MORPHOLOGICAL STUDIES IN THE GENUS NOCARDIA

II. Cytological Studies[1]

NORVEL M. McCLUNG[2]

Department of Botany, University of Michigan, Ann Arbor, Michigan

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Problems regarding the cytology of actinomycetes have been considered by Neukirch (1902), Drechsler (1919), Lieske (1921), and more recently by Grigorakis (1931), Badian (1936), Newcomer and KenKnight (1939), Von Plotho (1940), Carvajal (1946), Klieneberger-Nobel (1947), and others. Most of these investigations concerned organisms belonging to the genus *Streptomyces*. Such problems as the properties of the cell wall, septation of hyphae, the nature of the cytoplasm and its contents, and the manner of cell division and spore formation have been considered. The extreme narrowness of filaments (about 1 micron) renders internal structures difficult to study; thus decisions regarding their nature have in some cases been controversial and inconclusive.

The present studies were undertaken to add to the information of the cytology of actinomycetes by means of both light and electron microscopy and by the use of microchemical tests. The organisms investigated belong to the genus *Nocardia* (*Proactinomyces*), and for the most part one strain, *Proactinomyces ruber* (Casabó) Baldacci,[3] was used. This strain was selected for detailed study because it grows well and is intermediate in morphology between forms that are bacteriumlike and those that are *Streptomyces*-like.

METHODS AND RESULTS

Morphology of old cultures. Stained preparations of 6-month-old cultures of 46 strains of *Nocardia* were made in order to study their ultimate development. Basic aniline dyes were used as stains. A study of these preparations revealed that in strains which fragment (figures 1 to 10) pleomorphic rods, coccoid cells, and short hyphae were present. In some strains coccoid cells predominated (figures 1 and 5), but in others few coccoid cells were found and pleomorphic rods were more frequently present (figures 3, 6, 9, and 10). Preparations were made of strains that do not fragment by grinding the mycelium between two glass slides (Lieske, 1921). Uniform preparations were easily made with some of these forms, as the mycelium did not cling together in clumps but was well dispersed on the slide. Preparations of these strains showed weakly stained filaments that corresponded to the "ghost cells" of old cultures of bacteria in which the cyto-

[1] From a dissertation submitted to the Horace H. Rackham School of Graduate Studies, as a partial requirement for the Ph.D. degree.

[2] Present address: Department of Botany, University of Kansas, Lawrence, Kansas.

[3] This organism would now be placed in the genus *Nocardia;* however, the old name is retained here, as matters of redesignation are beyond the scope of the present work.

plasm has disintegrated leaving the cell walls (figures 14 and 15). Some filaments stained homogeneously, whereas in others condensations of the cytoplasm with weakly staining intervals between them were seen (figure 12). Colonies of these organisms have a tough, cartilaginous texture. Uniform preparations were difficult to make in other strains that do not fragment because clumps of mycelium persisted. Hyphal fragments 7 to 30 microns long were present, in some of which deeply stained areas alternated with faintly stained ones (figures 11 and 13). Colonies of strains that had this structure were waxy in texture.

General staining reactions. In order to study the staining characteristics of *Nocardia*, simple and acid-fast staining was done on both young and old material of 46 strains. Cells from young cultures of all strains were stained according to the gram method. All forms, even as old as 6 months, when grown on glycerol nutrient agar stained readily with the basic aniline dyes: basic fuchsin, methylene blue, and gentian violet. All 46 strains were non-acid-fast when stained as suggested by Umbreit (1939), in both young and old material. Young cultures were uniformly gram-positive.

The cell wall. The cell wall was studied by means of staining, electron microscopy of unshadowed material, and light and electron microscopy of material shadow-cast with a metal.

In preparations stained with basic dyes the wall does not retain the stain, hence is not visible. However, in strains of *Nocardia* having long filaments, empty, faintly stained hyphae can be found (figures 14 and 15). These are more prevalent in old cultures and represent walls that remain after the cytoplasm has disintegrated, corresponding to the so-called "ghost cells" found in old cultures of bacteria. In certain strains one can also see a faintly stained wall between cytoplasmic areas in filaments.

Preparations of *Proactinomyces ruber* were treated with Dyar's (1947) cell wall stain. The presence of a wall distinct from the cytoplasm was clearly demonstrated by this method. The deposit of dye on the wall exaggerated its thickness but clearly demonstrated its presence. When compared with *Bacillus subtilis* stained in the same manner, the wall of *P. ruber* appears about half as thick.

Specimens for electron microscopic observation were prepared by taking material directly from agar slants and suspending it in doubly distilled water. Dilutions were then made as necessary, and droplets of the suspension were placed on the collodion film over screens and allowed to air-dry. The specimens were either used for observations without further treatment or shadow-cast with a thin coating of a metal (Williams and Wyckoff, 1944).

