ELECTRON MICROGRAPH STUDIES OF TWO STRAINS OF PLEUROPNEUMONIALIKE (L) ORGANISMS OF HUMAN DERIVATION

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The organisms of the pleuropneumonia group have been set apart from other microorganisms because of their small size and curious morphology. Their extraordinary pleomorphism is well illustrated in the studies that Tang made by means of dark-field observation of living broth cultures (Tang *et al.*, 1934, 1936). He described granules, coccobacillary bodies, filaments, and "ring" forms; and he recognized that the latter were not actual rings, as some authors have supposed, but were solid bodies of the sort that have come to be called "large round bodies" or simply "large bodies." Tang noted that the large bodies were formed from the granules and came to contain one or more dots that increased in size to form new granules, which then budded off from the large bodies. In freshly isolated strains the various elements remained attached to one another in filamentous forms, and these filaments broke up into round or rod-shaped forms. When several granules remained attached by short filaments to different points on the surface of a single large body, the resultant structure had a starlike shape, which led to the name "*Asterococcus*," sometimes applied to these organisms.

Similar observations have been reported by other authors, as reviewed by Dienes (1945). Tang's studies were made with strains of *Pleuropneumonia bovis*. The importation of this organism into the Untied States is prohibited by law because of its virulence for cattle, but organisms of similar morphology have been isolated in this country and elsewhere from other animals. Some of these strains appeared as saprophytes upon mucous membranes, others as agents of spontaneous joint infections in rats. In human beings organisms of this sort are common inhabitants of the uterine cervix. They have also been isolated from various genitourinary infections, notably prostatitis. In a series of 23 men with prostatitis yielding these organisms, acute joint involvement occurred in 11 cases at the time the prostatic cultures were positive. The organisms were cultivated directly from joint effusions in two of these patients (Dienes *et al.*, 1948).

The strains from these diverse sources, differing in pathogenicity and in serological properties, have in common the fact that they are among the smallest organisms that have been grown on lifeless media, and that they resemble each other in their unusual morphology and distinctive, almost microscopic, colonies. For these reasons they have been classed together as organisms of a "pleuro-

pneumonia group," or "pleuropneumonialike organisms," or, as a brief designation, "L organisms" (Sabin, 1941).

Although morphology, including size as well as shape, is the basis of this classification, much debate has centered over the actual structure and nature of these minute, pleomorphic organisms (Klieneberger and Smiles, 1942). We felt that the greater resolution made possible by the electron microscope might be of some aid in this problem. The present paper contains electron micrographs of strains of human origin supplied by Dr. Louis Dienes. These will be referred to as L organisms.

METHODS

All electron micrographs were made at 55 kv.

L strains were grown on ascitic fluid peptic digest plates at 37 C and examined with the light microscope by means of wet cutout preparations stained with methylene blue and "azur II." This technique has been described elsewhere, together with notes on the preparation of the medium (Dienes *et al.*, 1948). The peptic digest was a modification of "Martin's peptone" (Wadsworth, 1947). It was prepared by adding 600 g of fresh, minced hog stomach and 36 ml conc. HCl to 3,000 ml water. After 20 hours' incubation at 50 C, the fluid was decanted and buffered with 4 g K_2HPO_4 and brought to pH 7.8 with 10 N NaOH. It was then filtered through gauze and cleared by filtration through paper. Sterilization was accomplished by passage through a Seitz filter. When plates were to be prepared, 15 per cent of this digest and 25 per cent of ascitic fluid were added to the sedimented boiled blood agar previously described. The digest affords more abundant growth, but its use is not essential. It is important that the ascitic fluid be buffered to pH 7.4 with KH_2PO_4 and that the plates be soft (1 per cent agar) and sealed to prevent drying during storage. L colonies grow down into the agar and hard plates can completely prevent growth.

Although the plates just described permitted excellent growth and serial passage, neopeptone infusion broth enriched with ascitic fluid and peptic digest failed to support continued propagation of two L strains which we isolated from the human uterine cervix. Rabbit serum proved equally unsatisfactory as an enriching material, but when 30 per cent horse serum was added to the broth the strains were successfully carried through 8 transfers. The tubes developed only faint turbidity, but subcultures to plates yielded abundant growth.

