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Chapter 12

Nonsymbiotic Nitrogen Fixation

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I. THE HISTORICAL BACKGROUND

A century has now passed since Jodin (1862) for the first time reported on experiments with "mucedinées" or "mycodermes" which he on apparently good evidence considered able to metabolize elemental nitrogen. Thus he formulated for the first time the conception of what has later come to be known as "nitrogen fixation" in microorganisms. Existence of this faculty in higher (leguminous) plants had long been surmised, but the final proof did not eventuate until about twenty-five years later. Jodin's discovery seems to have fallen into oblivion for a similar span of time, and no real interest was taken in the problem until the late 1880's when Berthelot conducted large scale experiments on the changes in nitrogen content of soils stored for long periods under the most varying conditions. The contents of nitrogen often showed notable increases that could not be ascribed to extraneous sources and which Berthelot (1888) first thought were due to biological causes and which he later (1890) ascribed partly also to electric discharges. The last hypothesis has never been confirmed, and in the light of our present-day knowledge of the biology of nitrogen-fixing microorganisms it seems next to impossible to imagine them as responsible for any gains of nitrogen under the conditions obtaining in Berthelot's experiments. Winogradsky (1895; 1949) expressed a strong scepticism in this respect both in the early and the concluding days of his scientific life, and contemporary experiments by Schloesing (1888) failed to confirm Berthelot's statements which nevertheless are still quoted in textbooks as the first experimental evidence of nonsymbiotic nitrogen fixation in soil.

Whether real or not, Berthelot's findings powerfully catalyzed the efforts of discovering agents of biological nitrogen fixation other than the partnership of legumes and root nodule bacteria, about whose ability to fix nitrogen there was no longer any doubt. The problem passed definitely into the realm of biology from the middle 1890's when Winogradsky first demonstrated nitrogen fixation in a strain of butyric acid bacilli (*Clostridium pasteurianum*). The next great step was taken around the turn of the century when Beijerinck discovered the aerobic nitrogen-fixing bacteria to which he gave the generic name *Azotobacter* and which were soon found to exceed the clostridia very considerably in nitrogen-fixing effi-

ciency. From then on research on nitrogen fixation began to develop along different lines.

(1). Fundamental studies on *Azotobacter* were conducted on a large scale. These were inspired partly by its extraordinary morphology that has played a decisive role in developing the concept of bacterial life cycles, and partly by the ease with which it can be cultivated under laboratory conditions; this latter property has made it a favorite object for studying the biological mechanism of nitrogen fixation which only today seems to be approaching its solution. In this respect the studies already have largely moved away from general microbiology into pure biochemistry.

(2). Much work has been spent in studying the distribution of the nitrogen-fixers and especially the azotobacter in relation to environmental and geographical factors, soil type, and origin, measures of cultivation, fertilizer treatment, etc.

(3). Soon after the discovery of the azotobacter there began an intensive search for other nitrogen-fixing microorganisms. This property was alleged in the most diverse groups of bacteria and fungi during the first two decades of this century, not least by the school of F. Löhnis in Leipzig. Many of these claims seemed untenable when other investigators failed to confirm the statements on nitrogen fixation in one important group of bacteria: the rhizobia when grown *in vitro*. From 1920 to 1930 there seemed to be a good reason to believe that only the azotobacter and the butyric acid bacilli were definitely endowed with nitrogen-fixing ability. The pendulum of opinion began to swing the other way when the previously controversial problem of nitrogen fixation in the blue-green algae was settled in the affirmative around 1930. Another great extension came in the 1940's from the Wisconsin school headed by P. W. Wilson and R. H. Burris: nitrogen fixing ability was found widely distributed among anaerobic bacteria, first the clostridia and later many others, photosynthetic (Thiorhodaceae and Athiorhodaceae) and chemolithotrophic (methane bacteria and sulphate-reducing vibrios). Finally the claims of the Leipzig school have in a way been vindicated, even if not with the same organisms then in principle, inasmuch as studies from the last decade, aided by the tracer technique with N^{15}-labeled nitrogen, has revealed nitrogen fixation in many otherwise "undistinguished" microorganisms.

(4). Great efforts were made to ascertain the "nitrogen-fixing power of the soil," often with none too clear a definition of this concept. The oldest method, the solution culture method of the Remy-Löhnis School, consisted in determining increments of nitrogen in selective liquid media inoculated with soil and incubated; this tells us little beyond the presence or absence of nitrogen-fixers and especially azotobacter in the soil. Winogradsky (1926) introduced the use of solid (silica gel) media for estimating the density of azotobacter and their amount of growth with defined energy sources; this method was in principle anticipated by Remy (1909) nearly twenty years before and does not show the gains that happen in the soil. A more real innovation was Winogradsky's (1926) principle of studying by direct microscopical methods the development of nitrogen-fixing bacteria in the soil itself as biological reaction upon the

introduction of non-nitrogenous energy material. This has subsequently led to the idea of correlating the multiplication of recognizable nitrogen fixers with chemically determined increments of nitrogen in the soil. The principle of determining the gains of nitrogen in the soil itself goes in principle back to Berthelot and Schloesing and would seem the only logical one. Countless experiments of this kind have demonstrated that very notable gains of nitrogen can be achieved if sufficient energy material be provided, but relations between gains of nitrogen and numerical representation of nitrogen-fixing organisms are still obscure, because two problems have not yet been fully solved. One of them is microbiological: the reliable numerical determination of metabolically active cell individuals (or more precisely, amount of living substance) of nitrogen-fixing bacteria. The other problem is a chemical one: the quantitative determination of nitrogen gains to be expected under natural soil conditions (comparable to the nitrogen requirements of a moderate or small agricultural crop). Under laboratory and still more under field conditions this is beyond the sensitivity of the current Kjeldahl method and presumably also the theoretically preferable Dumas method. The tracer technique has so far found very limited application but may provide the answer to the question in future investigations.

The present chapter brings a survey of our knowledge of the non-symbiotic nitrogen-fixing microorganisms and their activity in contributing to the nitrogen supply of cultivated soils. The literature of the last 25 years (until 1963) has chiefly been considered; many earlier papers have been reviewed by Winogradsky in his collected papers (1949), by Wilson and Burris (1953), Pochon and de Barjac (1958), and Jensen (1940a).

II. THE NITROGEN FIXERS *IN VITRO*

A. The Groups of Nonsymbiotic Nitrogen-Fixing Microorganisms

Among the heterogenous collection of potentially nitrogen-fixing soil inhabitants we may distinguish a group of organisms whose outstanding property is their ability to grow almost equally well with elemental and with combined nitrogen and which, as shown by Winogradsky (1926), will dominate the microscopic picture of the soil population under conditions selective for a nitrogen-fixing microflora. These are:

A. Organotrophic bacteria, including (a) the aerobic *Azotobacter* and one or two allied genera, and (b) the anaerobic *Clostridium butyricum* and some related species.

B. Photolithotrophic organisms including (a) many types of blue-green algae and (b) purple sulfur- and nonsulfur-bacteria which are probably of minor importance under soil conditions.

The Azotobacteraceae.—The aerobic members of group *A* belong to one family, Azotobacteraceae (Jensen, 1954); predominant among these is the genus *Azotobacter* Beij. whose morphological features are familiar. They are comparatively big, bluntly rod-shaped or oval to spherical, mo-

tile or non-motile cells whose size and shape vary greatly according to species, growth condition and age. The Gram reaction is essentially negative and endospores are to all appearances not formed, but the soil-inhabiting species mostly form thick-walled "microcysts" as resting cells. There is considerable evidence of a complicated life cycle comprising, *inter alia,* endogenously produced gonidia (Petersen, 1961). The species differentiation and even the unity of the genus are controversial. Some authors (Winogradsky 1938; Tchan, 1953; V. Jensen, 1955; Baillie et al., 1962) have advocated the creation of a separate genus (*Azomonas, Azococcus, Azotococcus*) for the types that form no thick-walled microcysts. Perhaps the most adequate classification is the one suggested by Baillie et al. (1962):

A. Microcysts formed: *Azotobacter* Beij.
   a. Motile with lateral flagella.
      1. Form dark brown insoluble pigment: *A. chroococcum* Beij.
      2. Form greenish fluorescent soluble pigment: *A. vinelandii* Lipman.
   b. Nonmotile, form yellowish, insoluble pigment: *A. beijerinckii* Lipman.

B. Microcysts not formed: *Azomonas* Winogr.
   a. Motile with lateral flagella: *A. agilis* (Beij.) Winogr.
   b. Motile with polar flagella.
      1. Big oval cells: *A. insignis* (Derx), V. Jensen (1955).
      2. Spherical or oval cells of varying size: *A. macrocytogenes,* H. L. Jensen (1955).

Azotobacter in this sense comprises typical soil inhabitants while *Azomonas agilis* and *insignis* seem to be purely aquatic organisms and *A. macrocytogenes* a very rare soil inhabitant. These species are in the following collectively called "the azotobacter."

Their carbon requirements are met by a wide range of substances from two-carbon compounds like ethanol and acetate over many aliphatic and oxy-acids, mono-, di- and polyvalent alcohols (e.g., mannitol), mono- and dihexoses (very rarely pentoses) up to starch, and in addition some aromatic compounds, e.g., benzoic acid. *Azotobacter* spp. as a whole utilize a wider range of carbon compounds than do those belonging to *Azomonas.*

The spectrum of available nitrogen sources is by comparison rather narrow. In addition to elemental nitrogen only ammonia, urea, some simple amino acids, nitrate and nitrite are readily available, the last two not even to all, for instance some strains of *A. agilis* (Becking, 1962; Green and Wilson, 1953). The azotobacter possess in addition to their nitrogen-fixing faculty only one outstanding physiological property: their respiratory activity is the highest in all kinds of living matter and may amount to Q_{0_2}-values (oxygen uptake μl/mg dry cell substance/hour) of 2000 to 4000, or 10 times as much if calculated on the basis of cell nitrogen. The oxidation of the non-assimilated carbon compounds normally proceeds quantitatively to carbon dioxide and water; acid formation from carbohydrates is an exceptional occurrence (H. L. Jensen, 1955; V. Jensen, 1959).

The amounts of nitrogen fixed *in vitro* under favourable conditions

usually range between 12 to 20 mg per g consumed carbon source of "optimal" nutritional value, like glucose, sucrose, mannitol or ethanol. Gains that essentially exceed 20 mg/g sugar do not seem to have been directly ascertained. The consumed energy material is largely spent in respiratory processes and no significant proportion seems to be required for the nitrogen fixation process *per se,* as discussed below. The fixed nitrogen is at least in the soil-inhabiting *A. chroococcum* and *A. beijerinckii* mostly present in cell substance and only to a small extent as excretion products.

The azotobacter usually show optimum growth at pH around 7.2 to 7.6 with either free or combined nitrogen, and most of them cease to grow at pH 6.0 or slightly below; some strains will grow at pH 5.0 or even somewhat lower (Jensen, 1955).

In relation to temperature all azotobacter are typically mesophilic; most soil forms have an optimum at 28-30°C while that of the aquatic forms (*Azomonas*) is often lower.

In agreement with the oxidative nature of their metabolism the azotobacter are all obligatory aerobes whose growth rate increases markedly by improved aeration (Alexander, 1954; Schmidt-Lorenz & Rippel-Baldes, 1957; Wilson, 1958). Growth may take place, however, at remarkably low oxygen tensions, according to Tschapek and Giambiagi (1954) and Lorenz and Rippel-Baldes (1957) at $pO_2 = 0.008$ atm, or even lower according to Meyerhof and Burk (1927). Tschapek and Giambiagi (1954) and Garbosky (1956) found that the oxygen demand of *A. chroococcum* decreased with decreasing organic nutrient concentration of the medium, as indicated by the formation of growth zones at varying depths below the surface in liquid media; at glucose concentrations less than 500 ppm it thus behaved like a microaerophile. This, indeed, seems to be true of aerobic bacteria generally (Zycha, 1932). Quispel (1947) found *A. chroococcum* unable to induce growth from a small inoculum at a redox potential (Eh) lower than ca. 400 mV or under special conditions 300 mV, but in growing cultures Eh could drop to about 75 mV. This was also observed by Döbereiner and Alvahydo (1959), and associated cultures of azotobacter and cellulose-decomposing bacteria may even show negative Eh-values (Jensen and Swaby, 1941). LeGall et al. (1959) have reported growth of *A. chroococcum* in a sulphide-containing medium at the expense of the oxygen impurities in commercial nitrogen gas.

Although improved aeration accelerates the growth of the azotobacter, the oxygen tension influences the efficiency of nitrogen fixation (expressed as the gain per unit of consumed energy material) in a different way, as discussed later.

The general mineral requirements of the azotobacter present nothing unusual. Calcium has often been considered specifically required for nitrogen fixation; it is now known to be generally essential for most species, but sometimes in very small amounts only (Bullock et al., 1960). For *A. agilis* it seems non-essential although stimulatory. Gerretsen and de Hoop (1954) claimed boron to be essential for *A. chroococcum,* while Anderson and Jordan (1961) found only a moderate stimulatory effect of this micronutrient which is not known to be required by other microorganisms. It

is unknown if the reported boron effect is specific to nitrogen fixation. The importance of some other trace elements specifically active in the nitrogen fixation process is discussed elsewhere.

Beijerinckia and *Derxia*. Closely allied to the azotobacter is a group of bacteria that some microbiologists include in *Azotobacter* while others (among them the present author) regard it as a separate genus *Beijerinckia* Derx (1950). These are typical rod-shaped bacteria, containing characteristic polar fat inclusions and considerably smaller than the azotobacter, nonmotile or more often motile with lateral flagella. Their growth is much slower than that of the azotobacter and is accompanied by copious formation of tenacious polysaccharide slime. Their range of available carbon sources seems a good deal narrower than in the azotobacter, particularly comprising fewer simple alcohols and organic acids. Their efficiency of nitrogen fixation is high and sometimes reaches 20-22 mg per g of consumed sugar.

The beijerinckias differ markedly from the azotobacter by their ability to grow within a pH-range from about 3 to about 9. Further they do not require calcium which even has an inhibitory effect (Bullock et al., 1960), and their geographic distribution is markedly limited, as discussed elsewhere. Some seven or eight species have been described but may not all be valid; Petersen (1959) considered three species well defined: *B. indica* (Starkey et De) Derx, *B. acida* (Roy) Petersen, *B. fluminensis* Döbereiner et Puppim-Ruschel.

A somewhat similar organism of apparently rare occurrence was found in Indian soils and was considered sufficiently characteristic to deserve rank as a separate genus *Derxia* (Jensen et al., 1960) with one species, *D. gummosa*. It differs from *Beijerinckia* by its considerably bigger rod-shaped cells, sometimes motile with a single polar flagellum and lacking the typical polar fat inclusions. It produces an even more tenacious growth than the beijerinckias and utilizes only a limited number of sugars in addition to ethanol. Its nitrogen fixation is very efficient and may exceed 25 mg per g glucose. Growth takes place between pH about 5.0 to 8.5, as in the more acid-tolerant azotobacter. Roy (1962) has very recently added another species *D. indica*.

Clostridium spp. The anaerobic nitrogen fixers of significance in natural soils are chiefly the clostridia of which the first representative was found by Winogradsky (1895). The next step was taken by Bredemann (1909) who showed nitrogen fixation to be common to butyric acid bacilli (*Clostridium butyricum*). Rosenblum and Wilson (1949) extended the range of nitrogen fixing clostridia further. The yield of fixed nitrogen per unit of fermented sugar was in earlier experiments mostly estimated at 2-3 mg/g sugar. This is considerably less than in the Azotobacteraceae but cannot be directly used as an index of "efficiency" because the clostridia leave a considerable part of the available energy behind in the fermentation products (butyric and acetic acid, butanol, acetone, hydrogen, etc.) in contrast to the complete utilization of the substrate energy by the oxidative azotobacter and beijerinckias. Later studies have revealed a much more economic nitrogen fixation in the clostridia. Already McCoy et al. (1928) found yields of 7 mg/g sugar in some strains,

and Wilson and co-workers (1950; 1959) as much as 12 mg/g under conditions where gaseous fermentation products were continuously removed. Parker (1954) even reported 27 mg/g in special media (which indeed needs confirmation). The range of carbon sources available for nitrogen fixation includes a large variety of simple sugars (also pentoses in contrast to the azotobacter), di- and polysaccharides and pectic substances, but no organic acids except possibly lactate; the pattern of fermentation upon the whole varies considerably in the different species. Their temperature range is wider than that of the azotobacter with optimum often around 35-40°C, and the lower end of the pH-scale extends to pH around 4.0 (Van Beynum and Pette, 1936) but approximately neutral reaction still seems to represent the optimum.

The clostridia tend to lose their nitrogen-fixing power when grown continuously in artificial media, but this power may be restored by passage through sterile soil (Bredemann, 1909). In this respect they resemble a somewhat neglected nitrogen fixer, the facultative anaerobic *Bacillus polymyxa*. Bredemann (1908), who studied it under the name of *Bac. asterosporus,* first detected a nitrogen fixation somewhat weaker than in *Cl. butyricum*. His discovery remained largely ignored for half a century until Wilson and co-workers (1958, 1962, 1963) confirmed it in experiments with labeled nitrogen.

Miscellaneous nitrogen fixing agents. Other well-documented observations of nitrogen fixation in organotrophic bacteria have been reported with increasing frequency during the last twenty years and include organisms of which it is at least credible that they could be active in the soil.

