The biology of nitrogen-fixing organisms

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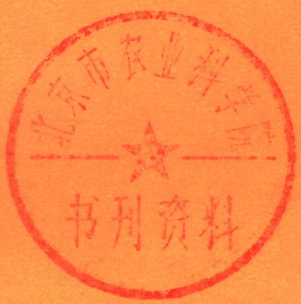

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Preface

Biologists and agriculturists have been interested in the processes of nitrogen fixation for many years. Recently, the use of nitrogenous fertilizers has become expensive in terms of hard cash, utilization of fossil energy, and pollution of water supplies. These facts, coupled to a requirement for high protein plant food, have led to renewed efforts in the field of nitrogen-fixation research. These efforts have been reported in numerous publications and have been the subject of international meetings of scientists at the rate of about one a year for the last decade. A number of excellent treatises containing review chapters by specialists have appeared in that time. These are, however, too costly and detailed for undergraduates, people in related research areas such as agriculture, forestry, ecology, or even, initially, for those newly entering the field of nitrogen-fixation research. It is for these people that the present book was written.

I have begun by describing the organisms that fix nitrogen, and then considered how they do it at the cellular level. Nitrogen fixation is then discussed in relation to agriculture, forestry, and ecology. Finally, there is a brief consideration of past and future evolution of nitrogen-fixing systems. An appendix on methodology is included. The chapters are necessarily of different size and emphasis, reflecting the particular aspects upon which research has been concentrated—legumes in agriculture, for example. The literature is enormous, and no attempt has been made to cover it exhaustively. Rather, material has been selected from recent publications to illustrate particular points and to act as an entry to the general literature.

I should like to thank my friends and colleagues who have encouraged me to write this book and have sent me manuscripts in advance of publication. Without the assistance of Angela Gallacher with typing, Yvonne Jones with photography, and Grant MacFarlane with preparation of drawings, the whole writing process would have ground to a halt.

Janet I. Sprent
March 1978

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I am indebted to Dr R. J. Summerfield for supplying the colour slide of a cowpea on which the cover design is based.

Abbreviations

ADP: Adenosine diphosphate
AMP: Adenosine monophosphate
ATP: Adenosine triphosphate
IBP: International Biological Programme
$NAD(P)^+$: Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H: Reduced form of $NAD(P)^+$
Pi: Inorganic phosphate
PPi: Pyrophosphate
PHB: Poly-β-hydroxybutyrate
RNA: Ribonucleic acid

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one

The range of nitrogen-fixing organisms

Introduction

Seventy eight per cent of the air we breathe in is nitrogen. Unfortunately, 78 per cent of the air we breathe out is also nitrogen. In this respect we are typical members of the plant and animal kingdoms—or eukaryotes—i.e., we cannot convert this very stable gas into a biologically useful form. All the organisms that can utilize nitrogen belong to the kingdom known as the prokaryotes, and the basic reaction appears to be the same in all cases, i.e., the reduction of nitrogen to ammonia with the aid of the enzyme complex, *nitrogenase*. Although this may sound simple, it is beset with all sorts of problems which may be dealt with in a variety of ways. This book aims to describe the organisms which can reduce or fix nitrogen, to show you how they tackle and solve their various problems, and to try to assess their overall significance and potential.

But first, let us put nitrogen fixation into perspective. Figure 1.1 is a simple version of the familiar nitrogen cycle, envisaged as three compartments. Outside we have the atmosphere, next the (soil + water) environment, and inside the organisms. If we accept (and not everybody does) that the world is short of protein, then clearly we need to increase the input into and decrease the output from the inner compartment. Taking the last point first: we know that some soils, particularly under climax vegetation such as forest, contain substances which inhibit specific stages in nitrification and thus tend to conserve nitrogen.[1] Some synthetic inhibitors are now being produced and introduced into agriculture.[2] These also reduce losses by leaching, since nitrate is the most easily leached of the nitrogen compounds.

