L-TYPE VARIATION AND BACTERIAL REPRODUCTION BY LARGE BODIES AS SEEN IN ELECTRON MICROGRAPHIC STUDIES OF BACTEROIDES FUNDULIFORMIS

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The division of one cell into two daughter cells has long been accepted as the mode of reproduction of the bacteria. A second mode of reproduction, however, has been observed in many strains, belonging to several common genera, and consists in the formation of large round bodies from which more than two daughter cells derive (Dienes, 1946, 1947). This process is exhibited very commonly by organisms of the pleuropneumonia group (Smith, Hillier, and Mudd, 1948). It is also often seen in the large anaerobic bacteria of the *Bacteroides funduliformis* group, and the production of multiple daughter cells from *Bacteroides* large bodies has been demonstrated by cinematographic records of the germination of individual living large bodies (Dienes and Smith, 1944). The daughter cells resulting from such germination usually resemble the relatively large bacillary *Bacteroides* cells; but under certain conditions much smaller elements are produced, and these form tiny colonies with the peculiarly distinctive appearance characteristic of colonies of the pleuropneumonia (L) group of organisms. The production by ordinary bacteria of such tiny L-type colonies has been termed L-type variation. The formation of large bodies has been a prominent feature in all strains of bacteria that have exhibited L-type variation, no matter what their genus, and the L-type of growth has derived from the large bodies.

The work reported in the present paper was undertaken with the aim of gathering further information about this process by means of the electron microscope. *Bacteroides funduliformis*, strain 132, was selected as the test organism. It had been isolated from a kidney abscess (Smith and Ropes, 1945). The paper just cited contains a review of the clinical aspects of *Bacteroides* infections.

METHODS

The 132 strain was grown in Brewer's thioglycolate broth. Cells for electron microscopy were obtained by diluting the broth with an equal part of Tyrode's solution and centrifuging the organisms down.

The sediment was resuspended in Tyrode's solution, spun again, and resuspended in the solution a second time. The washing freed the cells of material in the broth that otherwise clouded the electron pictures. This suspension was divided into 4 tubes, which were then centrifuged. The sediment in tube 1 was taken up in a small amount of Tyrode's solution and drops were transferred to parlodion films on screens. After drying, these screens were floated on distilled

water to dissolve salt crystals. The sediment in tube 2 was suspended in 0.1 per cent phosphotungstic acid (PTA) freshly made up from a stock 1 per cent solution; that in tube 3 was taken up in 5 per cent formalin. The sediment in tube 4 was suspended in Tyrode's and poured as a shallow layer into a dish that was put in a closed chamber together with a dish of 2 per cent osmic acid for 30 minutes, after which it was transferred to a tube. The suspensions of organisms treated by these various fixatives were spun down, resuspended in distilled water, spun again, taken up in a small volume of distilled water, and transferred to screens. For cultures on solid media, ascitic peptic digest plates incubated anaerobically were used (Smith *et al.*, 1948). All the micrographs were made at 55 kv.

Findings with the 132 Strain

On solid media this organism grows as a rod-shaped bacterium, rather uniform in shape and approximately the size of *Escherichia coli*. Large round bodies and bacillary cells with swellings toward the center or end do occur, but they are not numerous. The remarkable pleomorphism of the organism is best exhibited in liquid media, and the observations here reported were made with cells taken from Brewer's thioglycolate broth and fixed as noted in the legends accompanying the photographs. The pattern of pleomorphism described occurred equally well in meat tubes or in rabbit serum broth incubated in anaerobic jars. The pleomorphism of this strain was best seen in cultures transferred every 24 or 48 hours. Old cultures transferred at long and irregular intervals or kept in the icebox tended to grow as simple rods, rather uniform in size and shape.

The usual method for preparing bacterial cells for electron microscopy has been to suspend them in distilled water. *Bacteroides* cells when suspended in distilled water and transferred to screens gave preparations that were very cloudy; indeed, few cells could be seen in them. The cause for this was not far to seek, and is shown by the following experiment:

Cells from a 9-hour thioglycolate broth culture (figure 1) were washed three times in the centrifuge by spinning them down and resuspending them in Tyrode's solution. The final suspension was divided into two tubes, each of which was spun again. The sedimented cells from one of these tubes (tube A) were taken up in Tyrode's solution; those from the other (tube B) were taken up in distilled water. Within a few minutes there was a sudden clearing of the liquid in tube B, and its contents became slimy so that a gelatinous film was deposited on the glass when the tube was tipped. No such slime formed in tube A, which retained the swirling cloudiness shown by bacterial suspensions for as long as it was kept (5 hours). Obviously, the cells suspended in distilled water had undergone lysis, whereas those suspended in Tyrode's solution had not, as was confirmed by microscopic examination of drops of fluid from both tubes.

This experiment was repeated with four additional broth cultures between 9 and 30 hours old. In the younger cultures the cells were seen as simple rods or as rods bearing swellings upon them. The older cultures consisted almost entirely of large round bodies. When the organisms from these cultures were

suspended in distilled water, they underwent lysis just as had those in the first experiment. The slime that formed as a result of lysis absorbed so much of the electron beam that it was almost impossible to get clear-cut pictures of the occasional organisms that remained intact. To make more satisfactory preparations for electron microscopy, drops of organisms suspended in Tyrode's solution were transferred to the screens and permitted to dry, and the screens were then dipped or floated in distilled water to dissolve the salt crystals. Fewer organisms underwent lysis in these preparations, but the method was far from satisfactory because of the many salt crystals that remained. A means was therefore needed to make possible the transfer of unlysed organisms to the screens as free as possible of extraneous matter. It was found that when formalin was added to aliquots of Tyrode's suspensions of these organisms to give a final concentration of 5 per cent then the fixed cells could be spun down and resuspended in distilled water without lysing. The same preservation of cells was achieved by fixation with phosphotungstic or with osmic acid, and the technique given in the section on "Methods" was therefore devised.

