ELECTRON MICROSCOPE STUDIES OF CELLS BY THE METHOD OF REPLICAS*

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PLATES 16 AND 17

(Received for publication, December 15, 1948)

The technique of electron microscopy has failed thus far to find general application in the study of cells and tissues, chiefly because of the difficulties involved in the preparation of specimens of required thinness. Formed elements can be isolated from the cells and examined separately (2, 3) but in this case the connections with the other cell structures are lost and a study of the general cell organization is not possible. The use of tissue culture has offered an opportunity to examine certain portions of the cells, especially thinly extended cells margins (4), but in this case also a number of limitations must be recognized: the center of the cell, as a rule, remains too thick for study; cells in tissue culture, especially actively growing ones, have a tendency to dedifferentiate; and finally, the cellular arrangement existing in the whole organ is not retained by the outgrowing cells. It would seem, therefore, that the method of sectioning, so widely successful as an adjunct to light microscopy, would be the technique of choice because of its applicability to all cells and tissues, irrespective of their individual constitution or origin. The early attempts to adapt the method of sectioning to electron microscopy have not been completely satisfactory (5–7), and experience of recent years indicates that the problem does not reside solely in the difficulties in producing sections of sufficient thinness, but also in the fact that the current methods of fixation and embedding fail to preserve the fine structure of the cells (8–10).

As is known, the absorption and scattering of electrons are not affected by molecular differences, as is the case with light of various wave lengths, but are determined by variations in atomic densities. In biological material, therefore, where the major elements, *i.e.* carbon, nitrogen, and oxygen have nearly the same atomic weights, the absorption and scattering observed in the electron microscope is a measure, but in inverse proportion, of the relative amount of water originally present in different parts of the fresh specimen. The experiments reported in the present paper take advantage of this fact, and are based on the assumption that differences in the distribution and concentration of substance, as occurring in cells and tissues find expression, after

* The results recorded in this paper were presented at the annual meeting of the Electron Microscope Society of America, December 11 to 13, 1947, in Philadelphia (1).

drying, in variations in height and shape at the surface of the specimens. The results here reported indicate that surface replicas of dried cells and tissues can depict with surprising accuracy morphological details of their internal constitution.

Material and Methods

In the present work the material studied has been single cells, as in blood smears or tissue imprints, and bacterial cells. The preparations were fixed, usually in osmium tetroxide vapors, and allowed to dry in air, or over P_2O_5.

The technique employed consisted in preparing replicas of the cellular material and recording in the electron microscope the negative image so obtained, a method heretofore used in the study of crystalline structures or metal surfaces (11, 12).

Replicas were obtained by immersing glass slides supporting the dried specimen in a 0.5 per cent solution of formvar E[1] in ethylene dichloride and allowing the preparation to dry while in the horizontal position. The resulting plastic film presented a level surface on its upper side, while retaining the imprint on its under surface of the irregularities reflected at the surface of the specimen. The difficulties encountered in detaching the mold, prior to its transfer to the supporting wire mesh of the electron microscope, varied greatly with the nature, and especially the thickness, of the specimen. The plastic film must be thin to insure contrast in the image made of the replica and its fragility precludes the use of much mechanical force when freeing it from the glass support; hence, conditions must be such that it can be detached readily. This is usually accomplished when the cellular elements projecting into the film, and therefore weakening it in places, are not crowded, but have between them a sustaining network of relatively thick film. Favorable conditions of this sort are provided by using thinly spread blood or bacterial smears, where some free space is left between the cells.

The preparation of the mount, *i.e.* the lifting of the replica from the cells, and its transfer to the wire mesh screen of the electron microscope was carried out under the dissecting microscope. Formvar-coated smears of blood or bacteria were immersed in distilled water and an area, about the size and the shape of the supporting screen, was selected and outlined. This film disc was detached by means of sharpened watchmaker's forceps, and moved over a screen placed alongside beforehand. The screen and the film over it were then lifted from the water and the preparation was drained on blotting paper and allowed to dry. When resorting to shadowing of the replicas, the film was detached, turned over, and spread on a clean glass slide, in the inverted position. The exposed side of the replica was then shadowed in the usual manner.

The observations were made by means of a RCA, type E.M.U., electron microscope.

