

THE AZOTOBACTERIACEAE

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INTRODUCTION

The existence of bacteria capable of utilizing elementary nitrogen independent of a host plant has now been known for sixty years. A few earlier investigators probably had crude cultures of such organisms, but it was left for Winogradsky (1) to give definite proof of nitrogen fixation by pure cultures of an anaerobic bacterium. Beijerinck (2, 3) followed this with his discovery of the same phenomenon in aerobic bacteria, and in the following years many statements on the existence of other nitrogen fixing bacteria were made but not confirmed by subsequent research, as far as heterotrophic organisms were concerned. Two important groups of autotrophic nitrogen fixing forms of life have, however, been added: several blue-green algae, and more recently a number of photosynthetic bacteria (4, 5).

The heterotrophic bacteria that unquestionably are capable of independent nitrogen fixation and therefore commonly are known as free-living or nonsymbiotic nitrogen fixers include two broad groups: a family of aerobic bacteria, the Azotobacteriaceae, and numerous anaerobic spore-formers of the genus *Clostridium*, typified by *C. pasteurianum* Winogradsky (1).

The family Azotobacteriaceae is usually regarded as consisting of a single genus *Azotobacter*, with the type species *A. chroococcum* (6). Strong evidence has recently been presented for regard-

ing a group of strains typified by *A. indicum* Starkey and De (7) as a separate genus *Beijerinckia* Derx (8). This proposal has been adopted in the present paper which is an attempt to appraise the status of our present knowledge concerning the general biology of the aerobic nitrogen fixing bacteria. An enormous volume of papers has been published on their morphology, taxonomy, physiology, distribution in nature, and importance in the biological nitrogen cycle. Stapp and Ruschmann (9) and Waksman (10) have reviewed much of the earlier literature, and Kyle and Eisenstark (11) have compiled a more recent extensive bibliography. The mechanism and comparative biochemistry of nitrogen fixation have been fully discussed by Wilson (4, 5) and Wilson and Burris (12), and are not treated in detail here.

THE GENUS AZOTOBACTER

The genus *Azotobacter* forms an easily recognizable group of bacteria widely distributed in soil and water. The azotobacter are characterized morphologically by the large size and variable shape of their cells that sometimes resemble yeasts or unicellular algae, and physiologically by their strictly aerobic mode of life and their abundant growth in appropriate media free from combined nitrogen; their growth in routine media such as meat extract peptone broth and agar is feeble or even absent. The genus comprises at

least three well defined species, *A. chroococcum*, *A. vinelandii*, and *A. agile*, probably a fourth, *A. beijerinckii*, and possibly others.

Morphology and Reproduction

More papers on "pleomorphism" have dealt with the azotobacter than with probably any other bacterium. Löhnis and Smith (13) have gone farthest in this respect and described a complex "life cycle" comprising many different types of organisms as stabilized stages. This has not been confirmed by later investigators (14, 15, 16, 17, 18, 19, 20, 21), who have mostly explained Löhnis and Smith's results as due to latent contaminants that often are difficult to eliminate from the cultures of *Azotobacter*; this explanation, indeed, would hardly account for the reported regeneration of normal azotobacter from some of the aberrant forms, e.g., the so-called "fungoid form" studied by Löhnis and Smith. Yet the morphology of *Azotobacter* in reliably pure cultures is remarkably variable, and general agreement seems to exist that the following cell types occur:

1. Bluntly rod-shaped or oval cells, measuring very roughly $2 \times 4 \mu$; the size is subject to great variations and is largest in *A. agile*.

2. Approximately spherical cells or some 2-3 μ diameter, in short chains or clumps, arising by shortening of the rod-shaped cells. Cell types 1 and 2 may be taken to represent the "typical" cells of the azotobacter shown by young cultures in favorable nitrogen-free media and also in the soil (14, 15, 16).

3. Smaller rod-shaped or spherical cells, sometimes less than one μ in diameter, arising in aging cultures or under special conditions of nutrition, a process that Winogradsky (16) described as "nannocytosis".

4. Resting cells (cysts), of roughly spherical shape, with contracted cytoplasm and a double-contoured cell wall. Their formation, according to Winogradsky (16), is favored by simple organic compounds, e.g., butanol, as sources of carbon, and may be permanently suppressed by cultivation in glucose or mannitol media. At least one species, *A. agile*, lacks cyst formation and probably for this reason also the resistance to desiccation that otherwise is so characteristic of other members of the genus (16).

5. Large, often irregularly swollen or filamentous cells. These have played an important role

in discussions on pleomorphism in the azotobacter and were described as "gonidangia" by Löhnis and Smith (13). Their nature is not yet clear but upon the whole they seem to arise under conditions of nutrition different from those in simple nitrogen-free media, or generally under the influence of an unfavorable environment (16, 19, 20, 21, 22). Den Dooren de Jong (19) has given a very detailed description of the many bizarre cell types of *A. chroococcum*, including sometimes enormously swollen cells that arise in media with combined, especially organic, nitrogen. Winogradsky (16) regarded the foregoing mentioned cell types 1-4 as the only normal ones that solely are seen in media resembling the natural habitat of the azotobacter (soil and water); the arising of big swollen cells of azotobacter in glucose or mannitol media was interpreted as hypertrophy in "domesticated" azotobacter that had become adapted to "good" nutrients not represented in their natural surroundings. Eisenstark *et al.* (21) however, pointed out that "in nature, of course, azotobacter do not exist in a nitrogen-free medium as they do in the laboratory of artificial culture." Smit (J. Gen. Microbiol., 11, vii, 1954) found a variety of *A. agile* that appears exclusively as giant cells. The behavior of the big cells when transferred to fresh media has been described somewhat differently. de Regel (17) interpreted the swollen cells of *A. chroococcum* as involution forms incapable of reproduction, while Eisenstark *et al.* (21) found that similar cells of *A. agile* germinated with regeneration of normal cell and were as viable as these.

Dondero and Zelle (23) described a more complicated scheme of development: the big cells of *A. agile* were rarely viable, and if so, they either reproduced their own type or reverted to the normal; these either remained constant or sometimes reverted to production of big cells. The processes were ascribed to segregation of nuclear material in the big cells which were supposed to be heterocaryotic.

Cells of azotobacter often contain granular inclusions, partly fat and volutin (18, 24), partly bodies of a still unknown nature. Chromatinic material stainable by Robinow's method is certainly present at least at some stages of growth (20, 21, 22, 23), and Bisset and Hale (25) claim the existence of a vesicular nucleus in *A. chroococcum*. This question, however, is not yet settled.

The azotobacter are conventionally described as gram negative, and are essentially so, but may be somewhat unstable. Löhnis and Westermann (26) state that *A. beijerinckii* and the dubious *A. vitreum* show a certain number of gram positive cells, and others (25, 27) observed the same in other strains. Acid fast elements (22) are probably identical with fat inclusions.

Most azotobacter possess flagella although only *A. agile* and *A. vinelandii* show conspicuous motility. The arrangement of the flagella has been much disputed but is apparently lateral as shown by electron microscope observations by Hofer (28) and Krasilnikov *et al.* (29); the last authors mention an *A. nigricans* with a peculiar ring-shaped configuration of its flagella. Derx (30) described an aquatic organism, *A. insigne*, said to have constantly polar flagella.

