CYTOLOGICAL STUDIES OF *AZOTOBACTER*

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By

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I. INTRODUCTION

The maintenance of a nitrogen balance in the soil has long been recognized as vital to scientific agriculture. Among the lower organisms involved in this phase of agricultural economy, the Azotobacter group deserves close study. According to Bergey's Manual, (Breed, et al., 1948) the genus Azotobacter is characterized as follows:

> Cells without endospores. Relatively large rods or even cocci, sometimes almost yeast-like in appearance. The type of flagellation in this genus has been definitely established as peritrichous. Gram-negative. Obligate aerobes, usually growing in a film on the surface of the culture medium. Capable of fixing atmospheric nitrogen when provided with carbohydrate or other energy source. Grow best on media deficient in nitrogen. Soil and water bacteria.

The manual describes the species, Azotobacter agile, as rods four to six microns in length, almost spherical. The optimum temperature for the species is 25°C. to 28°C. Azotobacter agile was originally isolated from canal water at Delft, Holland. It occurs in water and soil. Bergey's Manual (Breed, et al., 1948) gives the following chemical analysis of Azotobacter agile:

> Four-day cultures grown upon mannitol agar when dried, contain more than four percent of hemicelluloses, more than 45 percent of crude protein, more than seven percent of ash, and less than four percent of lignin-like materials.

Much of the early knowledge of morphological variation and of life cycles of bacteria was derived from the study of Azotobacter. It has served as a standard of reference for the study of life cycles of other bacterial species, and the size of the organism makes it ideal for cytological study.

Because of the minuteness of the bacterial cell, knowledge of its structure had to wait for adequate development in optical instruments and in microscopic techniques. According to Knaysi (1938), the cytology of bacteria started with Cohn's work, first published in 1872, immediately following the introduction of Abbe's Condenser in 1870. Since then, the structure of the

bacterial cell has been the subject of numerous investigations.

This interesting phase of microbiology which deals with possible morphological life histories of bacteria has been subordinated in the past. Cytology in all applications of bacteriology was considered unnecessary and unimportant. According to Stevens (1949), Lohnis' studies upon bacterial life cycles were considered preposterous by most bacteriologists. Members of the National Academy of Science were profuse in their apologies that their organization should have sponsored such a paper as Lohnis'. Stevens further points out that today, however, every bacteriologist of standing whom he knows accepts the Lohnis ideas as a matter of course.

Currently a great deal of interest is being developed in this phase of bacteriology, and with our present methods there is a possibility of studying changes in bacterial structure with greater accuracy than has been possible in the past.

The present study is concerned with the cytology of Azotobacter with special reference to the cytological changes undergone with age by the cells of that organism. The nigrosin negative stain, the Robinow nuclear stain, and the electron microscope were employed in an attempt to determine those changes.

II. REVIEW OF LITERATURE

Morphological variation in bacteria was observed by many early bacteriologists. Some held the view that morphological differentiation was coupled with a complex life cycle, while others believed that bacteria exhibited constancy of form and size. These followers of the "monomorphistic" theory maintained that change of a culture in form was proof of either "contamination," or incorrect classification of an organism, or of the existence of "involution" forms.

Lohnis and Smith pointed out in 1916 that progress in bacteriology had been severely checked by the inclination to consider only conventional concepts of constant species characters as being worthy of investigation. A more scientific study of such "abnormal" forms long before would have revealed the fact that life cycles of bacteria are no less complicated than those of other micro-organisms, according to these authors. They further point out that the statement that bacteria multiply exclusively by fission has been sufficient to prevent thorough research in this direction, and the adherence to "standard methods" explains why the life cycles of the bacteria have been completely overlooked.

Lohnis and Smith (1916) made a comparative study of 24 Azotobacter cultures and eighteen strains of other bacteria. From this study they reached the following conclusions:

> All bacteria studied live alternately in an organized and in an amorphous stage. The latter has been called the "symplastic" stage, because at this time the living matter previously inclosed in the separate cells undergoes a thorough mixing either by a complete disintegration of cell wall, as well as cell content, or by a "melting together" of the content of many cells which leave their empty cell walls behind them. In the first case a readily stainable, in the later case an unstainable "symplasm" is produced.

According to the different formation and quality of the symplasm

the development of new individual cells from this stage follows various lines. In all cases at first "regenerative units" become visible. These increase in size, turning into "regenerative bodies," which later, either by germinating or by stretching, become cells of normal shape. In some cases the regenerative bodies also return temporarily into the symplastic stage.

Besides the formation of the symplasm, another mode of interaction between the plasmatic substances in bacteria cells has been observed, consisting of the direct union of two or more individual cells. This "conjunction" seems to be of no less general occurrence than the process first mentioned. The physiological significance remains to be studied.

All bacteria multiply not only by fission but also by the formation of "gonidia"; these usually become first regenerative bodies, or occasionally exospores. Sometimes the gonidia grow directly to full-sized cells. They, too, can enter the symplastic stage. The gonidia are either liberated by partial or complete dissolution of the cell wall or they develop while still united with their mother cell. In the latter case the cell wall either remains intact or it is pierced by the growing gonidia, which become either buds or branches.

