NITROGEN FIXATION AND ENCYSTMENT CREATED BACTERIAL CHARACTERISTICS

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Life in most ecosystems depends upon continual circulation of nutrient chemical from the earth to the biosphere, termed the "biogeochemical cycles." One of these cyclic element processes, the nitrogen cycle, is examined in relation to one bacterium, Azotobacter, which is capable of initiating the primary reactions of this cycle, namely the conversion of atmospheric nitrogen to ammonia. These bacteria, which are also capable of forming protective structures, termed cysts, are discussed in relation to their part of the perfect plan of God as revealed in life. The author also refers to the even more resistant forms produced by other independent, or non-symbiotic nitrogen fixers, of the genus Clostridium, and concludes that the resting cells which these bacteria have are a created advantage which is necessary for the perpetuation of the nitrogen cycle and life which is a result of it, and of the other cyclic processes.

Introduction

Azotobacter (meaning nitrogen rod) is the name assigned to several species of bacteria which possess the capacity to fix nitrogen, without necessary association with another organism; this is termed "non-symbiotic nitrogen fixation." These organisms were not of the early bacteria to be isolated since they are not responsible for disease production in any animal or plant, as far as is known. In fact, attempts to group members of this genus taxonomically by serological typing have met with only limited success, because of the feebleness of the organism in producing antibody in an experimental animal; they are poor antigens.[1] These organisms are members of a relatively small group of bacteria which are directly beneficial to man, especially in agriculture, and to the entire biosphere as well.

Elemental Circulation

On inspection of an ecosystem one finds that there is a continual flow of energy in the system and that the very existence of the individual members of the system depend on one another. To the observer, at least, it appears as if each contributor were following a small part of a larger, intricate plan for the continuance of life.

In a system in which plants and animals reside there is a continual flow of nutrient chemicals from the earth to the plants. These nutrients are then returned to the soil directly or indirectly after being digested and excreted by other organisms, or being assimilated into life and then returned to the earth upon death and decay of the organism. However, it is apparent a continual flow of these essential chemicals exists and that they are constantly being recycled.

The term biogeochemical cycle has been used to describe this circulation of chemical nutrients through biological organisms and the surface of the earth. A large number of the cycles have been studied, with the carbon and water cycles being best understood at present, and perhaps the nitrogen cycle being the most complex of these circulatory processes. Nitrogen, like carbon, phosphorus, and numerous other minerals, is necessary for the continuance of life, and is contained most importantly in amino acids, prophyrins, nucleic acid bases, and some vitamins.

Although a plentiful gaseous element, N_2 cannot be directly assimilated by the vast majority of living things. For nitrogen to be bio-utilized it must be transformed into an assimilable form. The process is termed nitrogen fixation, and is accomplished by some fungi, numerous algae, and to some degree by a number of bacteria.

The conversion of N_2 into fixed nitrogen, which appears initially to be ammonia (NH_3), is carried out with a relatively high rate of efficiency in two groups of bacteria: the symbiotic and non-symbiotic nitrogen fixers.

The **symbiotic** nitrogen fixing organisms which reside in root nodules of leguminous plants are members of the genus *Rhizobium*. This unique relationship, in which neither the plant nor the bacteria is capable of fixing nitrogen independently, illustrates cooperation found in nature and the delicate balance by which life functions. There is also a marked specificity between species of plant in the legume family and the strain of *Rhizobium* which resides sybiotically in the root nodules.

The second group of bacteria which are capable of nitrogen fixation is the **non-symbiotic,** or independent nitrogen fixers. Two bacterial genera, the anaerobic spore-forming members of the genus *Clostridium,* and the aerobic cyst-forming members of the genus *Azotobacter* already mentioned are well known examples of this type of nitrogen fixation.

The process of converting elemental N_2 into NH_3 is a difficult industrial task. The Haber process, developed during World War I, carries out this conversion as follows:

$$N_2 + 3H_2 \xrightarrow[200\ \text{Atm}]{500^\circ\text{C}} 2NH_3$$

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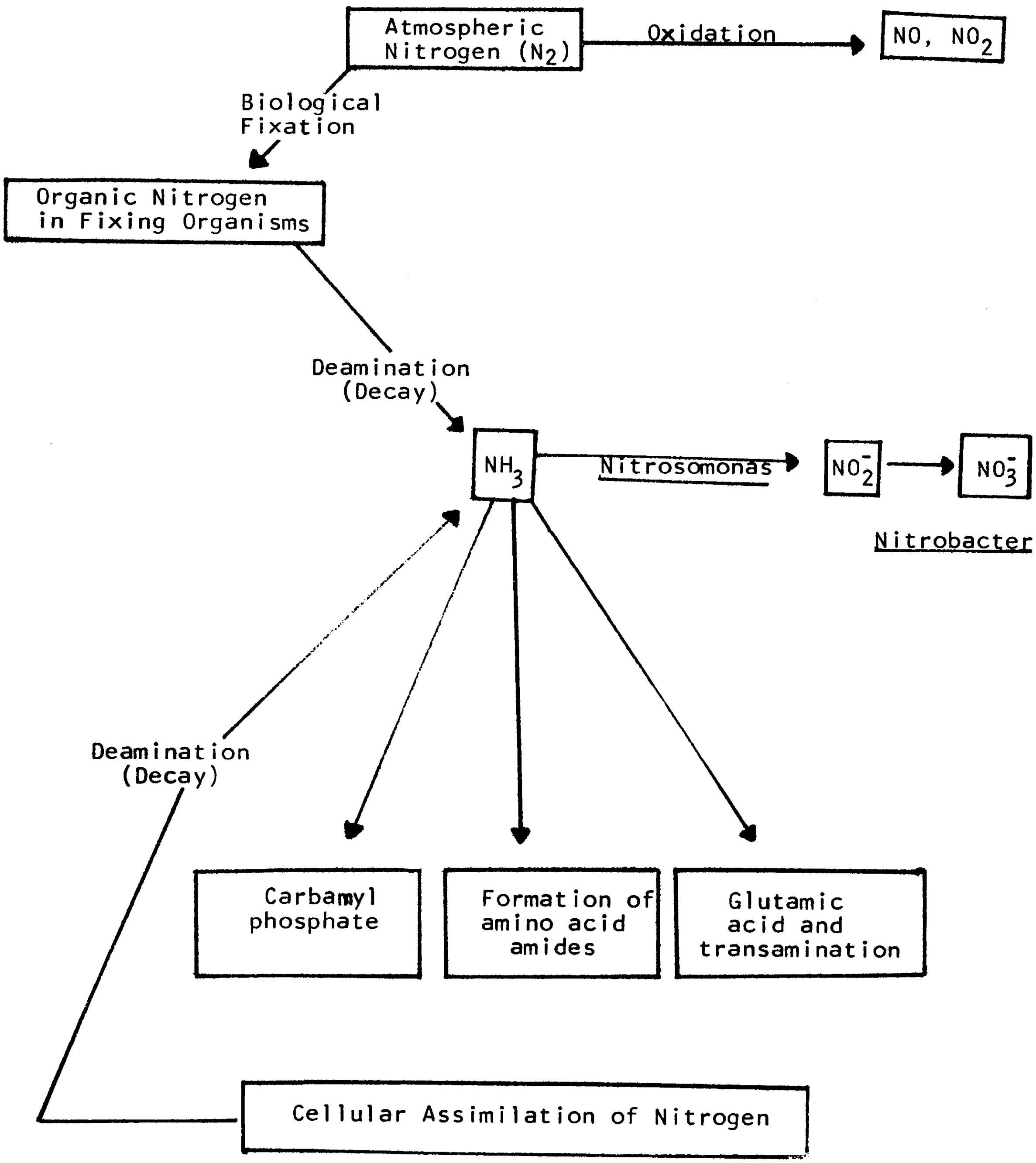

Figure 1. The nitrogen cycle.

Very high pressure and temperature are required to effect the transformation that single-celled organisms, which lack cellular refinement and definition found in eucaryotic cells, carry out independently at isothermal temperature.

It has been estimated[2] that some 225KCal are required to break the $N \equiv N$ bond, approximately three times the energy required to break the C - C bond. This process illustrates the wondrous adaptability of life and accounts for the respiratory rate of vegetative *Azotobacter* cells which is the highest of any living organism![3]

The enzymatic processes which result in the reduction of N_2 to NH_3 are still not understood. However, it appears that the enzyme nitrogenase, an enzyme requiring a metallic cofactor of molybdenum (Mo) or vanadium (V) for activity, is essential for the process to proceed. It also now appears that the N_2 to NH_3 conversion is progressively carried out enzymatically as illustrated below:[4]

$$\underset{\text{Enzyme}}{N \equiv N} \xrightarrow[2e]{} \underset{\text{Enzyme}}{NH = NH} \xrightarrow[2e]{} \underset{\text{Enzyme}}{H_2N - NH_2} \longrightarrow 2NH_3 + \text{Enzyme}$$

The energy for this reduction is, of course, supplied by adenosine triphosphate; however, the source of reducing hydrogens is not clear in *Azotobacter* nitrogen fixation. It appears that ferredoxin is involved in this reduction. Ammonia in the cell is transformed into organic nitrogen by conversion of α-ketoglutaric to glutamic acid. Subsequent transaminations among other amino acids occur and they are then incorporated into protein.

Excess NH_3 appears to be liberated extracellularly, with some liberation of peptides occurring, which serves to enrich the soil.[5] This nitrogen, in the form of liberated NH_3 or amino acids, is now available for another facet of nitrogen fixation, nitrification, or conversion to nitrate (NO_3). Nitrogen fixation in *Azotobacter* may be a relatively efficient process with a conversion of 0.1 moles of $N_2 \longrightarrow NH_3$ for each mole of O_2 consumed.[6] The cyclic relationships of nitrogen are illustrated in Figure 1.