EXPERIMENTAL

Replicas of Blood Cells.—Fig. 1 shows a micrograph of a replica of a smear of chicken blood. The replica was mounted on the screen of the electron microscope, and the photograph was made with an ordinary microscope, using visible light. The smears had been prepared in the usual manner by spreading thinly on a glass slide a drop of blood obtained from the comb of an apparently normal pullet, fixing it rapidly over osmium tetroxide vapors, and allowing it to dry in air. As shown in Fig. 1, the replica of the smear produces an image

[1] Formvar E (grade No. 15–95), obtained from the Shawinigan Products Corporation, New York.

of the blood cells, so faithful that it is difficult to distinguish, under the light microscope, between the original unstained cells and their plastic mold. As in the direct light microscope examination of the smear itself, the nuclei in the replicas of the erythrocytes are evident, and the leucocyte in the field appears to contain granules. Fig. 2 represents the replica of a chicken erythrocyte, photographed with the electron microscope under a magnification of 2200, and enlarged to 5300. Nearby is what appears to be the "ghost" of a red cell, with remnants of its nucleus. The fact that the body of the apparently intact erythrocyte appears granular, whereas the ghost cell is smooth and homogeneous, is evidence for difference in their surface conditions, or the properties of their membranes. The background of the preparation in Fig. 2 presents a fine granular structure, presumably produced by elements of the plasma.

Fig. 3 shows an electron micrograph of the replica of a mouse leucocyte, apparently a monocyte or a large lymphocyte. About it, can be seen the curved margins of a number of red cells. The replica reveals internal details, notably the shape of the nucleus, and cytoplasmic bodies with the appearance of rod-shaped mitochondria.

Fig. 4 is a micrograph of a chicken platelet, showing the vague outline of what is probably a nucleus, three vacuoles, and small bodies of various shapes which may be mitochondria. Fig. 5 is the replica of a leucocyte of chicken blood. The numerous dark bodies shown in the electron micrograph represent depressions in the surface of the dried cell and probably correspond to what were vacuoles. Differences in the density to the electron beam in these areas may reflect differences in the amount of water which existed originally in the various vacuoles.

Replicas of Bacteria.—Microorganisms are generally surrounded by a voluminous capsule or are encased in a relatively rigid covering. *A priori*, it would seem likely that structural details of their cell content would not be accessible for study by the method of replicas. In fact, it has been possible to obtain replicas, of *E. coli* for the most part, which picture a number of morphological features reflecting, presumably, details of cellular organization of the bacteria.

Figs. 6 to 8 are micrographs of such replicas. Fig. 6 shows a cell with two large bodies, one near the center, and the other near one end. Fig. 7 shows two bacterial cells, one of them appearing but faintly, obviously because it lay partly beneath the other and made but a slight impression on the formvar film. The upper cell shows two terminal bodies, like those of growing organisms, and a central body of smaller size. Fig. 8 represents a portion of a filamentous form of *E. coli*, such as develops in aging cultures. The white bodies which appear in the organism seem to be arranged along a spiral path extending from one to the other end of the filament. The same bodies have been noted in replicas of similar elongated organisms. They appear to be sufficiently

rigid to cause the cell wall to rupture over them, probably during desiccation; in the picture this is noticeable at both ends of the filament. The nature of inclusions of identical morphology detected with the ordinary microscope in specimens of various forms of *E. coli* is not known; the fact that they stain with methyl green might be taken to indicate that they contain in appreciable amounts substances related to chromatin and that they represent bacterial nuclei.

DISCUSSION

Replicas of the surface of certain cells have occasionally been obtained and photographed in the electron microscope (13, 14) but it seems that full advantage has not been taken of this interesting technique, and the fact that it can furnish information concerning the internal structure of cells has not been realized. These have been the objects of the work reported in the present paper. Replicas of blood cells and bacteria have been obtained which not only give the shape of the cells but show nuclear membranes and what appears to be chromatin structures, mitochondria, and vacuoles. An important feature of the method is that the thickness of the specimen, often a limiting factor in the electron microscopy of cells, may no longer be significant if replicas of the proper thinness can be prepared. The method would appear useful in the study of erythrocytes and bacteria, and in the case of the nuclear region, which remains too thick for direct electron microscopy even in thinly extended cells in tissue cultures. That the method of replicas, as applied to the study of cells, can be technically improved is probable, so that higher resolution may be obtained and even finer details of internal structure may be revealed. The hope seems warranted that the method may assist in the study of problems to which direct electron microscopy cannot yet be applied, as in the case of the intracellular growth of malarial parasites, the penetration and growth of bacterial viruses, and the morphology of chromosomes.

SUMMARY

The method of replicas has been applied to the study with the electron microscope of blood cells and bacteria.

The results indicate that the method can reveal details of intracellular structures. Nuclei can be perceived, and also cytoplasmic bodies such as mitochondria and vacuoles.