In order to enhance input, nitrogen compounds acceptable to living cells

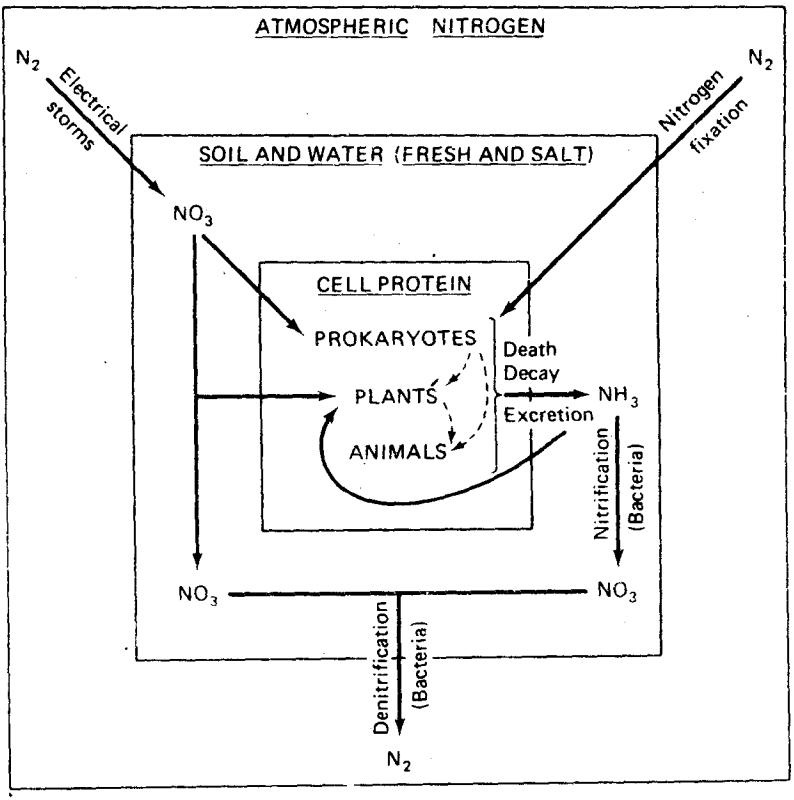

FIG. 1.1 The principal reactions of the nitrogen cycle.

must be produced, and this requires a considerable amount of energy. This energy may arise from atmospheric electrical discharge (or from the internal combustion engine), which causes nitrogen and oxygen to combine. Fortunately, we cannot yet control thunderstorms and although in some areas of the world significant nitrogen 'fixation' occurs in this way—values in the region of 30 kg ha^{-1} have been reported—it is likely to remain unpredictable in the foreseeable future. Nitrogen and hydrogen may be combined by the Haber–Bosch process to form ammonia which can be used for fertilizer but only at the expense of fossil fuel. About 1·5 kg of fuel oil is needed to manufacture and deliver 1 kg of fertilizer N to the farm.[3]

Nitrogen-fixing organisms use light energy, directly or indirectly, to produce ammonia and, since manufacture occurs on site, distribution costs do not arise. This energy-saving potential of biological nitrogen fixation may rank in importance with the end product, ammonia. A third aspect,

which has not yet been fully evaluated, stems from the fact that biological nitrogen fixation is tailored to the needs of the organism, unlike applied fertilizer, which cannot all be used immediately. Loss of fertilizer by leaching not only wastes energy and money, but is also a source of environmental pollution, particularly by contamination of fresh water supplies. Economic and sociological aspects of nitrogen fixation have thus added impetus to research in recent years.

It has been estimated that about 130 Tg Na^{-1} may be fixed by biological processes, compared with less than 50 Tg Na^{-1} by industrial and atmospheric fixation.[4]

The range of nitrogen-fixing organisms

Over the last century, many reports of nitrogen fixation have appeared in the literature. The organisms concerned have been both free living and in symbiotic association with other organisms. With a few exceptions, they have all been prokaryotic, that is, belonging to the bacteria and related groups. The exceptions almost all concern fungi, principally yeasts, but also some more complex fungi and higher plant/fungal symbioses (mycorrhizas—see also Chapter 6). None of these reports has survived critical rechecking.[5,6] One of the problems has been that some micro-organisms are excellent scavengers of traces of combined nitrogen (e.g., NH_3, NO_3^-) which may be present as impurities in culture media or as atmospheric contaminants (particularly in laboratories inhabited by smokers of tobacco). Unless or until we are successful in transferring genes for nitrogen fixation to eukaryotic organisms (prospects for this will be discussed in Chapter 6), it is probably safe to assume that all nitrogen-fixing organisms are prokaryotic. What are the distinguishing features of this group? Basically,

1. that their nucleoplasm is never separated from their cytoplasm by a nuclear membrane and is not associated with basic protein;
2. that their plasma membranes are frequently complex with intrusions into the cytoplasm; and
3. that they rarely have cytoplasmic organelles independent of the plasma membrane and, when they do (gas vacuoles and granules of various types), they are not enclosed by a unit membrane.