Stapp (1940) isolated a pseudomonas-like organism from rice shells and found it able to fix as much as 10 mg nitrogen per gram of added glucose. The organism, to which he gave the name *Azotomonas insolita,* does not seem to have been found again, and stock cultures examined by the present writer appeared to have lost their nitrogen-fixing power.

Earlier statements on nitrogen fixation in the facultative anaerobic *Aerobacter aerogenes* were confirmed by Hamilton and Wilson (1955), Pengra and Wilson (1958) and Jensen (1956) who found a maximum fixation of 4.5 mg/g added glucose. Other investigators who have detected nitrogen fixation in aerobic bacteria are Anderson (1955), Voets and Debacker (1956) and Paul and Newton (1961) in *Pseudomonas* spp., Jensen (1958) and Proctor and Wilson (1959) in *Achromobacter* sp., and Becking (1962) in a spirillum-like organism previously studied by Beijerinck who regarded the evidence for nitrogen fixation as inconclusive.

The reported yield of nitrogen fixed per unit weight of energy material is generally low but is rarely stated with precision. Fedorov and Kalininskaya (1961) have recently reported on an interesting bacterium called a "mycobacterium" but apparently more like an *Arthrobacter* sp. This bacterium lacked a glycolytic system and thus failed to utilize sugar, but when provided with growth factors it could fix as much as 10 mg nitrogen per g consumed pyruvic acid, and in association with sugar-fermenting bacteria the gain could rise to 13-16 mg nitrogen/g sugar. This

seems to be one of the few plausible reports of a nitrogen-fixing aerobic organism with an efficiency equalling that of the Azotobacteraceae.

Evidence for nitrogen fixation in the actinomycetes has mostly been negative or unconvincing. Recent statements by Fedorov and Ilina (1960) suggest gains so small as to appear dubious until verified by the labeled-nitrogen method. Two species of *Nocardia* studied by Metcalfe and Brown (1957) doubtless possess this faculty but tend to lose it on continued cultivation. One of them had the unique property of combining nitrogen-fixing and cellulose-decomposing power.

Much controversy has reigned concerning the existence of nitrogen fixation in fungi, particularly *Phoma* spp. Recent evidence according to Wilson and Burris (1953) is mainly negative except in certain species of *Saccharomyces* and *Rhodotorula* which Metcalfe et al. (1954) isolated from acid soil and found able to fix about 4 mg nitrogen per g of sugar as confirmed in experiments with labeled nitrogen.

Lithotrophic nitrogen fixation. This second main group of nonsymbiotic nitrogen fixers again includes aerobic and anaerobic organisms. The first is to all appearances by far the most important in the soil and consists of photosynthetic organisms: blue-green algae or Cyanophyceae. These represent a fascinating chapter in soil biology and plant physiology, inasmuch as their nitrogen-fixing power was first tentatively suggested, then denied on apparently good experimental evidence and finally proved to be real. Valuable reviews on these organisms are due to Fogg and Wolfe (1954), Fogg (1956) and Singh (1961). Nitrogen fixation has now been definitely shown to take place in at least 20 species belonging to the genera *Anabaena* (9 spp.), *Anabaenopsis* (1 sp.), *Aulosira* (1 sp.), *Calothrix* (2 spp.), *Cylindrospermum* (3 spp.), *Mastigocladus* (1 sp.), *Nostoc* (5 spp.) and *Tolypothrix* (1 sp.). They include aquatic as well as terrestrial organisms, and some occur as algal components in lichens and a liverwort (Bond and Scott, 1955). They appear to fix nitrogen under these conditions too and are thus capable of a kind of "facultative symbiotic" nitrogen fixation in a partnership less intimate than that of leguminous plants and rhizobia.

B. The Mechanism of Nitrogen Fixation

The biological mechanism by which elemental nitrogen is brought into the metabolic cycle seems to be fundamentally the same in symbiotic systems and the nonsymbiotic nitrogen fixers, as discussed in the recent review of Wilson (1958), on which this chapter is largely based. A number of features are common to all agents of nitrogen fixation so far studied.

(1). A hydrogenase is found in all nonsymbiotic nitrogen fixers, but possession of this enzyme does not necessarily convey nitrogen-fixing power. In the azotobacter the enzyme is of an inducible character, being only formed during growth with free nitrogen. Its precise function is unknown.

(2). Hydrogen gas competitively inhibits nitrogen fixation in the azoto-

bacter (Wilson, 1958), *Aerobacter aerogenes* (Pengra and Wilson, 1958), *Bac. polymyxa* (Grau and Wilson, 1962) and blue-green algae (Wilson, 1958). It has a similar effect in *Cl. butyricum,* but only at much higher partial pressure (Westlake and Wilson, 1959).

(3). Carbon monoxide likewise specifically inhibits nitrogen fixation in the azotobacter (Wilson, 1958), *Aerobacter aerogenes* (Pengra and Wilson, 1958), blue-green algae (Wilson, 1958) and *Cl. butyricum* (Virtanen et al., 1953). The effect is noncompetitive and is exerted at much lower partial pressures than that of hydrogen. Both gases incidentally act likewise in the symbiotic nitrogen-fixing systems.

(4). Oxygen gas has a remarkably complicated effect. In the clostridia and anaerobic nitrogen fixers upon the whole it would be expected generally to lessen the amount of growth at low pressures, although nothing definite is known about a specific effect upon nitrogen fixation. In the aerobes, increasing oxygen pressure tends to lower the *efficiency* of nitrogen fixation (although the *rate* of growth may be increased) by competing with gaseous nitrogen for the available hydrogen needed in the fixation process (Parker and Scutt, 1960). In some of the facultative anaerobes it abolishes nitrogen fixation, thus in *Bac. polymyxa* (Grau and Wilson, 1962; Hino and Wilson, 1958) in *Achromobacter* sp. (Jensen, 1958) and in *Aerobacter aerogenes* where it ceases already at $pO_2 = 0.05$ atm according to Pengra and Wilson (1958). Jensen (1956), on the other hand, found this species to fix nitrogen equally well aerobically and anaerobically. Meyerhof and Burk (1927) made a classical investigation of the influence of varying oxygen tension on the respiration of *A. chroococcum* and found a decreasing growth rate but increasing economy of fixation with decreasing oxygen tension down to approx. 0.1% oxygen ($pO_2 = 0.001$ atm), where the fixation would theoretically correspond to 50 mg/g consumed glucose. In more recent work, Schmidt-Lorenz and Rippel-Baldes (1957) found *A. chroococcum* to fix 18-20 mg N/g consumed glucose at 0.02-0.04 atm O_2, which efficiency declined gradually to 10-11 mg N/g glucose at 0.4 atm, and 0.6 atm O_2 stopped fixation completely. Similarly Parker (1954) found merely 7-8 mg nitrogen fixed per g (supplied) sucrose at pO_2 0.20 atm, but 22-23 mg at pO_2 0.04 atm. (Here, indeed, does the yield in atmospheric air seem abnormally low.) In later experiments where the N_2 tension was kept constant at the low value of 0.16 atm, Parker and Scutt (1960) found maximum fixation at 10% oxygen and estimated the Michaelis constant of fixation to be 0.0107 and 0.0229 at pO_2 0.10 and 0.20 atm, respectively.

The problem was approached from another angle by Tschapek and Giambiagia (1955) who found that *A. chroococcum,* when growing in a very dilute medium with only 10 to 30 ppm glucose, showed the maximum ratio between cell multiplication and glucose consumption at pO_2 0.01-0.02 atm. Unfortunately, the actual gain of nitrogen was not determined. The significance of this relationship between nutrient concentration and oxygen tension for the economy of nitrogen fixation in the soil is obvious, as pointed out already by Meyerhof and Burk (1927).

(5). Molybdenum is essential as an activator of nitrogen fixation. It was indeed the studies of Bortels (1930) on *A. chroococcum* that led to its

discovery as a micronutrient of general importance. It has been shown to be required for nitrogen fixation in the azotobacter and the clostridia (Wilson, 1958), the beijerinckias (Becking, 1962b), *Derxia* (Jensen et al., 1960), the blue-green algae (Fogg and Wolfe, 1954), *Azotomonas isolita* (Stapp, 1940), *Aerobacter aerogenes* (Pengra and Wilson, 1959), *Bac. polymyxa* (Grau and Wilson, 1962) and it is at least stimulatory in *Achromobacter* sp. (Proctor and Wilson, 1959). In addition it is essential for nitrogen fixation in the symbiotic systems so far studied and for nitrate assimilation in apparently all organisms capable of metabolizing this form of nitrogen; its function in the latter respect is that of an activator of nitrate reductase which has been identified as a molybdenum-flavoprotein (Wilson, 1958). Optimum concentrations of molybdenum for nitrogen fixation are mostly at the order of 0.10-1.0 ppm, whereas lower concentrations will suffice for nitrate reduction, and for utilization of fully reduced (NH_4) nitrogen it is to all appearances not necessary (Mulder, 1948).

Vanadium will serve as a somewhat inferior substitute for molybdenum in nitrogen fixation by some but by no means all types of azotobacter (Becking, 1962b; Wilson, 1958), likewise in some of the clostridia (Jensen and Spencer, 1947) and in *Azotomonas insolita* (Stapp, 1940) but not in the beijerinckiae (Becking, 1962b), the blue-green algae (Singh, 1961), or *Derxia* (Jensen et al., 1960). According to Becking it cannot replace molybdenum in nitrate assimilation by the azotobacter.

Recent evidence (Nicholas et al., 1962) suggests that very minute amounts of cobalt (0.001 ppm) may be necessary for optimum growth of *A. vinelandii* with free nitrogen as well as with nitrate; its function seems connected with the synthesis of nitrate reductase although it is not a constituent of this enzyme. It thus does not seem strictly specific in nitrogen fixation.

(6). Ammonia is to all appearances the first stable product of nitrogen fixation from which glutamic acid is formed as a primary organic compound by reductive condensation with α-ketoglutaric acid (Wilson, 1958). Other amino acids then arise by transamination and proteins by condensation of these. The intermediates in the actual process of nitrogen fixation ($N_2 \rightarrow NH_4$) are still not known with certainty; it is even an open question whether the process is purely reductive, proceeding via a hypothetic diimide (HN:NH) analog and hydrazine ($H_2N \cdot NH_2$) to ammonia, or whether the final ammonium formation is preceded by an intermediate stage of partial oxidation ($N_2 \rightarrow NH_2OH$), as has been maintained in the azotobacter. Progress into this problem has long been hampered by the difficulty of preparing actively nitrogen-fixing cell-free extracts, but in recent years this has been achieved in most nitrogen-fixing microorganisms (Carnahan et al., 1960; Grau and Wilson, 1963; Schneider et al., 1960; Wilson, 1958). The effect of azotobacter-extracts is mostly feeble and rapidly lost and a nitrogen-fixing enzyme has not been isolated. An important step towards the solution of this intricate problem is represented by the studies of Carnahan and co-workers on *Cl. butyricum*. This and much other recent work has been admirably reviewed in two articles by Mortenson et al. (1962) and Mortenson (1962).

Briefly the problem seems at present to stand as follows: Cell-free extracts of *Cl. butyricum* when prepared under proper conditions can fix elemental nitrogen actively with pyruvate as electron donor; practically all the fixed nitrogen can be recovered as ammonia within two hours, and approximately one μ-atom nitrogen is fixed per μ-mole of metabolized pyruvate (Mortenson, 1962). A particulate nitrogen-fixing enzyme could not be isolated by centrifugation at 144,000 $\times$ g, but it was possible to separate the extracts into two fractions individually unable to fix nitrogen but able to do so when recombined. One fraction seemed to contain the long-sought-for "nitrogenase," the nitrogen-activating system that combines with the elemental nitrogen, and the other fraction contained the pyruvate-dehydrogenating system responsible for the reduction (to the ammonia level) of the chemisorbed nitrogen. The nitrogenase is inducible and is probably an iron protein; the place of the molybdenum is still obscure (possibly in the hydrogen-donating system).

(7). Ammonia, as key compound of the nitrogen fixation, tends to inhibit this process by competition with the free nitrogen. No nitrogenase appears to be synthesized during growth with ammonium nitrogen. Cells grown with free nitrogen cease to fix nitrogen in the presence of ammonia. This happens very rapidly in *A. vinelandii* while *Cl. butyricum* may utilize the two nitrogen sources simultaneously (Wilson, 1958). Nitrate has a similar effect in organisms able to utilize it, but in the azotobacter the inhibition is preceded by a lag phase corresponding to the induction of biosynthesis of nitrate reductase. In some cases the azotobacter may utilize nitrate and gaseous nitrogen simultaneously (Becking, 1962a; Bortels, 1936).

(8). The process of nitrogen fixation as such, i.e., the reduction of elemental nitrogen to the ammonia level, seems to require very little energy (Wilson, 1958) or may even be accompanied by a decrease in free energy (Bayliss, 1956). Thus the nitrogen-fixing economy of an organotrophic organism will not be determined by the special nitrogen source but by the amount of cell material plus extracellular metabolites that can be synthesized at the expense of a given quantity of metabolized organic matter. If we choose glucose as an example of a standard energy source and imagine half of its carbon spent in respiration and the remainder utilized for synthesis of cell material that in its dry matter contains 50% carbon and 10% nitrogen derived from the atmosphere, we arrive at a yield of 40 mg fixed nitrogen per gram consumed glucose. Except perhaps in the experiments of Meyerhof and Burk (1927) at minimal oxygen pressure, such a gain of nitrogen has not been experimentally verified in pure cultures of azotobacter where the amount of synthesized cell substance is more like one-fourth of the amount of consumed carbon source—which agrees well with the normally observed maximum yield near 20 mg N/g carbohydrate. Higher yields than 40-50 mg/g carbohydrate could hardly be achieved unless metabolic products of high nitrogen content (e.g., ammonia) were continuously excreted and removed without interfering with the process of nitrogen fixation. In pure cultures of nitrogen-fixing bacteria the excretion of nitrogen compounds into the medium is usually small, at the most 50% in *Cl. butyricum* (Rosenblum and Wilson, 1950),

but it might reach a different magnitude in mixed cultures, although no direct evidence is yet available. Nevertheless, such an excretion could be possible if the fixation were to be regarded as a process alternative to respiration. This hypothesis seems attractive inasmuch as the fundamental processes are the same in nonsymbiotic and symbiotic nitrogen fixation, and the latter does indeed bear the character of a kind of respiration process accompanied by a copious transport of glutamic acid from the nodule tissue (Bond, 1936). Until further experimentation, however, this remains no more than hypothesis.

C. Energy Materials Utilized by Associated Growth

Winogradsky has repeatedly emphasized that the normal food substances of the azotobacter when growing in the soil are not compounds like glucose or mannitol that are currently regarded as "favorable" nutrients but are unlikely to arise in the soil in any quantity. Some sugars or related substances may occasionally become available, for instance in the form of young undecomposed plant materials, but in such cases the sugars are mostly accompanied by constituents like amino acids, proteins, etc., that provide nitrogen for the microflora and thus counteract the nitrogen fixers. By contrast, the natural carbon sources of the azotobacter are as pointed out by Winogradsky (1930) more likely to be very simple compounds like ethanol, butanol, acetate, propionate, butyrate, etc., that arise as products of fermentation or other incomplete dissimilation of various high-molecular substances. These are of course extremely varied but not all equally apt to provide energy material for nitrogen fixation. Some important organic materials have a narrow carbon-nitrogen ratio and are liable to decomposition with release of fixed mineral nitrogen: dead microbial tissue that arises in the soil, animal residues and excreta, farmyard manure or composts, and finally the soil "humus" itself. On the other hand the bulk of the organic matter continuously added to the soil is represented by dead plant residues that chiefly consist of nonnitrogenous skeletal substances: celluloses, hemicelluloses, and lignin. These are generally not directly available to the nitrogen fixers which can only utilize them in association with other organisms whose metabolic by-products they can intercept. An exception might be represented by the nocardias of Metcalfe and Brown (1957) which, however, do not seem to be prominent in nature. The yield of fixed nitrogen in such associated cultures depends upon (a) the quantity of the organic compounds that the "decomposer" releases from the high-molecular material, (b) the serviceability of these compounds as carbon food for the "nitrogen fixer," and (c) the availability of the fixed nitrogen that the latter puts at the disposal of its associate. In natural populations the relationships will be further complicated by the metabolic activity of other organisms interposing themselves between the "decomposer" and the "nitrogen fixer."

