m.

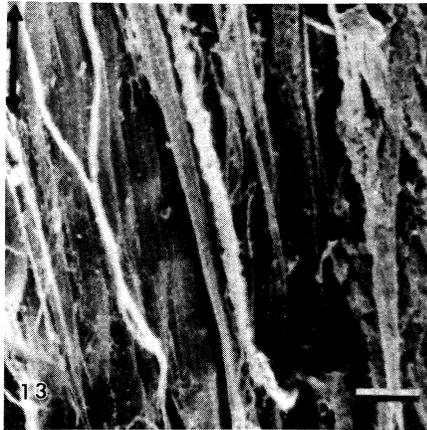


Fig. 13 Fractured interior surface of raw semitendinosus stressed parallel to fiber axis. The perimysium fibers are oriented in the direction of applied stress. (Arrow upper left.) S.M. = 30 μ m.

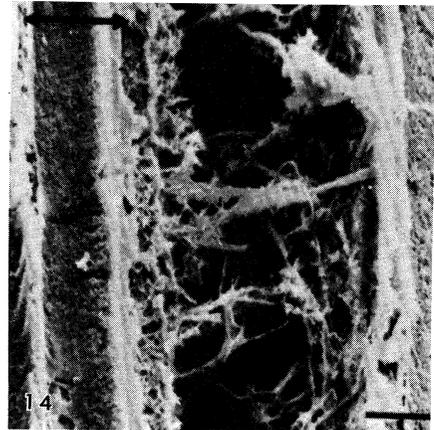


Fig. 14 Fractured interior surface of heated (90 C) semitendinosus stressed perpendicular to the fiber axis. (Arrow upper left.) Stress on the perimysium fibers is evident; muscle fibers undisturbed. S.M. = 20 μ m.

thermal stress. Such tensile properties, in turn, have a significant effect on both the subjective and objective evaluation of meat tenderness.

Future Directions

The strength of SEM for food science studies is its magnification range and large depth of field. The images of SEM often give information directly relating to textural properties of foods in a unique way and should be useful in assessing and predicting consumer appeal and in developing processed foods.

Operationally, improvements should be developed in techniques of sample handling which will preserve and protect the features of the viewed surface. Cryofracture techniques are useful and can be used routinely for the examination of a matrix structure such as muscle.

A potentially useful technique is low-temperature SEM of unfixed, frozen biological specimens. This technique avoids the drawbacks of chemical fixation, dehydration, and drying. Although different artifacts are introduced, cryofreezing may give an insight into structure determinations of lipids and lipid-protein systems that are difficult to preserve by conventional procedures. The cryo technique will also serve as a check on possible artifacts introduced by normal procedures. The use of a cryofracture device will expose additional sample surfaces for characterization at different levels within the sample without thawing. Use of a metal deposition device will permit specimen observation at higher accelerating voltages while minimizing specimen charging.

The use of energy dispersive X-ray analysis in conjunction with the SEM should provide needed information regarding chemical changes in food and food products associated with aging, processing, packaging, and other variables. This powerful technique gives elemental information related

to chemical changes in food tissues. Combined with the SEM structural observations, it should permit food investigators to obtain a more comprehensive characterization of food and food products.

References

1. M. J. Karnovsky. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**, 1965, 137A.
2. W. J. Humphreys, B. O. Spurlock, and J. S. Johnson. Critical point drying of ethanol-infiltrated, cryofractured biological specimens for scanning electron microscopy. SEM/1974, IIT Research Institute, Chicago, IL 60616, 275.
3. S. B. Jones, R. J. Carroll, and E. B. Kalan. A scanning electron microscope study of the host-pathogen interaction of *Phytophthora infestans* with potato tissue. SEM/1974, IIT Research Institute, 397-404.
4. D. C. Davenport, K. Uria, and R. M. Hagen. Antitranspirant film: curtailing intake of external water by cherry fruit to reduce cracking. *HortScience* **7**, 1972, 507-508.
5. W. O. Harrington, C. H. Hills, S. B. Jones, et al. Ethyl oleate sprays to reduce cracking of sweet cherries. *HortScience* **13**, 1978, 279-280.
6. B. B. Marsh and N. G. Leet. Studies in meat tenderness. 3. The effects of cold shortening on tenderness. *J. Food Sci.* **31**, 1966, 450-459.
7. S. B. Jones, R. J. Carroll, and J. R. Cavanaugh. Structural changes in heated bovine muscle: scanning electron microscope study. *J. Food Sci.* **42**, 1977, 125-131.
8. R. J. Carroll, F. P. Rorer, S. B. Jones, et al. Effect of tensile stress on the ultrastructure of bovine muscle. *J. Food Sci.* **43**, 1978, 1181-1187.
9. S. B. Jones, R. J. Carroll, and J. R. Cavanaugh. Muscle samples for scanning electron microscopy: preparative techniques and general morphology. *J. Food Sci.* **41**, 1976, 867-873.