Cyst Formation and Morphology

As if nitrogen fixation were not a sufficiently unique characteristic, members of the genus *Azotobacter* also possess the ability to form resting cells called cysts. Cysts differ from endospores, the most widely known biologically resistant structure, in that they are not as refractile to heat nor desiccation as the spore. However, cysts can withstand long periods (years) of desiccation without considerable loss of viability.

The *Azotobacter* cyst and the *Bacillus* endospore are contrasting structures, apart from their resistant properties, in that the cyst encompasses the entire cell and a portion of the capsule; while the endospore is formed from essential internal cellular components. The process whereby the vegetative cell is converted to a cyst is a phenomenon which has only recently been elucidated.

The morphology of these bacteria is interesting in that they are large, pleomorphic, and motile Gram negative rods. The size of the individual cell is large by bacterial standards, ranging from approximately 0.5 to 3.0 microns (μ)* in width by about 2 to 6μ in length.[7] When grown on media containing an organic nitrogen source the organism ceases to fix atmospheric nitrogen, although not all fixed-nitrogen forms are readily assimilable. Additionally, supplemental nitrogen also causes the morphology of the cell to be drastically affected, with the appearance of several types of bizarre forms in cultures.[8]

*A micron (μ) is 1×10^{-6} meters.

A large volume of research has appeared concerning this pleomorphism, probably more than for any other bacterial genus. At one time a complex life cycle including some thirteen different morphological forms had been proposed for *Azotobacter.* Many of these have been attributed to latent contaminants in the cultures of early workers; nevertheless the morphology of this genus remains complex.

When *Azotobacter* is grown in a closed system, a number of sequential morphogenetic stages are observed as the culture ages. In Figure 2 these forms are depicted. These include the vegetative cell, the precyst, and the cyst. *Azotobacter* is a chemoorganoautotroph, which requires only a fixed carbon source in addition to a balanced buffered salts solution for growth. The cellular form observed initially in this type of culture is the large coccobacillary vegetative cell.

In Figure 3, an electron micrograph of vegetative forms, this typically coccobacillary, or peanut-shaped morphology, is depicted. The electron dense areas represent polyphosphate (PP) inclusions, the cell wall-cell membrane complex (CW-CM) of the cells, is apparent, and a fibrous, continuous polysaccharide capsular areas (P) is observed to surround all of the cells.

The vegetative cell is motile by means of flagella located over the entire surface of the cell, termed peritrichous flagellation. When these same type of cells, vegetative forms, are observed by techniques of freeze-etching,[9] a highly ordered cell wall structure (S) is observed as shown in Figure 4. The surface of the vegetative cell appears composed of interwoven strands which form a mesh, or woven appearance, on the cell. Several flagella (F) are also depicted on the outer wrapping of the cell.

It is difficult to grasp the complexity of the earth, this solar system, or our galaxy, and the precise physical laws which keep each unit as a functioning part of the whole. On the other hand, one finds equal wonderment in the complexity of the minute, such as the order found in a microscopic bacterial wall, for the particles which give the wall the characteristic woven structure are only 6 to 8 nm* apart! However, in either direction, from the infinitesimal to the immense, it is apparent that an ordered, precisely functioning system exists.

The interesting process of encystment is initiated as nutrients are depleted in glucose-containing culture, or in cultures supplemented with

*A nanometer (nm) is 1×10^{-9} meters.

