THE EFFECTS OF NUTRITIONAL AND TOXIC AGENTS ON THE CYTOLOGY OF AN *AZOTOBACTER* SPECIES

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I. INTRODUCTION

Not since the days of early bacteriology when morphological studies were paramount, have so many bacteriologists applied themselves to problems of pure bacteriology as during the past two decades. These workers have, in considerable number, attempted to expand the knowledge of bacterial morphology, of microbial dissociation, of the megalomorphic phase of bacteria (i.e., large bodies), and of the Pleuropneumonia-like organisms.

Compared with most other sciences, pure bacteriology, nevertheless, must be regarded as a still relatively undeveloped science. This is due in part to restrictions placed upon its progress by the demands of the monomorphic conception many years ago, as well as to the demands of the field of applied bacteriology. The followers of the concept of monomorphism believed that a bacterial species existed in one and only one form. This monomorphism was confined largely to bacterial form, and alteration in the virulence of pathogenic bacteria was not regarded as inconsistent with an absolute constancy of form.

Topley and Wilson, in 1929, wrote:

> It is almost true to say that the present position of bacteriology is due to the fact that there have been no bacteriologists. From Pasteur onward bacteriologists have been more interested in what bacteria do than in what they are; and much more interested in the ways in which they interfere with man's health and pursuits than in the ways in which they function as living beings.

The organism used in the present study, Azotobacter agile, M.B. 4.4, is one from a genus of bacteria which are especially suited for cytological study due to their relatively large size. A great deal of the early knowledge of morphological and of life cycles of bacteria was derived from the

study of *Azotobacter*. This genus has served as a pattern for the study of variation in other bacterial species.

Azotobacter species are quite abundant in fertile soils where the acidity is not less than pH 6.0, and they are probably in a measure responsible for the accumulation of nitrogenous substances in the soil. They were first isolated by Beijerinck (1901), and named by him, *Azotobacter*. These bacteria are capable of fixing (or assimilating) atmospheric nitrogen; however, when a nitrogenous compound is readily available, as a rule the *Azotobacter* species do not assimilate gaseous nitrogen.

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 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 species *Azotobacter agile* is further described as being rods four to six micra in length, almost spherical. The growth on plain agar is yellowish-white, smooth, glistening, translucent with opaque center. In the presence of organic acids, a greenish or reddish pigment is formed, with occasional fluorescence. Its optimum temperature is 25° to $28^{o}C$. Bergey's Manual gives as the chemical analysis (dried) of *Azotobacter agile*:

> Four-day cultures grown upon mannitol agar when dried, contain more than 4 per cent of hemicelluloses, more than 45 per cent of crude protein, more than 7 per cent of ash, and less than 5 per cent of lignin-like materials.

Even though the *Azotobacter* cell is one of the largest among bacteria, it is still smaller than the cells of other microorganisms, such as molds

and yeasts. Because of this minuteness of the bacterial cell, knowledge of its cytology is awaiting further development of microscopic instruments and techniques. During the past ten years there has been a wave of interest developed in bacterial cytology due to the development of the electron microscope, the phase microscope, and nuclear staining techniques for bacteria. As a result of these developments, great strides are being made toward the uncovering of the "mysteries" of bacterial cytology.

II. REVIEW OF LITERATURE

A. The Bacterial Nucleus

The literature on the existence and state of a nucleus in the bacterial cell has been repeatedly reviewed (Knaysi, 1938 and 1944; Delaporte, 1939 and 1940; Lewis, 1941; and Dubos, 1945). Therefore, an attempt will be made to review only the important literature since 1945.

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. The literature of the subject is truly voluminous, frequently contradictory, and highly controversial; it is almost impossible to reconcile the numerous conflicting reports (Lewis, 1941). The uncertainty as to the existence and form of a nucleus in bacteria is usually attributed to the small size of the bacterial cell.

It has been assumed that bacteria, standing at the threshold of organized living matter, are devoid of any structure suggesting a nucleus. However, there were early claims of the existence of nuclei in bacteria, and these claims were based upon the results of staining reactions which were far from specific in character; volutin and other granules frequently staining like nuclear material and being mistaken for nuclei; in some cases the organisms studied were not true bacteria; the methods employed were not always suitable for cytological study; and some investigators, inadequately trained and without previous cytological experience, were not properly prepared for such a difficult undertaking.

Theories concerning the nucleus of bacteria may be divided into the following groups (Lewis, 1941):

1. Bacteria do not possess a nucleus or its equivalent.
2. The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm. This theory is based mainly on an assumption that the structure of bacteria is the same as that of the Cyanophyceae (blue-green algae).
3. The bacterial body is a nucleus devoid of cytoplasm: 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 bacterial cell as the homologue of a true nucleus.
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 protoplasm contains one or more true vesicular nuclei. This theory has been supported by many of the most able investigators; however, the numerous reports are not mutually confirmatory.
8. The nucleus is a naked, invisible gene-string, or a chromatin-encrusted gene-string analogous to a single chromosome. Lindegren (1935) formulated this theory.

In order to prove the existence of a true nucleus in bacteria, according to Lewis (1941), one must show that the organism belongs to the Eubacteriales or true bacteria rather than other lower 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, spore primordium, or an artifact. Such rigid criteria have not been applied generally.

Studies within the past ten years or so with improved staining techniques and also with the aid of the electron microscope have demonstrated the existence of nucleus-like bodies in many bacterial cells, these bodies dividing in characteristic manner before cell division occurs. These bodies have not been demonstrated in all species of bacteria studied so far,

either with the aid of staining reactions or with the electron microscope. It has been suggested that actively multiplying bacteria do not always form readily demonstrable resting nuclei since chromosome division proceeds so rapidly that there is no time for the formation of a discrete nucleus. Also, since vegetative cells in old cultures tend to be smaller than the young cells which are usually used for nuclear demonstration, the demonstration of a discrete nucleus in all stages of growth is a difficult task.

An entirely satisfactory explanation of the nature of the nucleus and of the nuclear apparatus of the bacteria remains as a problem for future studies. More evidence is accumulating all the time pointing to the existence of true nuclei in bacteria, at least in certain stages of their life history. Studies on induced mutations of bacteria also suggest that bacteria do undergo mutation in a manner similar to higher cells which possess true nuclei.

In spite of the difficulties encountered in this problem, there have been numerous recent studies of the bacterial nucleus which indicate the development of a sounder concept. Discrete nuclear bodies occurring in regular numbers and exhibiting characteristic division have been demonstrated using various staining techniques and also with the aid of the electron microscope, lending support to the hypothesis of a discrete nuclear apparatus in the bacteria. Ribonucleic acid, which occurs primarily in the cytoplasm of higher cells, is also present in bacteria. This material is basophilic and reacts so strongly with basic dyes as to obscure nuclear structures. When this material is hydrolyzed by the enzyme ribonuclease, with warm, dilute hydrochloric acid, or with cold, 10% perchloric acid, the bacterial cell no longer stains so deeply, and the nucleus can be demonstrated more readily, the desoxyribonucleic acid

of the nucleus being left intact to react with nuclear stains such as the Giemsa stain.

Robinow (1945) concluded that the basic chromatinic element in bacteria is a more or less dumbbell-shaped rodlet which divides lengthwise and usually parallel with the short axis of the cell, one "dumbbell" giving rise to two daughter "dumbbells". The first division of the rodlet may be followed immediately by constriction of the cell to form two cells, or, the division of the cell may not occur until after two or more divisions of the chromatinic rodlets.

Others have described the chromatinic elements as homogeneous, round bodies ("nucleoids") which divide by elongation in the direction of the long axis of the bacterium, followed by constriction hour-glass fashion. Robinow has pointed out that the words "chromosomes" (chromatinic rodlets) and "nucleoids" probably refer to two aspects of the same thing, the latter being optically unresolved groups of the former.

The presence in bacteria of a discrete nuclear structure has been considered necessary to explain the constancy of transmission of hereditary characters (Lindegren, 1935) and the results of experiments on the effect of ionizing radiations (Lea, 1947). Reasons for believing that these chromatinic bodies which have been demonstrated are nuclei or chromosomes include their great affinity for basic dyes, their positive reaction to the Feulgen test for desoxyribonucleic acid, their selective absorption of ultraviolet radiation (Malmgren and Heden, 1947), their loss of dye-affinity on treatment with desoxyribonuclease (Tulasne and Vendrely, 1947), and their appearance of undergoing division in relation to cell division (Robinow, 1945; Knaysi and Mudd, 1943).

Studies with the electron microscope have for the most part failed to reveal intracellular structures which could be considered as nuclei. However, by culture in a nitrogen-free medium, Knaysi and Baker (1947) were able to render Bacillus mycoides spores transparent to the electron beam so that relatively opaque intracellular bodies which they describe as the nuclei could be distinguished. Duguid (1948) has found that a deficiency of either phosphate or nitrogenous nutrient will prevent accumulation of ribonucleic acid in the cytoplasm, and that cells of Bacterium aerogenes grown on such media showed deeply stained nuclear bodies within weakly stained cytoplasm. When subcultured on nutrient agar the "nuclear" cells grew and multiplied rapidly, becoming transformed within a few hours into uniformly staining cells of the "normal" type.

Duguid (1948) has also demonstrated intracellular bodies in ordinary stained smears of Bacterium aerogenes grown on media of high carbohydrate content which seem to be identical with the nuclear bodies described by Robinow and others as being present in many common bacteria. The nuclear nature of the bodies observed was borne out by their high affinity for basic dyes, their position in the cell and their constant presence in all cells of a culture; they are not volutin granules since they do not give the metachromatic staining reaction; they are not shrinkage artifacts since they may be seen in unfixed wet films "vitally" stained; they do not represent the shrunken protoplasm of degenerate or plasmolyzed cells since on subculture the cells reproduce rapidly and normally, according to Duguid's report.

Evidence has been presented (Tatum and Lederberg, 1947) for the occurrence of character recombination (characters such as amino acid and vitamin requirements) in the bacterium, Escherichia coli. This suggests

the existence of a sexual phase and activities which are usually considered to take place in chromosomal material.

Knaysi, who in 1942 demonstrated a nucleus in the cell of a Staphylococcus using a number of morphological and microchemical criteria, in extending his work with the help of S. Mudd, was unable to find such nuclei in a number of other species, rods or cocci (Knaysi, 1946). They concluded that in certain species or strains the nuclear material may be in the diffuse state. Knaysi and Mudd admit that it is true that certain procedures gave pictures similar to those interpreted by some as showing nuclei, but that their long familiarity with the structure and behavior of the bacterial cell gave them sufficient ground to conclude that the results were negative. Knaysi described, however, a "possible" differentiation of a nucleus during the maturation of the spore of Bacillus cereus, C_3, as revealed by the Feulgen reaction.

Bisset (1948c) has concluded that two different types of nucleus occur in bacteria. One type is a spherical body occurring in some species of cocci, and in the genera Corynebacterium and Azotobacter. In Azotobacter, and possibly other genera, the nucleus may be of vesicular form, surrounded by chromatinic granules, and resembling the nuclear vacuole of certain yeasts. The second type of nucleus consists of paired chromosomes, usually one or two pairs, which form fusion nuclei within which chromosome divisions occur, followed by redistribution to daughter cells.

Bisset concluded that the majority of rod-shaped eubacteria, the lanceolate-celled streptococci, and the myxobacteria have the second type of structure, i.e., with paired chromosomes.

A review of the literature reveals that the study of the bacterial nucleus is a challenging one and that a keener interest is developing in this field. The significance of this phase of bacteriology grows as increased attention is being given to the genetics of microorganisms.

