Monochromatic Ultraviolet Microscopy of Microorganisms: Preliminary Observations on Bacterial Spores. By Tadayo Hashimoto* and Philipp Gerhardt. (*From the Department of Bacteriology, The University of Michigan Medical School, Ann Arbor.*)‡

An ultraviolet source may be used in light microscopy to excite visible fluorescence, increase resolution, or differentiate absorbing materials. The last two aims require ultraviolet-transmitting optics, usually of quartz, and have infrequently been sought by bacteriologists, especially since electron microscopy with its vastly increased resolving power became available. However, ultraviolet light has the special advantage of permitting the examination of aqueously mounted bacteria for cell structures that are strongly absorbing. If used in conjunction with a precision monochromator, the ultraviolet source can be resolved to narrow wave bands that may allow further qualitative or even quantitative identification of structural components.

We have sought to use this potential in conjunction with studies on the structure and function of bacterial spore membranes (1–3). A point of particular concern was the localization in the spore of dipicolinic acid (DPA), which is uniquely present in large amounts (4) and is believed to be associated with heat resistance (3, 5). Dipicolinic acid has absorption peaks at 263, 270, and 277 mμ and has been thought (6) to account for the uniformly dark appearance of spores in ultraviolet light, as reported in 1931 by Wyckoff and Ter Louw (7) for *Bacillus subtilis* and in 1938 by Piekarski (8) for an unidentified bacillus. Malmgren and Hedén (9) photographed *Bacillus cereus* at 257 mμ and noted that "there were present in the otherwise pale bacterial plasma more strongly absorbing grains," which apparently were spores. A similar picture of *Bacillus megaterium* spores may be found among the extraordinary but little known photomicrographs of microorganisms taken with filtered (275 mμ) ultraviolet light by Kruis in 1913 (10). The seemingly uniform absorption of ultraviolet light by bacterial spores and the inference that DPA is distributed throughout their structure seemed worthy of reexamination.

Materials and Methods

Bacillus megaterium was chosen for study because spores of this species characteristically have a high DPA content (4), which was confirmed for this strain by colorimetric analysis (11). It also possesses a prominent cortex in the multilayered wall (12). Virtually complete sporulation was attained by growing the culture in trypticase-soy broth on the shaker at 30°C. for 24 hours. After vegetative autolysis, the spores were separated and repeatedly washed in distilled water by means of differential centrifugation.

The ultraviolet source was a Sylvania type SH high pressure mercury vapor arc lamp, light from which passed through a Bausch and Lomb grating monochromator with both slit widths set at 1.5 mm. and with a chromatically corrected condenser. The emitted light was reflected into the microscope by a first surface aluminized plane mirror. Both the microscope objective and substage condenser consisted of an ultraviolet reflecting, Polaroid-Grey, water-immersion, catadioptric system (BL design VII, 94 ×, 1.00 N.A.). Specimens were mildly heat-fixed on a quartz coverslip and then mounted in water between it and a second coverslip.

The depth of focus was so small that it was found necessary to take a series of photographs, even though the monochromator was corrected and the approximate focus was precalibrated for the change from visible to ultraviolet light; even then only a portion of a field would prove to be in sharp focus. A minimum exposure time and lack of grain were obtained by using improved plus-X film processed with ethol ultrafine grain developer for 30 minutes. Adox KB-14, panatomic-X, tri-X, and Eastman spectroscopic type I-O films were also tried but found less satisfactory.

RESULTS AND DISCUSSION

The appearance of washed spores of *Bacillus megaterium* in transmitted 270 mμ ultraviolet light is shown in Fig. 1. When in sharp focus, the spores were seen to be differentiated internally with a transparent band discernible between a dense periphery and core. The internal non-absorbing region was interpreted as corresponding to the cortex of the wall. The banded appearance seen in mature free spores was absent or much less apparent in developing endospores, and the sporangium itself appeared uniformly and faintly ultraviolet-absorbing (Fig. 2).

The question immediately arose, however, as to whether the banded appearance of free spores was a consequence of the intense ultraviolet irradiation, which is known to trigger the release of DPA from the spore (13). In an attempt to resolve this inherent limitation, spores were photographed immediately upon being exposed and were seen to

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have the same internal differentiation; however, the photographic exposure time could not be reduced below 10 seconds and a way was not found to analyze the possible DPA release in the field immediately under examination. Thus the question of whether DPA originally was present in the transparent zone could not be definitely answered, although it seemed unlikely.

The possibility also existed that the zones of dense adsorption seen in the spores resulted not from DPA but rather from other ultraviolet-absorbing materials, especially if they occur dehydrated or bound as is often believed. Such was found to be the case, as shown most convincingly in spores autoclaved so as completely to remove DPA. As shown in Fig. 3, autoclaved spores showed much the same and an even more differentiated picture than did normal spores. A faint but again similar picture was seen in thin sectioned spores; here again, the slicing process is known to cause the release of internal DPA. Moreover, in photomicrographs taken at 250, 260, 277, 280, and 300 mμ, the banded appearance also was observable in sharply focused intact spores. Isolated spore coats could be visualized in ultraviolet light despite the fact that extraction by autoclaving of such preparations in a small proportion of water failed to show even traces of DPA either spectrophotometrically (4) or by colorimetric analysis (11).

