ELECTRON AND LIGHT MICROSCOPIC STUDIES OF BACTERIAL NUCLEI

I. Adaptation of Cytological Processing to Electron Microscopy; Bacterial Nuclei as Vesicular Structures[1]

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Electron microscopy has been most rewarding when used complementarily with other techniques of investigation. For revealing fine structure within cells the techniques of classical cytology and of electron microscopy are obviously complementary. In studying structure within the bacterial cell by light microscopy, the principal limiting factor is the *limit of resolution*, i.e., the capacity to distinguish adjacent elements of pattern as separate from one another. The preeminent advantage of the electron microscope is in reducing the limit of resolution to approximately 0.002 μ for suitable objects and 50 kv electrons.

However, adjacent elements of pattern to be distinguished in an electron image must differ appreciably in their capacities to scatter electrons; these are to a first approximation proportional to the products of thickness times density of the object along the electron paths in question. These products that principally determine *contrast* in the electron image are not related in any simple theoretical way to the factors that have been used to achieve contrast in light cytology, e.g., color, phase contrast, and double diffraction. The question of how far the techniques elaborated in light cytology may be useful in electron microscopy is therefore an empirical one.

In the present study we shall first describe a simple procedure by which bacteria may be prepared both for light and for electron microscopic examination. We shall then examine the effects on electron image contrast of the successive cytological steps currently utilized for demonstrating bacterial nuclei. In addition, the results obtained afford a basis for tentative conclusions concerning the constitution of the bacterial nuclei.

PREPARATIVE PROCEDURES

It is desirable for either electron or light microscopic study that the bacterial cells be altered from the state in which they are grown as little as may be compatible with other requirements for examination. The technique of growing bacteria on a thin membrane of collodion overlying nutrient agar (Hillier, Knaysi, and Baker, 1948) permits their cytological processing with a minimum of physical or chemical disturbance. Appropriate fixation is important for preserving the

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finer relationships in internal structure for electron as well as for light microscopic examination. Fixation merely by drying has served not too badly for electron microscopic examination of internal structure in gram-negative cells (Hillier, Mudd, and Smith, 1949). The present study demonstrates that fixation in OsO_4 vapors may add definition and sharpness of contrast between nuclear sites and surrounding cytoplasm without qualitatively altering the natural pattern.

Preparation of bacteria for light microscopy. Cells from a broth culture of the organism to be studied are spread in a not too heavy suspension over the surface of an enriched nutrient agar such as that described by Morton and Engley (1945). This medium was used throughout the present study. After incubation at 37 C from $1\frac{1}{2}$ to 2 hours, blocks of agar are cut out, fixed in OsO_4 vapor, and impression films are made on thin cover glasses as described by Robinow (1944). The fixed films are then subjected to treatment in normal hydrochloric acid at 60 C, after which the preparation is washed in distilled water and then mordanted in 1 per cent formaldehyde for from 2 to 4 minutes. This is followed by washing once more in distilled water, and finally the preparation is stained in 0.3 per cent aqueous basic fuchsin for from 15 to 30 seconds, washed, and mounted in tap water. The formaldehyde-mordanted basic fuchsin technique applied here is a modification by one of us (Smith, 1950) of Robinow's (1945) procedure for bacterial nuclei and a procedure described by DeLamater (1948) and DeLamater, Mescon, and Barger (1950) for staining the nuclei of fungi.

Preparation of bacteria for electron microscopy. An enriched nutrient agar is overlaid with collodion as described by Hillier, Knaysi, and Baker (1948). A broth culture of the selected organism is centrifuged, washed in distilled water, centrifuged, and resuspended in distilled water. A dilute suspension of the organism is then implanted on the collodion membrane with the aid of a capillary pipette and the excess inoculum withdrawn, leaving a thin circular film of suspension. When the moisture from this film evaporates, one should have left on the membrane a circular area about 5 mm in diameter containing well-dispersed single cells. Many such implantations may be made on the collodion membrane covering the area of the ordinary petri dish. After incubation the resulting young colonies may be subjected to various steps of cytological processing and then be studied with the electron microscope.