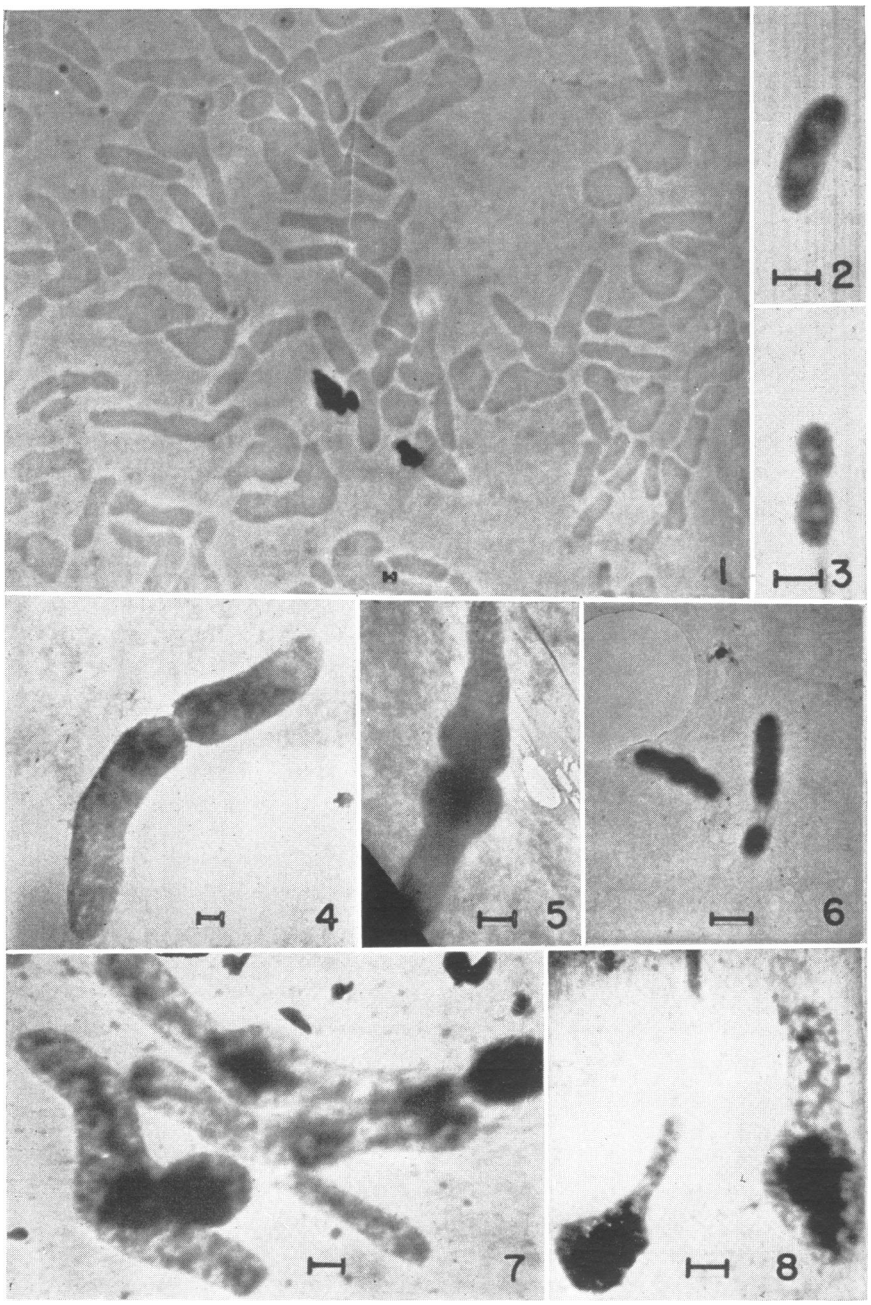
The lysis of these organisms when they were suspended in distilled water suggested the concept, later supported by the micrographs, that their cell walls were more delicate than those of other bacteria; for, with the exception of the organisms of the pleuropneumonia group, other bacteria studied by the electron microscope have been suspended in distilled water without this particular difficulty having been encountered.

The extraordinary pleomorphism of this strain is illustrated in figure 1, which shows organisms from a 9-hour broth culture. It can be seen that even in such a young and actively growing culture a great percentage of the cells already show swellings and a few large round bodies have formed. In preceding detailed studies of this strain, it was found that cells from cultures 3 to 6 hours old were mostly regular, uniform rods. Reproduction during this early phase evidently took place only by simple fission. By means of the electron microscope, it was possible to show such division by simple fission (figures 3 and 4). The point is mentioned because of the more complex mode of reproduction next described.

This more complex process began near the ninth hour and was signalized by the development of a bulge or enlargement in the middle or toward the end of the cell (figures 1 and 5 to 11). It not infrequently happened that this enlargement occurred at the adjacent ends of two cells that had resulted from a recent division by fission but that had not yet completly separated (figures 1 and 5). The bulbous, enlarged areas increased in size until they comprised most of the total cell area, only a small "tail" of the cell retaining the rod-shaped form (figures 10 and 11). Many cells thus became wholly round (figures 12 to 18), though often retaining a small stump of their original rod-shaped structure (figure 13).

By the twenty-fourth hour the cultures consisted chiefly of these "large round bodies." They measured 3 to 10 microns in diameter.

The manner of germination of these large bodies was best observed by transferring them to fresh medium. In previous studies large bodies of this strain were



All figures except 1 and 23 to 26 were made with an electron microscope at 55 kv. The scale on the photographs represents 1 micron.