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EXPLANATION OF PLATES

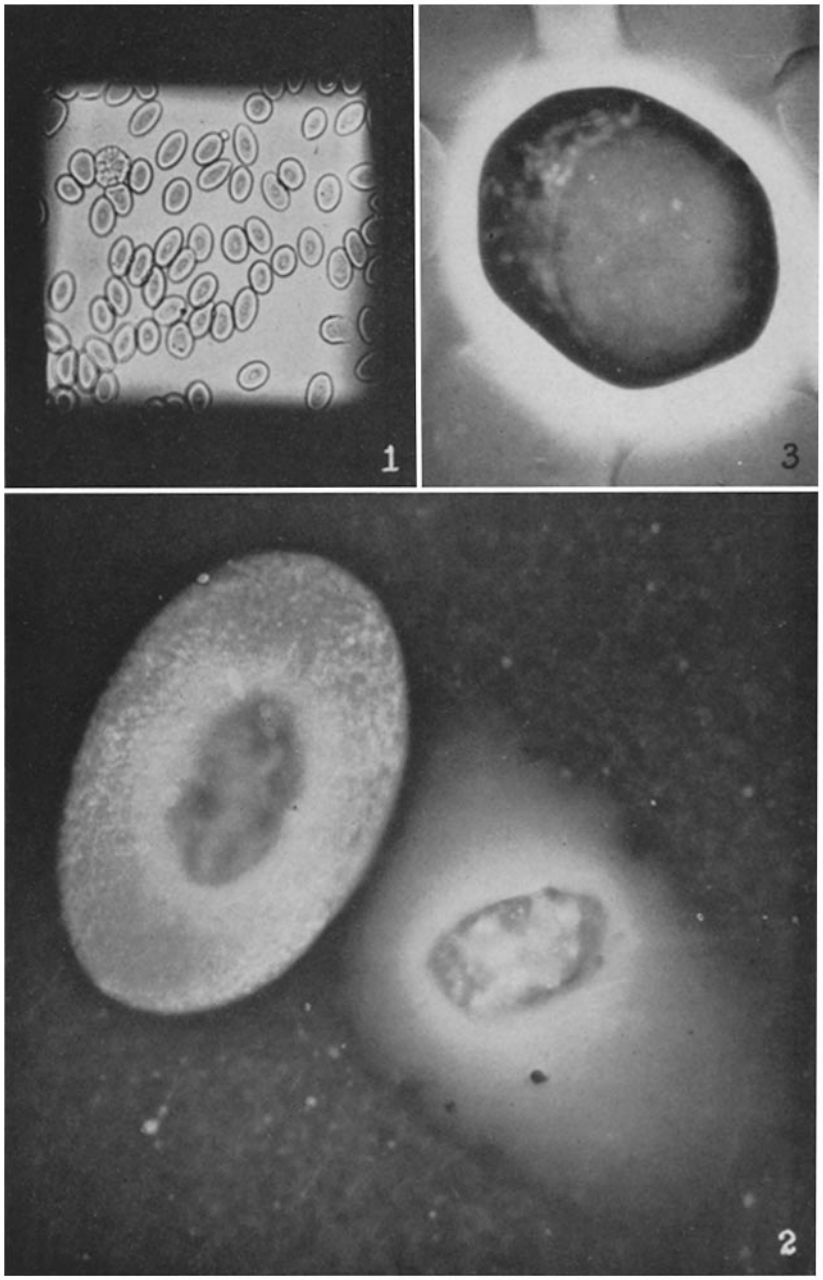
All preparations illustrated in Figs. 1 to 8 were fixed by exposure to osmium tetroxide vapors for 5 to 15 minutes, and subsequently were allowed to dry in air. Since the pictures were made from replicas, all illustrations are negative images of the original cells.

PLATE 16

FIG. 1. Formvar replica of a smear of chicken blood. The imprints left on the plastic film reproduce to a striking extent the appearance of the original blood cells as viewed without staining. The picture shows many erythrocytes, and one leucocyte. Photograph made with an ordinary microscope and visible light at a magnification of 250, enlarged to 450.

FIG. 2. Formvar replica of chicken blood cells. On the left, is an apparently intact erythrocyte; on the right, what would appear to be the ghost of a red cell. Electron micrograph taken at a magnification of 2200, and enlarged to 5300.

FIG. 3. Replica of mouse leucocyte, probably a monocyte, or a large lymphocyte. The elongated bodies in the cytoplasm correspond to mitochondria. Electron micrograph taken at a magnification of 2600, and enlarged to 4000.



