CONFOCAL SCANNING LASER MICROSCOPY OF CONTAMINATED PORK MUSCLE SURFACES

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SUMMARY: The purpose of this work was to demonstrate bacterial colonization of muscle surfaces using confocal scanning laser microscopy. A three minute stain in acridine orange proved sufficient for this purpose. Microcolonies developped at the surface of myofibers, capillaries and intramuscular adipose cells of porcine muscle inoculated with two species of *Pseudomonas* and a mixed flora. Biofilms were eventually formed which covered the entire surface. Our observations revealed that surfaces are a preferred site of activity for spoilage bacteria. The potential usefulness of this instrument for the study of microstructures such as myofibers and sarcomeres was demonstrated by examination of hot-boned muscle.

INTRODUCTION: The production of high quality fresh meat requires control over the physiological state of muscle and the proliferation of microbial contaminants. Despite extensive study of spoilage phenomena the actual site of microbiological activity on meat is not well defined. Bacteria are known to undergo irreversible attachment to a variety of surfaces, including those of muscle tissues (GILL, 1982). This behavior has obvious implications in sanitation and hygiene, but the role of attachment in the microbial ecology of meat spoilage is not clear. Evidence gathered from microscopic examination (YADA and SKURA, 1982; SCHWACH and ZOTTOLA, 1982) and studies carried out using cultural methods (DELAQUIS and McCURDY, 1990) suggests that growth and development of bacterial communities, a process referred to as colonization, is also a surface associated event. The most direct demonstration of such microbial behavior on meat should be derived from microscopy, but traditional light or electron beam systems are often impractical for this purpose since the preparative methods employed can alter the morphology of bacteria at surfaces. The highly hydrated polysaccharides believed to cement cell-surface interactions are particularly prone to damage or collapse during drying of samples for electron microscopy (FRAZER and GILMOUR, 1986; McMEEKIN et al., 1986). Interpretation of microscopic data is therefore difficult and subject to limitations. Confocal scanning laser microscopy (CSLM) has been touted as an attractive alternative to more conventional microscopies when it is desirable to observe fully hydrated biological tissues in a non-invasive fashion (CASSENS et al., 1990). The improved resolution in comparison to light microscopy and unique optical sectioning capabilities offered by CSLM (WILSON, 1985) suggested that the observation of bacteria on intact muscle is possible. Consequently, we used CSLM to study the spoilage of pork muscle inoculated with two species of *Pseudomonas* and a natural contaminating flora. Our purpose was to determine if exposed surfaces are actively colonized by spoilage bacteria. The usefulness of this technique for the study of meat microstructure was also assessed.

MATERIALS and METHODS: Fresh porcine Biceps femoris (pH 5.68) was placed inside a laminar flow hood. Exposed surfaces were flamed twice with alcohol and cores were removed from the muscle using a sterile honed stainless steel cylinder (9 cm ID). The cores were sliced to a thickness of 3 mm with a delicatessen meat slicer. Muscle pieces (2x2 cm) were cut from the slices with a scalpel blade. *Pseudomonas fluorescens* and *Pseudomonas frag* (DELAQUIS and McCURDY, 1990) were grown in 1/4 strength tryptic soy broth (Difco) at 21°C for 18 h. Water collected on a meat cutting table served as a source of natural contaminants. Inocula were prepared by addition of 1 mL of these cultures to 800 mL sterile tap water in separate metal trays. Sheets (20x20 cm) of sterile cotton fabric were soaked in the inocula and applied for 15 min to muscle pieces arranged evenly on a flat surface. The inoculated muscle was incubated at 4°C in disposable Petri plates and sampled periodically for bacteriological assessment and microscopic examination. Duplicate samples were homogenized in 0.1% peptone for 2 min with a Stomacher (Colworth) and surface cell densities were estimated by plating dilutions on standard plate count agar (Difco) incubated at 21°C for 48 h. Slices (2x2x.5 cm) of porcine Vastus medialis boned pre-rigor within one hour of slaughter and stored at 4°C for 24 h were also studied by CSLM.