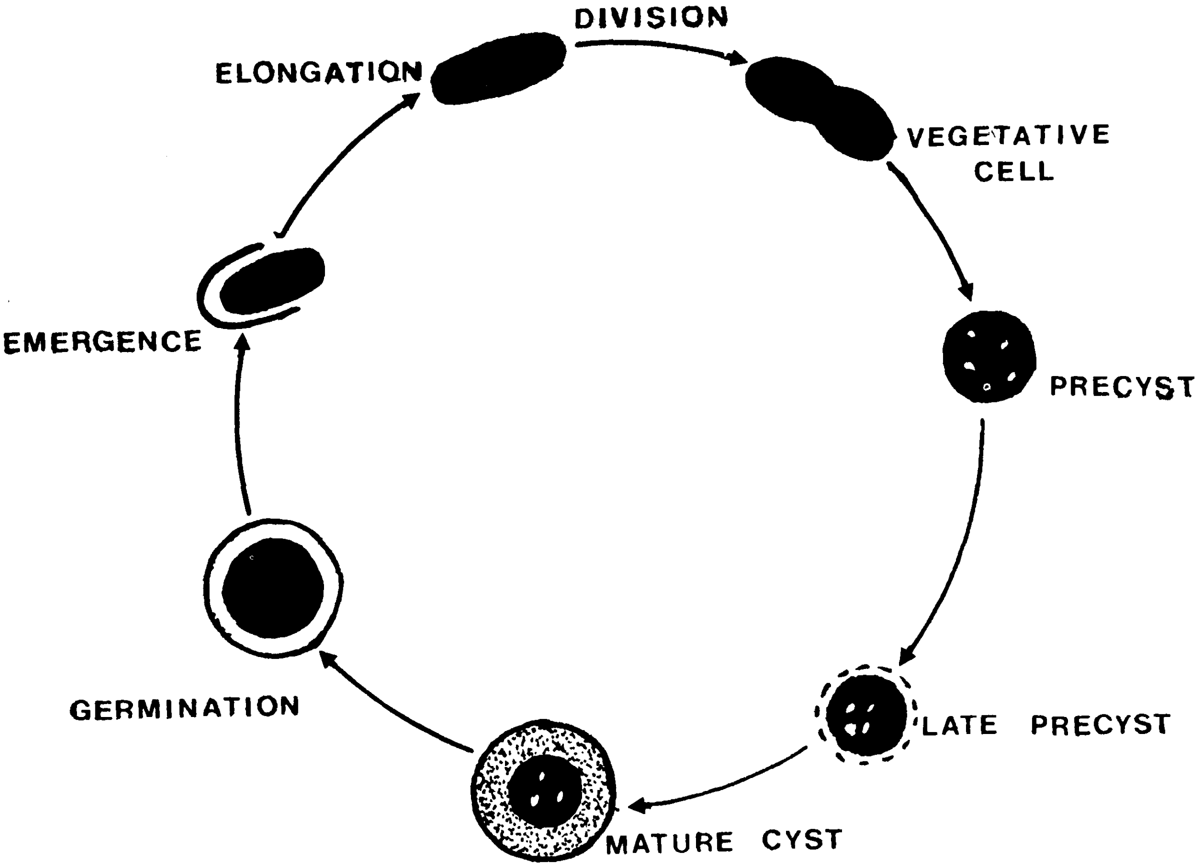

Figure 2. Schematic representation of the typical morphological forms encountered in *Azotobacter* culture. The vegetative cell, the precystic forms, and the mature cyst comprise the cellular forms through which the bacterium normally cycles. Arrows indicate the direction of normal progression of each form in nitrogen-free culture.

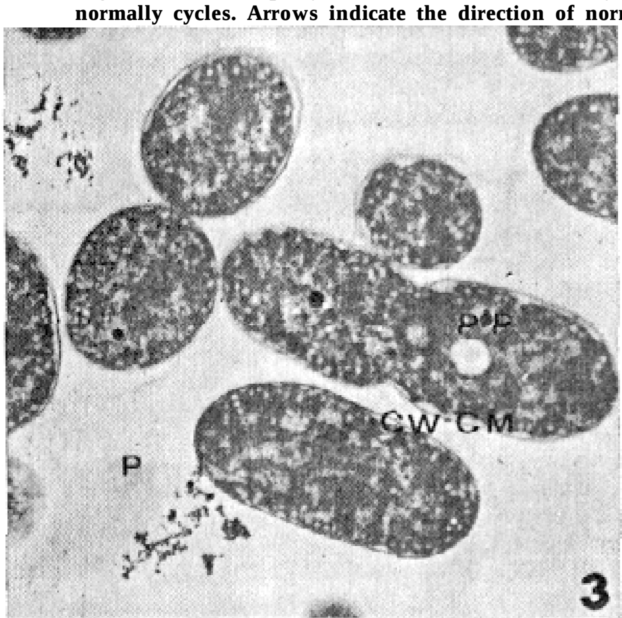

Figure 3. Electron micrograph of a thin section of vegetative cells of *Azotobacter.* A large amount of polysaccharide (P) surrounds the cells which contains polyphosphate (PP) inclusions. The cell wall-cell membrane (CW-CM) of each cell is also apparent. (x 17,000)

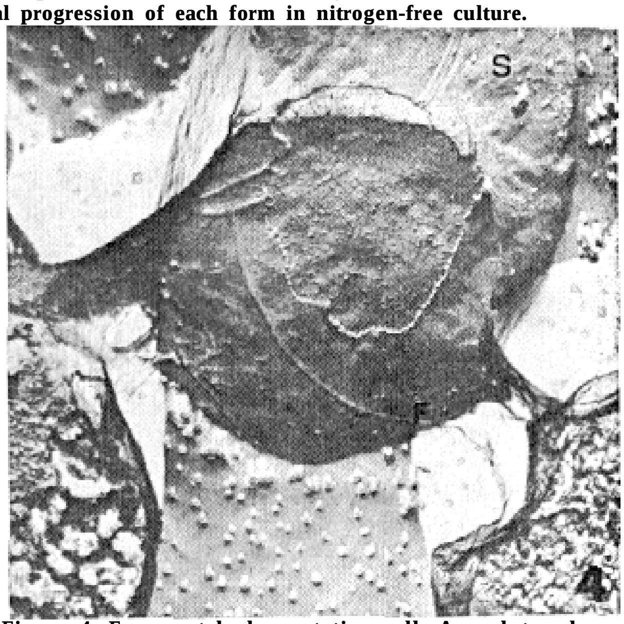

Figure 4. Frozen-etched vegetative cell. A sculptured (S) cell wall is apparent, beneath it the plasma membrane of the cell. Several flagella (F) are seen on the outer cell surface. (x 29,000)