Some of the gonidia are filterable. They also produce new bacteria either directly or after having entered the symplastic stage.

The life cycle of each species of bacteria studied is composed of several subcycles showing wide morphological and physiological differences. They are connected with each other by the symplastic stage. Direct changes from one subcycle into another occur, but they are rather rare exceptions. The transformation of spore-free into spore-forming bacteria seems to be dependent on the conditions acting upon the symplasm and regenerative bodies.

The discovery of the full life cycles of bacteria may be helpful in many directions. Systematic bacteriology now can be established on a firm experimental basis. Physiological studies will win considerably in conformity and accuracy when connected with morphological investigations along these new lines. Several problems in general biology are brought under more promising aspects. Agricultural bacteriology and medical also will derive much benefit.

A short time before the appearance of the work on bacterial life cycles by Lohnis and Smith, Bonazzi, (1915) made a study of Azotobacter chroococcum to determine the constitution of the cellular granules. At this time there were many different opinions as to the nature and function of these bodies. Beijerinck (1901) spoke of these granules as fat bodies. The presence of volutin was reported by Fischer (1906). Others have regarded the granules as

chromidia and the equivalent of a true nucleus (Mencl, 1911). Jones (1913) distinguished two types of granules on the basis of reaction to stains. He regarded the stainable granules as reproductive bodies and designated them as gonidia. The non-stainable granules appeared to be glycogen. In his cytological studies, Bonazzi used various microchemical tests. As a result of these, he pointed out that the granules found in Azotobacter chroococcum did not give the reaction for glycogen, but that they responded to all of the tests carried out to distinguish their metachromatic character. He further indicated that these granulations seem to have their origin from the nucleus, but they seem to have no relation to the reproduction of the cell since their disposition in the cells is not regular but changes in different individuals.

Whereas Bonazzi (1915) concluded that the granules of Azotobacter have no relation to reproduction, Jones (1920) reported the presence of granules which appear to represent reproductive bodies or gonidia. His conclusions are in agreement with Lohnis and Smith's theory regarding the fusion or mixing together of the protoplasm of Azotobacter cells which constitute symplastic clusters. However, what Lohnis and Smith refer to as conjunction of two individual cells is termed the incomplete fission of individual cells in the process of division by Jones.

Lohnis and Smith report the presence of normal heat-resistant endospores in fifty percent of all Azotobacter cultures. Their observations support the conclusion of Mulvania (1915) who noted the presence of spores in Azotobacter. From his studies, Jones showed that Azotobacter did not produce heat-resistant spores.

From his studies in 1920, Jones further reports that with Azotobacter there is an irregular occurrence of abnormal forms which merit the term "involution" forms. He found these forms to be common in old cultures (one

or two months) of Azotobacter grown in Ashby's solution or on Ashby's agar at 25°C. They were more numerous where grown at 37°C. In cultures more than fourteen days old, large, spherical thick-walled cells were common. These appeared to be resting cells or arthrospores.

Lohnis (1921) reviewed the literature from 1838 to 1918 on the life cycles of the bacteria. He concludes that numerous facts prove that all bacteria are pleomorphic. All well studied species, including Azotobacter, are able to grow in various, round, straight, and curved, small and large, regular and irregular cell forms. Budding and branching are common with all bacteria. He pointed out that the life cycles of bacteria are composed of several subcycles showing wide morphological and physiological differences. Successive transfers made repeatedly during several weeks from and to various substrates will reveal the pleomorphic character of a bacterial species. According to Lohnis, bacteria possess various organs of reproduction. These are gonidia, regenerative bodies, exo- and endospores, arthrospores, and microcysts. All of these are made up of nuclear substances. Gonidia and regenerative bodies participate actively in the process of multiplication, whereas the other reproductive organs are resting forms, according to the Lohnis report.

In continuing their studies upon life cycles, Lohnis and Smith (1923) made a study of the life history of Azotobacter. They pointed out that from every Azotobacter culture not less than seven different growth types can be developed and stabilized. These types are (1) large non-sporulating cells, (2) coccoid forms, (3) dwarfed cell types, (4) fungoid cell types, (5) small non-sporulating rods, (6) small sporulating rods, (7) and large sporulating cells.

According to Lohnis and Smith, the reproductive organs of Azotobacter are (1) gonidia, in part filterable, (2) regenerative bodies and exospores,

(3) arthrospores, (4) microcysts, and (5) endospores. They conclude that gonidia form the basis for the development of regenerative bodies, arthrospores, and endospores.

Conjunction was regularly seen in young cultures. Lohnis and Smith maintain that part of the regenerative bodies are zygospores.

This complicated life cycle described by Lohnis and Smith for Azotobacter has been partially confirmed and partially contested by subsequent investigation. Lewis (1939) made a study concerned with the structure and methods of reproduction of Azotobacter with special attention to the nature and function of the granular bodies which occur in the cells. On the basis of microchemical and solubility tests, Lewis concluded that the stainable granules of Azotobacter are volutin rather than chromidia, or gonidia, and that they are, therefore, not concerned in the reproductive process. The refractile granules of Azotobacter are lifeless storage products of a fat-like nature which function as reserve food. Knaysi (1938) is of the opinion that the existence of gonidia is still in doubt.

