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I. INTRODUCTION

The structure of bacteria is a subject which has stimulated the investigations of many workers since the earliest days of bacteriology; none of them, however, has provided a completely satisfactory explanation. The very long history of this question has been described in several reviews (among them, Guilliermond, 1907; Delaporte, 1939b and 1940, which contains references, numbering about 440, to almost every paper on bacterial cytology up to 1938; Knaysi, 1938, 1949; Lewis, 1941). I will mention here only that the very first papers written on the subject —between 1886 and 1890—gave the following diverse descriptions of bacterial structure: (*a*) bacterial cells do not have a nucleus; (*b*) the cell contains a small granule, which is the nucleus; (*c*) the entire cell is a

nucleus; (*d*) the greater part of the cell is a nucleus, and is surrounded by a thin layer of cytoplasm; (*e*) the nucleus is composed of a great number of small chromatic granules scattered inside the cell. Many of these opinions were still being supported quite recently.

A recent study by C. Robinow has attracted much attention. During the war Robinow (1942, 1944, 1945) used on several species of bacteria a technique described by Piekarski in 1937; namely, fixation of the cell by osmium tetroxide vapor, hydrolysis in a normal solution of hydrochloric acid at 60°C. for 7 to 10 minutes (the same as for the Feulgen technique), and staining with Giemsa solution. This technique, although not as specific as Feulgen reaction, is rapid, and results in remarkably clear and sharp figures, but the images of the nuclear substance are consistently larger than with other staining procedures, and in particular much larger than those given by the Feulgen reaction.

Robinow (1945) diagramed the changes in the nuclear structure of *Escherichia coli* occurring during the first hour after transfer from an 18-hour agar-slant culture onto a fresh agar plate. He has also described the early hours of growth after transfer of cells from old cultures of *E. coli* to a fresh medium, during which he observed 2, 4, or 8 chromatinic bodies having the shape of transverse rods or horseshoes, which divide lengthwise in a plane more or less parallel with the short axis of the bacterium.

II. Useful Methods of Studying Bacterial Cytology

The bacterial nucleus is not easy to identify, since it does not have the characteristic shape and appearance that we know in the cell nuclei of all other organisms. It is therefore desirable to examine first the various elements inside the cell that we can easily find and identify. The bacterial cell is not composed merely of a nucleus and some cytoplasm inside a membrane. There are in addition other elements, which are easily identified when stained, especially with basic stains. For example, some cytologists have described a "nucleus," which in actuality was simply a metachromatic granule; they had not made a critical study of what they had stained. Very few cytologists have made studies of these other structures in the bacterial cell.

1. *Living, Unstained Bacteria; Observations of Lipids*

Microscopic examination of a culture of living bacteria, between slide and cover glass in a drop of either plain water or broth, shows, in certain species, one or several spherical, somewhat refractive globules inside the cells. These globules are stainable by the specific stains for lipids: Sudan III, Sudan black, Sudan red, and nascent indophenol blue

(Nadi reagent). In a great many bacterial species, these lipids are formed, for the most part, by polymerization of β-hydroxybutyric acid (Lemoigne *et al.*, 1944). In sulfur bacteria (Beggiatoa) the lipids seem to be phospholipids (Delaporte, 1939b, p. 766). In some species we can also see refractive ovoid or spherical bodies which are spores. Round, extremely refractive globules of sulfur are visible in sulfur bacteria.

2. *Vital Staining; Metachromatic Granules and Vacuoles*

Living bacterial cells may also be observed in a very dilute solution of a nontoxic vital stain. There are, unfortunately, very few such vital stains for bacteria; almost all stains kill the cell at the time they enter it. We can use neutral red, which is nontoxic and stains the vacuoles only when the cell is living; at the moment of death the vacuoles lose their color and the whole protoplasm—cytoplasm and nucleus—becomes colored. Brilliant cresyl blue and methylene blue likewise stain vacuoles in living cells; they are slightly toxic, and they can be reduced to the leuco-base by living cells. They can also stain the living nucleus and the cytoplasm slightly. Because bacterial cells are so small, it is advisable, in order to understand the action of a stain, to study it first on cells whose structure is well known.