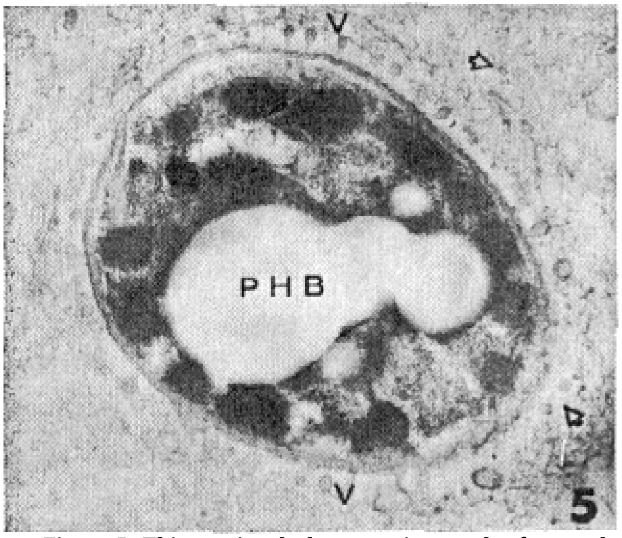

Figure 5. Thin sectional electron micrograph of an early precyst of *Azotobacter.* Numerous vesicles (V) and intracellular deposits of poly- b -hydroxybutyric acid (PHB) are observed. Extracellular polysaccharide (P) is also apparent. Some vesicles (arrows) are seen trapped in this extracellular polymer. (x 26,000)

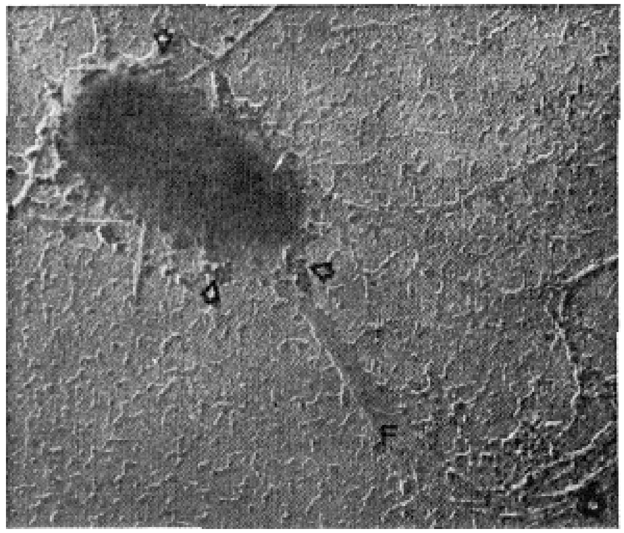

Figure 6. Electron micrograph of a shadow-cast preparation of encysting *Azotobacter* cells. The distribution of the vesicles (arrows) about the cell, the presence of flagella (F), and the rounding of the cell all indicate the occurrence of encystment. (x 11,500)

various short-chained organic compounds, as n-butanol, instead of glucose. The process of encystment[10] is marked by a change in morphology of the bacteria from bacillary to coccoid form (Figure 2). At the same time particles of membrane (arrows) are extruded from the shrinking cell as shown in Figure 5. The ejection of particles has been observed in a number of other Gram negative bacteria, each time in response to some unfavorable cultural condition.

The process of vesicle formation serves the cell at least two purposes: 1) to reduce cell volume, and 2) to provide membranous material for coat formation. The metabolism of the cell also joins in the integrated evolvement from cell to cyst. An endogenous metabolism source, poly- β -hydroxybutyric acid (PHB), is formed as the precyst stage occurs in *Azotobacter* cultures. In encysting cultures cells are observed to extrude large numbers of these vesicular elements which is shown in Figure 6. This shadow-cast electron micrograph depicts many of the vesicles produced during encystment, as well as the peritrichous flagellation of the cell.

