THE CYTOLOGY OF BACTERIA

I. M. LEWIS

Department of Botany and Bacteriology, The University of Texas, Austin

CONTENTS

It is quite impossible to include all of the literature of this subject in the space available here. Accordingly, this review is limited to the publications dealing with non-protoplasmic cell inclusions, the problem of the nucleus, the cytology of reproductive bodies, the cell wall and cytoplasmic membrane and the flagella. The last comprehensive review, "Die Zelle der Bakterien" by Arthur Meyer, was published in 1912. Since that time new methods of investigation have been developed, and many additional papers have appeared. A brief review by Knaysi (86) includes much of the more recent literature.

It must be understood at the outset that the subjects to be

treated have long been the cause of much controversy and that many conflicting reports have been published. The subject is difficult, and while it is obvious that many errors have been made in the past, there is probably no one who is fully competent to evaluate all the conflicting reports. My aim has been to present both sides of controversial matters as impartially as possible and to draw such conclusions as the evidence appears to warrant.

According to modern cytological terminology, a plant cell consists essentially of a nucleated mass of cytoplasm, the protoplast, surrounded by a cell wall. The surface of the protoplast is a semipermeable cytoplasmic membrane. Additional structures such as plastids, non-protoplasmic cell inclusion bodies, centrosomes, blepharoplasts and chondriosomes may occur in some cells. With some reservations concerning the presence of a visible nucleus, we may assume that the bacterial body is a cell, comparable to the cells of other fungi. Accordingly, the terms bacterial cell, protoplast, cytoplasmic membrane, cell wall, and cell inclusion bodies will be employed throughout this review.

I. CELL INCLUSIONS

The term cell inclusions is quite generally employed by cytologists to denote certain non-protoplasmic bodies which are deposited in the protoplast. Such bodies occur in cells of higher green plants, algae, yeasts, higher fungi and bacteria. They are generally absent from young actively growing cells but are formed *de novo* as the cells become older. Sap vacuoles, fat droplets, grains of volutin, starch, aleurone, glycogen, iogen, sulphur, and mineral crystals are appropriately spoken of as cell inclusions. Inclusion bodies may occur within the sap vacuoles or they may be embedded in the cytoplasm.

The occurrence of granules in bacterial cells has been known since the early work of Koch (90) who observed highly refractile bodies in unstained cells of *Mycobacterium tuberculosis*. Babes (10) and Ernst (41, 42) reported stainable granules in various species of bacteria. Many papers have since been published and almost every conceivable opinion has been expressed concerning the nature, origin and biological significance of bacterial granules.

They have been identified as spores, sporoids, spore primordia, gonidia, gametes, nuclei, chromatin granules, and non-protoplasmic inclusions.

Fischer (45) took the extreme position that all granules of bacteria are cell inclusions consisting of reserve materials. Although this view has been questioned by many investigators, there are very good reasons to believe that many of the bodies which have been described as nuclei, chromidia, and gonidia were nothing but non-protoplasmic cell inclusions.

Our knowledge of bacterial cell inclusions had its beginning in the work of Meyer (116) who introduced accurate microchemical methods of study. We now know that many bacteria contain fat bodies, grains of volutin, glycogen, iogen and sap vacuoles. Sulphur and mineral crystals are found in the sulphur bacteria. We may now consider the various inclusions in detail.

1. *Fat bodies.* Fat occurs in some bacteria as highly refractile, spherical, oval, or elongated bodies which resemble endospores and were erroneously identified as spores by some early investigators. The bodies are variable in size and form, but are generally spherical. They may reach a diameter equal to that of the cell or may even extend the dimensions of the cell body. They are usually lined up in a row in the median axis but may be scattered throughout the cytoplasm. When numerous, they become somewhat flattened and limit the cytoplasm to thin lamellae.

Fat bodies are not stainable by any of the methods usually employed for staining bacterial cells. They appear, therefore, as clear spaces in cells stained with aqueous solutions of aniline dyes and for this reason have been mistaken, at times, for endospores or vacuoles. Various methods are now known by which fat droplets may be stained selectively. Meyer (117) employed Sudan III, alkanin, and dimethylamidoazobenzol, but more satisfactory dyes are now known. Other dyes of the Sudan series have been found satisfactory for staining bacterial fat. The new compound Sudan black B, was tested by Hartman (66) and proved to be greatly superior to the earlier dyes. This new dye, when dissolved in pure ethylene glycol, is stable indefinitely, and is almost

free from the troublesome precipitates which are unavoidable in some methods of fat staining. It stains the fat droplets of bacteria and yeasts quickly, intensely and selectively.

The naphthol blue method discovered by Dietrich and Liebermeister (31) and modified by others (21, 38, 120, 164), has been employed extensively for staining fat bodies in bacteria, yeasts, and fungi. Eisenberg (38) found that certain aniline dyes, which stain the cell membrane and cytoplasm but not the fat bodies, may be treated with various precipitating agents to obtain useful fat dyes. When cells containing fat bodies are placed in a dilute aqueous solution of Nile blue sulphate, the bodies remain unstained, but upon addition of alkali, an orange-red precipitate is formed and the fat bodies are stained selectively. Similarly, the precipitates formed by treating an aqueous solution of basic fuchsin with iodine, picric acid, alkaline phenol, or alkaline α-naphthol may be used as fat dyes. Although the precipitates are soluble in alcohol and may be employed as dilute alcoholic solutions, better results are obtained by suspending the cells in the precipitating agent and adding an equal volume of dilute basic fuchsin. The staining action appears to depend on lipoidal solubility of the precipitates; they stain fat bodies by dissolving in them and saturating them with the dye. The same is true of indophenol blue, the dye synthesized in Dietrich and Liebermeister's method. It should be noted that dried fixed films are not suitable for any of these fat staining methods.

The species of bacteria which deposit fat are shown in table 1. The list is probably incomplete and there may have been some erroneous reports. The record shows that fat bodies occur in some species of *Bacillus* but not in others; that all species of *Spirillum*, *Azotobacter* and *Rhizobium* have been found positive; and that fat bodies have not been reported in the corynebacteria, clostridia or any of the coccaceae.

There is still some controversy concerning the occurrence of fat bodies in mycobacteria. Dorset (36) reported successful staining of tubercle bacilli with Sudan III and thought he had discovered a useful method for diagnosis. He described the stained cells as beaded rods. However, LeDoux (94) could not

TABLE 1

The occurrence of glycogen, fat and volutin in certain bacterial species

SPECIES	GLYCOGEN	FAT	VOLUTIN	REFERENCE
Azotobacter beijerinckii	−	+	+	98
Azotobacter chroococcum	−	+	+	98
Bacillus alvei	−	−	+	131
Bacillus anthracis	−	+	−	53
Bacillus asterosporus	+	−	+	117
Bacillus carotarum	+	−	−	50
Bacillus cohaerens	+	−	−	52
Bacillus ellenbachensis	−	+	+	131
Bacillus fusiformis	−	−	+	131
Bacillus graveolens	−	+	−	131
Bacillus lacticola	−	+	+	131
Bacillus lactis	−	+	+	131
Bacillus megatherium	−	+	−	131
Bacillus mycoides	−	+	−	50
Bacillus oxalaticus	−	+	−	131
Bacillus parvus	+	−	−	131
Bacillus petasites	−	+	−	50
Bacillus pumulus	+	−	−	131
Bacillus robur	+	+	+	131
Bacillus ruminatus	−	+	−	50
Bacillus silvaticus	−	+	−	131
Bacillus simplex	+	−	−	50
Bacillus sphaericus	−	−	+	131
Bacillus subtilis	+	−	−	50
Bacillus teres	+	−	−	131
Bacillus tumescens	−	+	−	117
Bacterium globiforme	−	−	+	126
Clostridium butyricum	+	−	−	117
Corynebacterium diphtheriae	−	−	+	10
Corynebacterium fimi	−	−	+	126
Corynebacterium hoagii	−	−	+	126
Corynebacterium simplex	−	−	+	126
Corynebacterium tumescens	−	−	+	126
Corynebacterium ulcerans	−	−	+	126
Corynebacterium xerose	−	−	+	41
Lactobacillus bulgaricus B	−	−	+	193
Mycobacterium leprae	−	−	+	12
Mycobacterium phlei	−	+	−	52
Mycobacterium tuberculosis	−	+	−	52
Pseudomonas sp.?	−	−	+	52
Rhizobium japonicum	−	+	−	99
Rhizobium leguminosarum	−	+	−	99
Rhizobium lupini	−	+	−	99
Rhizobium meliloti	−	+	−	99
Rhizobium trifolii	−	+	−	99
Sarcina ureae	−	−	−	39
Spirillum giganteum	−	+	+	39
Spirillum serpens	−	+	+	100
Spirillum tenue	−	+	+	100
Spirillum undula	−	+	+	100
Spirillum virginianum	−	+	+	100
Spirillum volutans	−	+	+	100
Streptococcus tirogenus	−	−	−	39

obtain satisfactory staining although he followed the same method. Dorset was not able to obtain positive results with several lots of dye and concluded that success depends on some unknown quality of the dye compound.

