The fundamentals of nitrogen fixation

J. R. POSTGATE, FRS

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Professor of Microbiology, University of Sussex

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PREFACE

Nitrogen fixation, as a subject of scientific research, has made enormous strides in the last two decades. Some developments have, indeed, revealed whole new areas of science: the chemistry of the metal complexes formed by the dinitrogen (N_2) molecule and the genetics of biological nitrogen fixation are both topics about which literally nothing was known in 1959. The biochemistry of the subject has blossomed too. I would not insult Professor Perry Wilson (see Wilson, 1969; Postgate, 1980*c*) by asserting that literally nothing was known before 1960 about the biochemistry of the enzyme nitrogenase (which catalyses the biological process) but, compared with what we know now, our knowledge of its enzymology was exiguous to say the least. Even the list of nitrogen-fixing systems – both free-living bacteria and symbiotic associations with plants – has changed so much since about 1960 that it is no exaggeration to assert that a revolution has taken place in our scientific thinking. Understanding of the ecology of the process has been transformed, whole new classes of nitrogen-fixing systems have been discovered, and today nitrogen fixation is becoming quantitatively one of the best understood steps in the natural cycling of the biological elements. It is fair to say that research on nitrogen fixation has been one of the scientific success stories of the 1960s and 1970s.

It is instructive to consider how all this scientific advance came about.

Of course, it can be argued that it came about because I gave the matter my personal attention, for I started research in this area in 1963. Despite my inclination to favour that argument I am regretfully compelled to dismiss it – or at least to reserve it for quelling the more bumptious of my colleagues.

More seriously, it came about mainly because of three funda-

mental scientific advances: the recognition of the oxygen sensitivity of the enzyme proteins, the discovery of the acetylene test, and the discovery of the dinitrogen complexes. I have illustrated elsewhere (Postgate, 1980*b*, *c*) how common sense about oxygen, coupled with exploitation of the acetylene test, transformed knowledge of the biology and genetics of nitrogen fixation; equally, the chemistry of the dinitrogen complexes underlies our present understanding of the chemistry of the action of nitrogenase itself, imperfect as that knowledge may be. All these statements will, I hope, become self evident from a perusal of the contents of this book. The underlying message, which I wish to emphasize here, is that all three seminal advances were at the fundamental level of scientific enquiry: they were all discovered as part of the pursuit of knowledge for its own sake. I do not wish to denigrate the contribution of studies at a more practical level, which did indeed contribute greatly to the advances of the last two decades (notably in the evaluation of the contributions of lesser-known symbiotic systems to the nitrogen cycle), but few authorities would dispute that it was fundamental science which underpinned the more dramatic transformations in our knowledge. Nor do I wish to disregard the importance of money: nitrogen fixation is crucial to world agriculture and even the most obtuse politician or scientific administrator was forced, in the third quarter of the twentieth century, to realize that the population explosion made research into agricultural nitrogen inputs, and therefore nitrogen fixation, imperative.

The surge of scientific information on nitrogen fixation continues undiminished, betokened by the plethora of primary papers, reviews, published symposia, handbooks and treatises which swelled the literature as the late 1970s progressed into the 1980s. What excuse, you may ask, have I for adding to this deluge of written words? My response is that the research scientist is indeed well served with reference works and so is the student at the preliminary or first-year level. But in between it is difficult to find a synoptic survey of more recent developments. Yet a substantial core of fundamental information has accumulated over the last two decades and, though this information can be extracted from reviews, compendia and treatises, it is not readily accessible except to the advanced specialist. In this monograph I have tried to bring it

together so that the student, lecturer or research worker entering the field can obtain, from one source, the background science which is conditioning our thinking about nitrogen fixation as research enters the 1980s. Some of the information will become obsolete; I hope rather little, because I have resisted the temptation to include 'stop press' items. I have tried to stick to basics and to illustrate the way in which chemistry, biochemistry, physiology and genetics have combined to give us a remarkably coherent understanding of biological nitrogen fixation. It has been, and is continuing to be, an inter-disciplinary study, *par excellence*.

A word about the character of this monograph. Firstly, I had planned a chapter on practical methods but Bergersen's (1980) *Methods for evaluating biological nitrogen fixation* has covered the topic admirably. Secondly, the chapter on ecology is briefer than it might have been because Sprent (1979) has covered much of the ground in her useful survey of the more biological aspects of nitrogen fixation. Finally, the bibliography of this book is in no way comprehensive.