All samples were stained in an aqueous 0.1% (w/v) acridine orange solution for 3 min, rinsed twice by gentle agitation in distilled water, and mounted on stainless steel slides with a central opening (5 mm ID). Surfaces were viewed directly on an inverted Nikon microscope interfaced with a MRC 600 confocal imaging system (Bio-Rad Microscience). Photomicrographs were recorded on black and white film (Ilford PF4).

RESULTS and DISCUSSION: Mean log bacterial densities (CFU/cm^{2}) on the surface of inoculated Biceps femoris sample duplicates stored at 4° C are given below:

Inoculum	Time (days) 0	2	4	6	8
P. fluorescens	3.402	4.905	6.443	7.875	8.989
P. fragi	3.356	4.667	6.000	8.169	9.494
Mixed flora	2.998	3.411	5.716	7.400	9.071

The surface of muscle pieces from each sampling period were examined for evidence of bacterial growth by CSLM. Individual cells were difficult to find immediately after inoculation because of low initial densities. Bacterial growth in the form of microcolonies at the surface and between adjacent myofibers was readily detected after 2 and 4 days of incubation. Other sites were also colonized including capillary walls (Plate 1 A,B,C) and intramuscular adipose cells (Plate 1 D).

Typical observations with *P. fluorescens* are shown in Plate 2. This species formed flat microcolonies without regular shape or size (Plate 2 A). In contrast, *P. fragi* microcolonies were larger, roughly circular and raised (Plate 3 A). Striking differences in the surface behavior of the strains are illustrated in Plate 2 B and Plate 3 B. The distance between *P. fluorescens* cells was considerable indicating that the organism had a tendency to spread over the surface, whereas stacks of cells in intimate contact were always observed with *P. fragi*. Microcolonies formed by the latter strain were so dense that dye could not penetrate the cell complex completely (Plate 3 B). Similar cellular arrangements have been reported by FRAZER and GILMOUR (1986) in a comparative study of conventional and cryo-SEM preparations of *P. fragi* growing on inert surfaces. These authors concluded that cellular morphologies observed in conventional SEM preparations are highly influenced by shrinkage during drying. The present work substantiates this conclusion but suggests that species specific surface behavior can also influence the microscopic appearance of attached bacteria.

Biofilm formation at the surface of colonized sites was observed with all inocula. *P. fluorescens* biofilms were less dense than those observed with *P. fragi* (Plate 2 C,D and Plate 3 C,D). The mixed flora biofilm was also quite dense and thick (Plate 1 A,B,C). These differences were probably related to the specific surface behavior of component species. Complete surface coverage was observed after 6 days of incubation for samples inoculated with *P. fluorescens*, but not until day 8 with *P. fragi* or a mixed flora. The spreading behavior of *P. fluorescens* may have resulted in more efficient colonization of available sites and to faster surface coverage. The high bacterial densities determined for these samples (about 10^8 CFU/cm^2) are usually associated with the appearance of a "slime layer" on spoiling meat (GILL, 1982), and the present observations demonstrate the involvement of biofilm formation in this phenomenon.

Hot-boned muscle was also examined in order to evaluate the usefulness of CSLM for the study of muscle microstructure (Plate 4). Cooling of unrestrained pre-rigor muscle at 4^0C caused the wavy appearance of some fibers in Plate 4 A. Microstructures within fibers, such as myofibrils and mitochondria, were readily viewed using the optical sectioning capabilities of the instrument (Plate 4 B,C). Sarcomeres could be measured quickly on-screen using the image analysis capabilities of the system. Traditional phase contrast methods for sarcomere length measurement require blending of the muscle and the use of a stage micrometer, both of which are eliminated in CSLM.

CONCLUSIONS: The study of contaminated muscle by CSLM revealed that surfaces are an important and possibly preferred site of microbial activity for spoilage bacteria. The unique properties of this instrument make possible the assessment of meat quality parameters as diverse as microbiological status and the microstructural state of muscle in rapid, non-invasive fashion.