Grimme (52) stained the refractile granules of *Mycobacterium phlei* and *M. tuberculosis* with Sudan III and dimethylamidoazobenzol and identified them, accordingly, as fat bodies. Meyer (129) confirmed Grimme's results and expressed the opinion that most of the fat extractable from tubercle bacilli is located in the bodies rather than in a fatty membrane, as had been generally believed. Knaysi (83) saw stained granules in cells of tubercle bacilli which had been treated with Sudan III. He found, however, that the granules remained stainable after treatment with fat solvents and denied their fatty nature. Hartman (66) stained the refractile granules of various mycobacteria with Sudan black B and identified them as fat bodies.

Although some workers have reported negative results, there is a preponderance of evidence that mycobacteria deposit fat in the form of definite bodies. When due allowance is made for differences in the quality of dyes and improper technique, there is little or no conflicting evidence.

The biological significance of fat bodies in bacteria appears to be the same as in other organisms. They probably function as reserve food in some cases while in others they may denote fatty degeneration of the cells. Grimme (52), Preisz (153), Meyer (123), Lewis (97) and others have shown that the fat bodies disappear when spore formation occurs and when cells are placed on agar devoid of nutrients. There is some evidence for the theory of fatty degeneration. Müller and Stapp (129) and Almon (5) proved that granulated bacteroids from root nodules are not viable. Fat formation in *Endomyces vernalis* has been described recently by Heide (67) who supports the theory that the fat bodies function as reserve food.

The conditions necessary for deposition in the form of definite visible droplets do not appear to be very well understood. Many species of bacteria which do not form visible droplets may contain relatively large amounts of extractable fats. This phase of

the subject has been reviewed by Meyer (123) and by Buchanan and Fulmer (22).

The occurrence of fat bodies in bacteria has been the cause of many erroneous interpretations of cell structure and methods of reproduction. Various workers, failing to recognize the true nature of the bodies, have identified them as endospores (90), spore primordia (23), sporoids (160), endoplasts (135), and non-stainable gonidia (48, 104).

Cells containing fat bodies do not stain uniformly when treated with protoplasmic dyes but present an appearance which has been frequently described as interrupted, speckled, granulated, barred, beaded, banded, vacuolated and alveolar. The writer (98-100) has shown that uneven staining in cells of species of *Azotobacter*, *Rhizobium*, and *Spirillum* is conditioned by non-stainable fat bodies embedded in the stained cytoplasm. The stainable compressed cytoplasm has been regarded by some investigators as chromatin, by others as reproductive bodies.

2. *Volutin.* Refractile granules, variously designated in the literature as Babes-Ernst granules, metachromatic corpuscles, and volutin grains, occur in many species of bacteria, yeasts, molds, higher fungi, and algae. According to Meyer (121), they are not found in any group of plants above the *Thallophyta.* Guilliermond (58) questioned this conclusion but offered no convincing proof to the contrary. Such granules have been known in bacteria since the early work of Ernst (1888) and Babes (1889). The latter (11) observed red granules in cells of bacteria which had been stained with methylene blue. He introduced the term metachromatic corpuscles to designate this peculiar staining reaction. The term volutin, now almost universally employed, was proposed by Grimme (52) to designate the stainable granules of *Spirillum volutans.*

Volutin is a viscous substance which may occur in the form of tiny droplets, large globoids, irregular bodies or elongated threads (121). The bodies are somewhat more refractile than cytoplasm, but they are less refractile than fat bodies and spores. The principal tests by which volutin is distinguished from other cell inclusions and protoplasmic structures were given by Grimme

(52) and Meyer (121). These granules dissolve and disappear from cells in water at 80° within 5 minutes, and still more quickly in boiling water. They are readily soluble in strong or dilute solutions of alkalies, in 5 per cent sulphuric acid, fresh Javelle water, and chloral hydrate; but not in picric acid, ether, chloroform, alcohol, trypsin, pepsin or 1.0 per cent sulphuric acid. When the cells are fixed by formaldehyde, osmic acid, alcohol, or the usual method of heating dried films, the granules resist solvents.

Volutin grains stain more intensely than cytoplasm with basic aniline dyes, but they do not stain with fat dyes. Heucke and Henneberg (71) have shown that neutral red, 0.001 to 0.005 per cent in water, stains the bodies *intra vitam* but has no staining capacity for cytoplasm. Similarly, dilute aqueous solution of methylene blue causes intense staining of the granules with little or no action on cytoplasm. They are markedly resistant to the destaining action of 1 per cent sulphuric, hydrochloric or acetic acid. Differential staining of the granules and cytoplasm may be accomplished by staining with methylene blue, destaining with 1.0 per cent sulphuric acid and counterstaining with a contrast dye. The replacement dye, vesuvin, may be employed as in Ernst's first method (41) to effect differential staining of granules and cytoplasm. He stained fixed films with methylene blue and, after rinsing with water, counterstained them with vesuvin. The deep blue granules stand out sharply in the yellowish cell body. Safranin may be substituted for vesuvin. Contrast staining is attained also by treating fixed stained films with methylene blue and Lugol's iodine solution which causes blackening of the volutin bodies.

Various special methods have been devised for staining the granules of diphtheria bacilli and have been widely used for diagnostic purposes. The methods of Neisser (133), Albrecht (2), and Albert (1), have been found useful.

The biological significance of volutin was not well understood by the earliest investigators, and almost every conceivable function was assigned to it. Ernst (41) spoke of the deeply stained bodies as spores. Neisser (132) reported acid-fastness and re-

garded the granules as true spores. Ernst (42) reversed his former opinion and introduced the term sporogenic granules. According to his conception, the granules consist of nuclear material and participate directly in the formation of spores by fusing together. This theory of spore formation has been widely held by many subsequent investigators, but there is no reason to believe that volutin bodies consist of nuclear material or that they participate in the formation of spores by fusion. Babes (10) saw no analogy between the granules and true spores. He seems to have reached no very definite opinion concerning their function, but suggested a possible relation to cell division and spore formation. Marx and Woithe (110) favored the idea that the granules function in cell division but opposed Ernst's theory of spore formation. They spoke in favor of a correlation between virulence and the presence of granules in pathogenic species. This theory of toxigenic granules was attacked and discredited by Krompecher (92), and others (44, 47). Volutin has been frequently confused with nuclei, chromidia, compressed cytoplasm, spore primordia and gonidia.

The true significance of volutin in bacteria appears to have been first recognized by Grimme (52) who spoke of it as a reserve food compound. Guilliermond (58) reached the same conclusion concerning the function of volutin in yeasts and other fungi. Meyer (121), who had previously discussed the function of fat, glycogen, and iogen in bacteria, supported Grimme's explanation.

It is now well known that volutin grains are not permanent organs of the cell; they arise *de novo* in cells of some species but never occur in others. The deposition of volutin in bacteria depends on the species, the age of the cells, and to some extent on the culture medium. They are not found in very young actively growing cells but may become abundant as the cells mature. Zikes (202) showed that the culture medium must contain phosphate. In spore-forming species, the granules reach their greatest abundance just preceding spore formation and disappear during the ripening of the spore. For these reasons, volutin must be considered as a storage product which functions as reserve food.

The chemistry of volutin has not been studied very extensively. Meyer (121) noted marked similarity between some microchemical reactions of volutin grains and nucleic acid extracted from yeast. He concluded that the substance is a nucleic acid compound which differs from the nucleoprotein found in the chromatin of nuclei. He reasoned logically that volutin from various species might differ in precise composition in much the same manner as fats differ. According to Schumacher (166), free nucleic acid becomes green when treated with methylene blue and chrysanilin while nucleoproteins become yellow. Since the granules of diphtheria bacilli take the green color when stained by this method, they should consist of free nucleic acid. Glaubitz (49) was not able to obtain a positive Schumacher test for nucleic acid in the volutin grains of yeast. Zikes (202) studied the chemistry of volutin by macrochemical methods and identified it as a nucleoprotein. Although the chemical nature of volutin may not be fully known, it can be identified by a series of microchemical tests which serve to distinguish it from all other cell inclusions and protoplasmic structures.

The bacterial species which deposit grains of volutin are shown in table 1. It is by no means probable that the list is complete or that all the original determinations were correct. Prior to the studies by Grimme and Meyer, methods of identification were not very reliable and errors are to be expected. It appears from the record that volutin occurs in all species of *Spirillum* and *Azotobacter*; in some species of *Bacillus*, *Aerobacillus*, *Lactobacillus*; *Corynebacterium*, *Pseudomonas*, and *Mycobacterium*; but not in *Rhizobium* and *Sarcina*.

The writer doubts the occurrence of volutin in mycobacteria although Babes (12) and Guilliermond (59) reported its presence in *Mycobacterium tuberculosis*. Hollande and Crémieux (75, 76) also described granules which resembled volutin but which differed from it in some respects. Similarly, Knaysi (83) saw hyperchromatic granules which were not soluble in hot water. According to my observation volutin does not occur in any of several species examined. Stainable granules have been reported in various species of cocci but no very critical investigation concerning their identity has been made.