10. D. W. Stanley, L. M. McKnight, W. G. S. Osborne, et al. Predicting meat tenderness from muscle tensile properties. *J. Text. Stud.* 3, 1972, 51-68.
11. P. E. McClain, G. J. Creed, E. R. Wiley, et al. Effect of postmortem aging on isolation of intramuscular connective tissue. *J. Food Sci.* 35, 1970, 258-259.

Discussion with Reviewers

R. G. Cassens: Does SEM of foods result in any well known or easily recognized artifacts?

Authors: Yes, food samples for the SEM require extreme care in all stages of sample preparation. One of the more common artifacts is shrinkage. Clamping of the meat tissue during fixation and dehydration kept shrinkage to a minimum. In addition, the loss of lipid from fat cells in meat samples processed by critical point drying is observed. Adequate metallic coating of the sample is essential to minimize charging in the SEM.

J. Chabot: Could you identify a structural component to resistance or susceptibility to infection by *P. infestans*?

A-M. Knoop: Did you find structural differences between races of fungi or between susceptible and resistant potatoes?

Authors: Work on structural relationships and host-pathogen interactions is being actively studied in our laboratory. In TEM studies of infected susceptible tuber cells, new structural features were observed in the region where cell penetration occurred. The significance of these structures and their frequency in resistant, as well as susceptible interactions, is not clear at this time.

J. B. Chabot: Why were penetrations of the leaf cuticle by hyphae not seen?

A-M. Knoop: Have you an explanation for the fact that no entering of hyphae into the stomata and into the cuticle of leaves was to be seen in your SEM work? Was the number of zoospores too small or were the intervals of sampling too large?

Authors: Penetration through the cuticle was expected, and it is not clear why it wasn't observed. It is possible that the geometry of direct penetration makes it difficult to detect by SEM. In general, entrance via stomata is much more readily documented although no such penetration was observed in this case. In this study, sporangia rather than zoospores were used; these sporangia germinate and presumably penetrate. In order to answer these important questions, work is continuing using both sporangia and zoospores and other sampling intervals.

J. B. Chabot: Is there other evidence that wax redistribution is important in preventing cracking of cherries in light of the lack of a major change observable in the SEM? What is the effect of ethyl oleate in stomatal function?

Authors: This is the only evidence involving rearrangement of surface wax and is offered as suggestive, not conclusive. These findings are subject to the qualifications imposed by random sampling as well as random deposit of spray on cherry surfaces. Nevertheless, the wax flow patterns were observed only on sprayed cherries. Although stomatal function was not measured in this study, the microscopy observations indicated that EO application by spraying had no effect on stomatal wax. Text reference 9.

R. J. Cassens: Why does heating to 90 C cause shortening of sarcomeres?

Authors: Application of heat to meat tissue causes (a) unfolding of peptide chains, (b) aggregation of protein molecules, (c) release of water, and (d) increased rigidity of physical structure. The combination of these factors results in a decrease in sarcomere length. (E. Laakkonen. Factors affecting tenderness in meat. *Advances in Food Research*, C. O. Chichester, Ed. Academic Press, New York, N. Y. 1973. 20, p. 313)

A-M. Knoop: In which way do the heat induced changes of the meat structure influence the meat tenderness?

Authors: When meat is heated above 60 C, the connective tissue components begin to denature, making the meat more tender. As the temperature is increased above 60 C, the induced changes in the actomyosin components (denaturation, aggregation, and loss of water binding capacity) cause the formation of "rigid blocks" making the meat less tender. The relative amounts of connective tissue, method of cooking, origin of muscle, age of animal, etc., all contribute to the ultimate toughness and/or tenderness of the meat tissue.

D. N. Holcomb: Can the authors estimate the magnitude of forces developed with their mini-stage? Microstructure-force correlations might be important.

A-M. Knoop: How large was the stress applied to the muscle fibers? Was the effect of stress different for heated and raw meat and how large were the differences?

Authors: Strain gauges were not incorporated into the minitensile stage. Therefore, it is not possible to determine the magnitude of the stress or microstructure-force relationships. The stage is driven by a D.C. power supply and the current was held constant as the voltage was increased to apply the stress at a more or less constant rate. A rough estimate of breaking force can be obtained from the relative voltage required to break the sample. In raw meat about twice the applied voltage was required to rupture parallel to the fiber axis than perpendicular to the fiber axis. Heated meat samples required half the voltage required to rupture raw meat in both instances. These estimates are in agreement with unpublished Instron tensile results on larger samples of unheated meat (cross-section = 6.35 mm x 12.7 mm). The tensile strength averaged 10.7 gm/mm² for parallel and 5.2 gm/mm² for the perpendicular stressed sample.