# NEW PREPARATION TECHNIQUES FOR THE ELECTRON MICROSCOPY OF BACTERIA

JAMES HILLIER, GEORGES KNAYSI, AND RICHARD F. BAKER[1]

*RCA Laboratories, Princeton, New Jersey, and Cornell University, Ithaca, New York*

Received for publication July 29, 1948

In the early application of the electron microscope to the examination of bacteria, the method of preparation involved the preparation of a distilled water suspension of the organisms and the evaporation of a drop of the suspension on a suitable supporting membrane. Although it was recognized that such treatment probably gave rise to considerable artifacts, this procedure nevertheless was used in most of the published work (Mudd and Anderson, 1944). Recently some of the artifacts introduced by this method of preparation have been investigated and a new method has been suggested (Hillier and Baker, 1946; Edwards and Wyckoff, 1947). This method involves the intact removal of the surface layer of organisms from a culture of a solid medium by means of a thin plastic membrane formed in place on the culture by the evaporation of a suitable solution. Further work on this technique has shown that although it is ideal for the examination of very young cultures of certain strains of organisms, it has numerous serious limitations that will prevent it from being generally useful. For instance, even for those types of organisms for which it can be used, it is not successful if the growing conditions are not "quite right." More serious, however, is that the physical organization of the culture is maintained only in the outermost surface of the growth. Thus, unless the preparations use single-layer growths of strong cells, the resulting specimen is confused by material pulled from inner layers in disorganized masses. This last difficulty was particularly troublesome in recent attempts by one of us to follow, by this technique, the infection of *Escherichia coli* by $T_2$ bacteriophage.

As a result of these difficulties a new technique has been developed that appears to have many advantages. Basically, it involves growing the organisms directly on the thin supporting membrane of the electron microscope specimen mount in such a way that any direct contact with the organism is avoided throughout the procedure. The only disturbance of the organisms that can introduce artifacts is a final desiccation. For organisms grown on ordinary media, such artifacts have not been observed. At the time of writing, this technique has proved uniformly successful in the preparation of specimens of *Bacillus mycoides*, *Bacillus megatherium*, and *Escherichia coli* grown under a number of different conditions and subsequently treated in several different ways. It would appear in principle at least that the technique should be successful for all organisms that produce surface colonies.

[1] Now at the School of Medicine, The University of Southern California, Los Angeles, California.

### TECHNIQUE FOR SOLID MEDIA

Figure 1 consists of a series of drawings showing diagrammatically the various steps in the new technique when it is used for a solid medium. Thinly poured agar plates were used in this work. In the first operation (a) the agar plate is flooded with sterile distilled water from a pipette with a fast delivery rate or, preferably, from a flask or a bottle. As soon as the entire surface of the plate is covered, a drop of collodion solution (0.5 to 1.0 per cent in amyl acetate) is dropped on the surface and allowed to spread (b). It is necessary to flood the surface and form the collodion membrane as rapidly as possible in order to minimize the time allowed for the agar material to go into solution. Too much agar material in the water will prevent the collodion solution from spreading sufficiently and will result in a thick membrane.[2] After the film is spread, sufficient ventilation of the surface is allowed to permit the solvent to evaporate. No traces of the solvent must be left as they tend to inhibit growth. When the solvent is completely gone, the water is pipetted from under the membrane, allowing it to settle in close contact with the agar (c). In the last stages of this operation, the plate is tilted in order to allow the water to drain completely from

