Recent Advances in Biological Nitrogen Fixation

Edited by N. S. Subba Rao

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FOREWORD

It is a familiar truism that the persistence of life on this planet depends on the cycling of biological elements in the biosphere. The biological cycles of carbon, oxygen, nitrogen, sulphur and other elements are fundamental to terrestrial and aquatic biology and the role of microbes in these cycles is crucial. A simple form of the biological nitrogen cycle is presented as Fig. 1. The global turnover times of these cycles may be extremely long because they are the resultants of a multitude of localized sub-cycles, which may have all possible turnover rates from extremely rapid to extremely slow. For example, the global nitrogen cycle has a turnover time somewhere in the region of 10^6 years, but in a rice paddy, where nitrogen fixation can take place in the water and denitrification in the mud, the cycle can turn very rapidly. Analogous examples could have been quoted for the biological cycles of sulphur and carbon (for instance, mankind is today enthusiastically re-cycling carbon fixed as coal

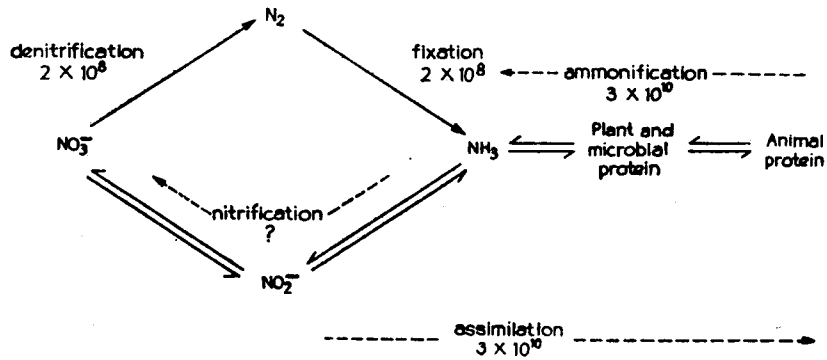

Fig. 1. The nitrogen cycle.
The simplest form of the nitrogen cycle is illustrated. The numbers beneath the various steps are orders of magnitude of turnover in tonnes/year. For more precise data see Delwiche (1977) and Hardy & Burns (1975).

in the carboniferous era) but the nitrogen cycle is of special interest here because of its significance in world agriculture.

In the early history of life on this planet it is probable that nitrogen transfer in the biosphere was non-cyclic: that supplies of fixed N, originally as NH_3, were adequate for the emergence of life and for many millenia of its evolution. However, as free oxygen appeared in the planetary environment, it would react, in the reducing conditions of the era, forming water, CO_2, sulphate and, of course, nitrogen and nitrate. It is probable that, by the time free oxygen became a *permanent* component of the planetary atmosphere, most of the planetary NH_3 had become N_2 or nitrate. Which of these came first, or in what proportions, we do not know; physical chemists (e.g. Broda, 1977) and biologists (e.g. Postgate, 1973) have different opinions. But it seems logical to assume that supplies of fixed nitrogen were not a general problem for living things until evolution had advanced considerably, at least in a physiological sense. We do not know when fixed N became the limiting nutrient for life on this planet but it could have been more recently than 10^9 years ago; thus, as I have argued elsewhere (Postgate, 1973), ability to fix nitrogen might be a relatively recent acquisition of living things. But this, too, is a matter of informed opinion, not scientific fact. Wherever the historic truth lies, the present day situation is that biological nitrogen fixation *has* emerged and, in consequence, N is no longer a limiting nutrient in equilibrium or climax ecosystems on this planet. In virgin forests, natural savannah, tundra and other fertile regions which are in ecological balance, biological fixation provides an adequate input of N and other nutrients—P, S, sometimes K or Mo—limits biomass production.

Why is nitrogen fixation so important, then? The reason is simple: perturbation of a balanced eco-system, whether by fire, vulcanism, inundation, drought or simple cultivation, renews or re-cycles all the biological elements. In consequence, fixed N frequently becomes limiting. In recent centuries the most persistent perturbations of the natural environment have been introduced by mankind as agriculture, so today it is fair to say that, on a global scale, world agricultural productivity is limited by the input of fixed N into agricultural soils.

