THE AZOTOBACTER.

A STUDY OF THE VARIATION WITHIN THE SPECIES

AZOTOBACTER AGILIS, M.B., 4.4

By

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I. INTRODUCTION

Under suitable conditions, the free living aerobic organism Azotobacter fixes nitrogen at a surprisingly rapid rate, considering that the fixation occurs at ordinary temperatures and pressures. Since the discovery and isolation of the first species by Beijerinck in 1901, the greatest interest in the genus Azotobacter has been of its possible economic importance in the maintenance of soil fertility. It has been estimated that an active flora of these organisms may fix from 15 to 40 pounds of nitrogen per acre per year in the soil (Martin, et al., 1937) and may act, therefore, as an economically important aid in maintaining the nitrogen content of the soil.

The biological fixation of nitrogen by Azotobacter has been subject material for numerous physiological and biochemical studies. Reports covering the years from 1901 to 1950 show great differences in opinions on certain specific characteristics and functions of this genus.

Of current and interest and importance is the field of bacterial genetics. Laws of inheritance as proposed by Mendel apply not only to the higher organisms, but also to microorganisms. The information gained from studies of the genetics of microorganisms aids in the understanding of the hereditary mechanism of all living things.

The numerous research projects in recent years dealing with mutations in microorganisms confirm their similarity to mutations in higher forms. As might be expected, such changes affect a great variety of characteristics; in fact, variants have been found representing all types of characters for which a search has been made. For a few examples of the wide variations which occur among bacterial species, one might mention those affecting pigment, enzymatic systems, morphology, cytology, colony form, nutrition, serology, and resistance to phages, drugs, and antibiotics.

The investigations of Beadle utilizing the mold Neurospora have indicated the feasibility of obtaining induced biochemical mutants differing from the normal or wild-type in their ability to synthesize specific growth factors. In general, these induced mutant strains have been shown to require only single substances for growth and to differ genetically from the normal strain by single gene changes (Beadle and Tatum, 1941, 1945; Beadle, 1945). By utilizing a procedure essentially similar to that developed for the Neurospora investigations, it has been demonstrated that analogous biochemical mutants can be obtained in bacteria (Gray and Tatum, 1944; Roepke, Libby, and Small, 1944; Tatum, 1945). In Escherichia coli, numerous mutants have been produced which require specific amino acids, B-vitamins, or nucleic acid components. The similarity between the results obtained with Neurospora and Escherichia coli following irradiation is taken as evidence that bacteria probably possess cellular elements comparable to the genes of some higher organisms. Further, it has been possible to utilize the biochemical mutant types induced in Escherichia coli, K-12, to obtain evidence for what appears to be genetic recombinations, presumably involving some sort of sexual process in this bacterium (Lederberg and Tatum, 1946).

In view of these results with Escherichia coli, K-12, it was felt that similar studies on induced mutations could profitably be extended to other kinds of bacteria. It also seems of interest to obtain comparative evidence as to the kinds of biochemical variants which could be obtained in a species quite different from Escherichia coli, K-12. Further, any such mutant strains should prove useful in a number of ways: they should make it possible to determine whether recombinational phenomena comparable to those found in Escherichia coli, K-12, occur in other bacteria; they might prove valuable as assay organisms; they should contribute to a solution of the many problems involved in an analysis of the biosynthesis of various growth factors.

The objectives of the present study are primarily concerned with variations within the species Azotobacter agilis, M.B., 4.4. These involved specifically: (a) an extensive review of the literature in an effort to become acquainted with the genus Azotobacter; (b) to study the viability of the so-called "involution" forms of the organism by means of single cell isolations of these forms; (c) the application of the serum reaction as a possible means of detecting antigenic changes in mutant strains of Azotobacter agilis, M.B., 4.4; and (d) the isolation and stabilization of induced motility, pigment, and biochemical (nutritional) variants of the organism.

II. REVIEW OF LITERATURE

A. Isolation of Azotobacter

Beijerinck (1901) isolated and described the first Azotobacter. He found two species, one of which he named Azotobacter chroococcum and the other Azotobacter agile. The former was isolated from the soil and the latter from a sample of water taken from one of the canals in the city of Delft. Two years later, Lipman (1903a) added a third species to the list, Azotobacter vinelandii, and the following year isolated and described two more, giving them the names Azotobacter beijerinckii and Azotobacter woodstownii. In 1909 Lipman isolated and named Azotobacter hilgardii, and Lipman and Burgess (1915) isolated and named Azotobacter smyrnii. According to Lohnis and Smith 1923), Azotobacter beijerinckii, Azotobacter woodstownii, Azotobacter hilgardii, and Azotobacter smyrnii are identical with Azotobacter chroococcum. Greene (1935) studied Azotobacter chroococcum and Azotobacter beijerinckii by chemical analyses and found the chemical composition of the cells to be practically identical, but different from that of Azotobacter vinelandii and Azotobacter agile. The results of Aso and Yoshida (1928) upon the basis of complement fixation tests are further indications that Azotobacter chroococcum and Azotobacter beijerinckii are the same organism. Smith, in a private communication with Hofer (Bergey, 1948) feels that Azotobacter beijerinckii is a non-pigmented rough strain of Azotobacter chroococcum.

In studies on the chemical composition of cells, Greene (1935) found Azotobacter vinelandii to be very similar to Azotobacter agile. Smith and Lohnis (1923) agreed and stated furthermore that the two are identical; they were also convinced that Azotobacter vitreum isolated by Lohnis and Westermann (1908) is another synonym of Azotobacter agile. Smith, in a private communication with Hofer (Bergey, 1948) states that Azotobacter vitreum is a very weak

growing, smooth strain of Azotobacter agile. Kluyver and van Reenan (1933), however, feel that a distinction should be made between Azotobacter agile and Azotobacter vinelandii. In regard to the former, Kluyver and van den Bout (1936) suggested that it be further subdivided into Azotobacter agile and Azotobacter agile var. atypica, the latter referring to an Azotobacter agile that fails to produce pigment.

Starkey and De (1939) isolated a new species which was named Azotobacter indicum.

The sixth edition of Bergey's Manual of Determinative Bacteriology (1948) recognizes only three speices of Azotobacter. These are Azotobacter chroococcum, Azotobacter agile, and Azotobacter indicum.

B. Description of Azotobacter

Beijerinck (1901) characterized the Azotobacter as stout bacteria, four to six microns or less in length, sometimes longer, occurring as large diplococci or short rods in young cultures, the hyaline cells often containing a vacuole and the entire organism enclosed in a mucilagenous wall of varying thickness. Beijerinck found no spores.

In classifying the species included in the genus Azotobacter, it was found that one point badly in need of clarification was the nature of the flagellation. Although the genus had been studied by many workers, the results were in disagreement. The determination of the number and place of origin of the flagella is difficult, and the usual flagella stains appear to be inadequate.

For instance, the type species, Azotobacter chroococcum, has been described as having one polar flagellum (Beijerinck, 1901), and as being peritrichous (Petschenko, 1930). Azotobacter agile has been described as having

bundles of polar flagella (Beijerinck), as having one polar flagellum (Lohnis and Westermann, 1908), and as being primarily monotrichous but with some cells carrying a bundle of flagella attached at the pole (Winogradsky, 1938). In the last instance, however, as in the first, examination of the plates published with the articles might cause doubt as to whether the flagella were really attached at the pole (Hofer, 1944). Hofer (1944) states that the general appearance of Winogradsky's plate is almost identical with that of a culture (No. 35) of Azotobacter vinelandii stained by Fisher and Conn (1942). Similarly (Hofer, 1944), the plate of Beijerinck shows that the flagellation of Azotobacter agile is not greatly different from those of Winogradsky and of Fisher and Conn. Other authors also believe that Azotobacter cells are peritrichous (Jones, 1913; Petschenko, 1930). Lohnis and Smith (1923), however, state that with the large cells of Azotobacter the flagella may be either monotrichous or peritrichous. An example of the confusion regarding the matter is Beijerinck's statement that he had been inclined to think that Azotobacter agile had peritrichous flagellation until he sent preparations to Professor Zettnow for staining. When the latter declared that the organism was lophotrichous, Beijerinck accepted his decision.

Hofer (1944a; 1944b) demonstrated clearly with the electron microscope and with the Hofer and Wilson (1938) modification of the Gray flagella stain that the genus Azotobacter is peritrichous, and that it possesses an unusually large number of flagella.

According to Bergey's Manual (Breed, et al., 1948) the genus Azotobacter is characterized as follows:

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

C. Cytology of Azotobacter

Since the Azotobacter are very pleomorphic they lend themselves well as tools for the study of cytological variation. Morphological variations within species of bacteria have been observed by most bacteriologists. Many have considered morphological differentiation to be coupled with some type of a complex life cycle; others have maintained that bacteria exhibit a constancy of form and size. These followers of the "monomorphistic" theory maintained that any changes of a culture in morphology was proof of "contamination", incorrect classification of the organism, or of the existence of "involution" forms.

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

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

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

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

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

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

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

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

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

A short time before the appearance of the work on bacterial life cycles by Lohnis and Smith, Bonazzi (1915) made a study of Azotobacter chroococcum to determine the constitution of the cellular granules. At this time there were many different opinions as to the nature and function of these bodies.

Beijerinck (1901) spoke of these granules as fat bodies. The presence of volutin was reported by Fischer (1906). Others have regarded the granules as chromidia and the equivalent of a true nucleus (Mencl, 1911). Jones (1913) distinguished two types of granules on the basis of reaction to stains. He regarded the stainable granules as reproductive bodies and designated them as gonidia. The non-stainable granules appeared to be glycogen. In his cytological studies, Bonazzi used various microchemical tests. As a result of these, he pointed out that the granules found in Azotobacter chroococcum did not give the reaction for glycogen, but that they responded to all of the tests carried out to distinguish their metachromatic character. He further indicated that these granulations seem to have their origin from the nucleus, but they seem to have no relation to the reproduction of the cell since their disposition in the cells is not regular but changes in different individuals.

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

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

From his studies in 1920, Jones further reports that with Azotobacter

there is an irregular occurrence of abnormal forms which merit the term "involution" forms. He found these forms to be common in old cultures (one or two months) of Azotobacter grown in Ashby's solution or on Ashby's agar at 25° C. They were more numerous when grown at 37° C. In cultures more than fourteen days old, large, spherical thick-walled cells were common. These appeared to be resting cells or arthrospores.

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

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

According to Lohnis and Smith, the reproductive organs of Azotobacter are (1) gonidia, in part filterable, (2) regenerative bodies and exospores, (3) arthrospores, (4) microcysts, and (5) endospores. They conclude that gonidia for the basis for the development of regenerative bodies, arthrospores, and endospores.

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

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

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

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

The studies of Lewis (1937) failed to reveal endospore formation in any of the strains of Azotobacter. He reported the failure to confirm the

occurrence of a filterable phase in the life history, and transmutation of cells to a different type cultivable as such to form culture phases different from the original.

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

The development of several new techniques, as well as some new concepts since these earlier workers debated the significance of the morphological cell types in Azotobacter species prompted several workers to study, cytologically, a pleomorphic strain of Azotobacter with the electron and phase microscopes and the Robinow nuclear-staining technique (McMahon, 1949; Eisenstark, McMahon, and Eisenstark, 1950; Eisenstark and McMahon, 1949).

These workers reported that four distinct morphological cell types of Azotobacter agile (Illinois strain ZN 350) were observed; other stages reported by Lohnis and Smith (1923) could not be easily distinguished. In addition to large rods, small rods, elongated and branched cells and minute coccoid cells, they described dwarfed cells, small sporulating rods, and large sporulating rods. Other investigations did not confirm the presence of heat-resistant endospores in this organism (Jones, 1920; Lewis, 1937).

Deep-staining intracellular bodies were observed in young cells when stained with Giemsa solution after hydrolysis in normal HCl. The presence of these bodies after hydrolysis, their strong affinity for nuclear dyes, and their disappearance from old cells suggest that these inclusions are similar to "chromatinic bodies" (Robinow, 1942). Eisenstark and McMahon (1949), using a special technique for phase-microscope observations of Azotobacter agile noted that the arrangement of the internal bodies in all preparations into central bodies and an external area (cytoplasm?) resembles somewhat the arrangement of the "chromosomes" in various bacteria described

by Robinow (1944).

Further study on the cytology of a pleomorphic strain of Azotobacter has been made by Ward (1950).

D. Distribution of Azotobacter

Nitrogen-fixing organisms are widely distributed, occurring in most soils. Lipman and Burgess (1915), who studied the nitrogen-fixing flora, especially those of the Azotobacter group, of 46 soils from Egypt, India, Japan, China, Syria, the Hawaiian Islands, Guatemala, Costa Rica, Spain, Italy, Russia, Mexico, Asia Minor, Canada, Unalaska, Samoa, Queensland and other parts of Australia, Tahiti, Belgium, and the Galapagos Islands, found every soil possessed the power of fixing nitrogen in mannite solution. About one-third of the soils contained Azotobacter; frequently the same soils showed the presence of two or three different species of Azotobacter. Asotobacter chroococcum, however, was the most prominent. It was also found most widely distributed in the various soils. Groenewege (1913) found Azotobacter in all but one of a series of Java soils.

Several hundred Utah soils have been examined and all found to fix nitrogen, many of them without the addition of carbohydrates (Greaves, 1914). Aerobic Azotobacter are present in nearly all Utah soils. Hutchinson (1912) found the Azotobacter in all the soils examined. They occurred in cultivated areas more frequently and in greater numbers than in virgin soils. This probably accounts for the much higher nitrogen-fixing power of cultivated soils.

Azotobacter were found in only two out of 64 localities in the soils of Danish forests (Weis and Bornebusch, 1914). Both of the soils which gave positive tests were from beechwood forests and contained calcium carbonates. Although the soils of these forests rarely contain enough carbonate to effervesce,

they are usually neutral or slightly alkaline. They contain calcium, but in forms other than the carbonate. It is generally understood that Azotobacter occur commonly in soils which contain sufficient calcium carbonate to effervesce when acid is added and that they scarcely ever occur in acid soils. Their disappearance from a soil is usually due to the absence of basic substances, especially of calcium and magnesium carbonate, and not to the presence of toxic substances (Christensen, 1915). However they are frequently not present in peaty soils, where their absence cannot be attributed to a lack of lime (von Feilitzen, 1910).

Other investigators in various parts of the world have examined groups of soils for the presence of Azotobacter. For convenience and conciseness, the results of some of these studies have been summarized in Table I, and other reports have been published by the following: Christensen, 1906a; 1906b; 1906c; Peterson and Mohy, 1913; Walton, 1915; Burgess, 1916; Aso and Yamagata, 1924; Mattern, 1928; Obraztsova, 1928; Selim, 1931; Aquino and Villegas, 1933; Paulie, 1934; Beck, 1935; Altson, 1936; Soriano, 1939; Uppal, et al., 1939; Sushkina, 1941; Peterson and Goodding, 1941; Stinger-Tulczynoka and Elze, 1941; Beaver, 1942; Vandecaveye and Moodie, 1942; Soriano, 1942; Lourerio, 1942; Fletcher and Martin, 1948; Gaw, 1940; Kononov, 1928; Castelli, 1938; Beijerinck, 1908; Arnaudi, 1932.

The data presented in Table I show that the Azotobacter are certainly widely distributed over the earth's surface. Every reference cited shows that some soils contained Azotobacter, although the percentages vary with the localities. On the average, however, about 50 percent of the soils examined contained the organisms. This finding verifies the independent estimates made by Winogradsky (1926a) and by Gainey (1927) to the effect that approximately half of all soils examined contain the nitrogen-fixing aerobic bacteria, Azotobacter.

TABLE I

The Occurrence of *Azotobacter* in Soils of Different Localities.