In applying the above-described light cytological procedure to bacterial specimens prepared for electron microscopy the following steps are utilized: A series of five aligned implantations is made on the collodion membrane; the preparation is then incubated at 37 C for from 2 to $2\frac{1}{2}$ hours. If the original inoculum has not been too heavy, examination with the high dry objective of a light microscope at the end of such an incubation period will show that suitable microcolonies, not too thick for electron microscopy, exist. With a sharp scalpel the section of agar and membrane containing the implantations is cut out. An implantation is cut off; the membrane is floated off on distilled water and picked up on a piece of carefully etched 200-mesh copper screen; it is designated the *normal control.* The remaining section of agar with its implantations is then placed in a suitably covered glass jar or tumbler containing several layers of glass beads and several

cubic centimeters of 2 per cent solution of osmium tetraoxide. The organisms are fixed in the osmium tetraoxide vapor for from 1 to 3 minutes. Another implantation is cut off, picked up on a section of screen as described, and designated the *osmium-fixed specimen.*

The remaining section of collodion membrane with its implantations is floated off on distilled water and picked up on a piece of wire screen cut slightly larger in length and width than the membrane. This is to allow for the cementing of the edges and cross sections of the membrane between the implantations to the wire screen with collodion (2 to $2\frac{1}{2}$ per cent is satisfactory). In this manner one makes certain that the collodion membrane will adhere to the screen throughout the remaining processing. This preparation is then immersed in normal hydrochloric acid at 60 C and allowed to remain there for the prescribed time. Under these conditions the acid does not etch the copper screen appreciably, and there is no apparent effect of copper ions on the bacterial specimen during this period as revealed by the electron microscope. After hydrochloric acid treatment and washing in distilled water, an implantation is cut off for study in the electron microscope. Following this, the two remaining specimens are treated in 1 per cent formaldehyde; they are then washed and one is prepared for electron microscopy. The last is stained in basic fuchsin, washed, and designated the *completed specimen.* It has been treated in all the steps of a given light cytological technique. One is now able to learn the effects of the successive cytological steps on the electron image contrast within bacterial cells.

The electron microscope used was the RCA standard EMU model. The pictures were taken by Mrs. Jean Minkin and Dr. Marshall D. Earle through the courtesy of the Franklin Institute, Laboratories for Research and Development.

EXPERIMENTAL RESULTS

Cells of *Eberthella typhosa* grown on collodion as described and subjected to no fixation other than drying are shown in figure 1. The cells are perceptibly darker toward their ends and in their centers, with lighter intermediate regions. The cells are still connected by their cell walls and are surrounded by peritrichous flagella several of which lie in close apposition side to side. The flagella in this reproduction cannot be seen as they approach the cells. The lighter nuclear sites are just distinguishable from the darker enveloping cytoplasm in the unfixed cells imaged with standard lenses. The positions of the nuclear sites, however, correspond to those in images of unfixed bacterial cells with contrast accentuated by the special high-contrast lens of Hillier (1949; Hillier, Mudd, and Smith, 1949).

In figure 2 cells of *E. typhosa* fixed by exposure for 3 minutes to the vapors of 2 per cent OsO_4 are shown. The pattern of lighter nuclear sites and darker enveloping cytoplasm is accentuated. In figures 3, 4, and 5 are shown microcolonies of *Escherichia coli,* strain B, after fixation with OsO_4 vapor. The nuclear sites appear as light areas in arrangements suggestive of division in process or completed. Figure 6 shows swarming cells of *Proteus* after fixation with OsO_4. Light nuclear sites alternate with darker cytoplasm along these filamentous cells.

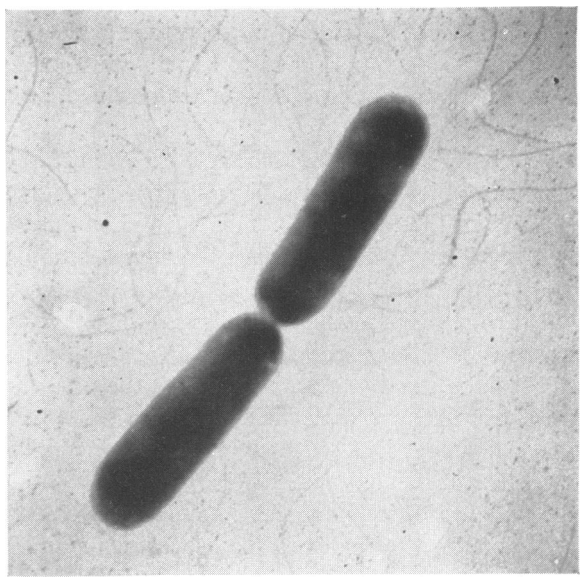

Figure 1. A cell from an *E. typhosa*, normal control specimen, grown for 2½ hours at 37 C on thin collodion membrane overlying Morton and Engley agar medium and prepared for electron microscopy as described in the text. (Magnifications in figures 1 to 12 are approximately 11,000 ×.)