LIST of PLATES:

Plate 1. Surface of pork Biceps femoris inoculated with a mixed flora. **A.** Highly magnified 8 day old biofilm on the surface of a capillary. The oblong white object is probably an endothelial cell. **B.** Lower magnification of the same capillary wall. **C.** Biofilm on the surface of the capillary and adjacent muscle fibers. **D.** Intramuscular adipose cells with attached bacteria in samples incubated 4 days.

Plate 2. Surface of pork Biceps femoris inoculated with *Pseudomonas fluorescens*. **A.** Highly magnified 2 day old microcolony. **B.** Four day old microcolony. **C.** Biofilm formation after 6 days of incubation. **D.** Same biofilm as in C but image is of a focal plane 10 um below the previous one.

Plate 3. Surface of pork Biceps femoris inoculated with *Pseudomonas fragi*. **A.** Highly magnified 4 day old microcolony. **B.** Same microcolony at lower magnification. **C.** Biofilm formation after 8 days of incubation. **D.** Same as C but image is of a focal plane 20 um below the previous one.

Plate 4. Hot-boned porcine Vastus medialis muscle fibers. **A.** Fibers displaying wavy appearance characteristic of rapidly cooled pre-rigor muscle. The dark bodies on the fibers are most likely unstained nuclei. **B.** Interior of a pre-rigor muscle fiber. **C.** Highly magnified view of the fiber shown in B. Note that mitochondria are visible.

