JOURNAL OF BACTERIOLOGY, Feb. 1968, p. 672-687

Vol. 95, No. 2
Printed in U.S.A.

Morphology and Reproductive Processes of the L Forms of Bacteria

II. Comparative Study of L Forms and *Mycoplasma* with the Electron Microscope

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Received for publication 17 November 1967

Representative electron micrographs, from the study of eight strains of L forms and one strain of *Mycoplasma*, are presented. A- and B-type L forms were derived from two strains of *Proteus*, two other L forms were derived from a diphtheroid and from a staphylococcus strain, and two strains (designated as LX) were isolated from L forms derived from a group A β-hemolytic streptococcus and from a staphylococcus. The *Mycoplasma* strain was isolated from goats. Sections were made of young colonies grown within agar and from parts of surface colonies embedded in the agar. B-type L colonies of *Proteus* were produced by inoculation of bacteria into media containing penicillin. The large bodies developing from the bacteria and the organisms in B-type L colonies of *Proteus*, like the parent bacteria, had a cell wall consisting of a plasma membrane and an outer cell wall. The loss of rigidity in the cell wall indicated an alteration in its structure. The A-type L cultures of *Proteus* consisted of irregular branching masses extending in several directions, of small dense organisms corresponding to the elementary corpuscles present in cultures of *Mycoplasma*, and of intermediary forms. In contrast to the B-type, all organisms in the A-type colonies were surrounded by a single unit membrane corresponding to the plasma membrane of bacteria. The structures inside the cell membrane, both in the A- and B-type, seemed to correspond to the structure of the parent bacteria, which contained ribosomes and threads of DNA. The elementary corpuscles formed chains and filaments, and, apparently, these corpuscles took part in the multiplication by gradual enlargement. The organisms seen in the cultures of all L forms and *Mycoplasma* studied, except in the B-type L forms of *Proteus*, corresponded in size, shape, and structure, as well as in the development of elementary corpuscles, to the organisms in the A-type L form of *Proteus*. In contrast to the spherical organisms usually seen in broth cultures, the organisms in young cultures of *Mycoplasma*, which were grown within the agar, were similar in morphology, as well as in the discernible structure of the organisms, to L forms. Significant morphological and structural differences were not apparent between the L forms and *Mycoplasma* (in cultures grown within agar media) under the conditions of this investigation.

In this paper, our purpose is to contribute to the knowledge of the morphology and structure of L forms of bacteria by publishing representative electron micrographs. We undertook this study to examine whether the similarities in morphology and most other properties between L forms and *Mycoplasma* are present also in the fine structures resolved by the electron microscope. The structure of bacteria and the morphology and structure of *Mycoplasma* have been clarified in many respects by the examination of thin sections in an electron microscope (1, 4, 8). This has not been the case with L forms (5, 10, 12–16). In most instances, the cultures were not appropriate or were not studied under the most favorable conditions. L forms are bacteria with cell walls altered by metabolic or structural defects. These defects are variable and can be multiple, and L forms isolated from different species, or even from a single strain of a bacterium, may vary con-

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siderably in their properties. For example, we obtained six varieties of L forms with markedly different properties from strain 52 of *Proteus*. One of these was, in many respects, more like *Mycoplasma* than the others. Furthermore, L forms often change their properties considerably during prolonged cultivation. Old cultures of L forms of *Proteus*, designated as strains 9 and 18, were found to have only a remote similarity to freshly isolated strains. The use of appropriate modes of cultivation is as important in the study of L forms as is the selection of strains. The physical properties of the medium exert a strong influence on morphology and on the mode of reproduction. Growth in the form of small granules, comparable to the granules of *Mycoplasma*, occurs only within agar media in most freshly isolated L forms. In liquid media and on the surface of various solid media, such as agar, gelatin, and coagulated serum, growth occurs in the form of large bodies (3). We saw multiplication of small granules in broth or on the surface of agar only in cultures of the old, altered L forms.

The main morphological difference between L forms and *Mycoplasma* is that *Mycoplasma* grows in the form of small granules, both in broth and on the surface of solid media. Large bodies develop under the same conditions as in L forms, but in *Mycoplasma* there is much less of a tendency to produce large forms. Young colonies of *Mycoplasma* and of some L forms, grown on and within agar media, are often indistinguishable with a light microscope. Thus, young colonies seemed to offer the best opportunity for the comparison of the two groups. It likewise seemed advisable to study, whenever possible, freshly isolated L forms grown within agar. The study of growth extending into agar from surface colonies appeared to be less appropriate because, for technical reasons, the earliest development cannot be studied. Unfortunately, it was only possible to find tiny colonies within the agar in densely populated cultures, and freshly isolated L forms often could not be induced to produce dense cultures.

The use of agar cultures offered technical advantages. In these cultures, young actively growing organisms can be found at the periphery of the colonies. The organisms remain in the position in which they have grown, they are less disturbed by manipulation, and they are protected during fixation and embedding.

Our first studies were made with the L forms of *Proteus*. These L forms were produced from bacteria and were maintained in cultures without difficulty. They offered the opportunity to study a variety of L forms isolated from the same strain of a bacterium, with properties markedly similar to or different from those of *Mycoplasma*. When *Proteus* was inoculated on the surface of appropriate agar plates, like several other groups of gram-negative bacteria, it produced two types of L colonies. The properties of one, which we called B-type (2), were similar, in several respects, to those of the parent bacteria. The growth requirements for a B-type L colony were simple: animal serum was not needed; growth was rapid and large colonies were produced; sensitivity to phages was present, and, if penicillin was eliminated, many organisms in the colonies resumed full bacterial structure. The organisms were fragile and without rigid cell walls, growth occurred in the form of small granules and large bodies, and the structure of colonies on the agar surface corresponded to that of other L forms. In addition to their large size, the B-type colonies also differed from the colonies of most L forms in that the granules producing the colonies grew into large bodies not only on the surface of the medium but also within it. Almost all of the bacteria in a culture of *Proteus*, when inoculated within nutrient agar and exposed to penicillin, grew into B-type L colonies. This made it possible to observe, in the electron micrographs, the way in which the granules of this L form developed from the bacteria.

A-type colonies, as well as a few B-type colonies, developed from *Proteus* on the surface of soft nutrient agar plates containing animal serum and penicillin. Only a few of the bacteria inoculated on the surface developed into colonies. The A-type colonies enlarged slowly and remained small. The appearance of young cultures was similar to that of *Mycoplasma*. Bacteria might not return at all in transfers made to penicillin-free medium or might return only after one or more days of incubation. Both the A- and B-types of L colonies lost the ability to revert to bacterial form after prolonged cultivation. The A- and B-types did not correspond to the so-called "stable" and "unstable" L forms. The loss of ability to revert to bacteria was due to aging of the cultures and may be comparable to the loss of virulence or of ability to form spores.

MATERIALS AND METHODS

Eight strains of L forms and one of *Mycoplasma* were studied. A- and B-type L forms were derived from *Proteus mirabilis* strains 49 and 52, originally isolated from urine specimens and used previously for various studies by us and by others (L. Dienes, in *Microbial Protoplasts, Spheroplasts and L Forms*, The Williams & Wilkins Co., Baltimore, *in press*). Two other L forms used were derived from diphtheroid strain NMI and *Staphylococcus aureus* ATCC 6538P, and two cultures, designated as LX, were isolated from L forms derived from *Staphylococcus* strain 6538P and from group A β-hemolytic *Streptococcus*

strain GL8. The K5 strain of *Mycoplasma* was isolated from goats.

The B-type L forms of *Proteus* were produced by inoculating the bacteria into melted nutrient agar containing 0.5% NaCl and 5 to 10% horse serum, and overlayering this mixture on agar plates of similar composition, which contained about 1,000 units of penicillin per ml. After 2 hr of incubation at 30 C, the bacteria were enlarged, and a few small granules were present around some bacteria. After 6 hr of incubation, the bacteria grew to large bodies and were surrounded by a dense growth of small granules. At 24 hr, the colonies were large and many granules in them developed into large bodies. Electron micrographs were prepared from these cultures after 2, 4, 6, 24, and 48 hr.

We did not succeed in observing the derivation of A-type L forms of *Proteus* from bacteria, nor did we succeed in obtaining a sufficiently dense growth of them by inoculating the culture within the medium. Sections were made from 2-day-old surface colonies embedded in and spreading within the agar. The colonies examined were those developing in the first transplants of the A-type colonies derived from bacteria.

In 1955, W. Hijmans isolated diphtheroid strian NMI from the human mouth in this laboratory. This strain corresponds to *Corynebacterium* in cultural properties and in morphology (no further identification was made), and it produced L forms abundantly when inoculated on nutrient agar media containing 10% horse serum, 3% NaCl, and penicillin. The young L colonies were very similar to colonies of *Mycoplasma*, but grew only when the concentration of salt in the medium was increased. They did not develop well in pour plates. For this study, 18-hr surface L colonies were used. The colonies were embedded in the medium and consisted of small granules at this stage.