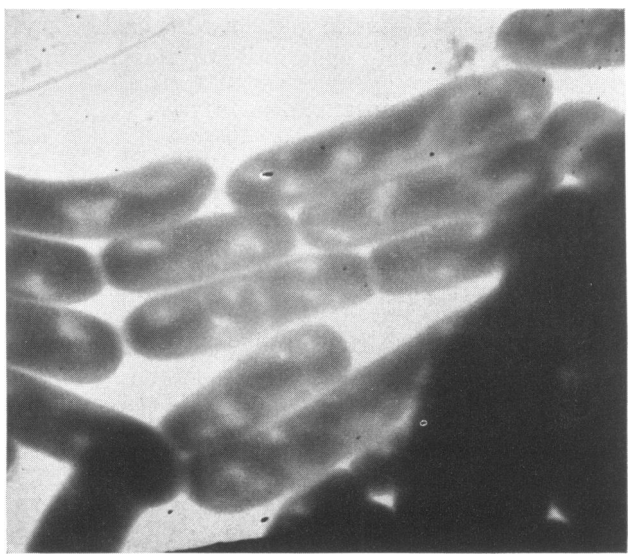

Figure 2. A microcolony of an *E. typhosa*, osmium-fixed specimen, grown for 3 hours at 37 C on thin collodion membrane overlying agar and prepared for electron microscopy as described in the text.

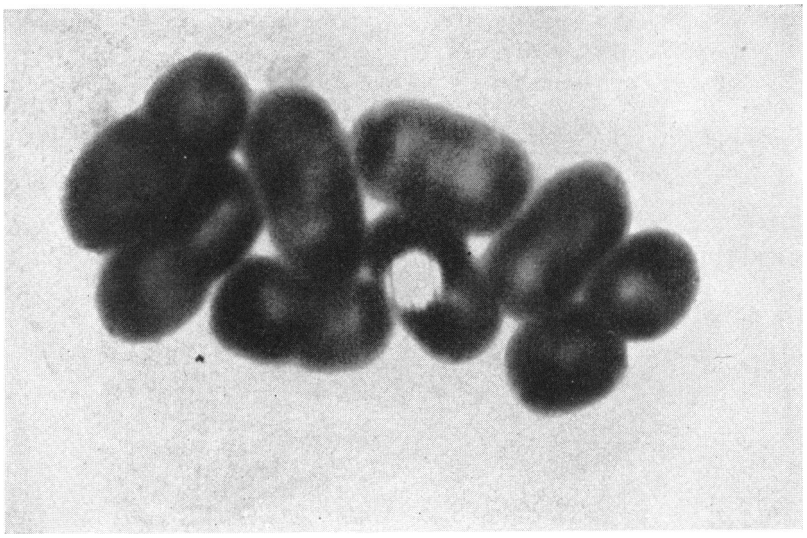

Figure 3. A microcolony of an *E. coli* B, osmium-fixed specimen, grown for 2½ hours at 37 C on thin collodion membrane overlying agar and prepared for electron microscopy as described in the text. The bright circle in the center of this picture is due to light coming directly from the electron microscope filament.

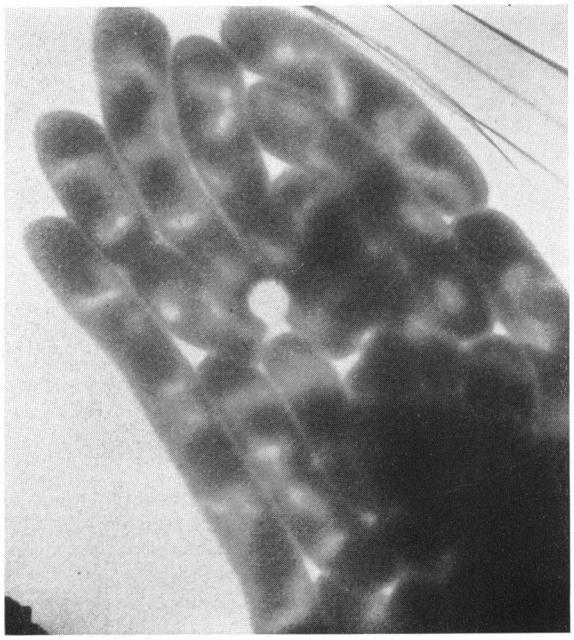

Figure 4. A microcolony of *E. coli* B from same osmium-fixed specimen as figure 3.