We are not specifically concerned with the occurrence of volutin in other groups of microörganisms but may call attention to its wide distribution in yeasts, actinomycetes, molds and higher fungi. It appears to be present in all species of *Cyanophyceae* where it is deposited abundantly in the central body which resembles the large sap vacuole of yeasts. It has been reported in various protozoa by several investigators. This wide distribution of the same substance in various microörganisms seems to indicate an important reserve material which functions in the same manner as fats, glycogen, starch and other storage compounds.

In the study of the bacterial cell, we are especially concerned with volutin as a cause of error in the interpretation of cell structure and methods of reproduction. This subject will be discussed in detail in a subsequent section.

3. *Glycogen.* Glycogen is a reserve or storage carbohydrate which occurs commonly in the cells of animal tissues, yeasts, molds, and some species of bacteria. According to Meyer (123), glycogen usually occurs in the form of viscous masses which are somewhat refractile. Microchemical tests for glycogen are less satisfactory than for other cell inclusions. The masses become reddish brown in strong Lugol's iodine solution and are, therefore, distinguished from the yellowish cytoplasm.

Glycogen has been reported in several species of *Bacillus* and in *Clostridium butyricum* as shown in table 1.

The refractile granules of *Azotobacter* were identified erroneously as glycogen by Heinze (68). Müller and Stapp (129) were not able to confirm this report by precise macrochemical methods of study. Similarly, Hiltner (72) reported glycogen in bacteroids of some rhizobia, but the identification was not confirmed (99, 171).

4. *Iogen.* Small refractile granules which stain blue with iodine have been known since the early work of Trécul (1865). Beijerinck (16) employed the term granulose which is still preferred by many bacteriologists. Meyer (117, 123) included all carbohydrates of bacteria under the terms iogen and glycogen and distinguished between them by differences in the reaction with iodine. Gray (51) identified as starch grains the granules of *Escherichia coli* which stain blue with iodine. Svartz ((179) and in earlier papers) studied the iodophilic granules of intestinal clostri-

dia. The occurence of this compound in various species, especially in the genus *Clostridium*, has been established. Iogen appears to serve as reserve food.

II. THE NUCLEUS

The question of the presence or absence of a bacterial nucleus, its nature, and the forms which it may assume, if present, has long been a subject of interest to cytologists, but no wholly satisfactory answer has yet been found. Many of the earlier workers assumed that the bacteria, standing as they do at the threshold of organized living matter, may lack some of the characteristic structures which occur normally in the cells of higher plants and animals. Haeckel introduced the term cytode to denote such cell-like organisms. This doctrine was acceptable to Fischer (45), Migula (124) and other early bacteriologists who regarded bacteria as non-nucleated organisms. The voluminous literature which has appeared during the past fifty years shows, however, that the theory has not been generally accepted. Henrici (69) said: "It satisfies the facts as we know them, but it does not satisfy the mind." There are some cytologists, perhaps many, who would challenge the statement, that the theory of no nucleus satisfies the known facts, but there is no doubt that it fails to satisfy the mind. Theoretically, it seems, we should expect to find a true nucleus or a functional equivalent in the cells of bacteria.

The literature of the subject is truly voluminous, frequently contradictory, and highly controversial; it is impossible to reconcile the numerous conflicting reports and theories which have been based on the study of *Bacillus anthracis* alone. This much, however, is certain: cell inclusions, immature spores, and cytoplasmic structures have been frequently mistaken for nuclei; in some cases the organisms studied were not true bacteria; and the methods employed were not always suitable for cytological study. It is also true that some investigators, inadequately trained and without previous cytological experience, were not properly prepared for such a difficult undertaking. For all of these reasons, much of the old uncritical work is of little value, and need not concern us here.

We may conveniently divide the theories concerning the nucleus of bacteria into the following groups:

1. The bacteria do not possess a nucleus or its equivalent.
2. The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm.
3. The bacterial body is a nucleus devoid of cytoplasm: a naked nucleus or nuclear cell.
4. The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm.
5. The form of the nucleus is not constant throughout the growth cycle; it may occur as a discrete spherical body, an elongated chromatin thread, or scattered chromidia depending on the stage of development: a polymorphic nucleus.
6. The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units: a diffuse nucleus.
7. The protoplast contains one or more true vesicular nuclei.
8. The nucleus is a naked invisible gene string, or a chromatin-encrusted gene string analogous to a single chromosome.

1. *No nucleus or equivalent.* Among the early workers, Fischer (45) was the most able advocate of the theory that a bacterial cell possesses no nucleus. He wrote as follows: "The bacterial cell then, interpreted in the light of the above facts is a simple protoplast enclosed within a cell-membrane but devoid of a nucleus." In another connection, he said: "The general conclusion to which all these observations lead us is that the bacterial cell-contents are a mass of protoplasm representing an osmotic system precisely like that of the cells of higher plants, but, unlike them, having no nucleus." He saw granules in various species of bacteria, but he regarded them as reserve inclusion bodies rather than nuclei. Migula (124) appears to have reached similar conclusions although he expressed his views in somewhat guarded terms. Wamoscher (191) studied several species by micrurgical methods, and saw nothing which could be interpreted as a nucleus. Roman (158) found no nucleus in *Mycobacterium tuberculosis.* Alexieff (3) denied the occurrence of chromatin in the cells of bacteria and *Cyanophyceae.*

2. *The central body.* Bütschli (25) advocated the theory that the bacterial cell, like the cells of *Cyanophyceae,* is differentiated into a dense nucleus-like organ, the central body, and a peripheral layer of cytoplasm. He saw complete analogy in the structure of *Spirillum volutans* and a species of *Oscillatoria* although, according to his observation, the cytoplasm in *S. volutans* is reduced to a thin outer layer and two polar caps from which the flagella arise. The occurrence of deeply stainable chromosome-like bodies in the central portion of bacterial and cyanophycean cells gave him complete assurance that the central body is the homologue of a true nucleus.

The nature of the central body, a prominent vacuole-like structure which occurs in all species of *Cyanophyceae,* has long been a controversial matter among botanists. This cell organ resembles a true nucleus in certain characteristics: it occurs in every cell as a definite, relatively large, spherical body; it contains chromosome-like granules; and it divides simultaneously with division of the cell. For these reasons, many botanists have regarded it as a primitive nucleus which differs from a true nucleus in one important respect, namely, the absence of a nuclear membrane. Others, notably Meyer (121), objected to this interpretation. According to Meyer, the central body is not a nucleus but a large central sap vacuole which contains volutin and is, therefore, comparable to the vacuole of yeasts. More recently, Hollande (73) described deeply stainable granules, *nucleosomes,* which occur among the volutin grains. According to his interpretation, the "nucleosome apparatus" is a protoplasmic secretion. Whatever may be the final outcome of the controversy concerning the central body in the *Cyanophyceae,* evidence is not sufficient to warrant the assumption that an homologous cell organ occurs in any of the true bacteria.

Zettnow, in his early writings (197-200), described the bacterial cell as a structure which consists essentially of a central chromatin-containing portion, the endoplasm, surrounded by a thin envelope, the ectoplasm. In large spirilla and in some bacillary species, he saw well-differentiated chromatin bodies, but in various species of small bacteria the chromatin appeared to be

more finely divided and diffused in the endoplasm. Later, Zettnow (201) concluded that the bodies he had formerly called chromidia were grains of volutin and that chromatin, if present, is in the form of minute invisible particles uniformly dispersed in the cytoplasm. The terms endoplasm and ectoplasm are still employed by some bacteriologists.

Petit (141) found volutin bodies in the cells of *Chromatium okenii* and other sulphur bacteria but no true nucleus or central body. A central body was present in *Oscillospira guilliermondi*, but he regarded this species as an alga. Guilliermond (60) reached similar conclusions concerning *Thiodictyon*. Some support was given to the theory of a central body in true bacteria by Guilliermond (61) who studied *Bacillus megatherium* and observed a central organ resembling that of the *Cyanophyceae*. According to Hollande (74) the structure described by Guilliermond is a metanucleosome. Lewis (100) studied the structure of *Spirillum volutans* and other spirilla. No support was found for the theory of a central body.

3. *A naked nucleus.* The fact that the staining capacity of bacteria is similar to that of the nucleus of ordinary cells has caused some investigators to regard the whole body as the homologue of a true nucleus. This theory has been adversely criticised as well as supported on theoretical grounds alone. The hypothesis has been put forward that the first living things to arise on the earth consisted of undifferentiated protoplasm similar to the substance of true nuclei, and that cytoplasm is the result of subsequent evolution. The opponents of this idea have contended that the most primitive living things consisted of undifferentiated protoplasm homologous with the cytoplasm of higher organisms, and that differentiation into cytoplasm and nucleus represents the final result of evolutionary development. According to these hypotheses, there could be a variety of cell or cell-like organisms with different degrees of differentiation: true cells with typical nucleus and cytoplasm; cell-like bodies devoid of a nucleus or devoid of cytoplasm; and cells differentiated into cytoplasm and a primitive nucleus.