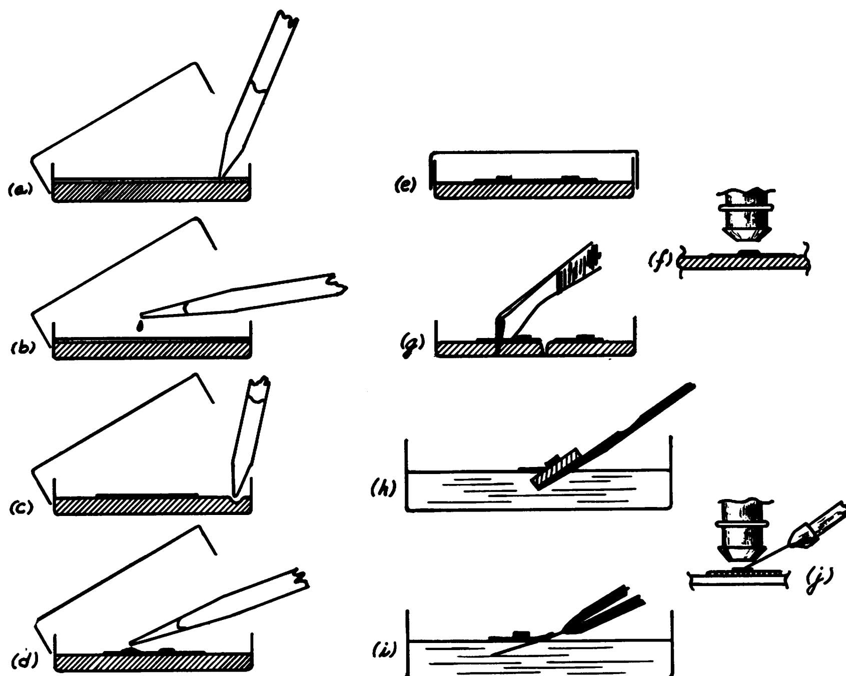


*Figure 1.* Explanation in text.

[2] More recently, in using this technique, Mr. Andrew Smith of the University of Pennsylvania has found that the collodion solution spreads much more satisfactorily if the surface of the agar is washed twice with sterile distilled water before being flooded with the water on with the collodion is spread.

between the membrane and the agar. At this point, the membrane should be checked for thickness by observing the reflection from it of light from a bright extended source. A sufficiently thin membrane should be completely invisible except for a thickening at its edges. A membrane that shows a definite sheen will be too thick.

Sufficiently thin portions of the membrane are inoculated with clean distilled water or saline suspensions of the selected organism, a small drop being placed on the membrane from a capillary pipette and immediately withdrawn (d). If the membrane is wet by the suspension, this procedure will leave a small disk of liquid—a portion of a millimeter thick and 1 to 2 millimeters in diameter. A direct correlation has been observed between wetting of the membrane and ability of the organisms to grow. In later work, it was found that suspensions that do not wet the collodion membrane may be made to do so by reducing their surface tension with as little as 0.05 per cent of tryptone. The tryptone added does not seem to produce an undesirable effect on the cleanliness of the background and is probably removed by dialysis in a later step when the film is floated. The preparation is then incubated (e); the cover may be left partly open so that the remaining moisture of the inoculum can dry rapidly if it is deemed desirable to prevent the growing cells from moving.

The condition of the inoculum and the growth of the organisms can be followed by examination with a light microscope, using a high-powered dry objective (f). It is desirable that the objective be warmed by keeping it in the incubator if condensation on the first surface is to be avoided.

Since by this technique all new cells and all other products of the growth are retained on the specimen, the period of incubation for normal cells is very short—usually between 1 and 3 hours. In fact, once established, the growth rapidly becomes too thick for electron microscope examination.

When the growth has reached a desired stage, as determined by light microscope observation, a selected area of the agar and membrane is cut out (g). In this operation it is desirable to cut the membrane by a sharp scalpel pushing perpendicularly into the agar some distance (8 to 10 mm) from the area selected for examination. The slab of agar bearing the membrane on its upper side is then lifted from the petri dish and slid under a clean surface of distilled water (h). The section of membrane floats easily on the surface of the water, bearing the growth on its upper side. The membrane is then picked up on a sufficiently large, carefully cleaned piece of 200-mesh screen, which is held in fine forceps and raised through the water surface under the floating membrane (i). The screen is held at a slight angle to the surface so that the membrane is kept smooth and taut by that part of it that remains floating. As soon as the membrane is lifted on the screen, the water remaining in the openings is absorbed by a hard filter paper so that the preparation dries very rapidly.

Since the growth is on the side of the membrane not in contact with the screen, it can be shadow-cast or treated in other ways with little difficulty.

