CELLULAR STRUCTURE AS REVEALED BY ULTRAVIOLET PHOTOLYSIS AND THE ELECTRON MICROSCOPE

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An important handicap of electron microscopy has been the inability of electrons to penetrate protoplasm. Thus, most bacteria are opaque when observed with this instrument unless special methods are used such as chemical treatment or growth in special media (Knaysi, 1951). Yeasts have appeared as completely opaque. In an earlier paper (Bartholomew and Mittwer, 1952) the authors reported that ultraviolet light induced the loss of gram positiveness in microorganisms. The present paper reports the results obtained when the electron microscope was used to observe morphological changes occurring during the conversion from a gram positive to a gram negative state by ultraviolet light. These investigations revealed that ultraviolet light had a marked effect of rendering yeast and bacterial cells more transparent to the electron beam, thus making possible the demonstration of internal structures.

EXPERIMENTAL METHODS

A Westinghouse sterilamp WL-15 was used as a source of ultraviolet light. This lamp has an output of 32 microwatts per cm^2 at 2537 A, at a distance of 1 meter. Exposures were made at 12 cm. Two types of electron microscope preparations were used: (1) cells were exposed in suspension in a quartz cell, followed by the preparation of collodion films on wire screens in the usual manner; (2) dry cells were exposed by preparing smears on glass slides which had been dipped in 0.2 per cent collodion. After irradiation the slide was again dipped in 0.2 per cent collodion and the preparation floated off on water. Screens were then prepared and observed in an RCA model EMU electron microscope. Most of the pictures presented here represent a final magnification of approximately 8,000×.

Similarly treated cells were also observed with the light microscope after staining with cell wall stains (Dyar, 1947; Welshimer and Robinow, 1949), toluidine blue (Lindegren, 1949), and Rafalko's modification of the feulgen reaction (Lindegren, 1949).

RESULTS

Figures 1, 2, and 3 represent the appearance of unirradiated cells of *Bacillus subtilis*. The influence of ultraviolet light on electron penetration can be readily seen by comparing figures 4, 5, 6, 7, and 8. These show the appearance of unfixed dried smears of *B. subtilis* after 3, 7, 20, 24, and 30 hours of irradiation. It is evident that the cells gradually lose their opacity. This is accompanied by a

[1] With the facilities of the electron microscope laboratory of the Department of Experimental Medicine.

general change of cell appearance from that of a normal rod shaped cell to that of a nearly empty sac. Similar experiments (Bartholomew and Mittwer, 1952) showed that about twenty per cent of the cells in figure 4 are gram negative. One hundred per cent are gram negative in figures 5, 6, 7, and 8. Compared to the controls (figures 1, 2, and 3), all the cells in figure 5 show some evidence of injury due to irradiation. The cells either are semitransparent or irregular in shape, and a general flattened appearance is observed as photolysis proceeds.

As the cells become transparent, clear nucleus-like bodies appear. These bodies start to become visible after 3 hours of irradiation (figure 4), become prominent after 20 hours (figure 6), and have nearly all disappeared by 24 hours (figure 7).

Further experiments were conducted, irradiating *B. subtilis* in distilled water and in 1 per cent formaldehyde suspensions. This treatment resulted in an entirely different type of cell change. Figure 9 shows unirradiated controls suspended in distilled water for 22 hours. Unirradiated controls in 1 per cent formaldehyde have an almost identical appearance. Figures 10, 11, 12, and 13 illustrate the changes occurring during irradiation. At 7 hours (figure 10) the cells suspended in distilled water were all gram negative. It can be seen that the cell wall has become extended and flattened in many areas. The cells in figures 11, 12, and 13 were suspended in 1 per cent formaldehyde and the irradiation continued for 22 hours. Here the cell wall has become even more extended and flattened, and a peculiar small opaque rod is left. The size of this opaque rod is much too small to represent the original cell of *B. subtilis*. It retains considerable third dimension as can be seen from the length of the shadow in figure 13. It can also be noted that the cell wall is less in evidence in the area near this small rod. Figure 14 shows an optical microscope picture of similarly irradiated cells stained by the gram method. Although all of these cells stain gram negatively, some are much smaller than others. The small cells bear the same size proportion to the large cells as do the small opaque rods in figures 11, 12, and 13 to the control cells in figures 1, 2, and 3.