Cellulose is probably that organic substance which exists in the greatest quantity in nature. Commensurate with this enormous mass, a legion of microorganisms are capable of decomposing cellulose: higher and lower

fungi, actinomycetes, myxobacteria, eubacteria (aerobic and anaerobic, spore-forming and non-spore-forming, mesophilic and thermophilic)—and in addition a few lower animals. For reviews of the cellulose-decomposing microorganisms see Siu (1951) and Imschenetzki (1953). Their metabolic activities are correspondingly varied and include formation of by-products like formic, acetic, propionic, butyric, lactic, pyruvic and succinic acids, ethanol, butanol, acetaldehyde and sometimes cellobiose and glucose. All these products seem to be formed more copiously by the thermophiles and the anaerobic bacteria (e.g., the rumen inhabitants) than by the aerobic organisms mainly active in the soil: cytophagas, cellvibrios, fungi, and some actinomycetes. Earlier investigations quoted by Jensen (1940a), Imschenetzki (1953), and Fedorov (1952) have shown that associations between strains of *Cl. butyricum* and cultures of anaerobic cellulose-decomposing bacteria (presumably impure) may fix 7 to 10 mg nitrogen per g fermented cellulose. In view of the fact that the share of energy material consumed by the cellulose decomposers, the nitrogen-fixers and the probable contaminants is unknown, this yield appears remarkably high and more than equal to what has later been observed in pure cultures of clostridia (Rosenblum and Wilson, 1950; Westlake and Wilson, 1959). The main results of more recent research may be summarized as follows:

(1). There is little evidence of nitrogen fixation by the azotobacter in association with the important aerobic soil inhabitants *Sporocytophaga* and *Cytophaga* that decompose cellulose via oligocelluloses, cellobiose, and glucose and are largely restricted to utilization of this class of compounds. The carbon sources are dissimilated oxidatively with formation of no other organic by-products than small amounts of acetic acid; a mucilage of polysaccharide- or polyuronide-like character is synthesized. Azotobacter may multiply to some extent in association with the cytophagas (Fedorov, 1952; Imschenetzki, 1953; Jensen, 1940b), but the gains of nitrogen are nil or scarcely significant.

(2). A second important group of cellulose-decomposing soil bacteria are the cellvibrios which are metabolically not very different from the cytophagas although they form less mucilage and utilize a wider range of carbon sources. Jensen (1940b) found no significant gains of nitrogen by azotobacter in association with *Cellvibrio* spp. Imschenetzki (1953) and Fedorov (1952) have later quoted experiments showing some multiplication of azotobacter in such cultures and sometimes (Fedorov, 1952) some acceleration of the cellulose decomposition, but the media were rich in nitrate, and nitrogen determinations were not reported.

(3). The azotobacter seem to behave similarly in association with several other aerobic cellulose decomposers. Vartiovaara (1938) found no gain or at best very small gains of nitrogen by *A. chroococcum* combined with cellulose-decomposing fungi under aerobic conditions but moderate gains at periodically alternating aerobic and anaerobic cultivation; probably the anaerobiosis favored the accumulation of intermediate hydrolysis products utilized by the azotobacter in the aerobic intervals. Jensen (1940b) likewise failed to find significant nitrogen fixation by azotobacter associated with strains of *Cellulobacillus, Streptomyces* sp., *Micromono-*

spora chalcea and *Trichoderma viride,* the last a typical cellulose-decomposing soil fungus. Apparently the metabolic by-products of these aerobic organisms are either unserviceable for the azotobacter or else quantitatively insufficient for appreciable gains of nitrogen.

(4). Certain facultative aerobic bacteria of a *Corynebacterium-* (or *Arthrobacter-*) type are able to promote nitrogen fixation by azotobacter (Jensen, 1940b). A quantitative investigation by Jensen and Swaby (1941) showed that *Azotobacter* spp. as well as a *Beijerinckia* under these conditions could fix almost as much nitrogen per gram of consumed cellulose as when utilizing a favorable carbon source in pure culture (Table 1). The actual economy of fixation must have been still higher, since a part of the cellulose would be consumed by the associate. Restricted aeration (Eh-values below zero) increased the yield of fixed nitrogen, partly due to increased formation of organic by-products of cellulose decomposition but probably also to more economic utilization of these products. The concentration of soluble organic matter in the cellulose medium would be very low and thus a low oxygen tension should favour efficient nitrogen fixation (Parker and Scutt, 1960; Tschapek and Giambiagi, 1955).

A similar "feeding" of azotobacter may generally be expected from organisms that decompose cellulose with formation of available by-products, not least from the anaerobes and thermophiles. Such organisms may not be prevalent in the soil, and little is known about their association with nitrogen fixers. Imschenetzki (1959) reported vigorous multiplication of azotobacter but no gains of nitrogen in such cultures (medium with high initial nitrogen content).

(5). The nitrogen-fixing clostridia seem quite apt to enter into association with certain aerobic cellulose decomposers. Vartiovaara (1938) indeed failed to detect nitrogen fixation by *Cl. pasteurianum* associated with cellulose-decomposing fungi in atmospheric air, but considerable amounts were fixed by impure clostridium-cultures + fungi in alternating atmospheric air and nitrogen gas. Jensen (1941) found that a cytophaga and a few cellvibrios, actinomycetes and fungi failed to support growth of *Cl. butyricum* with cellulose, but one cellvibrio induced nitrogen fixation approaching 7 mg per g consumed cellulose (Table 2). With allowance for the unknown demands of the cellvibrio, this indicates an efficiency as high as ever observed (Rosenblum and Wilson, 1950; Westlake and Wilson, 1959). The efficiency increases further in the presence

Table 1. Gains of nitrogen in associated cultures of aerobic nitrogen-fixing and facultative anaerobic cellulose-decomposing bacteria (Jensen and Swaby, 1941).

Associates		Incubation days	N fixed, mg/g cellulose decomposed
Nitrogen fixer	Cellulose decomposer		
A. chroococcum	Corynebact. 3	48	10.3
A. chroococcum	Corynebact. V.a	42	11.5
A. chroococcum	Corynebact. V.b	35	14.3
A. beijerinckii	Corynebact. V.b	45	12.8
A. vinelandii	Corynebact. V.b	42	12.2
A. vinelandii	Corynebact. 3	28	10.5
Beij. indica	Corynebact. 3	48	10.8

Table 2. Gains of nitrogen in associated cultures of anaerobic nitrogen-fixing and aerobic (obligate or facultative) cellulose-decomposing bacteria (Jensen, 1941).

Associates		Incubation days	N fixed, mg/g cellulose decomposed
Nitrogen fixer	Cellulose decomposer		
Cl. butyricum	Cellvibrio G. 2	14	5.2
Cl. butyricum	Cellvibrio G. 2	28	6.8
Cl. butyricum	Cellvibrio G. 2	16	1.7
Cl. butyricum	Cellvibrio G. 2	40	4.1
Cl. butyricum	Corynebact. V.b.	33	3.3
Cl. butyricum	Corynebact. V.b.	50	1.9
do + A. chroococcum	Cellvibrio G. 2	18	8.0
do + A. chroococcum	Cellvibrio G. 2	33	11.9
A. chroococcum	Cellvibrio G. 2	35	(1.5)*

* Not significant.

of azotobacter, presumably because this consumes the fermentation products of the clostridia, but the facultative anaerobic corynebacterium gives a lesser effect, probably because its by-products are mostly organic acids and more acceptable to the azotobacter than to the clostridia.

Hemicelluloses represent the second major constituent of plant residues, with xylan predominating in straw, wood, and root materials and probably second only to cellulose in quantitative respect. Xylan-decomposing microorganisms are even more numerous than the cellulose-decomposers and ability to decompose the two substances is very often combined, even in the cytophagas although these do not metabolize the hydrolysis products of xylan (Sörensen, 1957). These products are xylose and oligoxylans of which the former only in the rarest instances is serviceable for the azotobacter (Jensen, 1959). For this reason simple association between aerobic xylan-splitting bacteria will not result in nitrogen fixation, but this may be induced by the facultative anaerobic corynebacteria (cf. Table 5) that also produce acid from xylan (Jensen and Swaby, 1941). A xylose-fermenting in addition to a xylan-hydrolyzing organism may have the same effect (Jensen, 1942).

The clostridia are largely able to ferment xylose and therefore more favorably placed than the azotobacter in association with xylan-decomposers. Combinations of *Cl. butyricum* and cellvibrios will fix nitrogen with xylan (Jensen, 1941), and this might be expected from any xylose-utilizing nitrogen-fixer in association with any xylan-hydrolyzing partner, but very little is known about the efficiency of these processes.

Among other hemicelluloses, pectic substances are fermented by some types of clostridia, as is well known from the retting of textile plants; the typical flax-retting agent *Cl. felsineum* is capable of nitrogen fixation (Rosenblum and Wilson, 1949). Certain strains of *A. vinelandii* will utilize arabinose very economically (Jensen, 1942) and might well be expected to fix nitrogen on the basis of araban in association with an organism capable of hydrolyzing this hemicellulose. Pectin and araban may thus be potential energy materials for nitrogen fixation, but the existing amounts of these substances in nature are probably not large enough to make them a material of major importance. The same is true of the methyl pentose rhamnose (Jensen, 1961).

Whether lignin, the third large component of plant residues, ever becomes available for nitrogen fixation is an open question and must remain so until more information is gathered about the mechanism of biological lignin degradation. Winogradsky (1930) suggested that the readiness with which the azotobacter utilize benzoic acid might indicate a possibility of such compounds arising from lignin. This seems rather farfetched, since benzoic acid itself occurs as a product of bio-synthesis, and whether the lignin molecule ever gives rise to cleavage products available to the azotobacter is unknown. The clostridia are unlikely to be active in this respect, since they do not generally attack aromatic compounds, and lignin is even more resistant to decomposition under anaerobic than under aerobic conditions.

A quite different type of association, between *Azotobacter* and green algae (Chlorophyceae) has been regarded as a nitrogen-fixing partnership of some significance, where the algae, largely on theoretical grounds, have been assumed to provide the azotobacter with energy material in the form of photosynthetic products and to receive fixed nitrogen in return. Although photosynthesis is an intracellular process and its immediate products are not normally excreted, there is nevertheless experimental evidence that nitrogen can be fixed in this way, at least *in vitro*. Stokes (1940), however, found that *A. chroococcum* and *A. vinelandii* would only grow feebly with nine strains of green algae as sole providers of energy material and concluded that this source of fixed nitrogen might be real but unimportant. Dead algal cell material was unserviceable as energy source, and owing to its high nitrogen content its decomposition by mixed populations would be accomplished by others than the azotobacter. Sulaiman (1944) arrived at the same conclusion with other algae. Association between clostridia and photosynthetic organisms that excrete oxygen is difficult to envisage although the clostridia could conceivably be active in periods of darkness; this, however, is unknown.

Associations of azotobacter with non-nitrogen-fixing blue-green algae would presumably resemble those with green algae, and no activity of the azotobacter can be expected in the presence of the nitrogen-fixing cyanophyceae whose excretion products are largely polypeptides (Fogg and Wolfe, 1954). In agreement herewith De (1939) did not find any increased nitrogen fixation in cultures of blue-green algae when azotobacter was also present. A quite different matter is the stimulating effect of several non-nitrogen-fixing bacteria on the growth and nitrogen fixation by at least one blue-green alga (Bjälfve, 1962).

III. THE SOIL ECOLOGY OF THE ORGANOTROPHIC NITROGEN FIXERS

A. The Azotobacteraceae (*Azotobacter* and *Beijerinckia*)

More has probably been written about the occurrence and life activities of the azotobacter than about any other single genus of soil bacteria. The mere qualitative detection of the azotobacter in soil by means of selec-

tive media offers no particular difficulties, but the estimation of their numerical abundance is seriously limited by lack of reliable methods—a limitation that indeed applies to all soil microorganisms. Two methods in current use are (a) colony counts on some solid selective medium, such as agar or silica gel, with a suitable carbon source and no added nitrogen, and (b) determination of the dilution where growth ceases in some corresponding liquid medium. The plate method has chiefly come into use since Winogradsky (1926) introduced the technique of planting soil particles on silica gel plates or sprinkling larger plates of the same medium with weighed quantities of soil. This procedure yields only comparative results since the number of cells giving rise to each single colony is unknown and may be large. To some extent this objection remains valid when shaken soil suspensions are used for plate inoculation, although microscopic methods suggest that aggregates of azotobacter-like cells rarely consist of more than a few individuals. The method of dilution counts in its various modifications (Augier, 1956; Pochon, 1954; Tchan, 1952; Wenzl, 1934) usually shows somewhat higher figures than the plate counts, but rarely of an essentially higher order of magnitude (cf., e.g., Wenzl, 1934; Lochhead and Thexton, 1936); the same applies to various modifications in the preparation of the soil suspension (shaking with glass beads, etc., cf. Brown et al., 1962).

Direct microscopic studies of stained soil suspensions (Winogradsky, 1926) or of contact slides according to the Rossi-Cholodny method become fruitful when the population of azotobacter has become abundant owing to the selective effect of non-nitrogenous energy material, but can give little information about the unmodified soil microflora where the azotobacter-like cells are mostly few and not identifiable with any certainty. Hopf (1949) devised an ingenious combination of microscopic and cultural methods: removal of individual cells from unfixed and unstained contact slides by micromanipulation, and transfer to appropriate media. This method seems theoretically ideal but is probably biassed in favor of the conspicuous-looking azotobacter-cells.

The *Azotobacter* spp. proper are world-widely distributed, apparently rare in arctic soils but may occur even in desert soils; a very complete survey is given by Fedorov (1952). Their commonest representatives in temperate regions are *A. chroococcum* and *A. beijerinckii,* while *A. vinelandii* may be relatively common in tropical and subtropical soils and *A. macrocytogenes* appears extremely rare. In spite of their global distribution the azotobacter are by no means ubiquitous soil inhabitants as seen from some examples collected in Table 3.

The density of azotobacter in soil under normal conditions is of a strikingly low order in comparison with the tens or even hundreds of millions of bacteria (incl. actinomycetes) that cultural methods usually reveal. Winogradsky (1926, 1928) found densities between a few and 20,000 colonies per gram of soil, with 2,000 as "normal." These figures apply to silica gel plates sprinkled with soil particles, but even the use of shaken soil suspension in solid or liquid media does not result in much higher counts, as shown by various examples collected in Table 4.

These predominantly low counts that rarely exceed tens of thousands

Table 3. Occurrence of azotobacter in different parts of the world.

Geographic region and reference	No. of samples	% Az. positive
Europe: Germany (Niklas et al., 1926)	525	70
Denmark (Christensen, 1915)	142	49
Denmark (Jensen, 1950)	264	54
Switzerland (Stöckli, 1944)	1000	72
Italy (de 'Rossi, 1932a)	189	78
Soviet Union: Suschkina (1949)	3161	57
North America: Iowa (Martin et al., 1937)	287	75
Arizona (Martin, 1940)	213	51
Colorado (Gonick & Reuszer, 1949)	283	75
Australia: N.S.W., Vict. (Jensen & Swaby, 1940)	233	26
Queensland (McKnight, 1949)	146	43
Global: Becking (1961)	392	33

Table 4. Examples of the density of azotobacter in the soil.

Author and reference	Geographic region	Method	Azotobacter per gram of soil
Meiklejohn (1956)	England	Agar plate	10 - 700
Brown et al (1962)	England	Agar plate	1,600 - 18,000
Augier (1956)	France	Dilution	2,500 - 11,000
Rouquerol (1962)	France	Dilution	100 - 10^{11}
Jensen (1950)	Denmark	Agar plate	< 10 - 81,000
Stöckli (1944)	Switzerland	Agar plate	10 - 44,000
de'Rossi (1932)	Italy	Agar plate	<100 - 21,400
Abd-el-Malek (1962)	Egypt	Dilution	10 - 10^{8}
Uppal et al. (1939)	India	Agar plate	0 - 1.6×10^{6}
Lochhead (1936)	Canada	Agar plate	c. 100 - 1,000
Gonick & Reuszer (1949)	U.S.A.	Agar plate	<10 - 8,000
Jensen & Swaby (1940)	Australia	Agar plate	<10 - 2,300

per gram of soil suggest that the azotobacter represent normally only a very small fraction of the soil microflora. This has caused several investigators (de Rossi, 1932b; 1933; Lochead and Thexton, 1936; Jensen, 1940a) to become sceptical about the quantitative importance of the amounts of nitrogen gained by the normally occurring azotobacter populations—at least in comparison with the nitrogen requirements of crops from intensively cultivated soil. Three statements in Table 4 stand out in sharp contrast to the rest in showing counts that run into millions.* All three statements refer to somewhat unusual conditions: rice field soils and irrigated clay soils from the Nile Valley, with copious supply of plant residues. The possible significance of these factors are discussed later.

The azotobacter seem in a general way to be characteristic of well-cultivated soils, as pointed out already by Remy (1909), but the effect of cultivation is probably a complex one. Among individual soil properties, the reaction has long been recognized as a factor of prime importance

* Some of the highest counts (Rouquérol, 1962) would seem unrealistic. If the average volume of an azotobacter cell is estimated at merely 2 cubic microns (which is almost certainly too low), 10^{11} cells would occupy a volume of 0.2 cc. or 1/5 g of soil. Such figures are only imaginable if the growth were assumed to originate from ultramicroscopic filterable forms, the existence of which in the soil is possible but unknown.

in governing the distribution of the azotobacter, as probably first ascertained by Christensen (1915). Many earlier observations as well as more recent ones (Gonick and Reuszer, 1949; Jensen, 1940a, 1950; Suschkina, 1949) have shown that at least in soils of temperate regions the azotobacter rarely occur below a pH level of 6.0, in conformity with their usual behavior *in vitro*. If found below pH 6 they are usually few in number and are not stimulated to active growth when suitable energy material and mineral nutrients are provided. In tropical and subtropical soil there is some indication of more widespread representation at acid soil reaction (Döbereiner, 1953). The data in Table 5 illustrate the relation between soil pH and the occurrence as well as the density of the azotobacter population in cultivated soils from a temperate region.

Table 5. Correlation between soil pH and density of *Azotobacter* (colonies per gram) in 264 Danish soils (1950).