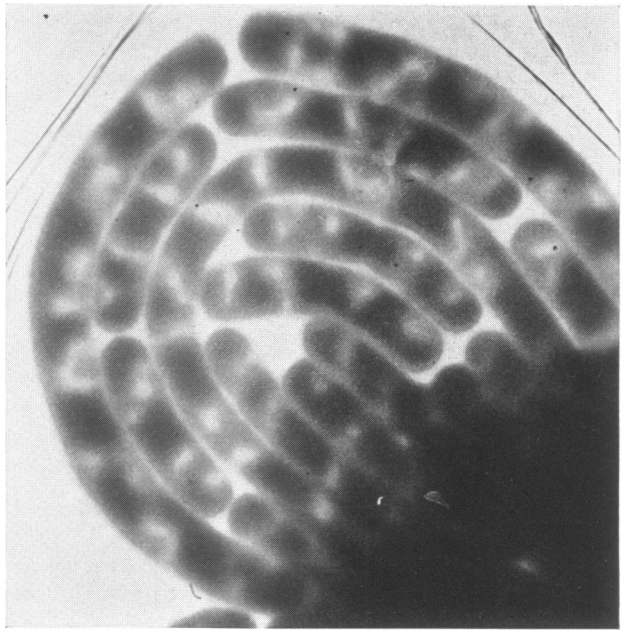

Figure 5. A microcolony of *E. coli* B from same osmium-fixed specimen as figures 3 and 4.

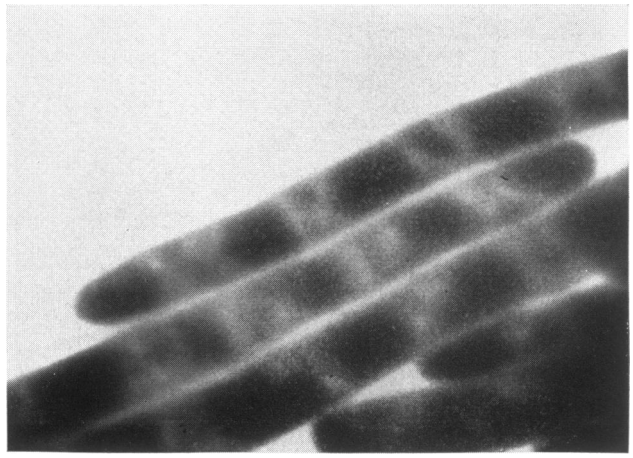

Figure 6. An impression film of *Proteus* P44 in the swarming stage made on collodion membrane overlying a section of copper screen. This variation in technique was resorted to because these organisms failed to pass through the swarming stage when grown on the membrane in the manner prescribed under experimental procedure. The culture was grown on Morton and Engley agar medium at 37 C. The organisms were fixed in OsO_4 vapor while still on agar. Swarming *Proteus* cells possess many flagella. The flagella that were present in the negative from which figure 6 was made were lost in preparing the print to show maximal contrast in internal structure.

In all these figures the contrast between nuclear sites and cytoplasm has been sharpened by the osmic fixation.

Treatment of the cells with normal hydrochloric acid completely reverses the pattern of contrast in the electron pictures (figures 7 to 11). The nuclear sites after HCl treatment are occupied by dense material appearing dark against a background of relatively light cytoplasm. The electron-scattering cytoplasm of the OsO_4-fixed specimens has lost in density, and the nuclear sites are occupied by very dense material apparently formed by coagulation of the nuclear contents. In figure 8 the dense material appears both as discrete particles, corresponding to the chromatinic bodies described by Robinow (1944, 1945), and in one elongated cell as a ribbon, as postulated by Delaporte (1939–1940); cf. also Johnson and Gray (1949) and Bisset (1949). Dark granules also appear in the cytoplasm of figure 8; their significance is not clear. Figure 9 is of HCl treated cells of *E. coli* in which the cytoplasm of the several cells has been cleared to varying degree by the acid treatment. The chromatinic bodies in some of these cells are seen to be composed of very dense granules in less dense surrounding material.

Figure 10 shows cells subject to OsO_4 fixation, HCl treatment, and mordanting with formaldehyde; the picture is little changed. Figure 11 shows a preparation in which the cytological processing has been completed by mordanting with formaldehyde and staining with basic fuchsin. The clear pattern of contrasting densities resulting from the osmic fixation and hydrochloric acid treatment is somewhat obscured by mordant and stain, despite the fact that the mordant and stain give brilliant color contrast in the light microscope.

Figure 12 shows the effect of fixation with Schaudinn's solution following OsO_4 vapors. Schaudinn's fixative, which is ordinarily used in Robinow's (1945) and in DeLamater's (1948) procedures for light microscopy, darkens and shrinks the cells, as was anticipated from earlier observations of the effect of bichloride of mercury on bacterial cells (Mudd and Anderson, 1942). Schaudinn's fixative is obviously unsuited for electron microscopic observations of internal structure.