About ten years ago I wrote a (now largely obsolete) short monograph on nitrogen fixation and was amused by one review of it, in which a learned contributor to the subject spent most of his space pointing out (albeit delicately) points where I had failed to cite his own work or give him priority. Let me therefore make this statement to my friends (and rivals) in this research area: you will not find your bibliography well represented here, any more than is my own. Only occasionally have I been concerned with scientific priorities; I have taken the view that, for a given detail, the most useful citation is usually to recent papers, or even reviews; the reader may then back-track from these if he wishes to go into the matter in depth. Yet in substance I have tried to keep a reasonable balance between the work of my own colleagues and that of other groups; if I have erred in our own favour it is familiarity with our home product rather than disrespect for the work of others which has led to bias. I hope any bias is minimal but, *sub speciae aeternitatis*, it is trivial anyway.

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John Postgate
July, 1982

CONTENTS

1

Dinitrogen fixation and the nitrogen cycle

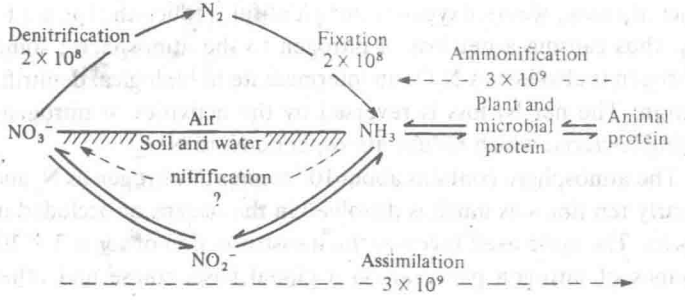
Though the early history of the earth involved massive geochemical and geophysical changes, living things are today responsible for the major chemical transformations which take place on our planet. It is convenient to express these changes, on a gross scale, in the form of hypothetical cycles of the biologically-active elements. The nitrogen cycle, the sulphur cycle and the phosphorus cycle were described by Svensson & Soderlund (1976). A version of the nitrogen cycle is shown in Fig. 1, to remind readers of its substance (elementary accounts may be found in most textbooks, for example Stanier, Doudoroff & Adelberg 1969; Postgate, 1979*a*; more sophisticated data may be found in symposia edited by Clark & Rosswall, 1981 and Stewart & Rosswall, 1982). Fig. 1 formalizes the information that plants and microbes make their component proteins (and other organic nitrogenous matter) from nitrates by way of nitrites and ammonia. Animals make their protein directly or indirectly from plants or microbes. Whether living or dead, such organic nitrogen is inaccessible to plants; it is sometimes called 'immobilized nitrogen'. Death, decomposition and putrefaction eventually lead to release of the protein nitrogen as NH_3, a process called 'ammonification' or 'mineralization'. Most of the NH_3 is rapidly oxidized back to nitrate by nitrifying bacteria. Denitrifying bacteria may, where oxygen is not plentiful, reduce the nitrate to N_2, thus causing a net loss of nitrogen to the atmosphere; some nitrogen is also lost as N_2O, an intermediate in biological denitrification. The net N_2 loss is reversed by the activities of nitrogen-fixing bacteria, which reduce atmospheric N_2 to NH_3.

The atmosphere contains about 10^{15} tonnes of nitrogen as N_2 and nearly ten times as much is dissolved in the oceans or occluded in rocks. The cycle itself involves the transformation of some 3×10^9 tonnes of nitrogen per year on a global basis (these and other

aspects of the nitrogen cycle are discussed by Delwiche (1970, 1977), Burns & Hardy (1975), Svensson & Soderlund (1976), Burris (1980) and Postgate (1980*a*)). In principle, the rate at which the cycle turns determines the biological productivity of a given locality. In undisturbed eco-systems (cl' max vegetation), nitrogen fixation usually imports adequate nitrogen for the biota, so other conditions or nutrients (phosphorus, sulphur, potassium etc.) are limiting. In cold climates, where the cycle turns but slowly, some climax eco-systems can accumulate very substantial reserves of nitrogen, often bound in microbial and plant debris as organic nitrogen compounds; examples are northern forests or upland moorlands. Such nitrogen is not readily available to plants; it is immobilized. When climax eco-systems are disturbed by fire, earthquake or other means, non-nitrogen nutrients become re-cycled and the local store of available nitrogen soon becomes limiting. The most radical disturbance of climax eco-systems today is mankind's agriculture and, in essence, world agricultural (food and fibre) production is limited by the nitrogen input into soils. Except in regions where sophisticated agriculture is practised and considerable use is made of chemical nitrogenous fertilizers, the rate-determining step is nitrogen fixation: the conversion of atmospheric N_2 to inorganic forms which are available to plants.