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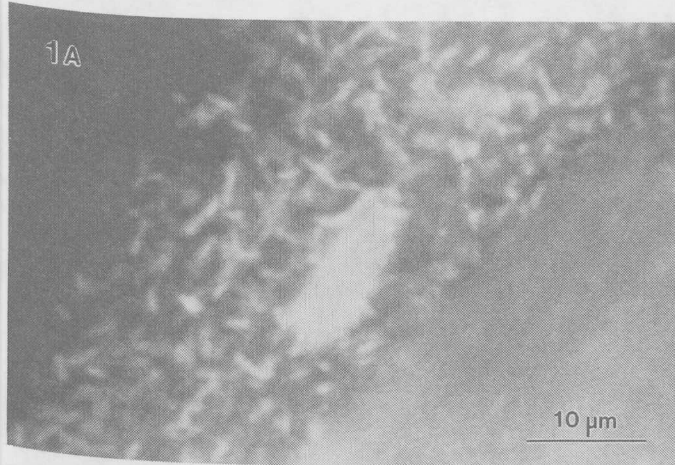
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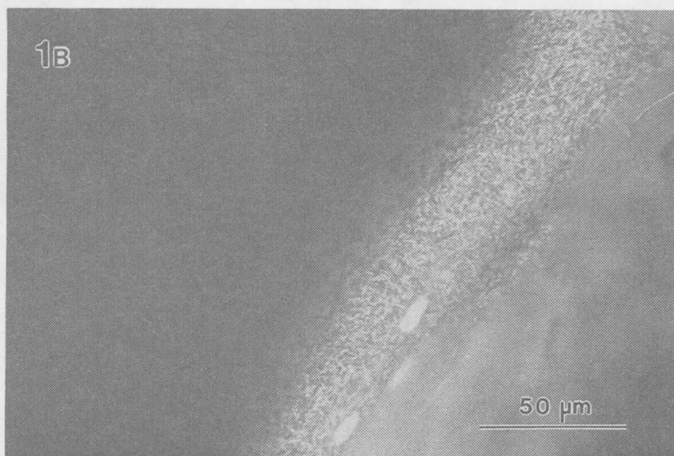
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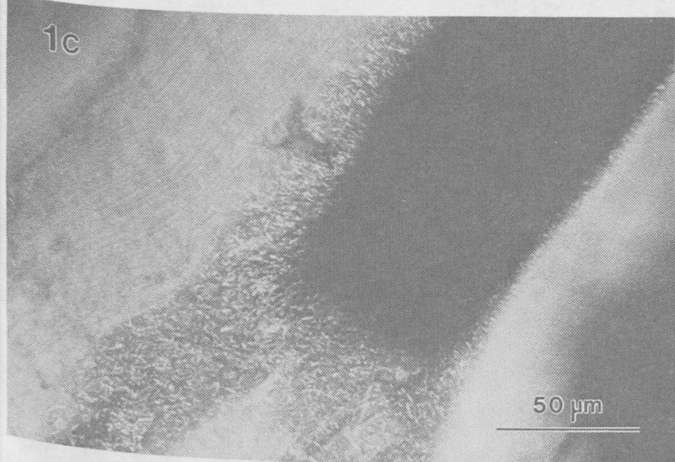
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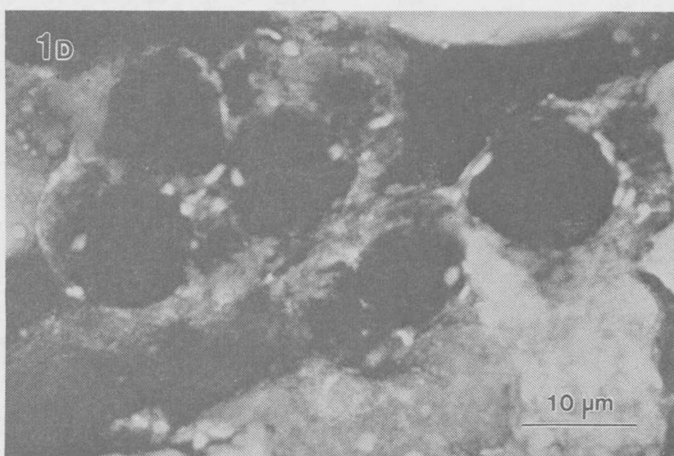
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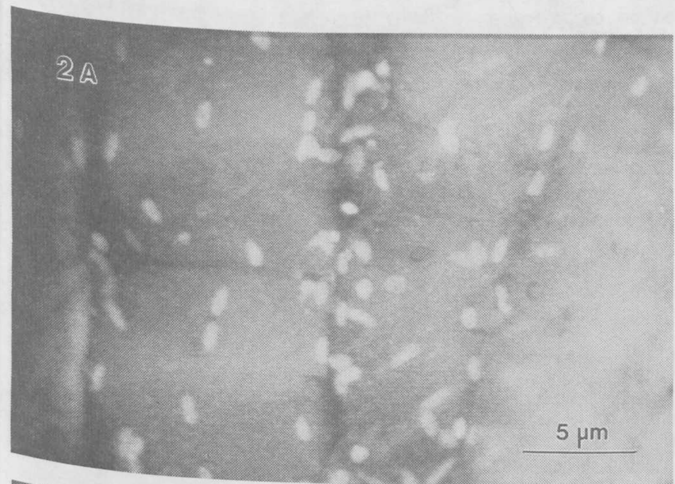