Simple binary fission seems to be the normal mode of reproduction in species of *Azotobacter* and was regarded by Winogradsky (16) as the exclusive one in nitrogen-free media with simple sources of carbon (lower alcohols and organic acids). A kind of budding similar to that in yeasts takes place as an accessory mode of reproduction in the hypertrophied cells that arise in glucose or mannitol media (16, 21, 22); this is probably the "exospore"-formation described by Löhnis and Smith (13). The cysts seem to germinate by local dissolving of the cell wall, upon which the cytoplasm emerges and assumes the rod shape typical of young cultures (22). Reproduction by endogenously produced gonidia (13, 17, 25) is still an unsettled question, and no evidence has been found for the existence of a filterable stage in cells of *Azotobacter* (17, 21, 31). The pictures of "conjugation cells", which Löhnis and Smith (13) regarded as evidence of sexual processes, have been explained as stages of incomplete cell division (118, 23). E. J. Petersen [private communication; paper to appear in Roy. Vet. and Agr. Coll. (Copenhagen) Yearbook 1955] has recently found direct evidence of cell fusion in *A. chroococcum*.

It remains to be added that den Dooren de Jong (19) found no evidence for the existence of bacteriophages in the azotobacter although he regarded their occurrence as likely.

Carbon Metabolism

Azotobacter as a whole show a great versatility in utilization of nitrogen-free organic compounds

as sources of energy. Numerous experiments by means of stationary cultures (9, 19, 32, 33) as well as by the manometric method (34, 35) have shown that the following compounds are utilized by one or more strains of *Azotobacter*:

Alcohols: Ethanol, propanol, butanol, 2,3-butylene glycol, glycerol, sorbitol, mannitol, inositol.

Organic acids (as sodium or calcium salts): Acetic, propionic, butyric, valeric, caproic, lactic, glyceric, pyruvic, malic, malonic, succinic, fumaric, tartaric, oxalacetic, citric, tricarballic, gluconic and saccharic.

Mono-, di- and trisaccharides: Glucose, fructose, galactose, sorbose, maltose, sucrose, lactose, melibiose, trehalose, melizitose, raffinose.

Polysaccharides: Dextrin, starch, glycogen.

In addition, the azotobacter have a remarkable aptitude for utilizing cyclic compounds: benzoic acid (36), salicylic acid and even phenol (37). Nonutilizable compounds include methanol, formic acid, oxalic acid, erythritol, xylose, arabinose [with exceptions (38)], rhamnose and surprisingly enough, mannose (9, 33).

The pattern of available carbon compounds varies a good deal in different strains and species. For instance, ability to use lactose and glycerol seems upon the whole to be rare (33), and several strains of *A. chroococcum* and *A. beijerinckii* fail to utilize mannitol (39, 40) and starch (27). *A. agile* seems somewhat more restricted in its choice of carbon compounds than other species, and does not utilize mannitol and benzoate (16). *A. insigne* is confined to ethanol and organic acids other than formic and benzoic (30).

The wide range in available carbon sources corresponds to a similar versatility of the azotobacter in adaptive enzyme formation, as shown by Burris *et al.* (35) and Harris (41), but the mechanism of carbohydrate dissimilation has been only recently intensively investigated.¹ Harris (41) observed that glucose-grown cells of *A. chroococcum* oxidized gluconate even stronger than the homologous substrate, and he assumed

¹ For example, L. M. Mortenson and his associates at the University of Wisconsin have completed a detailed study of the initial stages in the decomposition of carbohydrate by *Azotobacter vinelandii* O (private communication, papers are *In press* in: Arch. Biochem. and Biophys.; Biochim. et Biophys. Acta; J. Biol. Chem.)

the glucose dissimilation to proceed along the series glucose \rightarrow gluconate \rightarrow lactate \rightarrow pyruvate \rightarrow acetate. Mannitol is apparently metabolized via fructose since mannitol-grown cells of *A. vinelandii* are fully adapted to fructose (35), and *A. chroococcum* forms mannitol dehydrogenase adaptively (42). Lack of this enzyme may be responsible for the failure of some strains to utilize mannitol. Pyruvate seems to be utilized without adaptation (35, 41); acetate apparently precedes the formation of carbon dioxide in the oxidation of organic acids as well as ethanol, but the mechanism of its oxidation is only partially known. Karlsson and Barker (43) doubted the existence of a tricarboxylic acid cycle in *A. agile* because growth on the lower members of the acids in the cycle did not induce adaptation to the higher ones, and citric acid was not oxidized. Stone and Wilson (44), however, showed in experiments with cell-free extracts of *A. vinelandii* that oxidation of a given compound depends on cell "permeability" as well as on adaptive enzyme formation, and they warned against drawing premature conclusions from simultaneous adaptation experiments. They suggested the operation of a tricarboxylic acid cycle comprising the links of citrate, α -ketoglutarate, succinate, fumarate, malate, oxalacetate and acetate.

The respiratory activity of azotobacter is outstanding. Values of Q_{O_2} may under favorable conditions reach 2,000 in *A. chroococcum* (45) and even 4,000 in *A. vinelandii* (34), the highest observed in any kind of living matter. The respiration results in a practically complete oxidation of the substrate to carbon dioxide and water (34, 46).

Pure cultures of azotobacter, apart from certain mutants (19), have never been shown to form acids or other organic by-products, at least under optimal conditions for respiration. Fife (47) obtained evidence of intramolecular respiration at low oxygen tensions but did not determine the metabolic products.

Nitrogen Metabolism

Only a limited number of relatively simple nitrogen compounds seem available to azotobacter in addition to free nitrogen. Most earlier experiments in this direction are inconclusive since nitrogen fixation in the presence of combined nitrogen has not always been considered, and adequate media were not always used. Horner

and Allison (48) found that *A. chroococcum* used only ammonia and compounds readily convertible to this compound when free nitrogen was excluded. Nitrate, nitrite, urea, adenine, asparagine, aspartic and glutamic acid were readily available, and guanine, allantoin, cytosin and uracil to a slight degree. Oximes and nitrous oxide are unavailable, and hydroxylamine is toxic even in small concentrations (4, 5, 12). Assimilable organic nitrogen compounds, except urea, are utilized less readily than ammonia, nitrate and free nitrogen, according to Green and Wilson (49). The three last compounds thus command the chief interest, not least, from an ecological point of view.

Ammonia (4, 5, 12) is available without adaptation. It has an immediate inhibitory effect on nitrogen fixation, and apparently it represents the key compound of this process, where it reacts with α -ketoglutaric acid to form glutamic acid as the primary amino acid.

Nitrate (and nitrite) shows somewhat more complicated relationships, inasmuch as its utilization depends on previous adaptation. Experiments recorded by Wilson (4) show that nitrate adapted cells of *A. vinelandii* use nitrate practically to the exclusion of free nitrogen, and that adaptation takes place rapidly in this species. In stationary growth experiments Bortels (50) and Horner and Allison (48) found evidence that *A. chroococcum* fixed a certain amount of nitrogen in nitrate medium with sufficient molybdenum. The apparent disagreement possibly finds its explanation in recent experiments by Green and Wilson (49) who showed that the adaptation to nitrate takes place at different rate in different strains. They reported that one strain of *A. agile* and one of *A. chroococcum*, freshly isolated from soil, were unable to utilize nitrate although the latter could produce nitrate utilizing mutants.

Statements are sometimes made (6) that nitrate in amounts up to one gram per liter improves growth of the azotobacter, whereas higher concentrations are toxic. This belief seems based on earlier observations made with suboptimal (molybdenum-deficient?) media where conditions are less favorable for nitrogen fixation than for growth with nitrate. In recent experiments Green and Wilson (49) found similar growth rates of nitrate utilizing strains with free nitrogen and with 300 ppm NO_3-N , corresponding to 0.18 per cent sodium nitrate. The nitrate tolerance upon

the whole seems to vary considerably. Gainey (51), for instance, observed that among 65 strains of *A. chroococcum* the majority would grow in agar with 1,000 to 2,000 ppm $\text{NO}_3\text{-N}$, while a few were inhibited already by 100 ppm, and others could be adapted to 4,000 and even 5,500 ppm.