(Claude: Electron microscope studies of cells)

PLATE 17

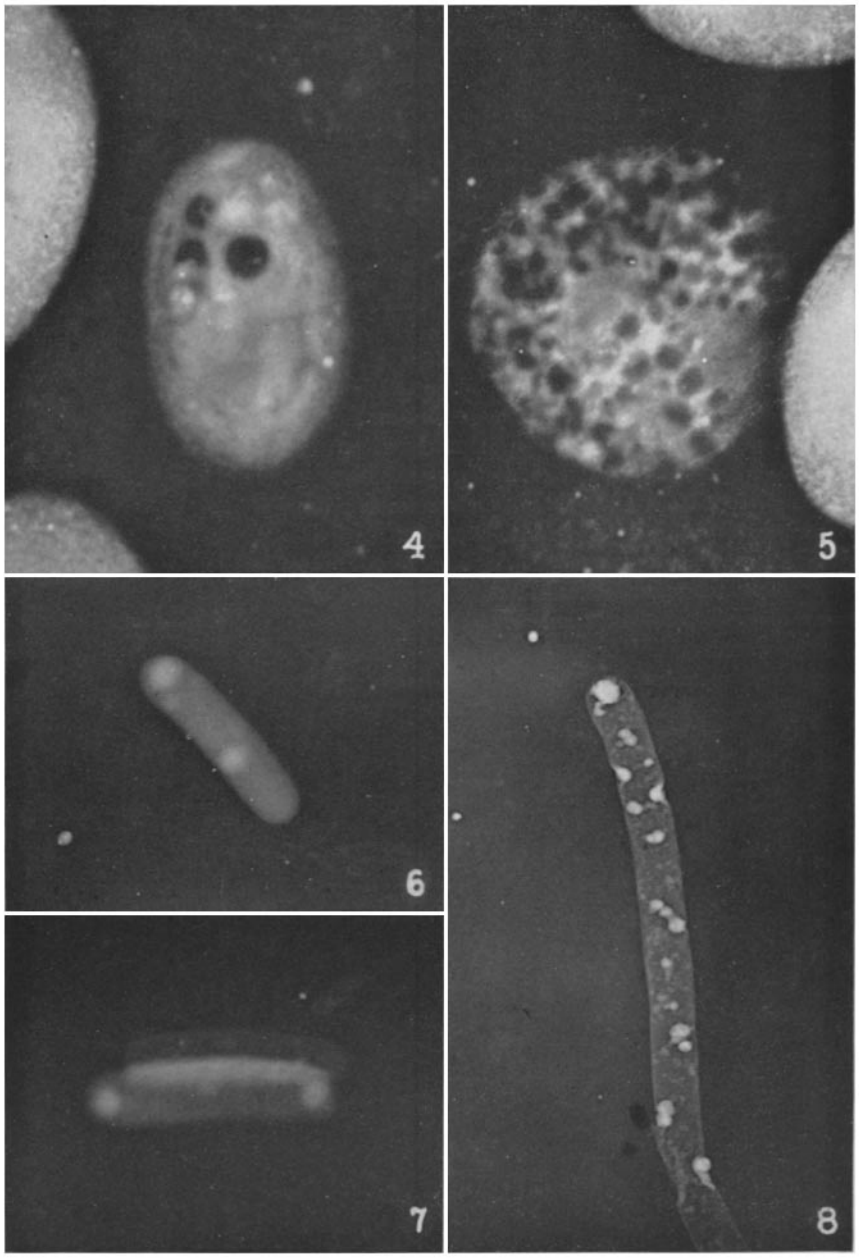
FIG. 4. Replica of a chicken platelet. A nucleus is faintly outlined. The dark cytoplasmic bodies presumably correspond to vacuoles, the light bodies probably to mitochondria, judging from the bulges and depressions, respectively, that they left on the replica. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.

FIG. 5. Replica of a chicken leucocyte. The areas free of granules correspond to two lobes of the polymorphic nucleus. Electron micrograph taken at a magnification of 2200, and enlarged to 4400.

FIG. 6. Replica of an organism from an *E. coli* culture, with two relatively large internal bodies, one near the middle of the cell, the other near one end. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 7. Replica of organisms from an *E. coli* culture, showing one cell lying partly over another. The upper cell presumably made the more effective replica. It has one rounded body at each end, and a central one of smaller size. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 8. Replica of a filamentous organism, of a type frequently found in "old" cultures of *E. coli*. The picture shows discrete bodies, apparently arranged along a spiral path. In ordinary microscopic preparations, methyl green stains bodies similar to those shown, and also to the larger ones of Figs. 6 and 7. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.



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