B. "Large Bodies" in Bacteria

The idea that complex reproductive processes exist in bacteria has persisted throughout the whole history of bacteriology. Lohnis (1921) has collected in a review many observations which suggest such processes. The observations most indicative in this respect were reviewed by Klieneberger (1930).

Klieneberger, in 1935, opened a new approach to the problem of reproductive processes in bacteria. She found that in cultures of *Streptobacillus moniliformis* tiny colonies developed which consisted of organisms with different morphology than the common form. Klieneberger called the organisms in these tiny colonies "L_1". They were similar to the Pleuropneumonia group of organisms which some authors put in a class different from bacteria. The organisms of the L_1 colonies, as in the colonies of the Pleuropneumonia group, are very soft and fragile, and in a certain phase of their life are small enough to pass through bacterial filters. Their morphology is bacillary. They tend to swell into large, round forms 3 to 10 micra in diameter ("large bodies") which in turn reproduce the small forms--a reproductive process also characteristic of the Pleuropneumonia group. In some strains of *S. moniliformis* normal bacilli are formed from the large bodies (Dienes, 1946).

Klieneberger explains the presence of these small colonies in the culture as a phenomenon of symbiosis between two organisms not belonging in the same class. However, the L_1 has been studied by various authors (Brown and Nunemaker, 1942; Dawson and Hobby, 1939; Dienes, 1939a; Heilman, 1941; Oerskov, 1942; and Smith, 1941). All have come to the conclusion that it is a growth form of the bacillus. This opinion is based on the following observations

(Dienes, 1946):

1. The morphology of L_1 as well as the whole Pleuropneumonia group, is distinctly bacillary.
2. The derivation of L_1 from the Streptobacillus can be directly observed. The bacilli swell into large, round bodies, which if appropriately transplanted, germinate and give rise to the L_1.
3. The L_1 is serologically similar to the Streptobacillus.
4. For a variable period of time following isolation up to many months, the L_1 will return to the usual bacterial form if it is transplanted to broth.
5. Ordinary bacilli are reproduced in some strains of S. moniliformis inside the large bodies formed by the bacilli.

These observations suggest that the L_1 is an intermediary phase in the reproductive process leading from large bodies to bacilli and from bacilli to large bodies. The large bodies apparently possess a double potentiality to develop either into Pleuropneumonia-like L forms or into regular bacteria.

The significance of these profound changes in the bacterium is not known. It is unlikely that such a drastic alteration as transformation into L_1 has no significance, and its discovery initiates a problem of great challenge (Dienes, 1946).

The L-type colony is probably identical with the so-called G-type colony which appears in earlier literature. Although apparently seen by earlier observers, the G colony was brought to general notice by Hadley (1931) and his co-workers. Jordan and Burrows (1945) describe the G colonies as follows:

> These colonies are very small, 0.05 mm. or less in diameter, and appear to be made up of small cells of diverse morphology. Since these colonies are the first to appear in cultures of filtered bacterial cultures, they are assumed by some workers to represent a virus-like phase in the life history of bacteria. At present, their significance must be regarded, however, as uncertain. Reversion of the G type to the parent form takes place gradually with apparently full resumption of the characteristic ancestral morphological, physiological and serological properties.

Development of large bodies, also called "megalomorphs", into both L-type and bacillary colonies has been observed in Bacteroides strains, in Proteus strains, in Escherichia coli, and in Hemophilus influenzae. The similarity to analogous processes in S. moniliformis indicates that they all represent the same fundamental processes. Dienes has recently (1949b) observed a similar process in a gram-positive, spore-bearing bacillus. This process is apparently widely distributed among bacteria in general, especially the gram-negative bacilli.

Dienes (1946) has found that as a rule the large bodies are produced only for a short period of time after original isolation of a bacterial strain. Large bodies may be artificially induced in many species by slightly toxic substances, such as gentian violet, lithium or mercury salts, penicillin, etc., in the medium (Dienes and Smith, 1944). These easily available large bodies have been studied extensively, while the naturally-occurring large bodies have received little attention. It is the general opinion of workers that large bodies produced by toxic influences never germinate and reproduce. They are similar in appearance to the large bodies that develop in ordinary cultures, but do not have the same potentiality for development. Dienes states that formation of large bodies under the usual conditions of cultivation is probably a property of the strain and is not necessarily caused by peculiarities of the medium. Dienes also has found that in a richer medium which permits more abundant growth, the pleomorphism develops earlier and is more pronounced.

Dienes makes the supposition that the transformation into large bodies is induced naturally by a secretion or by a metabolic product of the bacteria's diffusing through the medium. The two reasons he gives are: (1) often the majority of the bacteria in or on a medium are involved at

the same time, and (2) toxic substances, including penicillin, often induce the transformation of bacteria into large bodies, thereby demonstrating that diffusable substances do cause the development of large bodies, artificially.

It has been supposed that the formation of large bodies due to the presence of toxic substances is caused by the fact that the bacteria under adverse conditions retain the ability to grow longer than they retain the ability to divide. However, Dienes thinks that since similar large forms are viable under normal conditions of growth and are in all probability part of a complex reproductive process, it is more likely that the toxic substances start this reproductive process but are not able to bring it to its natural conclusion.

The majority of bacteriologists regard large bodies as products of degeneration, since large bodies in most cultures degenerate without further development. This is the case with all naturally-occurring large bodies unless they be transferred to fresh media under appropriate conditions. Large, round bodies of S. moniliformis left in the colonies do not multiply, but soon autolyse. If they are transplanted under appropriate conditions, however, they germinate into L forms. These elements are very small, but their morphology does not differ essentially from that of the bacterial form. L-type colonies invade the agar, however. As the colony becomes older the L_1 forms transform, by swelling, into large, round bodies. In some strains the L forms are capable of growing for many transfers without reverting to the regular bacterial forms.

The large bodies are very fragile structures and are readily destroyed by mechanical injury. In the case of all the bacteria discussed so far, except Proteus, the large bodies start with a swelling of the rod or fila-

ment (the swelling may be double). With Proteus, however, the large bodies are produced mostly by a process which has been described under the name "plasmoptysis" (Dienes, 1946).

> A small droplet appears on the side of the filament, and may increase within a few minutes to the size of a fully developed large body. The filament at the same time becomes thin and flexible. Evidently the content of the filament flows out and produces the large body.

The spreading filaments show a tendency to produce large bodies under various adverse conditions, such as refrigeration, or after having been transferred into tap water or into broth containing $HgCl_2$. According to Dienes the large bodies produced by refrigeration are apparently fully viable; those produced by tap water only occasionally viable; those produced by $HgCl_2$ not viable.

Dienes, who has done a tremendous amount of work with large bodies and L_1 forms, has observed a transformation in colonies of a freshly isolated colon bacillus (Dienes, 1939b) consisting of colonies which contained large bodies and fusiform bodies completely filled with small, regular-shaped bacteria. The membranes of the bodies were intact and they differed from large bodies in other colonies only in the fact that their content was made up of bacteria. The whole colony consisted of these large round and fusiform bodies; there was no bacterial growth outside of them.

Hutchinson and Stempen (1949), by means of time-lapse motion-picture photography, have observed large bodies of Proteus in the act of bursting. They state that almost immediately thereafter minute, refractile, spherical to ovoid granules appear at the edge of the ruptured body. The number of these granules rapidly increases. "These granules", state Hutchinson and Stempen, "would appear to be similar to those reported by Dienes, Smith, and others which develop into the L colonies of the Pleuropneumonia organism".

It was discovered by Pierce (1942) that the L_1 form of S. moniliformis is highly resistant to penicillin and could be readily isolated in pure culture by inoculating the bacterial form on media containing the antibiotic.

Bacilli in a culture of Bacteroides after loss of pleomorphism were induced by penicillin (Dienes, 1948) to swell to large forms. In early stages of transformation these large forms when transferred to penicillin-free media returned to the usual bacillary form; in a later stage they produced only L-type colonies.

Tulasne's observations (1949c) show that penicillin reduces considerably or completely inhibits cytoplasmic divisions in many bacteria, whereas the nuclear divisions take place at a normal rhythm, at least during a certain length of time. This leads to the appearance of giant forms ("large bodies") containing numerous normal or abnormal nuclei. In Proteus, microcolonies were derived from such large bodies. Tulasne is of the opinion that the L forms of bacteria may be considered as normal resistance forms which the microorganisms adopt against noxious agents. "They are selected by those agents but not produced by them". Tulasne also found that the Pleuropneumonia-like organisms which constitute the L colonies of Proteus consist of desoxyribonucleic granulations, isolated or clustered in unmembraned, round, plastic, ribonucleic masses. "The granulations have the aspect and dimensions of a normal Proteus nucleus".

Shanahan, Eisenstark, and Tanner (1947) observed that when Escherichia coli is put in the appropriate penicillin medium the fusiform bodies arise by direct swelling of a portion of an elongated rod. They also reveal intense granulation of fusiform and rod forms by electron microscopy. Shanahan and Tanner (1948) observed under oil immersion the mechanism of fusiform growth in E. coli:

> After two hours' incubation at 37°C cells were either of normal length or double normal length and thin. Elongated cells upon further incubation failed to divide and became thickened to some extent. First evidence of fusiform swelling, following elongation, was the appearance of two dark areas, half-moon-shaped and approximately in the middle of the cell. The dark areas were on opposite sides of the cell wall and indicated the spot where swelling would occur. Direct swelling of the cell followed the appearance of these areas that might or might not remain visible in the swollen fusiform. Young fusiform cells were homogeneous internally and became granulated only as the cultures aged.

Dienes has recently (1949c) cultivated L-type colonies from typhoid bacilli exposed to immune serum and complement.

The study of the phenomena reviewed above is in an early stage, and more revealing information may be expected from further study of these processes. According to Dienes (1946), "It appears of great importance to extend the investigation to as many other species as possible. Advance in this study depends in a large measure on finding strains appropriate for study". Different species, and even different strains of the same species are proving more valuable for the study of certain aspects of the process. Study of saprophytic strains appears especially promising, since a natural environment can be realized more easily with saprophytes.

With increasing attention being focused on genetics of microorganisms, interest has been stimulated in the problem of whether or not bacteria possess reproductive processes other than binary fission. The need for more research in this field is definitely indicated.

C. Effects of Irradiation on Bacterial Cytology

The radiation of cells of multicellular organisms has produced chromosome structural changes. These changes have been extensively studied, especially in the case of the large salivary chromosomes of *Drosophila melanogaster* (Lea, 1947). The chromosomes of microorganisms are too small for this type of observation with the instruments of today. Nevertheless certain cytological changes in the cells of microorganisms which have been irradiated may be observed.

It has been known for some time that morphological changes may be readily produced in fungi exposed to radiation. Schulze, in 1909, made the following observations after exposing *Mucor stolonifer* to sublethal doses of ultraviolet radiation of 2900A. About thirty minutes after irradiation the hyphae became densely granular, at the same time increasing to almost double their usual diameter, and the tips of the hyphae became bulbous or club-shaped. If the hyphae had not been irradiated too long there appeared after an interval, depending on the length and intensity of irradiation, a clear mass of protoplasm in the swollen end of each hypha from which arose a new hypha.

Radiations may be divided into two classes, ionizing radiations which eject electrons from atoms through which they pass, and non-ionizing radiations which are a form of radiant energy. "We shall take it for granted that the biological effects of ionizing radiations are due in some way to the chemical changes induced by the radiations", (Lea, 1947). In addition, Lea states that since even a very large dose of radiation produces a rather small percentage of chemical change in a cell, there have been several theories put forth to explain how such a small overall percentage of chemical change may be imagined to be effective. These are as follows:

1. Cell Poisons. The products of decomposition of proteins may be injurious in quite low concentration.
2. Activated Water Reactions. By the use of a sufficiently dilute solution a large percentage of chemical change in the solute can be accomplished by a moderate dose of radiation, whereas the same dose would have practically no effect upon concentrated solution or a dry preparation. Perhaps peroxides are formed; these of course would have a harmful effect.
3. Direct Action on Large Molecule. Direct ionization of large molecules in the cell, i.e., genes, might occur.
4. Localization of Ionization. If ionization is high locally, and if the structures through which the ionizing particle passes are sufficiently vital for changes in them to effect the cell as a whole, then a biological effect will be recorded.
5. Spread of the Effect of an Ionization. There is evidence which suggests that the passage of a densely ionizing particle anywhere through the chromosome thread causes a break (Lea, 1947).