The results for *Saccharomyces cerevisiae* were even more interesting. Figure 15 is an untreated control and represents the usual opaque yeast cell as seen with electron microscope. Figure 16 shows a cell suspended in distilled water and irradiated for 72 hours. By this time all of the yeast cells are gram negative with occasional gram positive granules. The same loss of cell substance as for *B. subtilis* is evident here. Also shown is a smaller internal area resistant to photolysis and leaching by this method.

Irradiation of dry smears of yeast gave the results shown in figures 17, 18, 19, and 20. An amazing amount of internal detail is visible. Figure 21 represents these internal structures diagrammatically, and can be directly compared with most of the cells shown in the photographs. Structure A is undoubtedly the cell wall, B is an area of cytoplasm, and C is a thin membrane-like structure which has not hitherto been observed in yeast cells. D is a membrane surrounding structure E which corresponds in size and position to the nuclear vacuole (Lindegren, 1949). Light microscope controls using the staining methods of Lindegren

confirmed the nuclear nature of structure E. It stained positively using the feul-
gen method of Rafalko (Lindegren, 1949), and the chromosomes(?) were seen.
The nuclear vacuole is in fact one of the last structures to be photolysed and
remains feulgen positive until apparently nothing remains but it and the cell
wall. Cell wall stains of irradiated yeast showed that both A and D took this
type of stain. Figures 17, 19, and 20 show a structure (F in figure 21) which corre-
sponds to the nucleolus demonstrated by Lindegren (1949), the "dancing body"
pictured by Henrici (1947), or the central volutin granule of Wager and Peniston
(Lindegren, 1949). The centrosome described by Lindegren is not seen in these
pictures. It is probably hidden by the dense area (probably heterochromatin)
generally seen at one end of the cell, since irradiated cells stained with the
toluidine blue stain of Lindegren (1949) showed this structure. As far as the
authors are aware, these are the first electron microscope pictures to show such
internal structures in yeast.

<div align="center">DISCUSSION</div>

The differences were marked between the results obtained on irradiation of
cells on slides and those in suspension. The empty cell areas appearing on irradia-
tion of cells in suspension can be explained on the basis of a leaching out of cell
material, due to a change in cell membrane permeability or to the production by
photolysis of substances of increased solubility. It is known that the ultraviolet
irradiation of bacterial and yeast cells results in the appearance of "growth
stimulatory" substances in the surrounding medium (Loofbourow and Morgan,
1940; Loofbourow, 1941). These substances were identified by Davidson (1940)
as being mostly nonprotein nitrogenous materials. Most of this was amino nitro-
gen, with about a third as nucleotides and nucleosides.

The sequence of events occurring on irradiation of cells in suspension could be
reconstructed as follows. First, material begins to leave the cell which would not
leave in the absence of ultraviolet light. Secondly, the cell becomes gram nega-
tive but maintains its normal size and shape. This agrees with earlier obser-
vations of Bartholomew and Mittwer (1952). Thirdly, the cell begins to flatten,
due to material loss, and a large cell wall area becomes spread out and easily seen.
Lastly, a residual small body is left which is within the old cell wall and which is
only about one-half the size of the normal cell. Many interesting postulates can
be made as to the nature of the residual body.

The small rods seen in figures 11, 12, and 13 are very much like the pictures
presented by Møller and Birch-Andersen (1951) to show the effects of plasmolysis
by salt, phenol, and mercuric chloride. It is impossible at the present time to
state whether these objects are actually morphological structures or simply
plasmolysed states. However, it seems peculiar that plasmolysis would result in
such a neat rod-like structure. The small rod bodies could represent the gram
negative medulla area of Churchman (1927). The small cells in figure 20 are
analogous in size to the small gram negative rod often seen in a chain of large
gram positive rods. These rods are also similar to the structures left in enzymati-
cally digested cells (Weidel, 1951). In the yeast cell the small body occupies a

position and is of a size analogous to the nuclear vacuole (Lindegren, 1949). Work is now in progress attempting to define more precisely the nature of these bodies in bacteria.