Preparations for electron micrography were made by touching a few colonies with a pin, which was then repeatedly thrust into a drop of distilled water upon a parlodion film covering the electron microscope screen. When it was desired to study fixed organisms, a drop of 2 per cent osmic acid solution was put upon the screen instead of a drop of water. This fixative volatilized without depositing crystals. A second method was to flush the osmic acid solution back and forth with a pipette over the surface of agar bearing colonies. A drop was then transferred to a screen. The latter method was much less satisfactory, since debris was carried over to the screen.

We are indebted to Dr. Keith Porter and Mr. Winfield Baker for assistance with some of the micrographs.

Findings with Organisms of the Pleuropneumonia Group (L Organisms) Isolated from Human Sources

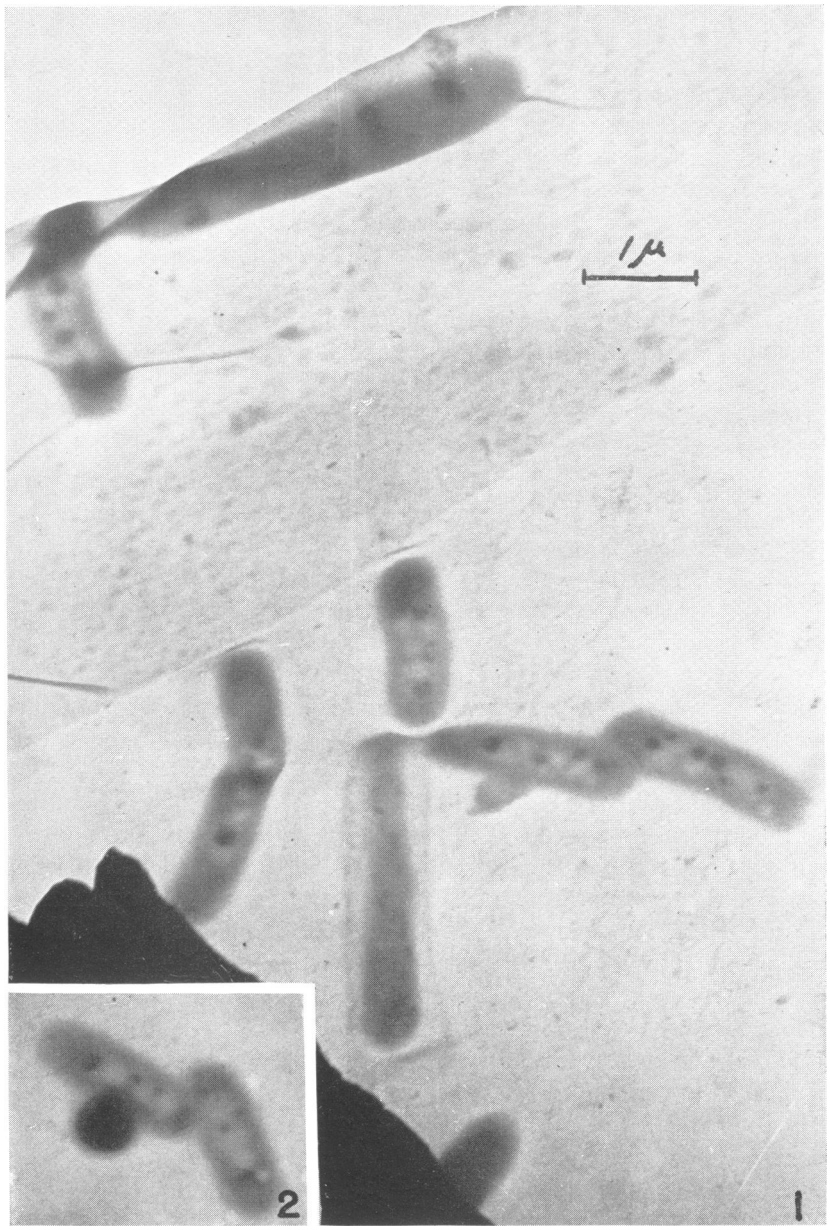
The L50 strain was the first to be examined. It had been isolated from the uterine cervix and carried for several years on ascitic peptic digest plates as a type culture representative of L organisms derived from the genital tract. A plate bearing colonies of this strain was provided by Dr. Dienes. The plate had been incubated 4 days, and a semiconfluent growth of tiny colonies approximately 0.2 mm in diameter had developed upon it. Stained wet cutout preparations, examined with the oil immersion lens, showed that these colonies were composed of round bodies of varying size and small rod-shaped bodies, and that they had the peculiarly distinctive appearance of L colonies, photographs of which have been published elsewhere (Dienes, 1945).