> Now the chromosome thread being (in the case of *Tradescantia*) of a diameter of about 0.1 micron, it must be made up of a very large number of chain molecules, and only a small fraction of these chains will be broken by the direct ionization or excitation of bonding electrons by impact of the ionizing particle. Some spread of the effects of ionization or excitation must therefore occur. Transference of energy from one part of a molecule to another is a process known to occur.

Irradiations are often accompanied by peculiar cytogenetic changes. What has been observed in many bacteria may be illustrated by the following statement of Haas, Wyss, and Stone (1948):

> When *Escherichia coli* are exposed to a limited dose of radiation, they form 'spaghetti-like' filaments which attain lengths up to 150 microns and diameters up to 3 times that of normal cells....As the filaments grow it was observed that the

nuclear bodies divide regularly and each filament soon contains pairs of nuclear bodies located 3 to 4 microns apart. After several hours many of the filaments recover from the effect of the irradiation and produce normally-dividing cells.

A temporary inhibition of cell division appears to be a general action of radiation (Lea, 1947). The duration of the delay increases with increasing dose. Therefore this effect of radiation differs from the other observed effects which have an all-or-none reaction. Lea (1947) states that it is not clear whether this division-inhibiting effect is due to the destruction of some component of the cell needed for division to occur, or to the production of some substance having an inhibitory effect.

The mechanism of cell division seems to be more sensitive to irradiation than that of growth, and doses of radiation which are too small to stop growth may be sufficient to stop cell division. This results in the production of giant cells (Duggar, 1936). Oster (1934) reports that after the exposure of yeast to ultraviolet rays giant cells and two-cell groups from 3 to 8 times the size of normal two-cell groups appeared. Gray, Marton, and Tatiun (1945) when observing mutants of Acetobacter after x-ray treatment found one or two darker areas of about 0.1 micron in diameter in each cell.

Eisenstark and Clark (1947), using the electron microscope, revealed "breaks" spaced periodically along the length of Escherichia coli filaments produced by x-ray treatment. Also these cells were seen to have a mottled and irregular appearance, certain areas of the cell being less dense than the rest of the cell. They suggest that the enzyme which is responsible for the pinching off of the cell wall in normal bacterial fission is destroyed while other enzyme systems continue to function.

According to Nickerson (1948) evidence which has accumulated in apparently widely different fields is leading toward a concept that cell division

in microorganisms, and probably also in other organisms, is under the control of a unit enzymatic mechanism. Among the investigations which Nickerson cites is that of Hinshelwood (1946) who views normal growth as the result of a balance between a division factor and an elongation factor; the latter has been shown to be diffusible. By incorporating into a nutrient medium traces of m-cresol or proflavine Hinshelwood has so disturbed the balance between division and elongation factors that cultures of Bact. lactis aerogenes consisted of tangled masses of long, thread-like elements. It was found by Gardner (1940) that sub-bacteriostatic concentrations of penicillin induce the growth of long, filamentous forms in many gram-negative and gram-positive bacteria even though higher concentrations are needed to achieve the same effect with the gram-negative group.

Henry and Stacey's investigations (1943 and 1945) on the chemical nature of the gram-positive complex have demonstrated it to be magnesium ribonucleate with associated --SH groups; oxidation to S--S abolished the gram-positiveness of the complex. Pratt and Dufrenoy (1948) have expressed the view that penicillin exerts its effect by oxidizing --SH to S--S in some manner. It is possible that peroxides produced by ultraviolet irradiation may act in the same manner.

Among other cytological effects which have been observed when bacteria are radiated is the observation by Haberman and Ellsworth (1940) that occasional streptococcus-like forms resulted from the treatment of Staphylococcus aureus with x-rays. Grainger (1947) when treating a strain of "Eberthella typhosa" with x-rays observed on one occasion that a high percentage of the organisms exposed showed a loss of motility. They were still viable.

Witkin (1946) states that strain B of Escherichia coli gives rise to a variant which is relatively resistant to the lethal effects of both ultraviolet and x-rays. This character is stable and heritable. Some additional properties of this radiation-resistant strain (B/r) are that this strain exhibits relative resistance to the division-inhibiting effect of radiation, and is also relatively resistant to the lethal and division-inhibiting action of penicillin and sodium sulfathiazole.

There is a marked similarity between certain biological effects produced by ultraviolet irradiation of nutrient broth and by the addition of hydrogen peroxide to the broth (Wyss, Clark, Haas, and Stone, 1948). The effects of both can be reversed by catalase (the enzyme which decomposes peroxide). Since the cells studied contained catalase, it was necessary to postulate that some reaction must occur such as the union of the labile substance (H_2O_2) with an anabolic (constructive) enzyme which protects the mutating molecule from the catalase of the cell.

Recently Kelner (1949) has shown that cells of Escherichia coli which have been irradiated with ultra-violet may have the lethal effects reversed by visible light below 5100A in wave length. How this fact affects work which has been done with the elongated cells produced by irradiation is not known. Perhaps the return to normal division of some of the elongated cells has been due to the action of light in reversing the effect of ultraviolet irradiation. At any rate experiments in the future should take "photoreactivation" into account.

D. The Cytology of Azotobacter

Variation in bacterial morphology was observed by many of the early bacteriologists. In spite of this, most of the bacteriologists were followers of the "monomorphistic" theory which maintained that any change in a pure culture of bacteria was proof of (1) "contamination", (2) incorrect classification of the organism, or (3) the existence of "involution" (degenerative) forms.

Lohnis and Smith (1916) pointed out that progress in bacteriology up to that time had been severely checked by the inclination to consider species as having a constant form. They further declare that the life cycles of bacteria are no less complicated than those of other microorganisms.

Since Azotobacter are larger than bacteria of most other genera, and have plainly visible cellular inclusions it is only natural that early bacteriologists should have strived to determine the nature of these granules. Beijerinck (1901) after studying these cellular granules in Azotobacter determined that they were fat bodies. Later, in 1906, Fischer reported that volutin granules were present in the cells of this species. Mencl (1911) described the granules as chromidia and the equivalent of a true nucleus. So it was that a wide difference of opinion developed as to the nature and function of these intracellular bodies in Azotobacter.

On the basis of staining reactions Jones (1913) distinguished two types of granules. The stainable granules he regarded as reproductive bodies and designated them as "gonidia". The non-stainable granules he thought to be glycogen. Bonazzi (1915), using various microchemical tests, pointed out that the granules found in Azotobacter chroococcum appeared to be metachromatic in character. He further indicated that they seemed to

have no relation to the reproduction of the cell since their apportionment in the cells was not regular but changed in the individual cells.

From a comparative study of twenty-four Azotobacter cultures and eighteen strains of other bacteria, Lohnis and Smith (1916) 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 the 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 latter case an unstainable 'symplasm' is produced.

Although Jones (1920) agrees with some, he does not agree with all the conclusions arrived at by Lohnis and Smith (1916) with regard to Azotobacter. Jones found that there was a fusion or mixing together of the protoplasm in symplastic clusters. However, he found no evidence for endospore formation in Azotobacter as reported by Lohnis and Smith (1916) and Mulvania (1915). Jones's viewpoint is held today in regard to there being no endospores formed in Azotobacter. From his studies in 1920 Jones further reports that in cultures of Azotobacter one to two months old grown in Ashby's solution or on Ashby's agar there is an irregular occurrence of "involution" forms. 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 bacteria. According to him, all well-studied species of bacteria, including Azotobacter, have been observed to grow in various, round, straight and curved, small and large, regular and irregular cell forms. He further states that budding and branching are common with all bacteria.

In 1923, Lohnis and Smith published a study of the life history of _Azotobacter_, pointing out that from every _Azotobacter_ culture they studied, 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, and (7) large sporulating cells.

The reproductive organs of _Azotobacter_ are, according to Lohnis and Smith (1923), (1) gonidia, in part filtrable, (2) regenerative bodies and exospores, (3) arthrospores, (4) microcysts, and (5) endospores. The basis for the development of regenerative bodies, arthrospores, and endospores are the gonidia, they conclude.

This complicated life cycle described by Lohnis and Smith for _Azotobacter_ has been partially confirmed and partially contested by subsequent investigation. In 1937, Lewis concluded that the so-called gonidia or chromidia of _Azotobacter_ are volutin, and therefore not concerned with reproduction. The refractile granules of this species, Lewis concluded, are storage bodies of fat-like nature. In the opinion of Knaysi (1938) the existence of gonidia is still in doubt.

Lewis (1937) could find no endospore formation in any of the strains of _Azotobacter_. In addition, he failed to confirm the occurrence of a filtrable phase, and of the transformation of cells to a different culture type. Lewis came to the belief that the life history of _Azotobacter_ is very simple depending for reproduction upon binary fission and arthrospores.

During the past ten years or so the cytological study of _Azotobacter_ has been almost completely neglected. However, Eisenstark, McMahon, and Eisenstark (1950), McMahon (1949), and Eisenstark and McMahon (1949) have made a cytological study of a pleomorphic strain of _Azotobacter_ using modern

instruments and techniques. They reached the conclusion that the species Azotobacter agile (Illinois strain ZN 350) exhibits four distinct morphological types of growth. "These types consisted of (1) large, plump rods, (2) small rods, (3) elongated and branched forms, and (4) minute coccoid cells".

As one can easily observe, there is to this day much disagreement as to the cytology of Azotobacter and of the bacterial cell in general. In view of the recent development of the electron microscope, phase microscope, and improved techniques of nuclear staining, a further study of the cytology of Azotobacter was deemed worthwhile at this time.

III. EXPERIMENTAL

A. The Normal Cytology of Azotobacter agile, M.B. 4.4

Purpose

The purpose of these observations was to obtain examples of the normal cytology of the organism so that comparisons with abnormal cytology could be made.

Materials and Methods

The procedures of (1) nuclear staining, (2) negative staining with nigrosin and subsequent observation under the phase microscope, (3) electron microscopy, and (4) fat staining were used for observing the cells in most of the experiments to be described. Therefore a brief discussion of the techniques used throughout will be presented here.

Nuclear stains were made according to the techniques presented in the papers of Robinow (1942 and 1945), Cassel (1950), and Smith (1950). According to Robinow's technique, smears or coverslip impressions taken directly from the agar are fixed in osmium tetroxide vapor for about 1 1/2 minutes, and then hydrolysed in 1/N HCl at $60^{o}C$ for 5 to 8 minutes. The smears are then stained for 20 to 30 minutes in Giemsa's solution at a dilution of about 1 to 6. If coverslip impressions are used, they may be mounted face down in tap water on an ordinary glass microslide, the excess water being absorbed with pieces of filter paper and the cover glass sealed to the slide with melted paraffin, applied with the aid of a camel's hair brush. Cassel found the action of cold perchloric acid on bacterial cells to be comparable to that of warm HCl. Cells were fixed with osmium tetroxide vapor, then put into 10 per cent perchloric acid at $4^{o}C$ for 30 to 40 hours. 0.1 per cent aqueous basic fuchsin (15 to 30 seconds) was used as the nuclear

stain. Smith uses osmium tetroxide vapor fixation and hydrolysis with warm HCl, but then mordants the cells in 1 per cent formaldehyde for 2 to 4 minutes. After this the preparation is washed and then stained with 0.3 per cent basic fuchsin for 15 to 30 seconds. Nuclear stains were made after 3 to 4 hours growth of the cells. The incubation temperature for all experiments was 27°C; the pH of the medium was 7.3.