According to Lohnis and Smith (1923), young cultures of Azotobacter show cells in the process of conjugation connected by a tube-like structure. Lewis points out that such pairs of cells are numerous in cultures, but the appearance is due to incompleted fission. This view was previously reported by Jones (1920).

According to Lewis, the symplasm consists of a mixture of empty cell membranes, fat, and volutin balls. He believes that the theory of a symplastic stage in the life history of bacteria is erroneous and deserves no further place in bacteriological literature.

The studies of Lewis (1937) failed to reveal endospore formation in any of the strains of Azotobacter. He reported the failure to confirm the occurrence of a filterable phase in the life history, and transmutation of cells

to a different type cultivable as such to form culture phases different from the original.

Lewis believes the life history of Azotobacter to be very simple depending for reproduction upon binary fission and arthrospores.

The question of the presence or absence of a bacterial nucleus has long been a subject of interest to cytologists, but no wholly satisfactory answer has yet been found. The difficulty of demonstrating a nucleus in a bacterial cell is accounted for partly by size, but also because of a lack of a sure criterion for the nucleus. Knaysi (1938) points out that most investigators aim at demonstrating a chromatin nature either from dyes or other physical and chemical tests. He further adds that confusion is introduced by the presence in the cell of volutin which is closely related to chromatin and is known to be reserve material. According to Knaysi, another criterion for the nucleus frequently used is division. He points out that other cell structures may divide, so therefore, division is not a sufficient criterion.

According to Lewis (1941), the theories concerning the nucleus of bacteria may be divided 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 a 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.

There is much conflicting evidence concerning these theories. Lewis

(1941) reports that in the present state of our knowledge no final conclusions concerning the nature of the nucleus can be drawn. 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 chromatin. Whether the protoplasm of the bacterial cell is undifferentiated, or consists of invisible genes and cytoplasm has not been fully determined.

Recently, extensive use has been made of the Feulgen reaction. Feulgen and Rossenbeck (1924) used the reaction as a test for thymonucleic acid and obtained negative results with yeasts and bacteria. Westbrook (1930) also obtained negative results with yeasts and bacteria. On the other hand, according to Knaysi (1938), Voit, Kuzela, Neumann, and others have obtained positive results.

There are differences of opinion as to the service which Feulgen's reaction has rendered in the study of bacterial structure. Knaysi (1938) questioned the specificity of the reaction. Also he pointed out that it is unsound to investigate the nucleus of bacteria with a positive assumption that it is made up of nucleo-protein. Lewis (1941) believes that the Feulgen reaction is difficult to evaluate because nucleic acid is frequently present as a reserve material and the reaction is not sufficiently intense to give a clear differentiation of minute structures. Schaede (1939) who stained bacteria and actinomycetes by Feulgen's method thinks that the stainable substance is not true chromatin or gene material and that it probably functions as reserve food.

Whereas Knaysi (1938) and others questioned the specificity of the Feulgen reaction, Stowell (1946) considers it to be very specific. According to him: "The preponderance of evidence indicates that with the proper precautions the Feulgen technic for thymonucleic acid is one of the most specific histochemical

reactions." Dodson (1946) concludes that our knowledge of the Feulgen reaction and its specificity leaves much to be desired, but as yet no serious experimental evidence against its practical usefulness in the identification of thymonucleic acid seems to have been brought forth. Barber and Price (1940) maintain that the Feulgen reaction is specific for the potential aldehyde groups of chromatin. Semmens (1940) suggested that the Feulgen reaction with chromatin may be due to the purine components of the nucleic acids. He observed that piperidine and pyridine restored to the Feulgen solution its "original" color, and that certain purines gave "positive" color reactions. The studies of Barber and Price (1940) show that the effect of piperidine and pyridine is not chemically equivalent to the Feulgen reaction, but is simply due to their basicity.

The Feulgen reaction, according to Hillary (1939), is carried out in two steps. The first is a mild acid hydrolysis, which breaks the binding between the purine bases and the carbohydrate complex of the nucleic acid. This splitting off of the purines frees the aldehyde groups of the aldo-pentose sugars. The second step involves a chemical reaction between the liberated aldehyde groups and luco basic fuchsin (fuchsin sulfurous acid). Stowell (1945) points out that desoxyribose sugars react with the fuchsin sulfurous acid to produce a reddish purple pigment. The reaction is based upon the Schiff reaction for aldehydes.

In an attempt to clear up some of the confusions and contradictions regarding the Feulgen stain, Hillary (1939), made some studies as to the effect of fixatives and time of hydrolysis on the reaction. According to Hillary, the length of the period of hydrolysis is perhaps the most critical point of the reaction. Normal hydrochloric acid is used, and 60°C. has been adopted as a standard hydrolyzing temperature because it allows the optimum to be reached

with a fairly short period of hydrolysis and is not high enough to damage the structure of the nucleus. As a result of his studies, Hillary concludes that the constituents of the commonly used cytological fixative have no deleterious effect on the Feulgen nuclear stain. He also concluded that for the production of a satisfactory stain, five minutes' hydrolysis with any of the used cytological fixatives is sufficient.