Nitrogen fixation is usually linked with cell proliferation and results, as is well known, in gains veraging some 15 to 16 mg nitrogen per gram consumed glucose, sucrose or mannitol under optimum conditions, and not materially exceeding 20 mg. Strain specificity influences to some extent the gain but particularly the rate of nitrogen fixation, as shown by Fischer (33) who compared 48 strains of *A. chroococcum*; the gain of nitrogen within 7 days varied between 13 and 19.5 mg per gram mannitol, with an average of 17.8 mg. The strains comprised two groups with a high and a low rate of fixation ("Anfangsintensität"); the first of these fixed an average of 8 and the second of 4 mg nitrogen within the first two days. A strain of *A. vinelandii* showed a high and one of *A. beijerinckii* a low rate of fixation. Jensen (52) found two similar groups of strains, the low rate group including all strains of *A. beijerinckii*. Some strains of *A. chroococcum*, however, grew rapidly on glucose though slowly on mannitol.

The economy of nitrogen fixation with substrates other than sugars and mannitol has not in recent time, when synthetic media permitting optimum growth are available, been studied to as large an extent as seems desirable, at least in view of Winogradsky's (53) repeated and forceful argument that the staple food of azotobacter in their natural environment would be chiefly simple organic acids and lower alcohols. Gainey (32) tested a number of fatty acids where the yield of fixed nitrogen per gram of acid increased steadily with the molecular weight and the heat of combustion from acetic to caproic acid. The medium, however, appears to have been suboptimal since the fixation reached only 6.5 mg per g with glucose and 2.5 mg per g with acetic acid, a value that appears remarkably small in view of the apparent key position of acetate in the normal respiration of azotobacter. In medium with adequate molybdenum, the gains may rise to 10-13 mg nitrogen per gram acetic, lactic and gluconic acids (54). Benzoic acid (33) appears to give a fixation much lower than expected according to its energetic value, probably because the

benzoate molecule is oxidized incompletely with formation of dark complex pigments.

Most of the nitrogen fixed by azotobacter is present as cell substance although nitrogenous compounds are usually excreted during active growth. Roberg (55) and Horner and Burk (56) estimated this extracellular nitrogen to be 10 to 25% of the total in young cultures. The amount increases considerably when the medium is depleted of energy material and autolysis takes place (55); it also varies in different species and is influenced by the composition of medium. Bortels (57) even found close to 40% extracellular nitrogen in 4 day cultures of *A. vinelandii* in medium of low iron content. The excreted nitrogen seems chiefly present as high molecular compounds (56), is available to certain bacteria growing in association with azotobacter (55), and includes small amounts of those simpler nitrogen compounds that have figured so prominently in discussions on the mechanism of nitrogen fixation *viz.*, aspartic acid and traces of hydroxylamine, free or bound as oxime (56).

The excreted nitrogen compounds also often include some ammonia, the origin of which has been the subject of much controversy. Winogradsky (36) regarded the ammonia volatilized from azotobacter cultures on silica gel with sodium salts of organic acids as a direct product of nitrogen fixation in excess over cell synthesis. Horner and Burk (56) found no excretion of ammonia until the medium was exhausted of energy material (glucose or mannitol), and then only to the extent of 10 to 12% of the excreted nitrogen, if the cultures were pure; contaminated cultures showed more rapid and copious ammonia formation. Winogradsky (53, 58) has, however, strongly upheld the view that although no ammonia arises in the presence of glucose or mannitol, this does not apply to cultures of *A. agile* on silica gel with ethanol, where a protracted liberation of ammonia begins well before the ethanol is completely used. This seems entirely at variance with the results of other studies on the competitive effect of ammonia in nitrogen fixation (4, 5) and would warrant retesting, especially in view of recent observations by Newton *et al.* (59) that *A. agile* and *A. vinelandii* under certain conditions may use ammonium and elementary nitrogen concurrently.

Proteolytic enzymes are lacking in azotobacter (9), and their deaminating ability is feeble (53).

Assimilable amino acids like aspartic and glutamic acid, or casein hydrolyzate, are much less readily assimilated than is inorganic nitrogen (49), and several amino acids, *e.g.*, glycine and leucine, inhibit growth of *A. chroococcum* at a concentration of 0.1% (19). The growth of azotobacter in ordinary peptone media without carbohydrates is therefore slight, particularly in nutrient broth which is the medium of choice in testing azotobacter for purity. A moderate growth usually takes place in meat extract peptone agar, which den Dooren de Jong (19) even found convenient for obtaining pure cultures of azotobacter. Peptone does not inhibit growth on glucose or mannitol agar (9), usually azotobacter tend to produce abnormal forms and rapidly to lose viability on cultivation in peptone media (19). The unreliable growth under such conditions probably depends both on strain specificity and on the amino acid composition of the peptone.

Mineral Nutrition

Phosphorus, sulphur, potassium, calcium, magnesium and iron, in addition to molybdenum under certain conditions, appear to be essential nutrients for azotobacter. A good deal of uncertainty exists in quantitative respect because many experiments have been performed in media of suboptimal composition, and distinction is not always made between rate and amount of growth. Krzemieniewska (60) first made a quantitative study of the general mineral requirements of *A. chroococcum* (grown with free nitrogen only). The following amounts were found necessary for optimum nitrogen fixation (13–15 mg per g glucose) in solution with humic acid as a growth activator:

Element	Amount required mg/g glucose
P (as K_2HPO_4)	2.46
S (as K_2SO_4)	0.49
K (as K_2SO_4)	0.38
Ca (as $Ca(H_2PO_4)_2$)	0.36
Mg (as $MgSO_4 \cdot 7H_2O$)	0.35

Phosphorus is thus required in by far the largest quantity. Recent experiments (61) even showed that in synthetic medium with 2% mannitol a supply of 2.25 mg P per g mannitol (0.025% K_2HPO_4) gave only half-optimal, but 4.45 mg optimal, fixation by *A. chroococcum*. Besides inorganic phosphates, the phosphorus in

glycerophosphate and 1,6-hexosediphosphate is also utilized (9, 61, 62) and actually seems to allow a higher rate of growth.

Sulphur appears to be available only as sulphate, according to Greaves and Anderson (63). Before the work of Krzemieniewska (60), potassium was often thought to be nonessential; Greene (64) found that amounts as small as 0.05 mg per gram of mannitol were sufficient.

Calcium was found by Burk and Lineweaver (65) to be replaceable by strontium only, and to be required in amounts of 20 to 50 ppm for optimum growth with free nitrogen. A good deal of controversy exists about its essentiality for growth with combined nitrogen. Several authors (9, 66, 67, 68) have maintained that it is not needed for assimilation of nitrate or ammonia. Bortels (50), however, was unable to confirm this; and also Burk and Horner (69), in contrast to their former views, concluded that equal concentrations of calcium are necessary for growth with free nitrogen, nitrate, ammonia, and asparagine. The actual physiological function of calcium is unknown.

Magnesium was found by Horner and Burk (68) to be required in equal amounts irrespective of the source of nitrogen. These authors found 3 to 5 ppm necessary for optimal and 1.0–1.5 ppm for half-optimal growth of *A. vinelandii*, and they calculated a similar value from the data of Krzemieniewska. The results were apparently obtained under conditions of partial molybdenum deficiency. Webb (70) found no less than 23–40 ppm magnesium required for optimal growth of *A. chroococcum*—an unusually high demand for a gram negative organism. Jensen (61) found similar requirements in medium with inorganic phosphate while 4–8 ppm seemed sufficient when phosphorus was supplied as glycerophosphate or hexose diphosphate.