Electron micrographs of organisms of this strain are given in figures 1 to 5. They show round bodies that vary in size from 0.5 (figures 2 and 3) to 3 microns (figure 5) in diameter. In addition, short curved or twisted and slightly nodular filaments were seen, the longest measuring 5 microns. Of particular interest was the observation of rod-shaped bodies. Many such were found. They averaged 1.3 microns in length and 0.5 microns in width. They thus had a distinctly elongated, bacillary shape. In figure 1 the protoplasm of one of these rod-shaped bodies has retracted slightly, and a well-defined cell wall is visible. The size and shape of these organisms, together with the demonstration of a cell wall, make it certain that they must be regarded as indubitable bacterial cells. The smallest cells seen in this strain were the 0.5-micron round bodies mentioned above.

The cytoplasm of most of the cells in figures 1 and 2 appeared vacuolated. In addition, in these pictures and in figure 3, well-defined intracellular granules 0.1 to 0.18 microns in diameter were seen. These granules occurred independently of vacuolation of the cells. The largest cell in figure 1 shows them distributed rather evenly along the course of the cell. Larger and less well-defined areas of increased density can be seen in the elongated cell shown in figure 3. A large, sharply defined area of increased density, 0.3 microns across, occurred at the enlarged end of the cell in figure 4. Short, twisted, nodular filaments or chains of small round cells are characteristic of the L type of growth. The long cell in figure 3 would appear to be such a filament. The large, rounded, dense knob at the end of the cell in figure 4 probably represents an early stage in the formation of a free round cell.

Of the three round cells shown in the micrographs, the smallest (figure 2), at the voltage employed, appeared as a homogeneous protoplast surrounded by a peripheral structure of relatively low density. The second, slightly larger (figure 3), contained five scattered areas of increased density. The large round body of figure 5 had a large diffuse area of increased density in its center. New round or elongated cells are known to arise from large bodies such as this. The body shown here was selected because its two stubby outpouchings seemed to represent the earliest stage in such germination. More will be said of the process of germination in this and the succeeding paper.

Round bodies were considerably less common in the electron micrographs than



Figures 1 to 17 were made with an electron microscope at 55 kv.
Figure 1. L50. Rod-shaped cells containing intracellular granules. In the big cell at the lower center of the photograph the protoplasm has retracted and the cell wall is visible. 15,000 ×.
Figure 2. L50. Two rod-shaped cells and a round cell. 15,000 ×.

in wet cutout preparations, and it was suspected that some procedure in the preparation of the screens may have acted to destroy them. Well-developed cell walls were not detected on the round bodies, a finding which suggested that their

surface might be less sturdy than the surface of the bacillary forms. Hence it seemed possible that the loss of round bodies had resulted from osmotic bursting occasioned by immersion in distilled water when the screens were prepared.