The L form derived from *S. aureus* was isolated in 1955. During the years of cultivation, its properties have changed considerably. At present, it grows abundantly on nutrient agar and in broth containing 3% NaCl, either with or without animal serum. The surface growth of agar colonies and broth cultures consisted of very large bodies, but growth extending from the surface of the colonies into the agar consisted of small granules. We were not able to induce sufficiently abundant growth of young colonies within the agar, so that the growth of 18-hr colonies embedded in the agar was studied.

The L forms designated as LX were isolated from L cultures of *Staphylococcus* and from the group A β-hemolytic *Streptococcus*. The colonies of these strains developed as secondary growth in aging cultures of the original L forms. We could not determine whether they derived from the L forms or were an admixture to the colonies. The properties of the LX forms are described in a recent publication (3). Their appearance corresponded to L forms, and they required increased salt concentration for growth, which, after a few days of incubation, was heavy and confluent. The cultures grew abundantly as small colonies within agar media and were studied after 18 hr of incubation.

Mycoplasma strain K5 was donated by Dr. Adler, who isolated it from an epizootic of goats. This strain grew abundantly within agar medium containing 10% horse serum, and the sections for electron microscopy were taken from such cultures incubated for 18 hr at 32 C.

A number of fixation and embedding methods were employed. The initial fixative was one of the following: 6.25% glutaraldehyde in Ryter-Kellenberger (RK) buffer (6) for 1 hr, or the vapor of 4% osmium tetroxide for 20 min, or the vapor of 37% Formalin overnight. When Formalin was used, the whole culture was exposed in a sealed container. For the other fixatives, a thin slice was cut from the agar containing the cultures, and this slice was divided by a razor blade into 1 to 2 mm^3 pieces.

This initial fixation was usually followed by 1 hr in 1% osmium tetroxide and 1 hr in 0.5% uranyl acetate, both made up in the RK buffer. Embedding was mainly in Epon, but prepolymerized methacrylate was found quite satisfactory for the B-type L forms. The fixative and embedding methods used are noted in the legend for each micrograph. The thin sections were stained with uranyl acetate and lead hydroxide and were examined in a Siemens Elmiskop I electron microscope.

RESULTS

Photomicrographs made with the light microscope (Fig. 18–26) indicate the appearance of the cultures when studied with low and high magnification. The extension of the growth within the agar is apparent, but the resolution of the light microscope is not sufficient to form a sharp image of the small granules. The pictures are useful, however, in comparing the morphology as seen with light and electron microscopes. The cultures studied, with the exception of those of the B-type L form of *Proteus*, consisted of small granules. The development and morphology of several L forms was described and illustrated in previous papers, the most recent dealing with gram-positive cocci (3).

The terms used to designate the structures in the micrographs, such as blebs, small granules, and irregular branching masses, are purely descriptive. The only exceptions are the "elementary corpuscles." These are characterized by their derivation from the larger forms and by their density. The "elementary corpuscles" were thought to be characteristic of *Mycoplasma*.

B-type L forms of Proteus. A large body developing from a bacillus and the earliest growth of granules from it are illustrated in the electron micrograph in Fig. 1. The contour of the large body is irregular, and small waves and irregular granules, about 1 μ or somewhat larger in size, can be seen separating from it. The contour of the granules is as irregular as that of the large body. It appears that ribosomes and nuclear areas were transferred into them from the large bodies. In

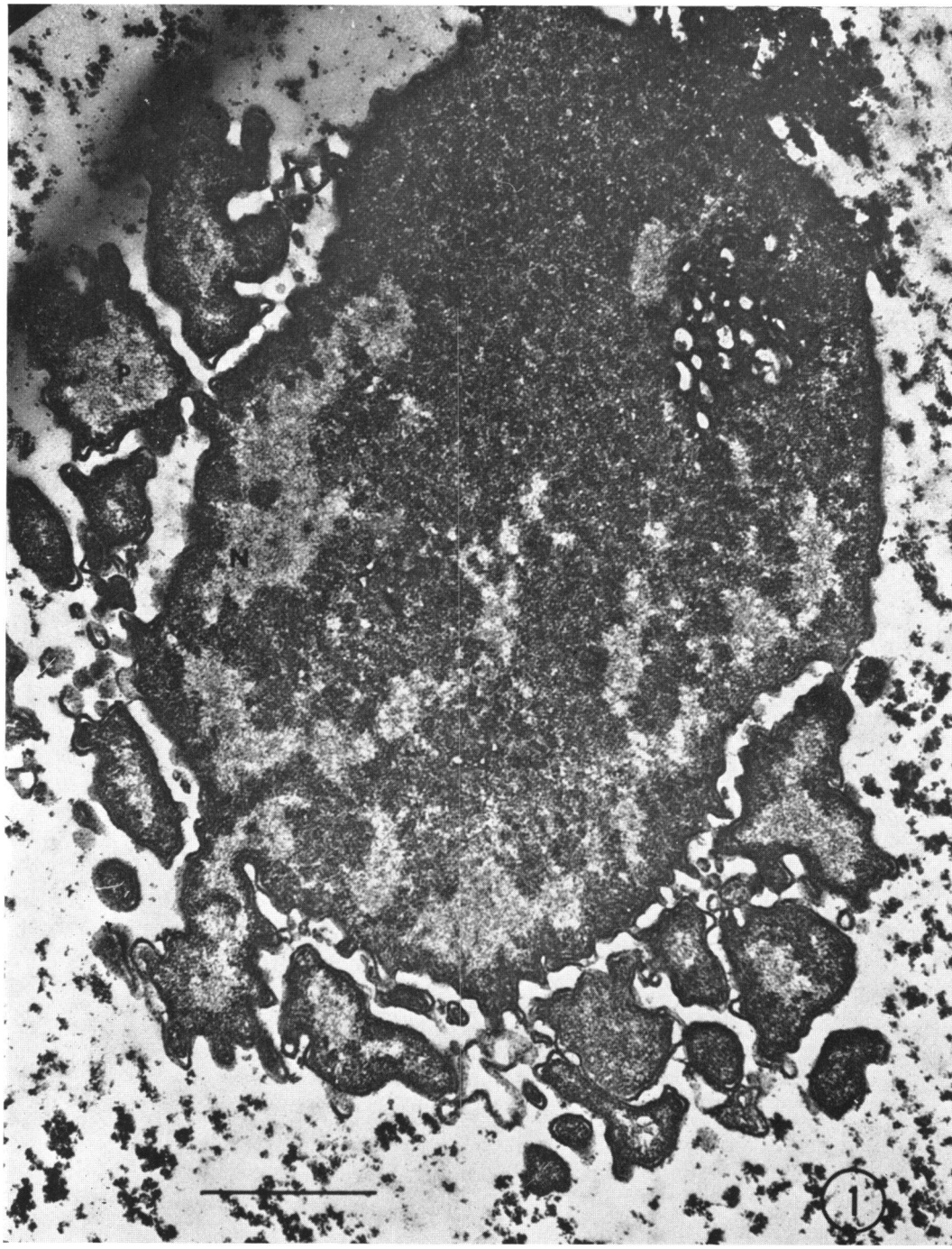


FIG. 1. *B-type L forms of Proteus after 4 hr of incubation of the bacteria in the presence of penicillin. Virtually the whole of the large body is shown. The DNA-containing nucleoids (N) are compact and similar in structure to those seen in normal bacteria. Many of the irregular projections (P) contain DNA. The large body and the projections are surrounded by a double-layered cell wall. This is seen better with higher magnification (Fig. 3). Fixation was in osmium tetroxide vapor, followed by uranyl acetate, according to the RK method (6). Embedding was in prepolymerized methacrylate. In the electron micrographs, the scale line is 1 μ in length, except where otherwise indicated.*

addition to these granules, very small, usually empty, vesicles may be seen detached from the wavy outside membrane of the large body. It is apparent with high magnification (Fig. 2, 3, 5) that the cell envelope of the large body and of the detached granules consists of a plasma membrane and an outer cell wall like those of the bacteria. The structures inside the cell wall are similar to those seen in the bacteria. In young large bodies, multiple distinct nuclear areas are usually arranged near the surface of the organism.