The mature cyst is formed as these vesicles migrate away from the cell which extruded them. *Azotobacter* cells are surrounded by a large capsule of polysaccharide, and as the vesicles diffuse away from the precyst their progress is hindered by the fibrous polysaccharide (Figure 7). The shingling of particles continues until the vegetative cell, approximately 2μ x 6μ, is reduced to a coccoid cell about 2μ in diameter. The particles, which appear to be doughnut-shaped, then

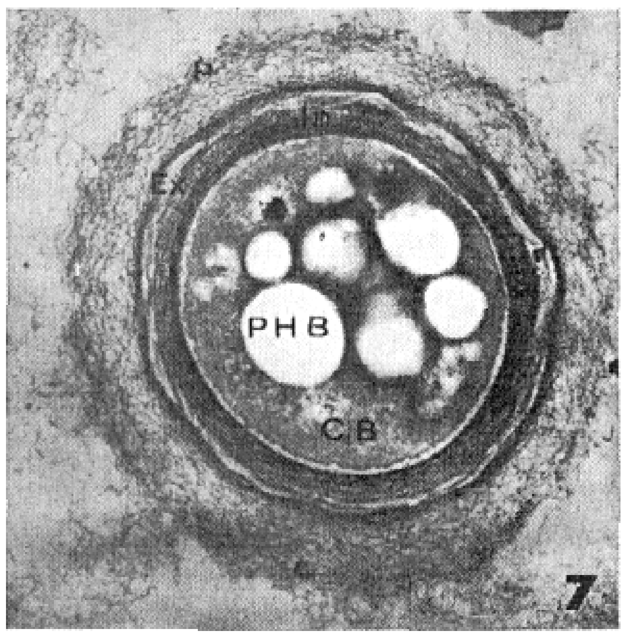

Figure 7. Electron micrograph of a thin section of a mature *Azotobacter* cyst depicting the general structure of the exine (Ex), with extracellular polysaccharide (P) extending beyond it; the intine (In); the central body (CB), with inclusions of poly- b -hydroxybutyric acid (PHB). (x 27,000)

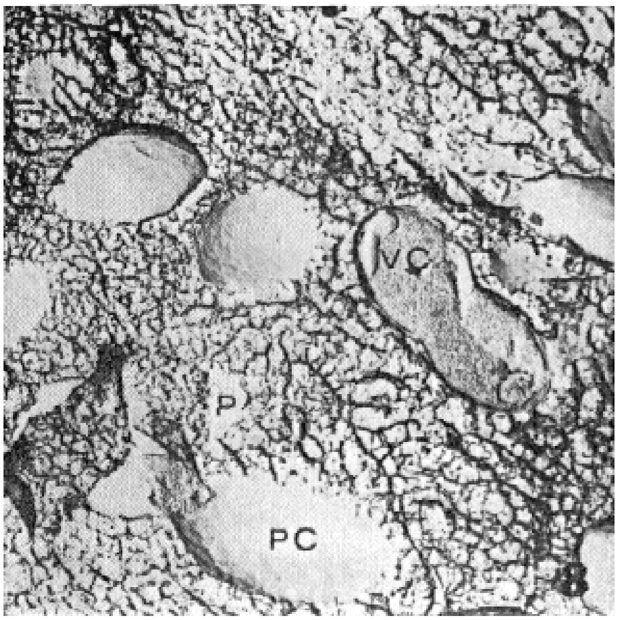

Figure 8. Electron micrograph of a frozen-etched preparation of vegetative cells (VC) and precysts (PC) embedded in a mass of polysaccharide (P). Note the slightly ordered surface structure of the precysts. (x 16,000)

open to form leaf-like units. From these units intact cyst coats appear to be formed, although the mechanism for opening of the vesicles is still unclear. Morphological features of the mature cyst (Figure 7) include: the cell or central body (CB), with large quantities of poly- β -hydroxybutyric acid (PHB); an area analgous to the capsule of the cell. termed the intine (In), an area which is amorphous and appears to buoy the central body and protect it; the cyst coat, or exine (Ex), the tough outer barrier to which the resistant properties of the cyst are attributed, and remnants of extraexinic polysaccharide (P).

Examination of the cell to cyst transition by freeze-etching reveals an equally precise process of morphogenesis.[11] The surface of the frozen-etched vegetative cell is smooth (Figure 4). As precysts are formed these cellular forms assume a surface with a slightly patterned surface which is almost geometric in nature (Figure 8). Each of the precysts illustrated have essentially the same surface appearance, although synchronous culture of these bacteria has never been achieved.

The mature cyst, as revealed by freeze-etching appears as a resistant biological structure (Figure 9). On first observation, the cyst seems more like a roughly hewn stone than a living, not inactive, metabolic organism. While cross sections of cysts (Figure 7) indicate only that the coat is multilayered, frozen-etched cells cleaved tangent to the exine indicate an architectural soundness of the structure. A ridged surface con-