In this form the specimen can be examined conveniently under a light microscope and suitable individual cells or colonies selected for examination in the

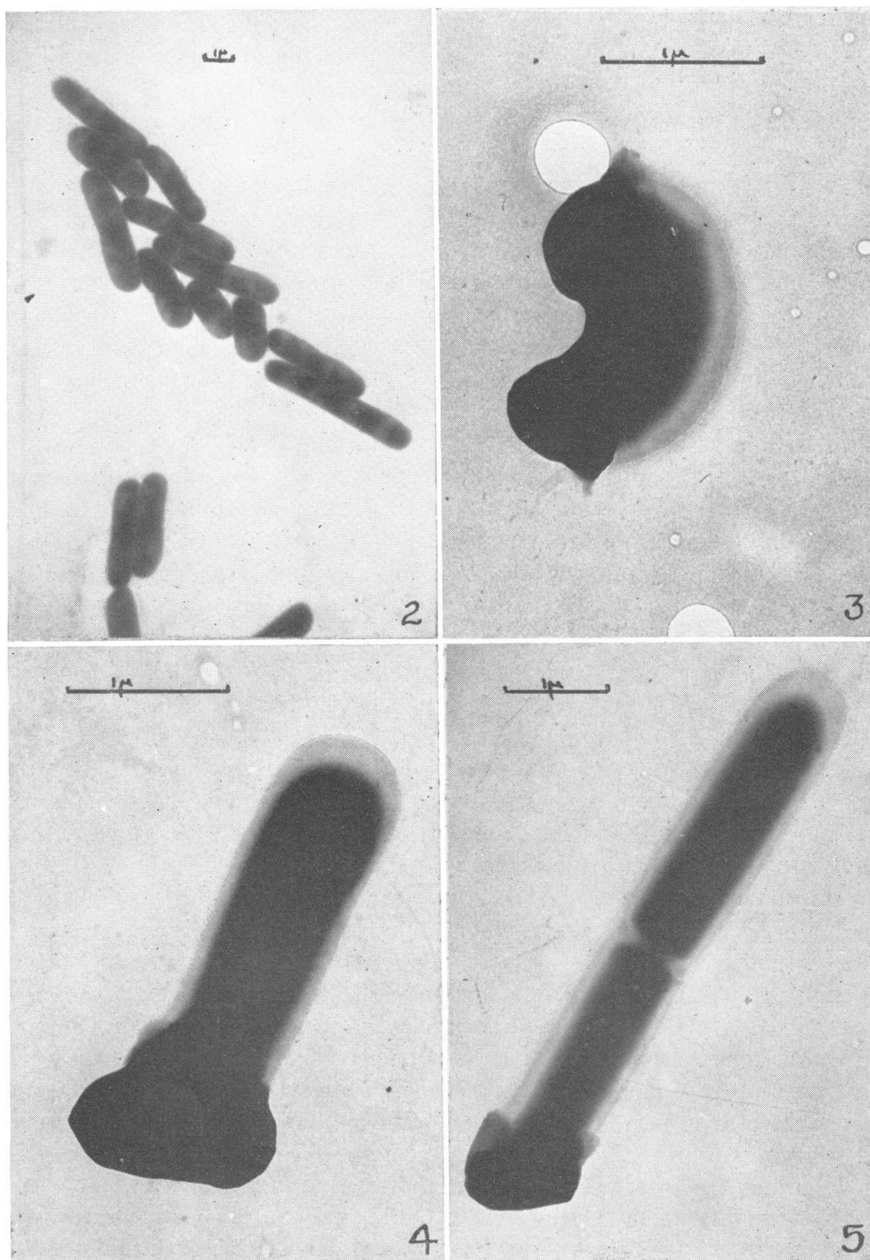


*Figure 2.* Small colony of *Escherichia coli* grown from single cell on thin collodion backed by nutrient agar (3 hours at 37 C). Magnification 3,500 ×.

*Figures 3* and *4.* Germination of spores of *Bacillus mycoides* on thin collodion backed by nutrient agar (2½ hours at 37 C). Magnification 21,000 ×.