In most unshadowed material of *P. ruber* used in electron microscopic studies

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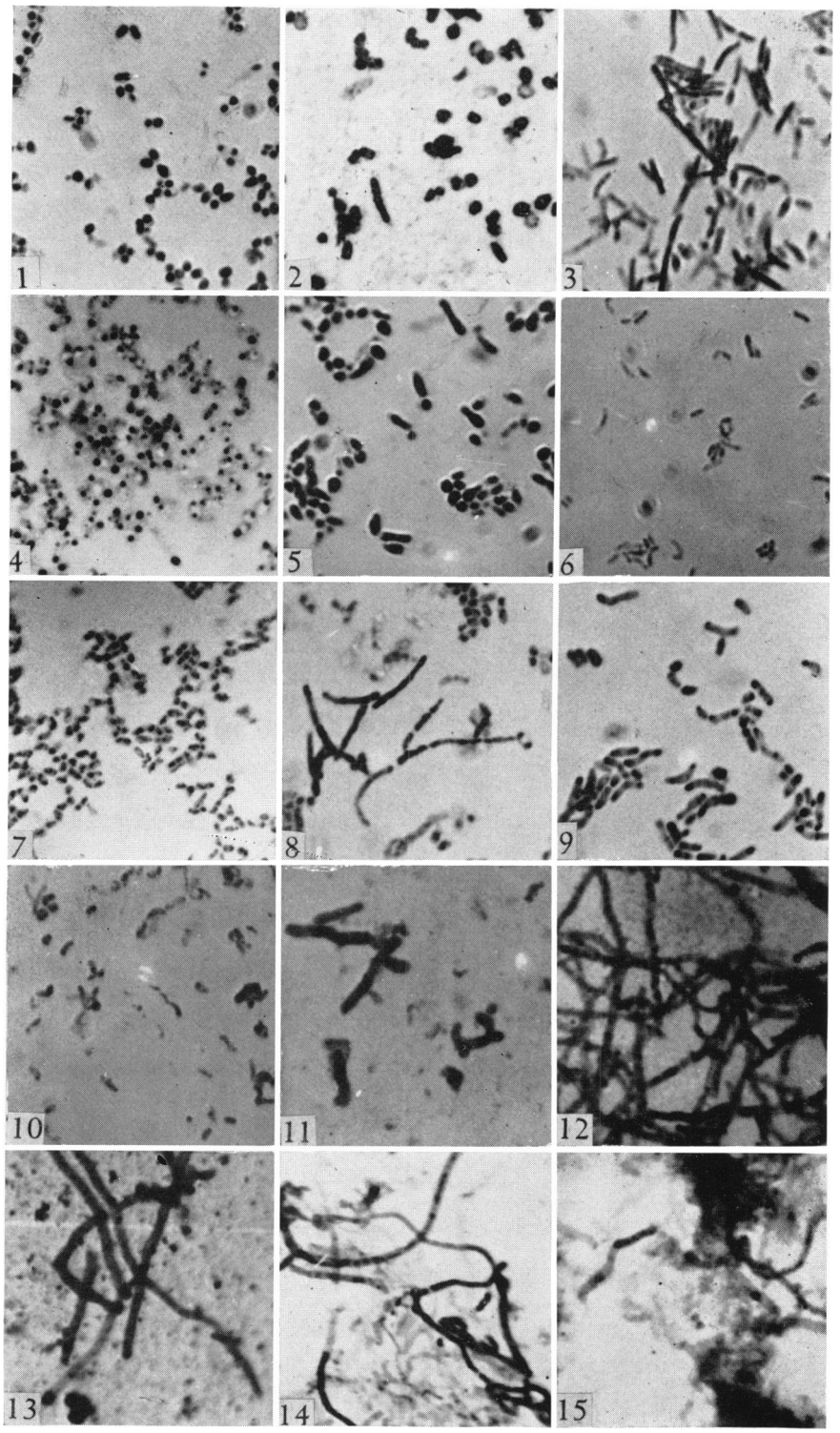
Figures 1 to 15. Photomicrographs of stained preparations of *Nocardia* sp. 6 months old grown on glycerol nutrient agar. × 1,600. *1*—Strain W-F, Ziehl-Neelsen. *2*—Strain B-B, fuchsin. *3*—*N. erythropolis*, gentian violet. *4*—Strain KLJ, methylene blue. *5*—*P. agnosus*, gentian violet. *6*—*P. polychromogenes*, gentian violet. *7*—Strain 13-20, methylene blue. *8*—Strain 43-8, gentian violet. *9*—*P. ruber*, methylene blue. *10*—*P. asteroides* var. *crateriformis*, gentian violet. *11*—Strain 13-10, gentian violet. *12*—Strain 7-7, gentian violet. *13*—Strain 13-3, fuchsin. *14*—Strain 18-2, gentian violet. *15*—Strain 13-16, methylene blue.



Figures 1 to 15

no wall distinct from the cytoplasm can be seen. Sometimes a thin extension of the cell wall was visible between the ends of dividing hyphal fragments. However, in material that had been shadowed by a metal, a lateral extension of filaments that represented a wall distinct from the cytoplasm was clearly visible (figure 39). Usually there was little differential shrinkage between the cytoplasm and cell wall in *P. ruber* when specimens were dried for electron microscopy. In figure 37 a cell wall is clearly visible where the cytoplasm happens to be in one end of the cell. In two other strains, 21–3 and 18–2, the cell wall can easily be demonstrated, in unshadowed as well as shadowed material at places between cytoplasmic areas in filaments (figure 41) and at the end of filaments that have been mechanically torn (figures 42, 46, and 47). It appears very thin and offers little resistance to the passage of electrons.