At this stage a point of nomenclature must be clarified. The term 'nitrogen' correctly refers to the nitrogen atom, N, and N_2 is cor-

Fig. 1. A simple form of the nitrogen cycle. The numbers beneath the various steps are orders of magnitude of turnover in tonnes yr^{-1}. For more details, see text; for variant forms of the cycle, see Clark, 1981.



rectly termed 'dinitrogen'. This terminology will be used henceforth except for certain colloquial usages (e.g. 'nitrogen fixation') which are inescapable. Biological systems able to fix dinitrogen are called 'diazotrophs'.

Dinitrogen fixation is not exclusively biological. Lightning generates oxides of nitrogen, and so does atmospheric pollution, particularly from fuel and internal combustion engines. Combustion (e.g. forest fires) is another source of atmospheric nitrogen oxide formation and was so for many millenia before anthropogenic pollution increased the nitrogen oxide content of the air. Rain washes these oxides of nitrogen into the soil as nitrates. The fertilizer industry also provides locally very important quantities of chemically-fixed nitrogen. Precise data on the relative contributions of these processes to the global nitrogen balance are subject to considerable imprecision (see Burris, 1980; Postgate, 1980*a*) but are probably accurate within a two-fold error. Fixation on a global scale has been calculated at about 2×10^8 tonnes of nitrogen per year. World production of fixed nitrogen from dinitrogen for chemical fertilizer was about 6×10^7 tonnes (i.e.25%) in the late 1970s; amounts of atmospheric oxides of nitrogen washed into soil suggest that this source may provide about 5×10^6 tonnes of fixed nitrogen per year (Burns & Hardy, 1975). Biological processes thus account for about 60% of the earth's newly-fixed nitrogen.

Though the immediate product of dinitrogen fixation is ammonia, whether formed by the Haber process or biological fixation, it is rarely the ammonia molecule which reaches the plant. Soil ammonia, whether 'natural' or added as chemical fertilizer, is rapidly nitrified to nitrate and, in fact, the majority of plants take up nitrate more readily than they take up ammonia. In diazotrophic symbioses the situation is different: nitrification does not normally occur and the fixed nitrogen is probably exported from the diazotroph as ammonia, though it is immediately assimilated into organic form (acid amides or ureides) for transport about the plant (this matter is discussed further in Chapter 6). Nitrification is an important step in the nitrogen cycle for two reasons: it provides the plants with fixed nitrogen in a form they seem to prefer and, secondly, it circulates fixed nitrogen, because nitrate tends to wash out of soil whereas ammonia tends to remain bound. Environment-

al problems can arise when ammonia, added as fertilizer or formed by decomposition processes, becomes washed by rain or flow of ground water into aquifers, rivers or lakes as nitrates.

In broad terms, the nitrogen cycle involves the annual turnover of about 3×10^9 tonnes of nitrogen, of which some 2×10^8 tonnes, nearly 10%, today passes into the atmosphere as N_2 or N_2O and is lost to the biosphere. Dinitrogen fixation, biological, spontaneous and industrial, compensates for this loss and is thus fundamental to the persistence of life on this planet, as well as being of critical importance in world agriculture.

2

The nitrogen-fixing bacteria

The agents of biological dinitrogen fixation belong exclusively* to the non-nucleate protista: the prokaryotes. Earlier reports of fixation by yeasts and *Pullularia* species, the only eukaryotes with an apparently substantial claim to be able to fix dinitrogen, are now discounted (Millbank, 1969, 1970).

The ability to fix dinitrogen is fairly widely distributed among the eubacteria and also among the class of bacteria called cyanobacteria, earlier known as blue-green algae; fixation by some streptomycetes also occurs. Some micro-organisms fix only in association with higher plants; among these associations are symbioses which are agronomically the most important dinitrogen-fixing systems. With the advent of the acetylene test for nitrogen fixation in the mid 1960s (see Chapter 3) the list of organisms believed capable of fixation underwent some abrupt changes and, in addition to the yeasts, reports of fixation by members of the genera *Azotomonas* and *Nocardia* were not substantiated (e.g. Parejko & Wilson, 1968; Hill & Postgate, 1969). The only diazotrophic *Pseudomonas* is *P. ambigua* (Golovacheva & Kalininskaya, 1968; Kalininskaya & Golovacheva, 1969), an organism of uncertain taxonomic status; the other putative member of the genus *Pseudomonas* which remains unchallenged (*P. azotogenesis* strain V) appears to be misclassified (Hill & Postgate, 1969).