Magnesium presumably functions as an activator of phosphorylation in the azotobacter as in other organisms, and may therefore be active at several different stages in the respiration. The idea is attractive, therefore, that the high magnesium demand may be connected with the extraordinary respiratory activity that would presuppose a high concentration of phosphorylating enzymes.

Manganese, known to replace magnesium in many enzymatic processes, also does so in the azotobacter. Stone and Wilson (44) found mag-

nesium unconditionally necessary for acetate oxidation but replaceable by manganese in the oxidation of malate and fumarate by cell-free extracts of *A. vinelandii*. Horner and Burk (68) reported magnesium irreplaceable by sixteen other metals (not including manganese), while Nilsson *et al.* (71) found that magnesium and manganese induced equally good growth of *A. chroococcum* in experiments of 7 weeks' to 5 months' durations. Growth with manganese, however, showed a longer period of latency. Experiments of shorter duration (61) showed that the growth induced by manganese in magnesium-free medium was so restricted that manganese could not be considered to be an equivalent substitute for magnesium. Clear evidence of adaptation to manganese was not found.

Iron is required in amounts that seem very large for a micronutrient, but the demands depend largely on the medium and the form of the iron. Burk *et al.* (72) and Bassalik and Neugebauer (73) found iron more readily available as colloidal (humus) or organic (hematin) compounds than as ferrous ion. Horner and Burk (68) obtained half-optimal rate of growth of *A. vinelandii* with free nitrogen or nitrate in the presence of as little as $0.1-0.16 \times 10^{-3}$ millimole, or approximately 0.006-0.009 ppm iron, probably under conditions of partial molybdenum deficiency. Bortels (57), Krzemieniewski and Kovats (74) and Rippel (75) found 20-40 ppm of iron as ferrous or ferric sulphate necessary for optimum fixation in media with adequate molybdenum content. Inhibitory effects of higher amounts were apparently owing to phosphate precipitation.

Molybdenum presents many intriguing questions. Bortels (76) first showed that it activates nitrogen fixation in *A. chroococcum*, and this was soon confirmed for this and other species (50, 66, 67, 74, 77, 78, 79, 80). The effective range of concentration is very wide. Stationary cultures usually show optimum effect of 0.1-1.0 ppm and a detectable effect of 0.0001 ppm molybdenum (50, 80); the effect of still lower concentrations may be observed in manometric experiments (78). It seems truly essential for growth of *A. chroococcum* with free nitrogen and at least stimulatory for *A. vinelandii* although some strains of the latter seem to fix nitrogen in its absence (57, 80). Molybdenum is not, however,

uniquely specific for nitrogen fixation since it also promotes growth with nitrate though not with ammonia (69). Mulder (81) has presented data showing that *A. chroococcum* requires the following concentration of molybdenum for half-optimal growth:

Free nitrogen.....	0.13 ppm
Nitrate.....	0.007-0.013 ppm
Ammonia.....	(nil)

With respect to nitrate assimilation the azotobacter thus show the same behavior as denitrifying bacteria, fungi and higher plants (81).

Vanadium alone has been found capable of replacing molybdenum in nitrogen fixation (50, 80). It is active at the same range of concentrations but produces only one-half to two-thirds of the effect of molybdenum, and appears unable to replace molybdenum in nitrate assimilation (50, 69). This suggests that although some pathways may be common to the processes of nitrogen fixation and nitrate assimilation, molybdenum does not act at the same stage in both; the higher concentrations of molybdenum required for optimum nitrogen fixation point in the same direction.

The essentiality of other elements for the azotobacter remains uncertain. Bortels (57) reported a stimulating influence of manganese in certain experiments when iron and molybdenum induced only a slight nitrogen fixation (in the opinion of Bortels because of unfavorable weather conditions), but Horner *et al.* (80) found no such effect either in the presence or the absence of molybdenum. According to Plaut and Lardy (82), manganese activates the oxalacetate decarboxylase of *A. vinelandii* more strongly than does magnesium. A "supporting" effect of tungsten at suboptimal molybdenum supply seems due to the difficulty of removing traces of molybdenum from tungsten salts (80). Schröder (67) considered copper and zinc to be necessary at least for growth with free nitrogen, while Mulder (83) found that *A. chroococcum* needed copper only for production of the dark pigment but not for actual growth. Lewis (84) concluded that an alleged beneficial effect of iodine was because of detoxication of small amounts of copper, and that boron had no stimulatory but on the contrary an inhibitory effect at 50 ppm. Gerretsen (85) has recently presented some evidence for boron as an essential element in *A.*

chroococcum although this still awaits confirmation.

Other Metabolic Features

Many earlier investigators have reported a favorable influence of undefined organic substances (plant, soil or manure extracts) on azotobacter. Most of these effects are probably largely due to molybdenum or vanadium. The very marked stimulatory effect of humus (46) has been clearly shown to be caused by readily available iron (72) and molybdenum (66, 73, 74, 79). Components of the B vitamin complex have been credited with similar effects. This appears generally unlikely since azotobacter will normally grow rapidly and abundantly in vitamin-free media, and synthesize large amounts of thiamin, riboflavin, nicotinic acid, pantothenic acid, biotin, pyridoxin, and inositol (86, 87). Jones and Greaves (87) found no effect of numerous growth compounds in the presence of molybdenum and only slight effects in its absence.

A few other observations remain. Birch-Hirschfeld (66) and Bassalik and Neugebauer (88) found some evidence that unknown organic substances from plant or soil extracts improved growth of *A. chroococcum* in the presence of molybdenum, and Rippel-Baldes *et al.* (89) showed that pyridine in small concentrations caused a minor but definite growth acceleration without itself being metabolized. According to Fischer (33), only strains with a slow initial rate of nitrogen fixation are stimulated by pyridine, and cell decoction of azotobacter produces the same effect. Greaves *et al.* (90) stated that various amino acids stimulate nitrogen fixation in *A. chroococcum*; with some exceptions, *e.g.*, tyrosine at 5 ppm, the effect was not very marked, and the medium appears to have been suboptimal (no molybdenum added).

Azotobacter thus normally seem to require no other organic compounds than an available energy source for optimum growth, but the possibility remains that certain strains may be stimulated by accessory growth factors or essential amino acids. In this connection it is worth noticing that Karlsson and Barker (91) produced a leucine dependent, although unstable, mutant of *A. agile*.

Azotobacter often form extracellular polysaccharides in copious amounts. Stapp (24) showed that the material produced in mannitol

medium was a protein-free hemicellulose hydrolyzable with weak acids, and Cooper *et al.* (92) later identified glucose together with small amounts of uronic acid among the hydrolysis products of polysaccharide from sucrose medium. Hestrin (93) has mentioned formation of levan and its hydrolysis to oligolevans and sucrose by adaptively formed levan-degrading enzymes.

Little is known about the chemical character of the dark insoluble pigment of *A. chroococcum* or the water-soluble greenish-yellow to purple pigments of *A. agile* and *A. vinelandii*; the character of the latter depends on the composition of the medium, *inter alia*, the proportion of iron and molybdenum (57). Winogradsky (36) first showed that many strains of *Azotobacter* produce a dark water-soluble pigment from benzoic acid. Wang (94) made a detailed study of this pigment and concluded that it largely resembles soil humus in its chemical and physical properties; its nitrogen is probably derived from the excretion products of the azotobacter. Wang regarded the formation of this pigment as a contributory factor in humus formation; his microbiological tests, however, indicate that the pigment is much more readily decomposed than is genuine soil humus.