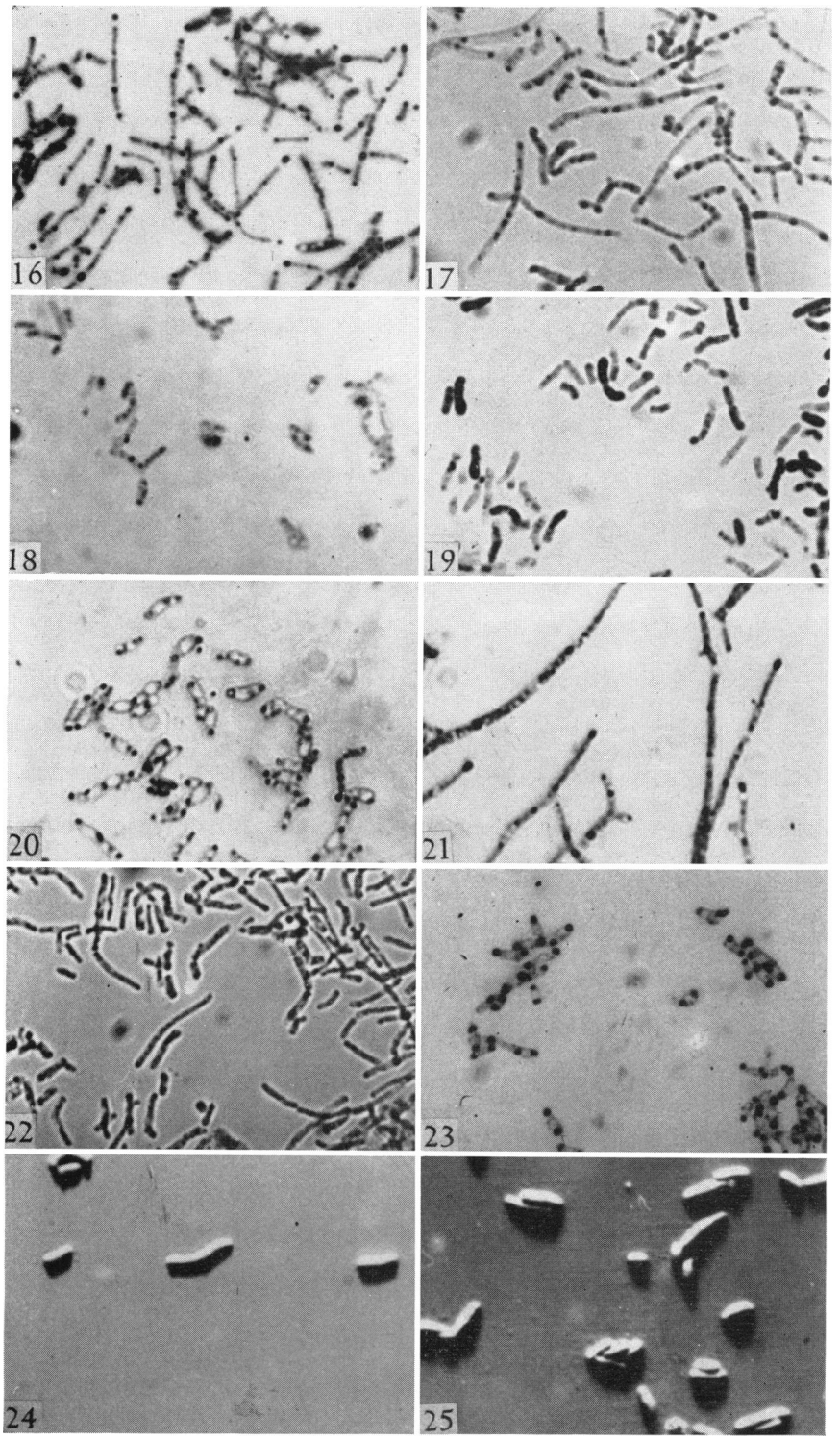
Preparations of *P. ruber* 24 hours and 12 days old that have been shadow-cast with chromium show that the contours of the outside of the cell are smooth (figures 24 and 25).

Further evidence of the nature of the cell wall of *P. ruber* was obtained by observing living cells in a mixed culture with motile bacteria. The movement of the bacteria caused the filaments of *P. ruber* to be bent and flexed. Some filaments about 20 microns in length were seen to bend almost double and then to straighten again. Thus the flexibility of the wall and cytoplasm was strikingly demonstrated.