Investigator	Locality	Number of soils Examined	Samples Containing Azotobacter	
			Number	Percent
Ashby (1907)	Rothamsted, England	13	4	31
Schalwyk and Hector (1932)	South Africa	--	--	60
Christensen (1923)	Denmark	3,161	1,806	57
Christensen and Jensen (1924)	Denmark	641	336	52
Weis and Bornebusch (1914)	Danish forest soils	64	2	3
Brenner (1924)	Finland	200	10	5
Niklas and Poschenrieder (1927)	Germany	500	200	40
Rossi and Riccardo (1924)	Italian volcanic soils	5	4	80
Yamagata and Itano (1923)	Japan	300	66	22
Itano and Arakawa (1930)	Japanese rice field soils	10	4	40
Ohmasa (1930)	Japanese forest soils	29	8	27
Kruyff (1910)	Java	100	5	5
Johnson and Murdoch (1919)	Eastern Ontario	29	22	76
Ziemiecka (1923)	Poland	28	14	50
Omeliansky and Solounskoff (1915)	Russia	26	12	46
Gainey (1927)	United States soils	154	42	27
Burgess (1930)	Arizona	33	29	88
Sackett (1911)	Colorado	30	22	73
Gibbs (1925)	Idaho forest soils	106	24	23
Martin, Walker, Brown (1937)	Iowa	287	101	35
Gainey (1927)	Kansas	269	157	58
Turk (1935)	Michigan	28	17	61
Skinner and Nygard (1930)	Minnesota peat soils	54	11	20
Wilson and Wilson (1933)	New York peat soils	54	50	93
Curie (1931)	Ohio	120	61	50
Vandecaveye and Anderson (1934)	Washington forest soils	16	15	54
Martin (1940)	Arizona cultivated soils	94	82	87
Martin (1940)	Arizona range lands	119	27	23
TOTAL.		6,470	3,111	3 - 93

There are numerous accounts of the occurrence of both aerobic and anaerobic nitrogen-fixing bacteria in fresh-water lakes, but whether they are indigenous species which are functional in lakes or are adventitious forms from the soil is another unsolved problem (ZoBell, 1946).

Benecke (1933) relates that Keutner was the first to make an extensive search for nitrogen-fixing bacteria in the sea, this being the subject of his doctorate dissertation at Kiel University. Species of the aerobic nitrogen-fixer, Azotobacter chroccoccum, were found (Benecke and Keutner, 1903). Azotobacter occurred primarily associated with the surface slime of algae. Azotobacter were found in the Baltic Sea, the North Sea, off the African coast, and in the Malay Archipelago; Keutner (1905) concluded that nitrogen-fixing bacteria are normal inhabitants of the sea. Azotobacter chroccoccum was found to tolerate up to eight percent sodium chloride.

Reinke (1903) believed Azotobacter to occur as an epiphyte on phytoplankton and larger marine algae, symbiotically obtaining utilizable organic matter therefrom while furnishing fixed nitrogen in return. Several other investigators have noted the occurrence of Azotobacter on marine as well as fresh-water algae, but a definite symbiotic relationship has never been established.

The strains of Azotobacter, which Keding (1906) found on the surface slime of algae near shore, appeared to be identical with those found in soil. The strains of Azotobacter which Issatchenko (1914, 1926) found associated with marine algae required enough salt for their development to lead him to believe that these nitrogen fixers were specifically adapted to sea water.

According to Korinek (1932) there are species in the ocean which closely resemble Azotobacter morphologically, but they do not assimilate free nitrogen. He found that Azotobacter chroococcum grew only poorly in sea-water media.

He admits that there may be marine strains of Azotobacter which fix nitrogen, but he failed to find one. Lloyd (1930) likewise regarded the existence of active marine nitrogen-fixing bacteria as problematical.

Azotobacter cells were only occasionally found by Davendamm (1932) in enrichment cultures of calcareous mud from around the Bahama Islands.

The occurrence in the sea of an abundant population of nitrogen-fixing Azotobacter is reported by Waksman, et al. (1933). In the presence of a favorable source of energy, the bacteria were capable of fixing appreciable quantities of nitrogen. However, the extent to which such a process actually takes place in the sea remains to be determined. von Brand, et al. (1942) concluded that, except for the sporadic presence of nitrogen-fixing bacteria, there is no evidence of nitrogen fixation in the sea.

B. Nutrition of Azotobacter

The distribution and physiological efficiency of the nitrogen-fixing organisms, especially of the genus Azotobacter, are governed by the physical and chemical properties of the soil, foremost among which is the basicity of the soil. The calcium or magnesium carbonate content is particularly important (Christensen, 1907). Numerous studies bear out the significance of this property to the activity of Azotobacter and its implications in soil fertility (Fischer, 1905a; Krzeminieniewski, 1906; Christensen, 1907; 1915; Koch, 1907; Ashby, 1907; Krainskii, 1908; Remy, 1909; von Feilitzen, 1910; Hoffman and Hammer, 1910; Larson, 1911; Mockeridge, 1912; Lipman and Burgess, 1914; Bear, 1917; Kopeloff, 1917; Greaves, 1918; Fred and Davenport, 1918; Johnson and Lipman, 1922; Gainey and Batchelor, 1922; 1923; Burk and Lineweaver, 1931; Burk, 1934; Klauss, 1940; Greaves and Jones, 1941; Gainey and Fowler, 1945; Gainey, 1948).

The nitrogen-fixing organisms probably require for their nutrition the

same elements as do the higher plants, namely: carbon, hydrogen, oxygen, nitrogen, potassium, phosphorous, sulfur, calcium, magnesium, and iron, and possibly aluminum and manganese (Greaves, 1918). In connection with their nutritional requirements there is a vast amount of literature (Hiltner and Stormer, 1903; Lipman, 1903a, 1903b; Stoklasa, 1908; Stransk, 1909; Heinze, 1910; Krainskii, 1912; McBeth, 1913; Hanzawa, 1914; Lipman and Burgess, 1915; Coleman, 1917; Barnes and Ali, 1917; Hills, 1918; Winogradsky, 1925, 1926a, 1926b, 1926c, 1927a, 1927b, 1928, 1930, 1935, 1938a, 1938b, 1941; Winogradsky and Ziemiecka, 1927; Ziemiecka, 1929, 1932; Joshi and Ram Ayyar, 1934; van Niel, 1935; Greaves and Anderson, 1936; Wieringa, 1939).

The influence of fixed nitrogen on Azotobacter both with respect to utilization and inhibition of nitrogen fixation has been studied by a number of investigators. The results have not always been conclusive, but it appears from the literature that Azotobacter does fix atmospheric nitrogen in the presence of combined nitrogen. The specific influence of this combined nitrogen depends upon the kind and quantity present (Hiltner and Stormer, 1903; Lipman, 1904; Jodidi, 1910, 1911; Headden, 1912; Dvorak, 1912; Hanzawa, 1914; Lohnis and Green, 1914; Greaves and Anderson, 1914; Brown and Allison, 1916; Murray, 1917; Mockeridge, 1917; Fulmer and Fred, 1917; Richards, 1917; Reed and Williams, 1917; Bear, 1922; Greaves and Nelson, 1923; Itano, 1923; Zoond, 1926; Telegdy-Kovats, 1928; Voitkevich and Runov, 1928; Guittonneau, 1929; Burk and Lineweaver, 1930; Baumgartel and Butenschon, 1930; Fuller, 1930; Iwasaki, 1930; Fuller and Rettger, 1931; Bassalik and Neugebauer, 1931; Thompson, 1932; Birch-Hirschfeld, 1932; Winogradsky, 1932; Smith and Coull, 1932; Itano and Arakawa, 1932; Smith and Brown, 1932; Burk, Lineweaver, and Horner, 1932; Wilson, Hull, and Burris, 1933; Dassai, 1933; Rigotard, 1935; Bortels, 1936; Gainey, 1936; Lochhead and Thexton, 1936; Stumbo and Gainey, 1938; Martin and Brown, 1938; Aso, et al., 1939; Starkey, 1939; Greaves and

Bracken, 1939; Rubenchik and Roizin, 1939; Winnik and Goldberg, 1939; Greaves, Jones, and Anderson, 1940; Tatunko, V.D., 1940; 1941; Hervey and Greaves, 1941; Wyss, et al., 1941; Lind and Wilson, 1942; Rippell-Baldes, Starc, and Kohler, 1942; Jones and Greaves, 1943; Burris and Wilson, 1943; Horner and Allison, 1944; Schroeder, 1945; Greaves, 1945; Fedorov, 1949; Kellerman and Smith, 1914; Kostychev and Schwezowa, 1926; Skinner, 1930; Vukherer, 1941; Bonazzi, 1931; Arnaudi, 1938).

The effects of trace elements of varying concentrations and combinations on nitrogen fixation or utilization, and on pigment production by Azotobacter has stimulated an interest on the part of many investigators (Olaru, 1915; Gregoric, 1916; Greaves and Carter, 1924; Stoklasa, 1926; Hirai, 1927; Bortels, 1930, 1936, 1937a, 1937b, 1939; Konishi and Tsuge, 1933a, 1933b; Greaves, 1933; Itano and Matsuura, 1933; Burk, 1934, 1935; Horner and Burk, 1934; Van Niel, 1935; Burk and Horner 1935, 1937; Krzemieniewski and Kovats, 1936; Kovats, 1938; Steinberg, 1938; Mulder, 1939, 1940, 1948; Lewis and Powers, 1941; Lewis, 1942; Horner, et al., 1942; Nilsonn, Alm, and Burstrom, 1942; Burema and Wieringa, 1942; Harris, 1943; Vinogradova, 1943a, 1943b; Harris, 1946).

The essentiality of molybdenum or vanadium in optimum concentrations for maximum nitrogen fixation and growth by many strains of Azotobacter has been established by Horner, et al, (1942).

Potassium is essential to the higher plants and cannot be replaced by related substances, yet Gerlach and Vogel (1902a; 1902b; 1903) early reached the conclusion that potassium and magnesium are not essential to the Azotobacter. Their results were, however, generally considered erroneous, for while as much nitrogen was fixed in twenty days without as with potassium, after forty days there was no further nitrogen fixation in the solution without

potassium, but in its presence the nitrogen gain nearly doubled. It was, therefore, argued that the traces of potassium left in the chemicals and dissolved from the glass during sterilization had been enough to permit development for a time. If these elements are essential, it must be in extremely minute quantities, for Vogel (1912), using the purest chemicals obtainable, was able to prepare potassium free media in which the Azotobacter developed. He did find, however, that potassium favors their development.

The Azotobacter require large quantities of phosphorous (Heinze, 1906; Wilfarth and Weinmar, 1907) for the building of the nucleo-proteins and phospho-proteins in which their bodies are extremely rich. Moreover, it greatly accelerates the reaction and economizes the carbohydrates; hence it is rather evident that phosphorous plays a very essential part in Azotobacter metabolism. The literature is filled with reports on the relationship of phosphorous to Azotobacter (Heinze, 1906; Wilfarth, and Weinmar, 1907; Voorhees and Lipman, 1907; Christensen, 1907, 1918; Lipman, 1908; Kaserer, 1910, 1912; Dzierzbicki, 1910; Harden and Young, 1911; Mockeridge, 1912; Hoffmann, 1913; Bear, 1917; Greaves, 1922; Greaves, Carter, and Lund, 1922; Winters, 1924;Niklas, et al., 1926; Truffant and Bezssonoff, 1927; Ziemiecka, 1928a; 1928b; Thompson, and Smith, 1931a, 1931b; Niklas and Poschenrieder, 1932; Jones, 1932; Greene, 1932, 1933; Smith, Brown, and Mensing, 1934; Halversen and Hoge, 1942). There is a definite relationship between the carbon and phosphorous content of a soil and the nitrogen assimilated. According to Stoklasa (1911) Azotobacter assimilates from 5.0 to 5.7 gm. of free nitrogen for every gram of phosphorous used. Although these organisms are directly dependent upon a readily-available supply of phosphorous to promote growth, they do not change it into the organic form as rapidly as do the ammonifying bacteria.

The common so-called soil "alkalies - sodium chloride, sodium sulfate,

and sodium carbonate" (Lipman and Sharp, 1912) do not stimulate the nitrogen-fixing organisms. They are quite resistant to these compounds (Barnes and Ali, 1917). Numerous other studies on the effects of soil alkalies, various types of soils, temperature and absolute reaction, and other physical and chemical agents and their effects on Azotobacter have been reported (Keutner, 1906; Lipman, et al. 1912; Hills, 1918; von Feilitzen, 1911; Fischer, 1905; Wilsdon and Ali, 1922; Werner, 1945; Walker, et al., 1930; Walker and Sullivan, 1929; Shunk, 1929; Sidorenko, 1940; Ryablkina, 1938, 1939; Niemeyer, 1925; Loew, 1927; Lebenbaum, 1932; Krasilnikov and Nikitina, 1945; Jensen and Swaby, 1940; Jensen, 1942; Hunter, 1923; Henckel and Zakharova, 1930; Greene, 1933; Allen, 1919; Greene, 1932; Greaves and Carter, 1920; Greaves, 1916; Gibbs, and Batchelor, 1927; Gibbs, et al., 1926; Gainey and Briscol, 1933; Gainey, 1944, 1943, 1927b, 1926, 1923, 1918, 1922; Flieg, 1937; Fife, 1931; Fedorov and Topper, 1945; Federov, 1949; Efendieva, 1944; 1942; Dhar and Tandon, 1936; Csaky, 1949; Cauda, 1916; Chuvaev, 1931; Bradley and Fuller, 1930; Blinkov, 1948; Berge, 1940; Bezrukov, 1928; Bassalik and Neugebauer, 1933; Omeliansky and Sieber, 1913; Greaves, 1910; Severin, 1917; Stoklasa, 1911; Lipman, 1904; Rippel, 1924; Vandecaveye, 1932; Wilson and Staker, 1932; Sackett and Stewart, 1931; Katznelson, 1940; Konishi and Tsuge, 1933; Kostnik, 1930; Rabotnova, 1942; Quispel, 1947; Pochon and Lajudie, 1948; McCalla, 1939; Molotkovskii, 1941; Lineweaver, Burk, and Horner, 1932; Richards, 1939; Rybalkina, 1937; Smith, 1944; Snyder and Wyant, 1932; Werner, 1935; Whelden, et al., 1941; Wilson, 1937a, 1937b; Wilson and Choudhri, 1948; Wilson et al., 1943; Young, 1933; Burk, Lineweaver, and Horner, 1934; Bassalik, 1913, 1932; Beijerinck, 1901, 1908; Curie and Batchelor, 1930; Cox and Martin, 1937; Christensen, 1923; Chodzicki, 1933; Castelli, 1932; Burris, Phelps, and Wilson, 1942; Bucksteeg, 1942; Bucherer, 1933; Blom, 1931; Baumgartel and Hartung, 1927;

Baumgartel and Bihler, 1930; Aristovskaia, 1941).

The nitrogen-fixing organisms differ widely from plants in their energy requirements. This is due to the fact that they carry on endothermic reactions. The fixation of nitrogen by Azotobacter necessitates a greater supply of energy than is required by most other bacteria. They are similar to most other bacteria in that this energy must be supplied by an organic compound, preferably one of the carbohydrates (Greaves, 1918).

The literature indicates that many vaired organic compounds such as simple carbon compounds, benzene derivatives, complex carbon compounds, and fatty acids, to mention a few have been studied as possible sources of energy for the genus Azotobacter. From the literature available it is apparent that simpler carbohydrates are the better energy sources; most of the naturally occurring glucosides and many benzene derivatives are unsuitable as sources of energy. Moreover, some species utilize one carbohydrate most effectively and another species a different one. The quantity of nitrogen fixed per gram of carbohydrate varies greatly with the species, and the efficiency of these organisms (Mockeridge, 1912) to utilize various carbohydrates as sources of energy is greatest when they are rapidly multiplying and it decreases as their metabolic products accumulate.