Photomicrographs were made of the nuclear, negative, and fat stains. Bausch and Lomb photomicrographic equipment, type J, was used, including the vertical camera and illuminating unit mounted on a common supporting base.

Marton's method (1941) was used in the preparation of material for the electron microscope. The organisms were suspended in distilled water, washed several times by centrifugation, and a small drop transferred to the parlodian film where it was allowed to dry. The R. C. A. type EMC electron microscope was used.

Fat stains were prepared according to Burdon's (1946) method for permanent fat stains. Smears were dried, heat fixed, and then stained with Sudan Black B for 10 minutes. After being cleared with xylol for 1 minute, the smears were counterstained with safranin for 10 to 15 seconds. The cells are red; the fat globules are blue-black.

The nitrogen-free media used in this and the following experiments were (1) a "basal" medium of the following composition: $MgSO_4$ 0.2 gm, K_2HPO_4 1.0 gm, $CaSO_4$ 0.1 gm, $FeSO_4$ 0.03 gm, $NaMoO_4$ 0.002 gm, glucose 10.0 gm., agar 15.0 gm., and distilled water 1000 ml.; (2) "Ashby's" medium; and, (3) "soil extract" medium of the following composition: K_2HPO_4 0.5 gm, glucose 1.0 gm, agar 15.0 gm, soil extract 100.0 ml., and tap water

1000 ml. The soil extract is prepared by heating 1000 grams of rich garden soil with 1000 milliliters of tap water in an autoclave for 30 minutes. A small amount of $CaCO_3$ is added and the soil suspension is filtered through a double paper filter until clear.

Results

When grown on nitrogen-free medium, as is customary, the cells of *Azotobacter agile*, M.B. 4.4 are relatively large, irregularly-shaped rods, so short that at times they may appear spherical (figure 1). The diplococcus-type cell arrangement is common (figure 2). Under the phase microscope the cells show inclusion bodies (figures 3 and 4). These bodies look similar to those seen when the cells are fat stained according to Burdon's (1946) permanent mount method (figures 5 and 6).

Stains for chromatinic material reveal a variety of patterns in young cells (3 to 4 hours old). Bisset (1948c) considers the nucleus of *Azotobacter* to be of vesicular form, surrounded by chromatinic granules, and resembling the nuclear vacuole of certain yeasts. A nucleus of this description is found in most of the cells of *Azotobacter agile* (figures 7 through 11); however, many other configurations are also found. Figure 12 presents diagrams of some of the patterns of chromatinic material found in the cells of this organism.

Summary

The cells of *Azotobacter agile* when grown on nitrogen-free medium are relatively large, short, irregularly-shaped rods which may appear spherical. Within the cells are clearly-visible bodies, internal structure being seen even when the cells are made visible by a simple stain. These bodies consist,

at least, of fat, and of chromatinic material. The arrangement of the chromatinic material varies considerably from cell to cell according to the observations made; however, most of the cells seem to contain a ring of 3 to 5 chromatinic granules.

B. The Effect upon Cytology of Growth on Nutrient Agar

Purpose

The purpose of this experiment was mainly to observe what effect the presence of nitrogen sources such as those found in the peptones and beef extract of nutrient agar would have on the cytology of *Azotobacter agile*, M.B. 4.4.

Materials and Methods

In this experiment the organism was merely grown on nutrient agar, or on an agar made up of different proportions of the two nutrient components of nutrient agar, beef extract and peptone. The cells were examined by means of the electron microscope, phase microscope (with negative staining), nuclear staining, and fat staining.

Results

When the test organism, *Azotobacter agile*, was grown on ordinary nutrient agar, very irregular forms were found. These consisted of extremely long filaments with no visible septae (figures 13, 14 and 15), elongated cells with swollen centers or ends (figures 16 through 20), or cells so swollen as to be spherical, or nearly so (figures 21 and 22). Figure 22 is an electron micrograph of a large body seen in some preparations. It was not possible to determine the nature of this object and it is possible that it may represent an artifact. Figures 23 through 27 show some of the shapes of the rods which are formed. It should be noted that some of the cells are quite transparent to the electrons despite their large size. These ghost-like cells which reveal an undetermined internal structure suggest degeneration forms. Other very large cells, however, are quite dense.

Kyle (1950) has tested 200 "balloon" and filamentous forms of this organism for viability by single cell isolation using the de Fonbrune micromanipulator. Of 200 isolates, 72 developed into colonies. As a means of comparison, 100 normally-shaped cells were isolated with 53 showing positive viability.

The "abnormal" forms were seen microscopically to produce normally-shaped cells. Reproduction of the swollen cells was observed microscopically after they had been transferred from nutrient agar to a nitrogen-free medium. These cells pinched off at the tips (figures 28 and 29) producing cells of normal morphology.

Kyle (1950) has, in an attempt to show a serological relationship between bizarre and normal forms of Azotobacter agile, inoculated rabbits, guinea pigs, chickens and hamsters with the cells of this organism. He found that antibody production could not be demonstrated by agglutination, precipitin, or complement-fixation tests.

Many of the above mentioned "abnormal" forms are similar to the "large bodies" described in other bacteria by Dienes (1946). Large bodies have been observed by Dienes to be formed by a swelling of the bacterial rod at some point (or points) along its length. With Proteus, however, (Dienes, 1946) the large bodies are also produced in the following manner: a small droplet appears on the side of the filament, and may increase to the size of a fully-developed large body; the filament at the same time becomes thin and flexible. Figures 30, 31, and 32 suggest that such a process might also occur in Azotobacter agile.

In order to determine what substance in nutrient agar might be responsible for the development of the large bodies nutrient agar was made

up lacking peptone and beef extract, respectively. The morphology of the Azotobacter cells grown on this agar was normal in every respect. However, when agars were compounded as follows, the results were:

Table I

Medium	Cell morphology
1/5 beef extr. plus 4/5 peptone	normal
2/5 beef extr. plus 3/5 peptone	many long rods
3/5 beef extr. plus 2/5 peptone	many long, swollen rods
4/5 beef extr. plus 1/5 peptone	extremely long, pleomorphic and swollen rods

(5 grams peptone and 3 grams beef extract were taken as 1/2 concentration of each).

From the above tabulation one can see that, as the concentration of beef extract increases, the morphology of the cells also becomes more abnormal, and yet with 100 per cent beef extract in the medium the cells show normal morphology. Some peptone is therefore necessary to produce the filamentous and large bodies. Because of the complexity of nutritional factors involved, this phase of the investigation was carried no further.

When first transferred to nutrient agar from nitrogen-free media Azotobacter agile grows slowly, growth being sparse in 24 hours. However, after several transfers growth becomes considerably more rapid. The morphological variants are more extreme and more numerous upon the early transfers, but continue to be found even after thirty transfers onto nutrient agar.

Fat stains of the "balloon" cells reveal relatively large globules of fat material scattered throughout the cells (figure 33).

Nuclear staining was attempted many times on the "balloon" cells, but the chromatinic material in these cells appears to be very finely divided and scattered throughout the cell so that an acceptable microphotograph was not obtained.

Summary

When cells of *Azotobacter agile* are grown on ordinary nutrient agar they develop into long filaments which may swell at one or more points into "balloon-shaped" forms, spheres, or they may produce a bud-like swelling from the side of the rod. These abnormally-shaped cells apparently may either degenerate or may reproduce normally-shaped cells by a pinching off of the ends.

The beef extract component of nutrient agar seemed to be the factor which stimulated the formation of large bodies, although beef extract alone in the agar would not cause the formation of the large bodies.

The chromatinic material in the large cells, unlike that of the normally-shaped cells, appears to be very finely divided and is scattered throughout the cell.

C. The Effect of Nutrition upon Morphology

Purpose

This experiment was carried out in order to test for possible single nutritional substances which might stimulate production of large forms in *Azotobacter agile*, M.B. 4.4. A few complex substances were also included.

Materials and Methods

Either one or the other of the following methods was used: (1) to a series of tubes of nitrogen-free medium a solution of the substance to be tested was added in amounts designed to vary the concentration from high to very dilute, and (2) plates of nitrogen-free agar were poured and the surfaces inoculated with a suspension of the organism by using a sterile camel's hair brush, and then small pieces of filter paper which had been soaked with the substance to be tested were placed on the surface of the agar, which had been incubated 2 to 3 hours in order to dry the surface somewhat. In the latter method all variations in concentration were present as the substance diffused outward some distance into the agar. Where a carbon compound was to be tested, the carbohydrate was omitted from the basic medium.

During the course of this experiment the following carbon compounds, amino acids, accessory growth substances, etc., were tested: glycine, proline, phenyl alanine, histadine, leucine, methionine, threonine, tryptophan, cystine, arginine, potassium nitrate, ammonium sulfate, nicotinic acid, thiamine, riboflavin, biotin, inositol, pyridoxine, pantothenic acid, para-amino-benzoic acid, yeast extract, sodium acetate, sodium oxalate, arabinose, maltose, lactose, sorbitol, galactose, xylose, raffinose, mannose, dulcitol, sodium citrate, guinea pig serum, and rabbit serum.

Results

None of the above substances produced any bizarre forms. Glycine, however, produced on one trial slightly enlarged cells. This effect was not seen again in repeated trials. Gordon and Gordon (1943) have reported involution forms of the genus *Vibrio* produced by glycine.

Summary

No production of large forms was observed in tests with any of the substances used. A slight enlargement of the cells was observed in the case of glycine, but this occurred in only one trial, and could not be repeated.

D. The Effect of Toxic Agents upon Morphology

Purpose

There have been many reports of the production of large bodies in bacteria by toxic agents (Shanahan, Eisenstark, and Tanner, 1947; Johnson and Gray, 1949; Tulasne and Vendrely, 1948). The purpose of this experiment was to determine whether large bodies could be produced in *Azotobacter agile*, M.B. 4.4 by toxic agents.

Materials and Methods

The toxic agents to be tested were added to the medium in the same manner as described under section C, "Materials and Methods".

The effects of these toxic agents were tested: ethyl alcohol, methyl alcohol, sodium azide, penicillin, streptomycin, mercuric chloride, bacitracin, tyrothrycin, sulfonamides, aureomycin, phenol, lithium chloride, colchicine, podophyllin, and nitrogen mustard.

Results

Large, abnormal forms were produced only in the case of various concentrations of bacitracin, gentian violet, tyrothrycin, ethyl alcohol, and sulfathiazole. Large bodies and filamentous forms were numerous and consistent only on the medium containing bacitracin. The other agents, ethyl alcohol, gentian violet and sulfathiazole, produced abnormally-shaped cells in an erratic manner, sometimes failing to produce any abnormal cells whatsoever on certain retrials.

It was attempted, in the case of bacitracin, to subculture the cells on the same medium. Apparently the large bodies produced by bacitracin are not viable, since several trials at subculturing failed. This fact is in

agreement with Dienes (1946), who states that large bodies produced by toxic agents are generally not viable. Microphotographs of the swollen forms produced by the toxic agents revealed forms very similar to those produced by nutrient agar. Figure 34 presents a swollen form produced by bacitracin.

Summary

Large, abnormal forms were produced by bacitracin, gentian violet, sulfathiazole, tyrothrycin, and ethyl alcohol. In general, the bizarre forms were far less numerous than those produced by nutrient agar. Also, on retrials, the effects were not always consistent, and abnormally-shaped forms often failed to appear.