According to Hillary, one of the greatest drawbacks to the use of the Feulgen stain is the fact that there have been frequent and often incorrect reports of a negative stain reaction. He points out that the various explanations possible for a negative nucleal reaction can be grouped under five headings, following Milovidor's classification:

1. Unsuitable general methods; that is, insufficient fixation and preparation, or unsuitable object (presence of thick cell walls or heavy cuticle or fats which hinder observation of the nucleus).

2. Incompleteness in the methods employed in the nucleal reaction; that is, wrong period of hydrolysis, temperature of hydrolysis, and concentration of HCl.

3. Presence of materials which hinder the normal process of the reaction; that is, neutralization or binding of HCl; binding of the liberated aldehyde groups; disturbances of the penetration of reagents by resins, tannins, and slime substances (a slight effect).

4. Strong dispersion of a small amount of chromatin in a large nuclear volume.

5. Chemical alteration of thymonucleic acid.

Even though the Feulgen reaction may have limitations, Robinow (1942) maintains that advances that have been made in our knowledge of the nuclear apparatus of bacteria have been mainly due to the application to bacterial cytology of the Feulgen reaction for the detection of thymonucleic acid. According to Robinow, the Feulgen method has shown that in many bacteria the nuclear apparatus conforms to a simple, ever-recurring pattern. He further points out that the use of the Feulgen method only, however, has sometimes

given misleading results. His studies of hydrolyzed, Giemsa-stained preparations have usually been more fruitful. Robinow mentions that in cells of various non-sporing organisms treated with 1/N HCl at 60°C., "nucleoids" can be demonstrated with Giemsa's stain, although such cells appear homogeneous if stained with Giemsa's stain without this preliminary treatment in acid. In his studies comparison of parallel preparations has shown a close correspondence between the structures stained by the two methods, but the Giemsa preparations are optically superior. From his studies Robinow (1942) concludes that the structural unit of the nuclear apparatus of aerobic, spore-forming bacteria is a dumbbell-shaped body, giving a positive Feulgen reaction, and possessing a strong affinity for nuclear dyes.

Several attempts have been made to establish the existence of a nucleus by studying bacteria with the electron microscope.

From their studies of Mycobacteria, Rosenblatt, Fullman, and Gessler (1942) point out that the tubercle bacillus consists of one or more dense nuclear masses within a granular cytoplasm. Knaysi and Mudd (1943) studied certain bacteria with the electron microscope and the results of their study support the view that different bacteria may contain nuclear material in different states, and that the state of the nuclear material may change with the development of the cell. Baylor, Appleman, Sears, and Clark (1945) made a study of some nodule bacteria with the electron microscope, and their results showed the presence of bodies which may be nuclei. Bacillus mycoides was grown in a nitrogen-free medium and studied with the electron microscope by Knaysi and Baker (1947). From their observations they conclude that there exists in the cell of Bacillus mycoides one to several nuclei. According to van Iterson (1947) the electron-microscopical observations of cells from some young cultures are in rather satisfactory agreement with the descriptions

given by authors using the Feulgen technique.

Several techniques have been employed in the study of bacterial cytology. The fact that the use of one should be coordinated with others is emphasized by Mudd, Polevitzky, and Anderson (1942).

> Granules in bacterial protoplasm have been variously interpreted as nuclei or nuclear equivalents, reserve food material, reproductive elements, and otherwise, without any of these interpretations having become established. The electron microscope shows fine structure within bacteria with clarity and detail not hitherto possible, and when coordinated with cultural and cytologic procedures, should contribute to eventual understanding of these fine structures.

A review of the literature on bacterial cytology indicates that there is much disagreement among investigators as to the structure of the bacterial cell. This interesting branch of bacteriology has been neglected in the past several years. Recently, however, this subject has again become one for numerous investigations. For these reasons, a study concerned with the cytology of Azotobacter was considered timely and worth while.

III. EXPERIMENTAL

A. Materials and Methods

This study is concerned with the cytological changes undergone with age by the cells of Azotobacter. In an effort to determine these cytological changes, the investigator made use of cultural and cytologic procedures. These procedures included: (1) the negative staining method with nigrosin, (2) the Robinow nuclear staining method, and (3) electron microscopy.

The organism selected for this study was Azotobacter agile Beijerinck. The organism was cultivated on a solid culture medium. The medium used was nutrient agar, and the pH prior to sterilization was 7.2. The organism was transferred daily over a period of 60 days. The incubation temperature for all transfers was 27°C. Cultures ranging in age from a few hours to 60 days were examined.

Each culture was stained with nigrosin and examined under the light microscope to determine the morphology of the organisms at different ages.

For the study of internal structure, smears were prepared and stained according to Robinow (1942). Smears were fixed in osmium tetroxide vapor for a period of 30 minutes. After fixing, the smears were hydrolyzed in 1/N HCl at 60°C. for five minutes and then stained for a period of one-half hour with Giemsa's stain diluted 1 to 10.

