IMPROVED VISUALIZATION OF BACTERIAL SPECIMENS FOR ULTRATHIN SECTIONING[1]

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Hashimoto and Naylor (J. Bacteriol., **75,** 640, 1958) have shown that enclosing bacterial cells in agar prior to embedding the specimens in butyl methacrylate greatly decreases distortion and explosion of the cells. In using an agar-enclosing technique to study the relationship of cells in colonies of *Bacillus anthracis*, it was necessary to expose the colonies to osmium tetroxide after enclosing them in agar, whereas Hashimoto exposed the cells to the fixative before enclosing in agar. As a result of this difference in the method of preparation of the specimens, it was quite difficult to see the specimens in the embedding medium (butyl methacrylate) and thus the cutting of ultrathin sections was exceedingly laborious.

A marked improvement in the visualization of the agar-enclosed specimens was achieved by utilizing the following procedure. The colonies examined were either subsurface colonies in pour plates or surface colonies on streaked plates which had been overlaid with melted, tempered agar. Blocks of agar (approximately 1 mm^3) enclosing selected colonies were cut out and fixed in 1 per cent osmium tetroxide (containing 0.0458 g per ml sucrose and buffered at pH 7.2 to 7.4) for periods of time ranging from 1 to 2 hr. The agar blocks were washed in two changes of Tyrode's solution for 1 hr. The Tyrode's solution was removed and the agar blocks were immersed in a 0.5 per cent aqueous solution of methylene blue for 5 min. Dehydration was accomplished by passage through a series of solutions of increasing ethanol concentration terminating the procedure with absolute ethanol. Some of the blue color of the agar blocks was lost in the process of dehydration, but enough of the dye was retained to make the blocks readily visible when embedded in butyl methacrylate. The marked improvement in the visualization of the specimen greatly facilitated the cutting of ultrathin sections.

Enclosing of colonies in agar before fixation has an advantage over previous methods of preparation of bacteria for electron microscopy in that it enables one to observe bacteria in their natural spatial relationships. The improvement in visualization of agar-enclosed specimens achieved through staining with methylene blue simplifies the cutting of sections and results in a practicable method of specimen preparation.

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ON STEROLS IN BACTERIA[1]

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The presence of sterols in bacteria has been investigated periodically since the studies of Gérard (Compt. rend., **126,** 904, 1898) on the lipids of *Micrococcus albus*. Many studies were performed before sensitive analytical methods and chemically defined media became available for the organisms tested. As a consequence, traces of sterols reported could have come from the media used. For example, claims that sterols were present in *Mycobacterium phlei*, *M. tuberculosis*, and *Escherichia coli* (Hecht, Z. physiol. Chem. Hoppe-Seyler's, **231,** 29 and 279, 1935)

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evoked counterclaims of contamination from unwashed hands and stopcock grease (Anderson and Chargaff, J. Biol. Chem., **85,** 509, 1930). Wherever careful precautions were taken, no sterols have been found except in *Lactobacillus arabinosus* and *L. pentosus* (Guirard *et al.*, Arch. Biochem., **9,** 361, 1946); in *E. coli* (Dauchy *et al.*, Compt rend. soc. biol., **150,** 1974, 1956); and in *Azotobacter chroococcum* (Sifferd and Anderson, Z. physiol. Chem. Hoppe-Seyler's, **239,** 270, 1936).

Major effort in the search for bacterial sterols has been concentrated on a few genera, notably *Bacillus* (Lemoigne *et al.*, Bull. soc. chim. biol., **31,** 1587, 1949), *Escherichia* (Williams *et al.*, J. Bacteriol., **37,** 301, 1939), *Lactobacillus* (Guirard *et al.*, Arch. Biochem., **9,** 361, 1946) and *Mycobacterium* (Anderson and Chargaff, J. Biol. Chem., **85,** 509, 1930). Miyoshi (Sei-i-kwai Med. J., **49,** 47, 1930) analyzed the lipids of Shigella, Staphylococcus, Pseudomonas, and Vibrio. Aerobacter (Zambruno, Boll. soc. ital. biol. sper., **22,** 337, 1946), Corynebacterium (Gubarev and Bakulenko, Biokhimiya, **10,** 285, 1945), and Azotobacter (Sifferd and Anderson, Z. physiol. Chem. Hoppe-Seyler's, **239,** 270, 1936) have also been examined.

From this list, it is apparent that, in addition to mycobacteria, only representatives of the eubacteria have been investigated. To determine whether other major groups of bacteria contain sterols, cultures of *Micromonospora* sp., *Streptomyces griseus*, *Sphaerotilus natans*, and *Rhodospirillum rubrum* were cultivated in appropriately defined media to yield approximately 30 to 40 g wet weight. The nonsaponifiable lipids were extracted (Klein, J. Bacteriol., **69,** 620, 1955), and assayed for sterols using a modified Liebermann-Burchard test (Idler and Baumann, J. Biol. Chem., **203,** 389, 1953), digitonin precipitation, and column chromatography (Johnston and Bloch, J. Am. Chem. Soc., **79,** 1145, 1957).

Micromonospora sp. gave a positive test equivalent to approximately 0.001 per cent (calculated as ergosterol) of the wet weight. This material behaved with digitonin and on columns like known sterols. We, therefore, conclude that Micromonospora contains traces of sterol, pending final characterization of this material. *R. rubrum* presented difficulties because of its high concentration of pigments, which were largely removed by chromatography. The resulting lipids yielded a positive Liebermann-Burchard test indicating a maximum sterol content (calculated as ergosterol) of about 0.001 per cent on a wet weight basis. Since this material did not yield a precipitate with digitonin, it is probable that the positive test was due, at least in part, to contaminating pigments. *S. natans* and *S. griseus* yielded negative results in tests that could detect as little as 0.00001 per cent of the wet weight as sterol.

USE OF A TETRAZOLIUM SALT FOR AN EASILY DISCERNIBLE SULFIDE-MOTILITY REACTION

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The hanging-drop technique employed for the detection of bacterial motility is tedious. Young cells are required, and, as it is difficult to observe motility when a limited number of cells within a culture exhibit motion, results are often confusing.

With semisolid media, motility is macroscopically manifested by a diffuse zone of growth spreading from the line of inoculation. The effects are cumulative, and localized outgrowths appear when only a small proportion of motile cells are involved, eliminating the possibility of overlooking motility. Growth and diffusion in semisolids, however, may be so slight as to necessitate comparison with a control tube for proper interpretation.

Colorless solutions of various tetrazolium salts are reduced to insoluble, pigmented formazans in the presence of viable cells. Kuhn and Jerchel (Ber. deut. chem. Ges., **74B,** 941, 1941) called attention to the fact that tetrazolium solutions could be used to stain yeast and bacteria. Wood