In the 1970's the world's human population passed the 4×10^9 mark and every concerned scientist recognizes that, assuming no global catastrophe intervenes, it will reach 8×10^9 in the next few decades. Whether it takes two, three or four decades to reach this figure depends on how acceptable and effective are current measures of population control; whether and where it stabilizes depends on similar questions as well as political and social factors. But the inescapable truth is that there are already sufficient children on this planet to ensure that the population will double in the early years of the next millenium, even if all programmes of population control could be immediately successful.

The explosive growth of the world's population in the twentieth century has been supported by increased input of N into the world's agricultural soils, largely as industrial N fertilizer produced by the Haber process. The net input of fixed N is today still sufficient to feed the world's population; local shortages, catastrophic though they may sometimes be, are a result of distribution problems, often exacerbated by political or social factors.

As far as can be judged, the world's agricultural productivity is still in modest excess of the world's *averaged* food requirements. But it will not remain so: to support the inescapable doubling of the world's population, mankind will be obliged effectively to double the N input into the world's agricultural areas (see also chapter 1).

Very important questions arise in discussing the ways and means to double the N input: whether chemically or biologically fixed nitrogen is most appropriate; to what extent the oceans can be exploited; how far new land can be brought into use; whether the obvious over-use of N-fertilizer in some countries is such that regulation of fertilizer use is desirable; whether the environmental consequences (in terms of eutrophication, effects of atmospheric N oxides, etc.) of such a massively increased N-input would be serious; whether the present mounting costs of energy (for transport or production) will price the products of the Haber process out of reach of many countries; whether agricultural procedures can be reformed without disastrous political and economic consequences. These are indeed vital questions, particularly for the developing countries, but they are questions that can only be touched upon in this volume. We must set demographic, socio-economic and political problems aside and accept the premise that we must exploit both industrial and biological nitrogen fixation more effectively and extensively than in the past. My personal view is that both economic (energy cost) and environmental considerations strongly favour biological nitrogen fixation as the process of choice in the future, but I recognize that a contrary view is tenable and I do not propose to argue either position here. It is sufficient to accept that biological nitrogen fixation, requiring a relatively simple and localized technology, and using largely renewable energy sources, has an important role to play in the immediate future of world agriculture.

* * *

Nitrogen fixation, then, is a subject of immense practical importance, and the research efforts now being put into its study could be justified for that reason alone. But it happens also to be a subject of fascinating academic interest. The chemical inertia of the N_2 molecule is legendary: "strong" reagents (molten potassium, red hot magnesium) or special catalysts, as well as anoxic, anhydrous conditions, are needed to induce the N_2 molecule to react chemically. Yet here on this planet are microbes which conduct this chemical miracle

daily, surrounded by air, water and living protoplasm. How do they do it? The full answer to this question is yet to be found, but our understanding of the process has progressed by leaps and bounds in the last two decades, as some of the chapters in this book make clear.

The history of the developments of the last two decades provide some excellent examples of purely academic, basic research having dramatic consequences in areas of applied research. The prime example is the acetylene test. It arose out of speculations on the effect of structural analogues of N_2 on nitrogenase (see Burris, 1976), was leaped upon by scientists as a test for nitrogen fixation and has dramatically altered our understanding of the process. Not only has it allowed substantial fundamental advances in the basic genetics and biochemistry of nitrogenase, it has also provided reasonably (if not absolutely) reliable data on the process in field conditions. Nitrogen-fixing systems once thought to be trivial, such as the woody (*Alnus*) type symbioses (see chapter 15) or the blue-green algae (see chapter 5), are becoming recognized as important and exploitable; indeed, because of this test, fixation is becoming quantitatively the best documented step of the biological nitrogen cycle (see chapter 2). Exploitation of the acetylene test has also revolutionized the list of accepted nitrogen-fixing systems, but this advance required a second fundamental development which has been less widely recognized: the role of oxygen. The oxygen-sensitivity of the enzyme was discussed in the early 1960's and recognition of its physiological importance (see, for example, Postgate, 1975) led directly to the discovery of several varieties of oxygen-sensitive micro-aerobic nitrogen-fixing systems and to confirmation of the reality of the grass associations. Table 1 is a list of nitrogen-fixing systems widely accepted in 1979 compared with a similar list compiled from publications around 1964 (Stewart, 1966, provides a critical review of the position about this time, with references). Though in some instances reclassification has altered the list (the family Azotobacteraceae is now subdivided further; *Desulfotomaculum* would then have been classified with *Desulfovibrio*; two systems were mixed cultures) several fundamentally important changes are obvious. Fixation by yeasts, other fungi or mycorrhiza is no longer accepted, so the only representatives of the eukaryotes have vanished; the genera *Pseudomonas* and *Achromobacter* no longer have accredited representatives in the list; with *Xanthobacter* (incorporating *Mycobacterium flavum*, *Corynebacterium autotrophicum* and probably other isolates of coryneform or arthrobacter-like appearance) a large new class of microaerobic heterotrophic nitrogen fixers is established; numerous non-heterocystous blue-green algae (e.g. *Plectonema*) are now recognized as microaerobic nitrogen fixers; the grass associations (e.g. *Azotobacter paspali* + *Paspalum notatum*) have been demonstrated as real nitrogen-fixing associations, even if their quantitative importance is disputed; many rhizobia have been shown capable of aerobic nitrogen fixation *ex planta* if the oxygen tension is low enough; a rhizobial association with a non-legume is known; the leaf