The gradual change from an opaque to a transparent condition with respect to electrons must be due to photolysis of cell substances. It cannot now be stated whether this photolysis causes a breakdown of electron scattering materials to compounds no longer scattering electrons, or whether volatile compounds are produced which then leave the cell. However, the effect of ultraviolet light here is very similar to that reported for electron beams by Knaysi et al. (1950) and Mudd et al. (1951) and which they attribute to volatilization. Ultraviolet light is, however, a more sensitive and easily controlled method of revealing internal structure than an intense electron beam.

Irradiation of dry slides gave no evidence of the small opaque rod observed in the irradiated cell suspensions. However, a different differential action occurs which does reveal a very transparent nucleus-like granule in the B. subtilis cells, and much interesting morphology in the yeast cells.

The structure of yeast revealed by irradiation of dry smears fits best into the morphological concepts of Lindegren (1949), and hence Lindegren's terminology has been used in the present discussion. However, further experiments are being conducted which may aid in clarifying the current controversy in the field of yeast cytology. This terminology is therefore used for convenience and is subject to change. Phase microscopy showed that the nuclear vacuole sometimes had continuity with a similar structure in a young bud. The very thin structure (figure 21, C) cannot be identified with certainty. It could be a cell vacuole or nuclear membrane, or it could be due to shrinkage of the nuclear structure during drying. This structure could be demonstrated by phase microscopy of irradiated dry cells suspended in water. Under these conditions the nuclear structure could be observed to move by brownian movement within this restricted area of the cell.

The attempts to follow the changes of morphology as cells were converted to the gram negative state were not successful. This was due to the fact that cells in suspension were converted before any obviously related morphological change was detected by the electron microscope. The photolytic power of ultraviolet light could be used to support either the chemical or membrane permeability concepts of the gram mechanism.

The morphological changes occurring on extended irradiation will undoubtedly become a useful tool in additional studies of cell structure.

SUMMARY

The exposure of cells in suspension, and as dried smears, to ultraviolet light was shown to result in an increased transparency to the electron beam. This

resulted in the revelation of various internal structures in bacterial and yeast cells.

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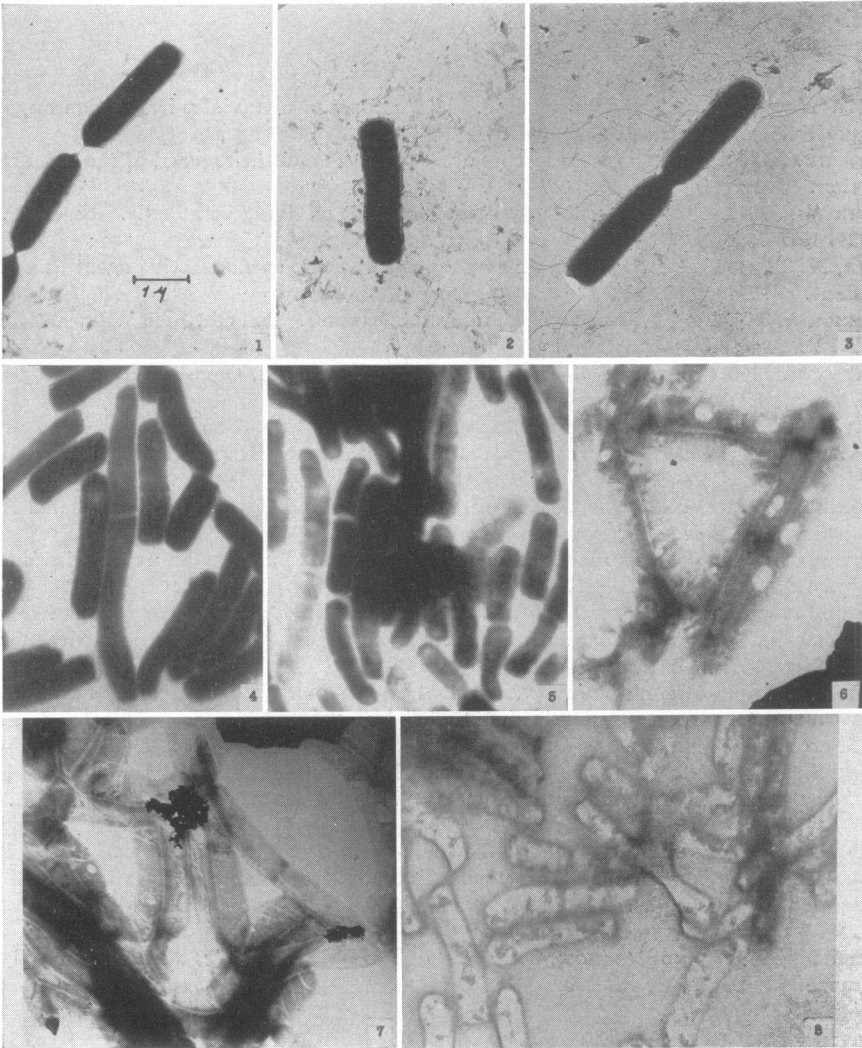
Figures 1, 2, and 3. Bacillus subtilis, untreated controls. Electron micrograph, 8,000×. Figure 3 is chromium shadowed.