E. The Effect of Ultraviolet Irradiation upon Morphology

Purpose

The production of bizarre cells by irradiation has been observed by several investigators (Duggar, 1936; Gray, et al., 1945, Oster, 1934; and Eisenstark and Clark, 1947). Therefore an attempt was made to produce cytologically abnormal forms in *Azotobacter agile*, M.B. 4.4 by use of irradiation.

Materials and Methods

A broth suspension of the organism was poured, in a 1 to 2 millimeter layer, into small, sterile dishes. The tops of the dishes were covered with cellophane, through which ultraviolet rays of about 2537A were passed for 5, 10, 15, and 30 minutes. A culture of *Escherichia coli* (strain B) was given the same treatment, serving as a control. Immediately after and at 2-hour intervals up to 18 hours after irradiation a smear and stain were made from the cultures in order to observe any morphological changes.

Results

Although growth was much slower in the cultures exposed to the longer periods of irradiation, the morphology of the *Azotobacter* culture remained unchanged. "Snake-like" forms were produced in the control cultures of *Escherichia coli*.

Summary

Irradiation of the cells of *Azotobacter agile* with ultraviolet rays of about 2537A produced no morphological change.

F. Observation of L forms of *Azotobacter agile*, M.B. 4.4

Purpose

L forms are the small organisms found in the tiny L colonies which have been observed to develop from normal cultures of many bacteria (Klieneberger, 1935; and Dienes, 1946). The organisms of the L colonies, which are similar to the Pleuropneumonia group of organisms, are very soft and fragile, and in a certain phase of their life are small enough to pass through bacterial filters (Dienes, 1946). When it was noticed that tiny colonies appeared on agar plates among the large colonies of *Azotobacter agile*, M.B. 4.4, the tiny colonies were studied to see whether or not they represented L forms. The strain in which this phenomenon was observed was not the parent culture that has been used in the other studies, but rather a non-pigmented mutant resulting after irradiation.

Materials and Methods

The tiny colonies were isolated and transferred onto nutrient agar and basal agar slants. The cells were grown in broth, and tested to see if they were filtrable.

Results

The cells from the tiny colonies grew well on both nutrient agar and nitrogen-free agar, and when examined microscopically appeared to be pure cultures of very minute *Azotobacter agile*, containing many paired forms (figure 35), as is usually the case with *Azotobacter agile*. Attempts at subculturing the minute cells failed. However, similar cultures were obtained again by going back to the original plates. When the tiny cells were transferred to Ashby's broth an interesting thing happened. In 48

hours the broth contained numerous cells which were the normal size and shape of *Azotobacter agile*. These broth cultures, nevertheless, when transferred to agar slants did not produce the normal *Azotobacter* cells, but only the tiny cells. Later, however, the broth cultures were streaked onto basal agar plates where large colonies (and no tiny colonies) containing normally-sized and normally-shaped *Azotobacter* cells developed. Attempts at subculturing the L forms were later successful, but not consistently.

Since these small cells were discovered just prior to the writing of this thesis, there was not time for other experiments which the investigator would liked to have carried out with these L forms.

Summary

L forms were isolated from tiny colonies produced by a pigmentless mutant of *Azotobacter agile* which had been treated with radioactive phosphorus. These cells appeared to be exact replicas of *Azotobacter agile* cells, except that they were relatively very small. Upon transfer of the L forms to Ashby's nitrogen-free broth, cells of normal *Azotobacter agile* morphology were produced.

G. The Effect of Age upon the Morphology of *Azotobacter agile*, M.B. 4.4

Purpose

Certain strains of *Azotobacter* have been shown to change morphologically upon aging (Lohnis and Smith, 1923; McMahon, 1949; and Eisenstark, McMahon, and Eisenstark, 1950). The purpose of this experiment was to observe the cells of this strain of *Azotobacter*, i.e. *Azotobacter agile*, M.B. 4.4, upon aging.

Materials and Methods

The organism was grown in screw-capped bottles which were placed on their sides. Ashby's agar and soil extract agar were used. Each day for 60 days a smear was made of the cells in the bottles. A few nuclear stains using Robinow's method (1945) were attempted at various ages of the cells.

Results and Summary

The morphology of *Azotobacter agile* remained extremely constant throughout the 60-day period. Old cells revealed no distinct chromatinic bodies.

H. Illustrations

Plate 1

<u>Azotobacter</u> <u>agile</u>, M.B. 4.4

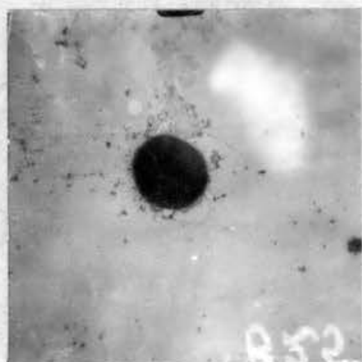
Figure 1. A twenty-four-hour-old cell grown in Ashby's broth. Electron micrograph, x 5,000.

Figure 2. Six-hour-old cells grown on soil extract agar. Electron micrograph, x 5,000.

Figures 3 and 4. Twenty-four-hour-old cells grown on Ashby's agar. Note inclusion bodies. Phase micrographs, x 1,250.

Figure 5. Six-hour-old cells grown on Ashby's agar, and fat stained by Burdon's method. Phase micrograph, x 1,800.

Figure 6. Six-hour-old cells grown on soil extract agar, and fat stained by Burdon's method. Phase micrograph, x 1,800.



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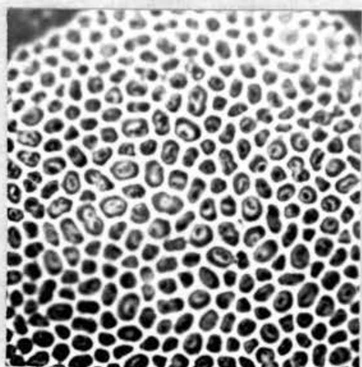

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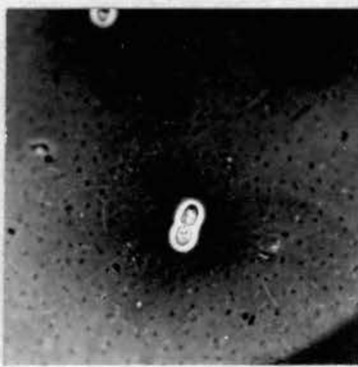

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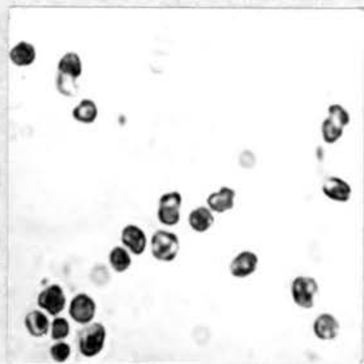
Plate 2

Azotobacter *agile*, M.B. 4.4

Figures 7 through 11. Three-hour-old cells grown on a nitrogen-free basal agar, fixed in osmium tetroxide vapor, hydrolyzed in 1/N HCl, and stained with Giemsa's solution. Note that some cells contain a ring of 3 to 5 chromatinic granules. Light micrographs, x 1,250.

Figure 12. Diagrams of some of the patterns of chromatinic material found in the cells of this organism.



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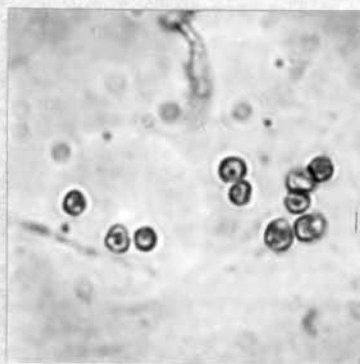

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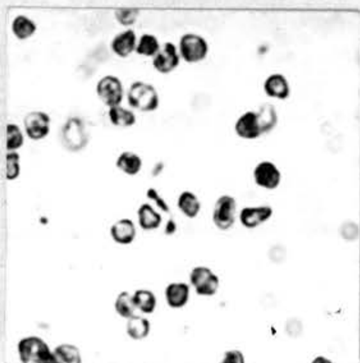

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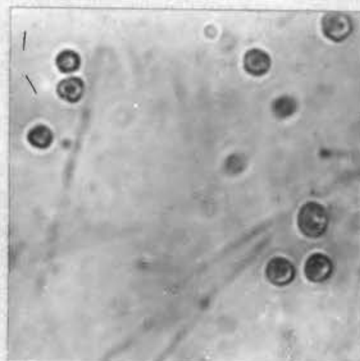

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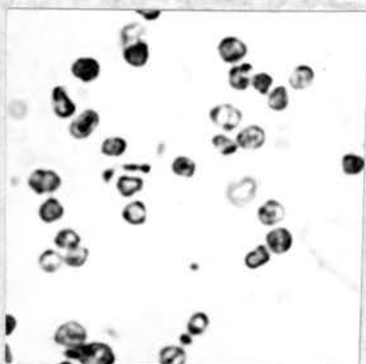

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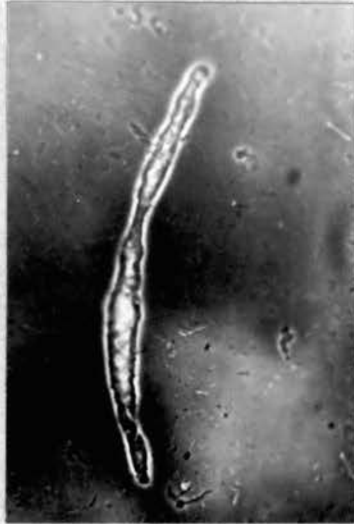
Plate 3

Azotobacter agile, M.B. 4.4

Figures 13, 14, and 15. Twenty-four-hour-old cells grown on nutrient agar. Extremely long filaments with no visible septae. Phase micrographs, x 1,250.



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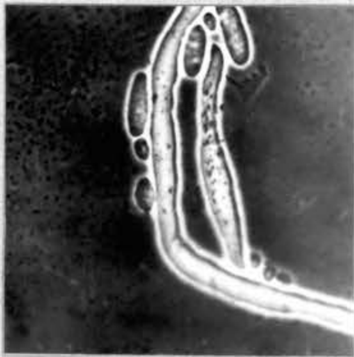

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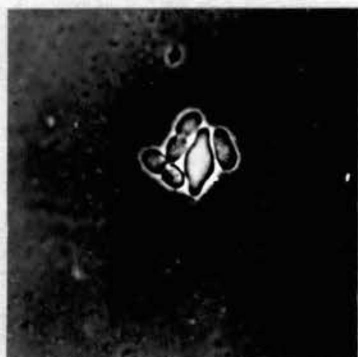
Plate 4

Azotobacter agile, M.B. 4.4

Figures 16 through 19. Twenty-four-hour-old cells grown on nutrient agar. Elongated cells with swollen centers or ends. Phase micrographs, x 1,250.

Figure 20. A forty-eight-hour-old cell grown on nutrient agar. Note the club-like appearance. Electron micrograph, x 5,000.