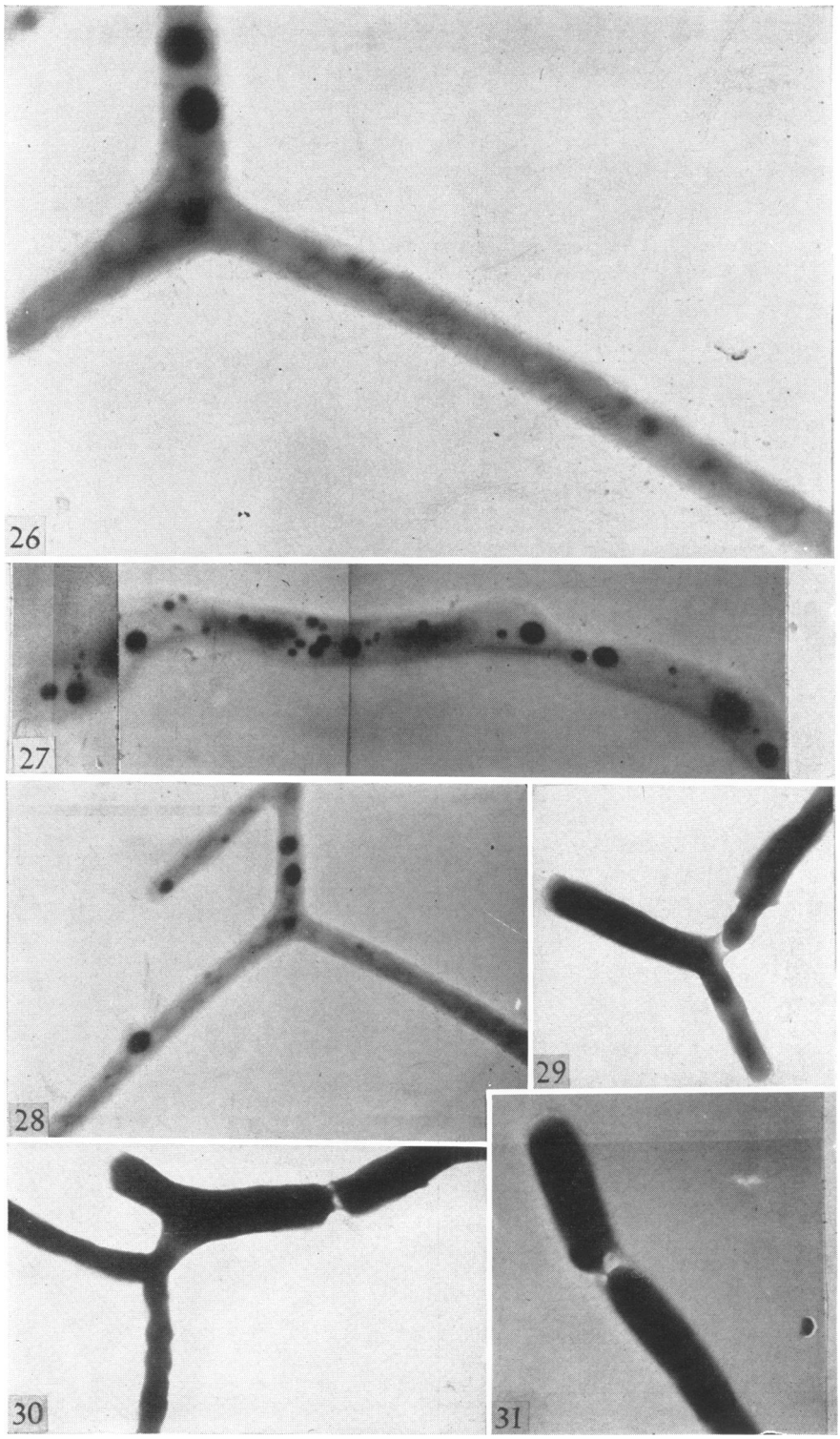
Cell division. A study of cell division in *Nocardia* was undertaken by the use of wall stains of impressions, by direct observation of living cultures, and by the use of the electron microscope.

Impressions were made by superimposing slides or cover glasses on young colonies of *Proactinomyces ruber* on agar medium. In this way slides can be made that show little disruption of the natural orientation of the hyphae. In cultures from 12 to 36 hours old, long filaments can be found, parts of which are breaking up into fragments. They were treated with either Knaysi's (1941) or Dyar's (1947) cell wall stain. Dyar's method gave more satisfactory results with this material. The cytoplasm stained blue, and Congo red dye was deposited on the cell wall. At places in the dividing filaments a clear separation of the cytoplasm within the still intact wall was seen. This indicates that division is initiated by a separation of the cytoplasm without visible changes in the wall. At no time could a deposition of dye be seen across the filament, as would be expected if a septum were deposited prior to separation.

Figures 16 to 25. Photomicrographs of *Proactinomyces ruber* (Casabó) Baldacci. *16*—Twenty-four hours, glycerol nutrient agar, 28 C, methylene blue, × 1,600. *17*—Twenty-four hours, glycerol nutrient agar, 28 C, Sudan black B, × 1,600. *18*—Two months, glycerol nutrient agar, 28 C, methylene blue, × 1,600. *19*—Two months, glycerol nutrient agar, 28 C, Sudan black B, × 1,600. *20*—Fourteen days, nitrogen-free agar, 28 C, gentian violet, × 2,000. *21*—Thirty-six hours, glycerol nutrient agar, 28 C, gentian violet, × 2,000. *22*—Forty-eight hours, glycerol nutrient agar, 28 C, Lugol's iodine, × 1,600. *23*—Fifteen days, nitrogen-free agar, 28 C, methylene blue, × 1,600. *24*—Twenty-four hours, nitrogen-free agar, 28 C, chromium shadow-cast, × 1,600. *25*—Twelve days, nitrogen-free agar, 28 C, chromium shadow-cast, × 1,600.