Azotobacter are able to produce auxin-like compounds that may either stimulate or inhibit the growth of plant roots or coleoptiles (95, 96, 97). Compounds of this type have figured largely in discussions on the relationship between azotobacter and higher plants, but their real nature and significance still remain to be ascertained.

Influence of Environmental Factors

Oxygen supply. Azotobacter are strictly aerobic organisms, as evidenced by their tendency to grow in pellicles on the surface of favorable liquid media, and forced aeration greatly accelerates growth. Meyerhof and Burk (45) studied the influence of varying oxygen concentration on *A. chroococcum* in manometric experiments and found optimum respiration in atmosphere with 20% O₂; the respiration still continued at 0.12% O₂. Williams and Wilson (97a) found that a *p*O₂ of 0.5 atm was necessary to insure maximum rate of respiration by *A. vinelandii* O even when as little as 100 micrograms of cell N per Warburg cup was used; they report Q_{O₂} (N) values as high as 10,000–20,000 on several substrates of the TCA cycle. Fife (47) found that the respiration in cultures with combined nitrogen increased up

to 100% O₂. den Dooren de Jong (19) found that only a few individuals appeared able to adapt themselves to growth with combined nitrogen in an oxygen atmosphere.

Temperature. As a group, azotobacter are typical mesophilic bacteria with minimum, optimum and maximum at approximately 10, 30 and 40–45 C (46). The optimum temperature may vary somewhat according to the source of nitrogen (78), and Greene (98) found evidence of a somewhat higher optimum temperature in strains from hot climates. *A. beijerinckii* appears to be adapted to a somewhat lower temperature than *A. chroococcum* and *A. vinelandii*. Fischer (33) stated that his only strain of this type grew slowly at 30 C but produced better growth than any other at 15 C where *A. vinelandii* hardly grew at all. A few strains of the *beijerinckii*-type tested by the present writer showed a slow but definite growth at 5–7 C where the typical *A. chroococcum* as well as *A. vinelandii* failed to grow.

Reaction. The marked sensitivity of azotobacter to acid reaction has been known ever since the work on their distribution in soil began in earnest. Many earlier investigators (9) agree about a limit near or slightly below pH 6.0 for visible growth in liquid or solid media. Burk *et al.* (99) arrived at somewhat different results in manometric experiments: growth of *A. chroococcum* and *A. vinelandii* ceased abruptly at pH 6.0 with free nitrogen; but with nitrate, ammonia and urea it continued at a reduced rate and did not appear to cease until pH 4.0–4.5. This has not been generally confirmed. Petersen (62) observed no macroscopic growth of *A. chroococcum* in nitrate medium below pH 6.0 although at pH 5.2–5.6 encysted cells showed delayed germination that sometimes resulted in abnormal cell types. The endogenous respiration of *A. chroococcum*, according to Harris and Gainey (100), has an optimum at pH 7.0 and continues in a slight measure at pH 5.5–5.8, where it is somewhat stimulated by calcium ions. Gainey and Fowler (101) observed no growth of 37 nonspecified strains of *Azotobacter* on agar with or without nitrate at pH 5.4; six or seven strains grew very feebly at pH 5.6, nine at pH 5.7, all at pH 6.0. Cell multiplication in liquid medium with ammonium ceased at pH 5.6–5.8, but nonproliferating cells showed some increase in volume accompanied by nitrogen uptake and sugar con-

sumption at pH 5.4–5.6. Csáky (102) obtained results in partial agreement with those of Burk *et al.*: *A. chroococcum* grew feebly with ammonium at pH 5.8, but equally well at pH 5.0 and 6.8 with nitrate; unfortunately the changes in reaction during growth were not reported.

Tchan (103) has very recently described a variety of *A. beijerinckii* capable of growth on agar at pH 4.8, but no data were given on nitrogen fixation. A strain kindly submitted to the writer induced growth in solid or semisolid agar of initial pH 5.1, and fixed 12 to 14 mg nitrogen per gram glucose in the pH interval 5.1–5.8 (considerable rise in pH took place during growth). Evidently this organism must have a nitrogen fixing enzyme system different from that of other azotobacter or else possess a special mechanism for regulation of intracellular pH. Uppal *et al.* (104) mention an organism, apparently a variety of *A. chroococcum*, that fixed nitrogen at pH 5.4–5.7, but upon the whole fixation was slight. Wyss and Wyss (105) obtained a mutant of *A. vinelandii* that was unable to fix nitrogen at pH below 6.5. The writer has recently isolated a new type of azotobacter, possibly related to *A. vinelandii* that fixes nitrogen at pH 4.5–5.0. The general rule that nitrogen fixation in azotobacter ceases at pH very near 6.0 thus admits of certain exceptions.

Growth on the alkaline side appears to stop between pH 9 and 10 (99, 106). The optimum in *A. chroococcum* and *A. vinelandii* lies near pH 7.5, with a considerable amplitude towards 7 and 8. In *A. beijerinckii*, Yamagata and Itano (106) found an optimum near pH 6.8; this together with the existence of strains like the one described by Tchan (103) suggests a somewhat greater acid-tolerance in this species.

Colloids. Various earlier statements on a favorable effect of different colloids on the growth of azotobacter are difficult to evaluate, owing to the possible effect of micronutrients carried as impurities (*cf.* Bassalik and Neugebauer (88) who found no effect of dialyzed silicic acid). Rippel (107) showed that the addition of 0.1–0.2% agar to a medium of adequate composition greatly increased the growth rate but not the final yield of *A. chroococcum*. This effect seems largely of a physical nature, partly due to lessened precipitation of iron, but perhaps chiefly to the increased viscosity of the medium which facilitates surface growth under ready access of oxygen and nitro-

gen. Mechanical agitation or increased surface/volume ratio of the medium can replace the effect of agar (12).

Meteorological factors. Bortels (108) has reported a remarkable correlation between weather conditions and growth of azotobacter (as well as other bacteria): growth with free or fixed nitrogen was stated to be rapid during the approach and persistence of a high-pressure area, while low-pressure areas had the opposite effect. The variations in growth seemed to depend on the movement of the air masses rather than the actual barometric pressure. The data were not treated statistically, but experiments in Berlin and Göttingen seemed to give concordant results. Experiments in Denmark (109) showed comparatively small variations in nitrogen fixation, without obvious correlation with the weather changes; certain long-term fluctuations seemed to depend on other factors.

Associative Growth

The earlier literature contains many statements to the effect that nitrogen fixation is stronger in impure than in pure cultures of azotobacter. Beijerinck and van Delden (3) observed such an effect of *Agrobacterium radiobacter*, and for a considerable time Beijerinck adhered to the view that pure cultures of azotobacter did not fix nitrogen. No definite proof seems to have been furnished that other organisms have a synergistic effect towards azotobacter in a really adequate medium. Lind and Wilson (110) mention a spore forming bacillus that promoted growth of *A. vinelandii* by making iron available from certain types of humate but had no effect in the presence of a sufficient iron supply. They concluded that previous reports of this kind probably are due to some improvement of a suboptimal medium by the associated organism. One imaginable way that such improvement could take place would be decomposition by the associate of the excreted nitrogen compounds that might, perhaps by chelation, render suboptimal amounts of heavy metals like iron or molybdenum unavailable, or the associate might actually digest azotobacter cells and thus release bound molybdenum. The last possibility might explain the stimulating effect of certain protozoa, as first shown by Cutler and Bal (111) and later by Hervey and Greaves (112). The last authors

observed some stimulation of *A. chroococcum* by the ciliate *Colpodia maupasi* both in the presence and the absence of molybdenum. Dead ciliates had the same effect as live ones; the active constituent appeared to be a somewhat heat-sensitive organic substance [cf. Fischer's (33) observation on stimulation by cell decoctions of azotobacter]. The strain of *Azotobacter* seemed to have a rather low nitrogen fixing capacity (an example of a strain favored by accessory growth factors in addition to iron and molybdenum?).