Photomicrographs were obtained from the slides prepared with the negative stain and the Giemsa stain. Bausch and Lomb photomicrographic equipment was used. That used was type J including the vertical camera and illuminating unit mounted on a common supporting base.

The method used in preparing the material for observation with the electron microscope was the usual method according to Marton (1941) of suspending the organisms, taken from a solid medium, in sterile distilled water in a test

tube and of transferring a small droplet to the surface of the collodion film over which it is spread and allowed to dry. The preparation was then introduced into the evacuated chamber of the electron microscope, a suitable field found and a picture taken by five seconds exposure to the electron beam. No fixation other than that of the drying and electron bombardment and no staining procedures were employed. The R.C.A. type EMC electron microscope was used.

B. Results

1. Negative staining: Smears were stained with nigrosin to determine the morphological changes undergone with age by the cells of Azotobacter agile. Cells from cultures one day old are predominately large plump rods (figs. 1 and 2). After two days of growth, cells are somewhat shorter and thinner (figs. 3 and 4). Smears made from cultures one week old show both short irregular rods and coccobacilli (fig. 5). Long branching rods, thread forms (fig. 6), and small short rods are observed in cultures two weeks old (fig. 7). Cultures three weeks old show long branching rods and thread forms (figs. 8 and 9). Cells from cultures one month old are predominately cocci, but a few short rods and long branching rods are present also (figs. 10 and 11). After two months of growth, cultures show mostly cocci with a few irregular rods (fig.12).

2. Robinow Nuclear Staining: Smears were fixed in osmium tetroxide vapor for 30 minutes, treated with 1/N HCl at 60°C. for five minutes, and stained with Giemsa's stain to determine the cytological changes undergone with age by the cells of this organism. Cells from young cultures (up to 24 hours old) show a definite internal organization when stained according to this method (figs. 13 and 14). In smears made from cultures four days old, a few deep staining intracellular bodies are still observed after acid hydrolysis and Giemsa staining (fig. 15).

By comparison with the nigrosin negative stain and other staining procedures, it is observed that only a portion of each cell shows typical reaction of desoxyribonucleic acid. The portion of the cell showing the reaction of desoxyribonucleic acid seems to be about one-fifth of the cell volume. The remaining portion of the cell seems to have been destroyed by hydrolysis, so that the "cytoplasmic" area of these Giemsa stained cells is not visible. It should be pointed out, however, that photography failed to record structures

that are faintly visible.

Careful observation of smears made from young cultures and stained according to the Robinow nuclear method indicates deep staining intracellular bodies conforming to a pattern similar to that described by Robinow (1942). Stains made from cultures less than 24 hours old (figs. 13 and 14) show cells with definite bands across them described by Robinow as "chromatinic bodies."

After cells have grown for more than 24 hours, the chromatinic bodies cannot be seen with clarity. Stains made from cultures up to four days old do, however, show cells with some internal differentiation (fig. 15). Cultures that are one week old show cells in which the chromatinic structures seem to have disappeared (fig. 16). Cells from cultures older than these (up to 60 days) did not reveal the chromatinic bodies, but a statement concerning these older cells is difficult because they are extremely small at this stage in the life cycle.

3. Electron Microscopy: The electron microscope was used to study further the morphology and cytology of Azotobacter agile. The routine procedure (Marton, 1941) was followed in preparing the material for observation. This method showed those changes in morphology with age that have been described by the observation of nigrosin negative stains. The electron microscope showed granulations within the cells that were not observed with the ordinary staining methods. These granules were so small that they were beyond the limits of the optical resolution of the light microscope. Young cells appear as large plump rods (figs. 17 and 18). Preparations made from slightly older cultures show both medium sized rods and small rods (fig. 19). Cultures that have grown for about a week show mostly small rods with a few coccobacilli present also (figs. 20, 21, and 22). Intracellular differentiation is observed in this group of cells. Granulation is particularly evident, but any attempt to describe the

nature of these granules would require further study. Cells from cultures two and three weeks old are mostly branching rods and thread forms (figs. 23, 24, 25, 26, and 27). Some internal differentiation is also evident. In these cultures there is observed an occasional group of cells resembling somewhat the symplasm described by Lohnis and Smith (1916, 1923) (fig. 28). Cells from cultures one to two months old are typically small coccobacilli and cocci (figs. 29, 30, 31, and 32).

4. Illustrations

Plate 1

Azotobacter agile

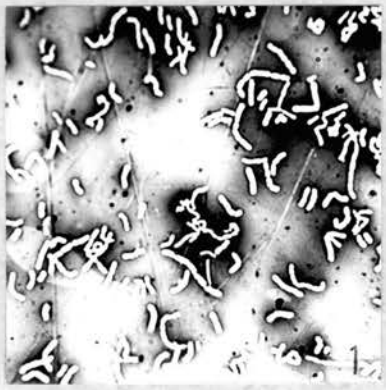
Figs. 1 and 2.--- 1 day old; stained with nigrosin. Large plump rods and a few small rods.

Figs. 3 and 4.--- 2 days old; stained with nigrosin. Medium sized rods, thin rods, and small rods.

Fig. 5.--- 1 week old; stained with nigrosin. Irregular rods and a few coccobacilli.