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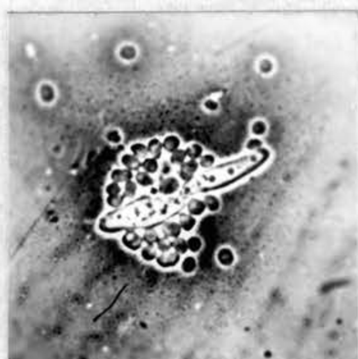

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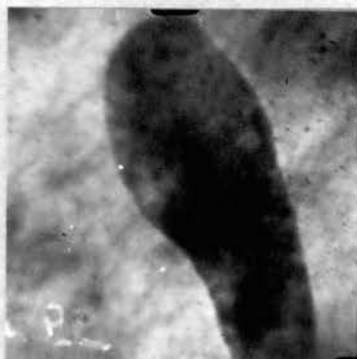

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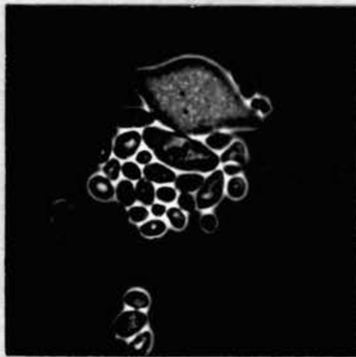
Plate 5

Azotobacter agile, M.B. 4.4

Figure 21. Twenty-four-hour-old cells grown on nutrient agar. Cell so swollen as to be nearly spherical. Phase micrograph, x 1,250.

Figure 22. A forty-eight-hour-old "cell" grown on nutrient agar. This "large body" may be an artifact. Electron micrograph, x 5,000.

Figures 23 and 24. Forty-eight-hour-old cells grown on nutrient agar. Some large cells quite transparent; others dense. Electron micrographs, x 5,000.



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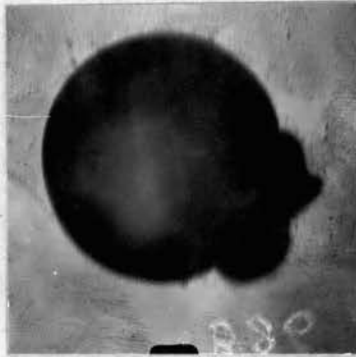

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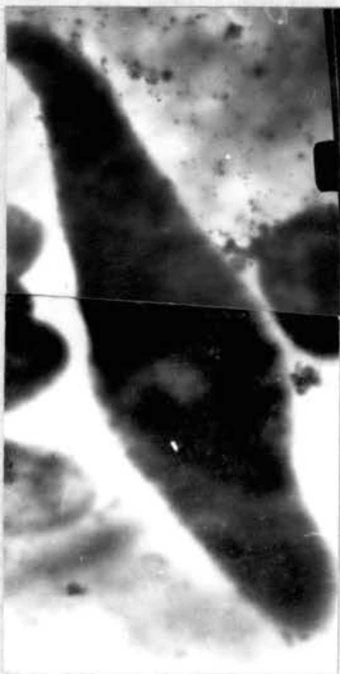

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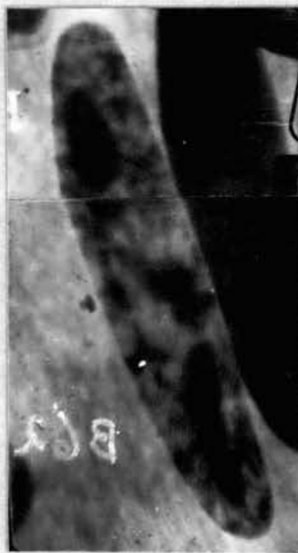

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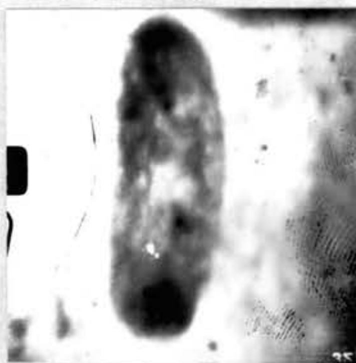
Plate 6

Azotobacter agile, M.B. 4.4

Figures 25, 26, and 27. Forty-eight-hour-old cells grown on nutrient agar. Large, transparent cells. Electron micrographs, x 5,000.

Figures 28 and 29. Twenty-four-hour-old cells grown on nutrient agar. Note production of normally-shaped cells from swollen bodies. Phase micrographs, x 1,250.



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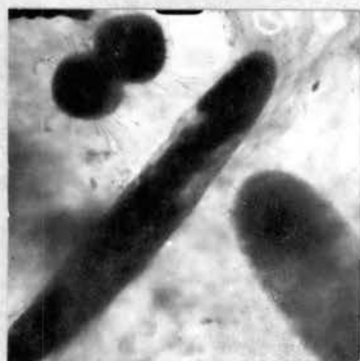

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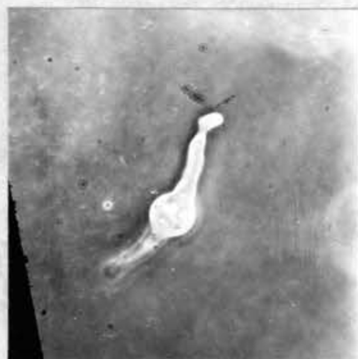

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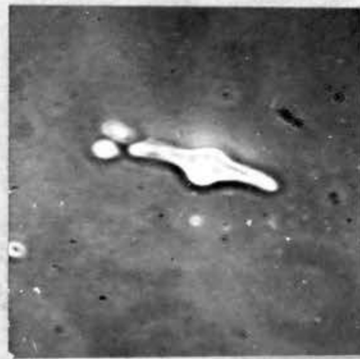

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Plate 7

Azotobacter agile, M.B. 4.4

Figures 30, 31, and 32. Forty-eight-hour-old cells grown on nutrient agar. Possible budding or "large body" production in Azotobacter. Electron micrographs, x 5,000.

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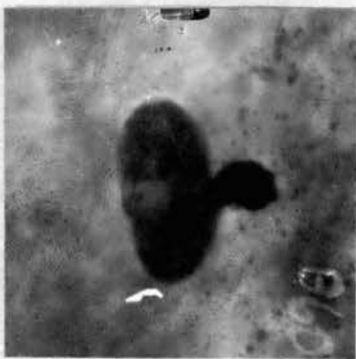

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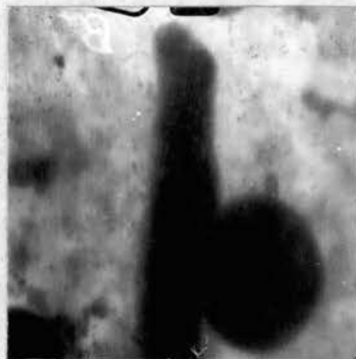

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Plate 8

Azotobacter agile, M.B. 4.4

Figure 33. Twenty-four-hour-old cells grown on nutrient agar, and fat stained by Burdon's method. Light micrograph, x 1,250.

Figure 34. Twenty-four-hour-old cells grown on Ashby's agar plus bacitracin. Note elongated cells with swollen ends. Phase micrograph, x 1,250.

Figure 35. Forty-eight-hour-old L forms grown on nutrient agar. These cells, except for size, are similar in morphology to the normal *Azotobacter agile*. Light micrograph, x 1,000.

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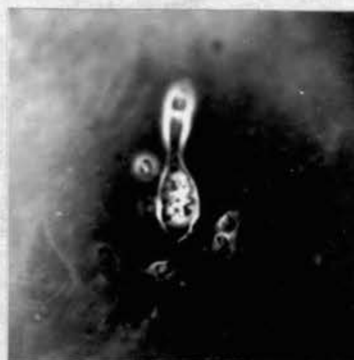

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IV. DISCUSSION

The findings in this thesis confirm the work of Bisset (1948c) that the nucleus of *Azotobacter* is of vesicular form, surrounded by chromatinic granules, and resembling the nuclear vacuole of certain yeasts. Bisset states that the nucleus of *Azotobacter* resists the acid-Giemsa technique of nuclear staining, but can be readily stained by the methylene-blue-eosin technique of Badian (1933), in which the cells are first stained with methylene blue and then differentiated with eosin. It was found in the present study, however, that *Azotobacter agile*, strain M.B. 4.4, does not resist the acid-Giemsa technique, especially when the cells are mordanted in 1 per cent formaldehyde according to the technique of Smith (1950).

Dienes (1946), who has done most of his work with pathogenic bacteria, has found that as a rule the large bodies are produced naturally only for a short period of time after original isolation of a strain. The strain of *Azotobacter* described in this thesis produced large bodies or very abnormal forms every time it was grown on nutrient agar, during the past eleven months. The formation of large bodies under the usual conditions of cultivation is probably a property of the strain and is not necessarily caused by peculiarities of the medium, according to Dienes (1946). However, it was found in this study that the beef extract in plain nutrient agar, which is not ordinarily considered a toxic substance for bacteria, is stimulatory to the production of large bodies in *Azotobacter agile*, M.B. 4.4.

Although Dienes (1939b), working with colon bacilli, observed the transformation of large bodies to normally-shaped bacteria, the method

of the transformation was not that observed by this investigator. Dienes found that large bodies and fusiform bodies were completely filled with small bacteria. During the course of the author's experiments, large, balloon-shaped bodies were observed to reproduce normally-shaped bacteria by a pinching off of the ends of the large cell.

Since the writing of section "F", under "Experimental", it has been found that the L forms of Azotobacter agile, M.B. 4.4, are filtrable. These L forms, it will be remembered, were the tiny organisms isolated from a culture of normally-shaped Azotobacter agile, and which reverted to the normally-shaped form upon growth in broth. The fact that the L forms are filtrable is in agreement with the observations of other workers (Dienes, 1946) upon L forms of other bacteria. The filtrability of some phase in the life cycle of Azotobacter has been claimed by Lohnis and Smith (1923).

The differences in the individual strains of Azotobacter agile is probably very great as verified by the fact that this investigator was able to confirm the observations of Eisenstark, McMahon and Eisenstark (1950) that Azotobacter agile (Illinois strain ZN 350) exhibits four distinct morphological types of growth upon aging, whereas he was unable to find any variation in morphology upon aging Azotobacter agile, M.B. 4.4 for two months.

The study of "large bodies" and L forms is in an early stage, and according to Dienes (1946), "It appears of great importance to extend this investigation to as many other species as possible. Advance in this study depends in a large measure on finding strains appropriate for study". Different species, and different strains of the same species

are proving more valuable for the study of certain aspects of the process. Also, with increasing attention being focused on genetics of microorganisms, interest has been stimulated in the problem of whether or not bacteria possess reproductive processes other than binary fission. The need for more research in this field is definitely indicated.

V. SUMMARY

Using the procedures of (1) nuclear staining, (2) negative staining and subsequent observation under the phase microscope, (3) electron microscopy, and (4) fat staining, the cytology of Azotobacter agile, M.B. 4.4, was studied before and after growing the cells on various nutritional and toxic media.

The normal morphology is described. Within the cells are clearly-visible bodies; these bodies consist of fat, and of chromatinic material. The arrangement of the chromatinic material varies from cell to cell; however, most of the cells seem to contain a ring of 3 to 5 chromatinic granules.

When Azotobacter agile is grown on ordinary nutrient agar, the cells develop into long filaments which may swell at one or more points into "balloon-shaped" forms, spheres, or they may produce a bud-like swelling from the side of the rod. These abnormally-shaped cells apparently may either degenerate or may reproduce normally-shaped cells by a pinching off of the ends. The beef extract component of nutrient agar seems to be the factor which stimulates the formation of large bodies, although beef extract alone in the agar does not cause the formation of large bodies. The chromatinic material in the large cells, unlike that of the normally-shaped cells, appears to be very finely divided and is scattered throughout the cell.

During the course of this work, the organism was grown in media containing various carbon compounds, amino acids, accessory growth substances, etc. No production of large forms was observed with the use of any of these substances.

Numerous toxic agents were added to the medium, but only five produced any change in cell morphology. Large, abnormal forms were produced with the use of bacitracin, gentian violet, tyrothrycin, ethyl alcohol, or sulfathiazole.

Irradiation of the cells of Azotobacter agile with ultraviolet rays of about 2537A produced no morphological change.