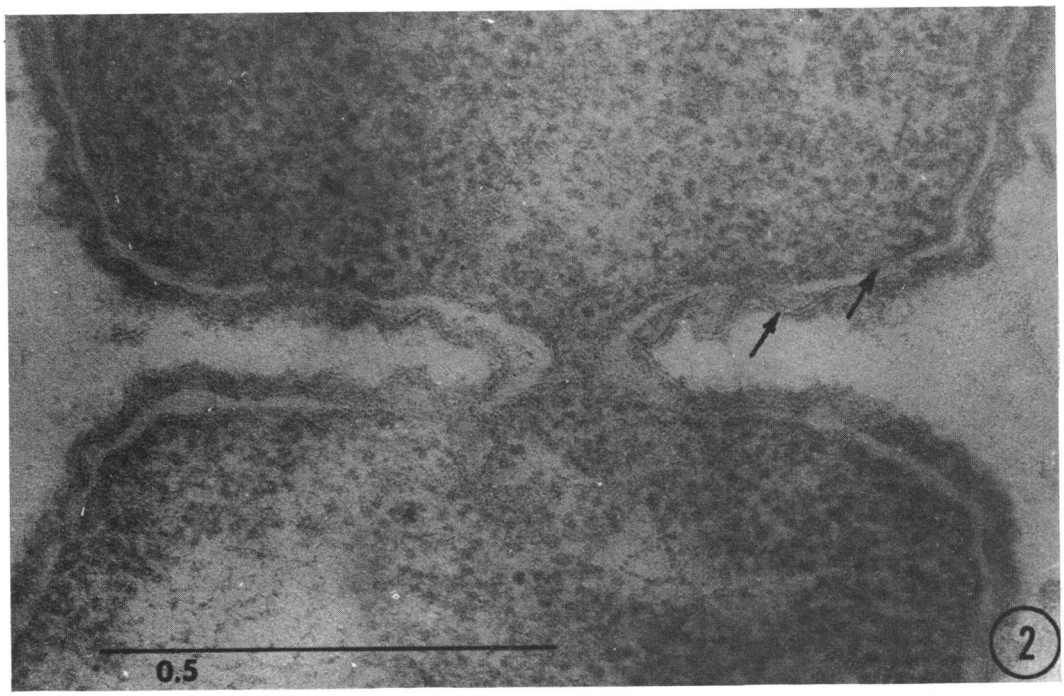
Multiplication of the granules, like their origin from the large bodies, seems to occur by irregular growth and segmentation. Some granules divide further into smaller granules, a few tenths of a micron in size (Fig. 5). Whether these granules take part in multiplication is not apparent. After 24 hr of incubation, many organisms in the colonies grew into large bodies (Fig. 20). Some of these organisms have (as pictured in the electron micrographs) smooth surfaces and regular shapes and probably do not continue to multiply. Other organisms have wavy membranes, seem to multiply like the original large bodies developing from bacilli, and are detached parts with a double cell wall around them. In addition, there are many very small granules present (some less than 0.1 μ in size); some are full, but many are empty. After 48 hr of incubation, these small granules were found in certain areas in masses. In many large bodies, the granules were found between the two layers of the cell wall, and most granules appeared to be full (Fig. 4).

A-type L forms of Proteus. With the electron microscope at low power, it was apparent (Fig. 6) that many organisms were empty or nearly so and collapsed membranes were present. Lysis of early growth was also seen with the light microscope. The size of intact organisms varied greatly. The largest intact organisms were irregular masses, several microns in size, extending in several directions (Fig. 6, 17). Elementary corpuscles, corresponding in size and density to those seen in cultures of *Mycoplasma*, and all transitional forms between these and the large organisms were present in varying numbers. These elementary corpuscles appeared to grow out from the larger forms and to be present as short chains and filaments (Fig. 8). With high-power magnification (Fig. 7, 8), the membranes of all organisms appeared as a unit membrane corresponding to the internal plasma membrane of bacteria. Inside the membrane, there were ribosomes and threads of DNA. The larger organisms had a smooth surface, and protrusions of the dense regions appeared to be the precursors of the elementary corpuscles (Fig. 6). The organisms at the edges of the colonies were variable. They were either irregular large masses filled with ribosomes, or membranes left after autolysis of these large masses, or preponderantly elementary corpuscles.

L forms of diptheroid NM1 and of a staphylococcus. Micrographs made from these strains were not as successful technically as those from other L forms and are not published here. A micrograph of the diphtheroid L forms is reproduced in another paper (L. Dienes, *in press*). It was apparent that the morphology and structure of these L forms were similar to those of the A-type L form of *Proteus* and of the LX cultures. The cultures consisted of irregular elongated large forms, elementary corpuscles, and transitional forms between them. Derivation of the elementary corpuscles from the larger forms and their growth in short chains and filaments were apparent. All organisms were enclosed in a single unit membrane.

LX from L forms of streptococci and staphylococci. The morphology and fine structure of the LX organisms were similar in the two strains, and only those obtained from *Staphylococcus* are illustrated. With low magnification (Fig. 9, 15), the majority of organisms were round or oval in some areas (between 0.5 and 1.0 μ), but irregular large forms, similar to the A-type L forms of *Proteus*, were also present. With higher magnification, all organisms appeared to be surrounded by a unit membrane (Fig. 10). In most cases, the ribosomes were located at the periphery of the organisms and the nuclear areas were located at the center. There was considerable variation in the appearance of nuclear areas, probably owing to fixation and staining. The origin of elementary corpuscles from the larger organisms was apparent (Fig. 10, 11), as was their arrangement in short chains. Fragments of thin filaments were often visible, and, in one micrograph, the growth of a thin dense filament from a large organism was seen (Fig. 11). In some areas of the micrographs, most of the elementary corpuscles were in short chains or were oval and elongated. This suggested that the elementary corpuscles were sections of filaments and that such structures were not accidental but an essential part of the colonies. The organisms at the extending edge of the colonies were as variable as in the A-type L form of *Proteus*. In some cases, small granules, in groups or in short chains, and fragments of very thin filaments were present (Fig. 15). Some of these groups, located far from the densely grown part of the colony, were connected with empty membrane-bound sacs and apparently originated from them.

Mycoplasma strain K5. Young colonies grown within the agar consisted of irregularly shaped,



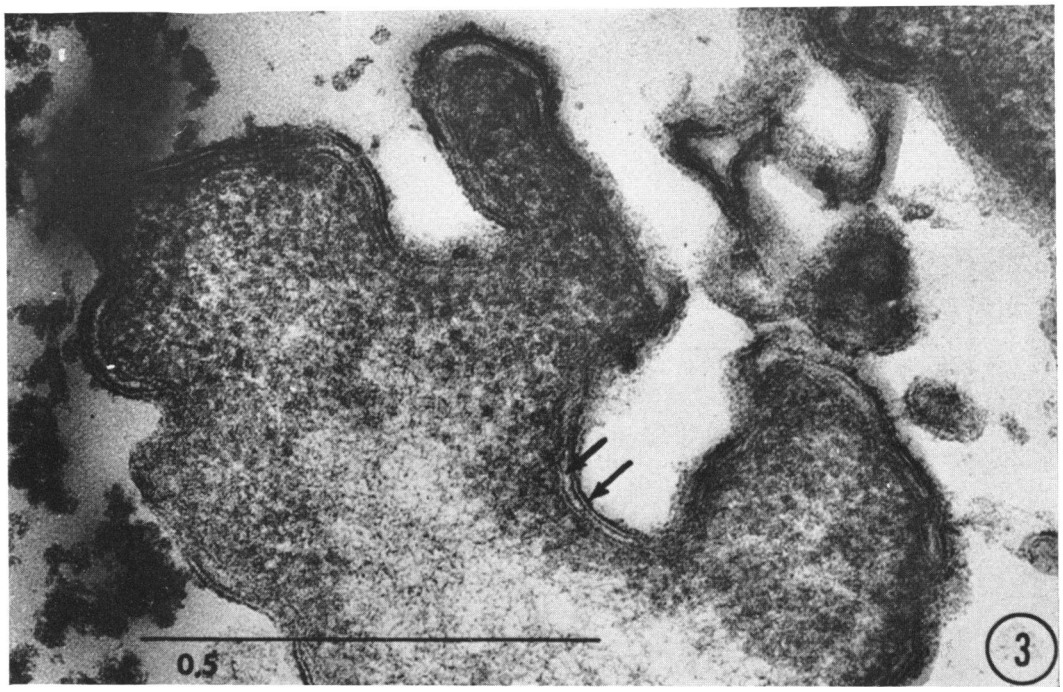
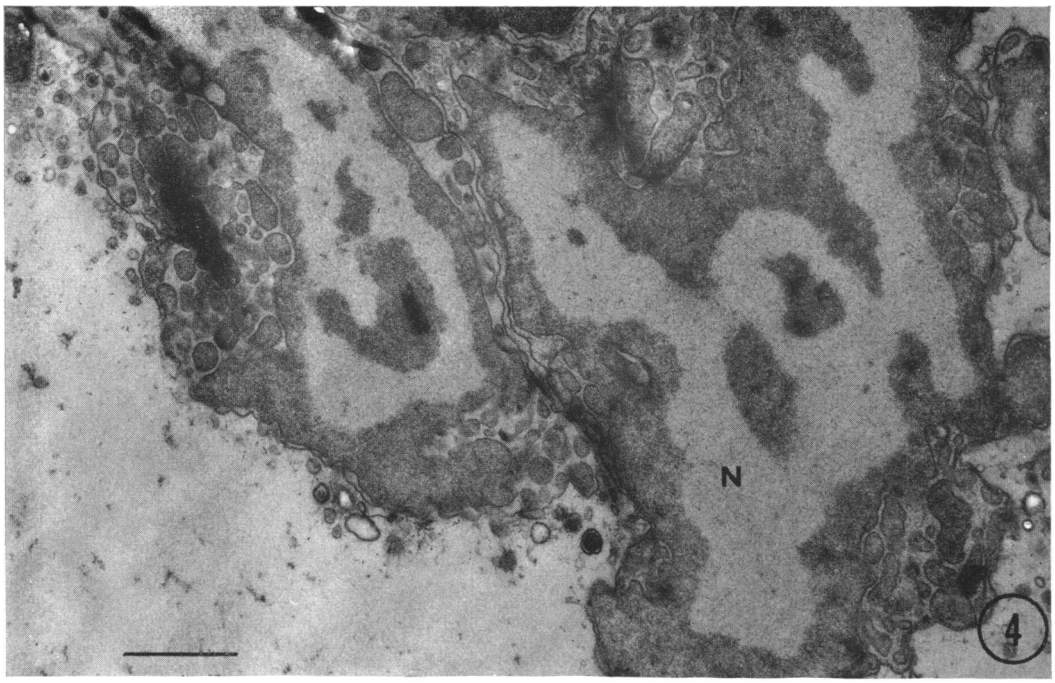