Figure 1. Cells from a young thioglycolate broth culture. The culture is only 9 hours old, yet the cells already show bulbous enlargements and a few large round bodies have formed. There are several pairs of bacilli bespeaking division by fission. One pair in the middle of the photograph shows enlargement of the adjacent ends. The organisms were placed in a thin film of agar and photographed unfixed and unstained with a light microscope. 1,110 ×.

placed upon a thin layer of anaerobic agar and covered with a sterile cover slip, and photographs were made at hourly intervals with an oil immersion lens (Dienes and Smith, 1944). This procedure provided a cinematographic record of the germination of individual living large bodies. It was thus shown that some large bodies segmented into four or five daughter bacillary cells, whereas others sent out filaments at two to six points on their surface, and these filaments then broke up into bacillary cells. Electron micrographs of such germinating large bodies are given in figures 19 to 22. In figure 22 it can be seen that outpouchings have occurred in six regions of the surface of the body, and it is possible to trace a continuous cell wall enclosing the body and these outpouchings. In other words, the protoplasm of the large round cell has pushed out to form short, twisted filaments extending from the cell but still sheathed with the wall of the cell. The body shown was selected because it shows the successive stages of the production of the new cells. The filament pushing out at the upper left of the body is short and has a rather smooth contour and homogeneous content. The longer filament just below it has a uniform width, as measured by the cell wall, but the protoplasm within the filament has divided into two spherical dense areas. The longest filament, at the bottom of the picture, shows not only the division of the inner protoplasm but also invagination of the cell wall around the spherical protoplasmic masses. From the electron pictures it was thus learned that the new cells produced by the large bodies were derived by simple fission of the multiple filaments that grew out of the bodies.

A striking feature of the cell wall of the large bodies was its veillike delicacy. In most of the photographs the cell wall could not be differentiated. It was visible only where the protoplasm had retracted somewhat from the surface of the cell, as in figure 22. The cell wall of the rod-shaped forms was also very delicate (figure 2) and again could be best identified only where the protoplasm had retracted (figure 6). It appeared as the same type of veillike sheath seen around the large bodies.

Internal Structure of the Cells

The electron micrographs disclosed striking inhomogeneities indicative of segregation within the cells of material with varying ability to scatter the elec-

Figure 2. Rod-shaped cell from 3½-hr culture. The delicacy of the cell wall is apparent. It is seen as a mere veil-like sheath. Osmic acid. 6,100 ×.

Figure 3. Division by simple fission. From the same preparation as figure 2.

Figure 4. Another pair of cells resulting from division by simple fission. Here the process is almost complete. Formalin. 3,300 ×.

Figure 5. A pair of bacilli with enlargement of adjacent ends. Nine-hour culture. Formalin. 4,900 ×.

Figure 6. The cell at left shows beginning of central enlargement in a single cell. In the other cell there has been segregation of the intracellular "dark" material and the veillike cell wall can therefore be seen. Formalin. 5,280 ×.

Figure 7. Three bacilli with enlargements in the middle. A small round cell is superimposed on the bacillus at the left; another lies at the extreme right of the photograph. Toward the lower right corner there is a small, regular rod-shaped cell. It contrasts greatly in size with the large bacilli bearing the swellings. Granules of dark material are seen throughout the cells. They are especially prominent in the central swollen areas. Formalin. 4,900 ×.

Figure 8. Two bacilli with enlargements toward the end. Dark intracellular material is distributed in granules in the enlarged areas of the cells. 15 hours. Formalin. 5,280 ×.

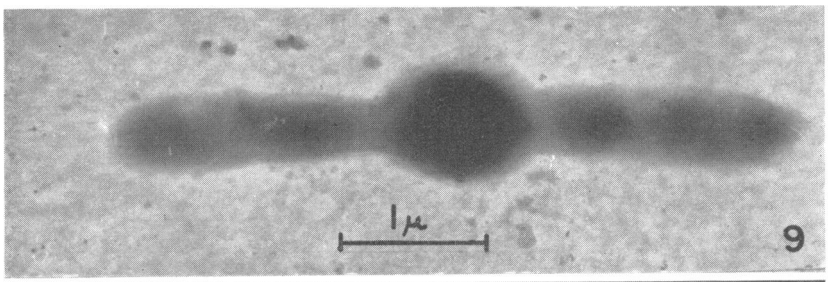


Figure 9. Enlargement of the middle of a cell. The cell membrane is visible in some places as a delicate veil. 9 hours. Formalin. 20,000 ×.

Figure 10. Two cells with enormous, but not uncommon, enlargements. The upper cell still bears the tags of the bacillary cell from which it developed. Bacillary tags are less evident in the lower cell, which is a more fully formed large round body. 15 hours. Formalin. 10,380 ×.

tron beam. The material that scattered the beam most strongly appeared dark in the photographs. For convenience it is referred to as the "dark material."

In the rod-shaped cells the dark material sometimes lay in large rounded masses distributed along the course of the cell or in a single mass in the enlarged area of a cell (figure 9). The dark material was often present in small granules, 0.14 to 0.5 microns in diameter, some of which were scattered throughout the cells, but for the most part these lay in the enlarged areas (figures 7 and 8).

Granules 0.1 to 0.5 microns in diameter were seen in the large bodies (figures 12 and 15). In some of these the dark material lay in delicately beaded, threadlike strands 0.1 micron wide and 1 micron or more in length (figure 16). In other large bodies it formed an area occupying much of the cell and showing a well-defined border against the lighter cell protoplasm surrounding it (figures 17 and 18). We have already described the manner in which the dark material pushed outward in the filaments that extended from germinating large bodies, how it pinched off within these filaments, and how the pinching off of the dark material was followed by invaginations of the wall of the filament to yield new cells, each containing a large rounded mass of the dark material (figures 20 and 22).