The prokaryotes are now frequently classified as a separate kingdom, divided into two divisions, Cyanobacteria and Bacteria. The taxonomy of both of these is hotly disputed. Take the Cyanobacteria, or blue-green bacteria—these are still more commonly known as the blue-green algae or Cyanophyceae and often placed in the plant kingdom. However, they possess all the attributes of prokaryotes, listed above. The main feature which distinguishes them from 'other' bacteria is that they carry out the higher plant type of photosynthesis, i.e., they use water as an electron donor and consequently evolve oxygen. They also show more cellular

differentiation. In this book, they will be regarded principally as prokaryotes, and the argument as to whether they are bacteria or plants left to others. We shall, however, adopt the common usage of 'blue-green algae'. Within the division 'Bacteria', the classification which has been adopted as far as possible is that of the latest (8th) edition of Bergey's *Manual of Determinative Bacteriology* (1974).[7]

Bacteria

Table 1.1 lists the species currently known to fix nitrogen. The first and largest section (a) includes the non-photosynthetic, non-filamentous forms, and even a quick glance shows the wide range of bacterial families

Table 1.1 *Genera of nitrogen-fixing bacteria*
(a) *Non-photosynthetic, non-filamentous forms.*

Family, Genus, Species	General Comments
Pseudomonadaceae	Soil, fresh water, and salt water:
Pseudomonas azotogensis	exact classification uncertain: fixation of N_2 anaerobic?
Azotobacteraceae	Soil, water, leaf, and root surface: all species fix N_2 aerobically, but generally more efficiently at low pO_2.
Azotobacter, *Azomonas*, *Azotococcus*	Alkaline soils: generally produce some extracellular slime.
Beijerinckia, *Derxia*	Acid soil: not temperate regions, produce abundant slime.
Rhizobiaceae	
Rhizobium	All spp fix N_2 in symbiosis with legumes, micro-aerophilically: some strains inactive: grow in soil on combined N, but have been induced to fix N_2 apart from legume in laboratory.
leguminosarum, *phaseoli*, *trifolii*	Rapid growth on yeast extract media.
japonicum, *lupini*	Slow growth on yeast extract media.
	Detailed classification controversial.
'cowpea miscellany'	Possibly the more primitive rhizobia.
Bacillaceae	
Bacillus	Widespread in occurrence: aerobic or facultatively anaerobic.
polymyxa	Most strains fix N_2 anaerobically.

Table 1.1 (a) *Non-photosynthetic, non-filamentous forms.* (continued)

Family, Genus, Species	General Comments
Bacillaceae (continued)	
Bacillus (continued)	
megaterium, *macerans*	Fixation less common.
Clostridium pasteurianum, *butyricum* and other spp	Soil, fresh water, salt water, sediments, intestines, faeces: some strains fix N_2 anaerobically or micro-aerophilically: some reduce Fe.
Desulfotomaculum mesophilic spp[a]	Intestines, rumens: strict anaerobes: convert SO_4^{2-} to S^{-}: some strains fix N_2.
Enterobacteriaceae	All originally isolated from intestinal flora, now reported from various habitats: only a few strains actively fix N_2. Nitrogenase synthesis and activity anaerobic or micro-aerophilic.
Klebsiella pneumoniae and other spp of uncertain classification	Leaf and nodule surfaces, faeces, rumen.
Enterobacter aerogenes, *cloacae*, *Erwinia herbicola*, *Citrobacter freundii*, *intermedius*, *Escherichia coli*, *intermedia*	
Spirillaceae	
Spirillum lipoferum[b]	Obligate aerobe associated with roots of grasses, etc., where it may fix N_2 micro-aerophilically.
Genera of uncertain family	
Desulfovibrio vulgaris, *desulfuricans*, *gigas*	Wet soils, fresh water and salt water with high organic content. Not all strains actively fix N_2.
Methylosinus trichosporium	Soils, water: utilizes methane: growth and N_2 fixation aerobic.
Thiobacillus ferroxidans	Acid waters with high iron content. Chemolithotrophic, oxidizing Fe^{++} and S compounds: growth aerobic and N_2 fixation microaerophilic

[a] Postgate[6] notes that no thermophilic bacteria have been shown to fix nitrogen.
[b] Krieg[9] has suggested re-classification of this species into the new genus *Azospirillum* with two species *lipoferum* and *brasiliense*.

***Table 1.1 Genera of nitrogen-fixing bacteria* (continued)**

(b) *Photosynthetic forms.* These genera have photosynthetic pigments associated with intracytoplasmic membranes formed from the plasmalemma and observed either as vesicles or lamellar stacks (Fig. 1.2). All are classified in the order Rhodospirillales which contains three families, members of each of which fix N_2.