The situation is of course radically different if an associated organism decomposes a carbon compound unavailable to azotobacter with formation of metabolic by-products serviceable for the latter, and in its turn utilizes the nitrogen fixed by azotobacter. Such symbiotic associations between azotobacter and certain types of cellulose decomposing bacteria (54) may lead to gains of 13-14 mg nitrogen per gram of consumed cellulose. Azotobacter do not seem able, however, to enter into real symbiosis with the commoner types of cellulose decomposing bacteria (113, 114, 115), or with fungi unless special measures are taken to insure the accumulation of breakdown products from the cellulose (116). Xylose and xylans may be rendered available in a similar way (38).

Mutations

Azotobacter often show mutation-like changes that may appear as dissociations in colony type or pigmentation (117) or may represent more profound modifications of morphological or biochemical properties. Most of these mutants, however, appear to be unstable. den Dooren de Jong (19) found one strain of *A. chroococcum* particularly prone to formation of morphological variants in glucose-peptone solution; one of these had lost its nitrogen fixing ability and produced acid from glucose but reverted occasionally to the original type. Also Stumbo and Gainey (118) obtained temporarily nonnitrogen fixing variants of *A. chroococcum* by cultivation in nitrate media. Other variants have been seen to arise under the influence of known mutagenic agents. Karlsson and Barker (91) exposed *A. agile* to x-ray treatment and obtained several mutants (filamentous, nonmotile, nonpigmented, or leucine-dependent) that proved unstable, and also a stable mutant that utilized only acetate,

ethanol and malonate. Karlsson (119) showed that its peculiarities arose from the loss of pyruvic oxidase and mentioned that it was not perfectly stable. Malate and fumarate could be oxidized to oxalacetate and pyruvate, and the energy thus released was available for growth with acetate. Wyss and Wyss (105) produced nonnitrogen fixing but partly unstable mutants of *A. vinelandii* by combined treatment with nitrogen mustard and ultraviolet light. Green *et al.* (120) obtained similar mutants of *A. agile* and *A. vinelandii* by cultivation with combined nitrogen and subsequent penicillin screening; two of these had become unable to fix nitrogen because of a blocking at the stage $N_2 \rightarrow NH_4^+$. Selection of unstable mutants like these may explain the observation of Fischer (33) on temporarily reduced nitrogen fixing power in *A. chroococcum* after growth in nitrate media.

Finally, Eisenstark *et al.* (21) observed rare instances when a nonpigmented mutant of *A. agile*, produced by exposure to radioactive phosphate, gave rise to apparent L-forms represented by dwarf colonies containing very small cells that gradually reverted to the normal type.

All these observations, together with our present knowledge of gene recombination in bacteria, suggest that although the claims of Löhnis and Smith (13) doubtless were largely due to latent contaminants, some reservation should be shown in invoking these as the sole explanation.

TAXONOMY OF THE AZOTOBACTERIACEAE

If we accept the two genera *Azotobacter* and *Beijerinckia*, as mentioned in the introduction, we may define the family Azotobacteriaceae as follows:

Cells with considerable variation in size and shape, not forming endospores, usually motile by means of lateral flagella; gram negative, sometimes with a tendency to positive reaction. Obligate aerobes that oxidize many carbohydrates, alcohols and organic acids to carbon dioxide and water. Gas is not formed; one genus forms acid from glucose. Feeble growth in sugar-free peptone media. No proteolytic activity. Capable of fixing elementary nitrogen. Grow best with free nitrogen and simple forms of combined nitrogen: ammonia, urea, and, as a rule, nitrate.

Validity of the Genus *Azotobacter*, and Species Differentiation

Azotobacter is distinguished by the following characters:

Large, rod-shaped, oval or coccoid cells with considerable pleomorphism, particularly in media with combined nitrogen, often forming thick-walled resting cells resistant to desiccation (cysts). Rapid growth, with oxidation of the substrate to carbon dioxide and water. No growth with free nitrogen below pH 4.5-5.0 and rarely below pH 6.0.

While *Azotobacter* is easily recognizable as a genus, the differentiation into species is less clear-cut. Beijerinck's two original species, *A. chroococcum* and *A. agile*, have remained unchallenged. *A. woodstownii*, *A. hilgardii* and *A. smyrnii* are not well defined and probably represent varieties of *A. chroococcum* (6). *A. vitreum*, Löhnis and Westermann (26), that should be characterized by its constant spherical shape, nonmotility and lack of pigment, was later considered a variety of *A. agile* (13). Aso and Yoshida (121) found it serologically different from other azotobacter, and its nitrogen fixing ability appears so slight (26) that doubts may be entertained about it being an *Azotobacter* at all. Some very recently described species are still so little known that they are perhaps better kept in abeyance for the present. These include *A. insigne* Derx (30), an aquatic organism with polar flagella, and two others mentioned by Krasilnikov *et al.* (29): *A. nigricans* and *A. fluorescens* (names that involuntarily suggest varieties of *A. chroococcum* and *A. vinelandii*, respectively).

We are then left with four species that may have a claim to be considered valid: *chroococcum*, *beijerinckii*, *vinelandii*, and *agile*. *A. beijerinckii* is often regarded as a variety of *A. chroococcum* (6). According to Lipman (122) it should be characterized by its yellow pigment and its tendency to form streptococcus-like chains. The pigment formation, however, gives no clear-cut separation since any larger collection of strains, as Winogradsky (53) points out, will show quite a spectrum ranging through every shade from very light to dark brown or nearly black, and further it is subject to mutation-like changes that sometimes manifest themselves as formation of white sectors in normally pigmented colonies (19, 117). Kluyver and van Reenen (77) called

attention to the larger cell size in *A. beijerinckii*, but it remains uncertain whether this as well as the tendency to chain formation exceeds the range of strain variation (27). Aso and Yoshida (121) considered it serologically identical with *A. chroococcum*. It seems possible, however, to retain the name *A. beijerinckii* for a group of strains characterized by nonmotility and yellowish or in some strains white growth on agar (26, 27, 106, 122, 123). These strains also sometimes lack cyst formation and starch hydrolysis (27), possess a somewhat greater resistance to acid reaction (27, 103, 106), and appear serologically different from *A. chroococcum* (E. J. Petersen, personal communication).

The two remaining species, *A. agile* and *A. vinelandii*, differ clearly from the previous ones by their lively motility, their formation of water-soluble fluorescent pigments, and their aquatic habits (16). Löhnis and Smith (13) considered them a single species (*A. agile*), a proposal that was adopted in the most recent edition of *Bergey's Manual* (6). Kluver and van Reenen (77), on the other hand, regarded them as different species on morphological grounds: larger, more rounded and more transparent cells in *A. agile*. Winogradsky (16) added the character of cyst formation, which took place in *A. vinelandii* (although it could be lost by prolonged cultivation in rich media) but was never seen in *A. agile*. On account of this difference Winogradsky suggested the placing of *A. agile* as the sole species of a new genus *Azomonas* [the use of *Azotomonas* by Orla-Jensen (124) as a new generic name for *Azotobacter* was disregarded by Winogradsky]. Tchan (123) shared the view of Winogradsky, but for linguistic reasons he substituted the name *Azotococcus* for *Azomonas* stating, "This generic name is not suitable, because *azo* in French does not mean nitrogen". Winogradsky's and Tchan's proposal for a separate genus based on the absence of cysts is difficult to accept since varieties of *A. beijerinckii* (27) may also lack cyst formation, but this property together with the differences in size and shape of the cells may well serve for differentiation between *A. agile* and *A. vinelandii*.