Aside from similarity in stainability, there is no substantial

evidence to support the theory that bacteria are naked nuclei. Růžička (159, 160) attempted to prove that the bacterial body is identical morphologically with true nuclei of other plants. His claims were based principally on the structure of *Bacillus anthracis* when cultivated on glycerol agar. The cells failed to form endospores on this medium; when stained, they simulated the structure of true nuclei. He described non-stainable masses of linin and net-like stainable chromatin. His drawings bear a superficial resemblance to diagrammatic drawings of true nuclei, but there is no reason to believe that the structures involved are related in any way. Růžička's work was severely criticized by Eisenberg (38), Meyer (123), and others. According to Meyer, the cells which failed to form endospores became filled with non-stainable fat bodies which were incorrectly designated as linin while the chromatin net was nothing but compressed masses of cytoplasm lying between the fat bodies. The criticism appears to accord with all known facts.

Some support for the theory was afforded by Ambroz (8), a student of Růžička's, who studied *Bacillus nitri* but was unable to add anything of value. More recently, Kůzela (93) studied the structure of *Bacillus anthracis* and various other species of bacteria by means of the Feulgen-Rossenbeck reaction. Support was found for the analogy between the bacterial cell and the nucleus of ordinary cells.

The hypothesis that the most primitive form of living things must have consisted of undifferentiated substance, analogous to that of true nuclei, appears to be logical enough since we know that the nucleus is the bearer of hereditary units and directs the activities of the cell. However, there is no evidence that bacteria are representatives of such a theoretical primitive organism. On the other hand, all the evidence indicates that the bacterial body possesses most, if not all, of the essential features of a true cell. We are not concerned here in a theoretical discussion of living things still more primitive than the known bacteria, although it is not denied that such organisms may have existed or may still exist.

4. *A chromidial system.* The theory that the bacterial nucleus consists of visible chromatin bodies, a chromidial system, scat-

tered throughout the cytoplasm has been advocated by many cytologists but has not gone unchallenged. The theory seems to have had its inception in the early work of Ernst (42) who observed stainable bodies in various species of bacteria. Support was given by R. Hertwig (70), who observed scattered chromidia in the cytoplasm of certain nucleated protozoan cells. Reasoning by analogy, he proposed that the nucleus of the bacteria and *Cyanophyceae* consists of a chromidial system. His exact language is of interest for it marked the beginning of a theory which attracted many advocates and which is at present one of the most generally accepted theories concerning the nucleus of bacterial cells. He wrote: "I have assembled above a number of examples in which chromatic material, which is diffusely distributed throughout the cell, is present in addition to the cell nucleus, and which temporarily substitutes solely for the nuclear apparatus. Herewith we have the possibility of organisms which have perhaps no true permanent nuclei but, in lieu of nuclei, chromatin bodies which are interspersed wholly or partly in the protoplasm. Bacteria and *Oscillatoria* seem to me to be such organisms."

Schaudinn (162) studied a giant species, *Bacillus bütschlii*, from the intestinal contents of the kitchen roach, *Periplaneta orientalis*. He saw no differentiation into central body and peripheral cytoplasm comparable to that described by Bütschli for *Spirillum volutans*. On the other hand, the cell contents consisted of alveolar cytoplasm in which there were scattered chromatin bodies during the greater part of the life history. A violent fountain-like streaming of the granules which continued for several minutes occurred in cells immediately preceding spore formation. As the streaming gradually subsided, the granules became arranged in the form of a wreath-like filament extending from pole to pole in the median axis of the cell. A few of the granules in the polar position then united to form a rather large nucleus-like body which he regarded as a true nucleus and the beginning of the spore. According to his interpretation, the cell nucleus consists of scattered chromidia during the vegetative phase of the life history, and a true nucleus is organized only at the time of spore formation.

There are very good reasons to doubt his identification of the

stainable granules as chromatin bodies. Unfortunately, he made no microchemical tests to determine their nature; and since the organism could not be cultivated and has not been since by other investigators who have searched for it in the animal host, the precise nature of the bodies is not known. Concerning the behavior of the granules he said: "During its development easy distinction is made between the nucleus and cytoplasm. During the period of the inactive spores, this differentiation is lost (how, we do not know), for the young sporelings show no separation of nucleus and protoplasm." We now know that such behavior is typical for spore-forming species which deposit volutin; and there seems, therefore, little reason to doubt that the granules of *B. bütschlii* were grains of volutin. More recently, Lindegren (102) suggested that the granules might have been true nuclei produced by multiple division, but their behavior, origin *de novo* in the vegetative cells, does not support this view. The organization of a true nucleus at the time of spore formation may also be questioned. This will be discussed in a subsequent section. In the light of more recent investigations and in view of the fact that Schaudinn's theory of the nucleus has been widely accepted, the severe criticism by Meyer (118, 119) together with the reply by Schaudinn (163) is of great interest.

Guilliermond (55–57, 59–62) over a period of many years has been the most able advocate of the chromidial theory. He studied various species and, at times, seemed to give some support to the notion of a central body, but he always maintained that the bacteria do not possess a typical nucleus like that of higher plants. In 1917, he said: "The conclusion, to my mind, would be that while some bacteria may contain a rudimentary nucleus whose existence is nowhere else precisely demonstrated, so far, in the great majority of species, nothing more has been found than a diffuse nucleus consisting only of grains of chromatin scattered through the cytoplasm."

In his study of various spore-forming species, *Bacillus mycoides*, *Bacillus radicosus* and others, Guilliermond (55) observed that young cells present a homogeneous appearance and are uniformly stained with no great differentiation. Toward the eighth

hour of development, the cells show clearly their structure which is changed in appearance; the cytoplasm becomes vacuolated and displays a fine alveolar structure. The web contains in its meshes small, highly stainable granules which seem to consist of chromatin. To one who has become familiar with the structure of *B. mycoides*, at different stages of the growth cycle, it is obvious that the description is accurate, but the identification of chromatin bodies is erroneous. The change from the homogeneous structure of young cells to the vacuolated alveolar condition of older cells, in this species, is due to the deposition of fat bodies, while the so-called chromatic bodies are compressed cytoplasm. Mencl (115) studied the cell structure of *Azotobacter chroococcum*. He observed that the cell body shows a characteristic honey-comb appearance in which the lattice-like walls are dull, while the enclosed spaces are filled with a highly refractile mass. This is a very good description of old, fat-filled cells of this species. He saw also deeply stainable grains of volutin which he mistook for chromatin bodies. In general, the evidence presented by other investigators (20, 138, 167) in support of a chromidial nucleus is not convincing.

5. *Polymorphic nucleus.* Amato (7) studied the structure of *Bacillus mycoides* in preparations stained *intra vitam* with brilliant cresyl blue. He reported a single large nucleus in the spores and young rods, but in older cells the nucleus appeared to break up to form numerous chromidia. He suggested that the variability in form might account for the various conflicting views concerning the nucleus.

Dobell (35) wrote at length on the nucleus of various unknown bacteria which occur in the intestinal contents of frogs, lizards and other animals. He concluded that the nucleus may occur as a single vesicular body, scattered chromidia, or chromatin filaments depending on different stages of development. There is no evidence that he made suitable tests to distinguish between nuclear structures and cell inclusions. The illustrations, reproduced in colors, suggest that many of the cells contained fat bodies as well as grains of volutin and that some of the cells were yeasts or other low fungi. His observations, limited to stained

films from raw mixtures, afforded no adequate basis for his conclusions.

Other papers which might be considered here are discussed in the section dealing with the diffuse nucleus.

6. *A true nucleus.* The theory that bacteria possess true nuclei has been supported by many of the most able investigators, but this does not mean that the several reports are mutually confirmatory. There is still much doubt concerning the real nature of some of the so-called true nuclei which have been described. In order to prove the existence of a true nucleus in bacteria, it must be shown that the organism belongs to the bacteria rather than yeasts or other low fungi; that a definite particulate body, differentiable from the cytoplasm, occurs constantly in each cell; that genetic continuity of the body occurs in nuclear and cell division; and that the body in question is not a cell inclusion, vacuole, spore primordium, a cytoplasmic body, or an artifact. It seems needless to add that such rigid criteria have not been applied generally and that many invalid claims for the discovery of true nuclei have been made.

The most generally accepted proof for the occurrence of a true nucleus in bacteria is found in the work of Vejdovsky (188) who studied *Bacillus gammari*, a species which he found in *Gammarus zschokkei*. There seems to be no doubt that the body described by Vejdovsky is a true nucleus which possesses a nuclear membrane, chromatin bodies, and divides karyokinetically. On the other hand, the identification of the organism as a bacterial species was seriously questioned by many (52, 123, 150, 162, 185). The consensus of best opinion appears to be that the organism is a yeast, *Cryptococcus gammari*. Mencl (114) described comparable nuclei in bacteria which occur in the intestinal content of the cockroach, but his work was severely criticized (57, 123) and has not been generally accepted.