*Figure 5.* Another preparation of *B. mycoides* after 3½ hours at 37 C. Magnification 13,000 ×.

electron microscope. Their general location can be marked by a sharp needle (j) and an area cut or punched out to fit the electron microscope specimen holder.

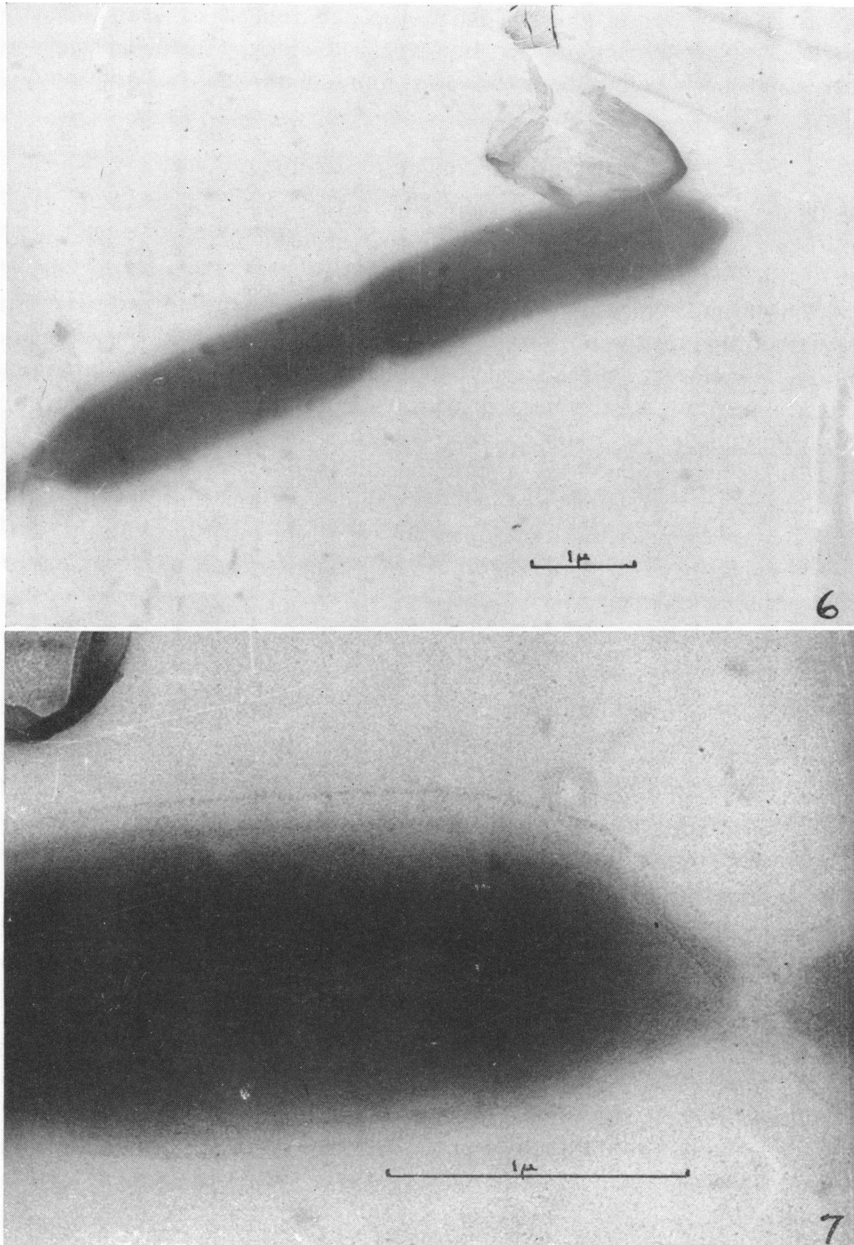


*Figure 6. Bacillus megatherium* grown on thin colloidion floating on broth (5 hours at 36 C). Magnification 13,000 ×.
*Figure 7.* Enlargement of another field of same specimen showing the preservation of delicate structures at the point of division. Magnification 40,000 ×.