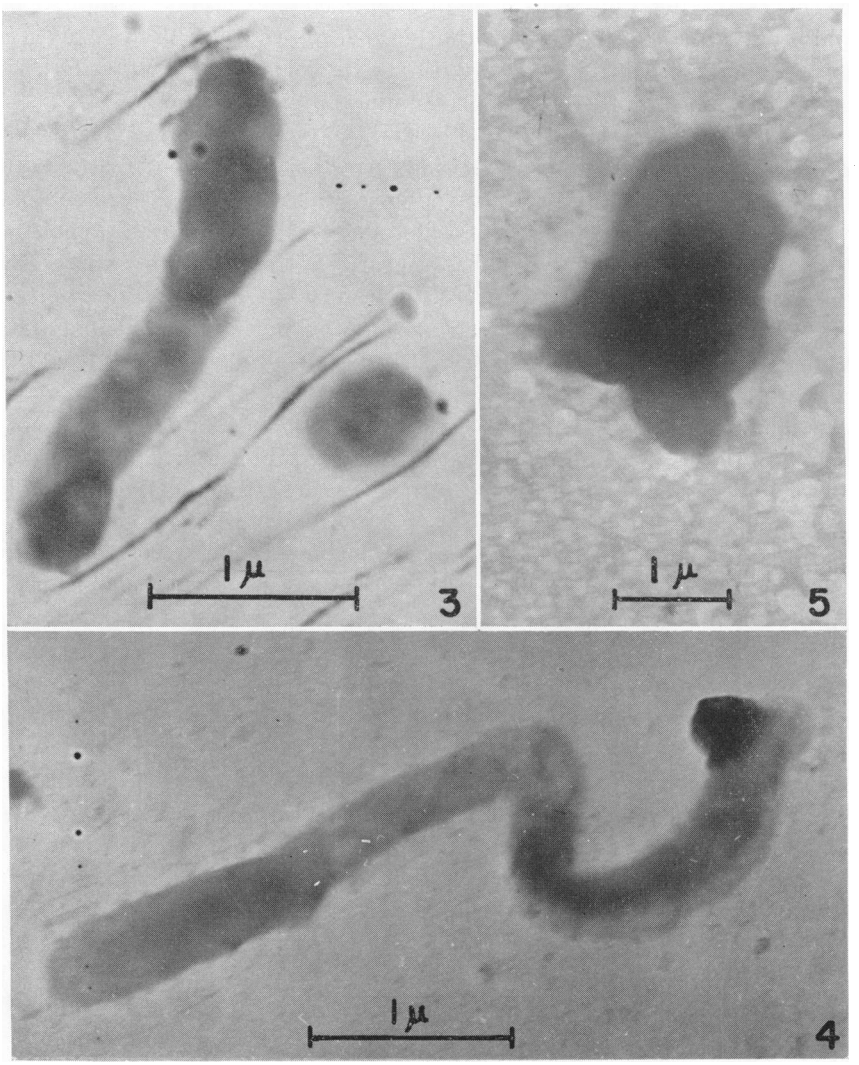


Figure 3. L50. A nodular, filamentous cell and a round cell. 27,000 ×.
Figure 4. L50. Filamentous cell with enlargement at one end. 27,000 ×.
Figure 5. L50. Large round body. Its uneven contour and the two blunt outgrowths at its lower pole illustrate the earliest stage of germination. 15,000 ×.

This hypothesis was borne out by subsequent observations reported in this and the accompanying paper.

The next strain examined, L4330, had been isolated from the uterine cervix in 1940. Except for intermittent periods of preservation in the frozen state, it had been carried since then on ascitic peptic digest plates by transfers every 4

days in Dr. Dienes' laboratory. In 1942 this strain was sent to Dr. Albert Sabin, who published an account of studies made with it in his laboratory, concluding that it should properly be classified in the pleuropneumonia group (Warren and Sabin, 1942). When sent to us in 1945, it formed colonies and exhibited pleomorphic forms like those shown in the photographs published by Warren and Sabin in connection with their study of it. In wet cutout preparations it appeared comparable to other L strains examined by this method.

Because of the small size of the colonies and their habit of burrowing down into the agar rather than heaping up as surface growths, transfers of L strains from plate to plate are customarily made by cutting out a piece of agar and rubbing it over the surface of the plate to be inoculated. L4330, transferred in this way, produced densely crowded growths that appeared as thin, delicately stippled films on the agar after 4 days' incubation. With the oil immersion lens, such a film was seen to be made up of tiny, discrete colonies, buried in the agar and composed of minute round forms that stained a deep blue in the cutout preparations. Pleomorphic forms were not in evidence.

Very different were the findings in less densely inoculated areas. Ascitic peptic digest plates were inoculated with L4330 by rubbing a small piece of agar bearing colonies over one-third of its surface (area 1), then cutting out a block from the inoculated area and rubbing it over another third of the plate (area 2), and finally inoculating the last third (area 3) in the same way with a piece from area 2. After 4 days' incubation, the growth in area 1 seemed confluent. In area 2 the colonies were almost everywhere discrete, separated by about 1 mm from each other. These colonies were about 0.2 mm across. In area 3 the colonies were well separated, 2 to 3 mm apart, and were about 0.5 mm in size.

Cutout preparations examined with the oil immersion lens showed that the colonies in the seemingly confluent part of area 1 were discrete, though almost touching each other. These colonies averaged 10 to 20 microns in diameter. Each was a rather compact mass of tiny, deeply stained granules growing down into the agar. None of the granules appeared to be larger than 0.5 microns. Large round bodies, bacillary forms, or filaments could not be found, but some granules were connected by a fine thread.