Meyer (116, 117, 122, 123) studied the cell inclusions and the nucleus of *Aerobacillus polymyxa* (*Bacillus asterosporus*), *B. tumescens*, and *Clostridium butyricum* (*B. amylobacter* Meyer and Bredemann). In each of these species, he observed a small, spherical, colorless, refractile body, about 0.3μ in diameter,

which could be readily differentiated from fat, volutin, glycogen, and cytoplasm by differences in microchemical reactions and stainability. According to his interpretation, the body should be called a true nucleus. The nucleus occurred in the spore primordia, mature spores, and in the young rods produced at germination of spores. In vegetative cells, there were usually two to six free nuclei, but he was not able to trace stages in nuclear and cell division. Meyer's methods deserve brief mention. The microchemical reactions for the determination of various cell inclusions have already been mentioned. In his first studies, he stained the cells *intra vitam* with formol-fuchsin, but in the later study of *C. butyricum* he employed various fixing agents and, after washing, stained wet mounts with iron alum hematoxylin. He stated with emphasis that dried fixed films are not suitable for the demonstration of nuclei.

Because of his great prestige, Meyer's conclusions were widely accepted but have not gone wholly unchallenged. Guilliermond (59) commented as follows: "It seems to be established, however, that the majority of the elements noted by Meyer are not nuclei but reserve products common among *Protista* and known as metachromatic corpuscles." This criticism could doubtless apply to the "nuclei" of *Aerobacillus polymyxa*, for volutin is deposited by this species. It could not, however, apply to *B. tumescens* which deposits fat bodies only or to *Clostridium butyricum* in which the cell inclusions are glycogen and iogen. Neither of these could be confused with Meyer's nuclei. The most damaging criticism was given by Zettnow (201) who studied *B. tumescens* by Meyer's method and confirmed his observations. However, Zettnow regarded the bodies in question as cytoplasmic structures rather than nuclei. Complete confirmation of Meyer's work was reported by several of his students (39, 52, 131). Preisz (153) reported similar nuclei in *Bacillus anthracis*.

Swellengrebel (180, 181) described zigzag and spiral filamentous nuclei in *Bacillus maximus buccalis*, *Spirillum giganteum* and other bacterial species; similar observations were recorded by Dobell (35), Dimitroff (32) and Paillot (138). Swellengrebel's work was however severely criticized (55, 123). Quite recently

Lewis (100) observed spiral arrangement of the stainable material in *Spirillum volutans* but was not able to confirm Swellengrebel's opinion that the filaments consist of chromatin. The spirally arranged substance appears to be nothing but compressed cytoplasm lying between the numerous non-stainable fat bodies which are present in all species of *Spirillum*.

Stoughton (177, 178) studied the structure and reproduction of a plant pathogen, *Phytomonas malvacearum*, which causes angular leaf spot of cotton. Each cell contained a single, spherical, centrally located body which could be differentiated from cytoplasm by *intra vitam* staining. Because of its constant occurrence, size, position in the cell, staining reactions, and division by constriction, he regarded the body as a true vesicular nucleus. The nuclear nature of this body has been questioned by various investigators: Dufrénoy (37) identified it as a vacuole; Petter (142) obtained diffuse staining of the rods by Feulgen's method; Guilliermond (62) regarded the body as a metachromatic corpuscle (volutin).

Hollande and Hollande (77) wrote at great length on the structure of various bacterial species including *Eberthella typhosa*, *Escherichia coli*, *Mycobacterium tuberculosis* and *Bacillus anthracis*. They introduced the terms nucleosome, paranucleosome, and metanucleosome to designate structures which were differentiated from cytoplasm by means of a special staining method. The nucleosome, a minute nucleus-like body, occurs in all cells, divides by constriction, and stains blue with the eosinate of methylene blue; the paranucleosome, an eosinophilic body, closely associated with the nucleosome and often obscuring it, divides into several small granules during cell division; the metanucleosome, an irregular basophilic body, surrounds the paranucleosome.

Hollande (74) observed these organules in the cells of various species from the intestinal content of animals as well as in pure cultures of well known species. He seems to have reached no very definite conclusions concerning the nature of the bodies but offered several possible interpretations. The constant occurrence of the nucleosome in all cells and its characteristics are indicative of a true nucleus. The transitory nature of paranucleosomes

and metanucleosomes suggests reserve substances elaborated by the cell protoplasm.

Barnard (14) photographed cells of *Bacillus mycoides*, *B. megatherium*, *Staphylococcus aureus* and *Serratia marcescens* by ultraviolet light and obtained images which, according to his interpretation, suggest that bacteria contain a nucleus which undergoes mitotic division. Wyckoff and Ter Louw (195) employed similar technique in photographing cells of *Bacillus subtilis* and called attention to the absence of any structures which could be regarded as nuclei. The theory of a true nucleus is supported by a number of observers (9, 18, 40, 130, 139, 165).

The theory of a true vesicular nucleus has received some support from investigators who employed Feulgen's reaction for differentiation. da Cunha and Muniz (29) observed, as a rule, two stainable granules in young cells of *Bacillus anthracis*. Stille (176) saw discrete stainable bodies in various species of spore-forming bacteria, *Azotobacter* and *Sarcina*. The number and arrangement in the cell were not influenced by methods of cultivation; the bodies appeared to divide by constriction and for these reasons were regarded as true nuclei. There is no indication that he distinguished between nuclei and inclusion bodies although we know that *Azotobacter chroococcum* deposits volutin.

Piekarski (143–145) demonstrated "nucleoid" bodies in cells of *Escherichia coli*, *Salmonella paratyphi*, and *Serratia marcescens* by means of Feulgen's reaction and the electron microscope. Cells from young cultures contained 2 nucleoids while other cells contained a single body. The bodies appeared to divide preceding cell division and to consist of thymonucleic acid, since the ultraviolet absorption spectra of nucleoids and known nuclear substance were identical. Piekarski and Ruska (146) studied the structure of several bacterial species by means of the electron microscope. Electron micrographs of cocci, sarcinae, and spore-forming species show very little or no structural differentiation. In cells of *Pseudomonas aeruginosa* and some other non-spore-forming bacteria, granular bodies similar to the nucleoids demonstrated by Feulgen's reaction are shown.

7. *The diffuse nucleus.* The theory that the bacterial nucleus

consists of finely divided particles of chromatin uniformly dispersed in the cytoplasm was proposed by Zettnow (201) although he had formerly spoken in favor of a central chromatin-containing structure, the central body. The term diffuse nucleus is generally employed to denote a "nucleus" which is so finely divided and dispersed as to become undifferentiable from the cytoplasm. Some writers have also spoken of scattered visible chromidia as a diffuse nucleus. In order to avoid ambiguity, the term diffuse nucleus is used here to denote only the condition in which chromatin in the finely divided state is uniformly dispersed in the cytoplasm. The term chromidial nucleus or chromidial system has already been applied to the condition in which the nucleus has been supposed to exist as scattered visible chromatin bodies.

Several investigators have supported the theory of a diffuse nucleus. Zettnow (201) based his conclusions on the fact that differentiated bodies invariably proved to be cell inclusions or cytoplasmic structures. Thomas (182) described a new species, *Bacillus calmette*, in which the chromatin was uniformly dispersed or, at times, became separated from the cytoplasm to form visible units of pure chromatin or chromatin mixed with other bodies.

The theory has received some support from investigators who employed Feulgen's reaction as a method of differentiation. Feulgen and Rossenbeck (43) obtained negative results and concluded that bacteria do not contain thymonucleic acid. Westbrook (192) was not able to obtain positive reactions with yeasts and bacteria. Stapp and Bortels (172) saw diffuse staining of *Phytomonas tumefaciens* with no morphological differentiation. Voit (190) obtained positive reactions with thick films. Pietschmann and Rippel (148) reported uniform distribution of the stainable substance in normal cells of *B. mycoides*. Cultivation in media containing lithium chloride or magnesium sulphate resulted in abnormal cell forms in which the stainable substance became separated from the cytoplasm and appeared in the form of definite bodies.

Imsenecki (78–80) maintained that a diffuse nucleus occurs in all bacteria and corresponds to a stage in the evolution of the nucleus at which chemical differentiation of nuclear substance has

already occurred, while the physicochemical conditions necessary for morphological differentiation of nuclear structures have not yet developed. He believed that the dispersed chromatin can aggregate at certain stages in the life cycle to form visible units which may again break up into fine granules.

Pokrowskaja (151) studied *Bacterium pestis*. Cells growing as parasites showed a diffuse reaction, while under saprophytic conditions the stainable substance became aggregated into a definite nucleus-like body which was capable of amitotic division. Milovidov (125) observed diffuse staining in young cells of *Bacillus mycoides*, *B. megatherium* and *B. anthracoides*, but in older cells the substance united to form compact bodies.