The following key to the species of *Azotobacter* can then be suggested:

- A. Feebly motile or nonmotile organisms, forming insoluble yellow to dark brown pigments. Typical soil inhabitants.

- a. Motile, pigment light to dark brown. *A. chroococcum*.
 - b. Nonmotile, pigment yellow, or lacking: *A. beijerinckii*.
- B. Vigorously motile organisms, forming soluble greenish-yellow to purple pigments, sometimes (125) none. Typical water inhabitants.
- a. Rod-shaped cells, forming cysts: *A. vinelandii*.
 - b. Very large oval to round cells, no cysts: *A. agile*.

Strain variation and strain specificity in *Azotobacter* leave plenty of material for future study, and until these have been worked out more fully, the species concept will probably remain subject to that diffuseness which find its expression in terms like "var. *atypica*" (125), "var. *acidotolerans*" (103), and "var. *achromogenes*" (27). A great extension of our knowledge could doubtless be attained through a study of the specific behavior of strains freshly isolated from the soil, as prescribed by Winogradsky (53), taking into consideration the nature and the extent of the morphological and biochemical changes that such "wild" strains of azotobacter may undergo through cultivation in laboratory media. The work of Green and Wilson (49) on nitrate metabolism is a noteworthy step in this direction.

The Genus Beijerinckia

Starkey and De (7) isolated from Indian rice soil an organism which they named *Azotobacter indicum*. It differs morphologically from other azotobacter by the smaller size of its cells which contain large polar fat inclusions, and physiologically by its slow growth and copious formation of tenacious slime, but particularly by its ability to grow and fix nitrogen over a pH range from approximately 3 to 9. The organism is motile, by means of lateral flagella according to Hofer (28), but forms no cysts. A rust-brown insoluble pigment arises in agar culture, and acid appears to be formed during growth. The nitrogen fixation, although slow, is very efficient and may exceed 20 mg per gram of sucrose (126). Ammonia and nitrate, the latter with accumulation of nitrite, are used at a much higher rate than is free nitrogen (49).

Kauffmann and Toussaint (127) described another new nitrogen fixing organism, *A. lacti-*

cogenes. According to their description it resembles *A. indicum* (which the authors did not mention) in its morphology, its cultural appearance, and its acid tolerance (optimum at pH 5.5, limits beyond 3.5 and 7.5), but is nonmotile. Lactic acid is produced from glucose. Observations by the present writer (61) showed *A. indicum* and *A. lactiogenes* to be morphologically similar: blunt, gram negative rods, approx. 0.5 to 1.0 x 2 to 3 μ , with many refractive granules, later irregular rods and cocci, often in short chains. The growth in meat extract peptone broth and agar was scant or barely visible. The main points of difference appeared to be the lack of motility in *A. lactiogenes*, and its less tenacious growth on nitrogen-free glucose and sucrose agar; it also produced a stronger acidity in glucose solution where pH dropped to 3.9 to 4.0 against 5.1 to 5.2 for *A. indicum*. Both species (61, 126) grew readily without added calcium, and magnesium demands were quite small; molybdenum was required for nitrogen fixation and could not be replaced by vanadium. Also in these respects the two organisms thus differ from *Azotobacter* proper.

Derx (8) proposed the creation of a new genus *Beijerinckia*, with *B. indica* (Starkey and De) emend. Derx as the type species, on the grounds of its striking morphological and physiological differences from other azotobacter. Derx (128) also described a *B. indica* var. *alba* that lacked the brownish pigment, and a very actively motile *B. mobile* (should be *mobilis*!) with amber-yellow pigment; it seems uncertain whether this is a real species or a variety. In older cultures of *Beijerinckia* Derx observed many irregular, sometimes branched cells distorted by fat accumulation, that made him suggest a possible relationship to the rhizobia.

Tchan (123) agreed with the views of Derx and suggested the transfer of *A. lactiogenes* to the genus *Beijerinckia*, with the approval of Kauffmann.

Derx's and Tchan's proposal for *Beijerinckia* as a separate genus of the Azotobacteriaceae seems well justified, in view of three major points of difference from *Azotobacter*: (a) smaller cells, comparable in size to the majority of the Eubacteriales, without cyst formation, (b) marked acid-tolerance, and (c) a carbon metabolism resulting in the formation of organic acids. The genus would include at least two species:

- a. Motile, growth on agar very tenacious: *B. indica*.
- b. Nonmotile, growth on agar pasty: *B. lactiogenes*.

Beijerinckia appears to be confined to tropical regions and so far has been found only in soils from India and Burma (7), Java (8, 128), Tropical Africa (127), Northern Australia (129), and South America (129). Quite special soil conditions thus seem necessary for the establishing of a *Beijerinckia* population in the soil. Derx (130) suggested an association (as rhizosphere organism?) with the root systems of tropical leguminous plants or possibly the Caesalpinioideae, and propounded the hypothesis that *Beijerinckia* represents an ancestral type of *Rhizobium* that has retained a supposed original power of non-symbiotic nitrogen fixation.

Addendum: The Genus Azotomonas (sensu Stapp)

The generic name *Azotomonas* was coined by Orla-Jensen (124) to replace Beijerinck's name *Azotobacter*, but it is clearly illegitimate according to generally accepted rules of nomenclature. Stapp (131) has later applied it to a nitrogen fixing organism isolated from a mixture of rice shells and cotton wool capsules. This organism was called *Azotomonas insolita* and differs in many important respects from *Azotobacter* as well as from *Beijerinckia*. It appears morphologically as small rod-shaped or coccoid gram negative cells with polar flagella, sometimes with large slime capsules, sometimes again irregularly swollen. Growth in routine media is vigorous and takes place over an interval from pH 3.3 to 9.5 (in potato extract medium). The organism is predominantly but not strictly aerobic, produces indole and hydrogen sulphide, and ferments many carbohydrates with formation of acid and gas. Gains of nitrogen in synthetic solution with two (?) % mannitol could reach in Stapp's experiments as much as 12 mg per 100 ml. A remarkably high iron concentration, in addition to molybdenum or vanadium, was found necessary for optimum nitrogen fixation.

In the last edition of *Bergey's Manual* (6) this monotypic genus *Azotomonas* is placed among the Azotobacteriaceae as an appendix to *Azotobacter*. Its morphology might not be irreconcilable with that of *Beijerinckia* although it lacks the characteristic fat inclusions and has polar flagella, but its cultural features and particularly

its fermentation reactions seem to place it well apart from the Azotobacteriaceae. Stapp himself regarded it as belonging to the Pseudomonadaceae. The ability to fix nitrogen nonsymbiotically does not *per se* place Stapp's organism in the Azotobacteriaceae since this ability is now known (132) to exist in heterotrophic bacteria other than *Azotobacter*, *Beijerinckia* and the clostridia. *A. insolita* seems to represent such an example. Its true systematic position warrants further study; unfortunately it appears to be a rare organism and has not been reisolated since first found by Stapp. Theoretically a homonym should not be used as valid name for another genus, but since the name *Azotomonas* has hardly been used since it was first coined by Orla-Jensen, probably without danger of confusion it could be applied to Stapp's organism unless this can be assigned to an already recognized genus.

Incidentally the observation of Hamilton *et al.* (132) with *Aerobacter aerogenes* shows that earlier unconfirmed statements on nitrogen fixation in many aerobic bacteria were not necessarily due to experimental errors. A re-examination by the isotopic nitrogen method might prove fruitful, *e.g.*, in organisms like *B. asterosporus* (133) and the "bacille gommeux" of Winogradsky (15, 53). Kleczkowska *et al.* (134) studied an organism related to the latter and found no satisfactory evidence of nitrogen fixation although their few data seem to speak for rather than against it.