Cells of yeast (for example, Saccharomyces) may be used for this purpose. When they are placed in a dilute solution of neutral red no staining is observed at first, but the large central vacuole (v) and many granules in the cytoplasm can be distinguished. After a short time, inside the unstained vacuolar sap, we see one or a few globules (m) of different sizes, which are stained red (Fig. 1a) and show Brownian move-

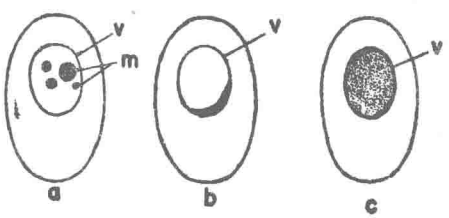

FIG. 1

ment. Apparently the dye has passed through the cytoplasm without staining it. After a few minutes these red globules migrate to the border of the vacuole and spread into the shape of a crescent (Fig. 1b). Later the stain diffuses completely through the vacuole, so that it is stained uniformly red (Fig. 1c); sometimes the whole vacuole stains red in this way without the previous staining of granules.

These red granules of yeast cells are metachromatic granules, formed by precipitation of the metachromatin that was in solution in

the vacuolar sap. (The name "metachromatin" is derived from the red color it gives with certain blue stains. Sometimes this substance is called "volutin," but the name metachromatin has priority.) It is very easy to confuse a metachromatic granule with a nuclear body, because their staining affinities are nearly the same; there is, however, a specific reaction for the identification of metachromatic granules developed by Meyer (1904). If, after staining with methylene blue, the cell is treated with a 1% solution of sulfuric acid, all the stain is removed except from the metachromatic granules, which change from red to black.

Except for unusually large cells (*Achromatium oxaliferum*), the bacterial cell has no large vacuole containing metachromatic granules, as in yeasts, but it has within the cytoplasm strongly staining metachromatic granules. These correspond to very small vacuoles, containing a high concentration of metachromatin. Another reason for considering the metachromatic granules as vacuoles is that they are stained black by silver impregnation with the Da Fano technique (Delaporte, 1939b). Metachromatin is a reserve substance composed—at least mainly—of ribonucleic acid (Van Herwerden, 1917; Delaporte, 1939a), probably in the form of ribonucleoprotein.

3. *Glycogen*

A third reserve substance of many species of bacteria is glycogen which is easy to recognize because of the mahogany color it takes with iodine. It is identified by its behavior when the material is warmed slightly. The glycogen always appears to be in a diffuse state inside the cytoplasm of the bacteria in which it is found; the quantity may vary greatly among cells. Some species of anaerobic bacteria (Clostridium) contain another type of carbohydrate (granulose) which is stained red-purple with iodine and occurs in the form of small granules.

It is necessary to consider these non-nuclear elements within the bacterial cell in order to understand and explain the structure and shape of the nucleus, because their presence can alter its shape. Some of these bodies have a surface tension greater than others. For example, we never see globules of lipid or sulfur that are deformed by nearby elements; they are always spherical since their surface tension is very high.

4. *Vital, or Sublethal, Staining; Observation of Nuclear Substance*

If we observe living bacteria (for example, *Bacillus cereus*, 16-hour culture; *B. enterothrix; B. camptosporus*) in a drop of a dilute solution of a vital stain (methylene blue or brilliant cresyl blue) over a period

long enough for the stain to enter the cell without killing it, we can often observe the presence of a reddish thread inside the light-blue cytoplasm. This thread is situated in the long axis of the cell (Figs. 30, 33); it is often a little wavy and sometimes slightly granular. It is the nuclear element of the cell.

Explanation of the Expression "Nuclear Element." By "nuclear element" I mean a cellular component which has many of the properties of a nucleus and which contains substances having the staining characteristics seen in every nucleus. The nuclear element of the bacterial cell, as understood here, has the following characteristics, which are also the characteristics of the true nuclei of the higher forms. It never appears *de novo,* but always is formed by the division of a preexisting similar element; it is found in every cell and spore; it divides when the cell divides; and it is deeply chromatic. It is stained by the Feulgen reaction, and with ferric and other hematoxylins, Giemsa, etc. The positive reaction with the Feulgen technique indicates that the nuclear element contains desoxyribonucleic acid, which is the nucleic acid characteristic of the nucleus of higher forms. Nothing is known, however, about structures in the nuclear element having hereditary functions. We do not know whether the nuclear element is composed of a single or of several nuclear units and consequently whether it is a simple or a multiple nucleus—in other words, whether it is one nucleus or a group of several nuclei. Therefore, it is advisable not to use the word *nucleus,* which designates something more precise than our knowledge justifies, but provisionally to use the more general expression "nuclear element." The extremely varied shapes it assumes, and the fact that no consistent structure is seen, render it difficult to identify this nuclear element of the bacterial cell with the true normal nucleus of higher forms.