The foregoing description of the several patterns in which the dark material was seen could have been made with cells treated with any one of the fixatives employed; in other words, the pattern was not dependant upon the fixative. Certain differences were, however, noted. Thus, the dark material appeared darker and the surrounding protoplasm lighter in cells fixed by phosphotungstic acid than in cells fixed by formalin or osmic acid. So sharp was this contrast that phosphotungstic acid seemed a "specific electron micrograph stain" for this material. In the concentration used, however, phosphotungstic acid made the material appear so black that fine detail within it was lost. The coarse, twisted threads seen in figure 15 are very similar to those seen in Giemsa-stained organisms. Fine details, notably the threadlike strands 0.1 of a micron wide, were best brought out by formalin or osmic acid.

It is of interest to compare these electron micrographs with previously published photographs of cells of this same strain of *Bacteroides* stained with Giemsa solution (Dienes and Smith, 1943). In the Giemsa preparations deeply stained granules were seen within the rod-shaped cells and in some of the large bodies. In other large bodies the deeply stained material lay in coarse, twisted threads or in a large, single mass. The patterns of the deeply stained material in the Giemsa preparations were, therefore, essentially similar to those of the "dark material" in the electron micrographs. The inference thus seems justified that the two methods revealed the same material within the cells. The observation of this material in the unstained cells studied by the electron microscope removed the possibility that the patterns seen in the Giemsa preparations were artifacts of staining. The finer granules and threads revealed by the much greater resolution of the electron microscope were, of course, not visible in the Giemsa preparations.

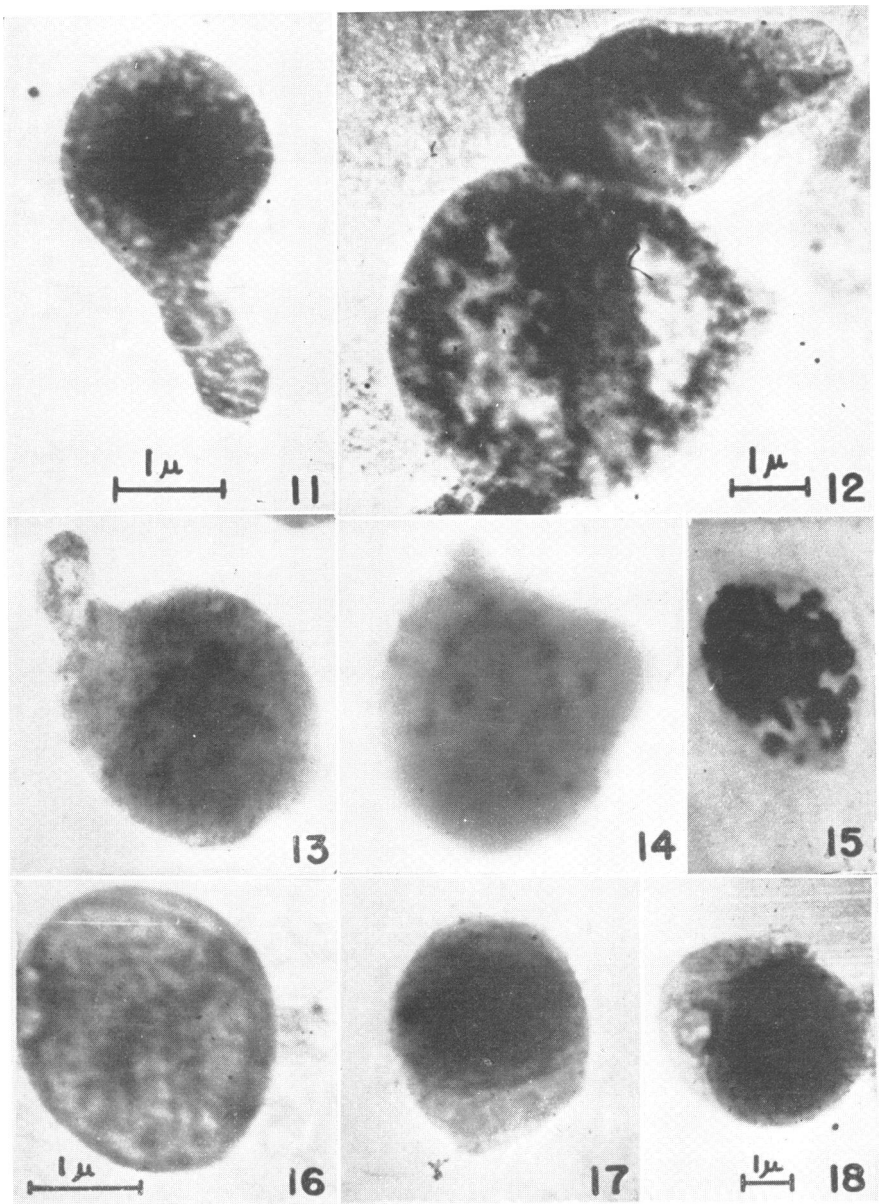


Figure 11. Large round body forming at the end of a cell. 15 hours. Formalin. 14,700 ×.

Figure 12. Fully formed large round body. The dark material lies in granules in the equatorial plane and around the periphery. A second cell with an enlargement in the middle lies above the body. 15 hours. Formalin. 10,000 ×.

Figures 13–22. Large bodies in various stages of development. Broth cultures were incubated 24 hours, by which time they had come to consist almost wholly of large round bodies. These were transferred to fresh broth and incubated 3½ to 4 hours in the fresh medium. Screens were then prepared for micrography.

Figure 13. Large body still retaining a part of its bacillary "tail." Finely stippled cytoplasm. Phosphotungstic acid. 6,1000 ×.

Figure 14. Large body from a Tyrode suspension. At least 15 dark granules lie scattered within it. No fixative. 6,100 ×.