Figures 16 to 25



Figures 26 to 31

Living cultures of one strain, B-B, were studied directly under the 1.8-mm oil immersion lens using Brown's (1942) culture slide technique. The cytoplasm was entirely homogeneous and showed little discernible change during division. Sometimes a less refractive zone and a slight constriction in the wall could be seen at places in filaments where division would occur. However, in most cases, cells divided without any visible preliminary structural changes. Sometimes plasmodesmata could be seen connecting two newly divided cells. The actual separation took place very rapidly, and it was difficult to predict just where the next division would occur.

Specimens for the study of cell division by means of the electron microscope were prepared as previously described. The first evidence of cell division revealed by the electron microscope is a separation of the cytoplasm within the cell wall (figures 29 and 32). In some instances thin cytoplasmic threads connect the dividing hyphae (figures 30 and 31). The delicate wall can usually be seen between separations of the cytoplasm. These observations indicate that division is initiated by a separation of cytoplasm within the intact cell wall.

The cytoplasm. The cytoplasm of *P. ruber* was investigated by means of both light and electron microscopy. In young living material of most strains of *Nocardia* the cytoplasm appears completely homogeneous. Now and then highly refractive areas can be seen in certain organisms.

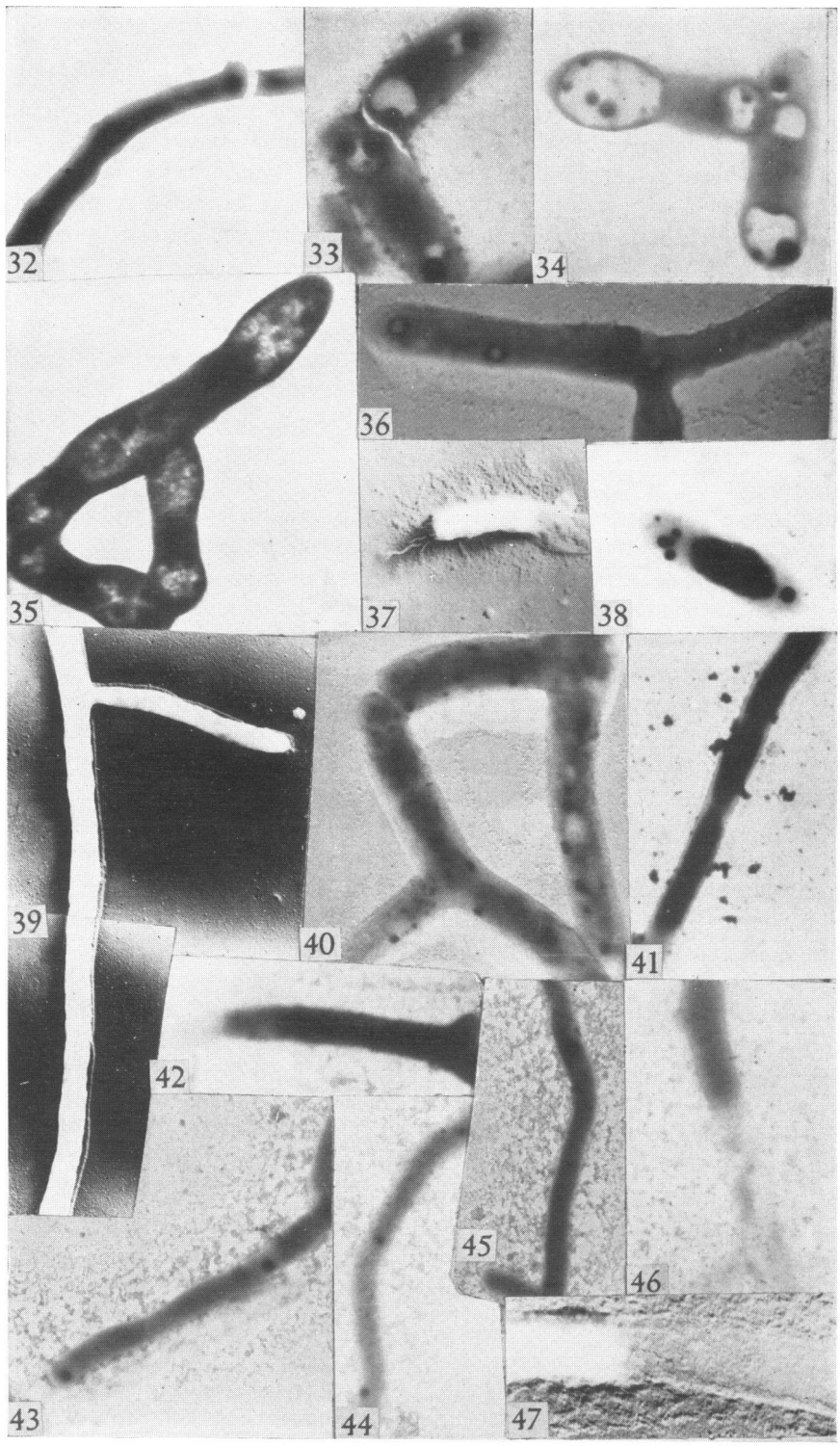
In cultures of *P. ruber* 12 to 36 hours old grown on glycerol nutrient agar and stained with methylene blue, the filament appears to consist of a network of cytoplasm having a honeycomb or alveolar appearance (figure 21). This structure can also be seen with the electron microscope (figures 26, 28, and 40). It is clearly visible in vitally stained hyphae and sometimes in unstained living filaments.