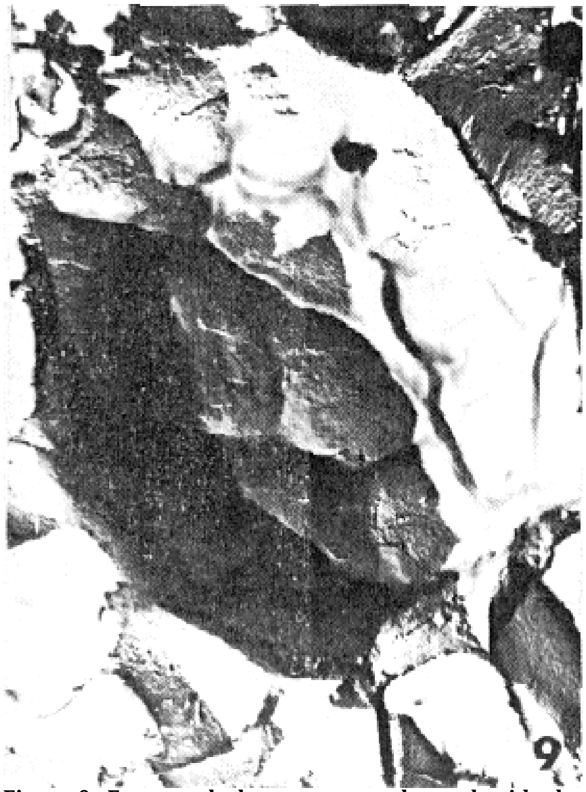

Figure 9. Frozen-etched mature cyst observed with the electron microscope. The cyst is composed of subunits which unite to form the intact coat. (x 32,500)

figuration, not unlike a geodesic dome, is observed. Each subunit of the exine is a hexagon and it is again interesting to observe that other resistant biological structures, including viruses and spores, possess the same basic structure.

One is even more amazed upon consideration that a structure such as this provides the greatest number of stable bonds and the lowest amount of free energy! And, these are found in the most primitive organisms, the bacteria, and in structures (viruses) whose viability has not been resolved. How much more complex then is the life observed in metazoan animals?

Consideration of Advantages of Cyst Formation

The above paragraphs represent basically the process involved in encystment. Once the cyst is formed it may remain unchanged for relatively long periods of time (in excess of ten years). That these microscopic cells contain all of the necessary genetic information to carry out two involved processes, nitrogen fixation and cyst formation, points out that God's plan in nature is a perfect one. The dependence of life on nitrogen-fixation has been mentioned.

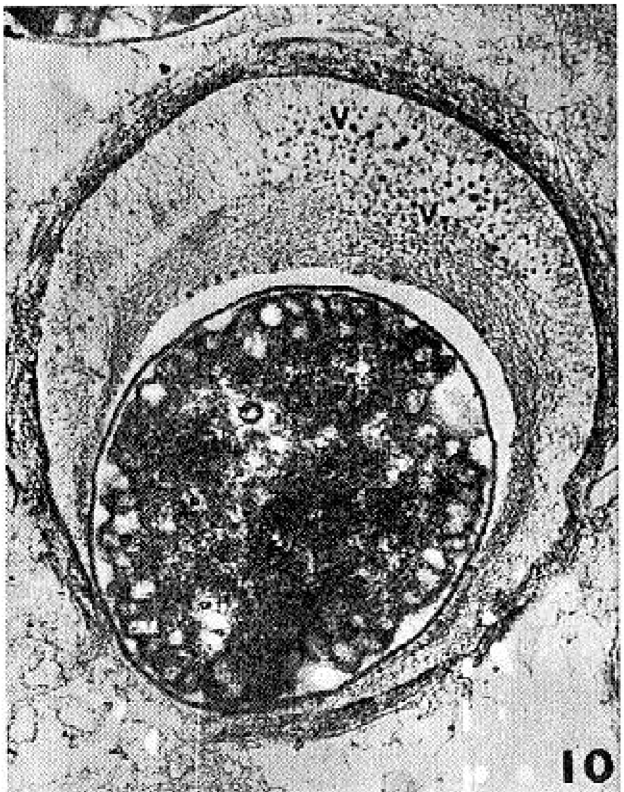

Figure 10. Electron micrograph of a thin section of a germinating cyst of *Azotobacter.* The cyst has been incubated in fresh media approximately four hours, The cyst coat is observed to be much thinner, and vesicles (V) are observed to reappear as the coat is digested. (x 30,500)

Both of the important non-symbiotic bacterial nitrogen-fixers have the capacity to form desiccation-resistant resting cells. It does not seem likely that this occurred simply by a random selective process, for too few cells form resistant forms, and too few are capable of converting N_2 to NH_3. Instead, it seems more likely that the trait of nitrogen-fixation is an important one and is intricately necessary for the cycling of nitrogen from the atmosphere to the biosphere.

Cyst and spore formation represent created advantages for other vastly different organisms, including the fungi, for example. Although very slow to grow, spores allow an inactive existence; and, when opportunity for growth is provided, the fungi are present.

The process by which encystment occurs has been studied broadly, and it has been noted that many other bacteria produce vesicular elements as cells start to die. However, no member of these genera is capable of taking this basic units of encystment and converting them into an intact resting cell, complete with cyst coat. This inability on the part of other Gram negative bacteria may be explained as due to lack of necessary enzyme compliment, which unites the vesicular subunits into an intact coat, along with sufficient extracellular polymer to hold the elements in proximity to the cell while the coat is being formed. Some bacteria may possess one of these characteristics, as a sufficiently large capsule, but not both.