Figure 4. Bacillus subtilis, irradiated with ultraviolet light 3 hours on slide. Electron micrograph, 8,000×.

Figure 5. Bacillus subtilis, irradiated 7 hours on slide. 8,000×.

Figure 6. Bacillus subtilis, irradiated 20 hours on slide. 8,000×.

Figure 7. Bacillus subtilis, irradiated 24 hours on slide. 8,000×.

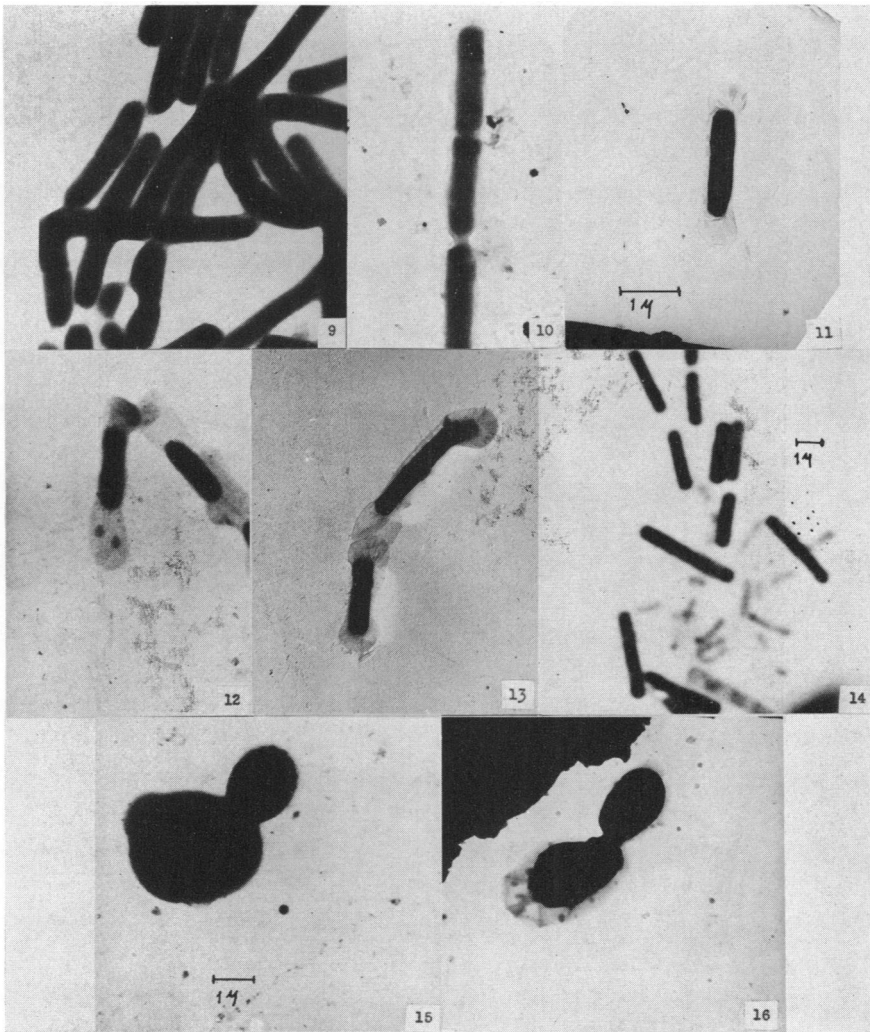*Figure 8. Bacillus subtilis*, irradiated 30 hours on slide. 8,000×.