"L forms", the small organisms found in the tiny L colonies which have been observed to develop from normal cultures of many bacteria, were isolated from an irradiated strain of Azotobacter agile. Upon transfer of the L forms to broth, cells of normal Azotobacter agile morphology were produced. These L forms were also found to pass the Seitz bacterial filter.

The effect of age on the morphology of Azotobacter agile was also observed, the morphology remaining extremely constant throughout a 60-day period. Old cells reveal no distinct chromatinic bodies.

V. BIBLIOGRAPHY

Almon, L. 1933 Concerning the reproduction of bacteroids. Z. Bakt., II Abt., 87, 289-296.

Badian, J. 1933 Eine cytologische untersuchung über das chromatin und den entwicklungszyklus der bakterien. Arch. Mikrobiol., 4, 409-418.

Beijerinck, M. W. 1901 Über oligonitrophile mikroben. Centr. Bakt., II Abt., 7, 561-582.

Belozerskii, A. N. 1944 On the nucleus of bacteria. Microbiology, 13, 23-31.

Belozerskii, A. N. 1945 The chemical nature of volutin. Microbiology (U.S.S.R.), 14, 29-34.

Bisset, K. A. 1948a The cytology of smooth and rough variation in bacteria. J. Gen. Microbiol., 2, 83-88.

Bisset, K. A. 1948b Nuclear reorganization in non-sporing bacteria. J. Hyg., Camb., 46, 173-175.

Bisset, K. A. 1948c Observations upon the bacterial nucleus. J. Hyg., Camb., 46, 264-266.

Bisset, K. A. 1949 Observations upon the cytology of Corynebacteria and Mycobacteria. J. Gen. Microbiol., Lond., 3, 93-96.

Boivin, A., and Vendrely, R. 1947 Sur le role possible des deux acides nucleiques dans la cellule vivante. Experientia, 3, 32-34.

Bonazzi, A. 1915 Cytological studies of Azotobacter chroococcum. J. Agr. Res., 4, 225-239.

Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1948 Bergey's manual of determinative bacteriology. 6th ed. Williams and Wilkins Co., Baltimore.

Brown, T. McP., and Nunemaker, T. C. 1942 Rat bite fever. Johns Hopk. Hosp. Bull., 70, 201-327.

Burdon, K. L. 1946 Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bact., 52, 665-678.

Cassel, W. A. 1950 The use of perchloric acid in bacterial cytology. J. Bact., 59, 185-187.

Commoner, B. 1949 On the interpretation of the absorption of ultraviolet light by cellular nucleic acids. Science, 110, 31-40.

Dawson, M. H., and Hobby, G. L. 1939 Pleuropneumonia-like organisms as a variant phase of Streptobacillus moniliformis. Third Intern. Congr. Microbiol., Abstr. Communications, pp. 21-22.

De Lamater, E. D. 1948 Basic fuchsin as a nuclear stain for fungi. Mycologia, 40, 423-429.

De Lamater, E. D. 1949 Nuclear cytology of fungi. Tr. N. York Acad. Sci., 11, 162-164.

De Lamater, E. D., and Ulrich, D. 1948 Basic fuchsin as a nuclear stain. Stain Techn., 23, 161-176.

Delaporte, B. 1939 Recherches cytologiques sur les bacteries et les cyanophycees. Rev. Gen. Botan., 51, 615-643, 689-708, 748-768.

Delaporte, B. 1940 Recherches cytologiques sur les bacteries et les cyanophycees. Rev. Gen. Botan., 52, 40-48, 75-96, 112-160.

Dienes, L. 1939a "L" organisms of Klieneberger and Streptobacillus moniliformis. J. Infect. Dis., 65, 24-42.

Dienes, L. 1939b A peculiar reproductive process in colon bacillus colonies. Proc. Soc. Exp. Biol. Med., 42, 773-778.

Dienes, L. 1941 The significance of the large bodies and the development of L type of colonies in bacterial cultures. J. Bact., 44, 37-73.

Dienes, L. 1943 Reproduction of bacteria from the large bodies of Streptobacillus moniliformis. Proc. Soc. Exp. Biol. Med., 53, 84-86.

Dienes, L. 1944 L type of growth in cultures of a hemolytic parainfluenza bacillus. Proc. Soc. Exp. Biol. Med., 55, 142-144.

Dienes, L. 1946 Complex reproductive processes in bacteria. Cold Spr. Harb. Symp. Quant. Biol., XI, 51-59.

Dienes, L. 1947a Isolation of Pleuropneumonia-like organisms from pathological specimens with the aid of penicillin. Proc. Soc. Exp. Biol. Med., 64, 165-166.

Dienes, L. 1947b Isolation of Pleuropneumonia-like organisms from H. influenzae with the aid of penicillin. Proc. Soc. Exp. Biol. Med., 64, 166-168.

Dienes, L. 1947c The morphology of the L_1 of Klieneberger and its relationship to Streptobacillus moniliformis. J. Bact., 54, 231-237.

Dienes, L. 1947d Further observations on the reproduction of bacilli from large bodies in Proteus cultures. Proc. Soc. Exp. Biol. Med., 66, 97-98.

Dienes, L. 1948a The influence of penicillin on the growth of Proteus cultures. Proc. Meetings Soc. Amer. Bact., 1, 3-4.

Dienes, L. 1948b The isolation of L-type cultures from Bacteroides with the aid of penicillin and their reversion into the usual bacilli. J. Bact., 56, 445-456.

Dienes, L. 1948c Isolation of L-type colonies from typhoid bacilli with the aid of penicillin. Proc. Soc. Exp. Biol. Med., 68, 589-590.

Dienes, L. 1949a The development of Proteus cultures in the presence of penicillin. J. Bact., 57, 529-546

Dienes, L. 1949b Isolation of a L-type culture from a gram-positive spore-bearing bacillus. Proc. Soc. Exp. Biol. Med., 71, 30-33.

Dienes, L. 1949c Cultivation of L-type colonies from typhoid bacilli following exposure to immune serum and complement. Abstracts of Papers, Soc. Am. Bact., 49th General Meeting, p. 28.

Dienes, L., and Smith, W. E. 1944 The significance of pleomorphism in Bacteroides strains. J. Bact., 48, 125-153.

Dubos, R. J. 1945 The bacterial cell. Harvard Univ. Press, Cambridge.

Duggar, B. M. 1936 Biological effects of radiation. McGraw-Hill Book Co., Inc., vol. I and II, New York.

Duguid, J. P. 1948 The influence of cultural conditions on the morphology of Bacterium aerogenes with reference to nuclear bodies and capsule size. J. Path. and Bact., 60, 265-274.

Eisenstark, A., and Clark, G. L. 1947 Electron micrographs of x-ray-treated Escherichia coli cells. Science, 105, 553-555.

Eisenstark, A., and McMahon, K. J. 1949 Some phase microscope observations of Azotobacter agile. Soil Sci., 68, 329-331.

Eisenstark, A., McMahon, K. J., and Eisenstark, R. 1950 A cytological study of a pleomorphic strain of Azotobacter with the electron and phase microscopes and the Robinow nuclear-staining technique. J. Bact., 59, 75-81.

Erickson, R. O., Sax, K. B., and Ogur, M. 1949 Perchloric acid in the cytochemistry of pentose nucleic acid. Science, 110, 472-473.

Fischer, H. 1906 Zweiter beitrag zur kenntnis der lebensbedingungen von stickstoff sammelnden bakterien. Centr. Bakt., II Abt., 15, 235-236.

Fisher, A. M. 1946 A study on the mechanism of action of penicillin as shown by its effect on bacterial morphology. J. Bact., 52, 539-554.

Gardner, A. D. 1940 Morphological effects of penicillin on bacteria. Nature, 146, 837-838.

Gates, F. L. 1933 The reaction of individual bacteria to irradiation with ultraviolet light. Science, 77, 350.

Giese, A. C. 1947 Radiations and cell divisions. Quart. Rev. Biol., 22, 253-282.

Gillespie, H. B., and Rettger, L. F. 1939 Bacterial variation: formation and fate of certain variant cells of Bacillus megatherium. J. Bact., 38, 41-61.

Grainger, Jr., T. J. 1947 The effects of x-rays on a strain of Eberthella typhosa. J. Bact., 53, 165-172.

Gray, C. H., Marton, L., and Tatiun, E. L. 1945 Observations of bulges on Acetobacter. J. Appl. Phys., 16, 265.

Guilford, R., and Orr, J. H. 1923 The influence of H ion concentration upon structure. I. H. influenzae. J. Bact., 8, 103-113.

Haas, F., Wyss, O., and Stone, W. S. 1948 The effect of irradiation on recombination in Escherichia coli. Proc. Nat. Acad. Sci., 34, 229-232.

Haberman, S., and Ellsworth, L. D. 1940 Lethal and dissociative effects of x-rays on bacteria. J. Bact., 40, 483-503.

Hadley, P. 1937 Further advances in the study of microbic dissociation. J. Infect. Dis., 60, 129-192.

Hadley, P., Delves, E., and Klimak, J. 1931 The filtrable forms of bacteria: I. A filtrable stage in the life history of the shiga dysentery bacillus. J. Inf. Dis., 48, 1-159.

Heilman, F. R. 1941 A study of Asterococcus muris (Streptobacillus moniliformis). J. Inf. Dis., 69, 32-44.

Henriksen, S. D. 1948 Pleomorphism in Hemophilus hemolyticus caused by V-factor deficiency. Acta Path. Microbiol., Scand., 25, 431-438.

Henry, H., and Stacey, M. 1943 Histochemistry of the Gram-staining reaction for microorganisms. Nature, 151, 671.

Henry, H., and Stacey, M. 1945 Nature of the Gram-positive complex in microorganisms. Nature, 156, 720-721.

Hinshelwood, C. N. 1946 The chemical kinetics of the bacterial cell. The Clarendon Press, Oxford.

Hollaender, A. 1946 Effects of ultraviolet irradiation. Ann. Rev. Physiol., 8, 1-16.

Hutchinson, W. G., and Stempen, H. 1949 The origin and development of large bodies in Proteus. Abstracts of Papers, Soc. Am. Bact., 49th General Meeting, p. 27.

Jillson, O. F., and Nickerson, W. J. 1948 Mutual antagonism between pathogenic fungi. Inhibition of dimorphism in Candida albicans. Mycologica, 40, 369-385.

Johnson, F. H., and Gray, D. H. 1949 Nuclei and large bodies of luminous bacteria in relation to salt concentration, osmotic pressure, temperature, and urethane. J. Bact., 58, 675-688.

Jones, D. H. 1913 A morphological and cultural study of some Azotobacter. Centr. Bakt., II Abt., 38, 14-24.

Jones, D. H. 1920 Further studies on the life cycle of Azotobacter. J. Bact., 5, 325-342.

Jordan, E. C., and Burrows, W. 1945 Textbook of bacteriology, 14th ed., W. B. Saunders Co., Philadelphia.

Kaufmann, B. P., McDonald, M., and Gay, H. 1948 Enzymatic degradation of ribonucleoproteins of chromosomes, nucleoli and cytoplasm. Nature, Lond., 162, 814-815.

Kelner, A. 1949 Photoreactivation of ultraviolet-irradiated Escherichia coli with special reference to the dose reduction principle and to ultraviolet-induced mutation. J. Bact., 58, 511-522.

Klieneberger, E. 1930 Bakterienpleomorphismus und Bakterienentwicklungsgänge. Weicherdt's Ergebnisse der Hygiene, etc. 11, 499-555.

Klieneberger, E. 1935 Natural occurrence of Pleuropneumonia-like organisms in apparent symbiosis with Streptobacillus and other bacteria. J. Path. Bact., 40, 93-105.

Klieneberger-Nobel, E. 1945 Changes in the nuclear structure of bacteria particularly during spore formation. J. Hyg., Camb., 44, 99-108.