In figures 13 to 19 the nuclei of young cells of *E. coli* in three stages of processing are reproduced, all at approximately the same magnification of 6,000 ×. Figures 13 to 15 show the nuclei after OsO_4 fixation as light vesicles enveloped in dark cytoplasm. In figures 16 to 18 the OsO_4-fixed cells have been treated with HCl. The cytoplasm has been greatly reduced in density and the coagulated nuclear chromatin appears dark relative to the cytoplasm.

Vendrely and Lipardy (1946) have shown that carefully graduated treatment of bacterial cells with HCl according to Robinow's procedure dissolves out selectively practically all the cytoplasmic ribonucleic acid but only a small proportion of the nuclear desoxyribonucleic acid. The cytoplasm in figures 7 to 11 and 16 to 18 appears shrunken, and the dark nuclear chromatin granules appear to be somewhat nearer the ends of the cells than do the corresponding nuclear vesicles in the fixed cells before HCl treatment (figures 3 to 5, 13 to 15). The decrease in density and volume of the cytoplasm resulting from the HCl treatment may well indicate extraction of cytoplasmic proteins, etc., as well as nucleic

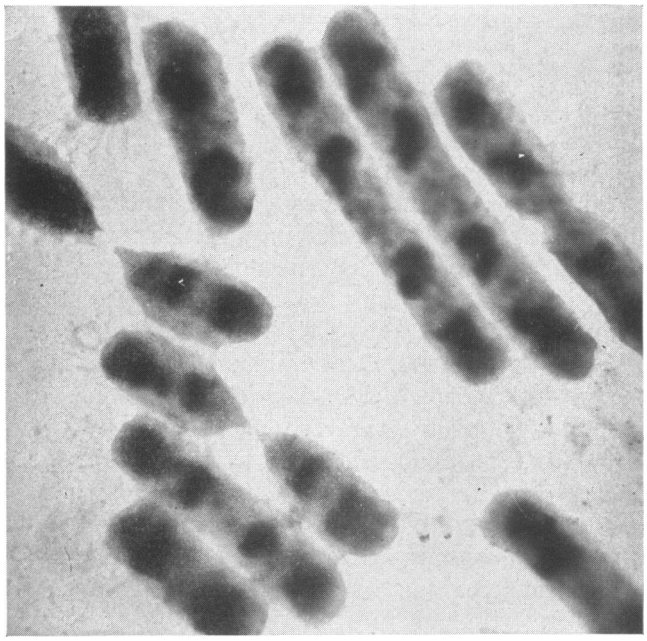

Figure 7. Cells from a microcolony of an *E. coli* B, hydrochloric-acid-treated specimen. After 2½ hours' growth at 37 C under conditions as described for figure 3, the specimen was fixed in OsO_4 vapor, transferred and cemented to a section of copper screen, and treated in N HCl for 6 minutes at 60 C. It was then washed in distilled water.

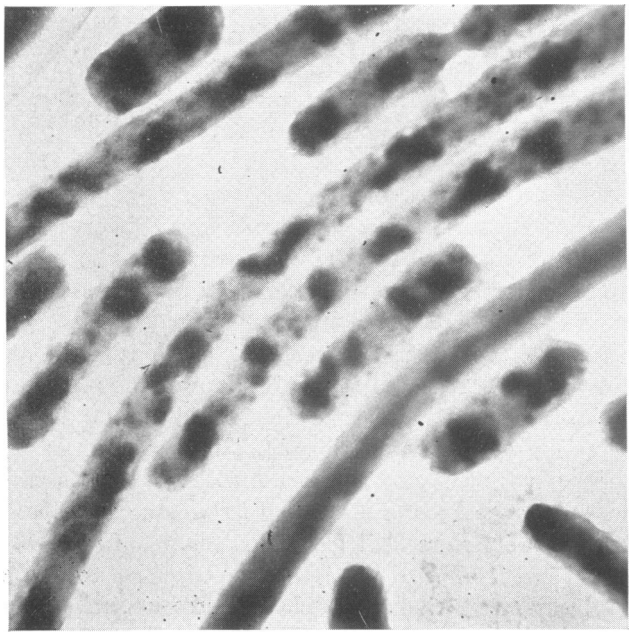

Figure 8. Cells from same hydrochloric-acid-treated specimen as figure 7.