At the time that the nuclear element can be observed with vital staining, the cell has already begun to deteriorate; it seldom recovers, and generally dies after a short while. At the moment of death there is a rapid and pronounced change; the cytoplasm congeals and stains dark blue, and the axial thread (in rod cells)—or central body (in cocci)—disappears from view, although it can sometimes be seen with the help of a green filter.

5. *Staining of Nuclear Substance after Fixation*

The most common method of studying the nucleus in bacteria is to fix the cells with one fixative, stain, and then make observations only with regard to the nucleus, entirely neglecting the other bacterial structures.

6. *The Comparative Method of Scrutinizing the Cytology of Bacteria*

A better method is to try a number of different combinations of fixatives and nuclear stains; to observe on the same culture not only nuclear structures but also metachromatic corpuscles, lipids, glycogen, etc.; and to consider all observations before coming to a conclusion. A more detailed study of observations based on such procedures can be found in earlier papers (Delaporte, 1934, 1935, 1936a, 1936b, 1939b, 1940).

III. Cytological Observations

1. *Cytology of Round Bacteria*

When these different techniques of studying and staining are applied to round-shaped bacteria (Micrococcus, Streptococcus, Sarcina, etc.) they suggest that all such cells have quite comparable structure: namely, that they may contain one or several metachromatic granules (Fig. 2, *m*), and one or several globules of lipid (Fig. 3, *l*); and that they have one nuclear body, in the shape of a large globule, which occupies the whole center of the cell (Fig. 4, *n*), or else is situated somewhat eccentrically (Fig. 4, cell at lower center with lipid globule, *l*, in the middle). In a very young culture—one with actively multiplying cells—there are a great many cells in which this nuclear element is dividing, by lengthening and constricting in the middle (Fig. 4, right); and often two nuclear bodies are visible in one cell, because the transverse membrane has not yet formed (Fig. 4, cells at lower right and left). In some species the nuclear element may have divided twice before transverse membranes are visible, so that three or four nuclear elements are seen in one cell (Fig. 5). In older cultures, however, one almost never observes division figures, and consistently finds only one nuclear element in each cell (Fig. 6, *Leuconostoc mesenteroides;* Fig. 7, Sarcina—two cells are dividing).

2. *Cytology of Ovoid Bacteria*

The large cells of ovoid shape (*Azotobacter agile, Achromatium oxaliferum, Chromatium weissei*) have their whole central region filled with globules. In *A. agile* the most numerous of these are fat globules (Fig. 9, *l*), some are metachromatic granules (Fig. 8, *m*), and the center of the cell contains much glycogen (Fig. 10, *g*). Stainings to demonstrate the nuclear substance in these cells show several small granules, more or less at the periphery of the cell (Fig. 11, *n*), making these cells very different in structure from coccus forms. The Feulgen reaction shows a few positive granules and an apparently diffuse positive reaction, chiefly in the peripheral part of the cell (Fig. 12). However, after a few trans-