FIG. 2. *Region between two dividing cells of normal Proteus grown within agar in a manner similar to the B-type L form, with the exception that there was no penicillin present. The bacterium is surrounded by an inner plasma membrane and an outer cell wall. The fixation is the same as in Fig. 1, but the material was embedded in Epon.*

FIG. 3. *Higher magnification of part of an outgrowth of the B-type L form as shown in Fig. 1. There are two limiting membranes (arrows) similar to those seen in the normal bacterium in Fig. 2. Fixation and embedding are the same as in Fig. 1.*



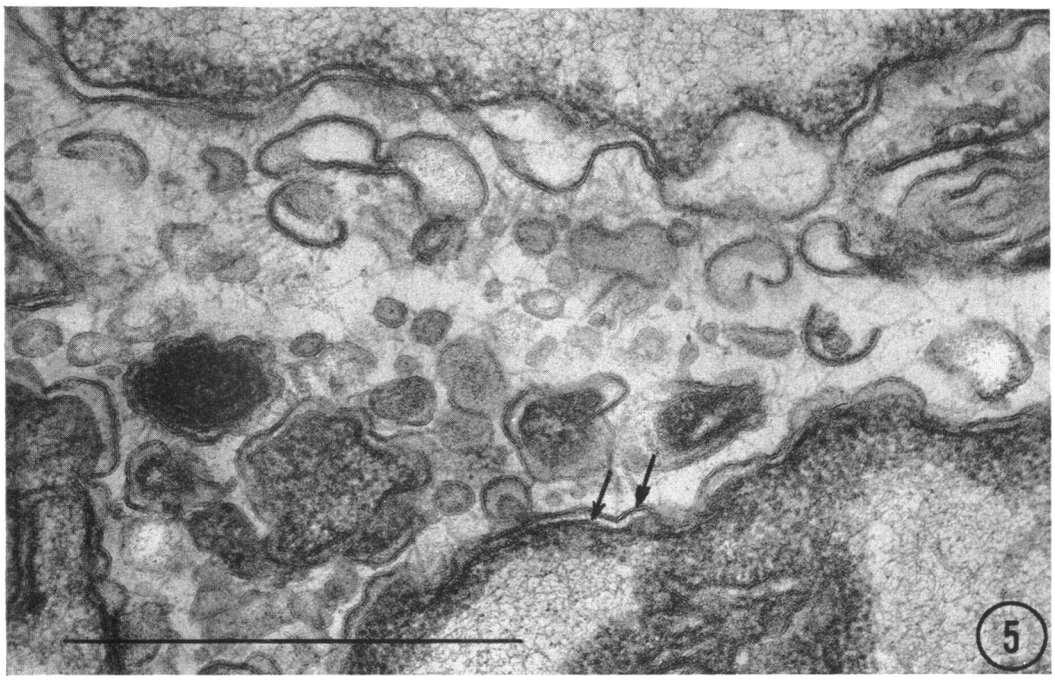
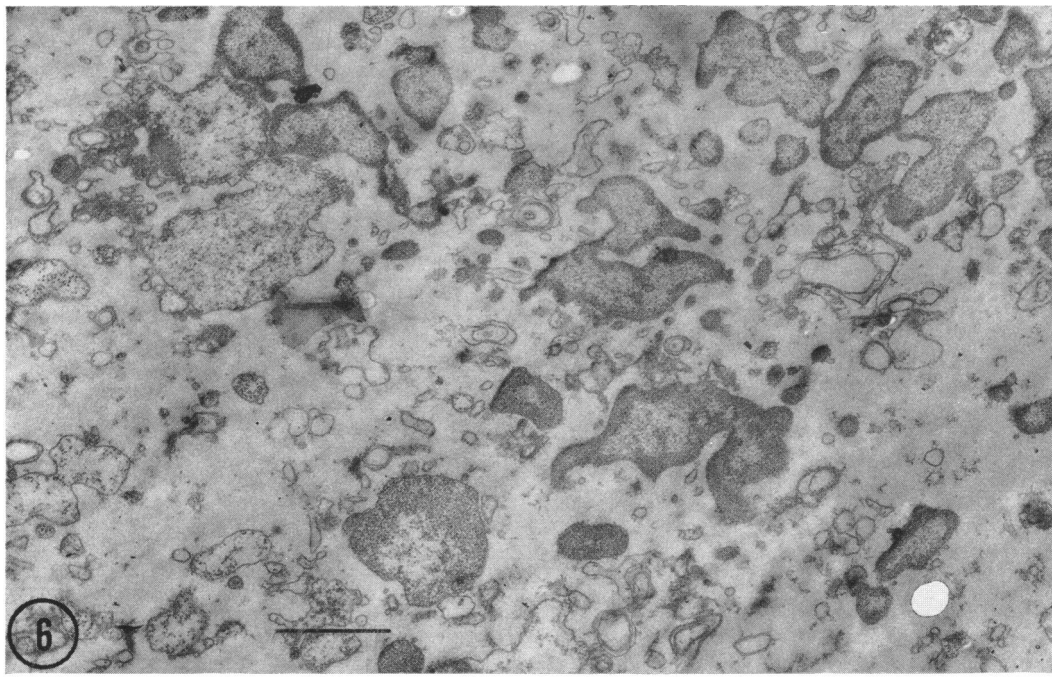


FIG. 4. *Two-day-old culture of B-type L form of Proteus showing part of a large body in a colony. The two limiting membranes are retained, and there are many small granules either free or between the two membranes. There is a large interconnected well-defined nucleoid (N). Fixation and embedding are the same as in Fig. 2.*

FIG. 5. *Higher magnification of part of a culture similar to that shown in Fig. 4. Two membranes (arrows) surround the organisms. Fixation and embedding are the same as in Fig. 2.*