A striking characteristic of the cytoplasm of *P. ruber* is the constant appearance of structures in both young and old material which stain deeply with the basic aniline dyes. These structures can also be observed by means of the electron microscope. Their size varies considerably in material of the same age, and they are distributed irregularly in the cytoplasm (figures 16, 21, and 26). They are almost always present at the tips of hyphae (figures 16, 23, 27, and 28) and occur frequently at the base of branches.

As filaments fragment, these bodies assume a more regular distribution. In all cases in which the cells were transparent enough to allow internal structure to be seen, opaque bodies were found at the dividing ends of cells. In fragments, one or more of them are present at each pole of cells (figure 38).

When the organism is grown on nitrogen-free medium, the cytoplasm becomes much more transparent to electrons at 50 kv (Knaysi and Baker, 1947). In material treated in this way the cytoplasm appears almost transparent and the contents are more easily seen (figures 20, 23, and 27). In this material two

Figures 26 to 31. Electron micrographs (50 kv) of *Proactinomyces ruber*. *26*—Fourteen hours, glycerol nutrient agar, 28 C, × 8,000. *27*—Four days, nitrogen-free agar, 28 C, × 9,000. *28*—Fourteen hours, glycerol nutrient agar, 28 C, × 3,000. *29*—Seventeen hours, glycerol nutrient agar, 28 C, × 3,500. *30*—Seventeen hours, glycerol nutrient agar, 28 C, × 3,500. *31*—Twenty-seven hours, glycerol nutrient agar, 28 C, × 8,500.



Figures 32 to 47

types of structures can be distinguished by their form and position. In short fragments one or more spherical opaque bodies with a definite outline appear at the poles. A larger elongated body having a more diffuse outline is located in the center of the fragment (figures 35 and 38). In longer hyphae the same two types of structures occur in a definite arrangement. Groups of the spherical opaque bodies alternate with a single elongate, diffuse body throughout the filament (figure 27).

Small vacuoles are present in the cytoplasm of young material, which probably account for its alveolar appearance. As the filaments get older, the vacuoles increase in size. They seem to originate at the poles of fragments and are associated with the opaque bodies located there (figures 33, 34, and 35). Under certain conditions two large vacuoles form at the poles of fragments. These become very large, and always appear toward the poles with the center of the cell remaining unvacuolated.

The cytoplasm of the strains that do not fragment is more homogeneous. There are localized cytoplasmic areas in the filaments separated by empty spaces (figure 41). Opaque spherical granules are present in filaments of strain 21-3 (figures 43, 44, and 45).

In an effort to determine the nature of these structures microchemical tests were conducted as follows:

Volutin. Since conspicuous structures that stain violet with methylene blue are always present in the filaments and fragments of *P. ruber* regardless of age, they might be regarded as metachromatic granules or volutin. Tests were performed on 14- and 15-day-old material grown on glycerol nutrient agar slants to determine whether or not the bipolar bodies possessed the solubilities of volutin. It was found that they are not soluble in water at 80 C for 2 hours, in 2 per cent nitric acid for 10 hours, or in a 0.02 per cent sodium bicarbonate solution for 2 hours, but are soluble in 10 per cent hydrochloric acid in 10 hours. The bodies then do not possess the solubilities known to be characteristic of volutin, but those of nuclear nucleoproteins (Knaysi, 1946).

Fat. Fat staining with Sudan black B dissolved in 70 per cent alcohol or ethylene glycol (Hartman, 1940) gives positive results in both young and old material. It is difficult to decide whether or not the number and distribution of the

Figures 32 to 47. Electron micrographs (50 kv) of *Proactinomyces ruber*. *32*—Seventeen hours, glycerol nutrient agar, 28 C, × 3,500. *33*—Four days, nitrogen-free agar, 28 C, ×9,000. *34*—Four days, nitrogen-free agar, 28 C, × 9,500. *35*—Twenty-seven hours, nitrogen-free agar, 28 C, × 9,000. *36*—Eighteen hours, glycerol nutrient agar, uranium shadow-cast, ×9,200. *37*—Seventeen hours, glycerol nutrient agar, 28 C, chromium shadow-cast, × 3,500. *38*—Four days, nitrogen-free agar, 28 C, ×9,000. *39*—Seventeen hours, glycerol nutrient agar, 28 C, chromium shadow-cast, × 3,500. *40*—Eighteen hours, glycerol nutrient agar, 28 C, uranium shadow-cast, × 8,800. *41*—Strain 18-2, 30 hours, glycerol nutrient agar, 28 C, × 9,000. *42*—Strain 18-2, 30 hours, glycerol nutrient agar, 28 C, ×8,800. *43*—Strain 21-3, 6 days, glycerol nutrient agar, 28 C, ×9,000. *44*—Strain 21-3, 6 days, glycerol nutrient agar, 28 C, ×9,000. *45*—Strain 21-3, 6 days, glycerol nutrient agar, 28 C × 9,000. *46*—Strain 18-2, 30 hours, glycerol nutrient agar, 28 C, ×8,800. *47*—Strain 18-2, 30 hours, glycerol nutrient agar, 28 C, chromium shadow-cast, × 8,500.