The service which Feulgen's reaction has rendered in the study of bacterial structure is difficult to evaluate, since nucleic acid is frequently present as a reserve material and the reaction is not sufficiently intense to give a clear differentiation of minute structures. Margolena (108), Knaysi (86) and others have questioned the specificity of the reaction. The investigations seem to prove that many bacteria contain thymonucleic acid which is generally in the form of minute granules but may, under some conditions, separate out and become aggregated into definite bodies which resemble true nuclei. It could be argued that the drastic treatment required in this method causes plasmolysis or other artificial alterations in the cell structure. This has been discussed at length by Stille (176). Schaede (161) who stained bacteria and actinomycetes by Feulgen's method thinks that the stainable substance, diffuse or in the form of granules, is not true chromatin or gene material and that it probably functions as reserve food.

8. *The chromosome theory.* Lindegren (102) has formulated a theory of the bacterial nucleus based on our knowledge of the cytology and genetics of higher organisms: the gene is the fundamental particulate living unit and without it life is impossible; a linear aggregate of genes is the chromosome and an aggregation of chromosomes is the nucleus; the chromatin itself is not the hereditary substance but consists of inert material in which the genes are embedded; the genes maintain a fixed position in regard to each other, synchronize in division, and are distributed in such a

manner that each daughter cell receives its full complement of genes. Accordingly, a nucleus reduced to its lowest essentials might consist of a single gene string encrusted with chromatin, or a single naked gene string. A nucleus of this type could occur in the bacteria, but a diffuse nucleus is not possible since the genes do not diffuse but maintain an orderly position in the gene string.

It seems probable that such a simple nucleus would take the form of a small granule or a rod-like body rather than a definite vesicle with a membrane separating it from the cytoplasm. Lindegren has shown by diagrams drawn to scale, that a space 0.2μ in diameter is sufficient to accommodate a gene string of maximum theoretical size, and provide ample room for an orderly transmission of genes to the daughter cells.

Some evidence favorable to this chromosome theory is found in the most recent investigations of the subject. Lindegren and Mellon (101) described a diplococcus in which the nucleus consisted of a single haploid chromosome which contained seven chromomeres.

According to Badian (13) the nucleus of *Bacillus subtilis* consists of a single rod-like, haploid chromosome which divides lengthwise preceding cell division. The daughter chromosomes move apart before the transverse membrane is formed and each of the new cells receives a single chromosome. In spore formation, the chromosome divides in the usual manner, the two segments arrange themselves end to end in the median axis of the cell, and fuse end to end to form a single bivalent chromosome. This is followed by two successive, longitudinal divisions resulting in four haploid chromosomes, one of which becomes enclosed in the young spore while the three remaining in the cytoplasm are eventually lost. Allen, Appleby and Wolfe (4) described a somewhat similar series of events in the division and spore formation of an unidentified species of *Bacillus* isolated from grass. Each vegetative cell contains a single haploid chromosome, but the spores may be either haploid or diploid depending on the manner of origin. Transition from diploid to haploid condition occurs in spore germination.

Summary of the theories. Much of the conflicting evidence

having been assembled, we are now faced with the obvious difficulty of reaching a satisfactory conclusion. There is probably no one fully competent to perform this difficult task. The writer believes that the claims for a naked nucleus, a central body, a chromidial system, a polymorphic nucleus, and a true vesicular nucleus are based on faulty evidence and must be rejected. Much of the confusion has been caused by failure to distinguish between volutin and chromatin. Due to their nucleic acid content these substances react to the usual nuclear stains in much the same manner. Suitable tests for volutin have long been known, but there has been a marked tendency among investigators to regard all deeply staining bodies as nuclei or chromidia. Unless specific tests for volutin have been made, the occurrence of stainable granules in the cells of bacteria is not significant. This criticism alone is sufficient to disprove the claims concerning the nucleus of such species as *Spirillum volutans*, *Azotobacter chroococcum Bacillus bütschlii* and many others.

Although the occurrence of volutin has been the principal cause of error, there has been much confusion concerning the nature of the stainable substance in fat-depositing species. When cells containing fat bodies are fixed and stained by the usual methods, the compressed protoplasm appears as deeply stained compact masses, and zigzag or spiral threads which in many cases have been described as nuclei. The picture is even more complicated in species which deposit both fat and volutin. It is impossible to escape the conviction that many observers, failing to recognize the true nature of the stainable structures, have projected a subjective element into the problem.

The more recent theory that the nucleus consists of minute particles of chromatin uniformly dispersed in the cytoplasm is based principally on results obtained by Feulgen's method of staining. No very definite conclusions can be drawn from the various conflicting reports which have appeared.

If the bacterial cell contains no demonstrable nucleus, the possibility still remains that the nucleus consists of an invisible structure essentially the same as the gene strings in the chromosomes of higher organisms, but devoid of the usual encrustation of chroma-

tin. In the present state of our knowledge no final conclusions concerning the nature of the nucleus can be drawn. It appears, however, that we may now discard much of the uncritical work of the past and begin to think in terms derived from the more certain knowledge of the genes of higher organisms. Whether the protoplasm of the bacterial cell is undifferentiated, as claimed by Fischer and others, or consists of invisible genes and cytoplasm has not been definitely determined. In the light of all the experience of the past it seems highly probable that any claims based on cytological methods will not be found wholly convincing. Whether our knowledge of the hereditary mechanism of bacteria can be enhanced by genetical technique is still a question.

III. REPRODUCTIVE STRUCTURES

1. *Gonidia.* The theory that bacteria reproduce by means of small coccus-like bodies, gonidia, borne within cells of normal shape or in large specialized cells, gonidiangia, has had many advocates. The theory, now regarded as an established fact by many bacteriologists, is based in part on the observation that bacterial cells frequently contain granular bodies which appear to escape from the mother cell and develop into cells of the parental form. Indirect evidence for the occurrence of minute reproductive bodies, smaller than ordinary vegetative cells, has been obtained by filtration experiments. Theoretically, there are some reasons to believe that such a method of bacterial reproduction is possible. We have long known that *Sphaerotilus dichotomus* reproduces by forming motile cells which escape and develop into the thread-like form. Similarly, reproduction by means of zoospores, reproductive cells formed in sporangia by free cell formation, is of common occurrence in many fungi and algae. Whether any of the true bacteria reproduce by the formation of internal cells, analogous to those produced by algae and fungi, is an open question although there is an ever increasing volume of evidence which supports the theory. It is true, however, that much of the evidence is not very convincing and that some of it has been discredited.

The opponents of the theory have maintained that the so-

called gonidia are not capable of germination; that they are, in many instances, cell inclusion bodies or compressed cytoplasm; and that growth in filtrates is not conclusive evidence of a gonidial method of reproduction. The literature of the subject has become rather extensive, and there is not sufficient space here for an adequate critical review. We shall be obliged, therefore, to select a few of the most thoroughly studied species and present the evidence for and against this theory of reproduction.

Jones (82) reported deeply stainable, non-filtrable, reproductive granules in the cells of *Azotobacter sp.* Löhnis and Smith (105, 106) described stainable and non-stainable, filtrable reproductive granules. Lewis (98) identified the granular bodies as grains of volutin and fat bodies. Jones (82), Roberg (157) and Lewis (98) obtained negative results by filtration methods. It appears that the advocates of gonidial reproduction in *Azotobacter* confused cell inclusion-bodies with gonidia and reached unwarranted conclusions.

Few genera of bacteria have been so thoroughly studied as *Rhizobium*, and there is probably no genus in which reproduction by gonidia has been so generally accepted. Support is found in the early work of Morck (127), and in later publications by Bewley and Hutchinson (19), Gibson (48), and others. It would appear that here, if any place, the evidence is so overwhelming as to compel acceptance. The case of gonidial reproduction was well stated by Thornton (183) who wrote as follows: "Thus, in *B. radicicola* the rod-shaped cells at first stain evenly, but later the stainable material becomes segregated into bands. These develop into spherical granules of which a single cell may contain from 1 to 6. Rupture of the mother cell releases the granules which at first are usually non-motile, but later swell in size and become actively motile. All stages in the elongation of the cocci to form the evenly staining rods can be found." The writer (99) attacked this theory on the ground that the banded condition is not caused by free cell formation but by the deposition of non-stainable fat bodies which restrict and compress the cytoplasm to form the stainable bands. The small cocci and ovoid cells seen in old cultures are small vegetative cells caused by fission during the period

of declining growth. There seems to be no reason to recede from this position.

Reproduction of *Bacillus mycoides* by means of filtrable gonidia was described by Nyberg (135), and Oesterle and Stahl (137), but this conclusion was opposed by Stapp and Zycha (173), Lewis (96) and den Dooren de Jong (30). The stainable bands and bars in this species, as in rhizobia, are conditioned by fat bodies. Haag (64) reported the occurrence of gonidia in *Bacillus anthracis*. Rettger and Gillespie (155) and Knaysi (85) found no evidence to support the theory of filtrable gonidia in other sporogenous species including *B. megatherium*, *B. vulgatus* and *B. mesentericus*. The evidence is, therefore, mostly against gonidial reproduction in this group of well known species. Quite recently Allen, Appleby, and Wolf (4) reported filtrable gonidia in an unidentified species of *Bacillus* isolated from grass.