Table 1. List of acceptable nitrogen-fixing systems in 1979 compared with 1964

	Asymbiotic	Commentary
Aerobic:	*Azotobacter*	No change: classification revised
	Beijerinckia	
	Derxia	
	Azomonas	
	Azotococcus	
	Methanosinus and other methane-oxidizing bacteria	New
	Aquaspirillum pengrinum A. fasciculus	Probably new[1]
	All heterocystous blue-green algae	No change
	Gloeocapsa	New
		1964 list would have included *Azotomonas, Pseudomonas, Nocardia, Arthrobacter* species; *Mycobacterium* species; species of *Saccharomyces, Rhodotorula, Pullularia*
Microaerobic:	*Xanthobacter* (*Corynebacterium*) *autotrophicum*	Related species include mycobacteria above
	Azospirillum lipoferum, A. brasilense	Almost new but reports of spirilla existed[1]
	Thiobacillus ferro-oxidans	New
	Many non-heterocystous blue-green algae	New, e.g. *Plectonema*
	Cowpea rhizobia, *R. japonicum* etc.	Earlier believed to be obligate symbionts for fixation
Facultative:	*Klebsiella pneumoniae, K. aerogenes, K. oxytoca, Bacillus polymyxa, B. macerans*	1964's *Aerobacter* is now *Enterobacter*
	P. azotogensis 'strain V'	'Strain V' was incorrectly classified and resembles an asporogenous bacillus
	Enterobacter (*Erwinia*) spp.	
	Citrobacter freundii	
	[*Escherichia coli*]	*E. coli* and *S. typhimurium* by laboratory transfer of *Klebsiella nif* genes
	[*Salmonella typhimurium*]	

(*Contd.*)

[1]Nitrogen fixing spirilla would have been in older lists but the assignment of the earlier types to *Azospirillum* or *Aquaspirillum* seems difficult.

	Asymbiotic	Commentary
Anaerobic:	*Clostridium pasteurianum* and other butyric clostridia	No change
	Propionibacterium species	New
	Desulfovibrio species	Doubtful before the acetylene test
	Desulfotomaculum (mesophilic)	New
	Chlorobium, Chromatium	No change
	Thiopedia, Ectothiospira	New
	Rhodospirillum, Rhodopsendomonas	No change
		Earlier lists would have included *Chloropseudomonas ethylica* and *Methanobacterium omelianskii*, now known to have been mixed cultures.
Symbiotic:	Legumes + rhizobia	Little change
	Parasponia + *Rhizobium*	New (host plant earlier called *Trema*)
	Woody plants + *Frankia*	No change; symbiont now named
	Paspalum notatum + *Azotobacter paspali*	New
	Other grass and weed associations	New; quantitatively minor
	Phyllosphere associations	Now established, significance disputed
	Lichens (alga + fungus)	No change
	Azolla + *Anabena azollae*	No change
	Cycads + *Anabena* or *Nostoc*	No change
	Gunnera + *Nostoc*	No change
	Ruminant animals + enterobacteria	Real but nutritionally trivial
	Termites + enterobacteria	Real but nutritionally minor
	Man + enterobacteria	New, significance disputed
		Earlier lists would have included the leaf nodule symbioses and Podocorpus + mycorrhiza, both now discounted.

nodule symbiosis is now discounted. So both the acetylene test and rationalization of oxygen sensitivity have contributed much of practical value to the study of nitrogen fixation. Recently the answer to a third fundamental question—why are hydrogenase and nitrogenase associated?—is deepening our understanding of the energy efficiency of the biological process (see chapter 3).