Klieneberger-Nobel, E. 1947 Morphological appearances of various stages in B. proteus and coli. J. Hyg., Camb., 45, 410.

Knaysi, G. 1938 Cytology of bacteria. Bot. Rev., 4, 83-112.

Knaysi, G. 1942 The demonstration of a nucleus in the cell of a Staphylococcus. J. Bact., 43, 365-386.

Knaysi, G. 1944 Elements of bacterial cytology. Comstock Publ. Co., Inc., Cornell Univ., Ithaca.

Knaysi, G. 1946 Further observations on the nuclear material of the bacterial cell. J. Bact., 51, 177-180.

Knaysi, G. 1948 The endospore of bacteria. Bact. Rev., 12, 19-77.

Knaysi, G., and Baker, R. F. 1947 Demonstration, with the electron microscope, of a nucleus in Bacillus mycoides grown in a nitrogen-free medium. J. Bact., 53, 539-553.

Knaysi, G., and Mudd, S. 1943 The internal structure of certain bacteria as revealed by the electron microscope -- a contribution to the study of the bacterial nucleus. J. Bact., 45, 349-359.

Knight, B. C. J. G. 1946 Growth factors and growth inhibitors for micro-organisms. Proc. Nutrition Soc., Camb., 4, 116-128.

Kritschewsky, I. L., and Ponomarewa, I. W. 1934 On the pleomorphism of bacteria. I. On the pleomorphism of B. paratyphi B. J. Bact., 28, 111-126.

Kyle, T. S. 1950 A study of the variation within the species Azotobacter agilis, M. B., 4.4. M. S. Thesis, Oklahoma A. and M. College.

Lea, D. E. 1947 Actions of radiations on living cells. The MacMillan Co., New York.

Lea, D., Haines, R. B., and Bretcher, E. 1941 The bactericidal action of x-rays, neutrons and radioactive radiations. J. Hyg., 41, 1-16.

Leonardi, G. 1949 Content and localization of nucleic acids in several bacterial species. Compt. Rend., 229, 393-395.

Lewis, I. M. 1937 Cell inclusions and the life cycle of Azotobacter. J. Bact., 34, 191-204.

Lewis, I. M. 1941 The cytology of bacteria. Bact. Rev., 5, 181-230.

Lindegren, C. C. 1935 Genetical studies of bacteria. I. The problem of the bacterial nucleus. Z. Bakt., II Abt., 92, 41-48.

Lindegren, C. C. 1936 Genetical studies of bacteria. III. Origin of G-type colonies by transgenation. Z. Bakt., II Abt., 93, 389-400.

Lohnis, F. 1921 Studies upon the life cycles of the bacteria. Part I. Review of the literature 1838-1918. Mem. Nat. Acad. Sci., 16, 2d mem., 1-335.

Lohnis, F., and Smith, N. R. 1916 Life cycles of the bacteria. J. Agr. Res., VI, 675-729.

Lohnis, F., and Smith, N. R. 1923 Studies upon the life cycles of the bacteria. Part II. Life history of Azotobacter. J. Agr. Res., 23, 401-432.

Luyet, B. J. 1932 The effects of ultra-violet, X-, and cathode rays on the spores of Mucoraceae. Radiology, 18, 1019-1022.

McMahon, K. J. 1949 Cytological studies of Azotobacter. M. S. Thesis, Oklahoma A. and M. College

Malmgren, B., and Heden, C. G. 1947 Studies of the nucleotide metabolism of bacteria. I. Ultraviolet microspectrography as an aid in the study of the nucleotide content of bacteria. Acta Path. Microbiol., Scand., 24, 417-436.

Marshak, A. 1948 Evidence for a nuclear precursor of ribo- and desoxyribonucleic acid. J. Cell. and Comp. Phys., 32, 381-405.

Marton, L. 1941 The electron microscope. A new tool for bacteriological research. J. Bact., 41, 397-413.

Mencl, E. 1911 Die kernaquivalente and kerne bie Azotobacter chroococcum und seine sporenbildung. Arch. Protistenk., 22, 1-19.

Mulvania, M. 1915 Observation on Azotobacter. Science, 42, 463-465.

Murray, R. G. E., and Douglas, H. C. 1950 The reproductive mechanism of Rhodomicrobium vanniellii and the accompanying nuclear changes. J. Bact., 59, 157-167.

Nickerson, W. J. 1948 Enzymatic control of cell division in microorganisms. Nature, 1948, 162, 241-245.

Oerskov, J. 1942 Streptobacillus moniliformis and the morphology of its variants. Acta Path. Microbiol., 10, 575-585.

Oster, R. H. 1934 Results of irradiating Saccharomyces with monochromatic ultraviolet light. J. Gen. Physiol., 18, 251-254.

Overend, W. G., and Stacey, M. 1949 Mechanism of the Feulgen reaction. Nature, 163, 538-540.

Palmer, J. L. 1946 Nuclear staining of Escherichia coli. J. Bact., 51, 586.

Peterson, W. H., and Peterson, M. S. 1945 Relation of bacteria to vitamins and other growth factors. Bact. Rev., 9, 49-109.

Pierce, C. H. 1942 Streptobacillus moniliformis, its associated L_1 form, and other Pleuropneumonia-like organisms. J. Bact., 43, 780.

Pollister, A. W., and Leuchtenberger, C. 1949 The nucleoprotein content of whole nuclei. Proc. Nat. Acad. Sci., U.S., 35, 66-71.

Pratt, R., and Dufrenoy, J. 1948 Cytochemical interpretation of the mechanism of penicillin action. Bact. Rev., 12, 79-103.

Pratt, R., and Dufrenoy, J. 1949a Cytochemical mechanisms of penicillin action. VIII. Involvement of ribonucleic acid derivatives. J. Bact., 57, 9-13.

Pratt, R., and Dufrenoy, J. 1949b Antibiotics. J. B. Lippincott Co., Philadelphia.

Rahn, O. 1945 Physical methods of sterilization of microorganisms. Bact. Rev., 9, 1-47.

Rice, C. E., and Guilford, R. B. 1931 Studies on variability of tubercle bacilli: influence of x-rays on dissociation. Can. J. Res., 5, 122-131.

Roberts, R., and Aldous, E. 1949 Recovery from ultraviolet irradiation in E. coli. J. Bact., 57, 363-375.

Robinow, C. F. 1942 A study of the nuclear apparatus of bacteria. Proc. Roy. Soc. B, 130, 299-324.

Robinow, C. F. 1944 Cytological observations of B. coli, Proteus vulgaris and various aerobic spore-forming bacteria with special reference to the nuclear structures. J. Hyg., Camb., 43, 413-423.

Robinow, C. F. 1945 Nuclear apparatus and cell structure of rod-shaped bacteria. Addendum to Dubos, R. J., The bacterial cell, Harvard Univ. Press, Cambridge.

Robinow, C. F., and Cosslett, V. E. 1948 Nuclei and other structures of bacteria. J. Applied Physics, 19, 124.

Schulze, J. 1909 Über die einwirkung der lichtstrahlen von 280 mu mu wellenlänge auf pflanzenzellen. Beih. Bot. Centralbl., 25, 30-80.

Shanahan, A. J., Eisenstark, A., and Tanner, R. W. 1947 Morphology of Escherichia coli exposed to penicillin as observed with the electron microscope. J. Bact., 54, 183-189.

Shanahan, A. J., and Tanner, F. W. 1948 Further studies on the morphology of Escherichia coli exposed to penicillin. J. Bact., 55, 537-544.

Smith, A. G. 1950 Electron and light microscope studies of bacterial nuclei. II. An improved staining technique for the nuclear chromatin of bacterial cells. J. Bact., 59, 575-586.

Smith, Wilson 1941 Cervical abscesses of guinea pigs. J. Path. Bact., 53, 29-37.

Sonneborn, T. M. 1946 A system of separable genetic determiners in the cytoplasm of Paramecium aurelia, variety 4. Anat. Rec., 94, 346-347.

Spencer, R. R., and Melroy, M. B. 1943 Principles of species adjustment. I. Continuous exposure. J. Nat. Cancer Inst., 4, 249-256.

Spencer, R. R., Melroy, M. B., and Calnan, D. 1943 Principles of species adjustment. II. Discontinuous exposure. J. Nat. Cancer Inst., 4 257-263.

Sterzl, J. 1949 Morphological variability of the nuclear substance and genetic changes induced by colchicine in Esch. coli. Nature, 163, 28.

Tatum, E. L., and Lederberg, J. 1947 Gene recombination in the bacterium Escherichia coli. J. Bact., 53, 673-684.

Thomas, Jr., A. R., and Levine, M. 1945 Some effects of penicillin on intestinal bacteria. J. Bact., 49, 623-627.

Tulasne, R. 1948 Cytologie des Proteus et ses modifications sous l'effet de la penicilline. Compt. Rend. Soc. Biol., 143, 286-288.

Tulasne, R. 1949a Sur une evolution possible des Proteus vers des micro-colonies et des formes inframicroscopiques sous l'effet de la penicilline. Compt. Rend. Soc. Biol., 143, 289-291.

Tulasne, R. 1949b Donnees nauvelles sur la cytologie des bacteries apportees par la microscopie de contraste. Compt. Rend. Soc. Biol., 143, 1392-1394.

Tulasne, R. 1949c Existence of L-forms in common bacteria and their possible importance. Nature, 164, 876-877.

Tulasne, R. 1949d Sur la cytologie des bacteries vivantes etudiees grace au microscope a contraste de phase. Compt. Rend. Soc. Biol., 143, 1390-1392.

Tulasne, R., and Vendrely, R. 1947 Use of ribonuclease in demonstrating the nuclei of bacteria. Compt. Rend. Soc. Biol., 141, 674-676.

Tulasne, R., and Vendrely, R. 1948 Cytology of staphylococci before and after treatment with penicillin. Nature, 161, 316-317.

Tulasne, R., Vendrely, R., and Minck, R. 1948 Bacterial nuclei and streptomycin. Comp. Rend. Soc. Biol., 142, 1012-1014.

T'ung, T., and Hsu, Chien-Liang 1940 Dissociation of pneumococcus by radon irradiation. Proc. Soc. Exp. Biol. Med., 43, 744-746.

van Niel, C. B. 1931 On the morphology and physiology of the purple and green sulphur bacteria. Verlag von Julius Springer, Berlin.

v. Euler, H., Ahlstrom, L., and Hogberg, B. Z. 1942 Veranderungen der hefezellen durch röntgenstrahlen und durch chemische substanzen. Physiol. Chem., 277, 1-17.

Wahlin, J. G., and Almaden, P. J. 1939 The megalomorphic phase of bacteria. J. Infect. Dis., 65, 147-155.

Wels, P. 1939 Observations on the irradiated cell nucleus. Strahlentherapie, 66, 672-676.

Williams, C., Sandholzer, L. A., and van Voorhis, S. N. 1940 The incorporation of radioactive phosphorus in the nucleoprotein and phospholipid of Escherichia coli. J. Bact., 39, 19-20.

Witkin, E. M. 1946 Inherited differences in sensitivity to radiation in Escherichia coli. Proc. Nat. Acad. Sci., Wash., 32, 59-68.

Wyss, O., Clark, J. B., Haas, F., and Stone, W. S. 1948 The role of peroxide in the biological effects of irradiated broth. J. Bact., 56, 51-57.

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ADDENDUM

The following references were discovered after the bibliography was prepared. Since they are pertinent, they are included here.

Gordon, J., and Gordon, M. 1943 Involution forms of the genus Vibrio produced by glycine. J. Path. Bact., 55, 63-68.

Topley, W. W. C., and Wilson, G. S. 1929 Principles of Bacteriology and Immunity. William and Wood Co., New York.

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