The effects of various organic compounds on Azotobacter as energy sources may be found readily (Berthelot, 1885; Bottomley, 1911; Brown et al., 1916; Dvorak, 1912; Dzierzbicki, 1910; Fulmer, 1917; Fulmer and Fred, 1917; Greaves and Carter, 1916; Hanzawa, 1914; Henry, 1902; Hoffmann and Hammer, 1910; Hoppe-Seyler, 1886; Hornberger, 1906; Koch, 1907, 1910; Koch, et. al., 1907, 1912; Krainskii, 1910; Krzemieniewski, 1908, 1909; Lohnis and Green, 1914; Lohnis and Pillai, 1908; McBeth, 1913; Mockeridge, 1915; Omeliansky and Solunskov, 1916; Peck, 1911; Pringsheim, 1910, 1913; Remy, 1909; Richards,

1917; Stoklass, 1906, 1908; Stranak, 1909; Suchting, 1905; Tottingham, 1916; Walton, 1915; Waksman, 1916; Zeimeicka, 1924; Stone and Werkman, 1937; Skinner, 1929; Sheloumova, and Menkins, 1935; Sanborn and Hamilton, 1929; Rippel-Baldes and Fischer, 1946; Reuszer, 1939; Makrinov, 1933; Lineweaver, 1933; Krishna, 1928a, 1928b; Kostytschev, et al., 1926; Jensen, 1942, 1940; Jensel and Swaby, 1941; Horvath and Kramli, 1948, 1947; Gorini, 1927; Georgi and Ettinger, 1941; Gainey, 1928a, 1928b; Fedorov, 1949; Diehm, 1930; Dhar, et al., 1936; Aso, et al., 1932; Burk, 1952).

As an example of the variety of carbon compounds which may be used by Azotobacter as energy sources, Lineweaver, 1933, found that Azotobacter vinelandii is capable of oxidizing, by means of molecular oxygen, a large number of compounds including the following: glucose, lactate, pyruvate succinate, acetate, malonate, malate, tartrate, fumarate, ethyl, propyl, and butyl alcohols, formaldehyde, and at least one amino-acid, l -tyrosine. He reported that in general, if a substrate is oxodized at all, it is oxidized practically in entirety (at least 95 to 100 percent) to carbon dioxide and water.

F. Protein Synthesis by Azotobacter

The biological fixation of atmospheric nitrogen has been the subject of many physiological and biochemical studies, particularly with reference to the metabolism of the nitrogen fixing bacteria. Of interest is the mode of protein synthesis by the genus Azotobacter.

Greene (1935) harvested and analyzed the growth of four species of Azotobacter which were grown on nitrogen-free mannitol agar. He found Azotobacter vinelandii and Azotobacter agilis to be very similar in composition; a close similarity was noted to exist between Azotobacter chroococcum and Azotobacter beijerinckii. He found this relationship to exist also in their nitrogen

fixing abilities.

Green found arginine and lysine were the amino acids present in the cells of the Azotobacter cultures in the largest amounts. Tyrosine, tryptophan, cystine, and histidine were found in smaller amounts. Approximately 40 percent of the total nitrogen was found in the non-basic fraction, which indicates the presence of simpler amino acids (glycine, alanine, etc.). He noted by qualitative tests the presence of a substance which gave a positive reaction with sodium nitroprusside and he reported it might be glutathione. From his results with semi-quantitative determinations he indicated that proteins were present chiefly as globulins, glutelins, and albumins.

The synthesis of vitamin B by such biologically separated genera of microorganisms as Torula, Oospora, Actinomyces and four genera of the order Eubacteriales reveals a general occurrence of vitamin B synthesis among widely separated groups of the lower plant forms. Vitamin B is a constituent prevalent in microorganisms (Sunderlin and Werkman, 1928).

Slanetz (1923) using mice found that Azotobacter chroococcum produced neither vitamins A nor B. Hunter (1923a, 1923b) concluded that Azotobacter synthesized vitamin B. Sunderlin and Werkman (1928) reported that Azotobacter chroococcum was found by supplementing the diets of white rats with the organism to produce vitamin B.

Azotobacter chroococcum when grown in a synthetic medium free from accessory food factors synthesizes biotin, inositol, nicotinic acid, pantothenic acid, pyridoxin, and thiamin as determined by assay procedures. The quantity synthesized compares favorably with the quantities found in other plant tissues including yeast (Jones and Greaves, 1943).

G. Biological Nitrogen Fixation

The faculty of using molecular nitrogen for the synthesis of protein is, so far as is known, confined to microorganisms, and is not shared by any of the more complex forms of life. It is true that periodically the power has been claimed for certain of the higher green plants, but so far it has always appeared subsequently that adherent symbiotic bacteria were the active agents. Indeed, even among the bacteria only a very few species possess the faculty of fixing nitrogen to any appreciable or useful extent.

It is indeed fortunate, not only for man, but for plants and animals in general, that certain species of microorganisms evolved the power of fixing atmospheric nitrogen, for nearly all animals and plants are dependent for their existence upon a supply of nitrogenous compounds.

Atmospheric nitrogen fixation is a fundamental phenomenon in the maintenance of life. It may be considered second in importance only to photosynthesis. Electrical discharges in the atmosphere, rainfall, etc., may be responsible for a small portion of nitrogen, but by far the greatest part of fixed nitrogen has been formed through the activity of microorganisms.

Evidence of early observation of biological nitrogen fixation is given by Waksman (1932):

> The actual discovery that nitrogen fixation occurs through the agency of microbes was due to Jodin. In a seldom quoted paper published in 1862, he demonstrated that a solution containing phosphate, and either sugar, tartaric acid, or glycerol, but no organic nitrogen, supported a vigorous growth of "mycoderms." If the culture fluid were enclosed in a sealed vessel, a decrease in nitrogen as well as of oxygen could be demonstrated, the nitrogen absorbed being six to seven percent of the oxygen used. It is difficult to tell whether this important observation was influenced by Pasteur's writings on the microbial cause of fermentation; in any case it stands as the first real biochemical evidence of the part played by microbes in nitrogen fixation.

Since the early observation by Jodin, many investigations have been made in the field of biological nitrogen fixation. The available literature on

the subject is tremendous and a complete review of all the literature is beyond the scope of this work. The two major theories of the mechanism for biological nitrogen fixation will be considered, and only briefly, as the primary emphasis will be placed on the work that has been done on the genus Azotobacter. However, in considering the hydroxylamine theory of Virtanen some of the work done on the Rhizobia must be presented.

1. The Ammonia Hypothesis

The classical view is that the mechanism for biological nitrogen fixation proceeds by way of ammonia (Wilson, 1940). Because of the apparent simplicity of the reaction and its obvious analogy both to the assimilation of combined nitrogen by plants and to the chemical process for fixation of atmospheric nitrogen, this scheme has always figured prominently in the speculation on mechanism (Wilson, 1940).

Winogradsky (1933, 1936) incubated excised pea nodules at 40° C., and found that a strip of litmus paper in the container soon turned blue. The amount of ammonia formed was appreciable; he states:

> Des racines de pois nodulees, detachees de la tige, introduites immediament dans des flacons-laveurs, relies a des barboteurs charges d'eau acidulee, laissent capter des quantites assez notables d'ammoniac. Elles sont d'ordinaire tres faibles durant les premiers 24 heurs, mais en continuant la ventilation avec de l'air tres sec (ce qui previent le developpement des moisissures), on recueille quelques dizaines de milligrames d'azote ammoniacal par 24 heures, degages par des racines nodulees d'un poids frai de 60 a 100 grammes (Winogradsky, 1938).

Ammonification was dismissed as a source of the ammonia because (1) a delay occurred in the evolution of the ammonia, which attained a maximum during the second day, and thereafter was evolved at a rapidly decreasing rate; (2) antiseptics and anaesthetics were ineffective in preventing the evolution; (3) if the nodules were dried at 50° C., ground to powder, and kept as long as three years, they would still evolve ammonia on being moistened;

(4) roots from maize or from peas free of nodules did not liberate ammonia on incubation. Most of these arguments could be used equally well in support of the view that the ammonia originated from organic nitrogen in the nodules (Wilson, 1940).

The evidence most vital to this theory--that the excised nodules were actually fixing nitrogen when the ammonia was being liberated--is lacking. Other workers have obtained no fixation with nodules kept under the conditions used by Winogradsky, and there is no reason to believe that his experiments were an exception (Wilson, 1940). The quantity of ammonia liberated is much too large to ascribe to fixation without definite proof. Also, nodules are relatively high in an organic nitrogen compound that readily loses a part of its nitrogen as ammonia. Finally, the behavior of the dried material would certainly suggest ammonification rather than nitrogen fixation (Wilson, 1940).

a. Ammonia as an Intermediate in Nitrogen Fixation by Azotobacter

Although the foregoing data in support of the ammonia hypothesis of the mechanism for symbiotic nitrogen fixation are not convincing, it is likewise true that few established facts recommend its rejection (Wilson, 1940). It is of interest, then, to determine whether there is any better proof for the view that ammonia is an intermediate in nonsymbiotic nitrogen fixation by Azotobacter. Numerous workers have suggested that ammonia is an intermediate in nitrogen fixation by Azotobacter, but Kostytschew and his co-workers (1925, 1926) were the first to obtain supporting evidence. They found from 4 to 13 mg. of soluble nitrogen in 400 ml. of filtrates from cultures of Azotobacter agile that had fixed a total of 30 to 70 mg. of nitrogen. About 25 percent was ammonia, and the remainder was amino nitrogen. Since the excreted nitrogen could be found in young cultures, and since the organism did not liberate ammonia from a glucose-peptone solution, Kostytschew claimed that the soluble

products represented intermediates in fixation rather than compounds formed by cell decomposition. He argued that if the ammonia nitrogen and amino nitrogen originated from decomposition, other forms of nitrogen would be present. His conclusion was that ammonia represents the "first" product of fixation and that the amino acids were the first step toward synthesis of protein (Wilson, 1940).

Succeeding investigators were unable to confirm these findings and many decided that the results must be ascribed either to impure cultures or to strain specificity. In 1930 Winogradsky demonstrated that Azotobacter cultures readily formed ammonia if the organisms were grown on silica gel medium containing lactate or succinate as the source of energy. Under these conditions the pH rose to 8 or 9, and ammonia distilled spontaneously from the culture. Other workers (Burk and Horner, 1935; Isakova, 1933) soon verified this finding and extended it to include production of ammonia when glucose was used as a source of carbon in a physiological range of pH. Burk and Horner (1936) have furnished a detailed account of the subject.

One of the most remarkable disclosures in this connection is the claim of Bach and his co-workers (1934) that they had successfully prepared from Azotobacter a cell-free "press-juice" that fixed nitrogen. In one experiment the total nitrogen in one ml. of an "Azotobacter juice," which orignally contained 1.98 mg. of nitrogen (0.622 mg. NH_3-N), rose in nine days to 2.78 mg (0.551 mg. NH_3-N); in the presence of glucose the nitrogen content increased to 14.68 mg., of which 10.68 mg. appeared as ammonia; with mannitol the total nitrogen was 10.65 mg. (8.26 mg. NH_3-N). This apparent realization of the ultimate aim of the research on nitrogen fixation, the isolation of a cell-free enzyme preparation that will fix nitrogen affords strong support for the ammonia hypothesis; but unfortunately the discovery awaits confirmation by

other workers (Burk, 1937; Roberg, 1936b), (Wilson, 1940).

Burk and Horner (1936) have attacked the conclusions of Winogradsky and others who support the ammonia hypothesis on the ground that the experiments have not been sufficiently well controlled to permit differentiation between the products of fixation and those of metabolism. They found that the liberation of ammonia from Azotobacter cultures was independent of the presence of free nitrogen; that is, cultures grown in fixed forms of nitrogen (nitrate, ammonia, amides, amino acids) behave essentially the same as those that secured their nitrogen from the air. They concluded that, therefore, the ammonia originated from oxidative deamination of cellular material.

Roberg (1936a) reported that from 10 to 20 percent of the nitrogen fixed by Azotobacter cultures appeared as soluble products in the medium, but that only a small portion of this nitrogen was ammonia; he agrees that such ammonia probably arises from cell respiration. Winogradsky (1938b), in reply to the criticism of Burk and Horner declares that the disagreement in the results of Roberg and those of Burk and his associates over the forms of nitrogen excreted indicates errors in their experiments--errors that he attributes to faulty cultures. He believes that many, if not all, of the previous findings of ammonia in Azotobacter cultures, including some of his own, are open to question because of the presence of contaminants. Azotobacter, he states, is incapable of ammonifying organic compounds, as was claimed by Burk and Horner, and one criterion of the purity of cultures used in studies on mechanism should be the lack of this function. Since he demonstrated formation of ammonia by Azotobacter cultures possessing no powers of deamination, Winogradsky concludes that this compound must be associated with the process of nitrogen fixation.

Horner and Burk (1939) admit that many of their former conclusions are vitiated because of the questionable purity of their cultures, but they state

that their major conclusion, that the form and extent of nitrogen (free or combined), has been verified in experiments with cultures of known purity. From 10 to 25 percent of the total nitrogen in the cells was excreted by young cultures in these experiments. Virtually all the excreted nitrogen was organic, and a large percentage of it was precipitable by trichloracetic and phosphotungstic acids, indicative of compounds of relatively high molecular weight.

A fundamental question in biological nitrogen fixation is whether the mechanism of fixation by the free-living Azotobacter is essentially the same as that of the symbiotic system (leguminous plants plus Rhizobium). Although some evidence exists in favor of an affirmative view, e.g., both are readily inhibited by combined nitrogen, convincing support has not been available (Wyss and Wilson, 1941). This deficiency arises mainly from a lack of suitable methods for defining the properties of the enzyme system concerned in the process, a deficiency that the physical-chemical studies of the past few years promise to overcome (Wyss and Wilson, 1941). Wilson (1939, 1940), for example, have demonstrated that molecular hydrogen acts as a specific, competitive inhibitor for nitrogen fixation by inoculated red clover plants. Wyss and Wilson (1941) reported that results from four types of experiments, involving both macro and micro estimation of nitrogen fixation by three species of Azotobacter, consistently indicated that H_2 acts as a specific inhibitor of the fixation reaction in these species. Azotobacter cultures grown in an atmosphere in which the pN_2 was reduced to 0.3 atm., the pO_2 kept at 0.2 atm., and the abstracted N_2 either unreplaced or replaced with helium or argon, fixed atmospheric N_2 at the same rate as that observed with cultures grown in air. If, however, H_2 is used to replace the N_2, a significant decrease is observed in both rate and extent of fixation. Since the

symbiotic nitrogen fixation system of red clover responds to H_2 in the atmosphere in essentially the same manner, Wyss and Wilson (1941) concluded that the mechanism of nitrogen by the symbiotic system is similar, if not identical, with that of the fixation system in the free-living Azotobacter. In an effort to determine the mechanism of this type of inhibition, cultures of the bacteria were tested for hydrogenase, the enzyme which Stephenson and associates (1939) found in several species of heterotrophic organisms catalyzing the reversible reaction: $H_2 \rightleftharpoons 2H$. The existence of hydrogenase, the enzyme activating molecular hydrogen was demonstrated by Phelps and Wilson (1941) in cultures of Azotobacter by a methylene blue reduction method as well as by measurements of H_2 uptake in the Warburg microrespirometer. The enzyme was found in cells grown in the presence or absence of molecular hydrogen and using either free or combined nitrogen.

The view that ammonia might be an intermediate in biological nitrogen fixation was suggested almost immediately following discovery of this process. As has been emphasized repeatedly by workers (Burk, 1937; Burk and Burris, 1941; Wilson, 1940), however, the evidence in support has been so lacking in specificity and proper control that it approaches irrelevancy. As a result an alternative mechanism based on hydroxylamine as the key intermediate has received increasing attention. This view, advocated mainly by Virtanen (1938), is based on experiments which deal with symbiotic nitrogen fixation brought about by association of leguminous plants and the root nodule bacteria. Workers have examined the evidence as it applies to this type of fixation and concluded that most if not all would support equally well the view that ammonia is an intermediate (Burris and Wilson, 1945; Wilson, 1940). Nevertheless, an increasing number of authors, especially of texts, present this proposal as a definitely proved mechanism rather than as a suggested hypothesis.