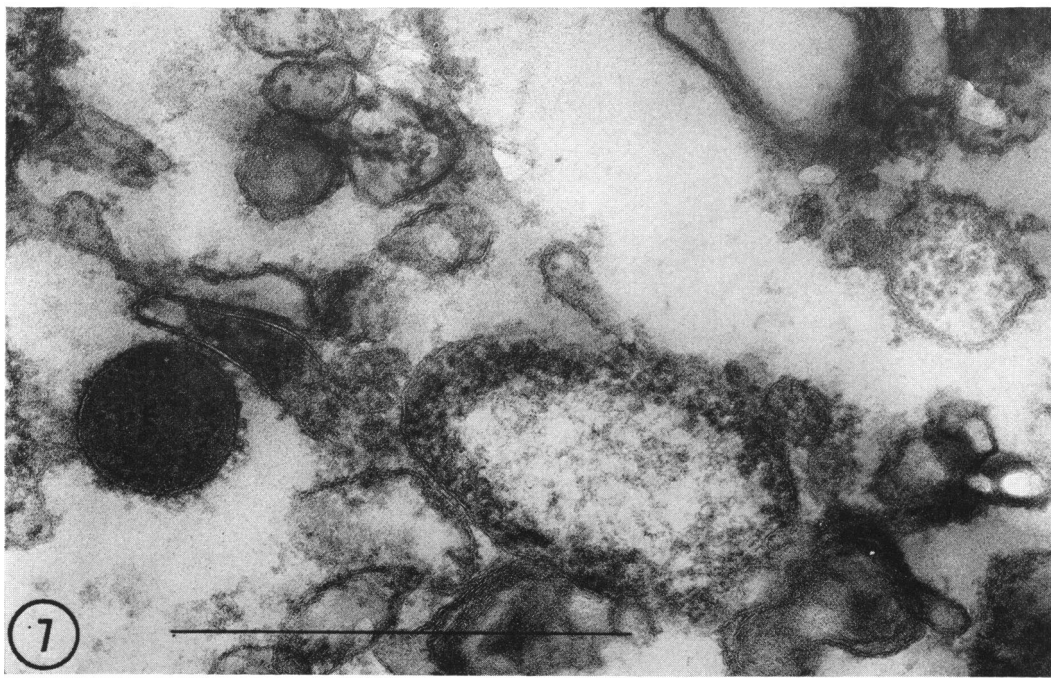
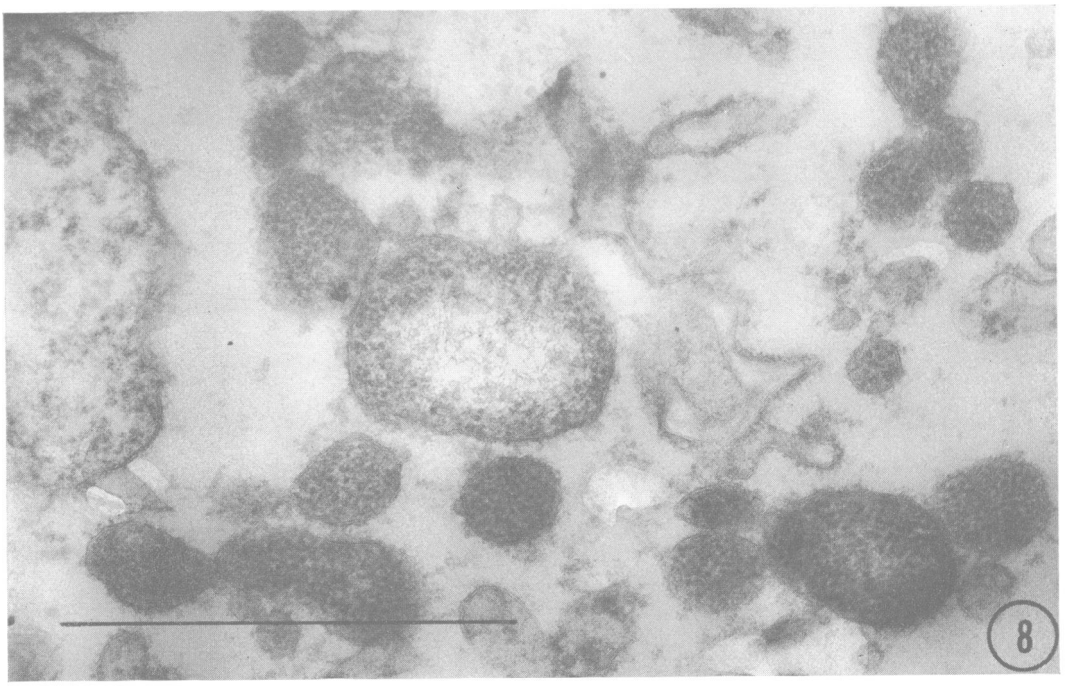


FIG. 6. *Part of a colony from a 2-day culture of the A-type L form of Proteus. There are large irregular forms with peripheral ribosomes and central DNA-containing areas. Elementary corpuscles can be seen. There are also large empty forms and a large amount of membrane-bound debris. Fixation was in Formalin vapor, followed by osmium tetroxide vapor and uranyl acetate, according to Ryter and Kellenberger (6). Embedding was in Epon.*

FIG. 7. *Similar culture to that shown in Fig. 6 but at higher magnification. A single unit membrane surrounds all forms. A well-defined elementary corpuscle (E) shows a very dense interior with ribosomes. Fixation and embedding are the same as in Fig. 6.*



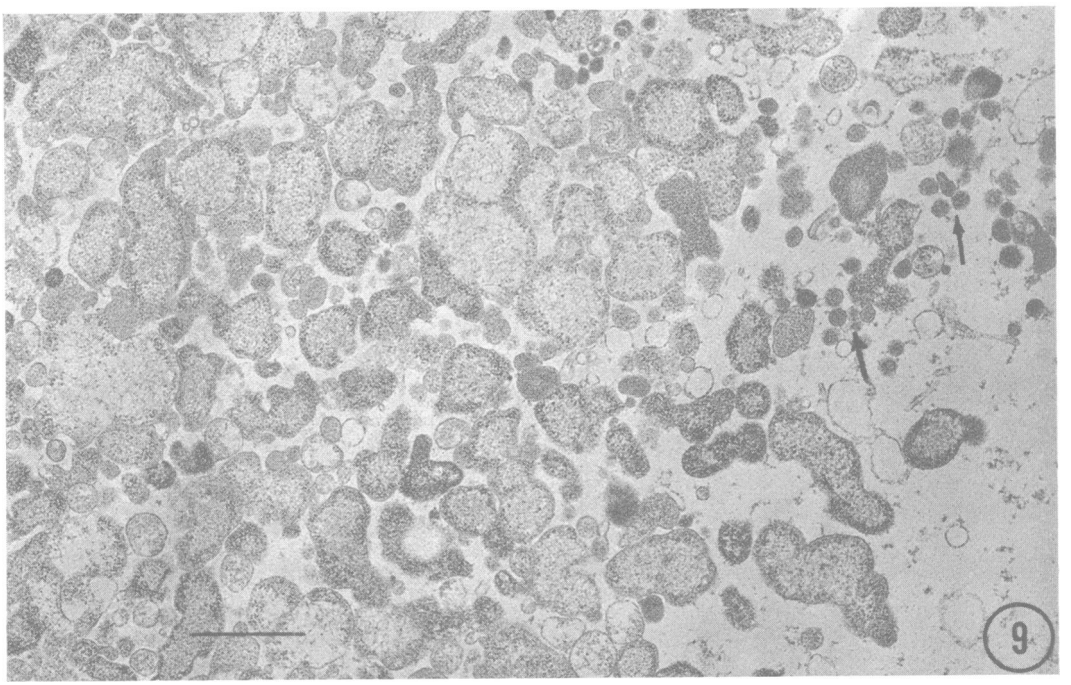
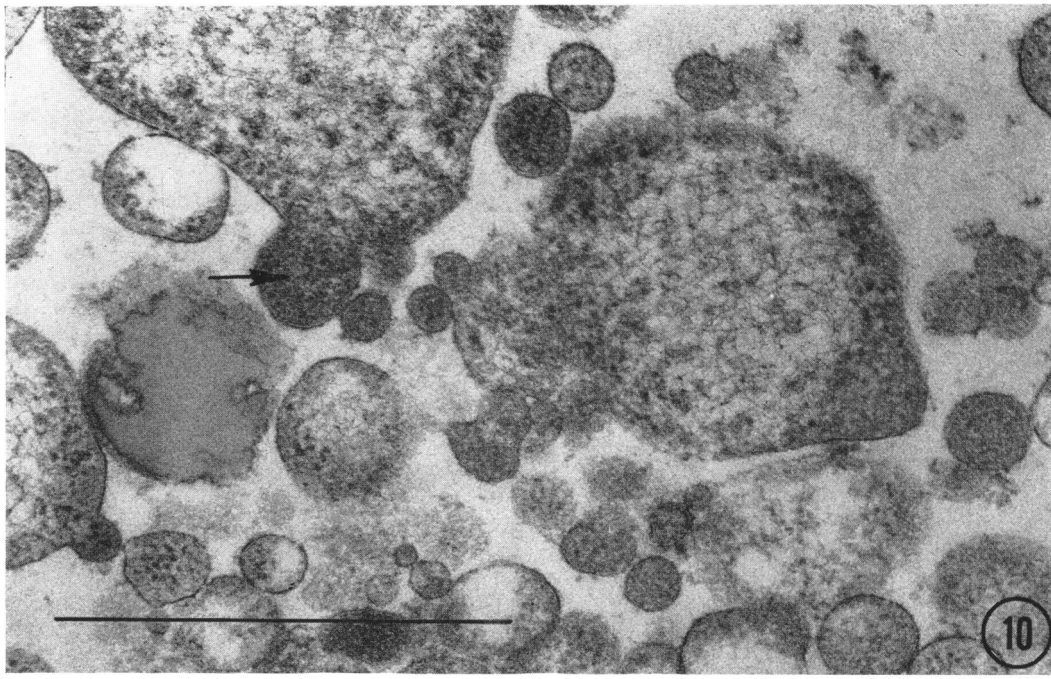


FIG. 8. *Similar culture to that shown in Fig. 6 but at higher magnification. A large number of elementary corpuscles are present. The group to the lower left consists of a short interconnected chain. Fixation and embedding are the same as in Fig. 6.*

FIG. 9. *LX form isolated from an L form of Staphylococcus. There are large forms with peripheral ribosomes, other slightly smaller forms which are denser and have a structure more like that of bacteria, and groups of elementary corpuscles (arrows). Fixation is the same as in Fig. 1, but was embedding in Epon.*