Range of pH	Number of soils with Az.-density					Total No.	Az.+ %
	0	$<10^2$	10^2-10^3	10^3-10^4	10^4-10^5		
<6.0	38	4	0	0	0	42	10
6.0 - 6.4	40	14	1	0	0	55	27
6.5 - 6.9	31	22	10	1	0	64	52
7.0 - 7.4	11	20	13	4	1	49	78
7.5 - 7.9	2	10	16	6	2	36	94
8.0 - 8.5	0	1	12	5	0	18	100

Total range of pH: 4.5 - 8.5. - Correlation coefficient between pH and log/azotobacter : r = 0.54.

Absence of paucity of azotobacter in soils of favorable reaction may be due to a multiplicity of factors. Important among these is the content of available phosphate for which the demand of the azotobacter is very marked. Christensen (1915) seems to have been the first to suggest the use of *A. chroococcum* for a bio-assay of available soil phosphorus on this basis. These earlier results have been borne out by more recent research. Ziemecka (1932), in England, found no development of azotobacter on plaques of soil containing less than 10 ppm water-soluble P_2O_5. Stöckli (1944), in Switzerland, found a marked correlation between azotobacter-density (by plate counts) and content of bicarbonate-soluble phosphate, and an examination of 121 cultivated soils from Denmark (Jensen, 1950) showed a partial correlation coefficient (pH eliminated) of 0.36 between log (azotobacter) and content of phosphate soluble in dilute sulphuric acid. Also Martin and co-workers (1937, 1940) found certain positive although less direct correlations between phosphate content and occurrence of azotobacter in soils from Iowa and Arizona. Van Niel (1935) found California soils that failed to support azotobacter owing to molybdenum deficiency, which is probably exceptional. Kaila (1954) suggested that toxic soluble aluminum compounds in addition to acidity might prevent the occurrence of azotobacter.

Whether antibiotic effect of other soil organisms will limit or suppress the growth of azotobacter in the soil is unknown but seems at least possible since many soil actinomycetes show antagonism towards azotobacter *in vitro* (Jensen, 1950; Nickell and Burkholder, 1947).

The content of total organic matter seems to be of relatively little consequence and would perhaps chiefly affect the azotobacter through its influence on the physical condition of the soil. Stöckli (1944) did find some correlation between azotobacter and humus content and Martin et al. (1937) likewise between degree of azotobacter-development and total nitrogen content which is mostly present in humus. No such correlation was found in Danish soils (Jensen, 1950) and could indeed hardly be expected, since the bulk of the "humus" compounds are unavailable to the azotobacter. The organic soil constituents of immediate significance are the simple compounds utilizable as carbon sources and those nitrogenous substances that are mineralized by the soil microflora with the release of ammonia. Biologically available nitrogen favors the bulk of the soil microflora in the competition with the nitrogen-fixers for the available energy material; therefore the carbon-nitrogen ratio of the organic material that at any moment is being mineralized becomes a most important factor in the soil ecology of azotobacter. It is indeed a common experience that nitrogenous fertilizers tend to reduce the density of the azotobacter-population. In laboratory experiments Winogradsky (1926) found that 50 ppm of nitrate-nitrogen strongly inhibited and that 100 ppm completely suppressed the spontaneous azotobacter development in soil + 1.0% glucose. Continued experiments (Winogradsky and Ziemecka, 1928) with big mannitol silica gel plates showed that 2.5 mg NO_3 – N per g mannitol had a notable inhibitory effect on azotobacter-growth and nitrogen fixation and this increased strongly with increasing doses of nitrate; nevertheless other experiments (cf. Table 6) have shown that multiplication of azotobacter in soil is not excluded even in the presence of considerable amounts of nitrate. Under field conditions Ziemecka (1932) estimated a density of some 8,000 azotobacter per gram of soil from plots that permanently received non-nitrogenous artificial fertilizers, but the figures dropped to a level of 30 to 300 per gram in plots that additionally received nitrate, ammonium sulphate or rape cake, the last an example of readily mineralizable organic nitrogen. Lochhead and Thexton (1936) observed consistently higher counts of azotobacter in unfertilized plots than in those receiving manure or nitrate, and Stöckli (1944) quotes examples of drastic lowering of the azotobacter-counts after application of nitrate, urea or liquid manure (essentially an ammoniacal fertilizer).

The density of azotobacter is nearly always reported to be highest in the top layers of soil and decreasing rapidly with the depth. Only in the most exceptional cases has a maximum density been observed in deeper layers of soils from arid regions (Garbosky, 1956; Sabinin and Minina, 1932). A complicated interaction seems to exist between moisture and access of air, i.e., oxygen tension. Jensen and Swaby (1940) studied the influence of moisture varying between waterlogging and a minimum corresponding to pF-values (logarithms of the capillary tension) of 3.8-3.9. The numbers of azotobacter in non-enriched soil were scarcely influenced, but addition of 0.2% glucose induced a maximum multiplication at pF 2.8. Dommergues (1962) found the limiting pF values to be between 3.6 and 4.4 for multiplication of azotobacter in tropical black clay soils; this

seemed to be of the same order as the wilting-point of higher plants. Obviously some very complicated relationships may be expected between the factors of moisture requirement, oxygen requirement and concentration of nutrient substances in the soil solution.

There is no evidence that the temperature affects the growth of azotobacter in soil differently from what it does *in vitro*. As growth of most azotobacter is poor or nil at temperatures below 8-10°C, it appears that long annual periods will be unfavorable for their activity in soil from cool regions though the population may be quite dense. A similar effect may be expected during the dry season in many tropical and subtropical soils. It is noteworthy in this connection that the *Azotobacter* proper (unlike *Azomonas*) are very resistant to desiccation owing to their microcyst-forming habit (Winogradsky, 1938).

When environmental factors like soil pH, supply of mineral nutrients, moisture, temperature etc. are favorable and the level of available mineral nitrogen is low or zero, the development of the azotobacter will largely depend on the supply of available organic matter to serve as source of carbon and energy. Incorporation of substances like glucose, starch, mannitol, ethanol, butanol or salts of several organic acids in amounts of $\frac{1}{2}$ to 2% will then stimulate the azotobacter to a multiplication that runs into hundreds or thousands of organisms per gram of soil and makes them the altogether dominant component of the soil microflora. This was first shown by Winogradsky (1926) and repeatedly confirmed (Gonick and Reuszer, 1949; Jensen, 1940a; de'Rossi, 1933); under these conditions the microscopic and cultural methods of counting may show figures of the same order of magnitude (Jensen, 1940a). This outburst of growth may happen with great rapidity, beginning after 24 hours and reaching a maximum after 3 to 4 days (Winogradsky, 1926). Darzniek (1961) found the multiplication of azotobacter (by plate counting) in sucrose-enriched soil beginning after a lag period of 3 to 6 hours at 25°C and continuing for the next 18 hours with a calculated (actually shorter) generation time of 106 to 232 minutes. The generation time was much longer, about 8 to 18 hours, in soil with addition of cotton root and leaf material and similar in irrigated field soil.

Concentrations of the order of 1% sugar and similar materials are of course not normally to be expected in the soil and microbial populations entirely dominated by azotobacter (or clostridia, for that matter) arising in response to such treatment may legitimately be considered artifacts that have no counterpart under natural soil conditions. However, much smaller amounts of simpler energy source may elicit a rapid proliferation of azotobacter, as shown in an experiment (Jensen, 1950) where 50 to 500 ppm ethanol was added to soils of varying character but all of adequate reaction (pH 7.2-7.6) and phosphate content. The soils were incubated in a moist condition at 25°C and azotobacter were counted on agar medium periodically within 72 hours.

The results in Table 6 show that the azotobacter may respond to ethanol doses as small as 40-50 ppm within 20 hours, and doses 10 times higher give rise to populations much denser than normally found in soil. Two features are noteworthy: firstly, azotobacter will multiply in the

Table 6. Multiplication of azotobacter in response to small doses of ethanol (Jensen, 1950).

Soil	Hours	Addition of ethanol ppm				
		0	50	100	200	500
A: Loam	0	140				
Azotobacter/g	18	180	370	1,940		5,000
	42	180	360	1,730		177,000
	70	100	650	1,540		750,000
NO_3-N ppm	70	43	44	35		17
B: Loam	0	440				
Azotobacter/g	18	490		2,100		79,000
	44	370		5,400		103,000
	72	340		700		67,000
NO_3-N ppm	72	45		44		45
C: Sand	20	40	27		170	2,300
Azotobacter/g	35	34	40		70	1,300
	72	63	30		80	1,700

presence of 20-40 ppm nitrate-nitrogen, contrary to the statement of Winogradsky (1926); secondly, the sandy soil supports a much less abundant azotobacter-population than the loam soils—possibly because the open structure permits an oxygen tension that is unfavorably high for growth in a dilute medium (cf. Tschapek and Giambiagi, 1954, 1955).

Among less well-defined environmental factors alleged to influence the growth of the azotobacter is the effect of atmospheric conditions, which should manifest itself in soil as well as *in vitro,* according to Bortels (1940) who has dealt with this topic in several publications. Final confirmation is still needed.

The second genus of the Azotobacteraceae, the beijerinckias, differ markedly from the azotobacter in ecological respect and were long thought to be confined to the tropical regions, having been detected only in India, Indonesia, Northern Australia, South America and Central Africa. Only in recent years have they been found to occur occasionally in subtropic and warm temperate countries (Mediterranean Europe, South Africa, Japan) as stated from the very complete review of Becking (1961). They appear largely to take the place of azotobacter in tropical regions where Becking found them present in 48% of the samples tested (especially in the pH-range of 5.5 to 5.9), while only 25% harbored azotobacter; the corresponding figures for nontropical soils were 9 and 39%.

The lower incidence of azotobacter in the tropical soils is largely a question of pH, but no adequate explanation has been found for this restricted occurrence of the beijerinckias. Their temperature interval for growth is rather narrower than that of the azotobacter especially at the upper end of the scale, and they survive remarkably well at low temperature (Becking, 1961). Their predominance in red lateritic soils typical of the tropic regions and very poor in exchangeable calcium has been thought to be due to the fact that the beijerinckias do not require calcium but are on the contrary sensitive to it; however, soils poor in exchangeable calcium also exist outside the tropics, and the quantitative

calcium demands of the azotobacter are only small. Becking (1961) suggested that the greater tolerance of the beijerinckias for iron and aluminum ions would favor their existence in the acid lateritic soils whereas in alkaline soils they would be outgrown by the more rapidly growing azotobacter. Still this does not explain why the beijerinckias should be absent from acid soils of cool regions although they do occur in tropical mountain soils under similar temperature conditions.

Derx (1953) suggested a possible association of the beijerinckias with certain tropical legumes or perhaps the Caesalpinioideae (rhizosphere organisms?), but Becking (1961) found no correlation with the type of vegetation. A notable aspect of the ecology of the beijerinckias is their copious growth on the leaf surface (to which Ruinen, 1956, gave the name "phyllosphere") of tropical trees whence they may be transferred to the soil. This agrees with the observations of Döbereiner and Alvahydo (1959) that washings from stems and leaves of sugar cane plants stimulate the beijerinckia-population of the soil and that sugar cane cultivation greatly favored the distribution of beijerinckia in Brazilian soils. Perhaps the limited occurrence of the beijerinckias has some connection with the presence of plant materials rich in sugars, since the beijerinckias as a whole seem much less apt to utilize the simpler organic acids and lower alcohols that are serviceable for most species of *Azotobacter*.

Little is known about the numerical abundance of the beijerinckias in the soils where they do occur. Agar plate counts (Derx, 1950; Döbereiner and Alvahydo, 1959; Tchan, 1953) suggest densities comparable to those of the azotobacter, and high soil moisture seems particularly favorable (Döbereiner and Alvahydo, 1959). Here again the relation of the cultural counts to the real number of cell individuals remains obscure. Virtually nothing is known about the soil ecology of *Derxia* because so far only four isolations have been reported (Jensen et al., 1960; Ray, 1962).

B. The Nitrogen-Fixing Clostridia

In contrast to the Azotobacteraceae, the large group of *Clostridium butyricum* and related nitrogen-fixing species are practically ubiquitous soil inhabitants whose presence *per se* thus does not seem to depend much on soil properties. This appears even in the early investigations of Bredemann (1909) who examined 134 soil samples of world-wide distribution and found butyric acid clostridia in all except 5, among which a sphagnum peat and an arctic beach sand. One obviously important element in this respect is their faculty of producing endospores that ensure their survival at least in a dormant form under adverse soil conditions but which on the other hand makes it difficult to estimate the size of the metabolically active population: the vegetative cells. Upon the whole there are no really adequate methods available for any accurate determination of numbers of anaerobic bacteria in general; all figures resulting from cultural counting methods therefore represent minimum values (as in the azotobacter, but probably more so). Dilution counts or some-

times anaerobic plate counts have mostly shown figures from sporadic to around 10^5 per gram (Augier, 1957; Jensen and Swaby, 1940; Meiklejohn, 1956). Far higher numbers even exceeding 10^8 per gram have been reported in other cases where particularly favourable conditions seem to exist (Abd-el-Malek and Ishac, 1962). Endospores sometimes seem to account for a very high proportion of the numbers (Jensen and Swaby, 1940), but this may partly be due to the difficulties of estimating the real size of the vegetative cell population.

As might be expected, anaerobic conditions will favor the growth of the clostridia. Winogradsky (1926) has shown that it is possible to encourage growth of clostridia as well as azotobacter even in moderately moist glucose-enriched soil simply by increasing the depth of the soil column, evidently because the azotobacter rapidly exhaust the oxygen of the soil atmosphere in the deeper layers. The level of anaerobiosis may similarly be raised or lowered by varying the moisture content, and the clostridia will at high moisture content entirely dominate the microscopic picture within two to three days and reach numbers estimated at hundreds or thousands of millions per gram. Microscopic counts in similarly treated soil (Jensen, 1940a) showed vegetative clostridium-cells numbering about 120 to 660 million per g or roughly 25 to 50% of the total bacterial flora. By contrast it is most rare to find cells that resemble vegetative cells of clostridia by microscopic examination of the normal soil microflora. To all appearances the clostridia are like the rest of the spore-forming soil bacteria typical "zymogenic" organisms that only enter a stage of active life when favorable conditions are created: provision of available energy material at a level of oxygen tension compatible with the anaerobic nature of the clostridia. As is well known, the clostridia may also co-exist with aerobic organisms, e.g., azotobacter, that lower the redox potential of the medium to a level where anaerobic organisms can initiate growth.

The clostridia have often been assumed to be more adaptable to varying soil conditions than the azotobacter owing to greater tolerance of acid reaction. Actually they resemble the azotobacter in showing optimum growth at approximately neutral reaction and although they can acidify the medium to pH around 4 it is uncertain how little or how much their nitrogen-fixing activity is impaired by acid reaction. The nitrogen-fixing enzyme system itself has a quite definite pH-optimum at pH 6.6 with rapid decline of activity on both sides (Mortenson, 1962).

There is little known about organic compounds specially favorable for the growth of clostridia in the soil. Their ability to utilize a wider range of carboyhdrates and related compounds may give them an advantage in comparison with the azotobacter, but on the other hand they will hardly avail themselves of simple alcohols and organic acids except in special cases: lactate + acetate by some strains of *Cl. butyricum,* ethanol + acetate by *Cl. kluyveri* which also fixes nitrogen (Rosenblum and Wilson, 1949). Since the azotobacter readily utilizes this class of compounds which the clostridia will produce by fermentation of compounds partly unavailable to the azotobacter, the two groups are very naturally fit to enter into symbiotic associations.

Upon the whole it must be admitted that our knowledge of the soil

ecology of the *Cl. butyricum*-group is still quite fragmentary and warrants further study.

C. The Miscellaneous Nitrogen Fixers

What has been said about the clostridia will probably also largely apply to the facultative anaerobic *Bac. polymyxa* whose physiology of nitrogen fixation is very similar to that of the clostridia (Grau and Wilson, 1963). Ecologically it is a widely distributed soil inhabitant (Bredemann, 1908) which may become active under the same conditions as the clostridia and simultaneously with these.

Hardly any information has been gathered about the ecology of the other potentially nitrogen-fixing microorganisms, their relative abundance in the soil or the opportunities that they may find for displaying their nitrogen-fixing effect. Their facultative anaerobic and somewhat oxygen-sensitive *Aerobacter* and *Achromobacter* might well like *Bac. polymyxa* become active together with the clostridia, although there is no positive evidence that they do so. It is equally difficult to judge the importance of the aerobic members of the group. None of them seems to have properties that would make them specially apt to flourish under conditions where the more specialized nitrogen-fixers will not. The yeasts could be imagined to fix nitrogen in soils too acid for the azotobacter and outside the region of the beijerinckias, but generally it seems possible to subscribe to the opinion of Winogradsky and Ziemecka (1928) that only the azotobacteraceae and the clostridia are quantitatively important agents of nitrogen fixation.

D. The Rhizosphere: A Special Habitat and a Side Issue

The conception that the immediate surroundings of the plant roots carry a particularly dense population of microorganisms and are the seat of a correspondingly high biological activity was developed nearly sixty years ago by Hiltner who coined the name "rhizosphere" for this zone. This "rhizosphere-effect" was naturally thought to apply also to the nitrogen-fixing bacteria—an idea that probably was behind earlier statements of stronger nitrogen fixation in soils planted to non-leguminous crops compared with unplanted soil. The first who definitely suggested organic root substances as nutrients for the azotobacter in the rhizosphere was probably Loew (1927) who regarded this association as important in maintaining the nitrogen status of Brazilian plantation soils. Subsequent work has given little support to this idea. Microscopic methods show only a very minor representation of azotobacter-like cells in the total rhizosphere population (Starkey, 1938), and cultural counting methods generally lead to the same results. Some increased density of azotobacter in the rhizosphere is sometimes observed (Brown et al., 1962b; Jensen, 1940a), but the effect is often nil or may even be negative (Darzniek, 1960; Katznelson, 1946), and when positive it seems slight in com-

parison with the effect on the rest of the microflora (Jensen, 1940a) and to occur in the soil adjacent to the roots rather than on the actual root surface (Brown et al., 1962b; Vancura et al., 1959; Vancura and Macura, 1959).