Studies made by Wilson and Workers since 1941 with N^{15} as a tracer have provided an increasing number of experimental findings pointing to the significance of ammonia in fixation by the free-living bacterium, Azotobacter vinelandii. One of the most critical of these is that extremely small concentrations of the ammonium ion will swiftly and completely suppress assimilation of free nitrogen by Azotobacter in contrast to the less rapid and less complete substitution noted when other available fixed compounds of nitrogen are supplied the organism. Wilson, Hull, and Burris (1943) reported a fundamental shift from fixation to assimilation of combined nitrogen, and Burris and Wilson (1946) examined in detail this shift. An aerated culture of Azotobacter vinelandii grown on N_2 was supplied NH_4^+ labeled with N^{15}; the culture immediately stopped fixing N_2 and used NH_4^+ as its exclusive source of nitrogen. When N^{15}-NO_3^- was supplied under the same conditions, no N^{15} was found in the cells for 30 minutes in contrast to its detection in one minute when N^{15}-NH_4^+ was supplied. The uptake of NH_4^+-N was at a constant rate, whereas the rate of assimilation of NO_3^--N increased with time. Aerated cultures of Azotobacter vinelandii previously grown either with N_2 or with KNO_3 assimilated NH_4^+ in preference to NO_3^- when supplied with NH_4NO_3. If NH_4NO_3 was added at the time of inoculation to cultures previously grown with KNO_3, the growing organisms used NH_4^+ preferentially and reduced NO_3^- to NH_4^+. Azotobacter vinelandii grown either with N_2 or NO_3^- has preformed enzyme systems capable of utilizing NH_4^+ immediately and to the exclusion of other nitrogenous compounds. To use NO_3^- the cells must first adapt themselves to it. From these and other studies made with isotopic nitrogen it is concluded (Burris and Wilson, 1946) that present information favors the view that ammonia is the first stable intermediate formed in nitrogen fixation by Azotobacter.

Further evidence for the ammonia theory is the work of Novak and Wilson

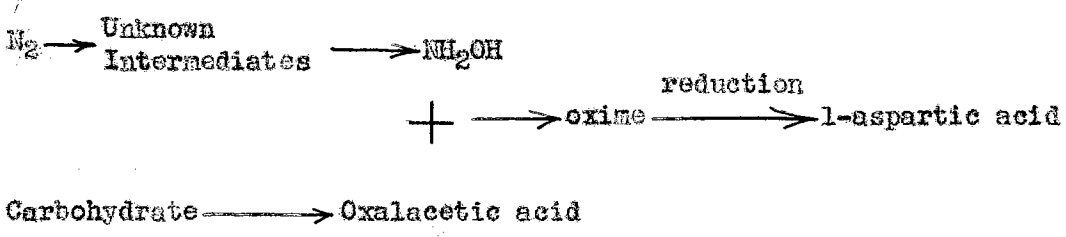
(1948). Free hydroxylamine disappears rapidly from sterile Burk's N-free medium. The compound is extremely toxic to Azotobacter, and its utilization in non-toxic concentrations (less than 1 microgram per ml) is doubtful (Novak and Wilson, 1948). A series of carefully controlled trials demonstrated that the nitrogen in the oximes of pyruvic, oxalacetic, and alpha-ketoglutaric acids is not available to Azotobacter vinelandii. This lack of utilization prevents the use of the specific critical tests that previously established ammonia as an intermediate in nitrogen fixation by this organism (Novak and Wilson, 1948). Until utilization of oxime-N is demonstrated (Novak and Wilson, 1948) so that the critical test can be applied, the hydroxylamine and oxime hypotheses should be rejected.

Segal and Wilson (1949), working with hydroxylamine, found that it disappears rapidly from Burk's N-free medium; its rate of disappearance is decreased by lowering the temperature and increasing the acidity. On prolonged incubation Azotobacter vinelandii was found to grow in Burk's medium plus hydroxylamine in a H_2--O_2 atmosphere; the growth was found to be proportional to the hydroxylamine originally present. The extent of growth could be accounted for completely by ammonia that arises from the decomposition of the hydroxylamine. No quantitative difference was noted in the inhibition by hydroxylamine of nitrogen fixation and the assimilation of nitrogen as ammonium nitrate; specificity for the nitrogen fixation reaction is therefore rejected (Segal and Wilson, 1949). Controlled experiments by these workers demonstrated that hydroxylamine is not utilizable as a source of nitrogen by Azotobacter vinelandii even in the non-toxic concentrations of one to two micrograms per ml.

2. The Hydroxylamine Hypothesis

An alternative theory of the mechanism for biological nitrogen fixation that has considerably more support than the ammonia hypothesis is that the

reduction of free nitrogen proceeds through hydroxylamine. Blom (1931) advanced in its favor several ingenious arguments based on theoretical consideration, but convincing experimental evidence was obtained more or less as a by product of the researches of Virtanen, v. Hausen, and Laine on the excretion of nitrogen by leguminous plants. The chemical nature of the excreted substances led Virtanen (1938) to propose the following mechanism for symbiotic nitrogen fixation:



The experimental evidence submitted by Virtanen and his collaborators will be considered, since it is much more diverse and extensive than that for any other mechanism that has been proposed. Moreover, their studies are noteworthy in that they are among the few in the field of biological mechanisms in which attempts have been made to associate the finding of a given compound with the actual occurrence of the reaction for which it is postulated (Wilson, 1940).

a. Hydroxylamine and Related Products in Symbiotic Nitrogen Fixation

Aspartic acid as the product excreted. - Virtanen attaches great significance to the fact that the initial product excreted by leguminous plants is almost entirely l-aspartic acid. His hypothesis is based primarily on this finding. Wilson (1939) questioned the validity of the conclusion that the excreted aspartic acid must represent an intermediate in the synthesis of N_2 to protein. His argument was that since aspartic acid (or asparagine) constitutes the key amino acid in the nitrogen metabolism of the plant, it

is impossible to state definitely whether a given molecule represents nitrogen that is being built into protein or nitrogen that has already passed through this stage. Virtanen (1939) objected to this interpretation. But the disagreement, as Wilson and Wyss (1939) have pointed out, is really a minor one and should not be allowed to overshadow the fundamental question at issue: does excretion of aspartic acid necessarily imply that fixation of atmospheric nitrogen by leguminous plants proceeds via hydroxylamine (Wilson, 1940)?

Consideration of the modern theories of nitrogen metabolism in plants suggests a negative answer (Wilson, 1940). Because aspartic acid occupies a fundamental position in the nitrogen transformations that occur in plants, it appears that unless the metabolism of legumes fixing nitrogen is definitely abnormal, nitrogen fixed in the nodule would eventually be converted to aspartic acid regardless of the path taken from free nitrogen to the stable form. The "first" product of fixation might be hydroxylamine, as suggested by Virtanen; ammonia as proposed by Winogradsky; or some other compound (amino acid, urea, etc.); but soon after it enters the metabolic stream of the host plant one would expect it to be transformed to aspartic acid (Wilson, 1940).

Occurrence of oxalacetic acid in pea plants. - Virtanen (1938) found that pea plants actively fixing nitrogen contained from 500 to 1000 micrograms of oxalacetic acid per gram of fresh material. Such a finding is somewhat unexpected, since even with muscle tissue, where oxalacetic acid is believed to play an important role in cellular respiration (Szent-Gyorgyi, 1937), its detection is difficult. In connection with studies on the respiratory enzymes of the root nodules, research workers at Wisconsin (Wilson, 1940) could not detect oxalacetic acid in either legumes or non-legumes grown at the Wisconsin station, although the added compound was readily recovered. Torstai peas (Finnish variety) inoculated with the Finnish culture of Rhizobium leguminosarum were examined at different stages of development, but in

no case was oxalacetic acid present (Wyss, Burris, and Wilson, 1939).

These contradictory results are not, however, critical for the hydroxylamine hypothesis, since the acid serves primarily as a convenient means of forming aspartic acid. The failure of Wyss and his associates does not necessarily invalidate the claims for its significance. Though the level reported by Virtanen (1938) is seldom reached, enough of the transient compound might be present to unite with the equally elusive first product of fixation. Nevertheless, it should be recognized that since formation of aspartic acid through oxalacetic acid is believed to occur in non-leguminous as well as leguminous plants, its presence is not necessarily significant for nitrogen fixation reactions. Even more important, its presence does not necessarily prove that the first stable product of fixation is hydroxylamine. The synthesis of aspartic acid from hydroxylamine and oxalacetic acid is only one of the mechanisms by which this amino acid is produced. If free nitrogen is fixed via ammonia, an amino acid, or any other plausible compound, the initial product could probably be converted directly or indirectly into aspartic acid by reacting with oxalacetic acid. Virtanen and Laine (1938) stress the fact that aspartic acid can donate nitrogen to alpha-keto acids, presumably by transamination, to form amino acids. Equally important is the fact that the reaction is reversible. Wyss obtained the following data at the Wisconsin station by adding oxalacetic acid to crushed nodules and incubating for 40 minutes at 37° C. They demonstrate that under these conditions aspartic acid is formed. The nitrogen being supplied either by donators already present or by added alanine (Wilson, 1940).

Fixation by excised nodules. - Interesting evidence in support of Virtanen's hypothesis is his report that fixation of nitrogen can be induced in excised nodules by adding oxalacetic acid. Virtanen (1939) states that in

twenty-five experiments pea nodules supplied with oxalacetic acid fixed nitrogen, but controls supplied succinic acid did not do so. Other investigators have been unable to confirm his finding, but this cannot be regarded in itself as evidence against Virtanen's hypothesis. Others have had difficulty in securing excretion of nitrogen, which undoubtedly occurs in the plants grown at Helsinki. The failure to duplicate the experiments on excretion may be related to the inability to secure fixation by excised nodules. Confirmation of the experiment would not, however, add greatly to its significance, since it might be held with equal reason that the function of added oxalacetic is to convert some other postulated initial product of fixation into aspartic acid. According to Virtanen's (1938) data the nodule already possesses more oxalacetic acid than would be required to combine with all the hydroxylamine likely to accumulate. It is not clear, then, why addition of the acid should induce fixation if it serves only to form aspartic acid from hydroxylamine by way of the oxime (Wilson, 1940).

Oxime of oxalacetic acid. - Isolation of small quantities of an oxime in the excreted products furnishes direct support for Virtanen's scheme. Virtanen and Laine (1939) announced its identification as oximinosuccinic acid, the oxime of oxalacetic acid. Oxime nitrogen has also been detected in the medium in which excised nodules are fixing nitrogen (Virtanen, 1939). Since oximinosuccinic acid is one of the postulated intermediates, the discovery constitutes the most convincing evidence yet presented in support of Virtanen's views. Of interest is the fact that oxalacetic acid and hydroxylamine *in vitro* combines very rapidly; this circumstance would prevent accumulation of the toxic hydroxylamine in the plant. Another line of evidence stressed by Virtanen is that, although hydroxylamine itself is toxic to plants, low concentrations of its oxime will support growth (Wilson, 1940).

b. Hydroxylamine as an Intermediate in Nitrogen Fixation by Azotobacter

Hydroxylamine has been a favorite intermediate in mechanisms proposed for nitrogen fixation by Azotobacter since Blom (1931) claimed to have found traces of the compound in cultures of the organism. Others have been unable to confirm this finding, but Endres (1934, 1935, 1936) detected the oxime of hydroxylamine in Azotobacter cultures. Since the oxime was found in the presence, but not in the absence, of free nitrogen, he concluded that it originated from nitrogen fixation rather than cellular metabolism. Burk and Horner (1935) disagreed with this interpretation on the ground that the organisms will not assimilate hydroxylamine even when present in non-toxic concentrations, and that oxime was found not only in Azotobacter cultures using molecular nitrogen, nitrite ion, and nitrate ion, where its presence might be expected, but likewise in cultures given ammonia nitrogen. Originally these workers suggested that the oxime represented a product of cell decomposition, but later (Burk, 1937) decided that it probably was an unspecific by-product of growth. The major reason for this conclusion is that its presence appears to be associated with the metabolism of growing rather than "resting" cells (Wilson, 1940).

Virtanen and Laine (1937) have reported that of the total nitrogen fixed by Azotobacter about five percent is excreted as aspartic acid with traces of oxime. Horner and Burk (1939) found in their experiments that, although aspartic acid formed a considerable portion of the alpha-amino acids excreted by Azotobacter, these acids constituted only a small fraction of the soluble nitrogen found in cultures of the organism. Since it was also excreted by cultures grown on fixed nitrogen they concluded:

> . . .the specificity of aspartic acid as an intermediate in the fixation mechanism is, therefore, of questionable significance, as far as Azotobacter is concerned (Horner and Burk, 1939).

There have been numerous other studies which pertain to the mechanism of biological nitrogen fixation. Some of these with direct application to the Azotobacter are: Wyss and Wilson, 1941; Wilson and Lind, 1943; Wilson and Burris, 1944, 1947; Wilson, Burris and Coffee, 1943; Wilson, Lee, and Wilson, 1942; Wilson and Wilson, 1942, 1943; Wilson and Lee, 1942; Tove and Wilson, 1948; Tove, Niss, and Wilson, 1949; Suomalainen, 1940; Stoklassa, 1908; Plaut and Lardy, 1949; Molnar, Burris, and Wilson, 1948; Lipman and McLees, 1940; Lineweaver, Burk, and Deming, 1934; Lee and Wilson, 1943a, 1943b; Lee, Wilson, and Wilson, 1942; Karlsson and Barker, 1948; Fife, 1943a, 1943b; Fedorov, 1949; Butkevich and Kolesnikova, 1941; Burris and Wilson, 1944; Burris and Miller, 1941; Burris, Eppling, Wahlin, and Wilson, 1943; Burris, 1942; Burk, Horner, and Lineweaver, 1932; Burk, 1930; Billen and Lichstein, 1949; Allison, Hoover, and Burk, 1933; Allison, 1947.

H. Azotobacter Inoculation of Crops

The interest that has been created by the rather extensive use in the USSR of "Azotogen" as a seed inoculant makes it desirable to consider the available scientific knowledge of the value of such inoculations with free-living bacteria for the purpose of increasing crop yields. It has been reliably reported (Balls, 1946) that "Azotogen" which is a commercial preparation of Azotobacter chroococcum in a peat-soil-calcium-carbonate mixture, was used on five million acres of crops in the USSR in 1942. The use of this material was initiated about 1932, and since that time active research on bacterial inoculants has been conducted by a number of Soviet scientists. These investigations represent a revival of interest in an old subject that at one time engaged the attention of a considerable number of soil bacteriologists.

Interest in the possibility of bringing about nitrogen fixation and increased crop yields through inoculation of soils and plants with free-

living bacteria dates back almost to the beginning of the science of soil bacteriology (Voorhees and Lipman, 1907). The first published claim of such benefits was probably that of Caron in 1895. He (1895, 1900) obtained marked increases in the yields of nonlegumes by inoculation with cultures of various organisms isolated from soils and composts. A preparation of Bacillus ellenbachensis was sold commercially under the name of "Alinit."

Following the discovery in 1901 of the nitrogen-fixing organism, Azotobacter, many new inoculation studies were started. As may be noted in Table II many workers have been involved in the inoculation experiments with Azotobacter, and it is quite readily noted from the table that the results are not in agreement. The strongest supporters of the value of Azotobacter as an inoculant are the Russian scientists.

Although there is no agreement among the Soviet scientists as to the reasons for the beneficial effects reported (Allison, 1947), three main ideas have been advanced. These are: (1) nitrogen is fixed by the bacteria living in the rhizosphere largely on the root excretions; (2) the added bacteria protect the higher plant against pathogenic microorganisms either by discouraging their growth or by destroying them; and (3) the bacteria stimulate plant growth through the production of auxins, hormones, vitamins, and other growth accelerators or regulators. A critical consideration of these ideas leads to the conclusion that if inoculation with Azotobacter is beneficial, the effect is not likely to be due either to nitrogen fixation or to the effect on disease organisms. The auxin-hormone explanation seems more plausible, but at present there are few data to support such a claim (Allison, 1947).