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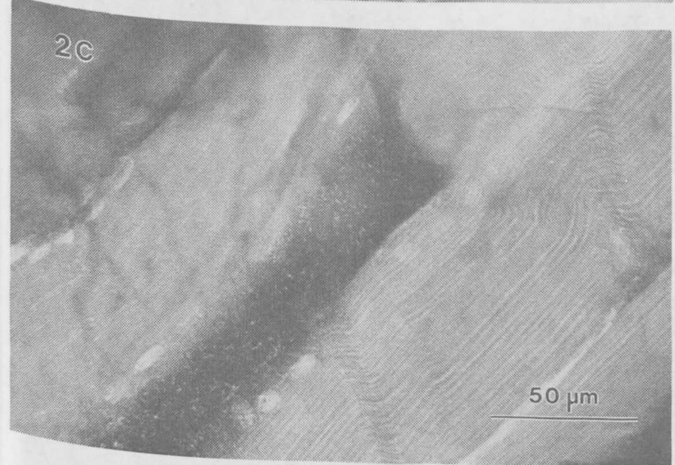
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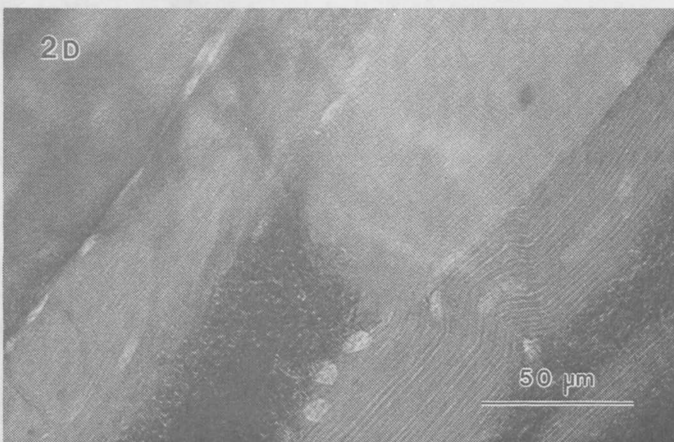
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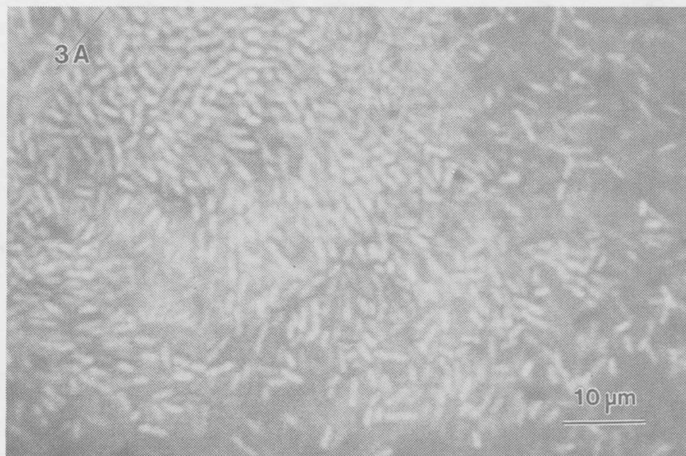
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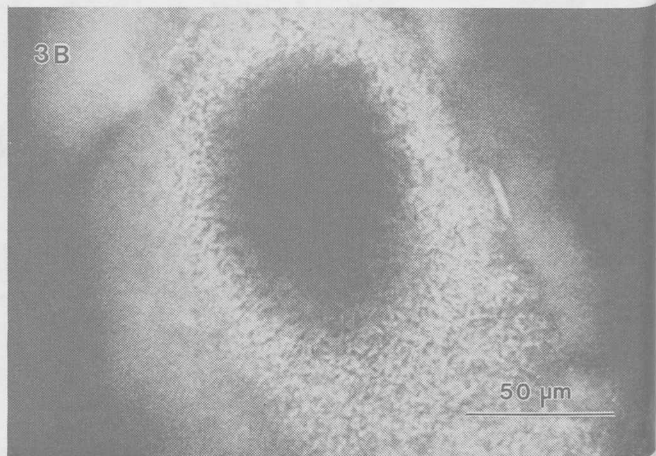
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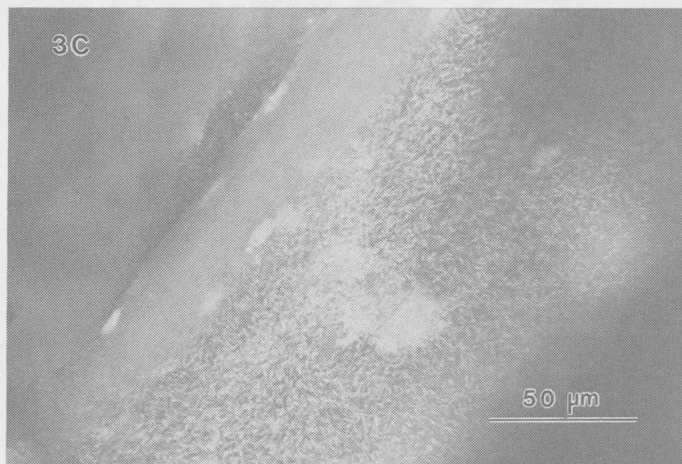