L-Type Variation

On solid media this strain formed large colonies up to 5 mm in diameter, comparable to colonies of *E. coli*. When examined by stained wet cutout preparations, such colonies were found to be composed of ordinary large bacillary forms (figure 26), very few of which bore swellings and, rarely, large round bodies. Here and there between these large colonies very different smaller colonies developed (figure 23). These never attained a diameter greater than 1 mm, and in stained preparations they were seen to be made up of small rounded organisms, singly or in twisted chains, and large bodies (figures 24 and 25). The very young colonies of this sort lay burrowed in the agar, but later growth heaped up on the surface of the agar and gave them a "halo" appearance. These differences between the large and the small colonies have been illustrated in previously published photographs (Dienes and Smith, 1944, figures 7, 9, 10). The elements composing the small colonies, their habit of growth into the agar, and their formation of the distinctive surface halo made them resemble very closely the colonies formed by organisms of the pleuropneumonia group (L organisms). They have therefore been called L-type colonies.

L-type colonies rarely developed on plates inoculated with cells from the regular large bacillary colonies or from broth cultures in which large bodies had not yet formed. When, however, cells from large bacillary colonies were transferred to broth and the cultures allowed to grow until large bodies developed in them, subcultures to plates then yielded many L colonies as well as large bacillary colonies. In microcultures photographed at hourly intervals, the L-type of growth was seen to derive from individual, living large bodies. Further, it was possible to influence experimentally the type of germination that took place. Thus, on plates incubated at 37 C, the majority of the large bodies developed into large bacillary cells. On plates incubated at 25 C, the majority of the large bodies underwent the L-type of germination (Dienes and Smith, 1944).

In order to study the germination of large bodies by means of the electron microscope, broth cultures were incubated until they came to consist predominantly of large bodies, and transfers to new tubes of broth were then made. After 4 hours' incubation of the newly inoculated tubes, the cells were spun down and fixed with formalin, and screens were prepared. In this way germinating large bodies were observed. As in the microcultures, many of these extended thick filaments destined to divide into ordinary bacillary cells (figures 19, 20, 22). From some of the bodies, however, very fine, tortuous filaments grew out. These seemed comparable to the large bodies seen undergoing L-type germination in the microcultures. One such body is shown in figure 21. The

Figure 15. Large body with dark material in granules and threads. Phosphotungstic acid. 6,100 ×.

Figure 16. Large body filled with dark material in reticulated pattern. 15 hours. Formalin. 14,700 ×.

Figure 17. A large round mass filling about two-thirds the volume of a large body. This mass contains one prominent large granule but is elsewhere delicately stippled. It seems delimited from the surrounding cytoplasm. Formalin. 6,100 ×.

Figure 18. Another body like that in figure 17. A large, well-delimited mass fills about two-thirds the volume of the body. Osmic acid. 6,100 ×.

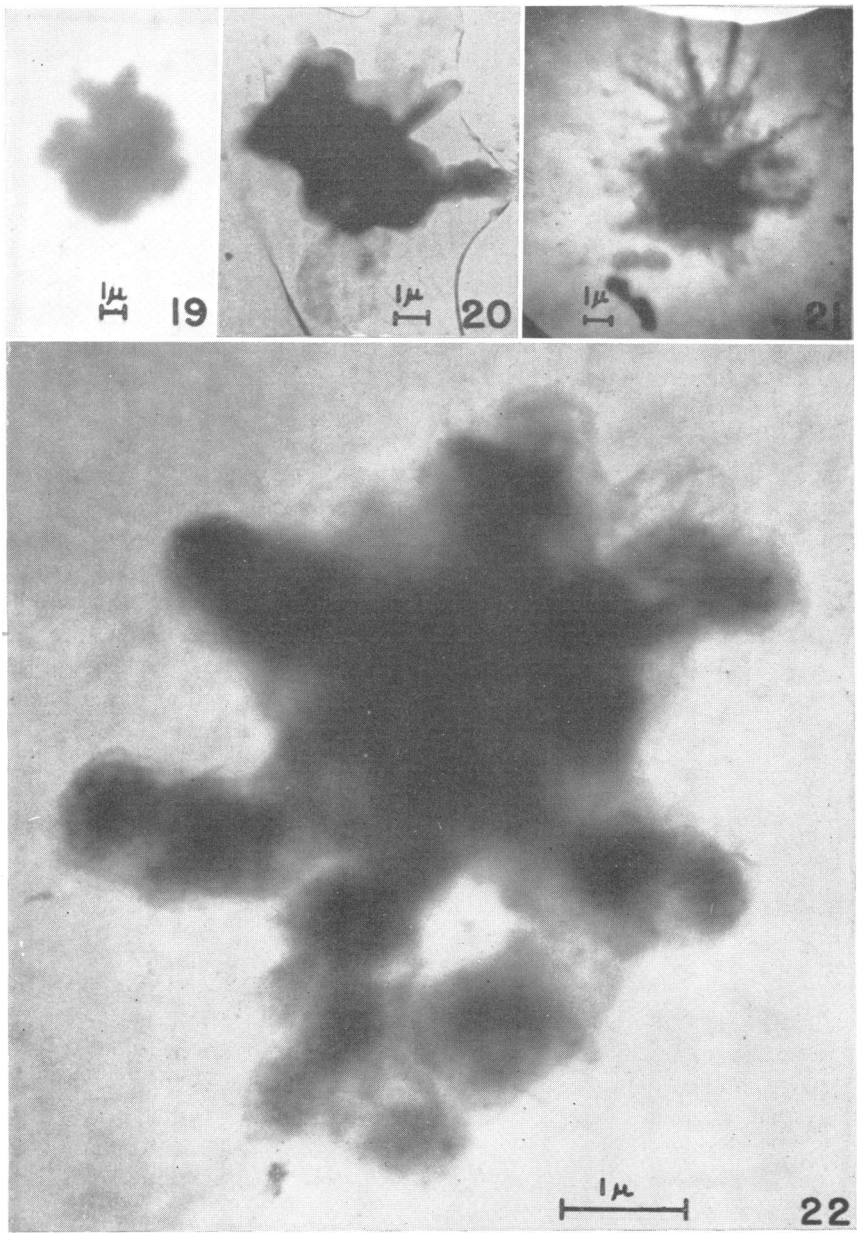


Figure 19. Large body with a bumpy, mulberrylike contour. This change in the surface indicates the beginning of germination. Two short, stubby filaments have begun to push out from the body at the top of the micrograph. Formalin. 3,460 ×.