This suggests that the rhizosphere conditions tend to favor other microorganisms rather than the azotobacter—a view supported by much information on the nature of root secretion products that has been gathered in recent years, as reviewed by Rovira (1962). These products include a mixture of reducing sugars, organic acids, numerous amino acids, nucleotides, vitamins, etc. Such a medium is quite non-selective for the nitrogen fixers and would place them very unfavorably in the competition with the general microflora. Vancura and Macura (1961) could separate the root secretions of barley and wheat into fractions that stimulated growth of azotobacter (glucose and organic acids) or inhibited its nitrogen fixation when added to glucose medium (amino acids). Varying proportion of such constituents may be the reason why root secretions of different plants will either stimulate or inhibit the growth of azotobacter and other bacteria (Krasil'nikov, 1961; Metz, 1955). Spicher (1954) found that the exudation products of barley roots could indeed serve as material for nitrogen fixation but lowered the pH of the rhizosphere to a level that inhibited growth of *A. chroococcum* unless extra buffer substance was present; this may well explain why the azotobacter seem to avoid the actual root surfaces but may be stimulated in the adjacent soil due to its buffer effect.

Experiments with aseptically grown plants associated with pure cultures of azotobacter would seem to give no true picture of what happens under natural conditions where the azotobacter must compete with non-nitrogen-fixing microorganisms in the rhizosphere. A few experiments of this kind have given little evidence of mutual benefit. Starc (1942) observed growth and slight nitrogen fixation by *A. chroococcum* grown together with corn in sand culture, but the fixed nitrogen was not taken up by the higher plant, and other bacteria tended to suppress azotobacter under non-aseptic conditions. Fedorov (1944) found under similar conditions that azotobacter could supply some nitrogen to corn plants receiving a small dose of nitrate, but the gain was only a very small fraction of the nitrogen content of plants with full nitrate supply.

The beijerinckias seem to show some particular features as rhizosphere inhabitants. Döbereiner (1961) observed low numbers of these organisms in Brazilian soils until sugar cane was planted, but then the numbers increased markedly during the following years, particularly in the immediate vicinity of the roots (the "rhizoplane"). This behavior of the beijerinckias in contrast to the azotobacter may well be due to their greater tolerance for the acid root exudates (cf. Spicher, 1954).

About the relationships of the clostridia in the rhizosphere we know very little (Katznelson, 1946, observed a strong stimulation in mangel rhizospheres) and about the rest of the nitrogen fixers scarcely anything.

"Bacterization"—a side issue. Although experimental evidence for any considerable nitrogen fixation in the rhizosphere is lacking, the subject has had a remarkable attraction for many soil microbiologists, particu-

larly in the East European countries. The interest seems largely to stem from investigations some forty years ago by Markrinoff (1924) who claimed that treatment of oat or barley grain with azotobacter before sowing resulted in notable increases of yield. Such "bacterization" of seed with cultures of azotobacter in peat, etc., has long been practiced on a large scale in Russia. An extensive literature exists on this subject, of which Allison (1947) and more recently Cooper (1959) have prepared valuable reviews (cf. also Fedorov, 1952). It is generally claimed that yields of cereals etc. may be increased by some 10 to 20%, but the effect is by no means universal and the crop yields appear to be generally low according to figures given by Fedorov (1952) and Mishustin and Naumova (1962).

Most attempts to reproduce these results in western countries have failed. Positive effects were rarely recorded and were then slight in comparison with the response to nitrogenous fertilizers. Among more recent investigators Wichtmann (1952) found small occasional gains in mustard, Vancura and Macura (1959) found statistically significant yield increases of 16 to 20% in oats (the actual yields were not stated), and particularly interesting is a brief report by Nutman (1962) on experiments at Rothamsted where increases of 28 to 40% were sometimes achieved in crops supplied with nitrogenous fertilizers.

The azotobacter-treatment of the seed seems sometimes to lead to the establishment of a permanent azotobacter-flora in the rhizosphere (Vancura et al., 1959; Vancura and Macura, 1959; Wichtmann, 1952). According to Brown et al. (1962b) this only happens in soil of pH above 6.7, but very high numbers may arise, amounting to millions of azotobacter per gram of soil even in the presence of nitrogenous fertilizers.

Among the Russian investigators there is no unity of opinion about the causes of the improved yields (Cooper, 1959). Stimulated nitrogen fixation is not generally regarded as important (although maintained by Fedorov, 1952), any more than protection against soil-borne pathogens which seems unlikely because the azotobacter have never been seen to produce antibiotic substances. A more plausible explanation would seem to be an improvement of early growth by some still unknown growth compound. Not only do the azotobacter produce large amounts of B-vitamins, but there is also clear evidence that culture filtrates from azotobacter will directly influence the growth of plant roots (Krasil'nikov, 1961; Metz, 1955; Spicher, 1954). The effect may be stimulatory or inhibitory (probably a question of concentration) and is not specific to azotobacter according to Krasil'nikov (1961). The active factor(s) is probably of a multiple nature. Spicher (1954) considered it to be a cationic compound that did not act by virtue of its nitrogen content. The presence of indolacetic acid (auxin) has also been reported (Kandler, 1951; Vancura and Macura, 1960), and Vancura (1961) detected gibberellic acid which he cautiously suggested might promote germination and early seedling growth.

The effect of "bacterization" thus appears to be qualitatively real but inconstant and unpredictable; it is mostly relatively small, probably not related to nitrogen fixation and not confined to the azotobacter. Its

future value for agricultural practice would seem almost entirely to turn upon the still undecided question whether it displays any beneficial effects in plants adequately provided with nutrients in the form of "orthodox" fertilizers.

E. The Direct Evidence for Nitrogen Fixation in the Soil

It has been shown many times that quantities of nitrogen large enough to be detected by the Kjeldahl method may be fixed in soils enriched with readily available energy materials in amounts sufficient to make the nitrogen fixers the predominant component of the soil microflora. The gains have not with certainty been shown to exceed 10 to 20 mg nitrogen per g spent energy materials corresponding to the nutritive value of glucose, which means that the economy of nitrogen fixation appears to be the same *"in terra"* as *in vitro*. The fixed nitrogen seems to be roughly accounted for in the cell substances of azotobacter or clostridia as determined by direct microscopic counting (Jensen, 1940a). Experiments with such enriched soils under laboratory conditions, however, only serve to demonstrate the potential "nitrogen-fixing power" of individual soils and the results are in nowise applicable to natural soil environments where low concentrations of simple organic compounds must be considered the normal state of affairs. An important question then poses itself: Is it permissible to extrapolate from the results of artificially stimulated nitrogen fixation in heavily enriched soils and to assume that the economy of nitrogen fixation under these conditions will also apply to natural soils?—There is *per se* no real evidence that low concentrations of nutrients will be utilized with particular efficiency, but several factors tend to obscure the picture. Firstly there is the important possibility that the economy of nitrogen fixation would be increased by a combination of low oxygen tension and low concentration of soluble organic matter in the soil solution. To this comes the possible effect of oxygen tension on the metabolism of the organisms that release nutrients from the insoluble organic substances, and finally as an opposing factor comes the inhibitory effect of combined nitrogen; at a constant level of nitrate- and/or ammonia-nitrogen this effect will obviously increase with decreasing concentration of organic matter, i.e., narrowing carbon-nitrogen ration.

An approach to these complicated problems is best obtained through nitrogen fixation experiments where natural plant materials are utilized in the soil under varying conditions. A most instructive contribution of this kind, although carried out with plant material alone, is due to Olsen (1932) who determined gains of nitrogen and loss of organic matter from forest litter during an 11-month incubation period at 2 levels of pH and 2 levels of oxygen access: "aerobic" (moderately moist) and "anaerobic" (water-saturated). Although losses of organic matter were greater under aerobic conditions, the gains of nitrogen were sometimes higher under water saturation, especially when calculated on the basis of organic matter lost. The nitrogen fixation under aerobic conditions

Table 7. Gains of nitrogen in leaf material + 2.0% $CaCO_3$, mg per g dry matter lost (calculated from the data of Olsen, 1932).

Beech leaves			Oak leaves		
Inc. days	Moist	Saturated	Inc. days	Moist	Saturated
0	0	0	0	0	0
95	4.0	5.2	92	7.7	6.5
161	4.3	7.8	159	5.7	6.3
229	2.6	9.0	221	5.5	4.8
337	2.7	10.0	327	4.8	3.9
Loss of dry matter, %	27.0	17.4		35.7	27.9
Gain of N, mg/100 g	78	180		170	109
pH	7.5-7.7	7.6-8.2		7.0-7.5	7.2-8.0

ceased when nitrate began to accumulate and appeared to be due to clostridia, since azotobacter could not be detected in the material. Some pertinent data are shown in Table 7. The fixation of nitrogen per unit weight of lost organic matter (i.e., disappeared as carbon dioxide, not the total amount metabolized!) is much the same or certainly not higher than in combined pure cultures of nitrogen-fixing and cellulose-decomposing bacteria (cf. Tables 1 and 2).

In essential agreement with Olsen's findings, experiments with straw in moderate moist nitrogen-poor soil or pure sand-kaolin-mixture (Jensen, 1940a) showed no gains of nitrogen in spite of vigorous multiplication of azotobacter, or only small gains at the expense of the water-soluble straw constituents alone. The failure of the microflora to utilize the rest of the straw appeared to be due to lack of formation of available metabolic by-products from the insoluble fraction, because addition of such compounds (acetate and lactate) immediately induced a further multiplication of azotobacter accompanied by a significant nitrogen fixation (Figs. 1 and 2). When the water content was raised to saturation level the growth of azotobacter on the soil surface became abundant and significant amounts of nitrogen were fixed with straw or root organic material, as shown in Table 8.

Table 8. Nitrogen fixation with plant residues in water-saturated soil or sand media (Jensen, 1940).

Medium	Incubation days	Gain of N ppm	Azotobacter per gram
Loam + oats straw 1.0%	70	18*	1.2×10^6
	120	13*	3.8×10^6
Sandy loam + oats straw 1.5%	62	16*	0.3×10^6
Sand soil + oats straw 1.5%	28	16**	3×10^6
	150	73**	4.7×10^4
	250	93**	6×10^3
Sand soil + wheat straw 1.0%	28	18**	9.5×10^6
	90	30**	5×10^6
Sand-kaolin mixture + wheat roots 2.5%	30	13**	3.3×10^6
	120	24**	0.3×10^6

* Significant at $P < 0.05$. ** do. at $P < 0.01$.

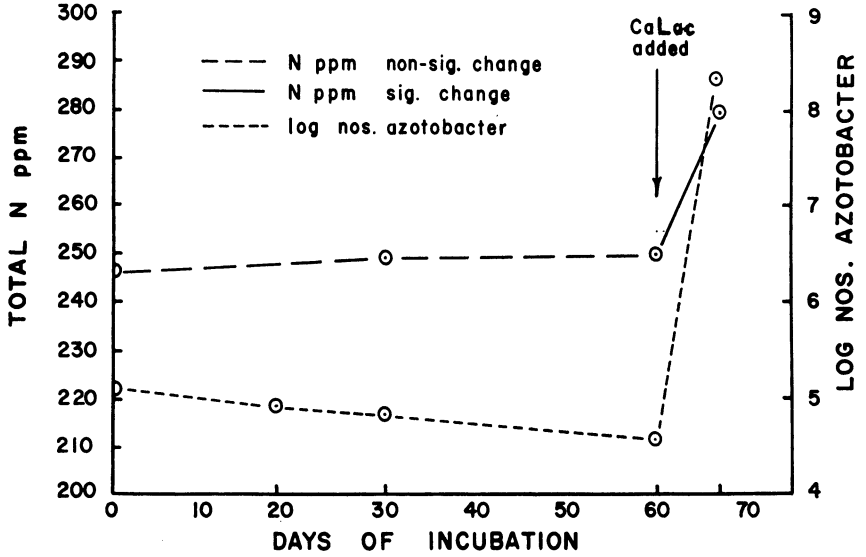

Fig. 1. Nitrogen fixation and numbers of azotobacter (by plate count) in moist sand soil + 1.0% filter paper cellulose followed by 0.5% Ca-lactate (indicated by arrow). (Gain of N following lactate addition significant at $P < 0.01$. (Jensen, 1940a).

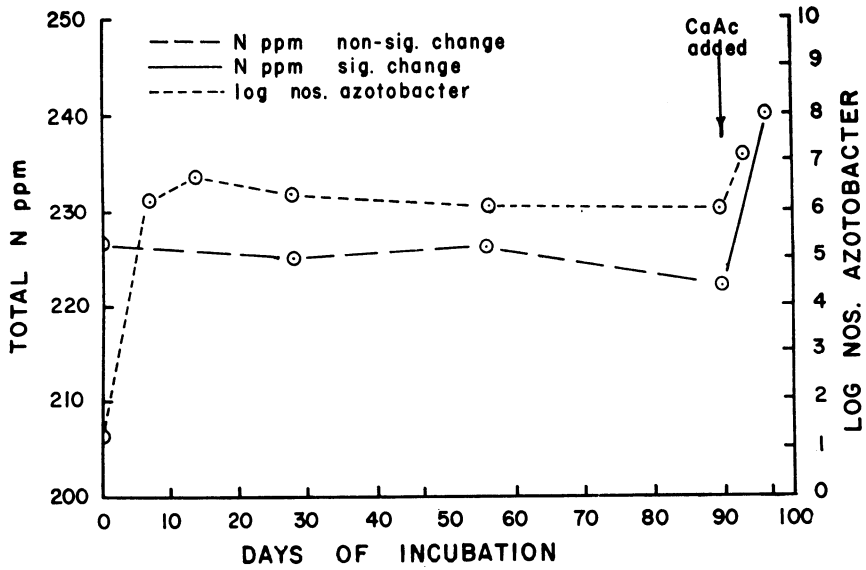

Fig. 2. Nitrogen fixation and numbers of azotobacter in moist sand soil + 1.0% wheat straw followed by 0.5% Ca-acetate (indicated by arrow). (Gain of N following acetate addition significant at $P < 0.01$) (Jensen, 1940a).

The gains of nitrogen, with a maximum of approx. 6 mg per g straw added, are accompanied by numbers of azotobacter that are not normally seen in natural soil but are comparable to those found by Uppal et al. (1939) and Rouquérol (1962) under similar conditions in the field

(cf. Table 4). No attempts were made to count the clostridia but microscopic examination failed to show more than at the most a sporadic occurrence of vegetative cells of clostridia; their significance in these experiments thus appears doubtful.

Bjälfve (1955) conducted similar experiments in moderately moist sand medium incubated in light or darkness. Gains of nitrogen with straw were only 1.0-1.2 mg per g in a year, but starch, which is available to most azotobacter, induced a "normal" fixation of 9-10 mg per g within 6 months (incubation in darkness). The loss of straw organic matter was determined in another experimental series and indicated a fixation of some 3.2-4.5 mg nitrogen per g lost dry matter.

Barrow and Jenkinson (1962) failed to detect nitrogen fixation in mixtures of soil and straw of moderate water content, but found gains of as much as 2.6-2.8 mg per g straw in water-saturated soil with restricted access of air. No bacteriological investigations were made, but it seems significant that no nitrogen was fixed in pure nitrogen atmosphere; this speaks strongly against clostridia as the agents of fixation.

Studies by means of the labeled-nitrogen (N_2^{15}) technique seem confined to a single contribution by Delwiche and Wijler (1956) who obtained negative results in various non-enriched soils or soils with addition of straw, grass, or grass roots. Nitrogen fixation was only detected (a) in soils enriched with glucose or sucrose which induced an intense proliferation of azotobacter, and (b) in decaying grass sods under restricted access of air; the gains of nitrogen in this medium corresponded to 12 pounds per acre at the most, while the method should permit detection of only 2 or 3 pounds per acre. Unfortunately Delwiche and Wijler's investigations have remained fragmentary, and further studies along this line are urgently needed.

Pot experiments with soils under growing crops represent a step towards field conditions and are numerous in the earlier and particularly the German literature but have led to no very definite results (Jensen, 1940a). More recently Moyer (1941) conducted pot and frame experiments (the latter a still closer approximation to field conditions) with fertile soils from semiarid North China and found no signs of nitrogen fixation after cropping for five years to nonleguminous crops; addition of oats straw or millet roots lessened the removal of nitrogen but did not lead to any net accretion. Fedorov (1952) describes a 3-year pot experiment with oats in soil variously treated with lime, straw (45 g per 2.5 kg soil), and azotobacter-inoculation. Fixation of 510 mg nitrogen per pot (= roughly 200 ppm or 11 to 12 mg per g added straw) was reported as the maximum gain, but this was calculated as difference between straw-lime-azotobacter-treated pots and uncropped control pots in which heavy but unexplained loss of nitrogen took place; the gain as difference between initial and final nitrogen in the treated pots appears to have been only 45 mg per pot (= 18 ppm or 1.0 mg per g straw).