In area 2, where the colonies were less crowded and bigger, their appearance was different. They still showed the deeply stained granules growing down in the agar, but each had a mound of surface growth that appeared as a lacy halo when observed with the low power of the microscope. With the oil immersion lens, this halo was seen to be composed of large round bodies 5 to 7 microns in diameter, as shown in many published photographs of L colonies. The colonies in area 3 were similar but larger, and in them new growths of granules down into the agar had begun at many places on the periphery of the halos. In these areas some of the "granules" were distinctly rod-shaped instead of round.

This experiment is cited in detail because bacillary forms were seldom seen in electron micrographs made from confluent areas of growth. To observe them it was necessary to employ screens prepared from well-separated colonies. They were sought in particular with the aim of elucidating the relationship between the rod-shaped and the round bodies.

It was soon learned that the round bodies in this strain, as in L50, were in large measure destroyed by immersion in distilled water. Figure 6 shows organisms plasmolysed as a result of such treatment. These plasmolyzed cells show clearly the reason for their destruction, for it is evident that they lack the sturdy outer structure that composes the wall of ordinary bacteria. One to five small dense granules lay along the limiting surface, presenting exactly the picture of "ring" forms long known to be characteristic of pleuropneumonia organisms. One such granule lay in each end of a rod-shaped cell residue (upper right of photograph), and two lay in the enlarged end of a longer rod-shaped cell residue with two smaller and fainter ones in the narrower portion of the same cell (lower left). The blurred appearance of the rod-shaped cells showed that they also suffered osmotic injury as did the round cells. The delicacy of their cell wall is apparent. The presence of granules in injured cells is obviously difficult to interpret. They are commented upon merely because they conform to the pattern observed in dark-field or stained preparations.

Because of the plasmolysis, it was determined to employ a fixative. For this purpose, a drop of 2 per cent solution of osmic acid was placed on the screen instead of a drop of distilled water, and the organisms were transferred to it by a pin. This procedure preserved them. Electron micrographs of L4330 made in this manner are given in figures 7 to 17.

The round forms observed in the fixed specimens ranged in diameter from 0.25 to 0.7 microns. They had a finely granular appearance, occasionally with a dense mass in the center (figure 7). These small round cells were found to be capable of division by simple fission in the manner of cocci (figures 8 and 9). Some of the small round cells became elongated, and it appeared that the rod-shaped cells arose in this way (figures 7 and 10). Rod-shaped cells from 0.5 to 1.3 microns in length were found (figures 11 to 15). The larger of these exhibited a swelling toward one end.

The formation of a new round cell ("large body") from this swelling is well shown in figures 11 and 15, which also show concentration of material with greater ability to scatter the electron beam in the forming large body, a picture reminiscent of that obtained with developing spores.

The germination of large bodies to form a new generation of small, round cells is shown in figures 16 and 17. Figure 16 shows a large body whose surface has become nodular, resulting in a mulberrylike appearance. Observation of living cultures has shown that such bodies break down into multiple new daughter cells without much further change in shape. The large body (A) in figure 17 had given rise to two thick outgrowths, B and C. B was a blunt mass, but C had begun to differentiate into small round cells, three of which are clearly defined. The invagination of the outgrowing filament to form these three cells marks the process as one of segmentation of the filament.

Observations on Nelson's Coccobacilliform Bodies

A further group of organisms of interest in connection with the present work were the "coccobacilliform bodies" isolated from the nasopharynx of rats, mice, and chickens (Nelson, 1939). Not all of these strains have been grown on life-