Figure 9. Bacillus subtilis, control suspended in 1 per cent formaldehyde 22 hours, not irradiated. 8,000×.

Figure 10. Bacillus subtilis, suspended in distilled water and irradiated 7 hours. 8,000×.

Figures 11, 12, and 13. Bacillus subtilis, suspended in 1 per cent formaldehyde and irradiated 22 hours. Figures 11 and 13 are chromium shadowed. 8,000×.

Figure 14. Bacillus subtilis, suspended in 1 per cent formaldehyde and irradiated 24 hours. Gram stained and photographed with light microscope. 4,000×.

Figure 15. Saccharomyces cerevisiae, untreated control. Electron micrograph. 6,000×.

Figure 16. Saccharomyces cerevisiae, suspended in distilled water and irradiated 72 hours. 6,000×.
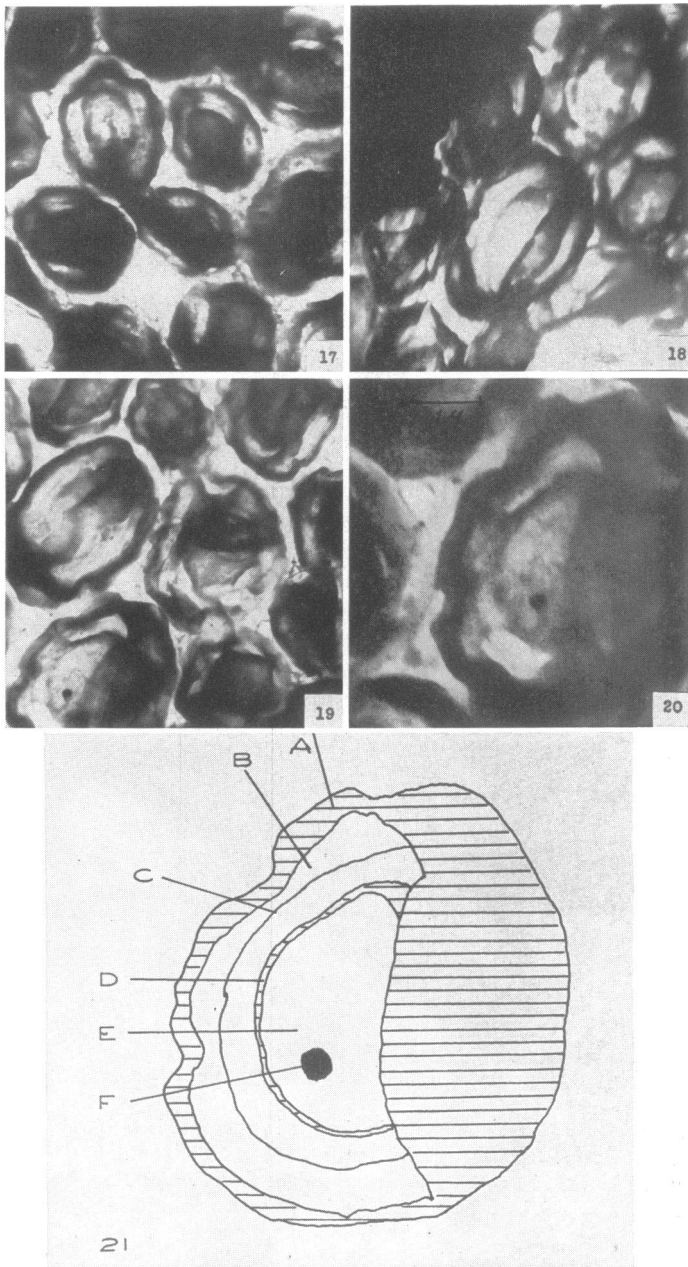
Figure 17. *Saccharomyces cerevisiae*, irradiated 72 hours on slide. 4,300X.

Figures 18 and 19. *Saccharomyces cerevisiae*, irradiated 72 hours on slide. 6,000X.

Figure 20. *Saccharomyces cerevisiae*, irradiated 72 hours on slide. 10,000X.

Figure 21. Diagrammatic representation of cells of *Saccharomyces cerevisiae* seen in figures 17–20. A cell wall, B cytoplasm, C membrane, D membrane of nuclear vacuole, E nuclear vacuole, F nucleolus.