In the 1930's, nitrogen fixation was studied at a dozen or so research centres. By the last meeting of the International Biological Programme on nitrogen fixation at Edinburgh in 1973 (Nutman, 1975; Stewart, 1975) interest in this research area had spread so much that representatives from well over 100 research organisations were present. Only seven of the research organisations could be regarded as engaged in fundamental or basic research. Whether this is a wise distribution of research effort is yet another subject which I choose not to discuss here; for present purposes it certainly emphasises the widespread recognition, throughout the World, of the practical importance of this subject (see chapters 13 and 14). Knowledge at both fundamental and practical levels is advancing rapidly and it is instructive to consider its implications for the future, both for research in and exploitation of nitrogen fixation. Where may our present knowledge lead us?

The biochemistry of nitrogenase has reached a stage at which the complex enzyme system is well understood at a descriptive level and a detailed mechanistic analysis is in hand in various laboratories throughout the World (see chapter 3). The site of N_2 reduction remains elusive in structural terms, but the view is compelling that a transition metal, probably Mo, is directly concerned. Thermodynamically, the process is more efficient than the Haber process, despite its consumption of ATP, so the possibility of developing substantially more effective catalysts than are used in the Haber process becomes more realistic. The major energy cost of the Haber process is the generation of reductant (H_2); if electrolytic reduction of N_2 could be developed, using a site comparable to that in the enzyme, one could hope for low technology process, comparable in energy balance with the biological system, for generating ammonia fertilizer. Environmental problems consequent on using that fertilizer would then have to be faced, but one solution to the N-input problem would have arisen from the joint advances in the chemistry and biochemistry of nitrogen fixation.

The genetics of nitrogen fixation has blossomed during the 1970's and, as with the biochemistry, it has revealed a system of unsuspected complexity. How lucky we were in the early 1970's that all 14 or so *nif* genes are clustered together in *Klebsiella*! A phage could package the whole lot, plasmids could be constructed and genetic analysis could proceed apace (see chapter 9). Biochemistry provided the geneticists with functions for a few genes (a regulatory site + K, D, H and more recently B—(see chapter 8) but now roles are reversed: the geneticist can justly ask the biochemist what do the other 10 or so genes specify? Why are they there at all?

As recently as 1975 there seemed to be several examples of conservation, transcription and translation of prokaryotic genetic information in plants, and to some it seemed a matter of but a few years' research to transfer *nif* to a eukaryote—a plant cell culture perhaps, or a mycorrhiza—and set up some kind of nitrogen-fixing system. Today most of those examples have crumbled

(see Cocking, 1977). Though it will soon be possible in principle to transfer *nif* genes to a eukaryote, the probability of their being expressed therein is remote indeed. Problems of conservation of the genes, their transcription and translation will arise, followed by the need to ensure protection of the nitrogenase, mobilization of Mo, ATP and reductant. Is the effort worth it? Is it not wiser to improve existing systems? To breed new ones by conventional procedures? The answer is that we do not know, therefore the scientific community as a whole must try all approaches. The natural grass associations may yet lead to a 'nitrogen-fixing' cereal sooner than somatic hybridization or genetic manipulation of *nif*: but one goal of the scientist must surely be to get nitrogen-fixing crop plants away from their dependence on microbes, which bring the attendant problems of specificity, competitiveness, effectiveness, persistence and so on.

The resultant of biochemistry and genetics is physiology: the functioning of the cell as a dynamic product of the regulated expression of its genome in response to its environment. The physiology of nitrogen fixation has advanced to a sophisticated level and we understand quite a lot about mechanisms to protect nitrogenase from oxygen, regulatory effects by the product, ammonia, as well as by oxygen and less well defined regulators such as molybdate. Molybdenum uptake and storage, too, are realised to be important factors. The ATP economy of the intact cell is influenced by the involvement of ATP in nitrogenase function both as a substrate and, with ADP, in control; it is also involved at the level of activation of glutamine synthetase, the primary regulator of nitrogenase synthesis. The apparently obligatory nature of the hydrogen evolution reaction of nitrogenase has imposed hydrogen re-cycling on the more efficient nitrogen-fixing systems and this system can assist O_2 exclusion as well as sparing reducing power and improving the ATP economy. Indeed, oxygen exclusion and hydrogen re-cycling are characteristic of the most efficient of nitrogen-fixing systems—exemplified by a good soybean nodule or *Azotobacter* culture—and the integration of these processes into the economy of the system represents a sophisticated physiology involving both genetic and functional regulation. In symbiotic systems, the links between the phase of plant growth and the nitrogen fixation process are exciting interest: the long-term regulation of fixation and the export of ammonia to the plant clearly have a profound influence on the efficacy of fixation in the field.