Table 1 lists the species believed to include diazotrophs at the time of writing (mid 1982). A commentary on Table 1 follows; a somewhat more extensive commentary on a similar table was presented by Postgate (1981).

* This dogma has been challenged by a report of fixation by a green alga (Yamada & Sakaguchi, 1980). The taxonomic position and nature of this organism need clarifying.

Table 1. *A list of diazotrophic bacteria*

1. Heterotrophs:

Habit when diazotrophic	Family or group	Genus	Species
Aerobic	Azotobacteriaceae	*Azotobacter*	*beijerinckii, chroococcum, paspali, vinelandii*
		Azomonas	*insignis, macrocytogenes*
		Azotococcus	*agilis*
		Beijerinckia	*derxii, indica, fluminensis, mobilis*
		Derxia	*gummosa*
		Xanthobacter	*autotrophicus, flavus*
Micro-aerobic	Spirillaceae	*Azospirillum*	*lipoferum, brasilense*
		Aquaspirillum	*perigrenum, fasciculus*
		Campylobacter	
	Corynebacteriaceae	*Arthrobacter*	*fluorescens*
	Streptomycetaceae	*Frankia*	(see text)
	Methanomonadaceae	*Methylosinus*	*trichosporum*
		Methylocystis	
		Methylococcus	
		Methylomonas	
		Methylobacter	
	Thiobacteriaceae	*Thiobacillus*	*ferro-oxidans*
	Rhizobiaceae	*Rhizobium*	*japonicum, meliloti, leguminosarum, phaseoli, lupini, trifolii*, 'cowpea miscellany'
	Uncertain	*Alcaligenes*	*latus*
Facultative anaerobic	Enterobacteriaceae	*Klebsiella*	*pneumoniae (oxytoca), aerogenes*
		Enterobacter	*aerogenes, cloacae, agglomerans*

2. Phototrophs:

	Family or group	Genus	Species
	Rhodospirillaceae	*Rhodospirillum*	*rubrum, tenue, fulvum, molischianum, photometricum*
		Rhodopseudomonas	*palustris, capsulata, acidophila, gelatinosa, viridis, sphaeroides, globiformis*
		Rhodomicrobium	*vannielii*
	Chromatiaceae	*Chromatium*	*vinosum, minutissimum, minus, violascens, gracile, weissei, warmingii*
		Thiocystis	*violacea*
		Thiocapsa	*roseopercina, pfennigii*
		Amoebobacter	*roseus*
		Ectothiorhodospira	*shaposhnikovii*
	Chlorobiaceae	*Chlorobium*	*thiosulfatophilum (=vibrioforme), limicola, phaeobacteroides*
		Pelodictyon	*luteulum*
		Erwinia	*herbicola*
		Citrobacter	*freundii*
		Escherichia	*intermedia*
	Bacillaceae	*Bacillus*	*polymyxa, macerans*
Anaerobic	Bacillaceae	*Clostridium*	*pasteurianum (pastorianum), butyricum, saccharobutyricum, acetobutyricum, beijerinckii, tyrobutyricun, felsinium, kluyverii, lactoacetophilum, madisonii, pectinovorum, tetanomorphum*
		Desulfotomaculum	*ruminis, orientis*
	Uncertain	*Desulfovibrio*	*desulfuricans, vulgaris, gigas*

3. Cyanobacteria:
(genera only quoted; for details see Stewart (1980) and Rippka & Waterbury (1977); not all strains in the genera are necessarily diazotrophic)

Aerobic:	Filamentous and heterocystous	*Anabaena, Cylindrosperma, Nostoc, Scytonema, Calothrix, Chlorogloeopsis, Fischerella*
	Unicellular	*Gloeothece*
Micro-aerobic:	Filamentous and non-heterocystous	*Spirulina, Oscillatoria, Pseudoanabaena, Lyngbia, Plectonema, Phormidium*
Micro-aerobic:	Unicellular	*Synechococcus, Dermocarpa, Xenococcus, Myxosarcina, Chroococcidiopsis, Pleurocapsa*