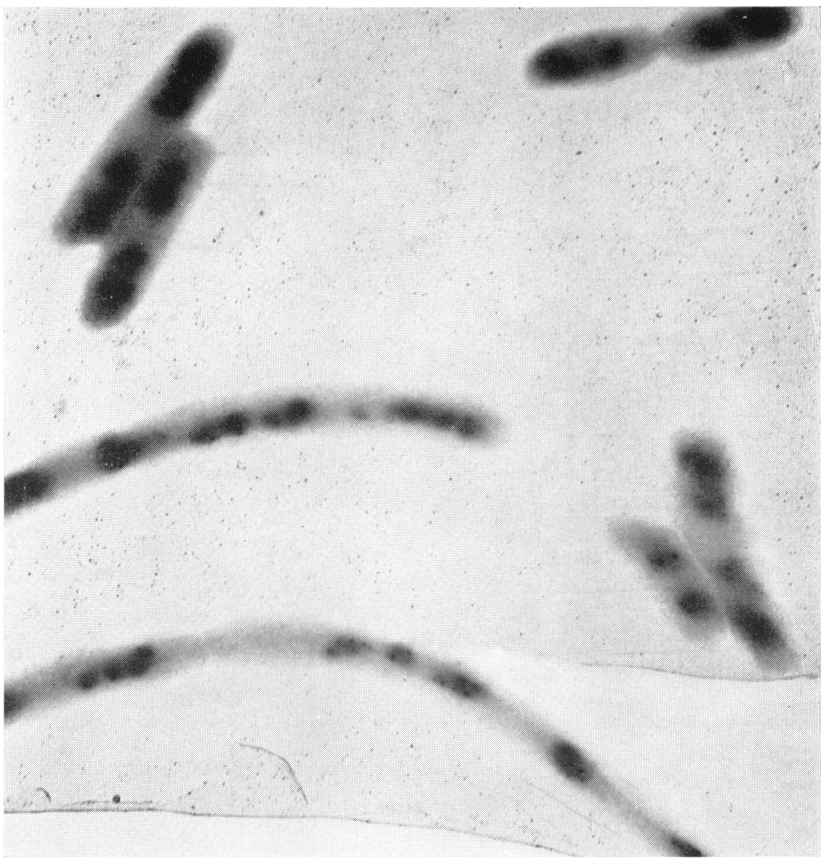

Figure 9. Cells from a microcolony of an *E. coli* B, hydrochloric-acid-treated specimen, treated similarly to those in figures 7 and 8.

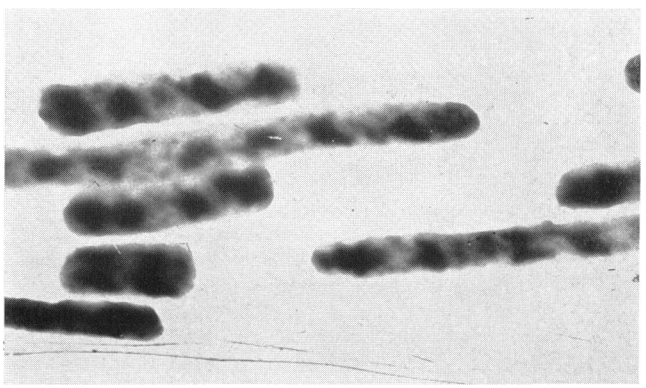

Figure 10. Cells from a microcolony of *E. coli* B. After OsO_4 fixation and N HCl treatment as described under figure 7, they were mordanted in 1 per cent formaldehyde for 3 minutes and then washed in distilled water.

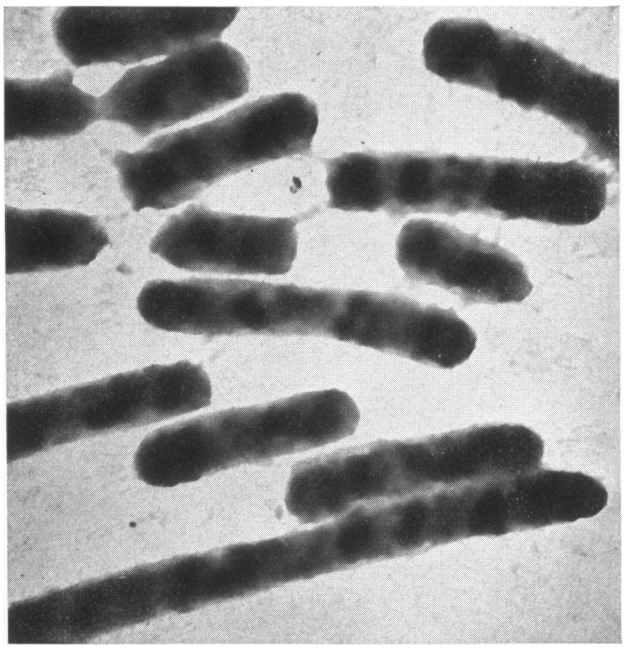

Figure 11. Cells from a microcolony of an *E. coli* B, completed specimen. They were grown for 2½ hours at 37 C in the manner described under figure 3. After OsO_4 fixation and N HCl treatment as described under figure 7, they were mordanted in 1 per cent formaldehyde for 3 minutes, washed in distilled water, then stained in 0.3 per cent aqueous basic fuchsin for 30 seconds, and, finally, washed in distilled water.