Figure 20. Another germinating large body in which the process is more advanced. Two relatively long filaments have pushed out from the lower pole of this body, and the body itself has lost the even, round shape. Extensions of the inner dark material have pushed out into the outgrowing filaments. There is some accordion-pleating of the lower filament due to retraction of the parlodion film, shown by the wavy line across the right side of the picture. Formalin. 4,900 ×.

filaments extending from it have a diameter of only 0.3 microns in contrast to the filaments extending from the other bodies in figures 19, 20, and 22, which have diameters of 0.7 to 1 micron. Most of the filaments extending from the large body of figure 21 appeared homogeneous, but segregation of the "darker" components of the protoplasm had occurred in the filament at the upper left of the micrograph. It would thus appear that the germination of some large bodies to yield ordinary large bacillary cells and the germination of others to yield small "L type" variants are essentially a similar process, the difference lying in the size of the filaments extended and the size of the cells produced.

The L-type colonies of this strain of *Bacteroides* have been carried for many transfers on solid media and have preserved their distinctive characteristics. Large colonies composed of the ordinary large bacillary cells never appeared in the transplants. Prolonged cultivation of the L-type variant in liquid media, however, resulted in the reappearance of the large bacillary cells (Dienes, 1948). After several years of cultivation in the laboratory, this strain of *Bacteroides* became steadily less pleomorphic and lost the property of producing L-type colonies. During this time it was found that L-type colonies could readily be got from strains of *Hemophilus influenzae* grown on media containing penicillin or from cultures taken from the respiratory tract when penicillin had been given to patients (Dienes *et al.*, 1948). For this reason the *Bacteroides* strain was plated on media containing penicillin. L-type colonies appeared in great numbers. They seemed wholly to resemble the L-type colonies that had been produced spontaneously during the first years of cultivation of the *Bacteroides*. These L-type colonies preserved their characteristics when they were transferred on solid media devoid of penicillin, but reverted to the large bacillary cells when they were cultivated in broth (Dienes, 1948).

Plate cultures of the L-type colonies induced from the *Bacteroides* by penicillin but carried on media free of penicillin were sent us by Dr. Dienes. In our hands they continued to grow in the L form on transplantation on solid media. Preparations for electron microscopy were best made by picking up tiny 20-hour colonies with a pin and transferring them to a drop of 2 per cent osmic acid on the electron microscope screen. Individual cells could be micrographed at the edge of the colonies in such preparations. Electron micrographs of organisms from these cultures are given in figures 27 to 34.

The electron micrographs disclosed that the small elements in the L-type colonies were in fact tiny round and rod-shaped bacterial cells, averaging

Figure 21. Large body undergoing the L-type of germination. Instead of thick, bacillary filaments, the filaments growing out from this body are narrow and tortuous. Formalin. 3,460 ×.

Figure 22. Germinating large body. Six filaments have grown out from the body and are still sheathed within the cell wall of the body. The dark material in the upper left filament is still homogeneous and continuous with the dark material that fills the large body. In the filament at the lower left, segmentation of the dark material into two rounded masses has occurred. In the filament extending out toward the bottom of the micrograph, the dark material has pinched off into separate masses, and invaginations of the cell wall of the filament have occurred around these masses. Completion of this invagination results in the formation of free daughter cells by simple fission of the filaments. Osmic acid. 17,000 ×.