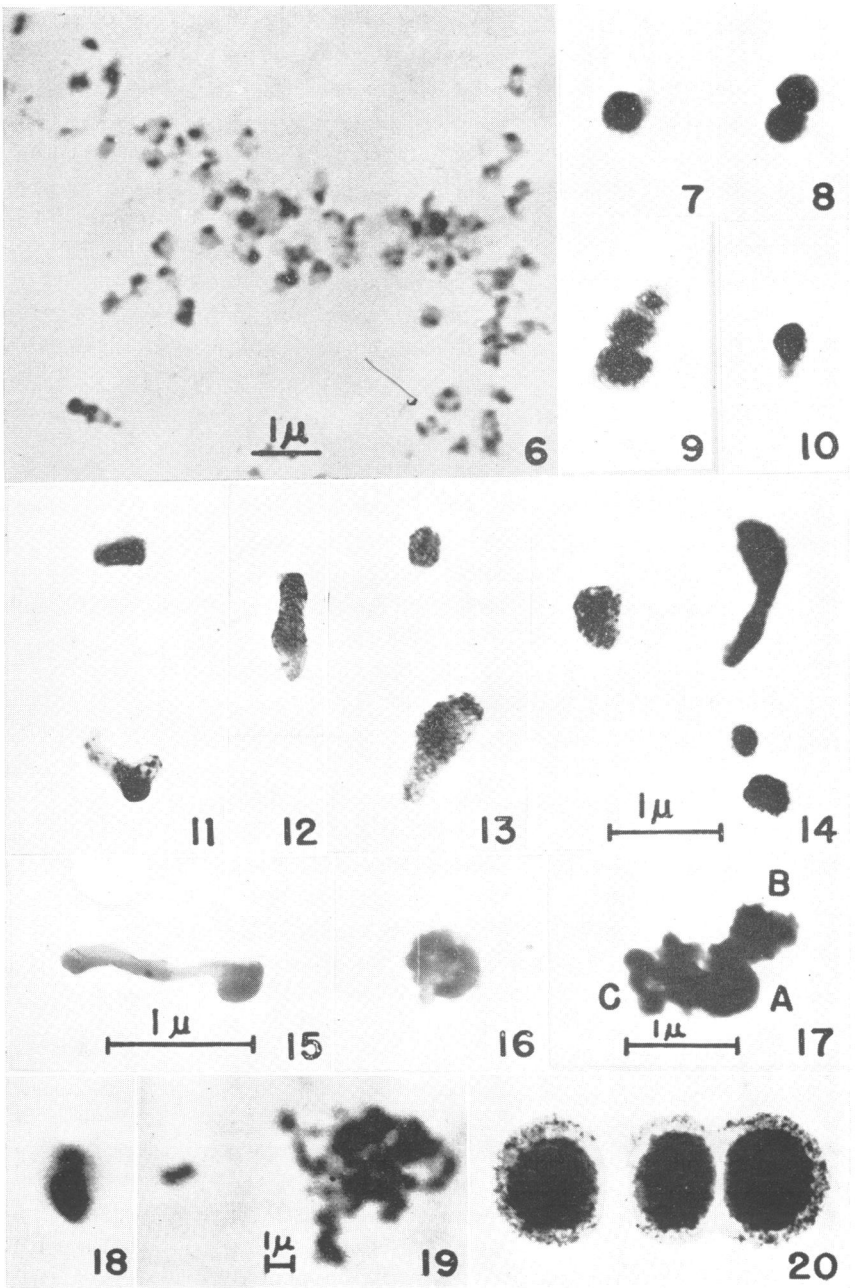


Figure 6. L4330. Cells plasmolyzed on a screen prepared from distilled water. Ordinary bacteria are not obviously damaged by similar preparation. Plasmolysis of the L organisms illustrates an important property of their surface, namely, its fragility. 9,000 ×.

Figures 7–17. L-4330. Osmic acid fixation. All are 15,000 × except figure 15, which is 20,000 ×.

Figure 7. Round cell with dark granule in center.

Figure 8. Two round cells dividing by simple fission.

less media, but it is probable that they should be included in the pleuropneumonia group (Sabin, 1941). A strain of these organisms isolated from chickens and adapted to *in vitro* cultivation was provided by Dr. Nelson. This was an avirulent strain. Nelson found that virulent strains failed to grow on media that supported growth of organisms of the pleuropneumonia group. When inoculated onto ascitic peptic digest plates, it produced abundant growths of tiny, glistening pin-point colonies. These proved readily transplantable, but the colonies were always tiny, never attaining a diameter greater than 0.3 mm. Growth was less abundant and the colonies tended to autolyze on horse serum or horse blood plates. In liquid media excellent growth was obtained in infusion broth enriched with 30 per cent horse serum, definite turbidity appearing after 72 hours' incubation and enormous numbers of colonies arising when a drop was transferred to a plate. No growth occurred in plain broth or in broth enriched with ascitic fluid. Good growth took place in Tyrode's solution containing bits of chick embryo tissue and buffered to pH 7.4 with Na_2CO_3. Although growth in this medium did not progress to the extent of producing turbidity, transfers from it to plates yielded great numbers of colonies. Giemsa-stained smears were prepared from the liquid media, and the tiny round coccoid and minute rod-shaped bodies described in the literature were observed.