Figures 2 to 5 are electron micrographs of a small single colony of *E. coli* and single cells of *B. mycoides* grown by this technique. In all such preparations the specimens have been singularly free of extraneous material and desiccation arti-

facts. Even the usual shrinkage of the protoplasm from the cell wall seems to be minimized. Of greater importance, however, is the lack of disturbance of sensitive structures such as flagella or the forms found at the point of division of the cells (figure 7).

## TECHNIQUE FOR LIQUID MEDIA

For liquid nutrient media, the technique (with some obvious modifications) can also be used for aerobic bacteria. In this case the membrane is formed on sterile distilled water in a petri dish. After evaporation of the solvent, the water is pipetted off and replaced by the liquid medium. In this process, the membrane is deposited on the bottom of the dish and then refloated when the liquid medium is introduced. Since sterile handling is essential and since large quantities of the medium are not needed for the small numbers of organisms grown by this technique, it is desirable to use petri dishes filled to a depth of 4 to 5 millimeters. The inoculation of the membrane, the incubation, and final mounting are similar to the preceding method with the exception that the medium is replaced by two changes of water before picking up the membrane on the screen. Figures 6 and 7 are electron micrographs of germinating cells of *B. megatherium* grown by this technique.

## DISCUSSION AND CONCLUSIONS

The technique described in the foregoing has achieved for three organisms some definite advantages over the earlier techniques used for the preparation of bacteria for the electron microscope. In this method the organisms are given a minimum of undesirable treatment. Although they are grown with a thin (100 to 200 A) membrane separating them from the nutrient medium, no harmful effects can be detected either from light microscope observation of the growing colonies or from the electron microscope results. During the remainder of the procedure, the organisms are given no unusual physical or chemical treatment with the possible exception of the short interval when the membrane is in contact with water. Even in this case the organisms are separated from the water by the membrane, and hence any interaction will be the result of diffusion—a relatively slow process. In no part of the procedure are the new cells subjected to the effects of mixing in water. When the organisms are grown under the near-dry conditions described, the surface film of liquid that surrounds them is everywhere nearly parallel to the contour of the growth so that surface tension effects are minimized. However, if more moisture is present, a droplet may form on the inoculated area. If this is the case—it can be identified by light microscope examination—there may be a rearrangement caused by surface tension during drying. This last case is to be avoided, of course, if the exact physical relationships in the growth are to be preserved.

Although the technique has been tried for only three species of organisms, those attempts have been uniformly successful. There seems to be no fundamental reason why it should not be equally successful for a large number of other species.