Determination of the nitrogen balance in field soil should theoretically give the final answer to the question but is beset with formidable difficulties owing to many uncontrollable factors: addition of nitrogen by

wild-growing legumes, precipitation and possibly ammonia absorption from the atmosphere, removals by leaching, denitrification and perhaps ammonia evaporation and erosion; finally the sampling error is much more serious here than in homogenized laboratory samples. Nevertheless, a good deal of evidence has accumulated to indicate that the process is of little significance in soils under intensive cultivation but is recognizable in pasture soils, particularly if grass crops are not carried away. Much of the earlier literature was reviewed elsewhere (Jensen, 1940a). Among later observations, Whitt (1941) calculated gains of no less than 100 pounds per acre per annum ascribed to nonsymbiotic fixation, in soil permanently under legume-free blue-grass. Similarly Parker (1957) estimated annual nitrogen gains of 60 to 70 pounds per acre in the upper 10 inches of a heavy, grass-covered loam soil in West Australia. The author offered the very plausible explanation that the fine texture of the soil and the dense sward would restrict the oxygen supply and consequently increase the efficiency of the nitrogen fixation at the expense of the plant residues. It is highly desirable that investigations like these should be combined with studies of the composition of the soil microflora and its changes during the process of fixation.

The possible role of the beijerinckias under similar conditions cannot yet be estimated. Opportunities for their activity doubtless exist, since at least one strain will work as effectively as the azotobacter in association with cellulose-decomposing bacteria (cf. Table 1). Their reaction to reduced oxygen pressure vs. substrate concentration is not well known, but a brief statement by Döbereiner and Alvahydo (1959) suggests a behavior similar to the azotobacter. The same authors (Döbereiner and Alvahydo, 1959) report fixation of 2.3 mg nitrogen per g dry sugar cane leaf in a soil inoculated with *Beijerinckia*. Further research in this direction is needed.

No means are yet available for estimating the actual amount of organic matter transformed by the nitrogen fixers in the soil. Garbosky (1956) has made an attempt hereat by measuring the level at which *A. chroococcum* grows in an aqueous soil extract, compared with solutions of known mannitol concentration. In this way he calculated a "potential nitrogen-fixing capacity" of 7 to 20 kg nitrogen per hectare. The principle sounds ingenious but the results are obtained in a very indirect way and rest upon many hypothetical assumptions. Further development of the method might bring valuable results.

A possibility finally to be considered is the fixation of nitrogen by nonbiological processes. Several investigators have made such claims, from Berthelot (1890) on silent electric discharges to Dhar (1937) in many publications on photochemical nitrogen fixation. No convincing proof of the reality of these processes has so far been presented. Dhar's claims find no support in experiments by Bjälfve (1955) who detected no gains of nitrogen in truly sterile light-exposed sand media with or without added organic matter. On the other hand, nitrogen was fixed and organic carbon accumulated in nonsterile light-exposed sand without organic matter (cf. **Fig.** 3).

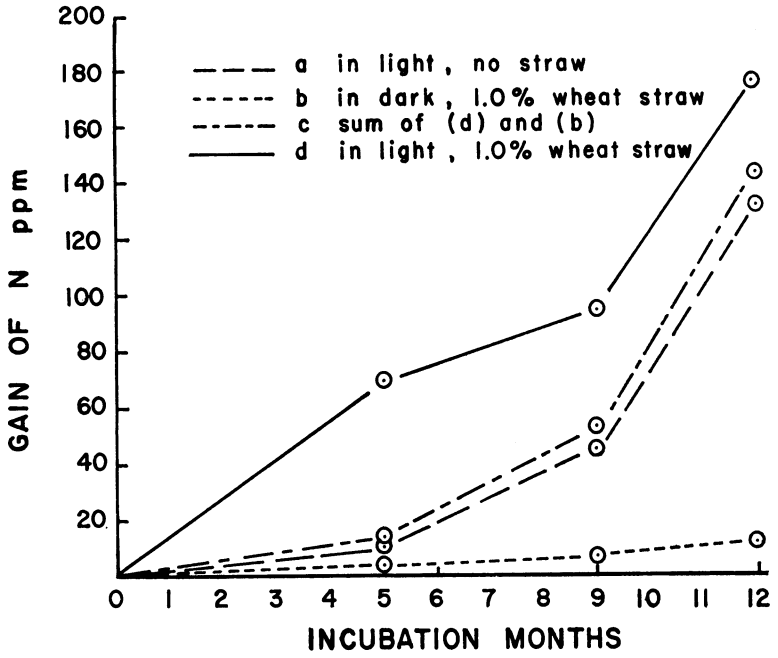

Fig. 3. Nitrogen fixation in moist sand ± 1.0% wheat straw incubated in light or darkness (Bjälfve, 1955).

IV. THE SOIL ECOLOGY OF THE LITHOTROPHIC NITROGEN FIXERS

The organisms of this group are, as far as soil inhabitants go, practically limited to the photosynthetic ones, among which the blue-green algae are predominant. They obviously differ profoundly in ecological respect from the organotrophs, inasmuch as they are independent of organic matter but can only display their activity at the soil-air interface and in water.

A. The Blue-Green Algae (Cyanophyceae)

The history of these organisms as authentic nitrogen fixers is little more than 30 years old, but the literature on their biochemistry, growth physiology and ecology is already very voluminous; for reviews see Fogg and Wolfe (1954), Fogg (1956), and Singh (1961). The nitrogen-fixing cyanophyceae are certainly the organisms most perfectly adapted for existence on the barest necessities of life: sunlight, air, water, and mineral salts, and consequently they are the classical pioneers on bare mineral soils, either independently or as constituents of lichens. In soil they are regularly found, but their abundance must vary enormously, and since they excrete relatively little of the fixation products during growth, it must be their bio-mass that determines their contribution to the pool of biologically fixed nitrogen.

Their importance in arable soils is probably slight, because frequent soil tillage will tend to disturb their growth on the soil surface, but they may be able to develop under dense cover crops owing to their low light intensity requirements. The total mass of cyanophyceae, including the non-nitrogen-fixing ones, is as difficult to estimate as that of the bacteria, but only the growth on the soil surface matters for the purpose of photosynthetic nitrogen fixation. According to Petersen (1935) the subterranean algae are relatively few and mostly present as resting cells; if active, they must depend on organic matter as facultative organotrophs, and any cyanophyceae that might fix nitrogen under these conditions would be counterparts of the azotobacter whose conditions they would share. Whether this happens at all is, however, unknown and perhaps unlikely.

The brilliant investigation by Schloesing and Laurent (1892) brought strong evidence that the epiterranean blue-green algae could become important nitrogen-fixing agents, although it could not then be definitely stated whether the algae or associated bacteria were the responsible agents. Verification of such algal activity under natural conditions came much later when Cameron and Fuller (1960) studied the algal crusts developing on both virgin and cultivated arid soils after rainfall; the crusts contained several species of nitrogen-fixing cyanophyceae and their nitrogen contents increased by some 240-400 ppm under illumination for 4 weeks in humid atmosphere. De (1936) was apparently the first to call attention to the importance of cyanophyceae in rice soils of India, where they grow abundantly in the flooding season. Later De (1939) proved the nitrogen-fixing power of several cyanophyceae isolated from such soils and considered it "legitimate to conclude that the fixation of nitrogen in the soils of the rice fields is mainly brought about by algae." De's pioneer work has been followed by a wealth of contributions, most of which are reviewed in a recent monograph by Singh (1961). Although world-widely distributed, the nitrogen-fixing cyanophyceae seem by no means always sufficiently abundant in rice soils to be detectable in small soil samples. Thus, Watanabe (1959a) found them in only 25 among 38 localities (851 soil samples altogether) from East and Southeast Asia; he therefore recommended their sowing as a green-manure crop and devised a method for large-scale cultivation of such algal inocula (Watanabe, 1959b). The very impressive gains of nitrogen that these organisms may achieve under favorable conditions have repeatedly been verified in laboratory experiments, although the relative effects of algae and bacteria are not always easy to separate. De and Sulaiman (1950) found that growing rice plants greatly increased nitrogen fixation in soil-water mixtures exposed to the light, apparently because the growth of algae was stimulated by the carbon dioxide production of the plant roots. As much as 40 mg nitrogen per rice plant could be fixed in a growth season, but decaying root material gave rise to a much smaller fixation apparently due to bacteria. In other experiments De and Mandal (1956) used closed respirometers and gasometric determination of nitrogen uptake. Six soils cropped to rice developed during 6 weeks a heavy growth of algae and fixed nitrogen in amounts corresponding to 14 to 44 pounds per acre, with addition of

phosphate and molybdenum even about 70 pounds per acre. Willis and Green (1949) found similar gains in pot experiments with rice, even in addition to the nitrogen carried away in the crops. Gains of this magnitude are not surprising in view of the growth rate that may be attained in pure cultures, where Allen and Arnon (1955) found a maximum daily production of 26 g dry matter of *Anabaena cylindrica* per m^2, with at least 1.0-1.2 g nitrogen; this corresponds to a fixation of 8.9-10.7 pounds per acre per day. Watanabe (1959) reported gains roughly one-third of this in cultivation on a technical scale.

The main difficulty of assessing the value of such figures is their translation to field conditions. There is as yet no ready answer to this problem, but recent work quoted by Singh (1961) gives some indication of the figures to be expected. The bio-mass of nitrogen-fixing algae in rice soils (among which *Aulosira fertilissima* is predominant in India) is difficult to estimate. Prahad, quoted by Singh (1961), calculated gains of 11.7 to 16.2 pounds per acre from nitrogen determinations in the algal mass collected from rice fields (this apparently in addition to the nitrogen removed in the crop). In other Indian soils the moist and undisturbed ground is always, according to Singh, covered by a growth of blue-green algae among which the shade-loving *Cylindrospermum licheniforme* is predominant, especially in sugar cane and corn fields but also on fallow soil and grassland. Singh developed a "micro-quadrat method" that consisted in removing the algal growth from the soil surface in randomly distributed quadrats of 225 cm^2 and determining nitrogen herein. By this obviously very approximative method Singh estimated gains of nitrogen in amounts of 64 to 96 pounds per acre within 75 days. Evidently much further study will be needed to give a precise idea about the reliability of such figures.

The cardinal ecological factors that allow optimum nitrogen fixation by the cyanophyceae would beside favorable temperature and light seem to be (a) sufficient moisture or even water-logging, (b) a soil surface undisturbed by soil cultivation for some length of time, (c) a soil reaction close to neutral or slightly alkaline, (d) adequate supply of mineral nutrients including molybdenum, and (e) absence or paucity of ammonia and nitrate nitrogen. The first three conditions are often fulfilled in Indian soils of which many according to Singh (1961) are neutral to strongly alkaline; in the latter ("usar") soils of pH around 9 *Nostoc commune* developed strongly under reclamation. The alternating drying and flooding to which rice soils are subject seems an essential factor in nitrogen fixation, as shown by Calder (1959) in experiments with an Uganda swamp soil where *Anabaena* were prominent. To this must be added the very small consumption of mineral nitrogenous fertilizers in Indian agriculture. It should be added, however, that not all investigators ascribe an equally great importance to algae as nitrogen-fixing agents in rice soils (Rouquérol, 1962; Uppal, 1939; Willis and Green, 1949).

In this connection it is worth noticing some laboratory experiments by Bjälfve (1955) in sand medium with addition of 1.0% straw or starch, sterile or nonsterile, and incubated for a year in light or in darkness.

No nitrogen was ever gained under sterile conditions, or in sand without organic matter and incubated in darkness, but fixation took place (a) in sand without organic matter incubated in light where it was accompanied by gains of organic carbon, (b) in dark-incubated sand with organic matter, and (c) in light-incubated sand with organic matter where it was sometimes stronger than in (a) and (b) together. Some of Bjälfve's data are shown in Fig. 3. The nitrogen-fixing agents were not identified but were almost certainly nitrogen-fixing cyanophyceae in (a), bacteria in (b) and a combination of both groups in (c). The obvious interaction between light and organic matter most probably consists in stimulated activity of the algae by organotrophic bacteria developing at the expense of the organic matter (cf. Bjälfve, 1962).

B. Miscellaneous Agents

It is difficult to form any opinion on the importance of the motley collection of organisms in this group, all anaerobes but not all obligatory lithotrophs: the Thiorhodaceae which are obligatory photosynthetic and use reduced sulphur compounds as hydrogen donors, the Athiorhodaceae which are facultative photosynthetic and use organic compounds as hydrogen donors, and the desulphovibrios and methane bacteria which are facultative lithotrophic. The two last groups might well display some activity in swamps and mud deposits but hardly in field soils. It is difficult to imagine the requirements of the Thiorhodaceae and Athiorhodaceae for anaerobic conditions combined with access of sunlight being fulfilled in surface soils but quite possibly in shallow water. Okuda et al. (1959) found *Rhodopseudomonas capsulatus* able to grow and fix nitrogen under conditions similar to those in rice fields, and particularly noteworthy is the subsequent observation (Okuda, 1960) that this bacterium will grow in the presence of oxygen when associated with *A. vinelandii*. It is too early to form an opinion of the significance of such organisms, but it should be remembered that although they are photosynthetic they demand organic compounds as hydrogen donors (butyrate and acetate in the experiments of Okuda and his associates).

V. AN EPITOME

The following ecological factors must be considered as chiefly regulating the activities of the three major groups of organotrophic nonsymbiotic nitrogen fixers (*Azotobacter, Beijerinckia, Clostridium* spp.):

(1). A temperature range at least 6° above 0 and probably optimal at 25 to 30°C.

(2). A range of pH extending from approx. 3 to 9 but for optimum growth of the azotobacter and clostridia not far from neutrality.

(3). An adequate supply of mineral nutrients including molybdenum and relatively large amounts of phosphate.

(4). A degree of moisture that permits vegetative cell growth and has wide implications because it is bound up with oxygen access which again affects (a) the balance between azotobacter and clostridia, (b) the production of organic metabolites from insoluble plant residues, and (3) the efficiency of the nitrogen fixation by azotobacter.

(5). The presence of available nitrogen compounds (nitrate and particularly ammonia) which restrict nitrogen fixation by competitive assimilation and by encouraging growth of non-nitrogen-fixing organisms, and finally

(6). The quality and quantity of organic compounds available as carbon and energy source. This is usually the limiting factor if conditions are otherwise adequate.

A certain degree of anaerobiosis seems necessary for effective nitrogen fixation on the basis of those plant residues that represent the bulk of organic matter returned to the soil. The effect of restricted aeration is doubtless of a dual nature: firstly increased production of organic metabolites from cellulosic materials and secondly a more efficient utilization of these by-products by the nitrogen fixers. For these reasons the process is unlikely to be of great importance in arable soils under intensive cultivation, at least in comparison with the nitrogen demands of the crops. Here the crop residues are mostly small and largely confined to the root material, and the frequent soil tillage together with drainage militates against efficient nitrogen fixation. This situation would seem to become extreme where cereal cultivation predominates and is combined with burning of the straw and copious use of nitrogenous fertilizers. Under such conditions we find azotobacter populations of a few hundred or at the most thousands per gram. We may draw certain conclusions concerning the relation between cell proliferation, organic matter consumption and nitrogen fixation with azotobacter as the model. An average azotobacter-cell may be assumed to have a volume of $5\mu^3$, a weight of 5×10^{-6} μg and a content of 20% dry matter with 10% nitrogen; a simple calculation then shows that the fixation of each ppm of nitrogen will mean the synthesis of 10^7 cells per gram of soil if *in vitro* conditions apply: consumption of at least 50 parts of organic matter (equivalent to glucose) per part of fixed nitrogen which is present as cell substance. If merely 1% of 10^7 cells were viable at any given moment, the result would be an azotobacter-density of 100,000 per gram, which is most exceptional (cf. Table 4).

A different state of affairs may obtain in soils where plant residues decay under partially or periodically anaerobic conditions, such as rice fields (Rouquérol, 1962; Uppal et al., 1939) or heavy-textured loams (Abd-el-Malek and Ishac, 1962); here the counts of azotobacter sometimes run into millions per gram as estimated by the same methods that elsewhere reveal numbers a thousand times lower. Something similar may apply to pasture soils and even temporary leys where annual nitrogen accretions may range from about 20 to about 100 pounds per acre (Jensen, 1940; Parker, 1957; Whitt, 1941). Restricted aeration in fine-textured soil under a dense greensward might well favor efficient nitrogen fixation as suggested by Parker (1957), but an "orthodox"

process of such a magnitude would still require a minimum consumption of approximately 0.5 to 2.5 tons of "first-class" organic matter largely derived from root materials—and corresponding high densities of nitrogen fixers. It seems necessary to consider the possibility that nitrogen fixation *"in terra"* may under some conditions cease to be strictly tied to cell proliferation and to be accompanied by copious excretion of fixation products (ammonia?). This is conceivable, since the fixation process *per se* requires little energy (Bayliss, 1956; Mortenson, 1962; Wilson, 1958). Verification of this possibility awaits a detailed study of the correlations between organic matter transformation, nitrogen accretion and the composition, density and turnover rate of the nitrogen-fixing microflora.