More definitive observations, for our purposes, were made by an examination of pieces of agar cut out of ascitic peptic digest plates bearing the colonies and stained as wet preparations by methylene blue and "azur II" in the manner employed for L organisms. With this technique the individual cells could be clearly seen lying on or in the agar, not shrunk by drying as in smears, and their relation one to the other could be clearly ascertained. During the first 48 to 72 hours of growth, the colonies consisted of tiny round bodies about 0.5 microns in diameter. These lay singly, in pairs, or in short, twisted chains, bespeaking division by simple fission. The colonies grew down into the agar. Larger round bodies, up to 2.5 microns in diameter, occurred, notably at the surface of the colonies or

Figure 9. One of the "short, nodular filaments" characteristic of L organisms. It is composed of 3 round cells that have divided by fission.

Figure 10. Elongated cell.

Figure 11. Two rod-shaped cells, one of which is enlarged at one end. This illustrates the beginning of large body formation.

Figure 12. Rod-shaped cell.

Figure 13. Rod-shaped cell with beginning enlargement toward one end. Also a small round cell.

Figure 14. Rod-shaped cell with bulbous enlargement at one end. Also 3 round cells.

Figure 15. Filamentous cell with well-formed large round body at one end.

Figure 16. Large round body showing beginning segmentation into multiple small round cells.

Figure 17. Large round body (*A*) showing outgrowths at *B* and *C*. The outgrowth at *C* has begun to segment into small round cells.

Figures 18–20. Light microscope photographs of a strain of Nelson's coccobacilliform bodies. Agar cutout preparations stained with methylene blue and "azur II."

Figure 18. Large round body on surface of agar. 4,000 ×.

Figure 19. Young colony composed of short tortuous chains of small round cells growing down into the agar. The central dark area was composed of a large round body lying on the surface of the agar and consequently somewhat out of focus. The chains of small round cells grew out from the large body. 4,000 ×.

Figure 20. Four-day-old colonies on agar. The dense center and lacy peripheral zone are characteristic of organisms of the pleuropneumonia group. 100 ×.

singly on the agar (figure 18). Although the colonies consisted for the most part only of the small cells of rather uniform diameter, it was evident that growth sometimes derived from the larger round bodies, one or more chains of small cells extending out from them. Figure 19 shows one such young colony that had developed from a large body. At least three twisted nodular filaments extended out from the body, and each filament had begun to segment into small round cells. These filaments had grown down into the agar, and the large body that lay on the surface was, therefore, slightly out of focus. Next to this young colony, two small round cells lay on the surface of the agar. They were evidently in the process of division by simple fission. The small round cells in this photograph were all approximately 0.5 microns in diameter. Older, better-developed colonies are shown in figure 20. They have the dark center and lacy peripheral halo associated with colonies of the recognized strains of pleuropneumonialike organisms. Each tiny dot in this halo was a single small round or minute rod-shaped cell, with larger round cells here and there, as higher magnification showed.

The appearance of the colonies, their habit of burrowing down into the agar, the size and morphology of the elements composing them, and the reproduction both by simple fission and by multipolar germination of large bodies led us to conclude that this strain of coccobacilliform bodies could properly be included in the group of pleuropneumonialike (L) organisms.

DISCUSSION

By means of the electron microscope it has been possible to observe the pleomorphic cells of two strains of L organisms with greater accuracy than had been possible with light microscopy. The rod-shaped cells of the L50 strain were thus found to have a cell wall that could be distinguished from the inner cell protoplasm. The somewhat larger, more filamentous cells developed nodular swellings, thereby revealing the manner of formation of the large round bodies. The organisms of the L4330 strain were smaller, and differentiated cell walls were not detected in them. Their pattern of development was nevertheless similar, and a series of micrographs is given showing the development of large bodies in this strain. When these bodies began to form in rod-shaped cells, one saw an enlargement in the middle or toward the end of the cell. This enlargement increased in size until it became very bulbous. Such enlarged areas usually caused more scattering of the electron beam than did the other portions of the cells bearing them.