Family, Genus, Species	General Comments
Rhodospirillaceae *Rhodospirillum rubrum* *Rhodomicrobium* sp *Rhodopseudomonas capsulata* *spheroides*	Predominantly aquatic, facultative anaerobes, purple, non-sulphur bacteria. Generally micro-aerophilic when grown on combined N. Require light if grown anaerobically and under these conditions some strains fix N_2.
Chromatiaceae *Chromatium* sp	Moist and muddy soils, salt and fresh water where sulphide high. Strict anaerobes: purple sulphur bacteria: fixation of N_2 rare.
Chlorobiaceae *Chlorobium limicola*	Habitats as for Chromatiaceae: strict anaerobes: green sulphur bacteria: fixation of N_2 rare.

***Table 1.1 Genera of nitrogen-fixing bacteria* (continued)**

(c) *Actinomycetes and associated spp. Filamentous forms.*

Family, Genus, Species	General Comments
Mycobacterium flavum[a] and other spp	Acid soils
Corynebacterium autotrophicum[a]	Soils, mud: several strains shown to fix N_2 autotrophically (H_2, CO_2), as well as heterotrophically (sucrose) in presence of O_2.
Frankiaceae *Frankia*[b]	Fix N_2 in symbiosis with non-leguminous angiosperms.

[a] Exact taxonomic positions uncertain.

[b] In Bergey's manual,[7] this genus is divided into a number of species, according to the host genera nodulated. The endophyte recently isolated from *Comptonia peregrina*[10] crosses these specific boundaries, and it seems clear that the classification will have to be revised.

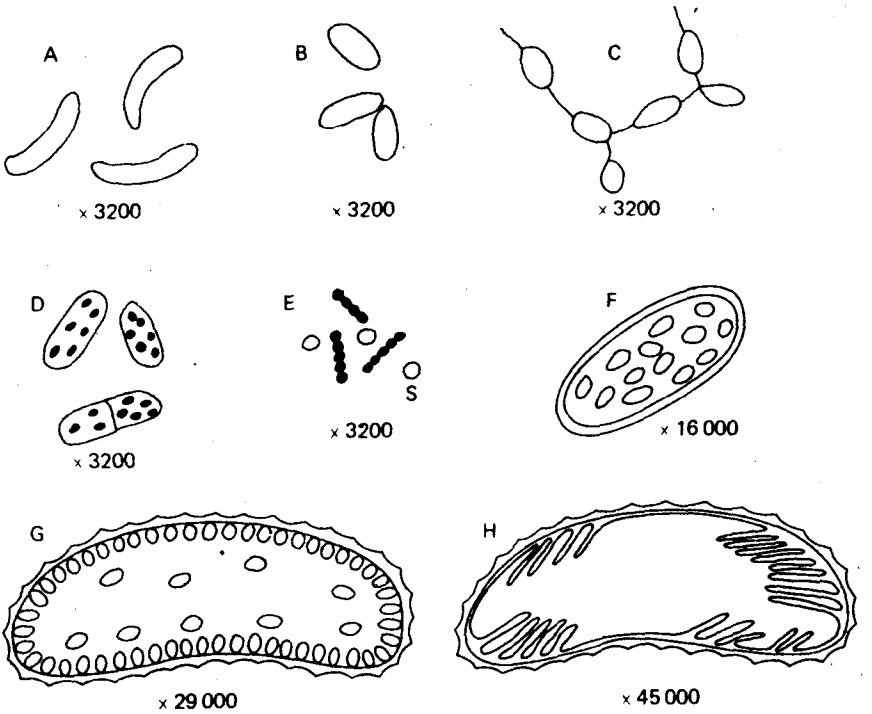

FIG. 1.2 Variation in form and structure in genera of free-living nitrogen-fixing bacteria. A, **Rhodospirillum molischianum;** *B,* **Rhodopseudomonas acidophila;** *C,* **Rhodomicrobium vannielii;** *D,* **Chromatium vinosum** *with internal sulphur granules; E,* **Chlorobium limicola** *with extracellular sulphur globules (S); F,* **Azotobacter chroococcum** *(note extensive internal membrane system found in cells grown on nitrogen gas); G and H,* **Rhodospirillum rubrum** *and* **R. molischianum** *grown anaerobically in the light showing, respectively, vesicles and lamellar stacks in intracytoplasmic membranes.*

represented. By the time this book is published, additional species may have been discovered: others may be reclassified or even deleted. The reasons for this apparently haphazard occurrence of the nitrogen-fixing habit may be evolutionary and this aspect will be considered in Chapter 6. Most of the genera are only active under anaerobic or micro-aerophilic (i.e., low concentrations of oxygen) conditions and only a few strains of a particular species show activity. The exceptions to this among the free-living forms are the members of the Azotobacteraceae, which have evolved several ways of living in an aerobic environment and yet carry out the strictly anaerobic process of nitrogen fixation, as we shall see in the next chapter.

All these non-photosynthetic genera are only indirectly dependent upon light energy for nitrogen fixation. They must be supplied with suitable