Concerning gonidial reproduction in mycobacteria, there have been so many publications that no attempt can be made to review them here. Many workers have regarded Much's granules as viable, filtrable units while others have identified them as products of cell degeneration. If it is true that the highly refractile granules, first described as endospores by Koch (90) are fat bodies, as claimed by Grimme (52), Meyer (123), and Hartman (66), then it would seem to follow that the stainable bands are nothing but compressed cytoplasm as in rhizobia and other fat depositing species. A still further study of this group is needed to establish the identity of the refractile bodies and the stainable elements.

The evidence in regard to gonidial reproduction in *Corynebacterium* is somewhat contradictory. Mellon (111) studied a species originally isolated from a case of Hodgkin's disease and described small motile bodies which became free and gave rise to new individual cells. Bergstrand (17) studied the same species but failed to confirm the occurrence of such reproductive bodies. More recently, Groh (54) reported that the granules which stain by Neisser's method are living units which burst the maternal rod and develop into new cells. This appears to contradict all that is known concerning the nature of Neisser's granules.

Löhnis and Smith (105), Enderlein (40) and Hadley, Delves and Klimek (65) maintained that gonidial reproduction occurs in all species of bacteria, but the evidence for this claim can not be regarded as very substantial. Whether gonidia occur in all, some, or none of the species of true bacteria is a difficult question to answer on the basis of our present knowledge. The writer believes that the positive results obtained by filtration experiments have little or no value and that the answer must be sought in the study of cell structure. Until we have seen a vegetative cell break up into granules, liberation of the granules from the mother cell, and development into new vegetative cells, this method of reproduction must be regarded as a theory rather than an established fact. There seems to be no doubt that many investigators have confused other structures with gonidia.

2. *Gametes.* The hypothesis that bacteria reproduce by sexual methods has received some support, but the evidence is not extensive and has not been very generally accepted. Among the early workers Schaudinn (162) observed an abortive division of the rods and violent streaming of granules preceding spore formation in the disporic species, *Bacillus bütschlii.* According to his interpretation, spore formation in this species is preceded by autogamic conjugation. Dobell (33) supported this conclusion but in a later study of the problem (34) reversed his former opinion; and still later (35), he rejected all theories of sexual reproduction in bacteria. Stewart (175) believed that asexual reproduction eventually comes to a close in a colony of bacteria and is followed by "an outburst of conjugation." He described the process as autogamous. The chromosome behavior observed by Badian (13) and Allen, Appleby and Wolf (4) supports the theory of autogamic conjugation.

Various investigators have described isogamic conjugation. Pothoff (152) described tube-like processes connecting the conjugating cells of *Chromatium okenii.* The cells of this species are very large, and if the process occurs the phenomena could probably be followed. His photographs are not convincing, and the conclusions were attacked by Krasil'nikov (91) who saw nothing but incompleted cell divisions. The same applied to the conjuga-

tion of *Azotobacter* described by Löhnis and Smith (105). Mellon (112) presented evidence to show that isogamic conjugation, similar to that of yeasts, occurs in *Escherichia coli*. More recently, Hollande and Hollande (77) described a somewhat similar fusion of isogametes in *Mycobacterium tuberculosis*. Nyberg (135) saw very tiny motile isogametes liberated from cells of *Bacillus mycoides*. Stoughton (178) reported isogamic conjugation in *Phytomonas malvacearum*.

Although most advocates of the theory of sexual reproduction in bacteria have supported the more primitive methods, autogamic and isogamic conjugation, Enderlein (40) claims to have observed fusion of heterogametes, sperm cells (spermits) and egg cells (oits) in *Vibrio cholerae*. According to his account, the gametes originate from gonidia by a process of reduction analogous to that of higher organisms. Almquist (6) reported somewhat similar reduction division in *Eberthella typhosa*. Sexual reproduction by conjunction, multiple fusion of cells, was reported by Löhnis and Smith (105) and Appleby (9).

If the bacteria reproduce by sexual methods, it should be possible to cross closely related strains or species and determine something concerning the genetical behavior. There is not, however, much evidence to support the theory that hybrids occur in bacteria. Almquist (6) reported success in crossing *Shigella dysenteriae* and *Eberthella typhosa*. Nyberg (136) believed that *B. mycoides* is a hybrid and that dissociation in this species is due to Mendelian segregation. Stewart (175) reported autogamic conjugation and segregation in *B. coli-mutabile*. Mellon (112) believes that rearrangement of chromatin by a sexual process is a chief cause of bacterial variation. Sherman and Wing (168) attempted to cross strains of *Escherichia* but abandoned the experiment with inconclusive results.

There appears to be no conclusive evidence that sexual reproduction occurs in bacteria. It may be noted also that botanists have not been able to prove sexual reproduction in the *Cyanophyceae*, although these plants are much larger than bacteria and more favorable for cytological study.

3. *Endospores*. Several conflicting methods of endospore for-

mation have been described by different investigators. According to Koch (88) spores result from the upgrowth of a tiny, refractile, non-stainable granule. Although some support for this theory is found in the literature, it is now generally regarded as erroneous.

Following the work of Ernst (41, 42) and Babes (10), the theory that spores are formed by the fusion of numerous sporogenic granules attracted many advocates and is still held by some bacteriologists. Support is found in the work of Bunge (23), Dobell (33–35), Schaudinn (162) and Nyberg (135). Much of the older as well as the more recent work is opposed to this theory. We now know that the so-called sporogenic granules of earlier writers are volutin grains, fat bodies, or glycogen and that they occur in many species which do not form spores. Moreover, there are many species in the genus *Bacillus* which do not contain granules and could not, therefore, form spores by this method. There is no sound cytological evidence that endospores are formed by the fusion of sporogenic granules.

A method of spore formation which seems to have been proved beyond reasonable doubt was described by Peters (140) and confirmed by Meyer (116, 117), and others (15, 52, 97, 153, 195). The spore is formed from a clear, hyaline, polar spore primordium which is set off from the remainder of cell by a membrane. The spore results from a condensation of the substance contained in the spore primordium. The granules, if present, are not concerned directly in spore formation, although they may furnish nutritive material which is digested and absorbed by the developing spore. During the early stages before the spore body has become fully condensed, it stains readily with aniline dyes and for this reason resembles a nucleus. There seems little reason to believe that the formed nucleus of *Bacillus bütschlii*, reported by Schaudinn (162) was anything but an immature spore.

IV. CELL DIVISION

Various methods of bacterial cell division have been described. Knaysi (86) classified the methods under three headings: (1) plate formation, centrifugal or centripetal; (2) cytoplasmic retraction and (3) constriction. Support is found in the litera-

ture for each of these methods. In the most recent study of the subject, Knaysi (87) found no indications of division by constriction or the formation of a cell plate with subsequent splitting. His observations appear to prove that the cell divides by cytoplasmic retraction and subsequent formation of two transverse cell walls which are separate from the beginning. A rather full review of the previous literature is given.

V. THE CELL MEMBRANE

There has been much confusion concerning the nature of the outer envelope of the bacterial cell and its relation to the protoplasm. The term membrane as generally employed has no very definite meaning, and the term ectoplasm or ectoplast is even less satisfactory. Since the early writings of Zettnow, some bacteriologists have employed the term ectoplasm to designate the outer portion of the cell and endoplasm to denote the cell contents. The following quotation from Rideal (156) is typical of the many brief descriptions which occur in recent textbooks. "In general, bacterial cells may be regarded as a chromatin network in an emulsoid protoplasm, the endoplasm, the whole being enclosed in a semi-permeable membrane, the ectoplasm." The term ectoplasm has also been used by Preisz (153), Eisenberg (38) and Gutstein (63) to denote the whole outer envelope. Churchman (26) introduced the terms cortex and medulla which have caused additional confusion.

The writer agrees with Knaysi (86) who suggested that we abandon this confusing terminology in favor of terms which are universally employed in dealing with other plant cells. It could be argued that the structure of the bacterial cell is not analogous to that of other vegetable organisms and that a different terminology is necessary. The evidence must therefore be considered. Knaysi (84) stained *Bacillus subtilis intra vitam* with dilute aqueous solution of crystal violet. He observed a purple outer cell wall surrounding a dark violet membrane which encloses a deeply staining cytoplasm but of much lighter shade. The cell wall could be seen much more clearly in cells plasmolyzed by mounting them in 25 per cent sodium chloride solution prior to staining.

According to his observations the cell wall has only a slight affinity for dyes; it stains a clear blue with methylene blue but has no affinity for iodine. The cytoplasmic membrane, on the other hand, is hyperchromatic and takes up dyes with great avidity. It is colored dark brown with iodine. More recently, Knaysi (87) has described a differential method of staining by which the cytoplasm, cell wall, and cytoplasmic membrane can be distinguished in fixed films.