AZOTOBACTER AS AGENTS OF NITROGEN FIXATION IN NATURE

The most remarkable gap in our knowledge of the Azotobacteriaceae is probably the fact that after half a century of study we have only vague notions about the quantities of nitrogen actually fixed by azotobacter under natural conditions and especially in the soil. It is significant that Winogradsky (53) never ventured to make any quantitative statement on this point. Determinations of the "nitrogen-fixing power" of soils, whether by the solution method of Remy and Löhnis or by the large silica plate method of Winogradsky (15), will serve for comparative purposes only and give no information on the extent of the processes in the soil itself. An impressive number of carbon compounds are known as potential foods for azotobacter, but we remain

practically ignorant about the quality and quantity of organic substances actually consumed by the azotobacter population of soils in their natural state. Comparative figures for the density of azotobacter in soil are legion (10, 11), but these tell us only about the "standing crop" of azotobacter (not even in terms of individual cells) and not about the rate at which new cells arise and replace the dying ones—in other words, the rate of turnover of the azotobacter population which (with the important proviso that free nitrogen only is used for cell synthesis) would be a real measure of nitrogen fixation. Gains of nitrogen detectable by the Kjeldahl method can indeed under adequate conditions be induced by addition of suitable energy material to the soil, but then the numbers of azotobacter rise to the order of hundreds or thousands of millions per gram of soil (15, 135) and become altogether incomparable to those observed in normal soils where they are rarely of an order of more than thousands per gram, as determined by cultural methods.

In fact, the numbers of azotobacter cells found in soil where nitrogen fixation has been artificially induced seem roughly to account for the amount of nitrogen fixed (135), and since cell proliferation and nitrogen fixation run parallel, we may form an estimate of the number of cells required to fix a given quantity of nitrogen. Cells of azotobacter seen by microscopic examination of soil vary considerably in size and shape (15, 135), but it may not be far out to regard their average volume as varying between 5 and 10 μ^3 , perhaps nearer the former which corresponds to a spherical cell slightly over 2 μ in diameter. If we assume the cells to have a specific gravity of unity and to contain 20% dry matter with 10% nitrogen, a simple calculation will show that fixation of one ppm of soil nitrogen corresponds to the formation of 5 to 10 millions of azotobacter cells per gram of soil, a figure that will not be essentially altered even with allowance for some nitrogen excretion. Detection of such a gain of nitrogen, even though it involves the synthesis of very high numbers of cells, is well beyond the range of the Kjeldahl and probably also the gasometric methods, but should be possible by the use of isotopic nitrogen. In this connection it is worth recalling an early experiment by Schloesing (136) who found no uptake of nitrogen by soil in a closed system when the gasometric method was used. For this

reason Schloesing disputed Berthelot's statements on copious nitrogen fixation, which today, as Winogradsky (53) points out, appear inexplicable. A repetition of Schloesing's experiments under application of the isotopic technique might go far towards answering the puzzling and very fundamental question: what gains of nitrogen may be expected from the activities of the azotobacter populations found in normal soils? This would be an extension of Winogradsky's ecological principle, and needless to say it would also be applicable to other groups of nonsymbiotic nitrogen fixers, not least the clostridia whose importance in the nitrogen balance of the soil perhaps has been underestimated.

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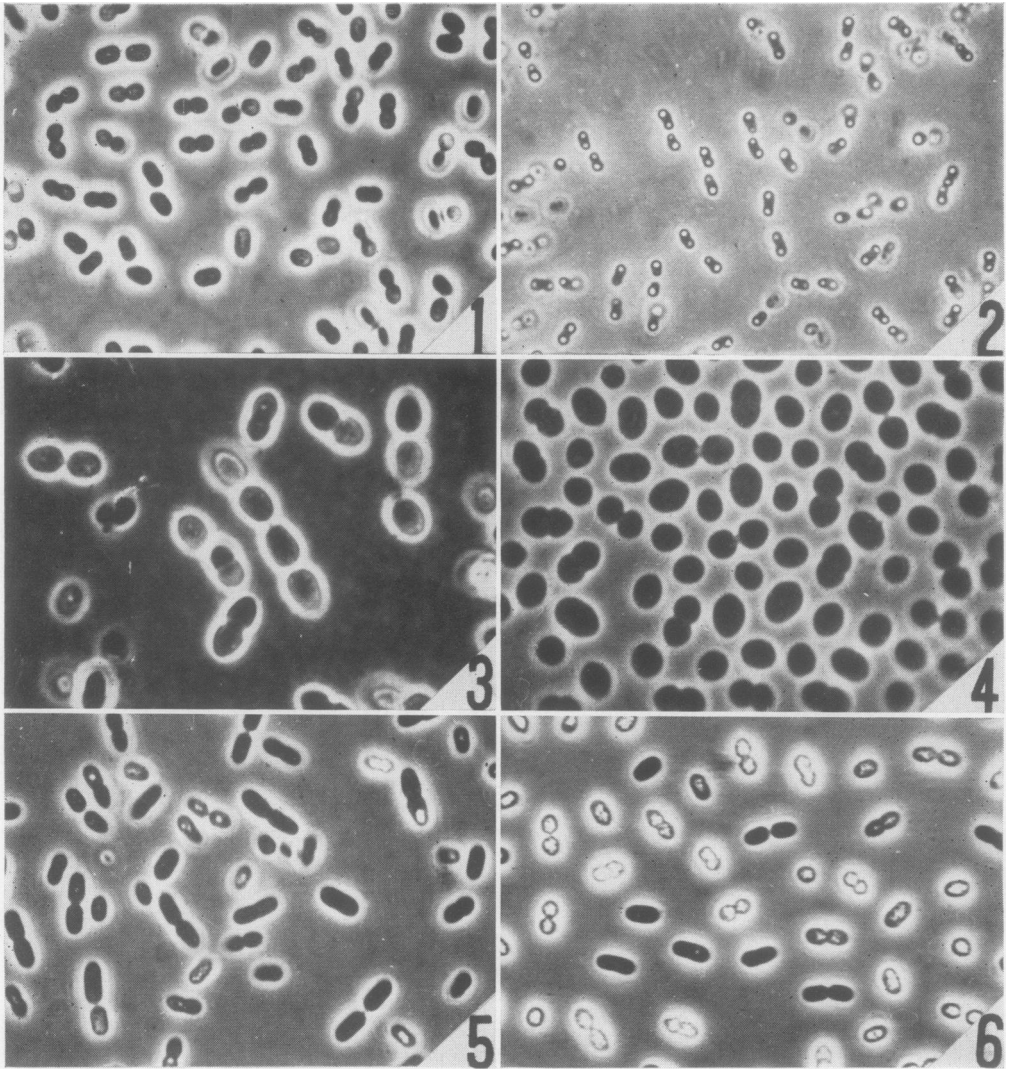


PLATE I. Species of *Azotobacter* and *Beijerinckia*

Fig. 1. *Azotobacter vinelandii* 0, (Wisconsin) 6 hr, 30 C.

Fig. 2. *Beijerinckia lacticogenes* (Kaufmann and Toussaint), 2 days, 25 C.

Fig. 3. *A. agile* (Professor J. Smit, Wageningen), 6 hr, 30 C.

Fig. 4. Same, 2 days, 25 C.

Fig. 5. *A. chroococcum* 210 (Lyngby), 20 hr, 25 C.

Fig. 6. *A. beijerinckii* BH (Lyngby), 20 hr, 25 C.

All photographs are by phase-contrast, magnification 1000 X. Cultures grown on nitrogen-free glucose agar except in figure 5 (sucrose agar).