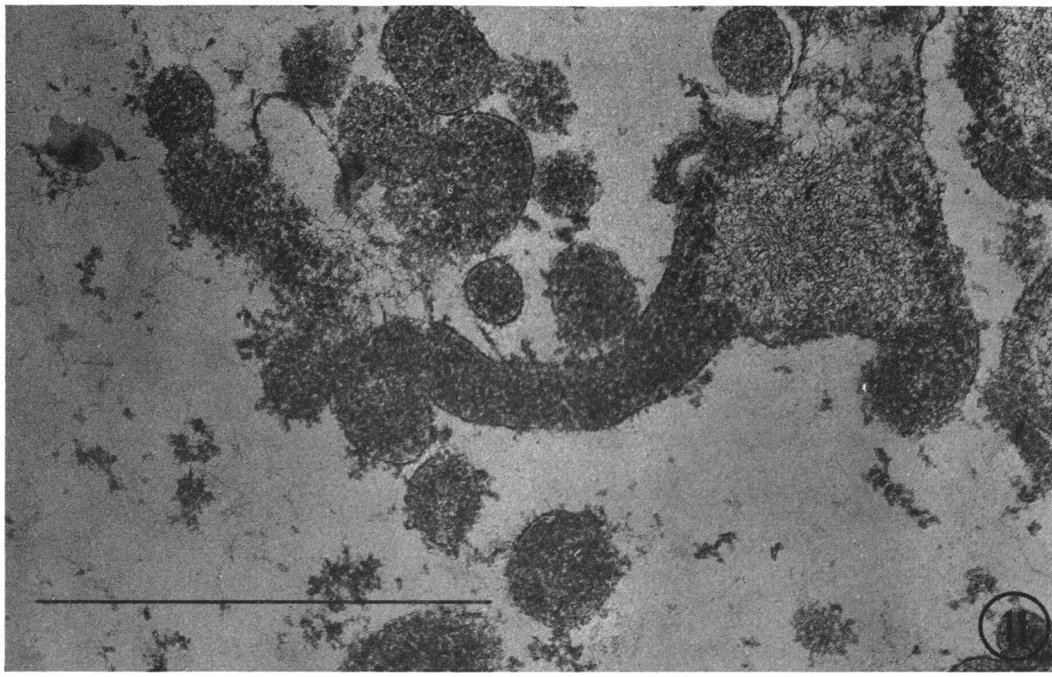


FIG. 10. *Similar culture to that shown in Fig. 9 but at higher magnification. The organisms are limited by single unit membranes. The arrow indicates the outgrowth of an elementary corpuscle from a large body. Fixation and embedding are the same as in Fig. 9.*

FIG. 11. *Similar culture to that shown in Fig. 9 but at higher magnification. There is a filamentous outgrowth (center) from a large body and there are numerous elementary corpuscles. Fixation and embedding are the same as in Fig. 9.*

very large organisms extending in several directions in the medium (Fig. 12, 16). Connected masses, up to the size of 6 μ (Fig. 16), were occasionally seen. They were surrounded by a unit membrane. Depending on the initial fixative, the DNA was either intimately mixed with the ribosomes, as after glutaraldehyde (Fig. 13), or appeared more in the central region, as after Formalin vapor (Fig. 14). The *Mycoplasma* showed a more marked response to variations in fixative than did the L forms. Elementary corpuscles were present but were not numerous at this stage of growth. Their origin from larger organisms was indicated (Fig. 13).

DISCUSSION

Excellent micrographs of *Mycoplasma* have been published by Anderson and Barile (1), Domermuth et al. (4), and Morowitz and Maniloff (8). These micrographs, together with those included in this paper, can be used to compare L forms and *Mycoplasma*. Our observations on *Mycoplasma* differ from those of the investigators mentioned only inasmuch as growth in irregular branching masses, which we saw in young cultures within agar, was not apparent in broth cultures. Very fine pleomorphic filaments were seen by several authors (1) on grids after negative staining. From our experience, we think that these filaments may be artifacts. Reuss (9) expressed a similar conclusion. Micrographs of L forms made by recently developed techniques were published by Weibull (14–16). The micrographs show the unit membrane and, what is of great interest, the development of granules inside the large forms. Among the observations made with less advanced techniques, our observations correspond most closely to those of Van den Hooff and Hijmans (13). They studied sections of colonies of L forms of *Streptococcus*. The structure is not as clearly visible in their micrographs as in those made with present techniques, but the shape of the organisms and the production of dense round granules, probably corresponding to the elementary corpuscles, are apparent.

The most interesting result of our studies is the observation of a close similarity between L forms, with the exception of the B-type, and *Mycoplasma* in young colonies developing within agar. They are similar in morphology, in fine structure, and in the suggested mode of multiplication. L forms and *Mycoplasma* produce similar large masses

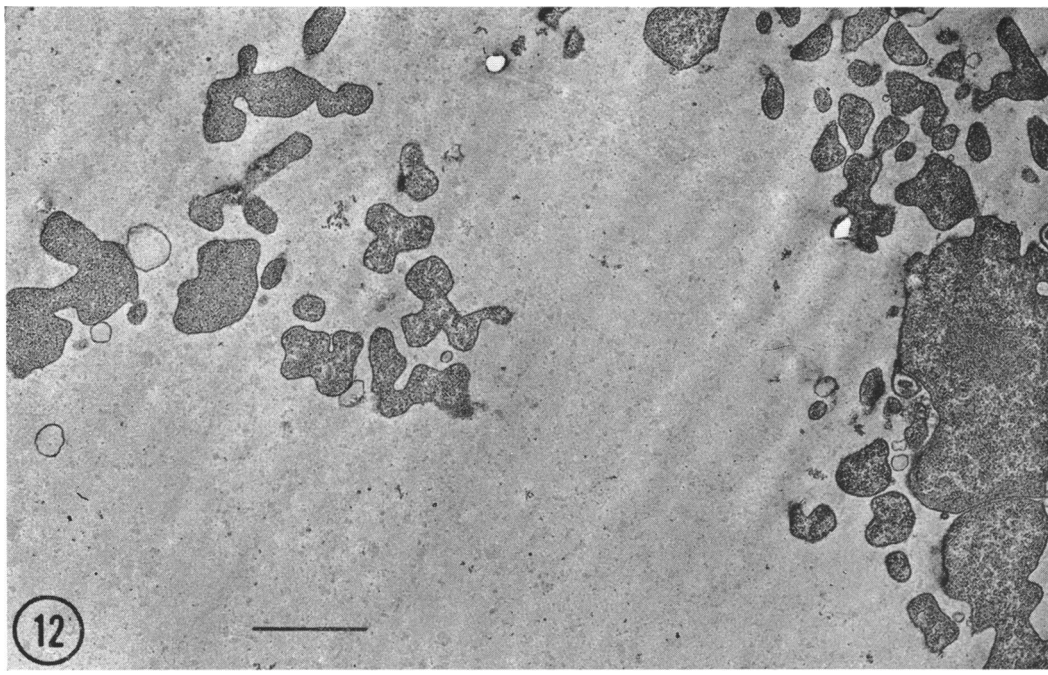
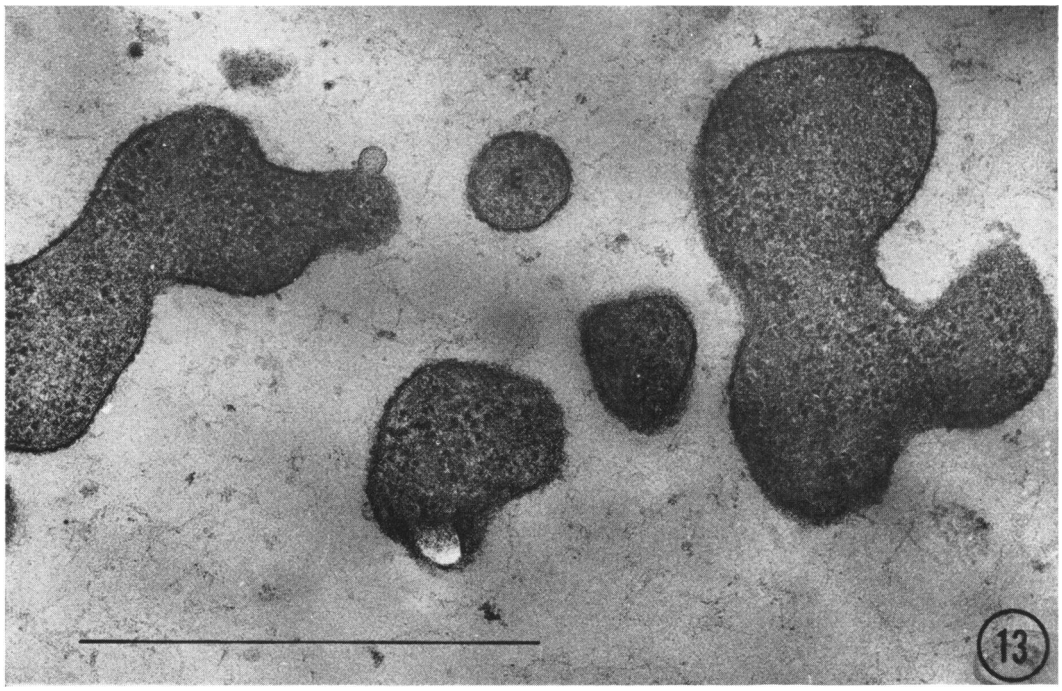