The relative importance of the groups of nitrogen fixers is difficult to assess. The azotobacter are absent from many soils, and in spite of some acid-tolerant members they are doubtless of little significance in soils of pH below 6 which account for a large part of the world's cultivated area; the compensating effect of the beijerinckias is still uncertain. In many soils the azotobacter are seriously handicapped by low phosphate supply and perhaps still more by combined nitrogen from industrial fertilizers. The clostridia have been thought to rival or exceed the azotobacter in effect because of their wider distribution and their lesser sensitivity to acid reaction. Widespread occurrence, however, is irrelevant in comparison with the mass of actively metabolizing cells, and until more becomes known about this aspect of the soil ecology of the clostridia, their contribution to the global pool of biologically fixed nitrogen must remain unknown. No positive evidence points to any major role of the "miscellaneous" nitrogen fixers.

Different conditions apply to the nitrogen-fixing cyanophyceae. While probably of little significance in arable soils, they are obviously important as pioneer organisms on bare ground and perhaps generally on the soil surface in warm and humid climates but from an agricultural point of view still more so under conditions existing in rice soils in Asiatic countries; here the nitrogen supply of rice crops has probably for many centuries been provided by these unique organisms that come near to having solved the formidable physiological problem of "living upon air." Future improvements of yield (which must perforce be achieved) will partly depend on synthetic nitrogenous fertilizers but perhaps also partly by a rational use of the cyanophyceae as a kind of green manure crop (Watanabe, 1959b).

Upon the whole the phenomenon of non-symbiotic nitrogen fixation appears to be a kind of long-term factor in the economy of Nature, compensating in part for losses of nitrogen from the biological cycle, slowly building up reserves of organic (humus) nitrogen in bare soils and others poor in organic matter, and contributing to the upkeep of these reserves. The cyanophyceae may be susceptible to cultivation, but the activity of the organotrophic nitrogen fixers depends on an excess of energy material in proportion to available nitrogen and is therefore particularly sensitive to removal of crops from the soil, which in any case is man-made intervention in the equilibrium of nature. As cultiva-

tion becomes intensified, the nonsymbiotic processes become unable to cover more than a fraction of the nitrogen requirements of the crops—and this a fraction that will gradually diminish as mankind for the sake of his survival will have to force ever-increasing amounts of protein material out of the biological cycle of nature.

REFERENCES

Abd-el-Malek, Y., and Ishac, Y. Z. 1962. Abundance of Azotobacter in Egyptian soil. Abstr. VIII. Intern. Congr. Microbiol. Montreal. 57.

Alexander, M., and Wilson, P. W. 1954. Large-scale production of the azotobacter for enzymes. Appl. Microbiol. 2:135-140.

Allen, M. B., and Arnon, D. I. 1955. Studies of nitrogen-fixing blue-green algae. Plant Physiol. 30:366-372.

Allison, F. E. 1947. Azotobacter inoculation of crops. I. Historical. Soil Sci. 64:413-429.

Anderson, G. R. 1955. Nitrogen fixation by *Pseudomonas*-like soil bacteria. J. Bact. 70:129-133.

Anderson, G. R., and Jordan, J. V. 1961. Boron: a non-essential growth factor for *Azotobacter chroococcum*. Soil Sci. 92:113-116.

Augier, J. 1956. A propos de la numération des Azotobacter en milieu liquide. Ann. Inst. Pasteur 91:759-765.

Augier, J. 1957. A propos de la fixation biologique de l'azote atmosphérique et de la numération des *Clostridium* fixateurs dans les sols. Ann. Inst. Pasteur 92:817-824.

Baillie, A., Hodgkiss, W., and Norris, J. R. 1962. Flagellation of *Azotobacter* spp. as demonstrated by electron microscopy. J. Appl. Bact. 25:116-119.

Barrow, N. J., and Jenkinson, N. S. 1962. The effect of waterlogging on fixation of nitrogen by soil incubated with straw. Plant and Soil 16:258-262.

Bayliss, N. S. 1956. The thermochemistry of biological nitrogen fixation. Aust. J. Biol. Sci. 9:364-370.

Becking, J. H. 1961. Studies on nitrogen-fixing bacteria of the genus *Beijerinckia*. I. Geographic and ecological distribution in soils. Plant and Soil 14:49-81.

Becking, J. H. 1961. Studies on nitrogen-fixing bacteria of the genus *Beijerinckia*. II. Mineral nutrition and resistance to high levels of certain elements, in relation to soil type. Plant and Soil 14:297-322.

Becking, J. H. 1962a. An aerobic heterotrophic Spirillum fixing atmospheric nitrogen. Abstr. VIII. Intern. Congr. Microbiol. Montreal. 57.

Becking, J. H. 1962b. Species differences in molybdenum and vanadium requirements and combined nitrogen utilization by Azotobacteriaceae. Plant and Soil 16:171-201.

Berthelot, M. 1888. Fixation de l'azote atmosphérique sur la terre végétale. Ann. Chim. Phys. Sér. 13:1-119.

Berthelot, M. 1890. Recherches nouvelles sur la fixation de l'azote par la terre végétale et les plantes et sur l'influence de l'électricité sur ce phenomène. Ann. Chim. Phys. Sér. 6, 29:434-492.

van Beynum, J., and Pette, J. W. 1936. Buttersäuregärung und Milchsäregärung im Silofutter. Centralbl. Bakt. (11) 94:413-433.

Bjälfve, G. 1955. Fixation of atmospheric nitrogen. I. Experiments in sand with and without addition of straw or starch and in straw alone in sterile and unsterile conditions in light and darkness. Ann. Royal Agr. Coll. Sweden 22:193-217.

Bjälfve, G. 1962. Nitrogen fixation in cultures of algae and other microorganisms. Physiol. Plantarum 15:122-129.

Bond, G. 1936. Quantitative observations on the fixation and transfer of nitrogen in the soy bean, with especial reference to the mechanism of transfer of fixed nitrogen from bacillus to host. Ann. Bot. 50:559-578.

Bond, G., and Scott, G. B. 1955. An examination of some symbiotic systems for fixation of nitrogen. Ann. Bot. (N. S.) 19:67-77.

Bortels, H. 1930. Molybdän als Katalysator bei der biologischen Stickstoffbindung. Arch. Mikrobiol. 1:333-342.

Bortels, H. 1936. Weitere Untersuchungen über die Bedeutung von Molybdän, Vanadium, Wolfram und anderen Erdaschenstoffen für stickstoffbindende und andere Mikroorganismen. Centralbl. Bakt. (II) 95:193-218.

Bortels, H. 1940. Meteorobiologische Untersuchungen an Azotobakter. Centralbl. Bakt. (II) 102-129-153.

Bredemann, G. 1908. Untersuchungen über die Variation und das Stickstoffbindungsvermögen des *Bacillus asterosporus* A. M. Centralbl. Bakt. (II) 22:44-89.

Bredemann, G. 1909. *Bacillus amylobacter* A. M. et Bredmann in morphologischer, physiologischer and systematischer Beziehung. Centralbl. Bakt. (II) 23:385-568.

Brown, M. E., Burlingham, S. K., and Jackson, R. M. 1962a. Studies on *Azotobacter* species in soil. I. Comparison of media and techniques for counting *Azotobacter* in soil. Plant and Soil 17:309-319.

Brown, M. E., Burlingham, S. K., and Jackson, R. M. 1962b. Studies on *Azotobacter* species in soil. II. Populations of *Azotobacter* in the rhizophere and effects of artificial inoculation. Plant and Soil 17:320-332.

Bullock, G. L., Bush, J. A., and Wilson, P. W. 1960. Calcium requirements of various species of Azotobacter. Proc. Soc. Exp. Biol. Med. 105:26-30.

Calder, E. A. 1959. Nitrogen fixation in a Uganda swamp soil. Nature (Lond.) 184:746.

Cameron, R. E., and Fuller, W. H. 1960. Nitrogen fixation by some algae in Arizona soils. Soil Sci. Amer. Proc. 24:353-356.

Carnahan, J. E., Mortenson, L. E., Mower, H. F., and Castle, J. E. 1960. Nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*. Biochim. Biophys. Acta 44:520-535.

Christensen, H. R. 1915. Studien über den Einfluss der Bodenbeschaffenheit auf das Bakterienleben und den Stoffumsatz im Erdboden. Centralbl. Bakt. Parasitenk. (II) 43:1-166.

Cooper, R. 1959. Bacterial fertilizers in the Soviet Union. Soils and Fertilizers 22:327-33.

Darzniek, Y. O. 1960. The effect of the cotton plant on the growth of *Azotobacter* in the soil. Mikrobiologiya 29:868-874. 1960. Engl. Transl. Microbiology 29:626-629.

Darzniek, Y. O. 1961. Reproduction rate of *Azotobacter* in soil. Mikrobiologiya 30:1042-1044. 1961. Engl. Transl. Microbiology 30:849-850.

De, P. K. 1936. The problem of the nitrogen supply of rice. I. Fixation of nitrogen in the rice soils under water-logged conditions. Indian J. Agr. Sci. 6:1237-1245.

De, P. K. 1939. The role of blue-green algae in nitrogen fixation in rice-fields. Proc. Roy. Soc. (Lond.) B. 127:121-139.

De, P. K., and Sulaiman, M. 1950. Fixation of nitrogen in rice soils by algae as influenced by crop, CO_2, and inorganic substances. Soil Sci. 70:137-151.

De, P. K., and Mandal, L. N. 1956. Fixation of nitrogen by algae in rice soils. Soil Sci. 81:453-458.

Delwiche, C. C., and Wijler, J. 1956. Non-symbiotic nitrogen fixation in soil. Plant and Soil 7:113-129.

Derx, H. G. 1950. *Beijerinckia*, a new genus of nitrogen-fixing bacteria occuring in tropical soils. Proc. Kon. Nederl. Akad. Wetensch. 53:140-147.

Derx, H. G. 1950. Further researches on *Beijerinckia*. Ann. Bogoriensis 1:1-11.

Derx, H. G. 1953. Sur les causes de la distribution géographique limitée des *Beijerinckia*. Communicazioni VI. Congr. Int. Microbiol. (Roma) 3:116-117.

Dhar, N. R. 1937. Nitrogen transformation in the soil. (Address of the President). Proc. Nat. Acad. Sci. India 6:3-82.

Döbereiner, J. 1953. Azotobacter em solos acidos. Boletim Inst. Ecol. Exp. Agricolas (Rio de Janeiro) Mo. 11:1-31.

Döbereiner, J., and Alvahydo, R. 1959. Influencia da umidade do solo na populacao de bacterias de genero *Beijerinckia*. Ciencia e Cultura (Rio de Janeiro) 11:208-218.

Döbereiner, J., and Alvahydo, R. 1959. Sobre a influencia da canade-acúcar na ocorrencia de "*Beijerinckia*." II. Influéncia das diverseas partes do vegetal. Rev. Brasil. Biol. 19:401-412.

Döbereiner, J. 1961. Nitrogen-fixing bacteria of the genus *Beijerinckia* Derx in the rhizosphere of sugar cane. Plant and Soil 15:211-216.

Dommergues, Y. 1962. Contribution a l'étude de la dynamique microbienne des sols (etc.). II. Fixation de l'azote par *l'Azotobacter chroococcum*. Ann. Agron. 13:302-303.

Fedorov, M. V. 1944. Der Einfluss der Wurzelausscheidungen der Maispflanze auf die

Bindung von atmosphärischem Stickstoff durch *Azotobacter* in Maiskulturen mit einer Bakterienrasse. (Russian with German Summary). Mikrobiologiya 13:199-210.

Fedorov, M. V. 1960. Biologische Bindung des atmosphärischen Stickstoffs. VEB Deutscher Verlag der Wissenschaften, Berlin. (Orig. Russian Ed. 1952).

Fedorov, M. F., and Ilina, T. K. 1960. The relations of individual forms of soil actinomycetes to various carbon sources during growth on nitrate and molecular nitrogen. Mikrobiologiya 29:363-364. 1960. Engl. Transl. Microbiology 29:495-500.

Fedorov, M. V., and Kalininskaya, T. A. 1961. Relationships of a nitrogen-fixing mycobacterium (Mycobacterium sp. 301) to various supplements of carbon and supplementary growth factors. Mikrobiologiya 30:832-838. 1961. Engl. Translation Microbiology 30:681-686.

Fogg, G. E., and Wolfe, M. 1954. The nitrogen metabolism of blue-green algae (Myxophyceae). Autotrophic Microorganisms (Fourth Symp. Gen. Microbiol): 99-125. University Press, Cambridge.

Fogg, G. E. 1956. Nitrogen fixation in photosynthetic organisms. Ann. Rev. Plant. Physiol. 7:51-70.

Garbosky, A. J. 1956. Distribution of *Azotobacter* in depth of soils from Tucuman Argentina). Rapp. VI. Congr. Intern. Sci. Sol. (Paris) C. 453-460.

Gerretsen, F. C., and de Hoop, H. 1954. Boron, an essential element for *Azotobacter chroococcum*. Plant and Soil 5:349-367.

Gonick, W. N., and Reuszer, H. W. 1949. The distribution of *Azotobacter chroococcum* and *Azotobacter vinelandii* in Colorado soils and surface waters. Soil Sci. Soc. Amer. Proc. 13:251-257.

Grau, F. H., and Wilson, P. W. 1962. Physiology of nitrogen fixation by *Bacillus polymyza*. J. Bact. 83:490-496.

Grau, F. H., and Wilson, P. W. 1963. Hydrogenase and nitrogenase in cell-free extracts of *Bacillus polymyxa*. J. Bact. 85:446-450.

Green, M., and Wilson, P. W. 1953. The utilization of nitrate nitrogen by the azotobacter. J. Gen. Microbiol. 9:89-96.

Hamilton, P. B., and Wilson, P. W. 1955. Nitrogen fixation by *Aerobacter aerogenes*. Ann. Acad. Sci. Fennicae (A.II) 60:139-150.

Hino, S., and Wilson, P. W. 1958. Nitrogen fixation by a facultative bacillus. J. Bact. 75:403-408.

Hopf, M. 1949. Untersuchungen über die natürliche Mikroflora des Bodens. Arch. Mikrobiol. 14:661-677.

Imschenezki, A. A. 1959. Mikrobiologie der Cellulose. Akademie-Verlag, Berlin. (Orig. Russian Ed. 1953).

Jensen, H. L. 1940a. Contributions to the nitrogen economy of Australian wheat soils. Proc. Linn Soc. N.S. Wales 65:1-122.

Jensen, H. L. and Swaby, R. J. 1940. Further investigations on nitrogen fixing bacteria in soil. Proc. Linnean Soc. N. S. Wales. 65:557-564.

Jensen, H. L. 1940b. Nitrogen fixation and cellulose decomposition by soil microorganisms. I. Aerobic cellulose decomposers in association with *Azotobacter*. Proc. Linnean Soc. N. S. Wales 65:543-556.

Jensen, H. L., and Swaby, R. J. 1941. Nitrogen fixation and cellulose decomposition by soil microorganisms. II. The association between *Azotobacter* and facultative-aerobic cellulose decomposers. Proc. Linnean Soc. N. S. Wales 66:89-106.

Jensen, H. L. 1941. Nitrogen fixation and cellulose decomposition by soil microorganisms. III. *Clostridium butyricum* in association with aerobic cellulose decomposers. Proc. Linnean Soc. N.S. Wales 66:239-249.

Jensen, H. L. 1942. A note upon the utilization of xylose and xylan by *Azotobacter*. Proc. Linnean Soc. N. S. Wales 67:316-320.

Jensen, H. L., and Spencer, D. 1947. The influence of molybdenum and vanadium on nitrogen fixation by *Clostridium butyricum* and related organisms. Proc. Linnean Soc. N. S. Wales 72:73-86.

Jensen, H. L. 1950. The occurence of *Azotobacter* in cultivated soils of Denmark (Danish with English Summary). Tidsskr. Planteavl 53:622-649.

Jensen, H. L. 1953. *Azotobacter* as a crop inoculant. Atti VI. Congr. Internaz. Microbiol. (Roma) 6:245-251.

Jensen, H. L. 1954. The azotobacteriaceae. Bact. Rev. 18:195-214.

Jensen, H. L. 1955. *Azotobacter macrocytogenes* n. sp., a nitrogen-fixing bacterium resistant to acid reaction. Acta Agr. Scand. 5:280-294.

Jensen, H. L., Petersen, E. J., De, P. K., and Bhattacharya, R. 1960. A new nitrogen-fixing bacterium: *Derxia gummosa* nov. gen. nov. spec. Arch. Mikrobiol. 36:182-195.

Jensen, V. 1955. The Azotobacter-flora of some Danish watercourses. Botanisk Tidsskr. Copenhagen. 52:143-157.

Jensen, V. 1956. Nitrogen fixation by strains of *Aerobacter aerogenes*. Physiol. Plantarum 9:130-136.

Jensen, V. 1958. A new nitrogen-fixing bacterium from a Danish watercourse. Arch. Microbiol. 29:348-353.

Jensen, V. 1959. Production of acid from arabinose and xylose by strains of *Azotobacter*. Nature (Lond.) 183:1536-1537.

Jensen, V. 1961. Rhamnose for detection and isolation of *Azotobacter vinelandii* Lipman. Nature (Lond.) 190-832-833.

Jodin. 1862. Du role physiologique de l'azote (etc.). Comp. Rend. Acad. Sci. (Paris) 55:612-615.

Kaila, A. 1954. Some observations on the occurence of *Azotobacter* in Finnish soils. J. Sci. Agr. Soc. Finland 26:40-49.

Kandler, O. 1951. Über den Einfluss von Bodenbakterien und deren Filtraten auf das Wachstum in vitro kultivierter Wurzeln, Arch. Mikrobiol. 15:430-438.