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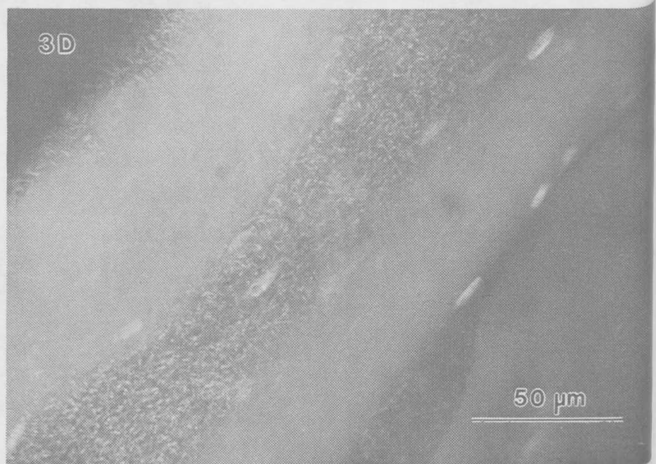
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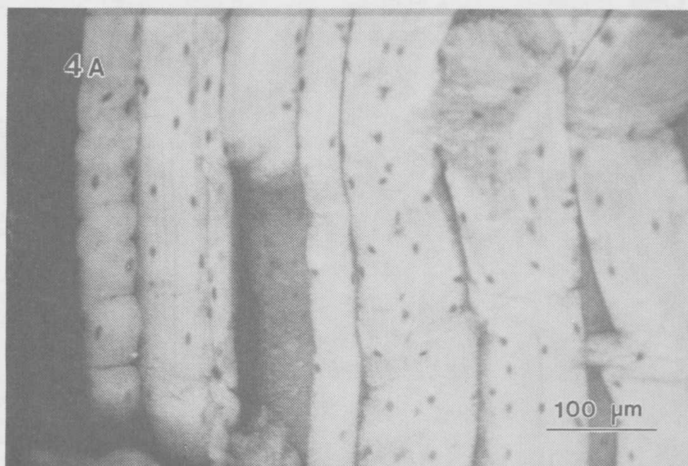
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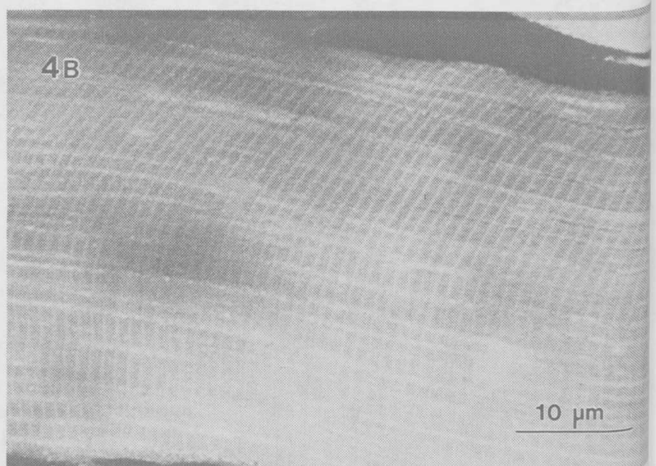
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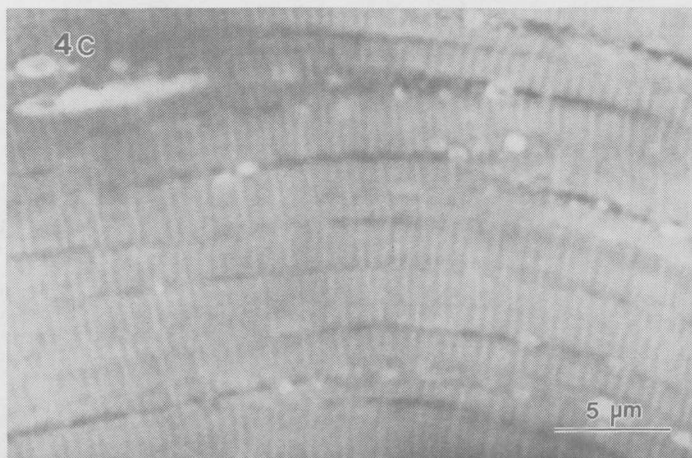
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