Reversion to Activity

As depicted in Figure 2, the life of these bacteria, or more specifically, of a single bacterium is a cyclic process. The first portion of the life cycle deals with the transition from cell to cyst. When cysts return to a favorable growth medium, the second portion of the bacterial life cycle is set into motion. The nutrients in the fresh growth medium diffuse across the external coat and initiate the metabolic activity of the vegetative cell.[12] In Figure 10 germination of an encysted central body of *Azotobacter* is illustrated.

This process is enzyme-mediated, as are nitrogen-fixation, cyst coat formation, and the myriad of other biological reactions which occur. As the transition occurs from cyst back to cell there is an accompanying increase in cell size and the thick, laminated coat begins to be enzymatically separated and dissolved, until finally the bacterium is liberated. The cycle is then repeated.

Conclusion

In conclusion, the presence of either of these systems, nitrogen fixation or encystment, in a single organism, much less the possession of both by single organisms, is more than can be explained by evolutionary concepts alone. This is true not only for the organism considered here, *Azotobacter*, but for the genus *Clostridium* as well. The Bible records the perfection of the works of God and states that in His works "there is no confusion." (I Cor. 14:33). Likewise reference is made to the cyclic nature of life—in the often quoted verse, "In the sweat of thy face shalt thou eat bread, till thou return unto the ground; for out of it wast thou taken: for dust thou art and unto dust shalt thou return." (Gen. 3:19). This constitutes only a small part of the perfect plan in nature.

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REPORT ON THE VELIKOVSKY SYMPOSIUM

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The first major interdisciplinary symposium on the works and theories of Immanuel Velikovsky was held at Lewis and Clark College, Portland, Oregon from August 16 to 18th, 1972. It was attended by over three hundred people who heard papers by ten invited speakers, and heard discussions of the papers led by twenty-six other invited scholars. Dr. Velikovsky himself contributed three keynote addresses.

There were six sessions spread over the three days, two on each day. On day one the main focus was on the physical sciences. On day two history and archaeology were emphasized. And on day three some of the relationships between Velikovsky's work and religion, psychology and sociology were explored. An open session on the final night was set aside for integration of the contributions from the three days of papers and discussion.

The idea for the conference emerged from publicity and response that resulted from publication of the May 1972 issue of *Pensée* magazine which was devoted to a review and assessment of Velikovsky's theories in the physical sciences.[1] This magazine has since been changed from a monthly to a quarterly publication and now constitutes a forum for critical comment, both favorable and unfavorable, on Velikovsky's work. Most of the papers presented at the Portland conference have been published in the issues of *Pensée* from the Fall 1972 through the 1973 numbers. No complete outline or description of the papers will be made here since they may be obtained from the Pensée office.

Velikovsky's Work and Theories

Velikovsky's theories have interested creationists ever since the publication[2] of his book, *Worlds in Collision,* in 1950. Velikovsky can be categorized as a catastrophist, overtly a secular non-theistic catastrophist,[3] who proclaims that the earth has interacted with other planets in a series of near collisions in the past. In *Worlds in Collision* he deals with two of these periods of near collision; (1) in approximately 1500 B.C. with two main phases of planetary proximity and attendant destruction around the Exodus from Egypt and around the "long day" of Joshua; and (2) from the mid-eighth to mid-seventh centuries B.C. associated with the earthquake in the days of King Uzziah,[4] the destruction of Sennacherib's army,[5] and the sundial of Ahaz episode in Isaiah 38:8. The earlier of these two cosmic events he contends was caused by the near approach of Venus to Earth and the latter by the approach of Mars.

Two earlier catastrophic periods, on which Velikovsky has books in preparation are linked with Saturn *(Saturn and the Flood),* and Jupiter *(Jupiter and the Thunderbolts).* The catastrophe of the Flood he considers to have been the most devastating of all in its effects on earth, and at the present not precisely datable, but to lie somewhere between five and eleven thousand B.C. The Jupiter influence on the earth received no further mention at the symposium. These books will be awaited with considerable interest and anticipation by creationists, but it would appear that considerable time may pass before they are published.

Velikovsky's theories also include a reworking of ancient Near Eastern chronology on the basis of his identification of the ten plagues and the Exodus at a period some six hundred years earlier in Egyptian history than according to conventional chronologies. When these events (and in fact all of ancient Egyptian history) are brought forward by several centuries to conform with the Biblical dating of the Exodus, an entire restructuring of ancient chronology is required since much of it hinges on synchronisms with events in Egypt. This was the theme of another work, *Ages in Chaos,*[6] Volume 1, which is to be followed in the relatively near future by at least three more volumes on the same topic. These books, and another volume on collective amnesia and the human race (his theorized explanation

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