Thus, it was concluded that ultraviolet-absorbing materials other than DPA account for the dark appearance of bacterial spores in ultraviolet light. The ultraviolet-transparent internal structure seen in these spores is not consistent with previous reports (7–10) of uniform absorption and indicates that DPA is probably not present in the cortex of the spore wall.

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Addendum.—We have become aware of additional observations, by Barnard and Welch (14) in 1936 and by Ernst and Wolbach (15) and Von Schrötter (16) in 1906, that bacterial spores seem to be uniformly absorbing in ultraviolet light.

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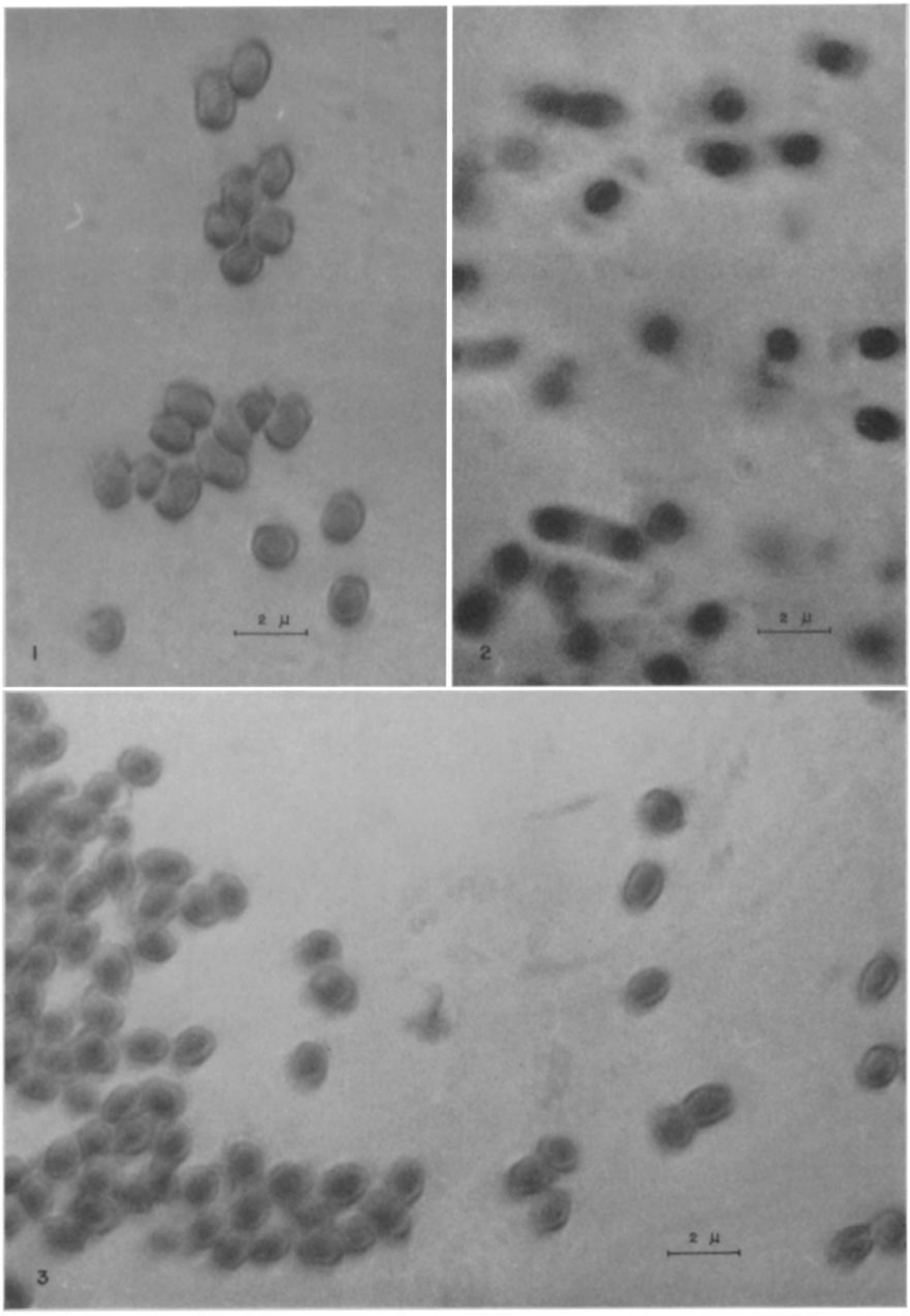
Explanation of Plate 102

Fig. 1. Washed spores of *Bacillus megaterium* in transmitted 270 mμ ultraviolet light. × 5,800.

Fig. 2. Sporangia and developing endospores. × 5,800.

Fig. 3. Autoclaved and washed spores, devoid of dipicolinic acid. × 5,800.



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