Fig. 6.--- 2 weeks old; stained with nigrosin. Long branching rods and thread forms.

All figures x 600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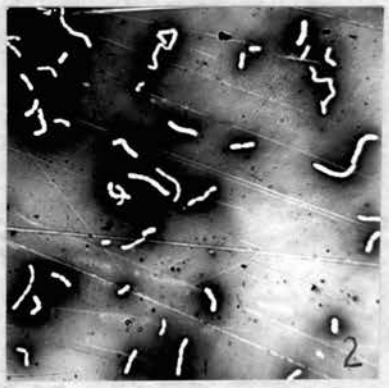
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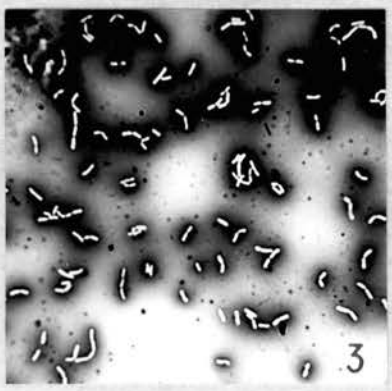
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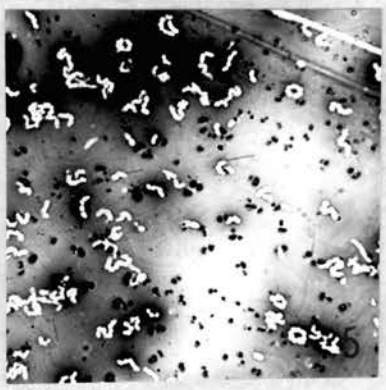
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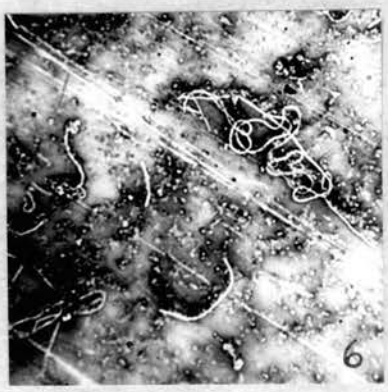
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Plate 11

Azotobacter agile

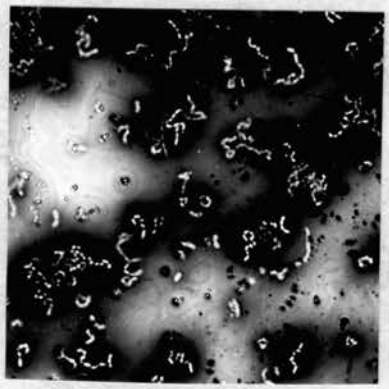
Fig. 7. --- 2 weeks old; stained with nigrosin. Small short rods.

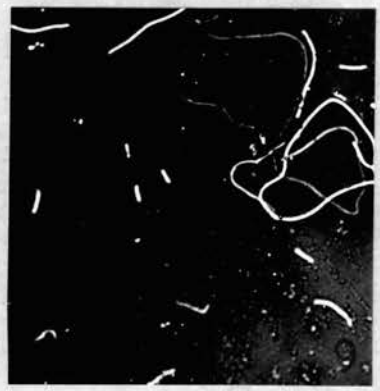
Figs. 8 and 9. --- 3 weeks old; stained with nigrosin. Long branching rods and thread forms.

Figs. 10 and 11. --- 1 month old; stained with nigrosin. Cocci, a few short rods, and a few branching rods.

Fig. 12. --- 2 months old; stained with nigrosin. Cocci and a few irregular rods.

All figures x 600





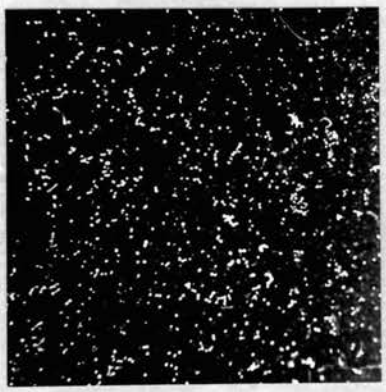


Plate III

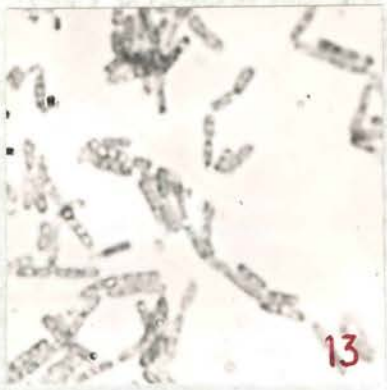
Azotobacter agile

Figs. 13 and 14. --- 20 hours old; fixed in osmium tetroxide vapor, hydrolyzed in 1/N HCl, and stained with Giemsa. Cells showing deep staining intracellular bodies.

Fig. 15. --- 4 days old; fixed in osmium tetroxide vapor, hydrolyzed in 1/N HCl, and stained with Giemsa. Cells showing some internal differentiation.

Fig. 16.--- 8 days old; fixed in osmium tetroxide vapor, hydrolyzed in 1/N HCl, and stained with Giemsa. Cells showing that deep staining intracellular bodies have disappeared.