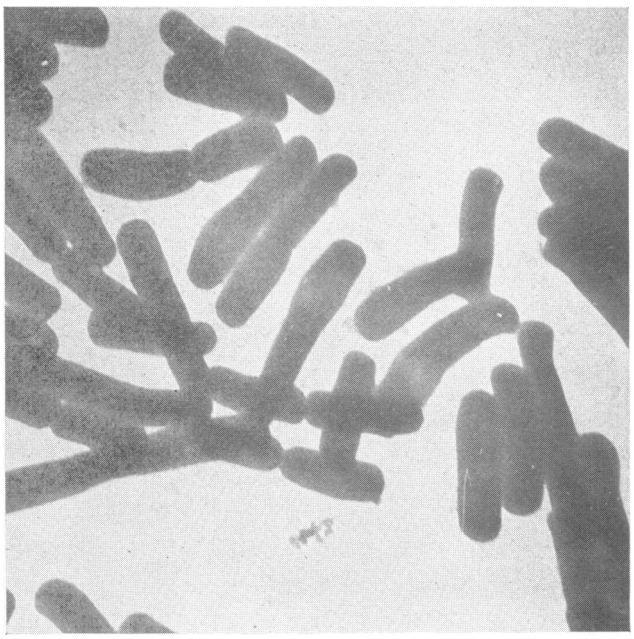

Figure 12. Cells from a microcolony of *E. coli* B. They were grown for 2½ hours at 37 C in the manner described under figure 3. After OsO_4 fixation, they were treated in Schaudinn's fixative (by covering the implantation with fixative before transfer to copper screen; otherwise one gets an amalgam effect) and washed in distilled water.