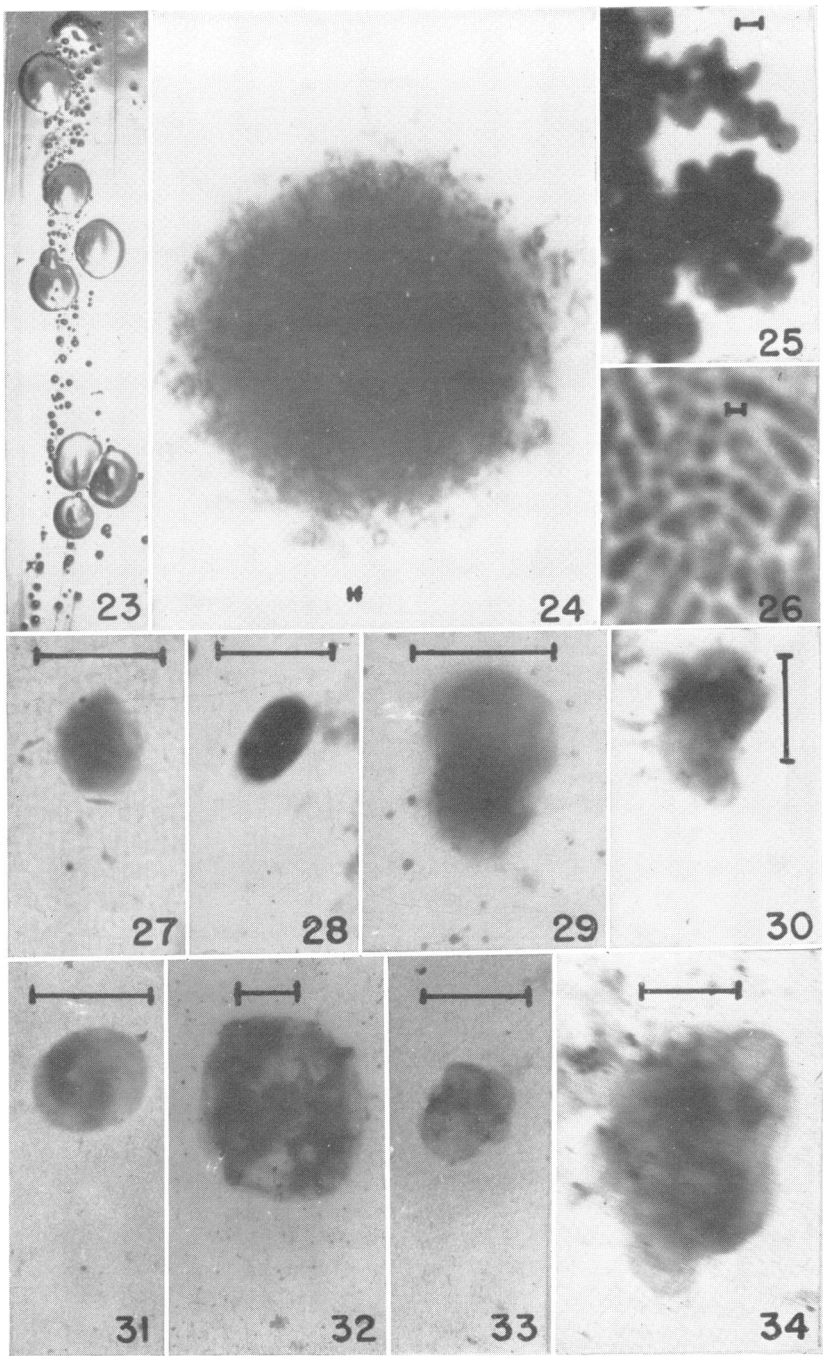


Figure 23. Ascitic peptic digest agar plate 4 days after inoculation from a broth culture of *Bacteroides* in which large bodies had developed. Note the large bacterial colonies and the tiny colonies of the L-type variant. 1.5 ×. Made with a conventional light microscope.

0.8 microns in size (figures 27 and 28). Larger rod-shaped cells, up to 1.5 microns in length, were also found. These bore bulbous enlargements at one end (figures 29 and 30). It seemed evident that the large round bodies so prominent in the wet preparations arose from such swelling of the cells. The large bodies seen in the micrographs measured 1 to 3 microns in diameter and masses of dark material could be seen within them (figures 31 and 32). Certain of the large bodies exhibited protuberances at several points on their surface that might indicate beginning germination (figures 33 and 34).

The forms seen were thus very similar to those encountered in the electron micrographs of organisms of the pleuropneumonia group (Smith, Hillier, and Mudd, 1948). It will also be noted that, though much smaller in size, the shapes of these forms mimic the pattern of pleomorphism of the large *Bacteroides* cells.

After 24 hours of growth much autolysis took place and the colonies came to consist chiefly of vacuolated large bodies. Electron micrographs made at this time showed much amorphous material, amid which were scattered many extremely minute round or elongated objects of about 0.1 micron in size. These were very opaque to the electron beam, doubtless owing to the osmic acid used to fix the preparations. Some resembled rod-shaped cells with a dark granule at each end, but the micrographs provided no certain evidence that any of these 0.1 micron objects were actual organisms.

DISCUSSION

The electron micrographs confirm the conception of the morphology of the 132 strain of *Bacteroides* gained by previous studies with Giemsa-stained organisms and show that the intracellular granules, large masses, and threads are not artifacts of staining. In addition, much finer granules and threads were found in the electron micrographs than had been visible with light micrography. These finer granules were of the order of size of the microsomes described in animal cells (Claude and Fullam, 1946). Similar fine granules were seen in the cells of the pleuropneumonialike organisms described in the preceding paper.

Figure 24. One of the tiny L colonies as seen in a piece of agar cut out of the plate and examined under a cover slip coated with methylene blue and "azur II." It shows deeply stained granules and large round bodies that stain very lightly. 1,000 ×.

Figure 25. Higher power view of edge of a young L colony before large bodies have become numerous. It is composed of small round organisms, some of which appear elongated. Stained cutout preparation. 3,000 ×.

Figure 26. Edge of one of the large *Bacteroides* colonies examined in same way. It is composed of big bacillary cells of ordinary shape. 2,000 ×.

Figures 27–34. Electron micrographs of organisms from L-type colonies, induced from *Bacteroides* by penicillin. This L variant was carried for 15 generations on media free of penicillin before cells were prepared for micrography. The transplants uniformly showed only L-type colonies. Osmic acid fixation.

Figure 27. Round cell. 16,800 ×.

Figure 28. Rod-shaped cell. It shows a delicate cell wall. 15,600 ×.

Figures 29, 30. Rod-shaped cells that have begun to enlarge at one end. They show that large round bodies develop from the rod-shaped cells of this L variant of the *Bacteroides* in the same manner seen in the large parent *Bacteroides* cells and in the organisms of the pleuropneumonia group. Figure 29 is 19,000 ×. Figure 30 is 14,200 ×.

Figure 31. Large body with two dark masses in its protoplasm. 16,000 ×.

Figure 32. Large body containing masses of dark material. The extreme delicacy of the surface is apparent in the areas between the dark masses. 8,400 ×.

Figures 33, 34. Large bodies with stubby outpouchings indicative of beginning germination. Figure 33 is 14,700 ×. Figure 34 is 13,300 ×.

Of paramount interest were the observations of germinating large bodies. Germination was observed to proceed by the extrusion of filaments from the large bodies. The outgrowing filaments were sheathed with the cell wall of the large body and contained within them masses of the dark material derived from the body. These filaments segmented by simple fission to yield new daughter bacillary cells. Germination was also observed in which large bodies segmented into multiple new daughter cells without marked extrusion of filaments or else with extrusion of only stubby filaments.

The swelling of the bacterial cells to yield large round bodies, much greater in size than the original cells, and the germination of these bodies into multiple daughter cells are very closely similar to the mode of reproduction described for two strains of pleuropneumonialike (L) organisms (Smith, Hillier, and Mudd, 1948). The *Bacteroides* cells, though much larger than those strains, exhibited essentially the same pattern of development.