TABLE II

SOME STUDIES ON *AZOTOBACTER* INOCULATION OF CROPS

Investigator	Agent Inoculated	Results Beneficial
Makrinoff, (1924, 1937)	Legumes, non-legumes	x
Gerlach and Vogel, (1902)	Oats, mustard	x
Gerlach and Vogel, (1902)	Carrots	-
Lipman and Brown, (1907)	Soil	-
Stranak, (1909)	Beets	x
Stoklasa, (1908, 1909, 1910)	Oats, potatoes	x
Bottomley, (1908, 1914a, 1914b, 1920)	Soil	x
Russell, (1917)	Soil	-
Emerson, (1918a, 1918b)	Soil	-
Nolte, (1919)	Oats, mustard	-
Zucker, (1928)	Soil	-
Duggeli, (1917)	Soil	x
Brown and Hart, (1925)	Wheat	x
Gainey, (1925)	Soil	-
Sheloumova, et al., (1929, 1933b, 1935a, 1935b)	Tobacco, mustard	x
Sheloumova and Protodiakonov, (1933b)	Tobacco, corn	x
Sheloumova, (1941, 1937)	Beets, potatoes	x
Savostin, et al., (1936, 1937, 1938)	Wheat, oats	x
Demidenko and Timofeieva, (1937a, 1937b)	Sugar beets, oats	x
Meshkov, (1945)	Maize	x
Henckel, (1933)	Wheat	x
Oknina, (1940)	Oats, barley	x
Isakova, (1937a, 1937b, 1938a)	Soil	x
Isakova, (1938b, 1940)	Soil	x
Berezova, et al., (1938, 1939)	Flax	x
Novogrudskij, et al., (1937)	Flax	x
Naumova, (1939)	Wheat	x
Turchin, (1944)	Oats, flax, barley	-
Karunaker, et al., (1936)	Sorghum seed	x
Uppal, et al., (1939)	Rice	x
Jensen, (1940, 1942)	Wheat	-
Greaves and Jones, (1942)	Soil	x
Allison, Gaddy, et al., (1947)	Kale, rape, rye	-

I. Stability of Azotobacter

Stable biochemical mutants of Azotobacter are difficult to obtain by traditional procedures (Karlsson and Barker, 1948; Lindstrom, 1948). The parent strain of the organism is very stable and not much literature is available to indicate many different variants from the normal. Morphological variants are readily noted when the organism is grown on various media which contain various organic nitrogenous compounds (Ward, 1940). These morphological variants may represent one of the various stages in the life cycle of the organism, as described by Lohnis and Smith (1923). Many have called these abnormal forms of the organism "involution" forms.

Stumbo and Gainey (1938) demonstrated that culturing Azotobacter on a high nitrate medium resulted in strains that failed to fix nitrogen even after continued transfers in the absence of fixed nitrogen. Wyss and Wyss (1949, 1950) reported that by culturing large numbers of organisms treated with mutagenic agents and incubation under hydrogen gas, mutants of Azotobacter were isolated that failed to fix nitrogen. They found, however, that the mutants were not too stable because they yielded reverse mutations in high frequency.

Since biochemical mutants have proved so useful in the study of the chemical processes in microorganisms, Lindstrom (1948) attempted to adapt this procedure to investigation of the mechanism of nitrogen fixation by Azotobacter. He was of the opinion that a mutant should be obtained that is unable to use molecular nitrogen but would use ammonia. Considerable difficulty could be experienced in characterizing such a mutant, as nitrogen fixation is the primary basis for identifying Azotobacter. The difficulty is increased because of the little recognized danger of contamination of Azotobacter cultures unless special precautions are taken. Lindstrom exposed pure

cultures of Azotobacter to ultraviolet radiation, and the treated cultures were incubated in a "complete" medium for 24 hours. This culture was plated out on a nitrogen-free medium and incubated in air to allow the nitrogen-fixing cells to appear. The resultant colonies were marked, and the plates transferred to an atmosphere containing ammonia to permit any non-fixing mutants to develop. Identification of the mutants was by specific biochemical tests such as use of sodium benzoate as a source of carbon. Using this technique Lindstrom recovered numerous morphological mutants, but no stable mutants that required ammonia in the presence of molecular nitrogen.

Smith (1935) reported the occurrence of a strain of Azotobacter chroococcum which failed to ferment mannitol. For detecting the presence of Azotobacter in soils, various methods are used, and almost without exception, media for culturing the Azotobacter have contained mannitol. Smith isolated a strain of Azotobacter chroococcum from various soils which could not utilize mannitol as an energy source by substituting a carbohydrate such as dextrin, starch, or sucrose for mannitol in Ashby's agar. He designated the strain as a mannitol-negative strain, since its only distinguishing characteristic seemed to be its inability to utilize mannitol.

Karlsson and Barker (1948) isolated mutants of Azotobacter agilis from X-ray-treated cultures. They isolated originally 7,884 colonies, tested 383 of these for mutants, and found only three biochemical mutants, one of which was lost because of unstability. The amino acid leucine was identified as a growth factor for one mutant. The third physiological variant was detected as an organism unable to utilize glucose for growth. When grown on alcohol, however, it was indistinguishable from the parent strain. They found morphological variants, involving colony form and color quite frequent. The parent strain forms a greenish-yellow water-soluble pigment, but Karlsson

and Barker found approximately 20 percent of the viable irradiated population in each experiment no longer able to form pigment. Similarly, a high proportion of rough colony variants, up to 40 percent, was found in the treated material, whereas the original strain consisted entirely of smooth colony variants.

H. Koeffler (1949) in a personal communication to A. Eisenstark, stated that he had completely negative results in efforts to obtain biochemical mutants in Azotobacter after ultra-violet irradiation.

In comparison with other bacterial species, Escherichia coli for example, most workers have found the Azotobacter to be a very stable group.

III. EXPERIMENTAL

A. An Improved Moist Micromanipulation Chamber Slide With Two Accessory Dissecting Pipettes

For quantitative single cell isolations with the De Fonbrune micromanipulator, an accessory chamber was developed to reduce the time required for the isolation of large numbers of single bacterial cells. The chamber slide to be described here and pictured in exploded diagram (Fig. 1), is essentially a modification and extension of that described by Richter (1948). Although the De Fonbrune micromanipulator was employed, the chamber slide is really best designed for use with the Chambers micromanipulator. Its basic construction features are readily adaptable to the De Fonbrune instrument, however.

The chamber slide consists primarily of three sheets of plastic (Fig. 1A, B,D), 2" x 3" x 1/16" thick, and one sheet of plastic (Fig. 1C), 2" x 3" x 3/8" thick. The operating chamber proper (Fig. 1E) is a centered recess in the front edge of the chamber slide. The base plate (Fig. 1D) and support plate (Fig. 1B) are cemented to the plate supports (Fig. $1C_1$,C_2,C_3) by means of plastic annealing compound, an acrylic cement. When cemented in proper position the manipulator disc chamber (Fig. 1J) will receive the manipulator disc (Fig. 1F). The guide plate (Fig. 1A) is cemented to the support plate (Fig. 1B) by means of the plastic annealing compound, and a coverslip chamber is formed (Fig. 1K). A rounded groove in the recess of the plate support (Fig. $1C_3$) holds a small roll of filter paper (Fig. 1I) and provides a moisture chamber. The manipulator disc (Fig. 1F) is held in the manipulator disc chamber (Fig. 1J) by a disc retainer pin which fits into the disc retainer pin holes (Fig. $1H_A$,H_B,H). The dissecting pipette fits into a rounded groove, the pipette guide (Fig. 1G), in the manipulator disc (Fig. 1F).

In use, the chamber is moistened by the periodic addition of drops of water to the filter paper roll. The filter paper roll, by capillarity, quickly conducts the water to the critical area of the operating chamber. Pipettes

are introduced into the manipulator discs, which have been removed from their chambers, and the tips bent to the desired angle. Syringes are fitted to the pipettes by means of plastic tubing and sterile broth is introduced into the pipettes. The manipulator discs are placed in their chambers and secured by the disc retainer pins. The coverslip is sealed in its chamber by means of vaseline.

This chamber has several distinct advantages over the standard moist chamber slide for isolating numerous single bacterial cells. It precludes the disarrangement of the pipette on the De Fonbrune micromanipulator proper since this pipette is used to select and place single bacterial cells on the coverslip. The accessory pipettes of the chamber may be used to aspirate the single cells from the coverslip, the manipulator disc removed from the chamber, and the cell forced from the pipette by the syringe into a suitable media for propagation. In this manner the manipulator disc and accessory pipette may be placed again into the chamber and reused without preparing a new pipette, or of necessity replacing and re-focusing the pipette on the De Fonbrune instrument. Further, the moisture chamber provides a measurable control of the relative amount of moisture within the chamber when this is essential. It dispenses with the interference so commonly occasioned by filter paper strips within the chamber and excess water on its floor.

The various properties of sheet plastic make it a material of choice and one very easily worked. Its uniform thickness virtually eliminates the necessary but usually laborious task of leveling the walls of the chamber.

Figure 1

An Improved Moist Micromanipulation Chamber
With Two Accessory Dissecting Pipettes

A . . . . . .Guide Plate

B . . . . . .Support Plate

C_1, C_2, C_3 . . . .Plate Supports

D . . . . . .Base Plate

E . . . . . .Operating Chamber

F . . . . . .Manipulator Disc

G . . . . . .Pipette Guide

H, H_a, H_b. . . . .Disc Pin Retainer Holes

I . . . . . .Moisture Chamber

J . . . . . .Manipulator Disc Chamber

K . . . . . .Coverslip Chamber

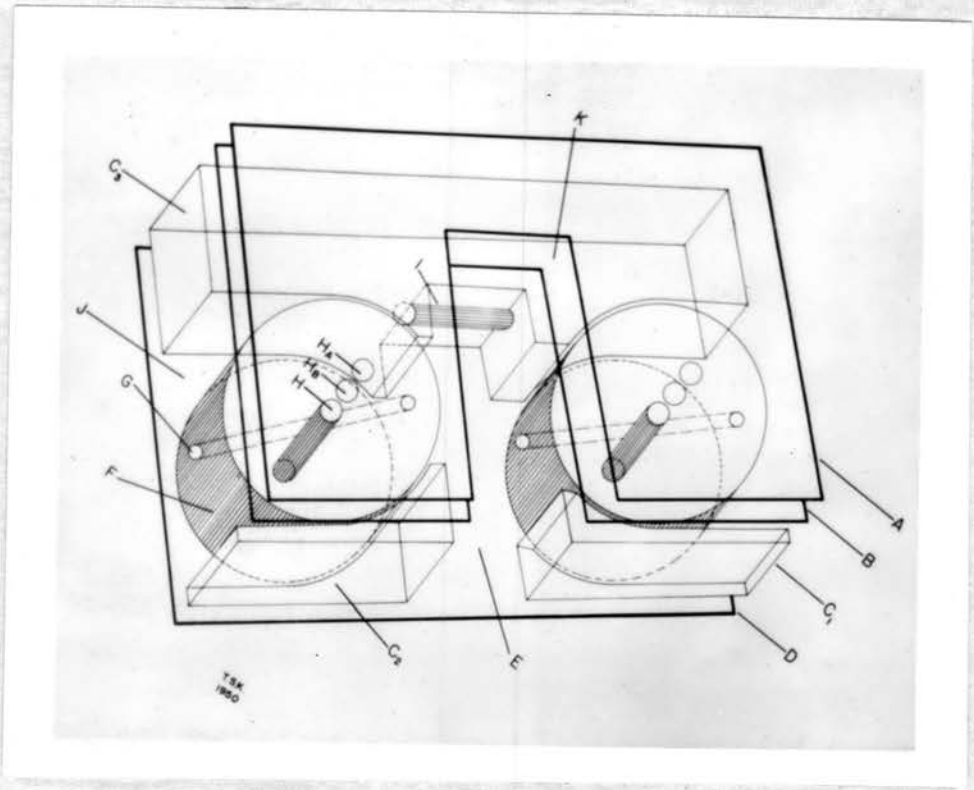


FIGURE 1

B. The Isolation of Single Cells of Abnormal and Normal Morphological Forms of Azotobacter Agilis, M.B., 4.4

Purpose

The interest in the variability and pleomorphism of microorganisms has shown the need for the study of cultures derived from single cells. Morphological variation has been observed by bacteriologists since the early founding of the science. Many have thought the pleomorphic forms represent various stages in a complex bacterial life cycle, while most believe that bacteria exhibit a constancy of form and size. The latter maintain that changes of a culture in form was proof of either contamination, incorrect classification of the organism, or of the existence of "involution" forms.

The Azotobacter species exhibit pleomorphism readily, and many morphological types may be seen. Lohnis and Smith (1923), in studying the life cycle of Azotobacter, pointed out that from every Azotobacter culture not less than seven different growth types can be developed and stabilized. Their work has not been completely accepted and most workers are of the opinion that the pleomorphic forms seen in cultures of Azotobacter are "involution" forms of the type noted when bacteria enter the "death phase" of their growth.

It was the purpose of this experiment to isolate and culture single cells of both normal and abnormal morphological forms of Azotobacter agilis, M.B., 4.4, and to determine if the so-called "involution" types are viable and stable in morphology. The preparation of pure cultures from single cells of the normal morphological type will serve as a possible index of the viability of the "involution" types, and will establish these types as contaminants or as true morphological variants.

Procedure

1. Prepare a suspension of the organism. To secure a large number of

the abnormal morphological types, the organism is cultured on nutrient agar for 24 hours.

2. Moisten the moisture chamber of the improved moist micromanipulation chamber previously described (Fig. 1I).

3. Clean a coverslip in cleaning solution and wipe dry with sterilized cheesecloth or gauze. The most satisfactory cleaning solution was found to be one consisting of:

80 percent ethyl alcohol . . . . 96 parts

Glacial acetic acid. . . . . 3 parts

Ether. . . . . 1 part

Greasing treatment recommended by some to insure isolated droplets is not necessary. Neither is heat sterilization necessary as one can always see any bacteria there may be in the condensation droplets. In hundreds of observations contaminated droplets were never seen.

4. A small loopful of the suspension is placed a few millimeters from one end of the coverslip near the center.

5. The coverslip is placed into the coverslip chamber (Fig. 1K) of the moist chamber with the hanging drop near the closed end and held securely by means of a vaseline seal.

6. A microscope lamp is centered on the mirror from the side and the light adjusted on the optical axis of the microscope. The heat from the lamp will help in the formation of the droplets. The rate of evaporation is readily controlled by varying the distance between the lamp and the mirror. In the moist chamber droplets 4 or 5 microns in diameter will hold an hour or more without evaporation. Usually droplets tend to become confluent only in the closed end of the moist chamber near the large culture drop, when the heat from the lamp is too great or the coverslip too clean.

7. It is of advantage to work with as low magnifications as possible

due to the size of the field and light intensity.

8. The under surface of the coverslip is now brought into focus. This is easily done due to the small droplets in the field. By means of the mechanical stage the edge of the large drop is now brought part way into the field from the closed end of the moist chamber.

9. Pipettes should be sterile and ready. These are best sterilized in a metal case with dry heat. Rigid shank pipettes with tips of 90° and 45° angles were found to be most satisfactory. A 45° pipette is used to isolate single cells in droplets and one of the 90° type is used to remove the cell from the moist chamber.