fers on very poor medium (agar with tap water, and possibly a few hours thereafter on very dilute nutritive medium), the cells show only one mass of nuclear substance (or two in dividing cells), located in the center of the cell (Fig. 13, *n*) and scalloped by fat globules (Fig. 13, *l*) which appear as empty spaces in such preparations. This mass divides by lengthening and constricting in the middle (Fig. 13, the cell at the top), as in cocci; it gives a positive Feulgen reaction. This leads one to suppose that the nuclear substance, which in this species theoretically consists of a single mass, is habitually deformed, displaced, and even fragmented into several parts, which are located wherever there is room for them between the fatty globules and other globules. The first diffuse Feulgen reaction is easily explained in this case: the nuclear substance, being displaced by globules, surrounds them in a thin layer, with tiny masses between the globules—thus giving the impression of a partially diffuse substance with Feulgen, and of granules with hematoxylin stain. This does not mean that there are several nuclei in the cell, but only that the nuclear substance does not have a definite shape and may be deformed and fragmented by other elements of the bacterial cell. Exactly the same thing is observed in cells of *Chromatium weissei* and *Achromatium oxaliferum*, but, in addition, these have sulfur globules, and the latter has concretions of calcium oxalate (or, according to certain authors, calcium carbonate). Figs. 14-17 show cells of *A. oxaliferum* enlarged 900 times. The central part of these cells contains large concretions of calcium oxalate (*o*), and around these are very refractive sulfur globules (*S*). Fig. 14 shows the vitally stained large vacuoles (*v*) found in this species. Fig. 15 shows stained lipid globules (*l*). In Fig. 16 the cell is fixed with Lenhossek's fluid, and the nuclear substance (*n*), situated between and around the calcium oxalate masses, is stained black with ferric hematoxylin; the cytoplasm (*c*), at the periphery of the cell, stains pink with erythrosin. Fig. 17, in comparison, shows a Feulgen positive reaction (*n*) only at the center, because this is a young cell from a very rapidly growing culture. In such a culture the nuclear substance is in one mass, centrally located. It divides by stretching and partition in the middle, as in cocci and Azobacter. (For other figures see Delaporte, 1939b, Pl. IV, Figs. 1-14, 29-36; Pl. VIII, Figs. 27-42; and Delaporte, 1940, p. 41.)

3. *Cytology of Rod-Shaped Bacteria*

Distribution of the nuclear substance in the form of several granules is usually also observed in rod-shaped cells when they contain a large amount of reserve material. The cytoplasm includes fat globules (*l*), sometimes very many (Fig. 23) or very large ones (Fig. 19), as well

as metachromatic granules (Figs. 18, 22, 26, *m*), and sometimes glycogen (Fig. 28). In these cells the nuclear substance is generally divided into several granules, located in the interstices between the globules (Figs. 24, 46); and the Feulgen reaction often seems to be diffuse, although occasionally the masses of nuclear substance are evident (Fig. 25). In a few cells of these cultures, however, particularly those that have a very small number of globules and a dense cytoplasm (often these are also smaller in diameter), the nuclear substance is in one single mass, and appears as a more-or-less granular thread extended in the center of the cell, its length proportional to the length of the cell (Figs. 24, 49, 74, 76—middle cell, 78—right).

In a rod-shaped species of bacteria (Figs. 18-21) that has a few small metachromatic granules (Fig. 18, *m*), a few large lipid globules (Fig. 19, *l*), and no glycogen, the nuclear element (24-hour culture) has the shape of an axial thread (Figs. 20 and 21). In Fig. 20, the nuclear element (*n*) is stained black with ferric hematoxylin. In Fig. 21, the nuclear element is stained purple by the Feulgen reaction; the lower cell of the two-cell rod is dividing. The axial thread is deformed by lipid globules; the unstained areas (*l*) show the location of lipid globules, which have been dissolved during the successive treatments. In another rod species (Figs. 22-25), which has a few small metachromatic granules (Fig. 22, *m*), many lipid globules (Fig. 23, *l*), and no glycogen, the nuclear element (Fig. 24, ferric hematoxylin; Fig. 25, Feulgen) appears most often as several granules scattered throughout the cell or between the lipid globules; exceptionally it appears as a thread. In a third species (Figs. 26-29), which has very few metachromatic granules (Fig. 26, *m*) and lipid globules (Fig. 27, *l*), but much glycogen (Fig. 28, *g*), the nuclear element is nearly always displaced (Fig. 29, 1 to 3-day cultures, stained with ferric hematoxylin).

When bacterial cells have little or no accumulation of reserve material, the nuclear substance is in the form of a single mass, rod-like or thread-like in long rod cells and more or less round in short rod cells (*Bacillus enterothrix*: Fig. 30, vital staining; Fig. 31, ferric hematoxylin; Fig. 32, Delafield hematoxylin; Fig. 36, Feulgen reaction; 900 X. *B. camptospora*: Fig. 33, vital staining, 900 X. *Bacterium* sp.: Fig. 38, Feulgen reaction, 1800 X. *Escherichia coli*: Fig. 34, 2700 X; and Figs. 82, 82A, and 82B.)