Katznelson, H. 1946. The "rhizosphere" effect of mangels on certain groups of soil organisms. Soil Sci. 62:343-354.

Krasil'nikov, N. A. 1961. Soil Microorganisms and Higher Plants. Acad, Sci. USSR. 1958.—English Ed. Off. Techn. Services, Washington, D.C.

LeGall, J., Senez, J. C., and Pichinoty, F. 1959. Fixation de l'azote par les bactéries sulfato-réductrices. Ann. Inst. Pasteur 96:223-230.

Lochhead, A. G., and Thexton, R. H. 1936. A four-year quantitative study of nitrogen-fixing bacteria in soils of different fertilizer treatment. Canad. J. Res. (C) 14:166-177.

Loew, O. 1927. Ueber die Ernährung des Azotobacter im Boden. Centralbl. Bakt. (II) 70:36-38.

McCoy, E., Higby, W. M., and Fred, E. B. 1928. The assimilation of nitrogen by pure cultures of *Clostridium pasteurianum* and related organisms. Cent. Bakt. (II) 76:314-320.

McKnight, T. 1949. Non-symbiotic nitrogen-fixing organisms in Queensland soils. Queensland J. Agr. Sci. 6:177-195.

Makrinoff, I. A. 1924. Is it possible to make a bacterial soil preparation for non-leguminous crops? Soil Sci. 17:31-38.

Martin, W. P., Walker, R. H., and Brown, P. E. 1937. The occurrence of *Azotobacter* in Iowa soils and factors affecting their distribution. Iowa. Agr. Exp. Station Res. Bull. 217.

Martin, W. P. 1940. Distribution and activity of *Azotobacter* in the Range and cultivated soils of Arizona. Arizona Agr. Exp. Sta. Tech. Bull. 83.

Meiklejohn, J. 1956. Preliminary numbers of nitrogen fixers on Broadbalk Field. VI. Congr. Intern. Sci. Sol. (Paris) 3:243-248.

Metcalfe, G., Chayan, S., Roberts, E. R., and Wilson, T. G. G. 1954. Nitrogen fixation by soil yeasts. Nature (Lond.) 174:841-842.

Metcalfe, G., and Brown, M. E. 1957. Nitrogen fixation by new species of *Nocardia*. J. Gen. Microbiol. 17:567-572.

Metz, H. 1955. Untersuchungen über die Rhizosphäre. Arch. Mikrobiol. 23:297-326.

Meyerhof, O., and Burk, D. 1927. Über die Fixation des Luftstickstoffs durch Azotobacter. Z. Phys. Chem. 139A:117-142.

Mishustin, E. N., and Naumova, N. A. 1962. Bacterial fertilizers, their effectiveness and mechanism of action. Mikrobiologiya 31:543-556. Abstr. Soils and Fertilizers 25:382.

Mortenson, L. E., Mower, H. F., and Carnahan, J. E. 1962. Nitrogen fixation by enzyme preparations. Bact. Rev. 26:42-50.

Mortenson, L. E. 1962. Inorganic nitrogen assimilation and ammonia incorporation. *In* I. C. Gunsalus and R. Y. Stanier: The Bacteria 3:119-166. Academic Press, New York and London.

Moyer, R. T. 1941. Non-symbiotic nitrogen fixation in soils of a semiarid region of North China. J. Amer. Soc. Agron. 33:980-992.

Mulder, E. G. 1948. Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. Plant and Soil 1:94-119.

Nicholas, D. J. D., Kabayashi, M., and Wilson, P. W. 1962. Cobalt requirement for inorganic nitrogen metabolism in the microorganisms. Proc. Nat. Acad. Sci. (U.S.A.) 48:1537-1542.

Nickell, L. G., and Burkholder, P. R. 1947. Inhibition of *Azotobacter* by soil actinomycetes J. Amer. Soc. Agron. 39:771-779.

Niel, C. B. van 1935. A note on the apparent absence of *Azotobacter* in soils. Arch. Mikrobiol. 6:215-218.

Niklas, H., Poschenrieder, H., and Hock, A. 1926. Über die Verbreitung des Azotobacter in den Böden Bayerns unter Berücksichtigung der Bodenreaktion, des Kalk- und Phosphorsäuregehalts derselben. Centralbl. Bakt. (II) 66:16-28.

Nutman, P. S. 1961, 1962. Soil Microbiology Department. Rept. Rothamsted Exp. Stat. 1960:91-92 and 1961:75.

Okuda, A., Yamaguchi, M., and Kobayashi, M. 1960. Nitrogen-fixing microorganisms in paddy soils. IV. Nitrogen fixation photosynthetic bacteria (*Rhodopseudomonas capsulatus* species) under various conditions. Soil and Plant Food 5:73-76.

Okuda, A., Yamaguchi, M., and Kobayashi, M. 1960. Nitrogen-fixing microorganisms in paddy soils. V. Nitrogen fixation in mixed cultures of photosynthetic bacteria (*Rhodopseudomonas capsulatus* species) with other heterotrophic bacteria. Soil and Plant Food 6:35-39.

Olsen, C. 1932. Studies of nitrogen fixation. I. Nitrogen fixation in dead leaves of forest beds. Comp. Rend. Trav. Lab. Carlsberg (Copenhagen) 19:(9) 1-36.

Parker, C. A. 1954a. Non-symbiotic nitrogen-fixing bacteria in soil. I. Studies on *Clostridium butyricum*. Aust. J. Agr. Res. 5:90-97.

Parker, C. A. 1954b. Effect of oxygen on the fixation of nitrogen by *Azotobacter*. Nature (Lond.) 173:780-781.

Parker, C. A. 1957. Non-symbiotic nitrogen-fixing bacteria in soil. III. Total nitrogen changes in a field soil. J. Soil Sci. 8:48-59.

Parker, C. A., and Scutt, P. B. 1960. The effect of oxygen on nitrogen fixation by *Azotobacter*. Biochem. Biophys. Acta 38:230-238.

Paul, E. A., and Newton, D. A. 1961. Studies of aerobic non-symbiotic nitrogen-fixing bacteria. Canad. J. Microbiol. 7:7-13.

Pengra, R. M. and Wilson, P. W. 1958. Physiology of nitrogen fixation by *Aerobacter aerogenes*. J. Bact. 75:21-25.

Pengra, R. M., and Wilson, P. W. 1959. Trace metal requirements of Aerobacter aerogenes for assimilation of molecular nitrogen. Proc. Soc. Exp. Biol. Med. 100:436-439.

Petersen, E. J. 1959. Serological Investigations on *Azotobacter* and *Beijerinckia*. Royal Vet. and Agr. Coll. (Copenhagen) Yearbook 1959:70-90.

Petersen, E. J. 1961. Studies on the morphology and Cytology of *Azotobacter chroococcum*. Royal Vet. and Agr. Coll. (Copenhagen) Yearbook 1961:115-155.

Petersen, J. B. 1935. Studies on the biology and taxonomy of soil algae, Dansk Bot. Arkiv 8:1-183.

Pochon, J. 1954. Manual Technique d'Analyse Microbiologique du Sol. Masson & Cie. Ed., Paris.

Pochon, J., and de Barjac, H. 1958. Traité de Microbiologie des Sols. Applications Agronomiques. Dunod Ed., Paris.

Proctor, M. H., and Wilson, P. W. 1959. Nitrogen fixation by *Achromobacter* spp. Arch. Mikrobiol. 32:254-260.

Quispel, A. 1947. The influence of the oxidation-reducetion potential of the medium upon the growth of *Azotobacter*. Antonie van Leeuwenhoek J. 13:33-43.

Remy, E. 1909. Untersuchungen über die Stickstoffsammlungsvorgänge in ihrer Beiziehung zum Bodenklima. Centralbl. Bakt. II. 22:561-651.

Rosenblum, E. D., and Wilson, P. W. 1949. Fixation of isotopic nitrogen by *Clostridium*. J. Bact. 57:413-414.

Rosenblum, E. D., and Wilson, P. W. 1950. Molecular hydrogen and nitrogen fixation by *Clostridium*. J. Bact. 59:83-91.

de'Rossi, G. 1932a. La fixation de l'azote élémentaire dans le sol. II. Les Azotobacters dans les differents terrains de 'Italie. Soc. Intern. Microbiol. Bol. Sez. Ital. 4:220-226.

de'Rossi, G. 1932b. La fixation de l'azote élémentaire dans le sol. III. Activité végétative et pouvoir fixateur des Azotobacters. Soc. Intern. Microbiol. Boll. Sez. Ital. 4:511-516.

de'Rossi, G. 1933. La fixation de l'azoté élémentaire dans le sol. IV. Activité des Azotobacters dans le sol. Soc. Intern. Microbiol. Boll. Sez. Ital. 5:27-32.

Rouquérol, T. 1962. Sur le phenomène de fixation de l'azote dans les rizières de Chamarque. Ann. Agron. 13:325-346.

Rovira, A. D. 1962. Plant-root exudates in relation to the rhizosphere microflora. Soils and Fertilizers 25:167-172.

Roy, A. B. 1962. A new species of *Derxia*. Nature (London) 194:604-605.

Ruinen, J. 1956. Occurence of *Beijerinckia* species in the "phyllosphere." Nature (Lond.) 177:220-221.

Sabinin, D. A., and Minina, E. G. 1932. Das mikrobiologische Bodenprofil als zonales Kennzeichen. Proc. Sec. Intern. Congr. Soil Sci. (Leningrad and Moscow) Comm. III. 224-235.

Schloesing, T. 1888. Sur les relations de l'azote atmosphérique avec la terre végétale. Comp. Rend. Acad. Sci. (Paris) 106:898-902; 982-987; 107:290-296.

Schloesing, T., and Laurent, E. 1892. Sur la fixation de l'azote libre par les plantes. Ann. Inst. Pasteur 6:824-840.

Schmidt-Lorenz, W., and Rippel-Baldes, A. 1957. Wirkung des Sauerstoffs auf Wachstum und Stickstoffbindung von *Azotobacter chroococcum*, Beijk. Arch. Mikrobiol. 28:45-68.

Schneider, K. C., Bradbeer, C., Singh, R. N., Wang, L. I., Wilson, P. W., and Burris, R. H. 1960. Nitrogen fixation by cell-free preparations from microorganisms. Proc. Nat. Acad Sci. (Wash.) 46:726-733.

Singh, R. N. 1961. Role of Blue-green Algae in Nitrogen Economy of Indian Agriculture. Indian Council of Agricultural Research, New Delhi. 174 pp.

Siu, R. G. H. 1951. Microbial Decomposition of Cellulose. Reinhold Publishing Co., New York.

Sörensen, H. 1957. Microbial decomposition of xylan. Acta Agr. Scand. Suppl. I:1-86.

Spicher, G. 1954. Untersuchungen über die Wechselwirkungen zwischen *Azotobacter chroococcum* und höherer Pflanze. Centralbl. Bakt. (II) 107:353-383.

Stapp, C. 1940 *Azotomonas insolita*, ein neuer aerober stickstoffbinder Mikroorganimus. Centralbl. Bakt. (II) 102:1-19.

Starc, A. 1952. Zur Frage der Rhizosphäre und Bodenimpfung mit *Azotobacter*. Arch. Mikrobiol. 13:164-181.

Starkey, R. L. 1938. Some influences of the development of higher plants upon the microorganisms in the soil. VI. Microscopic examination of the rhizosphere. Soil Sci. 45:207-249.

Stokes, J. L. 1940. The role of algae in the nitrogen cycle of the soil. Soil Sci. 49:266-275.

Stöckli, A. 1944. Die Verbreitung der Azotobacterorganismen in der Schweiz. Landw. Jahrb. Schweiz 58:67-105.

Sulaiman, M. 1944. Effect of algal growth on the activity of *Azotobacter* in rice soils. Indian J. Agr. Sci. 14:277-283.

Suschkina, N. N. 1949. Ökologisch-geographische Verbreitung von *Azobacter* in den Boden der Udssr. Quoted from Fedorov (53).

Tchan, Y. T. 1952. Studies of N-fixing bacteria. I. A note on the estimation of *Azotobacter* in the Soil. Proc. Linnean Soc. N.S. Wales 77:89-91.

Tchan, Y. T. 1943a. Studies of N-fixing bacteria IV. Taxonomy of genus *Azotobacter* (Beijerinck 1901). Proc. Linnean Soc. N.S. Wales 78:85-89.

Tchan, Y. T. 1953b. Studies of N-fixing bacteria. V. Presence of *Beijerinckia* in Northern Australia and geographic distribution of non-symbiotic N-fixing microorganisms. Proc. Linn. Soc. N.S. Wales 78:171-178.

Tschapek, M., and Giambiagi, N. 1954. The formation of Liesegang's rings by "Azotobacter" due to O_2-inhibition. V. Congr. Intern. Sci. Sol (Léopoldville) Act. Comp. Rend. 3:97-103.

Tschapek, M., and Giambiagi, N. 1955. Nitrogen fixation of Azotobacter in soil—its inhibition by oxygen. Arch. Mikrobiol. 21:376-390.

Uppal, B. N., Patel, M. K., and Daji, J. A. 1939. Nitrogen fixation in rice soils. Indian J. Agr. Sci. 9:689-702.

Vancura, V., Macura, J., Fischer, O., and Vondracek, J. 1959. The relation of Azotobacter to the root system of barley. Fol. Microbiol. (Prague) 4:118-129.

Vancura, V., and Macura, J. 1959. The development of *Azotobacter* in the oats rhizosphere and its effect upon the yield. Fol. Microbiol. (Prague) 4:200-202.

Vancura, V., and Macura, J. 1960. Indole derivatives in *Azotobacter* cultures. Fol. Microbiol. (Prague) 5:293-297.

Vancura, V., and Macura, J. 1961. The effect of root excretions on *Azotobacter*. Fol. Microbiol. 1961. (Prague) 6:250-259.

Vancura, V. Detection of gibberellic acid in *Azotobacter* cultures. Nature (Lond.) 192: 88-89.

Vartiovaara, U. 1938. The associative growth of cellulose-decomposing fungi and nitrogen-fixing bacteria. J. Sci. Agr. Soc. Finland 10:241-264.

Virtanen, A. I., Mustakalio, H., and Strandström, H. 1953. Einwirkung von Kohlenmonoxyd auf die anaerobe Stickstoffbindung. Suomen Kemistelehti B. 2:6-9.

Voets, J. P., and Debacker, J. 1956. *Pseudomonas azotogensis* nov. sp., a free-living nitrogen-fixing bacterium. Naturwiss. 43:40-41.

Watanabe, A. 1959a. Distribution of nitrogen-fixing blue-green algae in various areas of South and East Asia. J. Gen. Appl. Microbiol. (Tokyo) 5:21-29.

Watanabe, A. 1959b. On the mass culture of a nitrogen-fixing blue-green alga, *Tolypothrix tenuis*. J. Gen. Appl. Microbiol. (Tokyo) 5:85-91.

Wenzl, H. 1934. Zur Methodik der Keimzahlbestimmung von Azotobacter im Boden. Centralbl. Bakt. (II) 90:289-314.

Westlake, D. W. S., and Wilson, P. W. 1959. Molecular hydrogen and nitrogen fixation by *Clostridium pasteurianum*. Canad. J. Microbiol. 5:617-620.

Whitt, D. M. 1941. The role of bluegrass in the conservation of the soil and its fertility. Soil Sci. Soc. Amer. Proc. 6:309-311.

Wichtmann, H. 1952. Untersuchungen über die Beziehungen zwischen Azotobactergehalt und Bodenfruchtbarkeit. Arch. Mikrobiol. 17:54-78.

Willis, W. H., and Green, V. E. 1949. Movement of nitrogen in flooded soils planted to rice. Soil Sci. Soc. Amer. Proc. 13:229-237.

Wilson, P. W. and Burris, R. H. 1953. Biological nitrogen fixation—a reappraisal. Ann. Rev. Microbiol. 17:415-432.

Wilson, P. W. 1958. Asymbiotic nitrogen fixation. *In* Ruhland's Handb. d. Pflanzenphysiol. 8:9-47. Springer, Berlin.

Winogradsky, S. 1895. Recherces sur l'assimilation de l'azote libre de l'atmosphère par les microbes. Arch. Sci. Biol. (Pétersbourg) 3:297-352.

Winogradsky, S. 1926. Etudes sur la microbiologie du sol. II. Sur les microbes fixateurs de l'azote. Ann. Inst. Pasteur 40:455-520.

Winogradsky, S. and Ziemecka, J. 1928. Etudes sur la microbiologie du sol. III. Sur le pouvoir fixateur des terres. Ann. Inst. Pasteur 42:36-62.

Winogradsky, S. 1930. L'état actuel du problème de la fixation de l'azote atmosphérique et ses récents progres. Comp. Rend. Acad. Agr. de France 16:580-586.

Winogradsky, S. 1938. Etudes sur la microbiologie du sol. IX. Sur la morphologie et oecologie des Azotobacter. Ann. Inst. Pasteur 60:351-400.

Winogradsky, S. 1949. Fixation anaerobie. Avant-propos. *In* Microbiologie du Sol. Problèmes et Méthodes: 356-360. Masson et Cie., Paris.

Ziemecka, J. 1932. The Azotobacter test of soil fertility applied to the classical fields at Rothamsted. J. Agr. Sci. 22:797-810.

Zycha, H. 1932. Sauerstoffoptimum und Nährboden "aerober" Bakterien. Arch. Mikrobiol. 3:194-204.