Much depends on using pipettes with a very small aperture at the capillary end. The method of making these pipettes is as follows: One end of a thin-walled glass tube, 8 to 10 cm. in length and about 4 mm. in diameter, is drawn out in a small flame into a thin-walled capillary tube about 0.5 mm. in diameter. The capillary end is again drawn out over a very small, narrow flame produced by an improvised micro-burner using a 24-gauge hypodermic needle. This small flame should be only 3 or 4 mm. in height. If the capillary tube is held at a proper distance above the flame and drawn out with some force the instant the glass softens, apertures of a very narrow diameter and with smooth edges may be made. About 5 mm. of the tip is then bent to the desired angle in the flame and the pipette is ready to be filled with the culture medium. This may be taken directly from a test-tube. If, on inserting the tip into the liquid, it is found that there is no aperture present, one of sufficient fineness may often be made by scretching the tip very gently on the sides of the test-tube. The size of the aperture may be gauged approximately by the rapidity with which the liquid enters the pipette when suction is applied through the rubber tube attached to the pipette. One may

make and use pipettes having an aperture not exceeding a small yeast cell in diameter. Much coarser ones may be successfully used, but where the aperture is too large the labor is much increased and the accuracy of the work diminished.

10. The 45° pipette is filled with culture medium and placed in the pipette holder of the manipulator proper, with the tip up and the pipette shaft in a horizontal position. A rubber tube connected to a hypodermic syringe is attached to the pipette. Two straight pipettes are introduced into the pipette guides (Fig. 1C) of the manipulator disc (Fig. 1F) of the moist chamber which have been removed from the manipulator disc chamber. The tips of the pipettes are bent to the 90° in the flame. The manipulator discs are replaced in the chamber and the pipettes are connected to hypodermic syringes by means of rubber tubing.

11. Being careful not to make the pipette strike the coverslip or sides of the moist chamber the entire manipulator is moved into working position or until the pipette appears in the microscopic field on the side opposite the large drop. This can be most easily done with the lowest magnification, i.e., with the 16 mm. objective or even with the eyepiece removed. Final lateral adjustments can be made with the manipulator screws when the eyepiece is replaced.

12. Vertical adjustment is easily made with the coarse adjustment screw which is specific for this purpose.

13. With the tip of the pipette nearly touching the coverslip so that it is clearly in focus, the large drop is moved forward until it strikes the tip of the micropipette. The pipette will immediately fill with liquid and organisms. With the vertical adjustment the pipette is slightly lowered and the coverslip moved back by means of the mechanical stage. The pipette is now raised until it touches the coverslip when some of the liquid with the

microorganisms will be seen to wet the coverslip. Upon lowering the pipette a small droplet will be left with several organisms in it. This operation can be repeated again and again until parallel rows of droplets have been made. The first droplets usually contain several cells. The last ones may contain one or more or none. The adjustments are so easily controlled that a single cell can be transferred from one droplet to another with ease. The droplets formed by condensation are often convenient for this purpose as they are sterile.

14. When the cell selected is isolated in a droplet, the manipulator carrying the 45° pipette is toward the open end of the moist chamber by the coarse adjustment screw on the manipulator. A 90° pipette contained in one of the manipulator discs of the moist chamber (Fig. 1) is moved into the position previously held by the 45° pipette.

15. The advantage of the tubing and hypodermic syringe is now seen. If the plunger of the syringe is forced in slightly at the time the tip of the pipette enters the droplet and then gently pulled back, the droplet and the cell will be sure to enter the pipette.

16. By means of the mechanical stage the droplet and single cell are centered on the optical axis of the microscope. The 4 mm. objective is now focused on the droplet with the tip of the pipette immediately under it. With the vertical adjustment, achieved by rotating the pipette in its pipette guide, the pipette tip is made to enter the droplet and the droplet is aspirated into the pipette. The bacterium may be seen to enter the pipette tip.

17. The pipette is now lowered to a safe distance below the coverslip by rotating again in the pipette guide, and the manipulator disc is removed from the manipulator disc chamber after the pin has been removed from the disc pin retainer holes (Fig. 1H, H_a, H_b) of the moist chamber.

18. The isolated cell may be removed from the pipette in several ways. The tip of the pipette carrying the single cell may be broken off with a fine pointed sterile forceps and dropped into sterile culture media. It was found that an alternative method equally as satisfactory is to place the tip of the pipette over an agar medium and force the cell from the pipette by means of the plunger of the syringe. In forcing the cell from the pipette about 0.1 ml. of the culture medium in the pipette is forced onto the surface of the agar. This aids in furnishing a moist condition necessary for multiplication of the single cell. The bottom of the Petri dish is marked with a wax pencil at the site of inoculation with the cell. In quantitative isolations many single cells may be placed in one Petri dish.

Results

Single cells were isolated of 200 of the "involution" forms and 100 of the normal morphological forms in the manner described using the De Fonbrune micromanipulator. Of the "involution" forms 72 cultures were obtained; and 87 cultures from single cell isolations of the normal forms were obtained.

Microscopic examination of the cultures of the "involution" forms revealed a reversion to the normal form of the parent strain. Subsequent subculturing on nutrient agar produced the abnormal forms again in abundance. Macroscopic observation of the cultures of the normal forms showed a constancy of normals which produced an abundance of the abnormal forms when subcultures were made on nutrient agar. The ability of this medium to produce these morphological variants is considered by Ward (1950).

The failure to get all of the single cells to grow has been encountered by all who have undertaken to grow cultures from single cells of bacteria. The growth of isolated single cells of bacteria may involve a quantitative relation of the cell to the volume of the culture medium or a mutual action

of several cells on each other.

The success in culturing single cells of the abnormal type is indication of their viability. Their failure to produce stable abnormal types was readily noted and the reversion to the normal morphological form of the parent strain is evidence that they do not represent contaminants, but suggests they may possibly be one of the seven different growth types described by Lohnis and Smith (1923), but their stabilization as suggested by these workers was not confirmed.

C. APPLICATION OF THE SERUM-REACTION FOR THE PURPOSE OF DETECTING ANTIGENIC CHANGES IN MUTANT STRAINS OF AZOTOBACTER

Purpose

At present, the species of Azotobacter are classified according to the color of the pigments produced by them. This method of classification is not altogether satisfactory because it has been noted that the color of the pigment produced by the various species will vary directly with the age of the culture, as well as with many other factors.

A more satisfactory method of classification might be one based upon an antigen-antibody reaction. A search of the literature revealed one paper by Japanese workers, Aso and Yoshida (1927), in which an attempt was made of an application of the serum reaction in the classification of Azotobacter. No other literature was found to support or deny the validity of this work.

It was the purpose of this experiment to determine if antibodies to Azotobacter spp. could be readily produced for detecting minute changes in mutant strains of the Azotobacter. Since minute differences may be detected by the antigen-antibody reaction, it lends itself well to experimental investigations. The possibility of the serum-reaction as a method of classification of the Azotobacter spp. was also considered.

Procedure

Six different strains of Azotobacter were used for the preparation of antigen for the serological tests. These were:

Azotobacter agilis	M.B.	4.4
Azotobacter vinelandii	A.T.C.C.	9104
Azotobacter Illinois		
Azotobacter chroococcum	A.T.C.C.	9045
Azotobacter agile	A.T.C.C.	9042
Azotobacter indicum	A.T.C.C.	9037

As a control measure a multiple antigen of Azotobacter agilis, M.B., 4.4,

and Salmonella typhosa[1], which is known to produce a high titer of agglutinins, was used to inoculate a rabbit, guinea pig, hamster, and chicken.

Preparation of Antigens

Since variation is apparently not uncommon with bacteria, and since this phase of work was done to determine the readily availability of antibodies to the Azotobacter for purposes of detecting variation within the species, the cultures used in the preparation of the antigen were propagated from single isolated cells of the organism. These single cells were isolated by the De Fonbrune micromanipulator. Since the immune sera were to be used in determining variation it was thought that cultures from a single cell would better lend themselves to the production of a more normal serum, that is, a serum produced by an antigen with as little variation as possible.

Each of the Azotobacter organisms was inoculated into Ashby's nitrogen-free broth and incubated for 12-18 hours at 27° C.

Ashby's Nitrogen-Free Broth

Sucrose (Saccharose)	15.0 gm.
K_2HPO	0.2 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
NaCl	0.2 gm.
$CaSO_4 \cdot 2H_2O$	0.1 gm.
$CaCO_3$	5.0 gm.
H_2O (distilled)	1000.0 ml.

The organisms were then seeded on the surfaces of Ashby's nitrogen-free agar, slanted in four ounce medicine bottles, and incubated for 24-48 hours at 27°C. Two milliliters of sterile physiological saline were then added to these culture bottles, rocked back and forth gently, and allowed to set for twenty minutes.

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[1] Obtained from the Oklahoma State Public Health Department.

These saline suspensions of Azotobacter cells were transferred to sterile test tubes and placed in the Kahn shaking machine for two hours' vigorous shaking. The suspensions were heated in boiling water for two hours and replaced on the shaking machine for a second two-hour period of vigorous shaking, after which they were filtered through sterile cotton into sterile tubes. Motility and morphological studies were made of each suspension.

Each suspension was diluted with sterile physiological saline to a density equal to tube Number 3 of the McFarland nephalometer (approximately 9×10^7 cells/cc.). Formalin was added to a final concentration of 0.3% and the suspensions were placed in rubber-stoppered vaccine bottles, submerged in a water bath at 60° C. for thirty minutes and labeled. Each suspension was checked for sterility by: (1) adding 0.5 ml. to slants of Ashby's nitrogen-free agar; (2) adding 0.5 ml. into a tube containing Ashby's nitrogen-free broth; (3) incubating these for 48 hours.

TABLE III

Schedule for Animal Inoculations with Azotobacter Antigen

Day	Antigen dosage	Type of Antigen	Inoculation Route
1	0.5 c.c.	Killed antigen	Intradermally
3	0.5 c.c.	Killed antigen	Intradermally
5	1.0 c.c.	Killed antigen	Intradermally
7	0.5 c.c.	Killed antigen	Intravenously
9	1.0 c.c.	Killed antigen	Intravenously
11	1.0 c.c.	Killed antigen	Intravenously
13	0.5 c.c.	Live antigen	Intravenously
15	1.0 c.c.	Live antigen	Intravenously
17	1.0 c.c.	Live antigen	Intravenously
19	1.5 c.c.	Live antigen	Intravenously
19	Bleed and check titer for agglutinins		
20	Bleed and check titer for agglutinins - if sufficiently high, bleed the rabbit on the 21st day by cardiac puncture.		

TABLE IV

Schedule for Animal Inoculations with the Multiple Antigen of Azotobacter agilis, M.B., 4.4, and Salmonella typhosa

Day	Antigen Dosage	Type of Antigen	Inoculation Route
1	1.5 c.c.	Killed antigen	Intraperitoneally (Hamster and Guinea Pig)
3	1.5 c.c.	Killed antigen	"
5	1.5 c.c.	Killed antigen	"
7	1.5 c.c.	Killed antigen	Intravenously (Rabbit and Chicken)
9	1.5 c.c.	Killed antigen	"
11	1.5 c.c.	Killed antigen	"
13	1.5 c.c.	Killed antigen	"
15	1.5 c.c.	Killed antigen	"
17	1.5 c.c.	Killed antigen	"
19	1.5 c.c.	Killed antigen	"
21	1.5 c.c.	Killed antigen	"
28	1.5 c.c.	Killed antigen (as a booster)	"
35	Bleed by cardiac puncture.		

Immunization

Six rabbits were chosen and prior to inoculation 10.0 cc. of blood were obtained by cardiac puncture. This blood was used in determining initial titers to agglutinins and precipitins and, after immunization of the animals, used as controls for the titration of agglutinins and precipitins and as normal serum for the complement fixation reaction. The six rabbits were inoculated by the schedule shown in Table III.

Azotobacter agilis, M.B., 4.4, and Salmonella typhosa antigens were mixed and used as a multiple antigen for the inoculation of a rabbit, guinea pig, hamster, and chicken. The Salmonella typhosa was used as a control to determine the ability of the species to produce antibodies. These animals were inoculated according to the schedule shown in Table IV.

Titration of Agglutinins by the Macroscopic Method

The macroscopic method of agglutinin titration is the one usually employed in experimental investigations. The action of immune serum on homogenous suspensions of bacteria causes the cells to stick together forming flocculi which are easily visible to the naked eye, and which, on standing, settle to the bottom of the tube, leaving the supernatant fluid clear. The incubation period varies, but four hours in the 37° C. water bath is most desirable. The bacterial vaccine prepared for production of the agglutinins may be used as agglutinogens.

The scheme for the agglutination technique was used as outlined by Sherwood (1946), incubated at 37° C. for four hours and observed carefully by transmitted light. The tubes were placed in the icebox overnight and read again on the following day. Distinct clumps should be seen in a positive reaction. Settling alone is not agglutination. The control tube, # 11, is to guard against spontaneous agglutination of the antigen. Shaking the tubes

in an effort to resuspend the bacterial cells and checking with the control was done as a further check in reading the agglutination reaction.

The Precipitin Reaction

One of the serological reactions of considerable value is the precipitin reaction. If an antigen which is in solution such as an extract of bacterial cells is mixed under proper conditions with its appropriate antiserum (one which contains the specific precipitins), a visible reaction will take place. The antigen-antibody reactions may be demonstrated by the "ring test" method which involves the careful overlaying of tall columns of antiserum with successive dilutions of the antigen. A hazy zone of precipitation should occur at the interface of the two liquids.

The antigen for the precipitin test was prepared by suspending bacterial cells in physiological saline and breaking up the cells by repeated freezing and thawing.

In the titration of precipitin antiserum by the "ring test" method, the immune serum is undiputed and the antigen is used in varying dilutions. A positive test is indicated by a fuzzy white ring forming at the point of contact between the antigen and the immune serum. The ring is looked for after 2, 4, 10, 20, and 30 minutes incubation at room temperature. The tubes are then tipped so that the contents are mixed and they are then allowed to stand overnight in the icebox. The following morning, the test reading is rechecked, looking for the precipitate in the bottom of the tube.

Control tubes 7, 8, and 9 should remain clear at all times, and should not form a precipitate on overnight incubation. Antisera for the precipitin test must be perfectly clear, free from bacterial contamination, excess lipoids, or dissolved hemoglobin. Likewise the antigen must be stable in salt solution and perfectly clear.

The scheme for the titration of the precipitin antiserum was followed as outlined by Sherwood (1946).

Bacterial Complement Fixation

Bordet and Gengou (1901) discovered that one could ascertain whether a given sample of serum contained antibodies for a specific bacterial antigen by mixing the two together with complement and later testing with sensitized red blood cells to see if the complement had been bound. Much work has been done to improve on the original techniques of Bordet and Gengou. The scheme for bacterial complement fixation used on the six strains of Azotobacter was followed as outlined by Sherwood (1946).

Results

The six rabbits which were used for the Azotobacter strains in this problem were not all from the same dam, and were inoculated in the manner outlined. Initial bleedings of the rabbits showed no initial titer to the antigen. Agglutination tests, precipitin tests, and complement fixation reactions failed to demonstrate the presence of specific antibodies in the sera of the rabbits to the antigen of the Azotobacter species (Tables V, VI, VII).

The hamster, guinea pig, rabbit, and chicken inoculated with the multiple antigen were bled and the serum tested for agglutinins and precipitins of Azotobacter agilis, M.B., 4.4, and Salmonella typhosa. No antibodies to the Azotobacter sp. were demonstrated, but a high titer of agglutinins were produced to the antigen of Salmonella typhosa (Tables V, VI, VII, VIII, IX).

Conclusions

It would seem from the work done here that the Azotobacter species are poor antigens, but it can be easily demonstrated by staining techniques that the cells are rich in protein, Lewis (1937).

The results appear to indicate a negative response of the animal species

to the Azotobacter antigen and since good response was noted with the antigen of Salmonella typhosa, one may conclude that the Azotobacter antigen was not species specific. If this conclusion may be drawn it seems to be that in the inoculated animal structural features of foreign proteins which are duplicated in some of the animal's own protein do not elicit the production of antibodies. The very low titer of agglutinins, 1:20, to the Azotobacter antigen obtained in the chicken and rabbit sera is not significant. Since the results obtained by the complement fixation technique on the rabbits inoculated with the Azotobacter antigen were negative, it was thought that the multiple antigen did not warrant the use of the technique.