In *E. coli* we find cells of quite different lengths, depending on the strain and the culture conditions. Sometimes they are very short and almost spherical (Figs. 34, 39—bottom, 82—photograph, 82A top left—explanatory drawing), sometimes longer (Figs. 39-45). The nuclear element appears to divide by stretching. When the cell is short, the globular

nuclear element enlarges (Fig. 82—right and Fig. 82B), takes the shape of a dumbbell, and breaks in the middle (Figs. 35, 40, 44, 45). When the nuclear element is shaped like a thread (Fig. 39—top; Figs. 83 and 83A), only a break in the middle is visible (Fig. 40—top). Often a second nuclear division is accomplished before the formation of the transverse membrane that divides the rod after the first nuclear division (Figs. 44 and 45; 82 and 82B, which show beginning of division). Under different conditions (of strain, age, and culture medium), when very active multiplication occurs, the observed events are different. The nuclear substance takes the shape of transverse rods, as seen in Fig. 41-43 (strain B/r, 4¾ hours in nutrient broth without aeration), and in Figs. 79-81 (photographs) and Figs. 80A and 81A (drawings). Two or three nuclear divisions occur in one cell before the cell divides, and then four, six, or eight nuclear transverse rods are found in one bacterial cell. When these nuclear rods divide they divide lengthwise, forming V shapes or U shapes (Figs. 42 and 43).

a. *Formation of Spore in Bacillus.* A more complex cycle of development is seen in species that form spores. If we observe stained cells from a 16-hour culture of *Bacillus cereus,* for example, we see many cells in which several granules of nuclear substance are located between the globules; that is, the nuclear substance is fragmented between the globules in two to six (occasionally more) main centers. Each of these nuclear granules of a cell seems to be completely separate from the others, but perhaps they are linked by nonapparent ties. A few cells have only one nuclear element, in the form of an axial thread (Fig. 49); and many cells are just beginning to form their spores (Figs. 46-48). The successive stages of this process can be observed during the next few hours. At first, all the nuclear granules inside the cell seem alike, but at the very beginning of spore formation, one granule, situated nearest an end of the cell, appears a little larger, for example, the one at the extreme right in Fig. 46. Then the cytoplasm around this granule becomes more homogenous and denser, without globules, and stains pink with Giemsa (Fig. 47, two prespores, *p*, in the two-cell rod at right); this region, which is to develop into the spore, grows larger and becomes ovoid in shape (Figs. 46, 47, 48, and 51, showing twelve stages of spore formation; photographs in Figs. 84 and 85, and explanatory drawings in Figs. 84A and 85C). The nuclear element of the future spore stretches into a short rod (Fig. 51), which migrates to the periphery of the spore (Figs. 50, 52) and takes the shape of an oval ring with one side slightly larger than the other (Fig. 52). At this time the staining properties of the cytoplasm of the interior of the spore change; it stains sky blue with Giemsa, and the spore becomes refractive. Most often this oval

ring is seen in side view, appearing as a kind of curved rod with slightly thickened extremities. In Fig. 85, lower right corner, there are two free spores with dark peripheral nuclear element and ovoid grey cytoplasm; the spore coat is not stained. Fig. 53 shows spores after liberation; the spore coat is not stained and not visible.

At the beginning of spore formation, only a small portion of all the nuclear substance in the cell constitutes the nucleus of the future spore. In a cell that has a nuclear thread, only a small part of this thread—approximately a third in *B. cereus* (Fig. 47) or *B. macerans* (Fig. 54), and much less in species having longer cells (Figs. 30, 33, 36, 37)—becomes the prespore nucleus. As the prespore enlarges and occupies a larger part of the cell, it possibly assimilates by a chemical process more of the nuclear substance of the rest of the cell. Almost always, however, some remainder of nuclear substance is found outside the spore (Figs. 54, 55; *B. macerans*), sometimes the largest part of the axial thread (Figs. 33, 36, 37; bacteria from intestine of tadpoles). The spore is liberated after disintegration of the cytoplasm, the remainder of the nuclear substance, (Figs. 37, 52), and the cell membrane. (See also *Spirillum praeclarum*: (Figs. 98-101; and Delaporte, 1939b, Pl. VI, Figs. 1-17.)