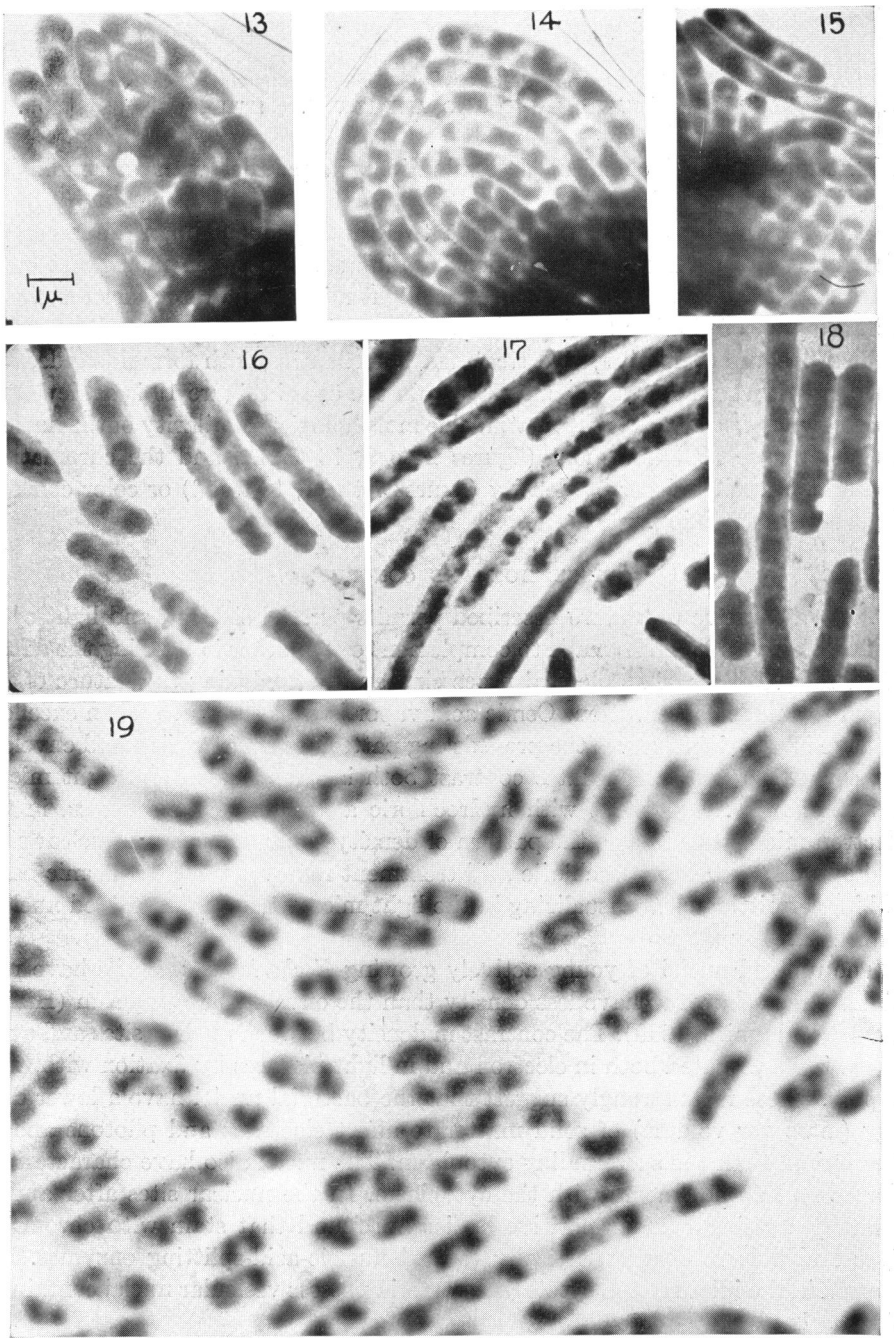

Figure 13. Contact print of the plate shown in figure 4.

Figure 14. Contact print of the plate shown in figure 5.

Figure 15. A microcolony of *E. coli* B, osmium-fixed specimen, grown for 2½ hours at 37 C on collodion membrane overlying agar.

Figure 16. Contact print of the plate shown in figure 7.

Figure 17. Contact print of the plate shown in figure 8.

Figure 18. A microcolony of *E. coli* B, hydrochloric-acid-treated as in figure 7.

Figure 19. Microphotograph of *E. coli* cells grown for 1¼ hours at 37 C on M and E agar. Fixed 1 minute in OsO_4 vapor; treated with N HCl at 60 C for 6 minutes; mordanted with 1 per cent formaldehyde for 3 minutes; stained with 0.3 per cent basic fuchsin for 3 minutes. Photograph on "panatomic X" film, 5 seconds' exposure, with Wratten G and B filters.

(Magnifications in figures 13 to 19 are approximately 6,000 ×.)

acid. More detailed chemical determinations in parallel with morphological studies would be desirable.

Contrast within the cells of figures 1 to 18 is dependent upon differences in electron scattering resulting from density differences between nuclei and cytoplasm. Figure 19 is an enlargement of the light micrograph reproduced as figure 1 in the paper following (Smith, 1950). These cells have been through the complete procedure of fixation, HCl treatment, mordanting with formaldehyde, and staining with basic fuchsin. Contrast within the cells in figure 19 is achieved by the staining of the nuclear chromatin. The morphological similarity of the nuclear vesicles before HCl treatment (figures 2 to 6, 13 to 15) and the chromatinic bodies as revealed either by density (figures 7 to 11, 16 to 18) or color contrast (figure 19) is striking.

DISCUSSION AND CONCLUSIONS

The preparative procedure described permits parallel electron and light cytological observations to be made to complement one another in learning the effects of successive steps of cytological processing and in analyzing the nature of the structures under observation. Osmic acid vapor is thus shown to be an excellent fixative in that it preserves the pre-existing pattern of nuclear sites and enveloping cytoplasm, and accentuates contrast both in the electron and light microscopic pictures. Treatment with hydrochloric acid completely reverses, in the electron microscope image, the pattern of density contrast between nuclear sites and cytoplasm. The hydrochloric acid treatment removes the cytoplasmic basophilia, permitting clear visualizing in the light microscope of the stained nuclear chromatin.

The unfixed nuclei of young actively growing *Escherichia coli* cells have previously been shown to have less density than the enveloping cytoplasm (Hillier, Mudd, and Smith, 1949). The contrast in density between nuclear sites and cytoplasm is accentuated both in electron and in light pictures by fixation with OsO_4 vapors. These facts strongly suggest that the bacterial nuclei have a lower density (mass per volume) of solid matter to affect electrons and photons and to fix osmium than the surrounding cytoplasm. The nuclei also have characteristic shapes and do not merge with the cytoplasm. In the nuclear sites after appropriate processing Feulgen-positive bodies are found that stain with chromatin dyes and behave characteristically toward nucleic-acid-splitting enzymes. Expressed in the idiom of the cytologist, bacteria possess vesicular nuclei containing chromatin.

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