Because of some peculiarity, antibodies were not produced to the Azotobacter antigen. The antigen-antibody reaction can not be used to detect mutant stains of Azotobacter, or as a method of classification, until further research has been done on the serological nature of the organism. This problem offers many possibilities for further research, probably in the preparation of the antigen for the inoculation procedures.

TABLE V

Titration of Agglutinins Produced by Six Different Domestic Rabbits to Antigens of Six Strains of Azotobacter

ANTISERA	Rabbit Number	ANTIGEN: Agglutinin Titer					
		Azotobacter agilis, M.B., 4.4.	*Azotobacter vinelandii* A.T.C.C. 9104	*Azotobacter* Illinois	*Azotobacter chroococcum*, A.T.C.C. 9045	*Azotobacter agile*, A.T.C.C. 9042	*Azotobacter indicum*, A.T.C.C. 9037
Azotobacter agilis, M.B., 4.4	1	20					
Azotobacter vinelandii, A.T.C.C. 9104	2		20				
Azotobacter Illinois	3			--			
Azotobacter chroococcum, A.T.C.C. 9045	4				--		
Azotobacter agile, A.T.C.C. 9042	5					20	
Azotobacter indicum, A.T.C.C. 9037	6						--
All Control Tubes		--	--	--	--	--	--

TABLE VI

Titration of Precipitins Produced by Six Different Domestic Rabbits to Antigens of Six Strains of Azotobacter

ANTISERA	Rabbit Number	ANTIGEN: Azotobacter agilis, M.B., 4.4.	Azotobacter vinelandii A.T.C.C. 9104	Azotobacter Illinois	Azotobacter chroococcum, A.T.C.C. 9045	Azotobacter agile, A.T.C.C. 9042	Azotobacter indicum, A.T.C.C. 9037
		Precipitin titer					
Azotobacter agilis, M.B., 4.4	1	100					
Azotobacter vinelandii, A.T.C.C. 9104	2		100				
Azotobacter Illinois	3			---			
Azotobacter chroococcum, A.T.C.C. 9045	4				---		
Azotobacter agile, A.T.C.C. 9042	5					100	
Azotobacter indicum, A.T.C.C. 9037	6						---
All Control Tubes		---	---	---	---	---	---

TABLE VII

The Complement Fixation Reaction of Antisera from Six Different Domestic Rabbits to Antigens of Six Strains of Azotobacter

ANTISERA	Rabbit Number	ANTIGEN: Reaction					
		Azotobacter agilis, M.B., 4.4.	*Azotobacter vinelandii* A.T.C.C. 9104	*Azotobacter* Illinois	*Azotobacter chroococcum*, A.T.C.C. 9045	*Azotobacter agile*, A.T.C.C. 9042	*Azotobacter indicum*, A.T.C.C. 9037
Azotobacter agilis, M.B., 4.4	1	---					
Azotobacter vinelandii, A.T.C.C. 9104	2		---				
Azotobacter Illinois	3			---			
Azotobacter chroococcum, A.T.C.C. 9045	4				---		
Azotobacter agile, A.T.C.C. 9042	5					---	
Azotobacter indicum, A.T.C.C. 9037	6						---
All Control Tubes		---	---	---	---	---	---

TABLE VIII

Titration of Agglutinins Produced by Four Animal Species to the Multiple Antigen of Azotobacter agilis, M.B., 4.4, and Salmonella typhosa

ANTISERA	ANTIGEN							
	Azotobacter agilis, M.B., 4.4				Salmonella typhosa			
	Agglutinin Titer							
	HAMSTER	GUINEA PIG	RABBIT	CHICKEN	HAMSTER	GUINEA PIG	RABBIT	CHICKEN
Azotobacter agilis, M.B., 4.4	---	---	20	20				
Salmonella typhosa					640	40	5120	2560
All Control Tubes	---	---	---	---	---	---	---	---

TABLE IX

Titration of Precipitins Produced by Four Animal Species to the Multiple Antigen of Azotobacter agilis, M.B., 4.4, and Salmonella typhosa

ANTISERA	ANTIGEN							
	Azotobacter agilis, M.B., 4.4				Salmonella typhosa			
	Precipitin Titer							
	HAMSTER	GUINEA PIG	RABBIT	CHICKEN	HAMSTER	GUINEA PIG	RABBIT	CHICKEN
Azotobacter agilis, M.B., 44	---	---	---	---				
Salmonella typhosa					---	---	100	100
All Control Tubes	---	---	---	---	---	---	---	---

D. An Experiment to Determine if a Rabbit will Retain or Excrete Radioactive Azotobacter Antigen

Purpose

Antigen prepared from cells of Azotobacter agilis, M.B., 4.4, did not elicit the production of antibodies when inoculated into a rabbit. The purpose of this experiment is to determine the retention or excretion of radioactive Azotobacter antigen.

Procedure

A rapidly proliferating culture of Azotobacter agilis, M.B., 4.4, was inoculated into Ashby's nitrogen-free agar medium, containing radioactive phosphorous as the only source of phosphorous for the organism. Since phosphorous is required in high concentrations for growth of the organism, the radioactive form is incorporated directly into the bacterial cell.

The culture is harvested while young and thoroughly washed to remove any of the soluble radioactive material taken from the medium in the harvesting procedure. The cells are diluted to a density equal to the number 3 tube of the McFarland Nephalometer (approximately 9×10^7 cells per milliliter) and 1.5 ml. used for intravenous inoculation of the rabbit.

The rabbit was sacrificed in 24 hours and the various tissues removed from the body, 1.0 gram samples of which were dried, and placed in the Berkley Decimal Scaler to determine the relative amount of radioactivity present in each tissue.

Results

High concentrations of the radioactive material were found in the kidney, spleen, adrenal gland, lung, liver, and lymph glands as shown in Table X. Fairly large quantities of the radioactive material were found in excretory products as well.

The experiment was used to determine if the cells of the Azotobacter

antigen or cellular material were being retained to any degree within the body of the animal, or if they were being excreted. The results indicate a good retention. From the degree of radioactivity of excretory products, however, it appears that some of the antigen is being excreted fairly rapidly and in good quantities. Whether the body is retaining the phosphorous from the cells of the organism for maintenance of proper salt balance or for use in the phosphorylation of glucose and intermediates in the body glycolysis mechanism of carbohydrate metabolism can not be concluded from this experiment. Further work is necessary for these determinations.

Relative Radioactivity of Various Tissues from a Rabbit Inoculated with Radioactive Azotobacter Antigen

1.0 Gram Wt. of Tissue	Ionizations Per Minute
Blood	362
Urine	4,879
Spleen	39,450
Kidney	9,830
Pancreas	5,620
Adrenal Gland	11,805
Thymus Gland	4,660
Lung	24,100
Heart	4,850
Liver	20,200
Bile	4,500
Gut (filled with fecal matter)	3,500
Sub-Maxillary Lymph Gland	11,150
Parotid Gland	6,190
Lachrymal Gland	6,800
Eye Ball	734
Cerebellum	7,100
Cerebrum	3,000
Yellow Bone Marrow	8,000
Background Radiation	72
Radium Standard	2,061
Antigen, 1/20 ml.	90,972

TABLE X

E. Induced Variations in a Strain of Azotobacter agile

Purpose

A field of intense study in recent years has been that of biochemical genetics (Williams, 1950). Through this field of study new light has been thrown upon the mechanism of gene action. This information together with previously known facts has made possible the investigation of the existence of gene-like determinants in microorganisms which had not readily lent themselves to common genetical analyses. These developments opened up the previously almost nonexistent field of bacterial genetics, and on the basis of recent evidence (Gray and Tatum, 1944; Lederberg, 1947) it has been fairly well established that at least some bacteria possess "organizers" that correspond to the genes in the higher forms.

Like the higher organisms a great hereditary stability, even potentially existing characteristics such as adaptive enzymes, characterize bacteria. Assumption then must be made of the existence of some precise mechanism whereby the characteristics of the parent strains are exactly duplicated in the offspring. A certain degree of mutability is exhibited by most bacterial species, and resultant new characteristics are readily noted in successive generations. The frequency of appearance of mutant forms is increased greatly by destructive and toxic agents that also increase mutation rates in higher organisms. And, as far as has been established, the function of the hereditary determinants of bacterial species seems to be the same as that of the genes in the higher forms. Indications of this are shown by the fact that artificially induced variants with specific growth factor requirements are obtainable by the same method as in sexually reproducing organisms (Gray and Tatum, 1944). It is assumed that these variants are unable to carry out a particular chemical process in a synthetic reaction chain as in the case of similar mutants of Neurospora, although this is yet to be specifically demonstrated.

A close resemblance between the basic hereditary mechanisms of bacteria and higher forms seemed so probable that this experiment was an effort to isolate known types of biochemical mutants, and also variants with specific genetic blocks in some major metabolic pathways, such as oxidative dissimilation of organic compounds. Since it was not certain that such mutations could be isolated from irradiated material, the process of nitrogen fixation was selected as a second choice, as it is known to be dispensable in the presence of nitrogenous compounds. The Azotobacter species represent suitable tools for the study of the mechanisms of both oxidative dissimilation and nitrogen fixation and was therefore chosen for this investigation. In an effort to induce variations in an Azotobacter species it was subjected to three mutagenic agents: ultra-violet irradiation, methyl-bis (beta-chloro-ethyl) amine hydrochloride, and radioactive phosphorous.

Procedure

The species imployed in this experiment was Azotobacter agilis, M.B., 4.4, obtained from C.B. van Niel. Uniformity in colony size was attained after several single colony isolations, and subsequently all experiments were started from single colonies. Azotobacter agilis was found to be more satisfactory than other Azotobacter species for these experiments because the cells are more easily separated and more easily identified by microscopic examination.

The basal medium composed of the following was used when nothing else is specified:

Ethyl Alcohol	10.0 ml.
K_2HPO_4	1.0 g.
$MgSO_4$	0.2 g.
$CaSO_4$	0.1 g.
$FeSO_4$	Trace

$NaMoO_4$	Trace
Distilled water	1000.0 ml.

The pH was adjusted to 7.2 with hydrochloric acid. In solid media 1.5 percent agar was included. The incubation temperature was 28 to 30° C.

For the isolation and irradiation procedures a complete medium was employed. This medium is a partially selective medium.

Ethyl alcohol	10.0 ml.
Glucose	0.1 g.
Yeast Extract	0.2 g.
K_2HPO_4	0.4 g.
$MgSO_4$	0.2 g.
$CaSO_4$	0.1 g.
$FeSO_4$	Trace
$NaMoO_4$	Trace
Distilled water	1000.0 ml.

A 0.04 percent aqueous bromthymol blue indicator solution was added to the medium, and hydrochloric acid was used to produce a grass-green color to the medium, with a resultant pH of approximately 7.2. The important features of this medium are: (a) alcohol is the only abundant energy source, so that colonies unable to utilize it for growth appear small; (b) the low buffering capacity and the indicator permit the detection of small amounts of acid or alkali if formed; (c) the total amount of nitrogen, added in the form of yeast extract, is so standardized as to permit only limited growth in the absence of nitrogen fixation; (d) the presence of yeast extract should permit development of variants requiring growth factors. This selective medium made the detection of variants with disturbances in the nitrogen fixation or alcohol oxidation mechanisms more probable, but did not discriminate

against any types of variants. Thus the probability of finding growth factor variants or mutants unable to oxidize glucose was the same as if the colonies were tested at random.

Exposure to Ultra-violet Irradiation

A broth culture of Azotobacter agilis, M.B., 4.4, four hours old was irradiated with ultra-violet for thirty minutes at a distance of three inches. The tube containing the culture was a celluloid centrifuge tube 10 x 2.7 cm. and contained the culture in broth to a depth of 1.25 cm, making a volume of approximately 7.2 ml.

A coat hanger was bent in such a manner that it could be used to hold the culture tube which was taped to it. Every five minutes the hanger was shaken to mix the culture being irradiated. Samples from the irradiated broth were streaked onto a complete medium immediately after irradiation, 30 minutes after irradiation, 60 minutes after irradiation, 3 hours after irradiation, 6 hours after irradiation, 24 hours after irradiation, and 21 days after irradiation.

Following a suitable incubation period growth supported by the complete medium was examined. Approximately 100 colonies were isolated and examined. The colonies appeared as the normal strain, and microscopic observation of the growth revealed cells undistinguishable from the normal. Upon transfer to a completely nitrogen-free medium, and incubation, growth was noted in 24 hours. A normal un-irradiated culture of the organism was used as a control, and the amount of growth on the nitrogen-free medium of the irradiated cultures was comparable to that of the normal.

Several attempts were made to induce variation by ultra-violet irradiation, but no success was obtained.

Exposure to Nitrogen Mustard (Methyl-bis (beta-chloroethyl) amine hydrochloride)

One milliliter of a 1:100 dilution of nitrogen mustard was added to a broth suspension of a rapidly proliferating culture of Azotobacter agilis, M.B., 4.4, and the mixture neutralized with hydrochloric acid, using brom-thymol blue as an indicator. Five minutes, 30 minutes, 60 minutes, 3 hours, 6 hours, and 24 hours after exposure to the nitrogen mustard, cells were plated out on the basal medium.

After colonies developed, they were examined for possible pigment and colonial variants. A photographic record of these plates were then made by placing plates in a photographic enlarger and projecting on sensitive paper. After this, an agar layer of the complete medium was placed on the surface of the basal medium, and incubated. New colonies represented possible variants with nutritional deficiencies, as indicated in Figure 2. The new colonies were transferred to agar slants of the complete medium and a microscopic study was made of each.

Approximately 200 colonies were isolated from cultures exposed to nitrogen mustard. Table XI lists the types of variants which may have been produced. Many of these were found to be unstable and were eliminated after further transfers.

Exposure to P^{32} Irradiation

A rapidly proliferating culture of Azotobacter agilis, M.B., 4.4 was grown in a complete medium containing P^{32} which was obtained as H_3PO_4 from the Oak Ridge National Laboratory, Oak Ridge, Tennessee. Radiation counts of this medium were kept at approximately 28,000 cpm./ml. when cups were placed on the second shelf of the Berkley decimal scaler. By comparison with a radium DEF standard, this represented approximately 1.23×10^{-1} MC per ml.

Figure 2

The Agar Layering Method Used for Detecting Possible Biochemical (Nutritional) Variants After Exposure to Mutagenic Agents.

A. This represents the petri dish containing the basal medium and colonies of the organism after exposure to the mutagenic agents. Note there are seven colonies.

B. This represents the same petri dish as in A. but has been layered with the complete medium. Note the original seven colonies and the six smaller colonies which represent possible biochemical (nutritional) variants.

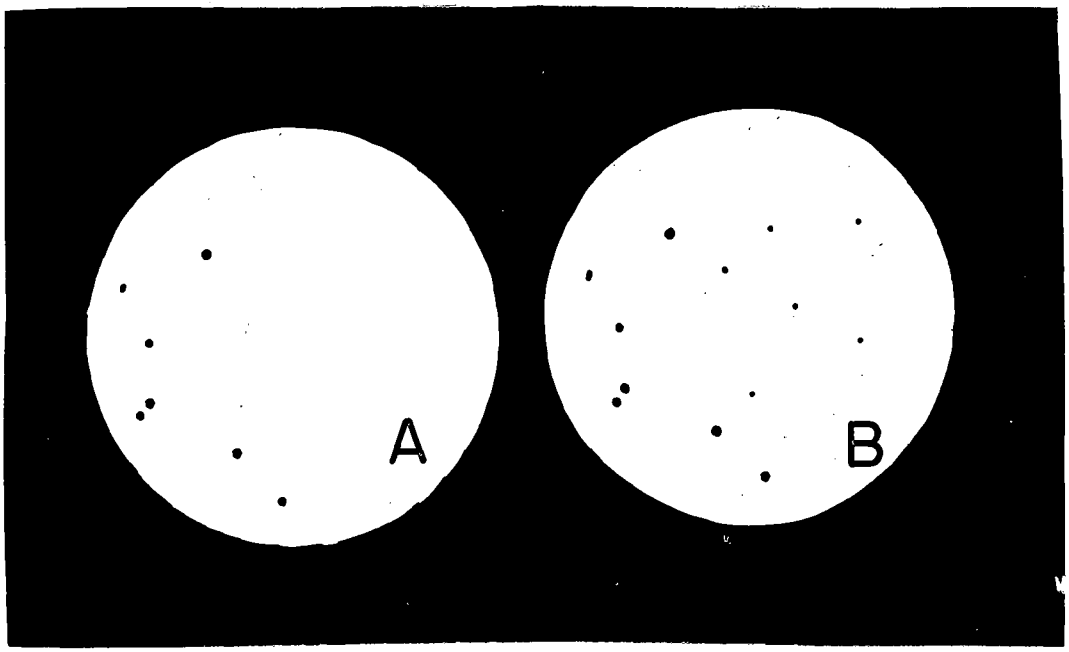

FIGURE 2

of medium. By initial trials this dilution was determined and was found to kill approximately 99 percent of the organisms.