structures that take this stain are the same as those that take methylene blue. A comparison is made of the distribution of particles in material of the same age stained in the two ways, shown in figures 16, 17, 18, and 19. In old material some cells stain homogeneously with Sudan black B. Cells fixed in 95 per cent alcohol for as long as 4 months, or glacial acetic acid for a month, still show particles staining with Sudan black B. Bacterial lipoids are generally soluble in these materials (Knaysi, 1946).

Carbohydrate. It was found that when *P. ruber* grown on nutrient agar for 48 hours and for 16 days was stained with Lugol's iodine solution, particles were present that stained a reddish brown. Prior to staining, the material was fixed in alcohol and glacial acetic acid in an effort to remove the lipoids (fats). Although stains with Sudan black B were still positive even after a long exposure to these fat solvents, fewer positive granules were present in material so treated. The reddish-brown particles in material treated with Lugol's solution are generally considered to be glycogenlike substances (Knaysi, 1946). Not all cells showed a positive test, but most of them did. They occur much less frequently than fat globules, and no regularity of distribution could be established. Figure 22 shows 48-hour material so treated.

The Feulgen reaction. Strains of *Nocardia* of various ages were treated according to the Feulgen method for the detection of thymonucleic acid. The leuco basic fuchsin was prepared according to Coleman (1938), and the material was hydrolyzed in N HCl at different times in the 3- to 10-minute range. Various fixing agents were employed: Nawaschin's fluid (Newcomer and KenKnight, 1939), saturated mercuric chloride solution and no fixative (Piekarski, 1940), 95 per cent alcohol (Delaporte, 1939–1940), and mercuric chloride and alcohol (Von Plotho, 1940). Material for testing was grown on different media, but all tests for thymonucleic acid in *Nocardia* were negative.

DISCUSSION

The strains of *Nocardia* that show a predominance of coccoid elements in old cultures are those that are most bacteriumlike in colonial characteristics. So far as the author is aware, the manner of formation of the coccoid cells is unknown. Krassilnikov (1938) reported that both vegetative and resistant coccoid cells are produced by *Proactinomyces*. Strains that have only pleomorphic rods in old cultures are those that are intermediate in morphology between strains that are bacteriumlike and *Streptomyces*-like.

Discrete cytoplasmic areas in hyphae, separated by empty spaces, have been seen and studied in *Streptomyces* by previous workers. Lieske (1921) considered them to be condensations of the cytoplasm. These cytoplasmic areas have been assigned a sporelike significance and were called chlamydospores by Krassilnikov (1938). They may be caused by an autolysis of portions of filaments, and "ghost filaments" may represent the completion of the autolysis of the cytoplasm.

The evidence from staining procedures and electron microscope studies indicates that the wall of *P. ruber* is very thin. Its existence is difficult to demonstrate with the 50-kv electron microscope because of its extreme delicacy and

plasticity. The latter quality apparently prevents separation of the wall from the cytoplasm in drying. The presence of a cell wall is more easily demonstrated in nonfragmenting strains of *Nocardia*, in which "ghost filaments" and empty portions of hyphae between cytoplasmic areas are frequently found. In these the wall can be shown to be very thin by studying shadow-cast preparations with the electron microscope (figure 47).

Efforts to determine the mechanism of cell division in *Nocardia* have resulted in showing that the first visible manifestations are separations of the cytoplasm within the cell wall. The cytoplasm may separate completely or threads may persist between the two daughter hyphae for a time. Thin extensions of a wall were seen between hyphae in which the cytoplasm had separated. Further details are not certain. It seems likely that following the separation of the cytoplasm the wall breaks and the hyphae separate. End walls may be deposited later. The initial stages in cell division, then, are similar to those described for bacteria by Knaysi (1941).

Most investigators have considered the actinomycetes to have a nonseptate mycelium. Drechsler (1919) was able to demonstrate the presence of septa irregularly and sparsely in the vegetative mycelium. Carvajal (1946) reported that septa are present only in old mycelium; Klieneberger-Nobel (1947) was able to show cross walls in all mycelium by special staining techniques. In the present studies no evidence of cross walls was found. Long empty hyphae have been studied, and in none of them was there a suggestion of a remaining septum.

The studies of the structure of the cytoplasm of *Nocardia* by microchemical staining methods and electron microscopy show that the cytoplasm is located inside a cell wall, without a demonstrable cytoplasmic membrane. In young filaments the cytoplasm regularly contains vacuoles that are distributed throughout their length so as to give them an alveolar appearance. This is thought not to be an artifact, as this structure appears in vitally stained material. With age the vacuoles increase in size and number, until in fragments of *P. ruber* two large vacuoles are formed at the poles of the cells. These are formed around the spherical granules in the ends of the fragments (figures 33 and 34) and in some instances replace most other cell contents (figure 35).