FIG. 12. *Low magnification of part of a colony of Mycoplasma strain K5. There are many irregular forms, some of them quite large (extreme right). Elementary corpuscles can also be seen. Fixation was in glutaraldehyde followed by osmium tetroxide followed by uranyl acetate, all made up in the RK buffer. The material was embedded in Epon.*



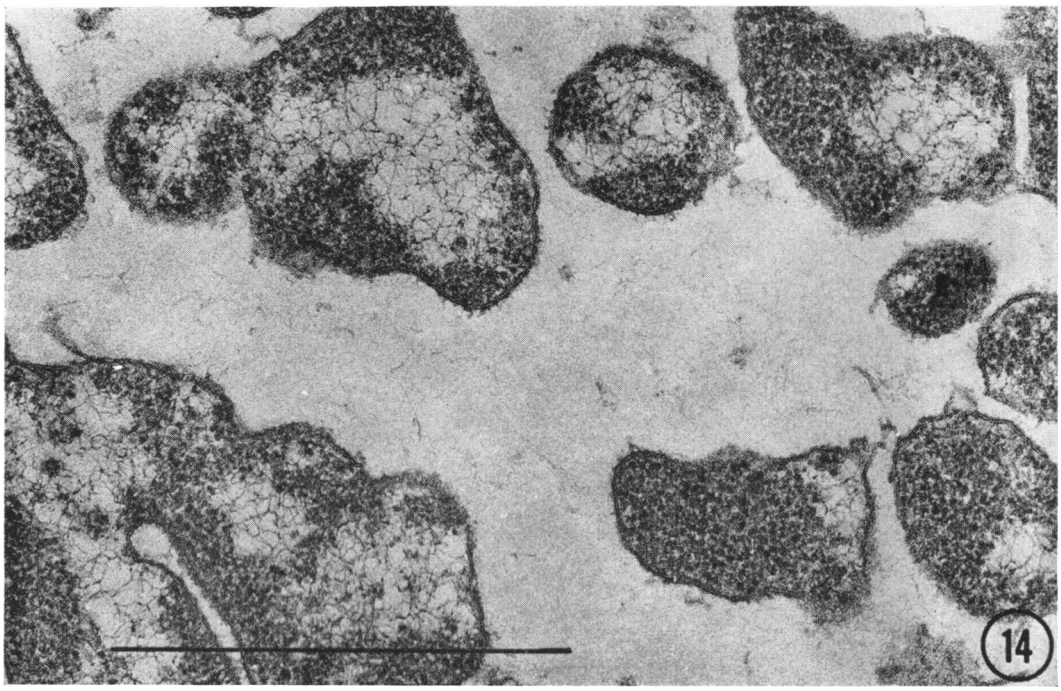


FIG. 13. *High magnification of a Mycoplasma colony similar to that shown in Fig. 12. All forms are surrounded by a single unit membrane. An elementary corpuscle (E) is shown. After this method of fixation, the ribosomes and DNA filaments appear to be intimately mixed. Fixation and embedding are the same as in Fig. 12.*

FIG. 14. *High magnification of colony of Mycoplasma K5. After this method of fixation, the DNA appears more separated into a central region. The unit membrane is well defined. Elementary corpuscle (E). Fixation and embedding are the same as in Fig. 12, but Formalin vapor, rather than glutaraldehyde prefixation, was used.*

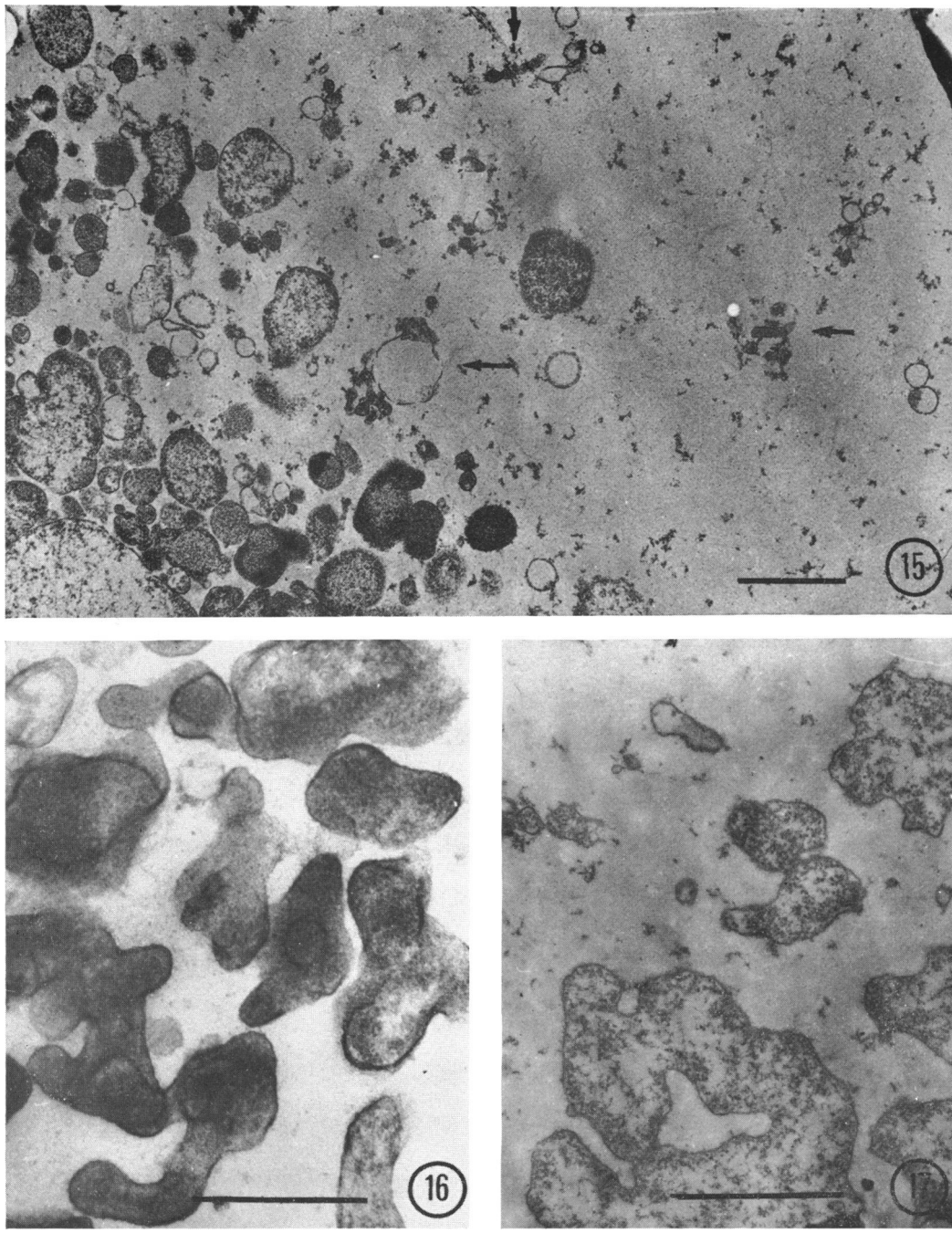


FIG. 15. *Edge of an 18-hr culture of LX of Staphylococcus. Groups of small granules and parts of chains and thin filaments are in the agar, away from the colony. They are marked with arrows. Fixation and embedding are the same as in Fig. 9.*

FIG. 16. *Thick section of a colony of Mycoplasma K5 indicating the tridimensional interconnected extension of the organism within the agar. The appearance of membrane-bound regions within the organisms was due to heavy metal staining on both sides of a thick section and does not indicate that there are bodies within. It does emphasize the three-dimensional structure of the organisms. Fixation and embedding are the same as in Fig. 12.*

FIG. 17. *Section through an A-type L colony of Proteus indicates the extension of the organisms similar to that seen in Mycoplasma in Fig. 16. Fixation and embedding are the same as in Fig. 6.*