Cells were plated out on the basal medium and after colonies developed, they were examined for possible pigment and colonial variants. A photographic record of these plates was made by placing the plates in a photographic enlarger and projecting on sensitive paper. After this, an agar layer of complete medium was placed on the surface of the basal medium, and incubated. New colonies represented possible variants with nutritional deficiencies, as indicated in Figure 2. The new colonies were transferred to agar slants of the complete medium and a microscopic study was made of each.

Approximately 2000 colonies exposed to P^{32} have been isolated by use of this method. Each culture which was studied was re-checked for possible contamination. Table XI lists the types of variants which may have been produced. Many of these were found to be unstable and were eliminated after further transfers.

Results

Possible Variants: As may be noted in Table XI several types of possible variants were noted and isolations of specific representatives of each type were made. The motility, morphological, and pigment variants were easily determined through macroscopic and microscopic studies of colonies that developed after the procedure of layering with the complete medium. The data are not extensive enough to warrant a statement as to the mutation frequency, particularly since the methods do not ensure detection of all variants.

Approximately 2400 single colony isolations were made from irradiated material, but only 162 colonies were specifically tested for altered physiological characteristics. The majority of these were small or otherwise aberrant colonies.

X Represents Possible types of Variants

TABLE XI

Types of Possible Variants Isolated

Isolation Number	Motility	Morphology	Pigment	Nutrition
P- 9		X		
P- 10		X	X	
P- 12			X	
P- 13			X	
P- 16			X	
P- 18			X	
P- 20		X	X	
P- 27			X	
P- 38		X	X	X
P- 42			X	
P- 47	X	X	X	
P- 54			X	
P- 58			X	
P- 66	X			
P- 68			X	
P- 82		X	X	
P- 84			X	
P- 85		X		
P- 92	X			
P- 96			X	
P- 98			X	
P-102			X	
P-114	X			
P-118	X		X	
P-120	X			
P-121			X	
P-122	X			
P-144	X			
P-154				X
P-155				X
P-165	X	X		
P-175				X
P-213	X			
P-216	X			
P-227	X			
P-243	X	X		X
P-246				X
P-282				X
P-283		X	X	
P-292				X
P-293				X
P-300	X			
P-301			X	
P-314	X			
P-317				X
P-324	X		X	
P-369		X		
P-380	X	X		X
P-381	X			
P-378				X

TABLE XI (cont'd)

Isolation Number	Motility	Morphology	Pigment	Nutrition
P-391	x	x		x
P-392			x	
P-398	x		x	
P-401			x	
P-405		x		
P-407		x		x
P-408	x	x		
P-414			x	
P-416			x	
P-423		x		x
P-426	x		x	
P-431	x			
P-437			x	
P-452			x	
P-467			x	
P-469			x	x
P-473	x			
P-481			x	
P-483		x	x	
P-484	x			
P-487		x		
P-499	x			
P-514	x		x	
P-515			x	
P-520				x
P-521				x
P-523			x	
P-527			x	
P-530			x	
P-531			x	
P-532			x	
P-533			x	
P-534			x	
P-537				x
P-544			x	
P-545			x	
P-549				x
P-561	x			
P-563				x
P-564	x		x	x
P-566	x			
P-572	x			x
P-581				x
P-584	x	x	x	
P-589			x	
P-592			x	
P-593			x	x
P-595		x	x	
P-604			x	
P-608	x	x	x	

TABLE XI (cont'd)

Isolation Number	Motility	Morphology	Pigment	Nutrition
P-610				x
P-617	x			
P-630		x		
P-631	x			
P-634	x			
P-635	x			
P-636				x
P-637	x			x
P-641	x	x		x
P-652	x			
P-653	x			x
P-657				x
P-673	x			
P-678				x
P-680				x
P-681				x
P-683			x	
P-684			x	
P-687			x	
P-689			x	
P-690			x	
P-695			x	
P-696			x	
P-698			x	
P-699			x	
P-700			x	
P-702			x	
P-703			x	
P-704				x
P-716			x	
P-719			x	x
P-720			x	x
P-722			x	
P-728			x	
P-732			x	x
P-742	x			
P-745			x	
P-752	x			
P-756			x	
P-762			x	
P-764			x	
P-765				x
P-768			x	
P-769	x			x
P-772				x
P-774			x	
P-777			x	
P-778				x
P-779				x

TABLE XI (cont'd)

Isolation Number	Motility	Morphology	Pigment	Nutrition
N- 5			x	
N- 8			x	
N-17			x	
N-34			x	
N-38			x	x
N-48				x
N-68	x			
N-75			x	
N-82	x			x
N-91	x			x
N-92	x			
N-97	x		x	x
N-98			x	

Results

Motility Variants

As was earlier discussed, the Azotobacter species are motile by peritrichous flagella. In the routine microscopic examination of many normal cultures of Azotobacter motility was noted in every instance. In the microscopic examination of the irradiated cultures of Azotobacter agilis, M.B., 4.4, non-motile cultures were noted which at first were thought to be contaminants. Subsequent transfers to nitrogen-free medium resulted in good, normal growth of the cultures. Further examination of isolated cultures showed many pure cultures of non-motile strains of the organism. These variants were found to be very stable and were never observed to revert to the motile form. Twenty-seven non-motile strains were found and recorded.

Colonial Variants

Of interest was the high degree of variation with regard to colony form and size. Although no record was kept of the number of colonial variants, the mucoid, rough, smooth, and small punctiform (the L_1 types) types of colonies were represented.

Morphological Variants

Many morphological variants were noted in the microscopic examination of isolated colonies. Sixteen morphological variants were isolated and studied. These variants were found in isolations that represented motility, pigment, and possible nutritional variants, as well as morphological variation alone. In most cases solid media supplemented with yeast extract (complete medium) supported good growth of the colonies representing morphological variants. The growth that was obtained on the solid basal medium appeared glossy, and microscopic examination revealed abnormally shaped cells which were filled with large, refractile globules. These morphological variants

were considered by Ward (1950).

Pigment Variants

The most common variants that occurred were those lacking the ability to form diffusible greenish pigment that characterizes the parent strain of the organism. These mutants appeared like the normal strain in growth except for the absence of pigmentation. These were found to be the most stable of all variants isolated. Karlsson and Barker (1948) found the pigment variants which they isolated to be the most stable. They suggested that the fact that they differ from the other kinds of physiological mutants, occurring more frequently and being more stable, suggested that they may be formed by a different mutation path.

The pigment variants were individually streaked on a basal medium agar and following suitable incubation periods none of the colonies were pigmented. Numerous subsequent transfers failed to yield any reversions, suggesting these strains were quite stable.

Filtrate Experiment: A filtrate from a broth culture of the normal, pigmented strain of Azotobacter agilis, M.B., 4.4 was added to basal broth which contained one ml. of a broth culture of the mixed non-pigmented variants. After 24 hours incubation with occasional shaking, this broth culture containing the filtrate was streaked onto 15 plates. After a suitable period of incubation no pigmented colonies were noted. The filtrate control plate showed no growth. This experiment was stimulated by the work of McCarty, et al. (1946) who added extracts of capsulated pneumococci to non-capsulated varieties to obtain a genetically stable capsulated variety.

Heat-Killed Cell Experiment: The non-pigmented strains were mixed in a basal broth culture and a suspension of heat-killed normal pigmented parent strain was added to the mixture of the non-pigmented strains. This was

incubated 24 hours and streaked upon basal agar plates. No pigmentation was observed after a suitable incubation period. This experiment was a continuation of the Filtrate Experiment; it was felt there might be some cell constituent in the normal strain which could produce genetically stable types from the non-pigmented variants.

Tyrosine Experiment: The non-pigmented strains were mixed in a basal broth to which was added tyrosine as a possible precursor of the pigment. This pigment was intermittently shaken and plated on basal medium agar plus tyrosine. After 72 hours incubation, no pigmentation was noted. Fat stains of these cultures using Sudan Black B stain were made since there is contention among some workers that pigments in microorganisms are responsible for the conversion of carbohydrates into fats within the cells of the organisms. The cells were found to be filled with fat bodies.

Many of the suspected pigment variants originally isolated were contaminants, but 26 definite Azotobacter pigment variants were isolated and maintained as stable variants.

However, among the large non-pigmented colonies, many very small punctiform colonies were noted. These were thought to be possible biochemical variants. Further observation demonstrated that the morphology of these cells, however, was not the same as that of the parent strain of the organism, but consisted of minature diplococci which appeared as small replicas of the parent strain. The morphology of the small cells was found by Ward (1950) to be constant on both nutrient agar and on basal medium agar. Other studies of these variants were considered by Ward (1950).

Biochemical (Nutritional) Variants

As may be seen in Table XI a number of suspected biochemical (nutritional) variants were isolated. These were double checked microscopically

for purity of culture, and maintained on complete medium agar, as it was early noted that basal agar induced only a slight amount of growth, which indicated their carbohydrate source which was ethyl alcohol in the basal medium was not too available, but successive transfers on the complete medium which contained both alcohol and glucose resulted in reversions of most of the suspected variants to large, normal appearing colonies; these were discarded, and the non-reverting types were further tested by the auxanographic method for their growth reaction to 12 different nitrogen-containing compounds, specifically vitamins and amino acids.

The vitamins and amino acids specifically used in the testing procedure were biotin, thiamin, paraaminobenzoic acid, niacin, threonine, methionine, leucine, cystine, arginine, histidine, proline, and phenylalanine.

The suspected nutritional variants were added to basal medium agar, which had been melted and cooled to 45°C., and poured into four Petri dishes. After 24 hours initial incubation, small filter paper pads which had been soaked in the nitrogenous compounds, were placed upon the surface of the poured plates containing the cultures. Each pad represented a different compound, and three were placed in each Petri dish. Stimulation by specific growth substances should manifest itself around the specific pad. This may be seen in Figure 3. These plates were examined periodically for a period of one week and the results were noted.

Out of the 28 suspected variants tested three were noted with definite zones of simulation; reversion must have occurred in the other cultures, for they overgrew the plates. The zones of stimulation were not limited to one compound alone, however. Each of the three suspected variants were tested three times and the same compounds were found to be stimulatory. Isolate P-537 was stimulated by niacin; isolate P-722 was stimulated by both arginine and proline; and isolate P-906 was stimulated by both arginine and histidine.

The organisms were shown to be capable of growth on basal medium agar after a long initial lag period, which indicates their instability. The stimulating effects observed from the different compounds tested may indicate that the organisms fail to fix atmospheric nitrogen in the presence of these compounds, and that the compounds are interchangeable with each other as a source of nitrogen for the Azotobacter.

Of interest was the inhibitory effect on the organisms, in every case, produced by cystine. Zones of inhibition about the auxanographic pad measured 10 to 20 millimeters in width; the reason for this inhibition is not known.

Figure 3

The Auxanographic Pad Method Used in Testing for Specific Growth Promoting Factors.

Note the increased growth supported by one of the three filter paper pads which had been soaked in a specific substance before being placed on the surface of the agar.

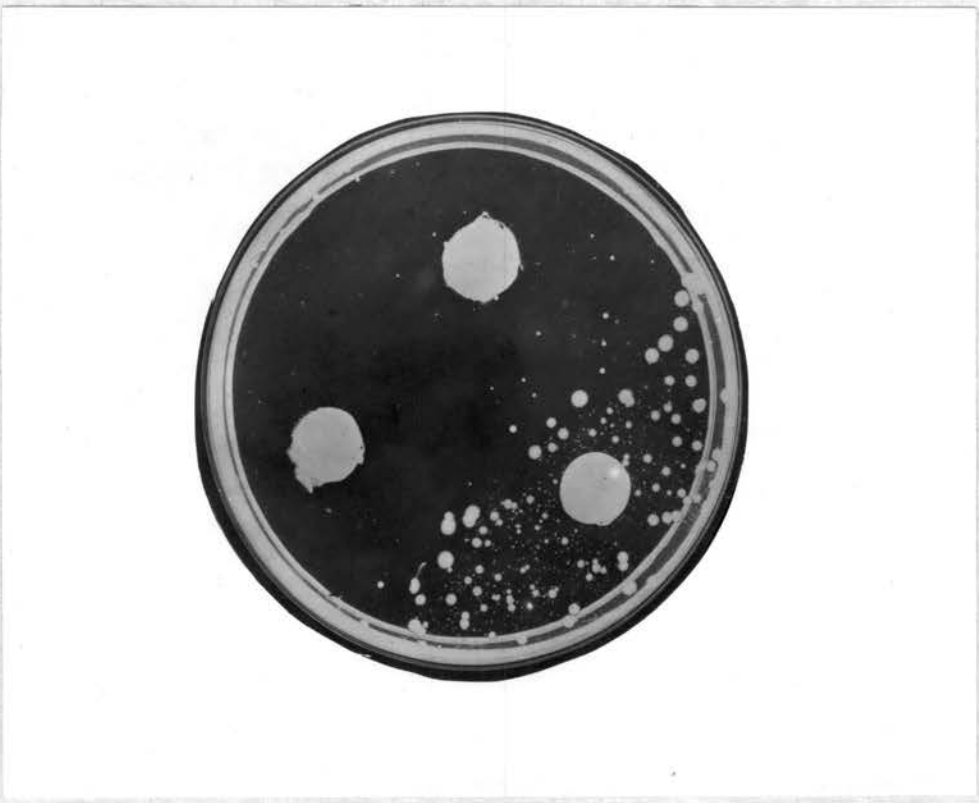

FIGURE 3

IV. SUMMARY

Variations within the species Azotobacter agilis, M.B., 4.4, were studied.

The viability of the so-called "involution" forms of the organism was established by isolating single cells of these forms and successfully culturing them. These were shown to yield pure cultures of the parent strain, thus demonstrating they did not represent contaminants or dying cells as suggested by many workers, but rather supports the view that the Azotobacter may have a complex life cycle as proposed by Lohnis and Smith (1923).

An effort was made to apply the serum reaction to this species of the Azotobacter for the purpose of detecting antigenic changes in mutant strains of the organism. Using six different strains of Azotobacter as antigen, the agglutination, precipitin, and complement fixation tests failed to demonstrate the presence of antibodies to the antigen in the serum of immunized animals. In an effort to determine if a rabbit was retaining or excreting the Azotobacter antigen, it was shown, using radioactive antigen of the organism, that fairly good retention was possible; from the degree of radioactivity of excretory products, however, it appeared that some of the antigen was being excreted fairly rapidly and in good quantities.

In an effort to induce variations in the species, three mutagenic agents were used: ultra-violet irradiation, methyl-bis (beta-chloroethyl) amine hydrochloride, and radioactive phosphorous. In studying approximately 2400 isolates, variations were found after exposure to these agents, but the indications are that the frequency of stable mutation is in a lower order of magnitude than in other bacteria. Stable motility, pigment, and colony variants were isolated. Morphological variants were also noted, but this phase is given consideration by Ward (1950). Three possible biochemical

variants were isolated.

In comparison with other bacteria this species of Azotobacter appears to be a very stable organism.

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