b. *Germination of Spore.* In subcultures from an old culture, one made up mostly of spores, the spores swell, and during this process nuclear changes may be observed inside them. In *Bacillus mycoides*, for example, the nucleus, which was at the periphery (Figs. 56, Giemsa; 57, Feulgen; 65, vital staining), migrates to the center of the spore and enlarges (Figs. 58, Feulgen; 59, Giemsa; 66, vital staining; 60, Giemsa, one hour later). Then the new cell comes out like a bud, grows bigger, and emerges from the envelope of the spore, while the nucleus undergoes a first and a second division (Fig. 60, 3-hour culture, Giemsa; Fig. 61, 4-hour culture, Giemsa; Fig. 62, 4-hour culture, Feulgen). The first transverse membrane is sometimes formed as soon as the first nuclear division is completed, but more often after the second or the third division. The nuclear element is larger at this time than it was inside the resting spore.

In the interior of many spores, however, before the new cell emerges, the nuclear element is in the form of a mass of three or four granules (Fig. 59, bottom row, *B. mycoides*); and later, in the first cells that emerge, the nuclear element is granular (Figs. 60-62). In many other spores the nuclear element has the shape of a thick thread, often greatly distorted, or of a mass that seems to be pulled in many directions (Fig. 59, several spores); such nuclear elements may divide before the cell emerges (Fig. 59, right). In some spores, for example, we see two short, bent threads, transversely and symmetrically situated (Figs. 58, 59);

the rods emerging from these spores have two nuclear elements, each consisting of a twisted, angular thread (U-shaped, square, Z-shaped, or V-shaped), with granules located especially at the angles (Fig. 60). When the nuclear element does not divide before germination, there is only one nuclear axial thread (Fig. 61, right). Rods that are a little older and longer have four similar nuclear elements (Figs. 61, 62). Although these rods have no visible transverse membrane, they must be considered as virtually two or four cells, because they are *dividing* and not *resting* cells, and cell division has not been completed at this time. Consequently, each actual cell is very short, and is square or slightly rectangular in shape. In such cultures there are a few rods that consist of only one cell, and in these the nuclear element is a wavy thread situated in the long axis of the cell (Fig. 61, lower right).

It can nearly always be shown that scallops in the outline of the nuclear element are made, or appear as if made, by globules of unknown nature (Fig. 63, 5-hour culture, Giemsa). What are these globules in the very young rods? Probably they are not yet true reserve substances, as are the lipid globules in older cells; possibly they are centers of more rapid metabolism, which enlarge locally and thereby displace the nuclear substance and other nearby substances.

Remnants of sporangium: In certain old cultures having living resting spores (particularly in *B. mycoides*, either unstained, or, better, with vital staining), it is possible to observe that the spore remains, perhaps permanently, inside the remnant of the sporangium membrane (*spg*), which can be seen, like two short fingers, protruding beyond the poles of the spore (Figs. 64, 65). Occasionally, a slightly refractive granule (*a*) is seen inside the sporangium membrane, most frequently located at a pole on the surface of the spore; very rarely, there are two such granules. This granule is vitally stained purple with cresyl blue. No change occurs in it during germination, and it is discarded on the surface of the spore coat. It stains red-purple with Giemsa after hydrolysis, and shows a positive Feulgen reaction. It is probably a remnant of nuclear substance of the sporangium. It is seen (*a*) on resting spores in Fig. 56 (Giemsa after hydrolysis), Fig. 64 (beginning of a vital staining), and Fig. 65 (vital staining of spores); and during germination in Fig. 66 (1-hour culture, vital staining), Fig. 59 (2-hour culture, Giemsa after hydrolysis), and Fig. 61 (4-hour culture, Giemsa after hydrolysis).

Vital, or sublethal, staining of the nuclear element (*n*, Fig. 65) in resting spores may be obtained only in certain nonrefractive spores, such as may be found, for example, in cultures of *B. mycoides*. It is never possible to stain the nuclear element, without previous hydrolysis, in refractive spores.