The presence of bodies in the cytoplasm of actinomycetes has been noted and studied by many workers, who have interpreted them as volutin, waste products, or nuclei. Neukirch (1902) found granules in hyphae stained with dilute methylene blue that he regarded as nuclei. The presence of such granules was denied by Gilbert (1904) but confirmed by Drechsler (1919) and Lieske (1921). Drechsler thought that the deeply staining granules in the serial spores were nuclei, and those in the vegetative mycelium were occluded wastes, because they appeared more frequently in the older parts of the mycelium. Lieske held that the polar granules were perhaps volutin, and the granules that he was able to demonstrate in the vegetative mycelium by using Neukirch's method of staining with dilute methylene blue were nuclei. He found the latter structures infrequently present. More recently the problem of a nucleus in ray fungi has been investigated by Grigorakis (1931), who reported the presence of nuclei dividing amitotically in

Actinomyces bovis and providing each particle with a nucleolus; he also reported the spores to be mononucleate. Schaede (1939) obtained a diffuse reaction when applying the Feulgen stain for the presence of thymonucleic acid. Newcomer and KenKnight (1939) constantly obtained a positive Feulgen reaction in discrete bodies in several strains of actinomycetes. Von Plotho (1940) reported a positive Feulgen reaction in strains of actinomycetes grown under very different environmental conditions. Using the electron microscope, Carvajal (1946) found opaque granules in the mycelium of *Streptomyces griseus* which he considered nuclei. Klieneberger-Nobel (1947) reported not only the presence of nuclei but also their fusion and division in four strains of *Streptomyces*.

Structures having two different forms were found to occur in the cytoplasm of *P. ruber*. One type visible under the light and electron microscopes is spherical or oval in shape, has a very definite outline, and does not permit the passage of electrons. These structures are believed to be identical with those that stain deeply with basic dyes, thus are easily demonstrated with the light microscope. They are present irregularly throughout the length of long filaments of young material and almost invariably occur at the tips of hyphae. Their size varies; they are smaller when they occur in groups. In older hyphae they come to be located at the poles of fragments, and may occur singly or in groups. There is evidence that these bodies have a vacuolated center, as they occasionally appear as rings (figure 36). The second type of structure can be seen only with the electron microscope and in material grown on nitrogen-free medium, which renders the cytoplasm less dense. These are generally larger than the previously described structures, have a more diffuse outline, and are less resistant to the passage of electrons. A regularity of distribution of these two types of structures can be seen in filaments grown on nitrogen-free medium. The smaller, more opaque ones alternate in a filament with the larger, diffuse bodies. In fragments a similar distribution is seen; the center of the cell contains the larger, diffuse mass, the smaller, opaque bodies being located at the poles of the fragment. Studies of dividing and recently divided cells show that the spherical, opaque bodies are almost always seen in the recently divided ends. Hyphae from nitrogen-free medium show that division occurs in the part of the cell in which the spherical opaque bodies are located.

Tests of solubility show that the spherical, polar structures are not volutin, but they possess the solubilities of nuclear nucleoproteins. However, all efforts to demonstrate the presence of thymonucleic acid by the Feulgen reaction were negative. These spherical bodies stain deeply with methylene blue, basic fuchsin, and gentian violet. In old material they appear at the poles of cells and may be referred to as bipolar granules. The fact that they exist as bodies and are not due to polar thickenings of the cytoplasmic membrane is demonstrated by the electron microscope.

From evidence presented by these studies it must be concluded that the nature of the structures in the cytoplasm of *P. ruber* is complex but unknown.

The internal structure of only two strains of *Nocardia* that do not fragment was investigated by means of the electron microscope. One strain, 18-2, when

grown on glycerol nutrient agar, presented a homogeneously opaque cytoplasmic structure with some separations in the cytoplasm (figure 41). The other strain, 21-3, showed a less opaque cytoplasm containing irregularly distributed opaque spherical granules (figures 43, 44, and 45).

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SUMMARY

Three types of development were revealed by a study of stained preparations of six-month-old cultures of 46 strains of *Nocardia*. Strains that are most bacterial in colonial texture produce a preponderance of coccoid cells. Pasty-textured forms develop pleomorphic rods for the most part, and waxy- and cartilaginous-textured forms show "ghost filaments," homogeneously stained hyphae, and filaments with discrete cytoplasmic condensations separated by clear areas.

The 46 strains studied are gram-positive and non-acid-fast and have a marked affinity for the basic aniline dyes.

The cell wall of *Proactinomyces* (*Nocardia*) *ruber* is thin, plastic, and flexible.

Cell division in *P. ruber* is initiated by a separation of the cytoplasm, without a previous deposition of a septum.

The cytoplasm of *P. ruber* is alveolar in structure; large vacuoles develop as cultures age.

Two types of granules or bodies are present in the cytoplasm of *P. ruber*. Although they seem to have a regular pattern of distribution in fragments and filaments, no definite role could be assigned to them on the basis of microchemical tests.

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