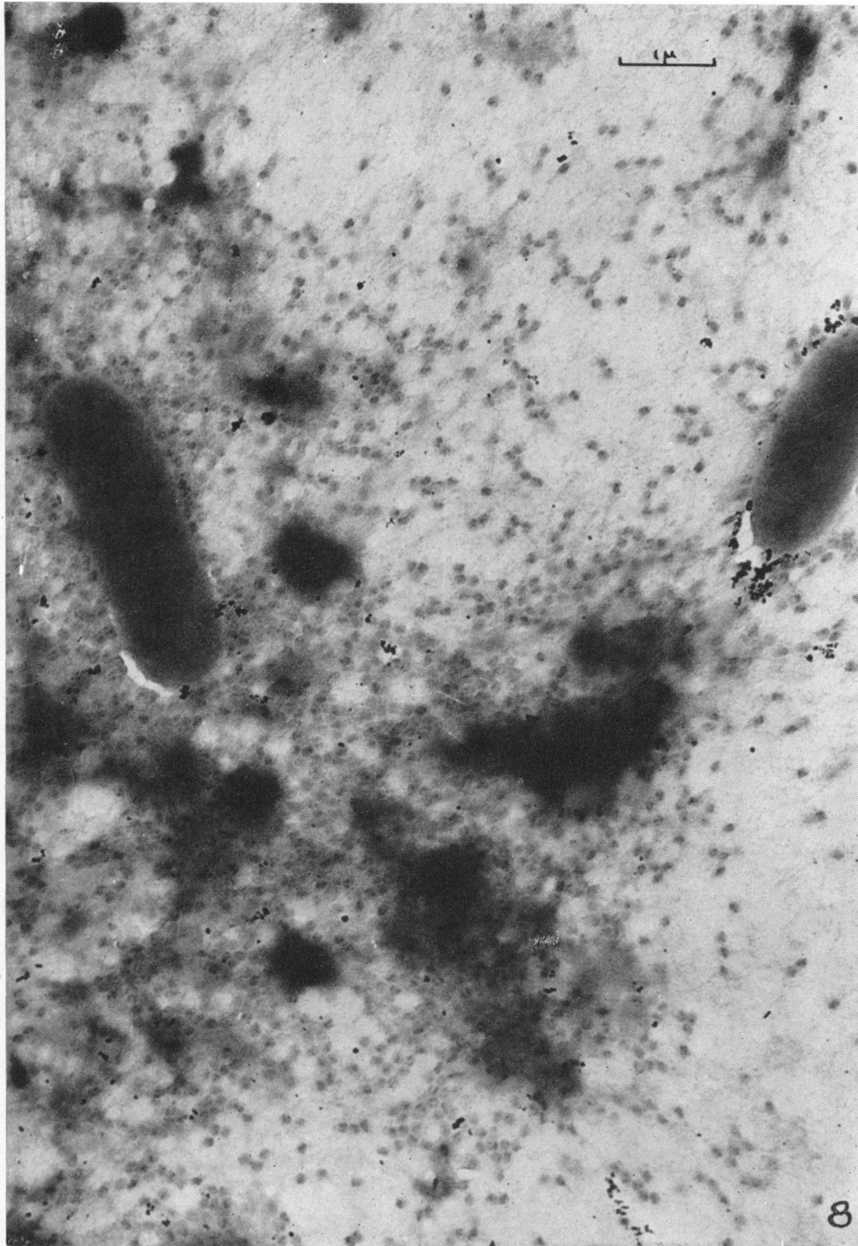


*Figure 8.* *E. coli* strain B grown on thin collodion backed by nutrient agar, inoculated after $1\frac{3}{4}$ hours at 37 C with $T_2$ bacteriophage, and then incubated 20 hours at 37 C. Large numbers of bacteriophage particles, various types of cell debris, and a few unlysed cells are all retained near their original positions. Magnification 12,000 ×.

Since in this technique the physical relationships between the organisms are preserved, it is particularly useful for studying the morphological changes that occur as the organisms multiply. The method should also be useful for studying

the effects on bacteria of various chemical agents. The procedure in this case would be to float the film on a solution of the agent before taking it up on the supporting screen. Only the reagent absorbed by the membrane and the organisms would remain in the specimen.

An application of the technique that is being found particularly useful in this laboratory is the study of the action of bacteriophage. The host organisms are grown to any desired stage, inoculated with a bacteriophage suspension with only slight, if any, disturbance, and then examined at any subsequent time. Since all the organic products of lysis are retained, such micrographs are very informative (figure 8).

ACKNOWLEDGMENT

Figures 2 and 8 were obtained during a subsequent research by one of us in collaboration with Drs. Stuart Mudd and A. G. Smith of the University of Pennsylvania, and it is with their kind permission that they are reproduced here.

SUMMARY

*Escherichia coli*, *Bacillus mycoides*, and *Bacillus megathaerium* have been grown successfully on the exposed surface of a thin collodion membrane deposited on a nutrient agar surface. *B. megatherium* has been grown on the exposed surface of a membrane floating on a synthetic liquid medium. Such preparations can be transferred to the electron microscope specimen screen without disturbing the cells in any way. Electron micrographs of specimens prepared in this way show little, if any, evidence of artifacts.

## REFERENCES

EDWARDS, O. F., AND WYCKOFF, R. W. G. 1947 Electron micrographs of bacterial cultures infected with bacteriophage. Proc. Soc. Exptl. Biol. Med., **64**, 16–19.

HILLIER, J., AND BAKER, R. F. 1946 The mounting of bacteria for electron microscope examination. J. Bact., **52**, 411–416.

MUDD, S., AND ANDERSON, T. F. 1944 Pathogenic bacteria, rickettsias and viruses as shown by the electron microscope. J. Am. Med. Assoc., **126**, 561–571.