* * *

There are numerous benefits for mankind which can be foreseen from the developments in knowledge which the future will bring. Possibilities have been discussed frequently (Brill, 1977; Evans, 1975; Gutschick, 1977; Hardy, 1976; Postgate, 1977a, b) and attempts have been made to assign research priorities (e.g. Brown *et al.*, 1975). Naturally, the application of present know-

ledge has immediate priority, but some of us must concern ourselves with medium and long term possibilities. The scientist's dream may be of fields of cereal, unencumbered by symbiotic bacteria, fixing nitrogen with their own nitrogenase, regulated in economical concordance with photosynthesis, and lasting from seedling to seed formation. The plants would be tolerant of cold and water stress, with *nif* stably integrated into their genome, and *nif* would cause no more drain on the plant's economy than exogenous nitrate; there would be fewer environmental problems from run-off, because *nif* expression would cease at seeding...and so on! The desiderata are many, and can be modified to suit one's taste for cereals, other vegetables and science fiction. For such dreams to become reality, and there is still no compelling reason why some at least should not, we need a lot of research of the kind outlined in this volume. The application of what we already know will suffice for a decade or so, but a complete solution to the nitrogen problem requires imaginative research at both fundamental and applied levels.

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PREFACE

The combination of nitrogen and hydrogen to form ammonia through biological means is known as biological nitrogen fixation. It is a unique process restricted only to certain microorganisms and plant-microbe interactions capable of harnessing atmospheric nitrogen for the growth of plants. This natural process of 'nitrogen fixation' has been going on for centuries as part of the nitrogen cycle. With the advent of inorganic nitrogen fertilizers, the world has witnessed dramatic increases in food production in the last two decades, which could not have been achieved by biological fixation of nitrogen alone. The dependence of fertilizer nitrogen production on fossil energy resources and the prospects of diminished availability of this costly input for fertilizer production in years to come has obviously brought the subject of biological nitrogen fixation to the forefront.

Rapid advances have been made in our knowledge of the mechanisms of enzymes involved in nitrogen fixation, the 'nif' genes and the genetic 'engineering' of microorganisms to tailor new or altered microorganisms with highly efficient genomes for nitrogen fixation. Equally innovative has been the research work on new methodology for assessing the quantum of nitrogen fixed in any natural microhabitat or plant systems. As a sequel to this, new plant-microbe systems with potentiality for use in agriculture have been highlighted.

In advanced countries, fertilizer nitrate pollution has been a major concern while in developing countries, the concern has been that continuous use of fertilizer nitrogen may endanger soil structure. There is a consensus that organic fertilization not only improves soil structure but also contributes to general soil health. A second factor to be reckoned with in developing countries is that fertilizer nitrogen is used at minimal levels even for the cultivation of cereals and other cash crops. In many countries, fertilizer nitrogen is not made indigenously and has to be imported. Therefore, the future of agriculture in developing countries lies in judicious combinations of organic and

inorganic fertilization of soil to obtain required yields. It is in this context that biological nitrogen fixation and recycling of organic wastes are considered to be vital ingredients of agriculture in Asia and Africa.

In recent years several books on biological nitrogen fixation have been written. They are expensive and are also not readily available to students and research workers in developing countries. This book is the fructification of an idea which emerged three years ago to bring out a reference volume on the latest developments in this exciting area of biological nitrogen fixation. Inflation has hit all countries during the last few years and has not spared this effort also. However, an attempt has been made to keep the price relatively modest.

I am grateful to all the contributors who readily agreed to write for this volume. The proof reading was done by me and I trust that it would meet with the authors' requirements. This was done to facilitate quick publication of the volume. The publishers have been extremely cooperative and it was a pleasure to work with them. Special thanks are due to Prof. J. Postgate for writing a foreword and to Drs. A.H. Gibson and J. Bergersen of C.S.I.R.O., Australia for their useful suggestions in the preparation of the volume. I record my appreciation of the encouragement provided by Dr. H.K. Jain, Director, Indian Agricultural Research Institute, Dr. O.P. Gautam, Director-General, Indian Council of Agricultural Research and Dr. M.S. Swaminathan, Secretary, Department of Agriculture, Govt. of India.

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