All figures x 2000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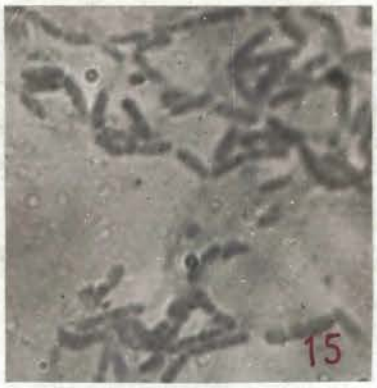
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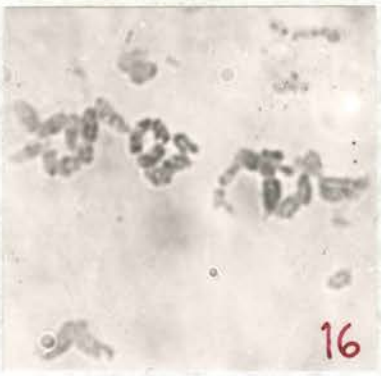
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Plate IV

Azotobacter agile

Figs. 17 and 18. --- 1 day old; electron micrographs. Large plump rods.

Fig. 19. --- 2 days old; electron micrograph. Medium sized rod and small rod.

Figs. 20, 21, and 22. --- 1 week old; electron micrographs. Small rods and coccobacilli.

All figures x 5000


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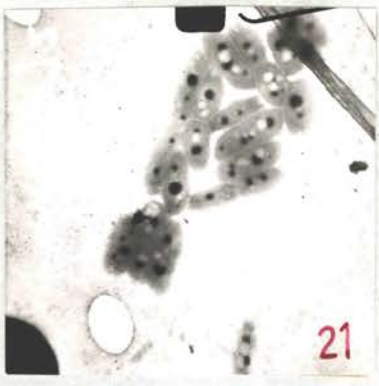
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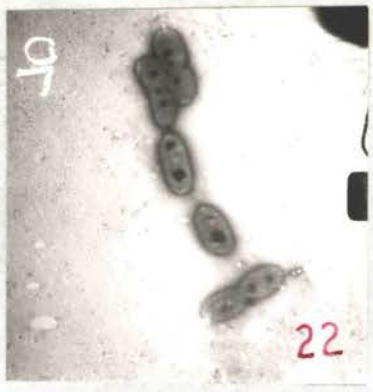
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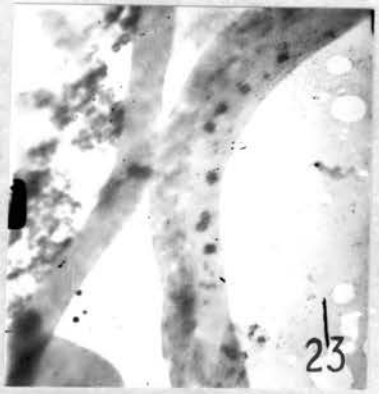
Plate V

Azotobacter agile

Figs. 23, 24, 25, 26, and 27. --- 2 and 3 weeks old; electron micrographs. Long branching rods and thread forms.

Fig. 28 --- 3 weeks old; electron micrograph. Cells twisted together resembling symplasm.

All figures x 5000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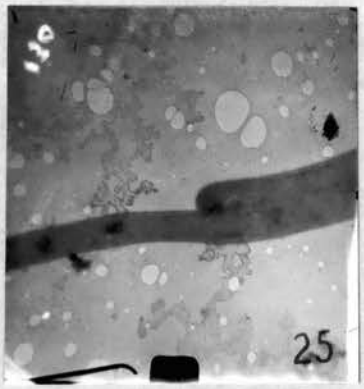
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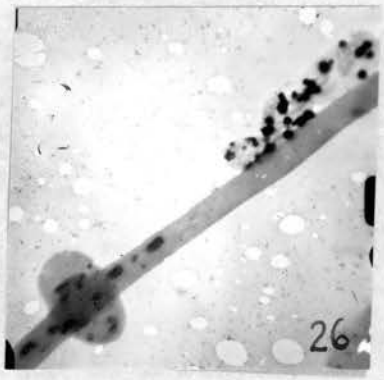
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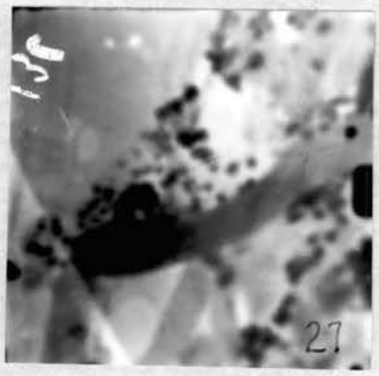
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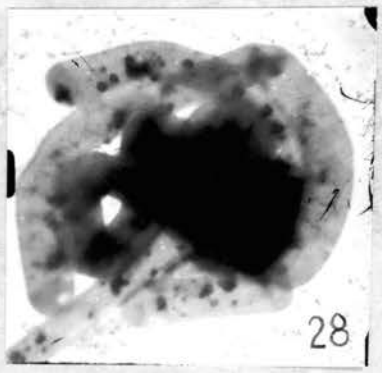
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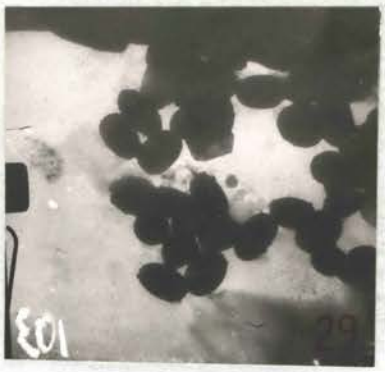
Plate VI