The germination of large bodies to yield a new generation of small round cells was also observed. When this occurred, the protoplasm pushed outward at 2 to 3 points on the surface of the body. These outgrowing filaments then became constricted in several places along their course, and the new cells were produced by fission of the filaments. This type of germination might be compared to the germination of certain *Actinomyces* spores from which four filaments grow out and then segment to yield new cells (Knaysi, 1944). It differs from the

germination of most bacterial spores in that it is multipolar rather than unipolar. Sometimes the whole surface of a large body became nodular, only very stumpy filaments were extended, and segmentation into new cells occurred while the body was still essentially round.

Consideration of the differences between large bodies and bacterial spores appeared to throw some light upon the manner of their germination. The resistance of spores to heat, changes in osmotic environment, drying, and aging is considered to be a property conferred upon them in large part by the character of their cell walls, which have been shown by various techniques, including electron micrography (Knaysi *et al.*, 1947), to be well-developed structures. Upon germination, the wall cracks, and the new vegetative cell then pushes out. On the other hand, our electron micrographs showed that the large bodies did not have the well-formed, sturdy cell walls possessed by spores. The lack of this structure doubtless accounts in part for the fact that they are not resistant to heat, drying, or aging. That their limiting membrane is indeed delicate was shown by the finding that they were readily burst by immersion in distilled water. This quality of the surface of the large bodies would seem to go some way toward explaining the multipolar character of their germination; for, at the time of germination, an increase in the volume of protoplasm contained within such a delicate membrane might be expected to weaken the membrane at many points, through which the protoplasm could push outward. Individual differences in elasticity of the surface may perhaps determine whether a given large body will send out a few relatively long filaments, which then segment, or whether its surface will become nodular and segmentation occur without the development of filamentous outpouchings. The latter process is well described in the case of the breakdown of large bodies of L1 into multiple small round forms (Klieneberger and Smiles, 1942).

It is abundantly clear that reproduction by means of large bodies is not the sole means of growth of the L organisms. Under certain conditions, notably crowding of colonies, one sees no forms other than small round cells, and these are obviously reproducing by simple fission in the manner of cocci. Our studies with the electron microscope show that these small round cells, as well as the rod-shaped cells, are capable of division by simple fission. It would seem likely that large round bodies also developed from the small round cells, for large bodies often formed in colonies in which rod-shaped cells were not in evidence.

Indubitable bacillary forms were demonstrated by Weiss (1944) in electron micrographs of a strain of organisms of the pleuropneumonia group isolated from a spontaneous joint infection in a rat. Multipolar germination of a large round form is well seen in figure 10 of her paper. The frank bacillary forms detected in her cultures resemble those seen in the L50 strain, in which such forms were larger and more numerous than is usually the case in strains isolated from the human genital tract. The smaller cells of the L4330 strain are more typical of freshly isolated human strains.

Recently, Hesselbrock and Foshay (1945) published studies on the morphology

and filterability of *Bacterium tularense* that led them to conclude that that organism should be included in the pleuropneumonia group. Their photographs show well-defined bacillary forms, and also large round bodies from which filaments, sometimes branched, have grown out.

The identification of rod-shaped cells in the two human L strains described in this paper, and their presence in the photographs of Weiss and of Hesselbrock and Foshay, support Dienes' view (1945) that the organisms of the pleuropneumonia group are essentially small, pleomorphic bacilli. The development of large round bodies as swellings within bacilli or bacillary filaments and the manner of subsequent germination of these bodies afford an explanation of the significance and function of the pleomorphism. The main point that distinguishes the germination of the large bodies is that they divide into multiple daughter cells. Formation of such large bodies and reproduction by means of them are not limited, however, to the tiny organisms now lumped together in the pleuropneumonia group. It has been observed in large bacteria belonging to several of the common genera. In the accompanying paper electron micrographs are presented that show this mode of reproduction in a large anaerobic bacterium, *Bacteroides funduliformis* (Smith, Mudd, and Hillier, 1948).

SUMMARY

Two strains of pleuropneumonialike (L) organisms derived from the human genital tract were studied. The morphology and mode of reproduction of these organisms as revealed by the electron microscope are described. One strain of Nelson's coccobacilliform bodies was examined. Its morphology and colonial appearance correspond to those of recognized strains of pleuropneumonialike organisms.

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