The thick filaments extruded from *Bacteroides* large bodies segmented into large bacterial cells, but it was known from previous work that certain of the *Bacteroides* large bodies gave rise to very small cells that formed colonies similar in appearance to those of pleuropneumonialike (L) organisms. Colonies composed of these small cells have been called "L-type variants." Large bodies developing in this way were seen in the electron micrographs to germinate by extrusion of multiple filaments, but in their case the filaments were very much thinner. Segmentation of such thin filaments by fission obviously results in cells of a much smaller size.

Electron micrographs were made of organisms from L-type colonies induced by cultivation of this *Bacteroides* strain on media containing penicillin. These showed cells much smaller than those of the ordinary *Bacteroides* but otherwise resembling the pattern of pleomorphism exhibited by the large *Bacteroides* cells.

When the phenomenon of L-type growth was first described, it seemed so very different from the growth of the large bacillary cells that the relation between them was obscure. However, as illustrated in the present paper, large bacterial cells, though ordinarily dividing by simple fission, can also reproduce by the formation of large bodies followed by multipolar germination of these bodies, which is the mode of reproduction distinguishing the pleuropneumonia (L) group. Secondly, as shown in the preceding paper, organisms of the pleuropneumonia group, though ordinarily reproducing by means of large bodies, can also grow as regular tiny bacillary cells. And, in the case of the *Bacteroides* exhibiting L-type variation, it appeared that large bacillary cells resulted when the segmenting filaments extended from the large bodies were thick, that small L-type cells were formed when the filaments were thin. The morphological difference between the large bacillary cells and the L-type variants is thus not as great as it originally appeared. It seems essentially a matter of cell size.

How do these findings bear on the classification of organisms of the pleuropneumonia group? The situation can be summarized as follows: There are certain strains of L organisms that always grow in the small cell, pleuropneumonialike form. These are the strains from cattle, goats, and mice, from rats, and from the human genital tract. Then, in addition, there are the L organisms

that arise as variants in cultures of various species of bacteria, notably *Streptobacillus moniliformis*, *Bacteroides*, *E. coli*, and *H. influenzae*. In the case of *Streptobacillus moniliformis* and *Bacteroides* the variation is reversible: the L variants have been induced to revert to the original large cell size by transfer from solid to liquid media (Dienes, 1939, 1948; Brown and Nunemaker, 1942). In the instances of L-type variation observed in other strains of bacteria, the variation has been irreversible under all conditions thus far studied: the L variants have continued to grow in the small cell, L-type form on transfer. Whether any of the L-strains from the genital tract or from the various animal sources originated by such irreversible variation is not known.

Several years ago the *Bacteroides* strain (no. 132) employed in the present work was sent to Klieneberger. In a recent article she expressed general agreement with our account of its pleomorphism and stated that the L colonies appearing in its cultures were comparable to those of other L strains isolated in her laboratory (Klieneberger, 1947). She made the reservation that final decision as to whether the L colonies were variants of the *Bacteroides* or were symbiotic viruslike organisms associated with it must await demonstration of their bacterial nature and of their reversion to the large *Bacteroides* cells. This evidence now seems to have been provided.

In the present studies it has been found that two strains of pleuropneumonialike organisms (L50 and L4330) and a *Bacteroides* strain that exhibits L-type variation possess a property in common that seems to set them apart from other bacteria. This is the plasticity and fragility of their cell walls. The delicacy of their walls is apparent from the electron micrographs. Further, bacteria examined by electron micrography have usually been suspended in distilled water for the preparation of the screens. This procedure resulted in lysis of most of the *Bacteroides* cells and many of the cells of the two L strains. It seems likely that the plasticity of the cell wall is a considerable factor in accounting for the tendency of these organisms to develop swellings and to assume the shape of large round bodies.

One of us has pointed out (Mudd, 1944) that "evidence is slowly accumulating from many sources to indicate that the specific, pathogenic types of bacteria represent highly differentiated phases which are characteristically found under conditions of active and successful parasitism." The relative rigidity of the cell wall is unquestionably one factor that makes possible the deviation of ordinary bacteria from the spheroidal shape which the naked protoplast would tend to take under the action of surface forces. The minute bacterial cells with plastic cell walls that are characteristic of naturally occurring pleuropneumonialike strains it is tempting to regard as either undifferentiated or dedifferentiated forms, and the minute cells of pleuropneumonialike variants as dedifferentiated forms that have failed to maintain some essential differentiating factor or factors. Incidentally there is much to recommend a similar conception of rough variants as dedifferentiated forms.

The clinical application of L-type variation cannot be evaluated at present. There is evidence that the *Streptobacillus moniliformis* exists in the L form in certain infected human and animal tissues (Brown and Nunemaker, 1942).

This seemed to be the case in the patient from whom the O.H. strain of *Bacteroides* was recovered (Dienes and Smith, 1944). The apparent induction of L variants in patients given penicillin and the resistance of these variants to penicillin should be borne in mind (Dienes *et al.*, 1948).

SUMMARY

Electron micrographs are presented that show a mode of bacterial reproduction differing from that of binary fission, seen in a large anaerobic bacterium, *Bacteroides funduliformis*. Bulbous enlargements develop in the cells and form large round bodies from which multiple filaments grow out. These filaments then segment to yield new cells.

It had previously been noted that certain cultures of this bacterium threw off variant colonies which resembled colonies of pleuropneumonialike (L) organisms and which were called, for this reason, L-type colonies. The minute size of the cells of the L variant had made previous estimation of their nature difficult. A strain of L-type variant induced from this *Bacteroides* by penicillin was examined by electron micrography. It exhibited cell forms similar to those of the large parent bacillus, but smaller in size.

The large *Bacteroides*, its L variant, and the organisms of the pleuropneumonia-like (L) group all have in common the ability to reproduce by means of large round bodies.

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