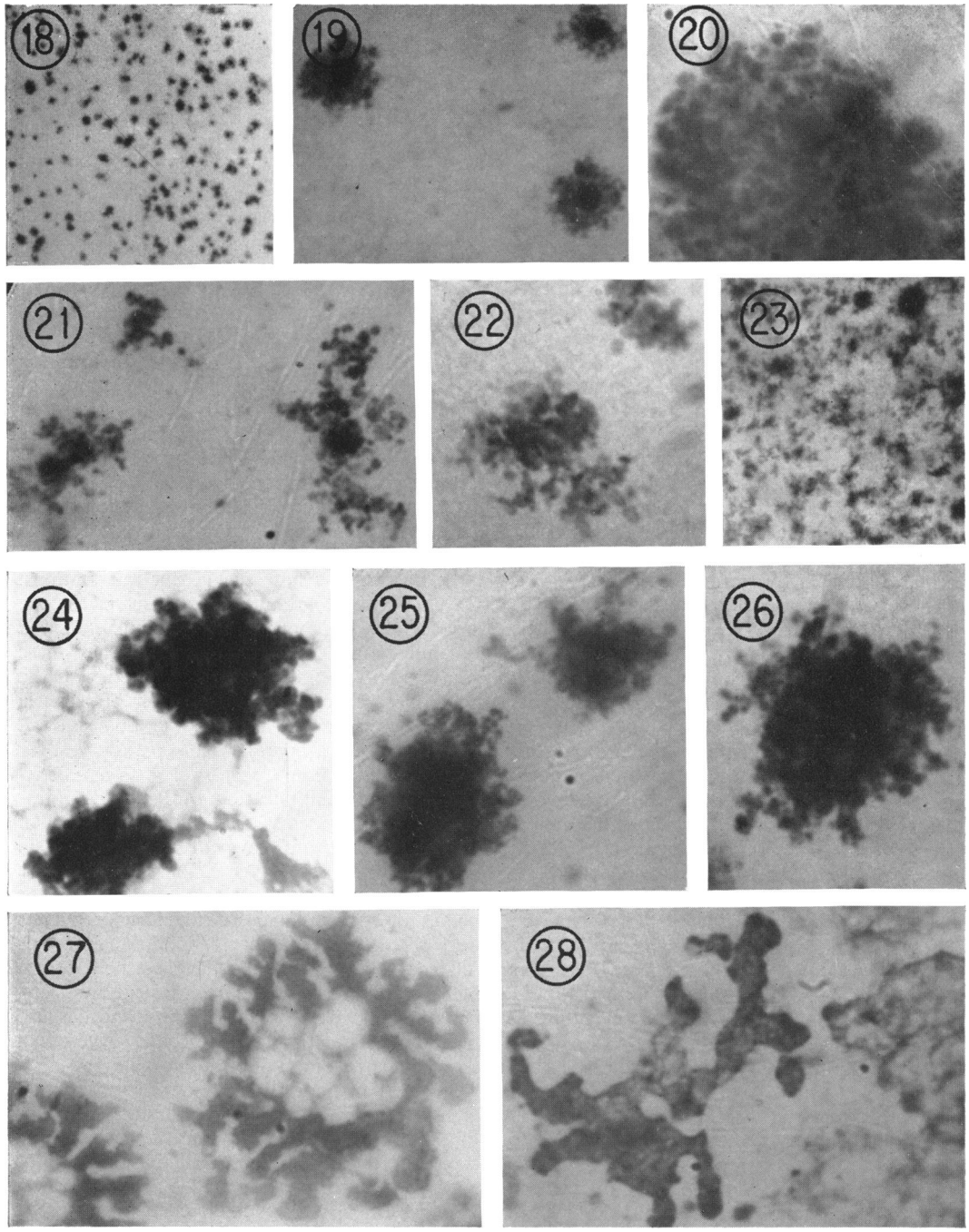


FIG. 18–28. *Figures 18–26 were taken from dried, stained agar preparations, Fig. 27 from an impression from the surface of gelatin, and Fig. 28 from a stained membrane filter.*

FIG. 18. *B-type L colonies of Proteus grown within agar medium for 6 hr.* × *250.*

FIG. 19. *B-type L colonies, like those in Fig. 18, with high magnification.* × *2,250.*

FIG. 20. *Colony similar to those in Fig. 18 and 19, after 24 hr of incubation.* × *2,250.*

FIG. 21 and 22. *A-type L colonies of Proteus developing on agar medium containing horse serum. The colonies are embedded in the medium, and were incubated for 24 hr.* × *2,250.*

FIG. 23. *Colonies of Mycoplasma strain K5 grown within agar media for 18 hr at 32 C.* × *250.*

FIG. 24. *Colonies of LX of Staphylococcus, grown for 18 hr within agar.* × *2,250.*

FIG. 25 and 26. *Colonies of Mycoplasma strain K5 grown within the agar as in Fig. 23.* × *2,250.*

FIG. 27. *Vacuolated large body of the L form of Staphylococcus, extending irregularly without division on a medium containing 30% gelatin.* × *2,250.*

FIG. 28. *Large body of the L forms of Streptococcus, extending irregularly on the surface of a 0.3-μ(pore size) membrane filter.* × *2,250.*

extending in various directions in the agar. To emphasize this similarity of the large organisms, a micrograph from strain K5 *Mycoplasma* (Fig. 16), made from a thick section, is presented beside a micrograph from an A-type L form of *Proteus* (Fig. 17). Elementary corpuscles, which are regarded as characteristic of *Mycoplasma*, are produced by the L forms as well. The cell membranes and the structures inside them are similar, and both differ from those seen in other groups of microorganisms. The morphology of L forms varies considerably after prolonged cultivation, and the environment exerts a great influence on it. Considering this potential for great variability, the similarity of agar cultures of L forms to *Mycoplasma* is of greater significance than the differences observed under less favorable conditions. Study of L forms under variable conditions indicates that characteristics thought to be specific to *Mycoplasma*, such as growth in filamentous form, also occur in L forms (L. Dienes, *in press*).

The structural basis of one variant of the L forms of *Proteus*, which we called B-type (2), is apparent in the micrographs. In this type of L form, the outer cell wall of bacteria is retained, although the loss of rigidity indicates that its structure is altered.

Two types of reproduction of the L forms and *Mycoplasma* are suggested in the micrographs. One is growth in irregular masses extending in different directions. Sections of such masses, between 4 and 6 μ in size, are seen in both L forms and *Mycoplasma*; the actual interconnected tridimensional growth extending in several directions can, of course, be much larger. Segmentation of these large organisms evidently occurs. Similar multiplication of L forms is apparent with the light microscope on the surface of solid media (L. Dienes, *in press*) and was seen in broth cultures in L forms of gram-positive cocci (3). On the surface of membrane filters, large bodies may extend as irregular branching masses (Fig. 28), and narrow outgrowths from the large bodies embed themselves into the pores of the filter and continue to extend in it. The growth of large bodies on the surface and the extension of small granules within the agar appear to be essentially analogous (L. Dienes, *in press*).

Another type of multiplication suggested is the development and growth of elementary corpuscles. The short chains and the fragments of filaments of similar thickness seen in the micrographs indicate that the elementary corpuscles develop as an outgrowth from the large organisms. The presence of forms transitional in size and structure between the elementary corpuscles and the large organisms probably indicates enlargement of the corpuscles. It was observed in *Mycoplasma* that elementary corpuscles are present in largest number at the end of the growth period (1). This may be the result of their enlargement within a short time during the period of fast growth, whereas later they fail to enlarge. From observations of the growth of granules from large bodies on the surface of agar and on membrane filters with the light microscope (L. Dienes, *in press*; 7), it is apparent that condensations develop on their surface and growth starts from them. The division of elongated granules embedding themselves into the agar also seems to be preceded by development of condensations at the two ends and disintegration of the organisms between them. These processes may correspond to the development of elementary corpuscles within the agar.

One process of multiplication apparent in broth cultures of L forms of gram-positive cocci, the development of small viable granules inside the large bodies, was not seen in our micrographs. Development of granules was observed in the large bodies by Thorsson and Weibull (12) and Weibull (14). It is not known whether these granules correspond to those observed with the light microscope. The reproduction of bacteria which was observed inside large bodies of L forms (L. Dienes, *in press*) is of great interest for further investigation.

The initial multiplication of B-type L forms within the agar seemed similar to the growth of irregular masses and segmentation observed in other L forms. The B-type L form differed from other L forms since it tended to grow to large bodies of regular shape, elementary corpuscles were absent, and it frequently produced empty or full small blebs or granules. The presence of these blebs or granules, sometimes in large numbers, between the two membranes of the cell wall is also of interest. The micrographs do not suggest that they have a role in multiplication (Fig. 4).

There is no indication in the micrographs of either L forms or *Mycoplasma* as to which type of organism is able to penetrate the agar and extend in it. In many cases, fairly large organisms are visible at the extending edge of the colonies; in other cases, numerous elementary corpuscles are visible. Full and autolyzed large organisms may be at some distance from the colony, together with a few elementary corpuscles. In a few micrographs, small groups of granules, smaller than the elementary corpuscles, and fragments of short chains and filaments of similar thickness are visible outside the colony (Fig. 15). Occasionally, fragments of such thin filaments are visible inside the colonies. It may be that if colonies are studied at an earlier stage of development and if more attention is given to the edge of the colonies,

information will be obtained as to whether these very small organisms play any part in growth.

Whether physical processes, such as streaming of liquid inside the agar, exert an influence on the extension of the colonies is not known. Streaming inside the colonies was observed (11). It also occurred on the surface of the agar by alteration of surface tension. With the light microscope, the growth of whole tiny colonies could be observed, and extension of the colonies seemed to occur only by contiguous growth.

We called attention only briefly to a few points that seemed of interest in the micrographs. The morphology and various other properties of L forms and *Mycoplasma* are complex, and opinions expressed on the significance of similarities between the two groups vary considerably. We refer the reader to a recent paper (L. Dienes, *in press*) for a detailed discussion of pertinent observations in this regard.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-05625, from the National Institute of Allergy and Infectious Diseases, and GM 11427, from the National Institute of General Medical Sciences.

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