Azotobacter agile

Figs. 29, 30, 31, and 32. --- 1 and 2 months old; electron micrographs. Coccobacilli and cocci.

All figures x 5000

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C. Discussion

(McMahon, 1949? noticed) Four distinct morphological cell types in the life cycle of Azotobacter agile were observed during this investigation. These types were large plump rods, small rods, branching rods and thread forms, and small cocci. A more complicated life cycle was described by Lohnis and Smith (1916, 1923). They described seven different cell types characteristic of the life cycle of Azotobacter. In addition to large rods, small rods, fungoid cells, and cocci; these authors described dwarfed cells, small sporulating rods, and large sporulating rods. ~~Lohnis and Smith's description of the first four cell types is similar to that given in the present paper.~~ Their fungoid cells have been called branching rods and thread forms in the present study. According to Lohnis and Smith (1923), separate development of the dwarfed cells is usually slow and inconspicuous, and the isolation of this type of growth, therefore, is not easy. They further point out that pure cultures once established are inclined to reproduce larger cells, especially rods and fungoid growth. Lohnis and Smith (1923) observed both large and small sporulating rods in the life cycle of Azotobacter. Subsequent investigation has not confirmed the presence of heat-resistant endospores in this organism, Jones (1920), Lewis (1937).)

There has been much disagreement among investigators as to the nature of the granules in the cells of Azotobacter. They have been described as fat bodies, volutin, chromidia, and gonidia. Bonazzi (1915) and Lewis (1937) concluded that the granules of Azotobacter were not concerned in the reproductive process. Mencl (1911) regarded the granules as chromidia and the equivalent of a true nucleus, and Jones (1913) maintained that the stainable granules of Azotobacter were reproductive bodies.

In this study, the intracellular bodies observed in hydrolyzed Giemsa stained smears of Azotobacter agile seem to be similar to the chromatinic bodies described by Robinow (1942) as being present in many common bacteria. It is believed that the nuclear nature of the bodies observed is borne out by their presence after hydrolysis in 1/N HCl, their strong affinity for the nuclear dyes, and their disappearance from cells stained from old cultures. Some smears were stained with Giemsa's solution without a preliminary treatment in acid. Such cells showed no intracellular differentiation. According to Robinow (1942), it is not clear why treatment with hydrochloric acid should be necessary for the satisfactory demonstration of the chromatinic bodies. He points out, however, that it is extremely unlikely that the chromatinic bodies are merely artefacts. The reasons he gives for this are firstly, the chromatinic bodies may also be demonstrated by differentiation of Giemsa-stained films with one percent watery solution of eosin. Secondly, hydrolysis is a gentle treatment and does not alter the size and shape of fixed chromosomes. Finally, the fact that, like chromosomes, the chromatinic bodies of bacteria are constant in appearance and distribution and pass through a regular series of changes, strongly suggests that they represent an actual component of the living cell. Robinow concluded that the chromatinic bodies represent a true nuclear structure.

It was observed in the present study that the intracellular bodies began to disappear after the cells had become 24 hours old. Robinow (1942) reports the almost complete disappearance of the chromatinic bodies from cells after 24 hours incubation on nutrient agar.

Some investigators claim that in demonstrating a chromatin nature confusion is introduced by the presence in the cell of volutin (Knaysi 1938). Lewis (1941) maintains that volutin grains are not found in very young

actively growing cells, but may become abundant as the cells mature. In the present investigation, however, intracellular deep staining bodies were observed in young cultures, but they disappeared as the cells became older.

The cytological evidence, therefore, suggests that the deep staining intracellular bodies observed in the young cells of Azotobacter agile during the course of this investigation are similar to the nuclear bodies described by Robinow (1942) as being present in many common bacteria.

IV SUMMARY

The form and structure of the cells of Azotobacter agile were studied with the nigrosin negative stain, the Robinow nuclear stain, and the electron microscope.

Over a period of two months, four distinct morphological cell types were observed in the life cycle of this organism. These types consisted of large plump rods, small rods, branching rods and thread forms, and small cocci.

Intracellular bodies were observed in young cells which stained with Giemsa solution after hydrolysis in 1/N HCl. The presence of these bodies after hydrolysis, their strong affinity for nuclear dyes, and their disappearance from old cells suggests a nuclear nature.

The results of the present investigation support the view that the Azotobacter may have a complex life cycle, and the intracellular bodies found in the cells of this organism may be similar to the nuclear bodies that have been described for some of the other bacteria.

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Typist -- Carol Ealy