These experiments, together with the earlier work of Fischer (45), Grimme (52), Ellis (39), Swellengrebel (181), Meyer (123), and others proved that the protoplast of the bacterial cell, like other plant cells, is not attached to the cell wall but lies free within it. In the fully turgid cell, the cytoplasmic membrane is in close contact with the outer wall and is not readily distinguished. When the cells are suspended in a hypertonic solution, the cytoplasmic membrane is drawn in with the contracted cytoplasm while the more rigid cell wall retains its original form.

Much has been written on the permeability of the bacterial membrane especially in its relation to dyes. We know that the cellulose wall of higher plant cells is very permeable while the cytoplasmic membrane is semipermeable. It is known that the protoplast of the bacterial cell functions as an osmotic system, precisely like that of other plant cells, and that the cytoplasmic membrane is semi-permeable. According to Fischer (45), such a degree of division of labor as occurs in the higher plants has not been reached in the bacteria where communication between the organism and the outer world is regulated by two layers of medium permeability. To what extent, if any, the true cell wall of bacteria functions as a selective membrane is difficult if not impossible to determine.

The chemical composition of the cytoplasmic membrane as well as that of the cell wall is not very well known. Knaysi (86) believes that the cytoplasmic membrane is made up chiefly of surface active materials, lipoids and lipoproteins, which accumulate to form a rather firm surface structure which may consist of several layers. The cell wall is a firm, rigid, somewhat elastic structure as was shown by Ellis (39) for *Spirillum giganteum* and by the

ingenious needle dissection experiments performed by Wamoscher (191).

Claims have been made that the wall substance of some bacteria consists of cellulose, but there is little or no convincing evidence that such claims are valid. The earlier literature was reviewed by Meyer (123) who advanced the theory that the substance is a hemi-cellulose. The subject has been discussed more recently by van Wisselingh (187). The occurrence of chitin in the cell walls of various bacteria was denied by van Wisselingh (186) but was supported by Iwanoff (81), and Viehoever (189). van Wisselingh (187) found that chitin is a common component of the cell walls of molds but is not present in bacteria.

The mucoid substances deposited as a clear zone external to the cell wall and variously designated as the slime layer, sheath or capsule have been studied more extensively than the wall itself. The precise origin of the capsular material has long been a more or less controversial matter. The earlier investigators generally regarded the substance as a product formed by swelling and gelatinization of the so-called ectoplasm. Meyer (123) and Zettnow (201) attacked this theory and held that the substance is a secreted product. In the light of more recent studies on the chemical nature of the gums produced by various species, this view is much more likely. The chief interest manifested in capsules by cytologists has been in the matter of successful methods of staining. There is little doubt that low affinity for dyes is due to the carbohydrate nature and that the capsular substance may adsorb stainable materials from blood serum or even from milk. The extensive literature dealing with the chemistry of the capsular material and its relation to virulence and to immunological reactions, though matters of great importance, does not concern us here.

Of considerable interest to the student of bacterial cytology are the investigations of Churchman (26) who maintained that the gram reaction is due to a protein-like, gram-positive substance, the cortex, deposited as a sheath around the inner gram-negative portion, the medulla. Eisenberg (38) and Gutstein (63) described a similar surface layer differentiable from the remainder of the cell

body by various staining methods. Churchman's conclusions were attacked by Burke (24) who could find no evidence that gram-positive bacteria possess a cortex which stains differentially by Gram's method. Stearn and Stearn (174) expressed some doubt concerning the presence of an external cortex. Knaysi (84) regarded the cytoplasmic membrane as the structure which corresponds to Churchman's cortex and is responsible for the gram reaction.

Although our knowledge of the structure of the bacterial cell may not be perfect, enough is known to warrant a more uniform usage of the terms employed in the general domain of plant cytology. Accordingly, the bacterial cell consists of a protoplast encased in a non-protoplasmic cell wall composed of ergastic substances of unknown identity. The surface of the protoplast is a differentiated semi-permeable cytoplasmic membrane which encloses the cytoplasm. Within the cytoplasm there are sap vacuoles and in some cases various granular inclusion bodies. The nucleus of the protoplast probably consists of a structure analogous to a chromosome rather than to a true vesicular nucleus.

VI. FLAGELLA

The earlier investigations concerning flagella were directed principally at the problem of staining. Robert Koch (1877) discovered the first successful method by which flagella could be made visible by staining. He employed "Extractum campech," a crude extract of logwood (*Lignum campechianum*). The staining action was enhanced and made permanent by treating the stained films with dilute chromic acid.

Loeffler (103) discovered a better method based on the use of a mordant consisting of a mixture of tannic acid, ferrous sulphate, and basic fuchsin. He stained the mordanted cells with carbol fuchsin. Zettnow (200) improved the silver impregnation method which had been previously employed and devised a valuable method especially suitable for photographic purposes.

Modifications of Loeffler's method have been made by Shunk (169), Gray (51), Leifson (95), Maneval (107) and many others. The most complete study of mordants is probably that of Maneval

who prepared 24 different solutions which gave excellent results with various species. Successful mordants contain much material in a colloidal state, and it appears probable that the principal factor involved in staining is adsorption. Maneval suggested that the size and electrical charge of the colloidal particles as well as the H-ion concentration are important factors. It is of interest to note that Loeffler recommended the use of caustic soda or dilute sulphuric acid as a "corrective solution" for staining some species.

The several factors causing variable results with flagella stains have been discussed in considerable detail by Wright (194). A factor which appears not to have been fully appreciated concerns the nature of the glass surface. Erwin F. Smith (170) called attention to the fact that thoroughly cleaned cover glasses sometimes give trouble because the surface of the glass itself is at fault. More recently, Conn and Wolfe (27) stressed the importance of flaming the slides until an orange color appears in the flame. The surface appears to be improved by this method. Shunk (169) recommended chemical rather than heat fixation of films.

The discovery of successful staining methods stimulated investigation of other problems concerning flagella. Much interest has been manifested in the manner of origin from the cell body. Migula (124), Zettnow (199, 201), Schaudinn (162), Marrassini (109) and others believed that flagella originate from the membrane, while Fischer (45), Ellis (39) and Meyer (123) held that they originate in the cytoplasm and grow out through openings in the cell wall. This latter view was modified somewhat by Reichert (154), Fuhrmann (46), and Yamamoto (196) who described the origin from an internal granule comparable to the blepharoplast of other flagellated cells. More recently, Leifson (95) supported cytoplasmic origin, and Enderlein (40) reported the occurrence of a blepharoplast (centriolit) in the sperm cells (spermits) but not in ordinary vegetative cells. It is probably impossible to obtain any very definite cytological evidence as to the precise manner of origin, but in the light of our present knowl-

edge of the cell structure, there is little or no support for the theory that flagella originate from the cell membrane. The occurrence of blepharoplasts appears to be doubtful.

The application of dark field illumination to the study of bacteria has enhanced our knowledge of the mechanism of flagellar motion. This subject was thoroughly studied by Reichert (154) and more recently by Neumann (134), who stressed the importance of a suitable viscous mounting fluid. According to Reichert (154), the force which propels the bacterial body is not due to lashing of the flagella as was supposed by Fischer (45) but to a rhythmic contraction which moves helicoidally over the surface; the action is comparable to that of a screw rather than an oar. Pijper (149) has shown that the movements to right or left are controlled by changes in the angle which the flagella make with the cell body; they act as a rudder as well as a propeller.

Recent studies by dark field methods have caused some doubt concerning the occurrence of peritrichous flagellation. According to the observations of Pijper (149) *Eberthella typhi*, *Proteus vulgaris* and similar species swim by means of a long "tail," formed by the twisting together of two rather broadly coiled flagella which are attached to the cell near its middle. He regards as artifacts the usual appearances of peritrichous flagellation seen in stained fixed films. Pietschmann (147) observed subpolar flagella on living cells of *Bacillus subtilis*, *B. ellenbachensis*, *B. ruminatus*, *E. coli*, and *Serratia marcescens*. He regards peritrichous flagellation as an illusion which is due to the appearance of subpolar flagella on chains of cells.

Although we are not concerned here with the literature dealing with flagellar and somatic antigens, the dark field observations of agglutination made by Pijper (149) may be noted. In the presence of flagellar antigen, the flagella become encrusted with a substance which causes them to adhere rather loosely when fortuitous entanglements occur. This results in the formation of clumps which are readily broken down by shaking. There appears to be no effect on the cell body. On the other hand, the somatic antigen has no such effect on the flagella but acts on the

cell bodies causing them to unite in compact masses which are not readily separated by shaking. In this connection the experiments of Craigie (28) on *Eberthella typhosa* are of special interest.

Investigations by Piekarski and Ruska (146) and by Mudd, Polevitsky and Anderson (128) indicate that the electron microscope is valuable for the study of flagella. Evidence obtained by Polevitsky suggests that the flagella of *Eberthella typhosa* and coliform bacteria may be tubular structures.

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